Hydroxylation of Phenol to Catechol by *Candida tropicalis*: Involvement of Cytochrome P450

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Abstract. Microsomal preparations isolated from yeast *Candida tropicalis* (*C. tropicalis*) grown on three different media with or without phenol were isolated and characterized for the content of cytochrome P450 (CYP) (EC 1.14.15.1). While no CYP was detected in microsomes of *C. tropicalis* grown on glucose as the carbon source, evidence was obtained for the presence of the enzyme in the microsomes of *C. tropicalis* grown on media containing phenol. Furthermore, the activity of NADPH : CYP reductase, another enzyme of the microsomal CYP-dependent system, was markedly higher in cells grown on phenol. Microsomes of these cells oxidized phenol. The major metabolite formed from phenol by microsomes of *C. tropicalis* was characterized by UV/vis absorbance and mass spectroscopy as well as by the chromatographic properties on HPLC. The characteristics are identical to those of catechol. The formation of catechol was inhibited by CO, the inhibitor of CYP, and correlated with the content of cytochrome P450 in microsomes. These results, the first report showing the ring hydroxylation of phenol to catechol with the microsomal enzyme system of *C. tropicalis*, strongly suggest that CYP-catalyzed reactions are responsible for this hydroxylation. The data demonstrate the progress in resolving the enzymes responsible for the first step of phenol degradation by the *C. tropicalis* strain.

Key words: Environmental pollutants — Phenol — Biodegradation — Yeast – *Candida tropicalis* — Cytochrome P450

Abbreviations: CYP, cytochrome P450; HPLC, high performance liquid chromatography; K_m, Michaelis constant; V_{max}, maximum velocity.

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Introduction

Phenol and its derivatives are found in a wide variety 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. Bacteria are most likely to be responsible for aerobic breakdown of phenol in activated sludge. Nevertheless, fungi including *Trichosporon cutaneum* and *Candida tropicalis* (*C. tropicalis*) are also capable of utilizing phenol as the major carbon source (Neujahr and Varga 1970; Neujahr et al. 1974; Krug et al. 1985; Krug and Straube 1986; Stephenson 1990; Komárková and Páca 2000). Although examples are known in which yeast *C. tropicalis* utilizes phenol for growth or metabolism, much less information on the nature of the phenol-oxidizing enzymes in this microorganism are known.

Three types of enzymes known to oxidize phenol in various eukaryotic organisms might be considered enzymes responsible for the first step of phenol degradation (the formation of catechol) in *C. tropicalis*: (i) the mixed function monooxygenase system localized in the membrane of endoplasmic reticulum and containing cytochrome P450 (CYP) (EC 1.14.15.1) as a terminal oxidase (Porter and Coon 1991; Ohkuma et al. 1995; Nelson et al. 1996), (ii) cytosolic NADPH-dependent phenol hydroxylase (EC 1.14.13.7) (Neujahr and Varga 1970; Neujahr et al. 1974; Krug et al. 1985; Krug and Straube 1986; Xu et al. 2001) and (iii) membrane-bound or soluble peroxidases (EC 1.11.1.7) (Klibanov et al. 1983; Eling et al. 1990; Nicell et al. 1993; Dec and Bollag 1994).

In *C. tropicalis*, alkane-inducible CYP oxidizing alkanes (CYP52) were detected (Sanglard et al. 1984, 1986, 1993; Seghezzi et al. 1992). Moreover, another CYP, sterol 14-alpha demethylase (lanosterol 14-alpha demethylase (CYP51)), participating in metabolism of ergosterol, has also been found in this microorganism and genes of both CYP families have been characterized (Sanglard et al. 1984, 1986, 1993; Seghezzi et al. 1992; Ohkuma et al. 1998; Zimmer et al. 1998). So far, it has not been known, whether CYP enzymes oxidizing phenol are present in this microorganism. Therefore, we investigated in this study whether CYP capable of oxidizing phenol is present in microsomal preparations isolated from *C. tropicalis*. The *C. tropicalis* yeast cells grown on different media (in the absence or the presence of phenol) were used in the study.

The present paper reports for the first time the results providing evidence that CYP is an essential component of the enzyme system which might participate in hydroxylation of phenol in *C. tropicalis*.

Materials and Methods

Chemicals

Chemicals were obtained from the following sources: NADPH, cytochrome c, catechol, hydroquinone and bicinchoninic acid (2,2′-biquinoline-4,4′-dicarboxylic acid)

from Sigma Chemical Co., (St. Louis, MO, USA), and NADH from Roche Diagnostics, (Mannheim, Germany). Other chemicals were supplied by Lachema (Brno, Czech Republic). All chemicals were of reagent grade purity or better.

Organisms and cultivation methods

The yeast *C. tropicalis* was isolated from soil contaminated with aromatic hydrocarbons and identified using the culture collection in Research Center (Brno, Czech Republic) (Komárková and Páca 2000). The yeast culture was maintained on slope agar with mineral salts and glucose as a carbon and energy source at 4 ℃.

Media and cultivations

The growth medium contained (in $g \cdot l^{-1}$): 1.7 yeast nitrogen base (YNB-Difco), 5 $(NH_4)_2SO_4$, 1 KH_2PO_4, 0.5 $MgSO_4 \cdot 7H_2O$ and 0.1 NaCl. The inoculum was prepared in two stage precultures implemented in shaking flasks at 30 ℃ and pH 5.2. The medium was supplemented with (i) 4.0 $g \cdot l^{-1}$ glucose (glucose medium), or (ii) the same amount of glucose and 350 $g \cdot l^{-1}$ phenol (glucose-phenol medium) (Martius et al. 1996; Páca and Martius 1996). The third medium was BSM medium (4.3 $g \cdot l^{-1}$ K_2HPO_4, 3.4 $g \cdot l^{-1}$ KH_2PO_4, 2 $g \cdot l^{-1}$ $(NH_4)_2SO_4$, 0.34 $g \cdot l^{-1}$ $MgCl_2 \cdot 6 H_2O$) containing 350 $g \cdot l^{-1}$ phenol as a sole carbon and energy source (phenol medium).

Cell cultivations were carried out in shaking flasks using fed batch process with three different growth media (see above) at 30 ℃ and pH 5.2 (Martius et al. 1996; Páca and Martius 1996). After separation, the cells were washed three times with distilled water and disintegrated by sonication to obtain the cell-free homogenate.

Preparation of microsomes

The isolation of the microsome fraction from the *C. tropicalis* cell-free homogenate was carried out by differential centrifugation by the procedure used for isolation of rat liver microsomes (Stiborová et al. 1995, 2001a,b). The 105,000 *g* sediment was taken as microsomes (Stiborová et al. 2001a,b) and used for studies presented in the paper. Microsome samples were stored at −80 ℃.

Analytical methods

Protein concentrations in microsomal fractions were assessed by the bicinchoninic acid protein assay (Pierce, Rockford, IL) with serum albumin as a standard (Wiechelman et al. 1988). The concentration of CYP was estimated according to the method by Omura and Sato (1964) based on the complex of reduced CYP with carbon monoxide. The activity of NADPH : CYP reductase was measured according to Sottocasa et al. (1967) using cytochrome c as substrate (i.e. as NADPH : cytochrome c reductase).

Phenol hydroxylase activity assay

The efficiency of microsomes in oxidation of phenol was followed by HPLC. The incubation mixtures used for oxidation of phenol contained 0.1 ml of 40 mmol·l^{-1} sodium phosphate buffer (pH 7.5), 1.5 mg microsomal protein, 1.0 mmol·l^{-1} phenol and 1.0 mmol·l^{-1} NADPH. Control incubations were carried out either without NADPH or with this cofactor, but with the heat-inactivated microsomes. Incubations were performed at 37 ℃ for 20–180 min.

Incubations to study the oxidation of phenol with microsomes of *C. tropicalis* were stopped with 20 μl of 0.6 mol·l^{-1} $HClO_4$, centrifuged and 50-μl aliquots of supernatant were injected directly onto the HPLC. 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 % after 20 min incubation in the presence of microsomes without a cofactor (NADPH). 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).

Results

Microsomal preparations of *C. tropicalis* grown on three different media with or without phenol were isolated and characterized for the content of CYP. While no CYP was detected in microsomes of *C. tropicalis* grown on glucose as a carbon source, evidence was obtained for the presence of the enzyme in microsomes of *C. tropicalis* grown on media containing phenol (Table 1). As shown in Fig. 1, the carbon monoxide difference spectrum of the dithionite-reduced preparation, with a peak close to 450 nm (448 nm), is typical of this heme enzyme. Studies on the inducibility of this enzyme system are summarized in Table 1. The highest content of CYP was found in microsomes of *C. tropicalis* cells grown on a medium containing phenol alone as a carbon source. Furthermore, both the content of NADPH : CYP reductase, another enzyme of the microsomal CYP-dependent system, and the phenol hydroxylation activity were markedly higher in cells grown on phenol (Table 1).

The phenol hydroxylation activity of microsomal preparations was estimated by an originally developed HPLC method. Employing this method, we were able to quantitate the phenol hydroxylation activity of microsomes of *C. tropicalis* cells

Table 1. Inducibility of the CYP-dependent system by growth of *C. tropicalis* on phenol

Carbon source for growth	CYP [a] ($nmol \cdot mg^{-1}$)	NADPH : CYP reductase activity [b] (nmol cytochrome $min^{-1} \cdot mg^{-1}$)	Phenol hydroxylation activity [c] (nmol phenol $min^{-1} \cdot mg^{-1}$)	(nmol phenol $min^{-1} \cdot nmol^{-1}$)
Glucose	–	0.096 ± 0.01	–	–
Glucose + phenol	0.121 ± 0.01	0.216 ± 0.01	1.146 ± 0.12	9.471 ± 0.98
Phenol	0.359 ± 0.03	1.520 ± 0.14	3.790 ± 0.54	10.557 ± 1.05

[a] the cytochrome CYP content was estimated from CO difference spectrum using the molar absorption coefficient of 91 $cm^{-1} \cdot l \cdot mmol^{-1}$ for absorbance at 450 nm (Ohkuma et al. 1998); [b] the activity of NADPH : CYP reductase was measured according to Sottocasa et al. (1967) using cytochrome c as substrate (i.e. as NADPH : cytochrome c reductase); [c] the phenol hydroxylation activity (phenol disappearance) was determined by the procedure utilizing HPLC for separation and quantitation of phenol and its metabolite, described in Materials and Methods. The values in the table are averages ± S.E.M. of three experiments; – not detectable.

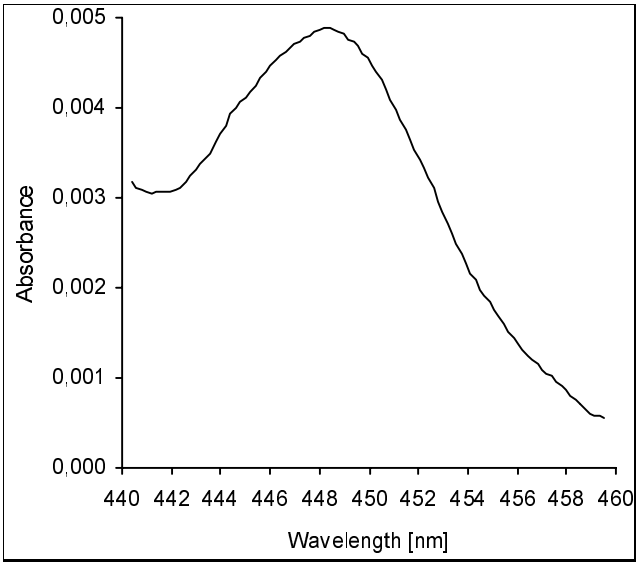

Figure 1. Reduced carbon monoxide difference spectrum of microsomal fraction of *C. tropicalis* grown on phenol. The preparation, containing 5 mg of protein per ml, was reduced by addition of 0.1 mg solid dithionite. The sample cell was bubbled with CO for about 30 s, and the CO difference spectrum was recorded on a Specord M-42 spectrophotomer (Carl Zeiss, Jena, Germany) with 1-cm cuvettes.

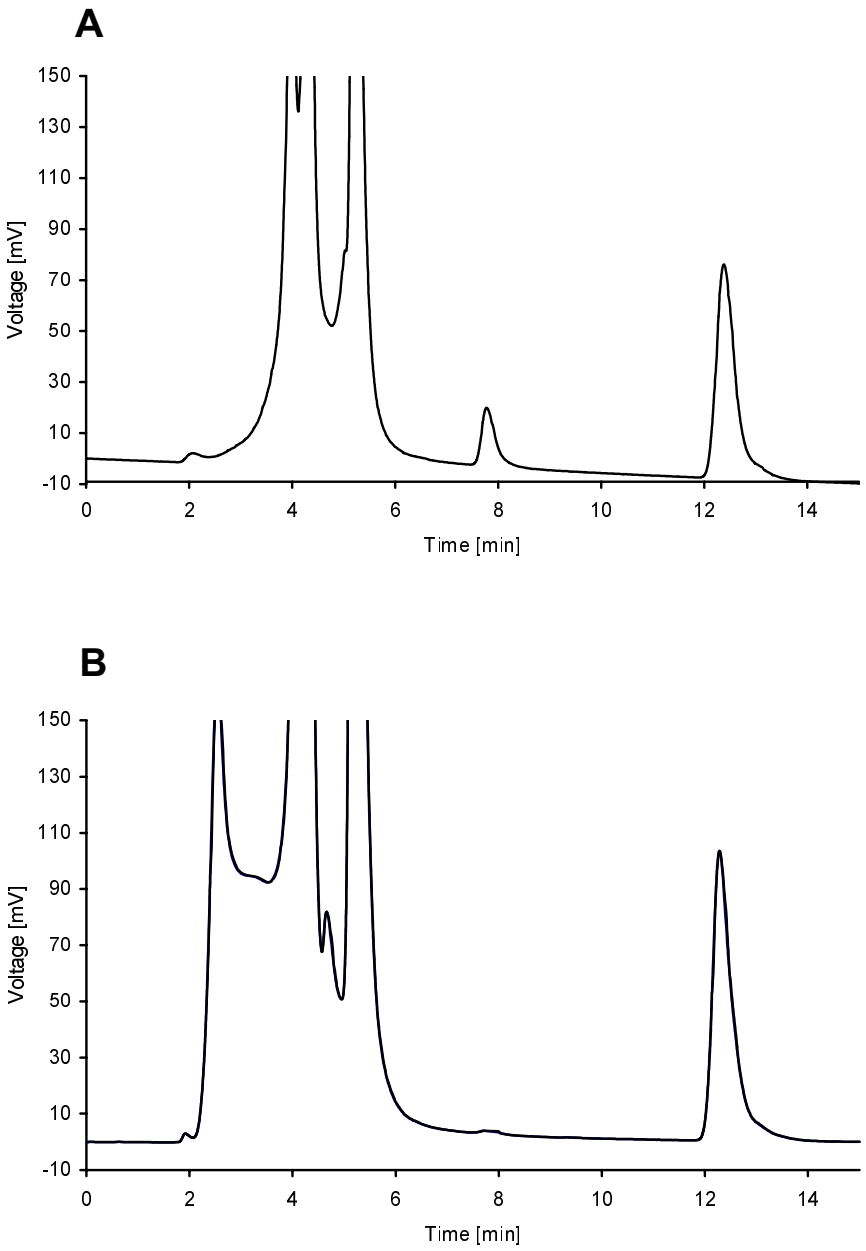

Figure 2. Chromatograms showing the separation of phenol from its metabolite, catechol. Chromatograms of the full reaction mixture (A) and the mixture containing heat-denatured microsomes (B). Peaks eluting between 2.0–5.5 min, solvent front, NADPH and protein components of microsomes; peak eluted at 7.9 min, catechol; peak eluted at 12.3 min, phenol. Experimental conditions and procedures are described in the text.

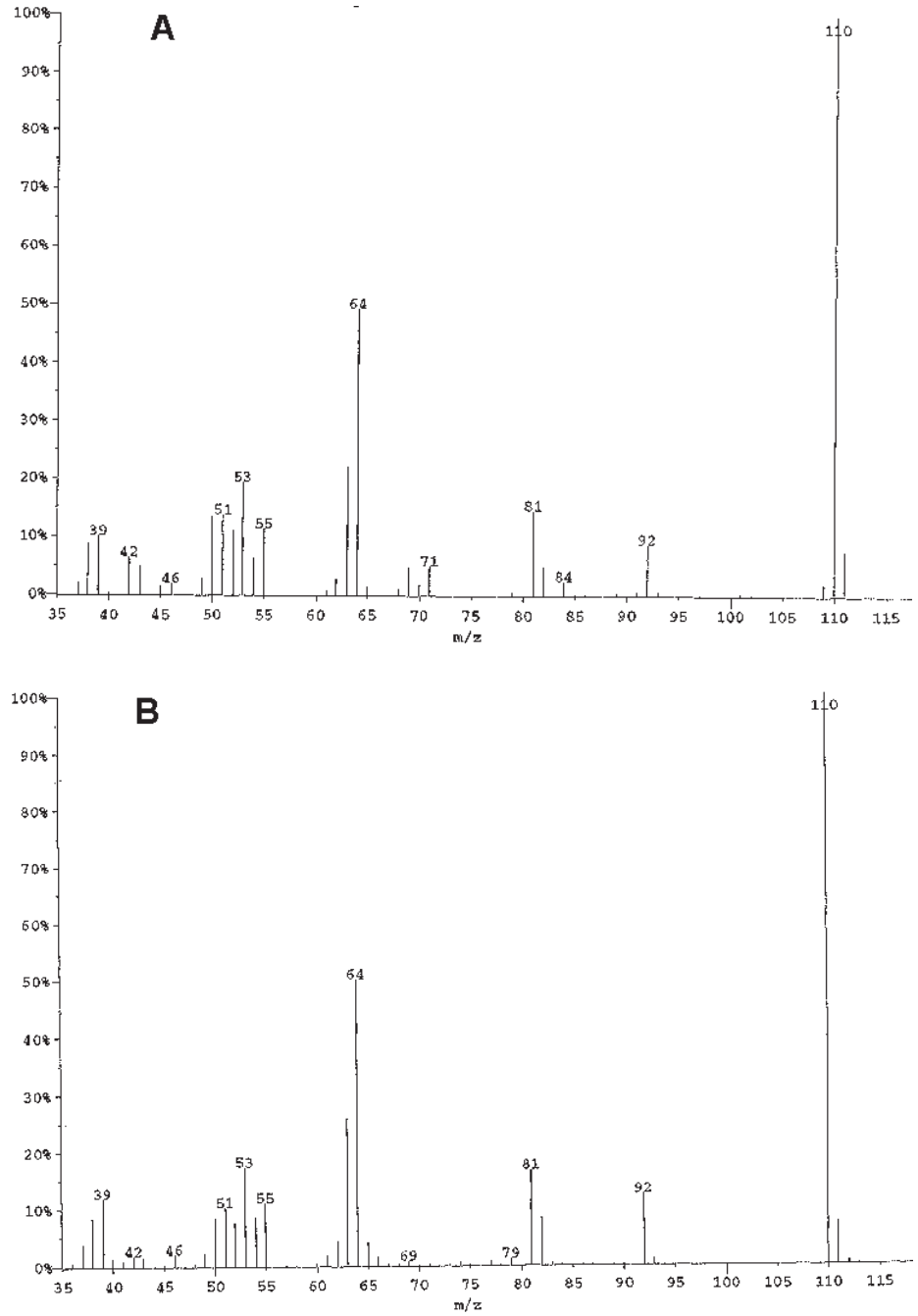

Figure 3. Mass spectra of a phenol metabolite formed by microsomes of *C. tropicalis* (A) and catechol (B).

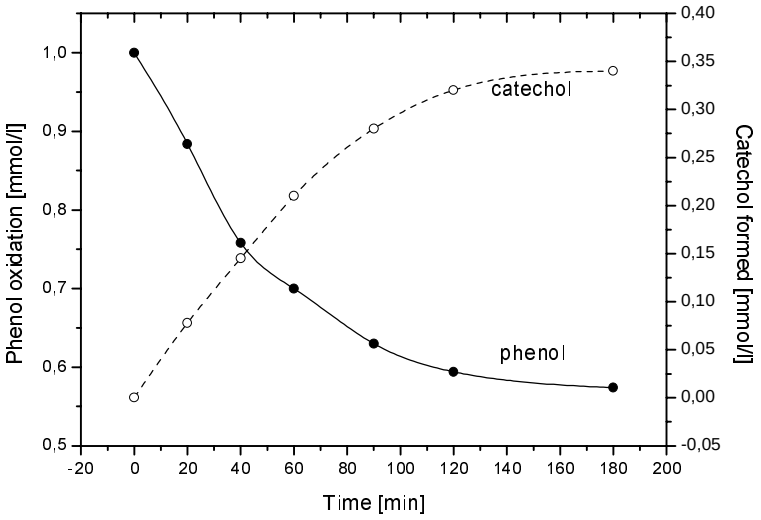

Figure 4. Time dependence of phenol consumption and catechol formation by microsomal enzymes of *C. tropicalis*. Incubations were carried out by the procedure described in the text, except that incubation times were 0–180 min.

(Table 1). We found that phenol is oxidized with microsomes of *C. tropicalis* to a major reaction product having the retention time 7.9 min (Fig. 2). This metabolite was identified by co-chromatography with an authentic standard, catechol, having the same retention time 7.9 min (Fig. 2), 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, are identical to those of a standard, catechol (Fig. 3). In addition, UV/vis absorbance spectrum of the metabolite, having an absorption maximum at 277 nm was identical to that of catechol (data not shown). All these results indicate that catechol is the phenol metabolite formed in microsomes of *C. tropicalis*.

Testing chromatographic properties on HPLC of another possible oxidative product of phenol, hydroquinone, we excluded this compound being formed by the microsomal system of *C. tropicalis* under the conditions used. No product having the retention time identical to that of hydroquinone (6.12 min) was detected. Similarly, a possible reaction product of catechol hydroxylation, 1,2,3-trihydroxybenzene (having the retention time of 5.82 min), was not found.

A time-dependent decrease in phenol followed by an increase in an oxidation metabolite, catechol, was detected in microsomes grown on media containing phenol (Fig. 4). Control incubations carried out in parallel either with heat-inactivated microsomes (Fig. 2), or without NADPH (not shown) were essentially free of catechol even after prolonged incubation times. The phenol hydroxylation was dependent on the presence of oxygen, being decreased under anaerobic conditions

Table 2. The effect of enzyme cofactors on phenol-hydroxylation catalyzed by microsomes of *C. tropicalis* grown on phenol

System	Gas phase	The rate of phenol consumption (nmol phenol $min^{-1} \cdot mg^{-1}$)	
Complete[a]	Aerobic	3.91 ± 0.43[b]	(100.0)[c]
Complete with heat-inactivated microsomes[d]	Aerobic	–	
Complete[e]	CO-O_2	2.46 ± 0.21	(62.9)
Complete[f]	N_2-O_2	2.81 ± 0.25	(71.9)
No cofactor	Aerobic	0.10 ± 0.01	(2.5)
NADPH replaced by NADH[g]	Aerobic	1.15 ± 0.12	(29.4)

[a] experimental conditions for incubations: the incubation mixtures used for oxidation of phenol contained 0.1 ml of 40 $mmol \cdot l^{-1}$ sodium phosphate buffer (pH 7.5), 1.5 mg microsomal protein, 1.0 $mmol \cdot l^{-1}$ phenol and 1.0 $mmol \cdot l^{-1}$ NADPH. Incubations were performed in open tubes at 37 °C for 60 min. The reaction mixtures were analysed by HPLC as described in Materials and Methods. The rate of phenol consumption was determined from a decrease in amounts of phenol; [b] the values in the table are averages ± S.E.M. of three incubations; [c] percentages of the rate of phenol consumption are shown in parentheses; [d] the same composition of the reaction mixtures as in [a], but microsomes were heated (100 °C) for 10 min before their addition into incubations; [e] bubbled (saturated) with CO (20 min). Incubations were carried out in closed tubes; [f] bubbled (saturated) with N_2 (20 min). Incubations were carried out in closed tubes; [g] 1 $mmol \cdot l^{-1}$ NADH instead of NADPH; – not detectable.

(Table 2). These results suggest that the microsomes tested in this study contain enzymatic systems capable of catalyzing the oxidative metabolism of phenol utilizing oxygen. CYP present in microsomes (see above) is the major candidate for the hydroxylation of phenol. To investigate this possibility, the influence of compounds serving as cofactors of the CYP-dependent system on phenol oxidation catalyzed with *C. tropicalis* microsomes were examined. As shown in Table 2, the formation of catechol required NADPH, a known cofactor of a CYP-dependent enzyme system. The production of this metabolite was negligible when NADPH was omitted from the incubation mixture. NADH, a cofactor of the microsomal NADH : cytochrome b_5 reductase serving as a second electron donor for CYP-dependent systems (Schenkman and Jansson 1999), was much less efficient as a cofactor than NADPH (Table 2). Nevertheless, the stimulating effect of this cofactor on the phenol hydroxylation supports suggestion that CYP might be responsible for phenol hydroxylation. The stimulation is caused by a NADH : cytochrome b_5 reductase-mediated electron transfer from NADH to the Fe ion of CYP in the second reduction step of the CYP reaction mechanism (Schenkman and Jansson 1999). Moreover, the phenol hydroxylation was inhibited by CO, the inhibitor of CYP binding to a heme iron ion, and correlated with the content of CYP in microsomes (Table 2). Taken together, these data strongly suggest that cytochrome P450-catalyzed reac-

tions are responsible for the hydroxylation of phenol to catechol in microsomes of *C. tropicalis*.

Oxidation of phenol was measured in the reaction mixture, which contained *C. tropicalis* microsomes, NADPH and various concentrations of phenol. The values of a maximal velocity (V_{max}) and an apparent Michaelis constant (K_m) for oxidation of phenol were 5.4 nmol·min^{-1} *per* mg of protein and 0.23 mmol·l^{-1}, respectively.

Discussion

Several fungi including *C. tropicalis* are capable of utilizing phenol as the sole carbon and energy source. These organisms might therefore be useful for biotechnological applications such as decontamination of phenol in wastewaters. The hydroxylation of phenol to catechol is the first and rate-determining step in the phenol degradation pathways. The question which of the yeast enzymes are responsible for this first step of phenol degradation has not been fully answered yet. The attempts to identify such enzymes in fungi have been carried out by Neujahr and co-workers (Neujahr and Varga 1970; Neujahr et al. 1974; Sejlitz et al. 1990). A soluble flavoprotein monooxygenase, NADPH-dependent cytosolic phenol 2-hydroxylase (EC 1.14.13.7) was detected in *Trichosporon cutaneum* and found to be capable of hydroxylating phenol in this species (Neujahr and Varga 1970; Neujahr et al. 1974; Sejlitz et al. 1990). Although phenol as a xenobiotic chemical can be easily oxidized with CYP in mammalian organisms including humans (Porter and Coon 1991; Ohkuma et al. 1995; Nelson et al. 1996), no proof of phenol oxidation with this enzyme in *C. tropicals* is known.

The presence of two CYP multigene families in *C. tropicalis* has been demonstrated (Sanglard et al. 1984, 1986, 1993; Seghezzi et al. 1992; Ohkuma et al. 1998). The isoforms of alkane-inducible CYP52 hydroxylating alkanes and/or fatty acids and constitutive CYP51 demethylating lanosterol were found in this organism (Sanglard et al. 1984, 1986, 1993; Seghezzi et al. 1992; Ohkuma et al. 1998). Seven members of the CYP52 gene family are located in four different chromosomes and four of them are arranged in tandem on the *C. tropicalis* genome. The products of these seven genes, *alk1* to *alk7*, were compared and a high degree of divergence was found: the two most diverged proteins exhibit a sequence identity of only 32 %. Six of the seven genes were shown to be induced by a variety of aliphatic carbon sources but suppressed when the organism was grown on glucose. Some of the CYP52 genes were found to be successfully expressed in *Saccharomyces cerevisiae* and to display different substrate specificities in *in vitro* assays with model substrates, aliphatic alkanes and fatty acids (Seghezzi et al. 1992). However, so far, no evidence has been documented of the presence of genes of CYP or expressed enzyme proteins catalyzing hydroxylation of aromatic xenobiotics. The CYP multigene families seem to be typical also for some other fungi, e.g. basidiomycete fungi *Phanerochaete chrysosporium* contains at least 78 CYP genes (David Nelson's web site, http://drnelson.utmem.edu/CytochromeP450.html). The results shown in the

present paper suggest that CYP induced by phenol, which catalyzes ring hydroxylation of an aromatic compound, phenol itself, is an essential component of the enzyme system of *C. tropicalis*. Catechol is formed as the major reaction product. Expression of CYP oxidizing phenol in *C. tropicalis* is strongly dependent on the presence of an inducing agent, because no evidence for the occurrence of CYP protein and the phenol hydroxylation activity was detected in cells grown on glucose as the sole source of carbon. Similarly to other eukaryotic organisms, the enzyme is located in the microsomal subcellular fraction of *C. tropicals* cells, in a membrane of endoplasmic reticulum (Porter and Coon 1991; Ohkuma et al. 1995; Nelson et al. 1996). Because the CYP enzyme oxidizing phenol found in our study is the inducible enzyme, it presumably might be the enzyme similar to or identical to some members of a CYP52 family, but this remains to be established.

Conclusions

The results presented in this paper demonstrate the ability of *C. tropicalis* microsomes to hydroxylate phenol, which is a contaminant of a wide variety of wastewaters. Analyses of our data suggest that CYP-mediated reaction is responsible for the phenol hydroxylation reaction. We found that the enzyme is induced by phenol itself. This is highly significant in view of the fact that the content of this enzyme in *C. tropicalis* grown on media containing this environmental pollutant increases and stimulates the potential of the organism to degrade phenol. Here we assume that organisms rich in such an enzyme might be able to degrade phenol and might be utilized in bioremediation technologies. Characterization of the protein of this CYP as well as of genes coding such an enzyme is the aim of our future work.

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