

## Toluene monooxygenase from the fungus *Cladosporium sphaerospermum*

Dion M.A.M. Luykx,<sup>a,\*</sup> Francesc X. Prenafeta-Boldú,<sup>b</sup> and Jan A.M. de Bont<sup>c</sup>

<sup>a</sup> Division of Industrial Microbiology, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

<sup>b</sup> Fungal Biodiversity Center, P.O. Box 85167, 3508 AD Utrecht, The Netherlands

<sup>c</sup> Department of Environmental Biotechnology, TNO Institute of Environmental Sciences, Energy Research and Process Innovation, P.O. Box 342, Apeldoorn, The Netherlands

Received 10 October 2003

### Abstract

Assimilation of toluene by *Cladosporium sphaerospermum* is initially catalyzed by toluene monooxygenase (TOMO). TOMO activity was induced by adding toluene to a glucose-pregrown culture of *C. sphaerospermum*. The corresponding microsomal enzyme needed NADPH and O<sub>2</sub> to oxidize toluene and glycerol, EDTA, DTT, and PMSF for stabilization. TOMO activity was maximal at 35 °C and pH 7.5 and was inhibited by carbon monoxide, Metyrapone, and cytochrome *c*. TOMO preferred as substrates also other aromatic hydrocarbons with a short aliphatic side chain. Its reduced carbon monoxide difference spectrum showed a maximum at 451 nm. A substrate-induced Type I spectrum was observed on addition of toluene. These results indicated that TOMO is a cytochrome P450. TOMO and its corresponding reductase were eventually purified by a simultaneous purification revealing apparent molecular masses of 58 and 78 kDa, respectively.

© 2003 Elsevier Inc. All rights reserved.

**Keywords:** Cytochrome P450 monooxygenase; Cytochrome P450 reductase; *Cladosporium sphaerospermum*; Toluene monooxygenase; Toluene

Toluene and other related aromatic hydrocarbons are abundant environmental pollutants. Bioremediation of such pollutants is an attractive method because of its environmental and economical advantages [1]. The use of fungi instead of bacteria in the bioremediation technology offers specific advantages under conditions of reduced water activity and low pH, which often prevail in air biofilters. The first fungus that was isolated growing on toluene as carbon and energy source was *Cladosporium sphaerospermum* [2]. It was isolated from a trickle bed reactor used in the removal of toluene from contaminated air. Recently, additional fungal strains were isolated growing on toluene [3]. For bacteria the complete aerobic biodegradation of toluene to carbon dioxide, water, and biomass has been studied exten-

sively. Different initial oxidative reactions have been identified for a variety of bacteria, and the biodegradation of the resulting oxygenated compounds is well documented [4–6]. Although it is not well documented yet for fungi, it has been proposed for fungi growing on toluene that they assimilate toluene only by an initial oxidation of the methyl group [2,7].

Cytochrome P450s are a superfamily of heme-containing enzymes that are often involved in the initial oxidative metabolism of a wide variety of endogenous and xenobiotic compounds [8–10]. These enzymes are found in prokaryotic and eukaryotic organisms and have mostly been studied in mammalian liver [11,12]. From microbial sources a number of P450s have been identified [13–15]. Knowledge on fungal cytochromes P450 is limited, although their involvement in several important conversions has been shown: hydroxylation of progesterone at specific sites [16], demethylation of lanosterol [17], hydroxylations relevant for biocatalysis [18], and hydroxylation of xenobiotic compounds [19]. Despite the relevance of these conversions, the putative

\* Corresponding author. Present address: Center for Biological Medicines and Medical Technology, National Institute for Public Health and the Environment (RIVM), P.O. Box 1, 3720 BA Bilthoven, The Netherlands. Fax: +31-30-274-4421.

E-mail address: [dion.luykx@rivm.nl](mailto:dion.luykx@rivm.nl) (D.M.A.M. Luykx).

cytochromes P450 have not been isolated so far. This is due to the low amounts of cytochrome P450 present in these organisms and the instability of these enzymes during purification. Microsomal P450s are part of a membrane-bound multienzyme complex in which they function as a substrate binding terminal oxidase. A flavoprotein acts in this complex as NADPH-cytochrome P450 reductase (CPR) transferring electrons from the reduced coenzyme to the heme-protein [20]. Until now only two membrane-bound cytochrome P450s have been isolated successfully from filamentous fungi. The first one, however, concerns a benzoate-*para*-hydroxylase from an overproducing, recombinant *Aspergillus niger* strain [21]. The other one is a benzo[*a*]pyrene hydroxylase from *Pleurotus pulmonarius* which was only purified to near homogeneity [22]. Besides the microsomal P450s, cytosolic P450s from the fungus *Fusarium oxysporum*, yeast *Trichosporon cutaneum*, and *Trichosporon montevidense* have been purified and characterized [23–25]. These cytosolic enzymes, which have also been found for bacteria, are more stable and therefore more easy to purify.

This paper describes the identification and characterization of the microsomal cytochrome P450 from the fungus *C. sphaerospermum* that is involved in the degradation of the aromatic hydrocarbon and environmental pollutant toluene. A simple method that was eventually developed for a simultaneous purification of this enzyme and its corresponding reductase has been included.

## Materials and methods

**Cultivation of fungus.** *C. sphaerospermum* was grown aerobically at 30°C on a mineral medium, pH 7, supplemented with a trace-element solution and 2 g of glucose and 0.2 g of yeast extract per liter. A 20 L culture was started by using 16 × 5 L Erlenmeyer flasks (shaking speed 110 rpm) containing each 1.25 L of medium. At the end of the stationary phase the mycelium was harvested with a cheesecloth, washed with cold distilled water and mineral medium, and suspended in mineral medium to a total volume of 2.0 L. Subsequently, the suspension was divided over 20 serum bottles of 1 L. By introducing to the suspension a tube containing toluene (0.5 ml) dissolved in dibutylphthalate (1.5 ml) consumption of toluene by the fungus was possible via gas-phase. The bottles were placed at 30°C and shaken with 110 rpm. The conversion of toluene was followed by the production of CO<sub>2</sub> on GC (HP 6890 series (Hewlett–Packard); column: poraplot Q (25 m) (Chrompack)). After 15 h the mycelium was harvested, washed, and frozen in liquid nitrogen.

**Preparation microsomal fraction.** The frozen mycelium was disrupted in a Braun dismembrator and subsequently suspended in 250 ml of 0.1 M Hepes buffer containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM FAD, and 1 μM FMN, pH 7.2 (Buffer A). The suspension was centrifugated at 12,000g for 20 min at 4°C. This centrifugation step was repeated again for the supernatant, yielding the cell-free extract. Via ultracentrifugation (200,000g) of this extract for 2 h, at 4°C, a cytosolic and microsomal fraction were obtained. The microsomes were washed and resuspended in Buffer A. For solubilization of the

microsomes a 10% CHAPS-solution was added to the suspension to a final concentration of 1% CHAPS. After 2 h of stirring at 4°C, followed by another ultracentrifugation step, the supernatant represented the solubilized microsomes.

**Enzyme assays.** Toluene monooxygenase (TOMO) activity was measured in a screw capped vial of 5 ml with a Teflon Mininert valve preventing toluene evaporation. The optimized assay mixture (1 ml) consisted of 25 μM FAD, 25 μM FMN, an enzyme fraction, and 50 μM toluene, all in 0.1 M potassium phosphate buffer, pH 7.5. Before 100 μl of NADPH (10 mM) was added to start the reaction, the assay mixture was first incubated at 30°C for 7 min with a shaking speed of 200 rpm. The conversion of toluene was followed (30°C, 200 rpm) by injecting every 2 min 100 μl of headspace sample on a GC (HP 6890 series (Hewlett–Packard); column: 10% Silicone SE-30 (1.2 m) (Chrompack)). The column was operated at 110°C.

The substrate specificity of TOMO in the microsomes was examined by the standard enzyme assay in which the microsomes represent the enzyme fraction and toluene was replaced by the substrates tested.

The pH optimum was determined by using the following buffers in the standard assay mixture; sodium citrate (pH 4–5), Mes (pH 5–7), Hepes (pH 6.5–8.5), potassium phosphate (pH 6.6–8.3), and sodium pyrophosphate (pH 8–10). For determining the temperature optimum the standard assay was incubated at temperatures varying from 25 to 60°C. The optimal shaking speed during the reaction was determined by shaking the vial containing the assay mixture with 30, 90, 150, 210 or 270 rpm. The enzyme stability was followed by storing the enzyme at temperatures of –80, –20, 4, 21, and 30°C for 21 days.

A suitable detergent for solubilization of the microsomes was obtained by solubilizing the microsomal fraction with Triton X-100, Tween 20, CHAPS, cholate or deoxycholate. Therefore a 10%-detergent solution was added to the resuspended microsomes to obtain a final detergent concentration of 1% followed by stirring for 2 h at 4°C. Subsequently, the enzyme activity was measured.

The possibility of hydroperoxides to replace the requirement of CPR and NADPH for TOMO activity was checked by testing the following hydroperoxides: hydrogen peroxide, perchlorate, periodate, *t*-butylhydroperoxide, and cumenehydroperoxide. Concentrations varied from 0.1 to 10 mM and temperatures tested were 30 and 37°C.

NADPH-cytochrome *c* reductase activity was determined at 25°C by following the reduction of cytochrome *c* at 550 nm, using a Perkin–Elmer lambda-10 spectrophotometer [26]. The reaction mixture used for this purpose contained 0.1 M potassium phosphate buffer, pH 7.5, 5 μM FAD, 5 μM FMN, 1 mM KCN, an enzyme fraction, 0.05 mM cytochrome *c*, and 1 mM NADPH in a total volume of 1 ml. The activity was determined from the difference between the initial rate of cytochrome *c* reduction obtained with a complete reaction system and its control without NADPH. The amount of reduced cytochrome *c* was calculated by the use of an extinction coefficient at 550 nm of 21 mM<sup>-1</sup> cm<sup>-1</sup>.

**Protein determination.** Protein concentrations were determined by using the Bio-Rad detergent compatible protein assay with bovine serum albumin as a standard.

**Enzyme purification.** All purification steps for TOMO and CPR were performed at 4°C on a GradiFrac purification system from Pharmacia. During the purification of TOMO the eluate was always monitored at 405 nm. Solubilized microsomes were first loaded on a DEAE-cellulose column (1.6 × 35 cm) equilibrated with 20 mM Hepes buffer containing 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.25 mM PMSF, and 0.4% CHAPS, pH 7.2 (Buffer B). Proteins were eluted with 2 bedvolumes of a linear gradient of NaCl (0–1 M) in Buffer B, at a flow rate of 1 ml/min. Fractions exhibiting a P450 spectrum and a Type 1 substrate binding spectrum on addition of toluene were pooled and applied to a Q-Sepharose column (1.6 × 15 cm) also equilibrated with Buffer B. The adsorbed enzyme was eluted with 1 bedvolume of a linear NaCl gradient (0–1 M) in Buffer B, at a flow rate of 1 ml/min. Fractions exhibiting spectra as were observed after the previous purification-step were pooled and applied onto a hydroxyapatite column

(1 × 10 cm) equilibrated with Buffer C (Buffer B with 10 mM potassium phosphate instead of 20 mM Hepes). The enzyme was now eluted with 3 bedvolumes of a linear potassium phosphate gradient (10 mM–1 M), at a flow rate of 0.5 ml/min. Fractions exhibiting P450 spectra were pooled, concentrated, and stored at –80 °C.

The purification of CPR was modified compared to a previous method [27]. As described for the purification of TOMO again the DEAE-cellulose and Q-Sepharose column were applied. During these purification steps active fractions were pooled and subsequently applied to a 2',5'-ADP-Agarose column (1 × 5 cm) equilibrated with Buffer B. CPR was eluted with Buffer B containing 2 mM of 2'-AMP. Active fractions were pooled, concentrated, and stored at –80 °C.

**Gel electrophoresis.** The homogeneity and molecular mass of the enzymes were determined by SDS-PAGE on commercial gradient gels (10–15%, Pharmacia) using the Phast system equipment (Pharmacia). The gels were calibrated with low molecular mass protein standards from Pharmacia ( $\alpha$ -lactalbumin (14.4 kDa); trypsin inhibitor (20.1 kDa); carbonic anhydrase (30.0 kDa); ovalbumin (43.0 kDa); serum albumin (67.0 kDa); and phosphorylase *b* (94.0 kDa)).

**Spectrophotometric analysis.** UV/Vis-spectra were recorded in Quartzglass Suprasil cuvettes (chamber volume 160  $\mu$ l) on a Perkin-Elmer lambda-10 spectrophotometer at 20 °C. The presence and content of cytochrome P450 ( $\epsilon = 91 \text{ mM}^{-1} \text{ cm}^{-1}$ ) were determined by reduced carbon monoxide difference spectra as described previously [11]. For this method cuvettes as described for the UV/Vis spectra were used. Spectra were recorded on a SLM Aminco DW2000 spectrophotometer (American Instrument, Urbana). Substrate binding spectra were obtained by recording difference spectra between 350 and 500 nm with the same cuvettes and spectrophotometer. To eliminate interference from absorbance of toluene (dissolved in dimethylformamide) at these wavelengths, the sample cuvette contained the enzyme fraction and the reference cuvette buffer after which the baseline was recorded and toluene was added to both cuvettes.

**Amino acid sequence.** SDS-PAGE was performed with a nearly homogeneous TOMO fraction on a 12.5% (wt/vol) polyacrylamide gel. The band which corresponded to TOMO (based on its molecular mass) was cut out of the gel. The protein was eluted out of the gel and internally sequenced. Parts of internal sequences were compared with sequences in protein sequence databanks using the program FASTA3.

## Results

### Growth of *C. sphaerospermum* with toluene

A two-step cultivation procedure for *C. sphaerospermum* was performed to obtain sufficient biomass for experimentation. It included: (i) production of biomass by using glucose as growth substrate and (ii) induction of TOMO by adding toluene. Fig. 1 shows the production of CO<sub>2</sub> in time when the pre-grown fungal biomass was incubated with toluene. The shape of the curve indicates that TOMO activity was induced. Via a 20 L culture of *C. sphaerospermum*, 50 g of mycelium (squeezed cheesecloth) was harvested. In the cell free extract 1.7 g of protein was determined.

### Isolation and localization of TOMO

TOMO activity was detected both in the microsomal and cytosolic fractions after disruption of the mycelium. The specific and total enzyme activities in the microsomes were 20.5 nmol/min/mg and 6478 nmol/min,

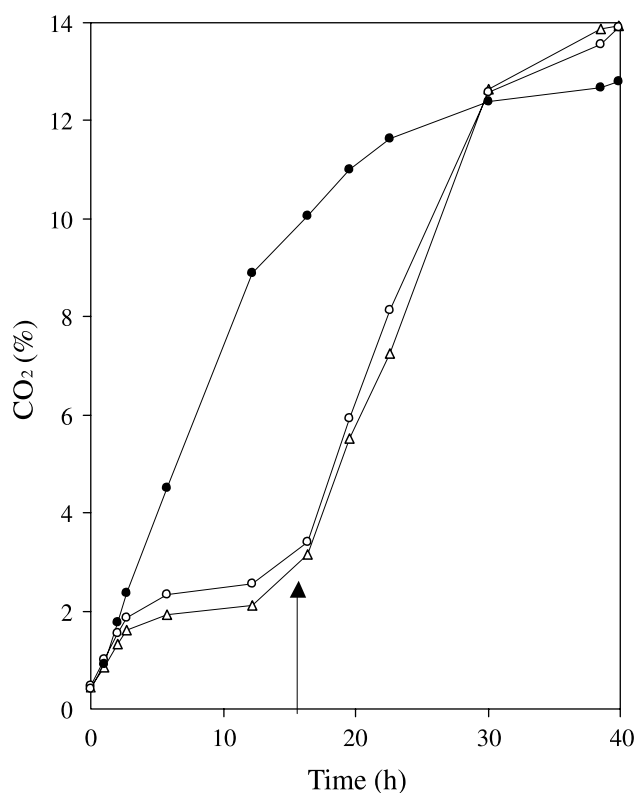


Fig. 1. Production of CO<sub>2</sub> during incubation of *C. sphaerospermum* with toluene. CO<sub>2</sub> production by glucose pre-grown *C. sphaerospermum* after induction by toluene dissolved in dibutylphthalate (●), in the presence of dibutylphthalate but without toluene (○), and in the absence of toluene and dibutylphthalate (△). In the latter two cases toluene dissolved in dibutylphthalate was added after 15 h.

respectively. Since these activities in the cytosolic fraction were 2.8 nmol/min/mg and 1210 nmol/min, respectively, it was concluded that the major toluene degrading activity was present in the microsomes.

### Enzyme assay and stability

The pH optimum of the microsomal TOMO assay was 7.5 (potassium phosphate buffer) whereas the temperature optimum was 35 °C. The shaking speed optimum was 200 rpm. The highest TOMO activity was measured in the presence of NADPH, O<sub>2</sub>, FAD, and FMN. The following TOMO activities were measured in the presence of different compounds: Standard, 100%; No NAD(P)H, 4%; NADPH (1 mM), 84%; NADH (1 mM), 16%; No O<sub>2</sub> (but N<sub>2</sub>), 16%; No FAD/FMN, 77%. Upon isolation, it was noted that the enzyme activity was quite labile. TOMO activity was retained for samples that were stored at very low temperatures (–80 °C) and in the presence of glycerol, EDTA, DTT, and PMSF. Storage either at temperatures higher than –20 °C, outside the pH range, 6.5–8.0, or in the absence of stabilizing compounds, caused inactivation of the enzyme within a few hours.

### Identification of TOMO and CPR

Microsomal TOMO activity was inhibited by CO, Metyrapone, N<sub>2</sub> or cytochrome *c*, and almost not inhibited by KCN. The following TOMO activities were measured in the presence of different inhibitors: Standard, 100%; CO (2.5%, v/v), 64%; CO (10%, v/v), 34%; Metyrapone (1 mM), 77%; Metyrapone (10 mM), 14%; cytochrome *c* (0.01 mM), 56%; cytochrome *c* (0.1 mM), and 32%; and KCN (1 mM), 92%. This indicated that TOMO is presumably a cytochrome P450 monooxygenase. This assumption was supported by the fact that optimal enzyme activity was measured in the presence of O<sub>2</sub> and NADPH instead of NADH as electron donor. These results also demonstrated the presence of CPR. The identification of TOMO as a P450 enzyme was established by measuring a reduced CO difference spectrum of CHAPS dissolved microsomes (Fig. 2A). In this spectrum a peak maximum was observed at 451 nm. A type 1 substrate binding spectrum was observed with a maximum at 386 nm and a minimum at 421 nm on addition of toluene (Fig. 2B). This means that toluene was bound to the P450 apoprotein causing a shift in the heme iron to a high spin state.

To add evidence for the P450 nature of TOMO, the spectral amount of P450 and TOMO activity were also measured in the isolated microsomes from fungal cells that were harvested after 0 (uninduced mycelium), 5, and 10 h of incubation with toluene. Normally, the fungal cells are harvested after 15 h of incubation with toluene to obtain a maximum amount of P450 and maximum TOMO activity (standard cultivation procedure, as described in the Materials and methods). The following TOMO activities were measured in the microsomes after the different incubation times: 0 h, 0%;

5 h, 36%; 10 h, 87%; and 15 h, 100%. With respect to the spectrally determined P450, the following amounts were measured in the microsomes after the different incubation times: 0 h, 0%; 5 h, 32%; 10 h, 84%; and 15 h, 100%. The proportional increase of the P450 amount and TOMO activity supports the P450 nature of TOMO.

### Purification of TOMO and CPR

The effect of different detergents for solubilization of the microsomes on TOMO activity was tested before the purification of TOMO and CPR was started. The following TOMO activities were measured in the presence of different detergents (final concentration 1%): No detergent, 100%; Triton X-100, 3%; Tween 20, 14%, CHAPS, 15%; cholate, 19%; and deoxycholate, 7%. All detergents caused a decrease in enzyme activity. Although a higher enzyme activity was retained in the presence of cholate, CHAPS was used for further purification of the enzymes because it is easily removed upon dialysis.

To simplify the purification of TOMO, several hydroperoxides were tested for an alternative enzyme assay. Neither hydrogen peroxide, perchlorate, periodate, *t*-butylhydroperoxide nor cumenehydroperoxide was able to replace the requirement for CPR and NADPH. TOMO activity was not observed in the presence of these hydroperoxides.

Tables 1 and 2 show the purification schemes for TOMO and CPR. The final preparations were purified 106- and 120-fold, respectively. Determination of the molecular masses of TOMO and CPR by SDS-PAGE yielded 58 and 78 kDa, respectively.

An internal peptide sequence, SQEEIDAVI, of TOMO showed similarity to published sequences of

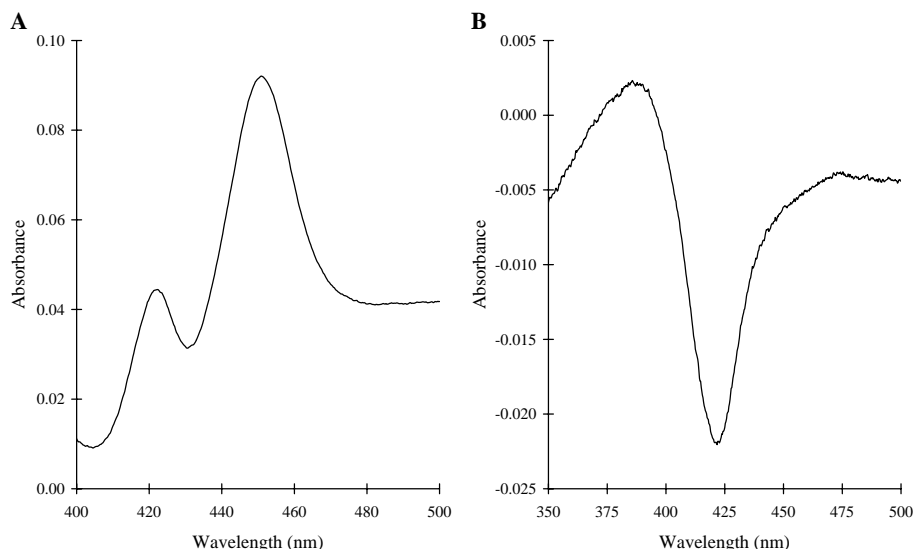


Fig. 2. (A) Reduced carbon monoxide difference spectrum of TOMO. (B) Substrate binding spectrum for TOMO and toluene.

Table 1  
Purification scheme of microsomal TOMO

Purification step	Total TOMO (nmol) <sup>a</sup>	Total protein (mg)	TOMO/total protein (%) <sup>b</sup>	Yield (%)	Purification (fold)
Microsomes	33	239	0.8	100	1
Solubilized microsomes	27	173	0.9	81	1.1
DEAE-cellulose	11	1.6	40	33	50
Q-Sepharose	9.4	0.8	67	28	84
Hydroxyapatite	3.4	0.2	85	10	106

<sup>a</sup> Determined by the spectroscopic method of Omura and Sato ( $\epsilon = 91 \text{ mM}^{-1} \text{ cm}^{-1}$ ) [11].

<sup>b</sup> (mg TOMO/mg total protein)  $\times$  100%. The enzyme purity was calculated with a molecular mass of 58 kDa for TOMO.

Table 2  
Purification scheme of microsomal CPR

Purification step	Total protein (mg)	Specific activity (nmol/min/mg)	Total activity ( $\mu\text{mol}/\text{min}$ )	Yield (%)	Purification (fold)
Cell-free extract	1664	66	110	100	1
Microsomes	239	179	43	39	2.7
Solubilized microsomes	173	241	42	38	3.7
DEAE-cellulose	38	714	27	25	10.8
Q-Sepharose	4.5	4198	19	17	63.6
ADP-Agarose	1.2	7911	9	8	119.9

several mammalian P450 enzymes, a soybean P450, and eukaryotic alkane-inducible P450 enzymes (similarity of 89%). However, this finding is not a good evidence for the P450 nature of TOMO since it is not one of the highly conserved amino acid sequences of P450 proteins.

#### Substrate specificity

The apparent  $K_m$  value for toluene, determined in the standard assay and calculated in a Lineweaver–Burk plot, was  $0.9 \mu\text{M}$ . Attempts to reconstitute the multienzyme complex of TOMO and CPR were not successful. Therefore, microsomes were used to study the substrate specificity of TOMO. Microsomal TOMO catalyzed the oxidation of several aromatic hydrocarbons (Table 3). It is clear that TOMO prefers alkylbenzenes. Ethylbenzene appeared to be a better substrate for TOMO than toluene. Remarkable is the preference for *m*-xylene in comparison with *o*-xylene and *p*-xylene. Benzene was

not converted significantly despite using a much larger amount of microsomes.

#### Discussion

In this report we describe the involvement of a microsomal cytochrome P450 from the toluene-degrading *C. sphaerospermum* in the initial oxidation of toluene. It was possible to identify TOMO as a P450 enzyme by first obtaining rather high amounts of protein produced by the fungus. By applying a two-step cultivation procedure a much higher amount of fungal biomass was obtained than by growing the fungus only on toluene [2]. Instead of a few milligram, 50 g of cells was harvested now. After optimizing the extraction of the microsomes, enzyme activity, and inhibition studies already indicated that TOMO was a cytochrome P450. This was established by spectral analysis of TOMO (Fig. 2) and a proportional increase of the spectral amount of P450 and TOMO activity in the microsomes during growth of the fungus in the presence of toluene.

Since cytochromes P450 are labile outside the cell, the isolation and purification of these enzymes are very complicated. As long as TOMO was present in the microsomal fraction, the enzyme was relatively stable although the presence of stabilizing compounds was necessary. The specific enzyme activity in the microsomes was determined to  $20.5 \text{ nmol}/\text{min}/\text{mg}$ . This value is somewhat higher than the specific enzyme activity observed for benzo[ $\alpha$ ]pyrene hydroxylase from the fungus *Pleurotus pulmonarius* [22] which was  $13.6 \text{ nmol}/\text{min}/\text{mg}$ . As soon as the microsomal fraction containing

Table 3  
Substrate specificity of microsomal multienzyme complex of TOMO and CPR

Substrate	Substrate concentration ( $\mu\text{M}$ )	Activity (%)
Toluene	50	100
Ethylbenzene	50	156
Propylbenzene	50	114
Styrene	50	75
<i>o</i> -Xylene	50	83
<i>m</i> -Xylene	50	117
<i>p</i> -Xylene	50	52
Benzene	50	1

TOMO was solubilized, TOMO activity decreased dramatically implying the importance of the membrane. Furthermore, the observation of a peak at 420 nm in the reduced carbon monoxide difference spectrum of the solubilized microsomes (Fig. 2A) revealed partial inactivation of TOMO. The fact that TOMO did not show any activity with hydroperoxides is unfortunate. Since these hydroperoxides make CPR unnecessary for the reaction it is a very useful tool for purifying P450 enzymes and in measuring kinetic parameters of P450s [28]. Concerning this aspect TOMO differs from other eukaryotic P450 enzymes although not all cytochromes P450 display activity with hydroperoxides. During purification attempts of TOMO, the enzyme did not bind to  $\omega$ -amino octyl-Sepharose. In this way TOMO also differs from other P450 enzymes because cytochromes P450 usually bind to this column material [29,30]. According to earlier results, the use of  $\omega$ -amino octyl-Sepharose proved to be even a very efficient purification step.

In spite of all difficulties described above, TOMO was eventually purified to near homogeneity by three chromatographic steps (Table 1). Because of the low yield and because reconstitution of TOMO activity with purified TOMO and CPR was not successful, the substrate specificity of TOMO was investigated by using the microsomal fraction (Table 3). The preference of TOMO for alkylated benzenes as substrates indicates an initial oxidation at the side chain. This is in agreement with earlier results [7]. Although *C. sphaerospermum* was not able to grow on xylene [3], TOMO catalyzed the oxidation of xylene. The soil fungus *Cladophialophora* sp. Strain T1 neither showed growth on xylene as single substrate but xylene was cometabolized when the fungus was grown on toluene or ethylbenzene [31]. The fact that TOMO showed a higher activity for *m*-xylene than for *o*-xylene and *p*-xylene indicates that the location of the second methyl-group is very important for activity.

On the basis of our results we conclude that TOMO is a membrane-bound cytochrome P450 coupled to its membrane-bound CPR. The presence of CPR was clearly demonstrated by the need for NADPH for optimal TOMO activity and the inhibition of TOMO activity by cytochrome *c*. Microsomal CPR was purified to electrophoretic homogeneity using three chromatographic steps as described in Table 2. The final specific enzyme activity was determined to be 7.9  $\mu\text{mol}/\text{min}/\text{mg}$ . This value is somewhat lower than the specific enzyme activities observed for purified mammalian reductases, but is comparable in its value to some other fungal reductases [21,32]. The lower CPR activity could be explained by possible loss of the flavine coenzymes during the purification procedure. In eukaryotic microsomes CPR is the major electron-donating enzyme. Since reconstitution of TOMO activity with purified TOMO and CPR was not successful it appears that CPR is not

the only electron-donating enzyme. For some cytochromes P450 NADH:cytochrome  $b_5$  oxidoreductase is also involved in electron donation. However, the use of this alternative system seems to be limited.

Cytochromes P450 are considered to be interesting enzymes to be used in environmental technology and as biocatalysts. Their applications, however, are hampered for several reasons. The fact is that P450 enzymes have, in general, low specific activities. This results in low conversion rates of an environmental pollutant and low production rates of the compound of interest in case of biocatalysis. Furthermore, as was shown in this report, cytochromes P450 prefer whole cell systems, as the enzymes are labile outside the cell, need electron donating enzymes, and need expensive NADPH for the reaction. The use of whole cell suspensions is no problem for bioremediation of environmental pollutants. In case of biocatalysis, however, cell suspensions may cause metabolization of the product of interest, that the mycelium may be depleted of reducing equivalents and that contaminating substances may be excreted in the medium, resulting in a more difficult downstream processing. All the problems described here may be overcome by introducing multiple copies of a cytochrome P450 and CPR into the organism, which results in higher specific activities [33]. This may also be accomplished by a covalent attachment of CPR to the P450 enzyme [34]. Moreover, in contrast to yields of several nanomole enzyme, heterologous expression in yeast or *Escherichia coli* can yield several hundred nanomole per liter of material. This means that further characterization of TOMO and CPR needs molecular biological approaches. In this way abundant protein can be obtained to perform successful reconstitution experiments and to investigate the structure, substrate specificity, and detailed spectral characteristics of the enzymes. Therefore, an important objective for future work remains the cloning and characterization of the genes encoding TOMO and CPR.

## Acknowledgments

Helpful discussions during this work with Dr. B.W. Faber are gratefully acknowledged. We would also like to thank M.G. Boersma and W.A.M. van den Berg for making available their ultracentrifuge and DW2000 spectrophotometer. This project was supported by the EC (BIO4 CT 972295).

## References

- [1] D. Muncnerova, J. Augustin, Fungal metabolism and detoxification of polycyclic aromatic hydrocarbons: a review, *Bioresour. Technol.* 48 (1994) 97–106.
- [2] F.J. Weber, K.C. Hage, J.A.M. de Bont, Growth of the fungus *Cladosporium sphaerospermum* with toluene as the sole carbon and energy source, *Appl. Environ. Microbiol.* 61 (1995) 3562–3566.

- [3] F.X. Prenafeta-Boldú, A. Kuhn, D.M.A.M. Luykx, H. Anke, J.W. van Groenestijn, J.A.M. de Bont, Isolation and characterization of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source, *Mycol. Res.* 105 (2001) 477–484.
- [4] M.J. Worsey, P.A. Williams, Metabolism of toluene and xylenes by *Pseudomonas putida* (arvilla) mt-2: Evidence for a new function of the TOL plasmid, *J. Bacteriol.* 124 (1975) 7–13.
- [5] M.S. Shields, S.O. Montgomery, S.M. Chapman, S.M. Cuskey, P.H. Pritchard, Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4, *Appl. Environ. Microbiol.* 55 (1989) 1624–1629.
- [6] G.M. Whited, D.T. Gibson, Separation and partial characterization of the enzymes of the toluene-4-monooxygenase catabolic pathway in *Pseudomonas mendocina* KR1, *J. Bacteriol.* 173 (1991) 3017–3020.
- [7] F.X. Prenafeta-Boldú, D.M.A.M. Luykx, J. Vervoort, J.A.M. de Bont, Fungal metabolism of toluene: monitoring of fluorinated analogs by <sup>19</sup>F nuclear magnetic resonance spectroscopy, *Appl. Environ. Microbiol.* 67 (2001) 1030–1034.
- [8] F. Gonzalez, The molecular biology of cytochrome P450s, *Pharmacol. Rev.* 40 (1988) 243–288.
- [9] M.T. Williams, Cytochrome P450. Mechanisms of action and clinical implications (Review), *J. Florida Med. Assoc.* 79 (1992) 405–408.
- [10] C. Von Wachenfeldt, E.F. Johnson, Structures of eukaryotic cytochrome P450 enzymes, in: P.R. Ortiz de Montellano (Ed.), *Cytochrome P450: Structure, Mechanism, and Biochemistry*, Plenum Press, New York, 1995, 183–223.
- [11] T. Omura, R. Sato, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature, *J. Biol. Chem.* 239 (1964) 2370–2378.
- [12] D. Ryan, A.Y.H. Lu, W. Levin, Purification of cytochrome P450 and P448 from rat liver microsomes, *Methods Enzymol.* 52 (1978) 117–123.
- [13] D.P. O'Keefe, P.A. Harder, Occurrence and biological function of cytochrome P450 monooxygenases in the actinomycetes, *Mol. Microbiol.* 5 (1991) 2099–2105.
- [14] S. Masaphy, D. Levanon, Y. Henis, K. Venkateswarlu, S.L. Kelly, Microsomal and cytosolic cytochrome P450 mediated benzo[*a*]pyrene hydroxylation in *Pleurotus pulmonarius*, *Biotechnol. Lett.* 17 (1995) 969–974.
- [15] D.L. Zhang, Y. Yang, J.E.A. Leakey, C.E. Cerniglia, Phase I and phase II enzymes produced by *Cunninghamella elegans* for the metabolism of xenobiotics, *FEMS Microbiol. Lett.* 138 (1996) 221–226.
- [16] K.E. Smith, F. Ahmed, T. Antoniou, Microbial transformations of steroids, *Biochem. Soc. Trans.* 21 (1993) 1077–1080.
- [17] H. VandenBossche, in: D. Berg, M. Plempel (Eds.), *Sterol Biosynthesis Inhibitors, Pharmaceutical and Agrochemical Aspects*, Ellis Horwood Ltd., Chichester, UK, 1988, pp. 79–119.
- [18] H. Honeck, W.H. Schunck, H.G. Muller, The function of cytochrome P-450 in fungi and prospects of application, *Pharmazie* 40 (1985) 221–227.
- [19] D.G. Kellner, S.A. Maves, S.G. Sligar, Engineering cytochrome P450s for bioremediation, *Curr. Opin. Biotechnol.* 8 (1997) 274–278.
- [20] C.H. Williams, H. Kamin, *J. Biol. Chem.* 237 (1962) 587–595.
- [21] B.W. Faber, R.F.M. van Gorcom, J.A. Duine, Purification and characterization of benzoate-para-hydroxylase, a cytochrome p450 (CYP53A1), from *Aspergillus niger*, *Arch. Biochem. Biophys.* 394 (2) (2001) 245–254.
- [22] S. Masaphy, D.C. Lamb, S.L. Kelly, Purification and characterization of a benzo[*a*]pyrene hydroxylase from *Pleurotus pulmonarius*, *Biochem. Biophys. Res. Commun.* 266 (1999) 326–329.
- [23] H. Shoun, T. Tanimoto, Denitrification by the fungus *Fusarium oxysporum* and involvement of cytochrome P450 in the respiratory nitrite reduction, *J. Biol. Chem.* 266 (1991) 11078–11082.
- [24] Y. Yang, D. Zhang, C.E. Cerniglia, Purification and characterization of a cytosolic cytochrome P450 from the yeast *Trichosporon cutaneum*, *FEMS Microbiol. Lett.* 154 (1997) 347–353.
- [25] U.M. Stündl, D. Patzak, F. Schauer, Purification of a soluble cytochrome P450 from *Trichosporon montevidense*, *J. Basic Microbiol.* 40 (2000) 289–292.
- [26] T. Omura, S. Takesue, A new method for simultaneous purification of cytochrome b<sub>5</sub> and NADPH-cytochrome c reductase from rat liver microsomes, *J. Biochem.* 67 (1970) 249–257.
- [27] Y. Yasukochi, B.S.S. Masters, Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography, *J. Biol. Chem.* 251 (1976) 5337–5344.
- [28] E.G. Hrycay, J.A. Gustafsson, M. Ingelman-Sundberg, L. Ernster, Sodium periodate, sodium chlorite, and organic hydroperoxides as hydroxylating agents in hepatic microsomal steroid hydroxylation reactions catalyzed by cytochrome P-450, *FEBS Lett.* 56 (1975) 161–165.
- [29] T. Shimada, K.S. Misono, F.P. Guengerich, Human liver microsomal cytochrome P-450 mephenytoin 4-hydroxylase, a prototype of genetic polymorphism in oxidative drug metabolism, purification and characterization of two similar forms involved in the reaction, *J. Biol. Chem.* 261 (1986) 909–921.
- [30] U. Scheller, T. Zimmer, E. Kärge, W. Schunck, Characterization of the n-alkane and fatty acid hydroxylating cytochrome P450 forms 52A3 and 52A4, *Arch. Biochem. Biophys.* 328 (1996) 245–254.
- [31] F.X. Prenafeta-Boldú, J. Vervoort, J.T.C. Grotenhuis, J.W. van Groenestijn, Substrate interactions during the biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX) hydrocarbons by the fungus *Cladophialophora* sp. Strain T1, *Appl. Environ. Microbiol.* 68 (2002) 2660–2665.
- [32] T. Makovec, K. Breskvar, Purification and characterization of NADPH-cytochrome P450 reductase from filamentous fungus *Rhizopus nigricans*, *Arch. Biochem. Biophys.* 357 (1998) 310–316.
- [33] J.M. van den Brink, C.A.M.J.J. van den Hondel, R.F.M. van Gorcom, Optimization of the benzoate-inducible benzoate p-hydroxylase cytochrome P450 enzyme system in *Aspergillus niger*, *Appl. Microbiol. Biotechnol.* 46 (1996) 360–364.
- [34] T. Kitazume, A. Tanaka, N. Takaya, A. Nakamura, S. Matsuyama, T. Suzuki, H. Shoun, Kinetic analysis of hydroxylation of saturated fatty acids by recombinant P450foxy produced by an *Escherichia coli* expression system, *Eur. J. Biochem.* 269 (8) (2002) 2075–2082.