Appl Microbiol Biotechnol (2006) 72: 323–329
DOI 10.1007/s00253-005-0264-7

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Heterologous expression of the benzoate *para*-hydroxylase encoding gene (*CYP53B1*) from *Rhodotorula minuta* by *Yarrowia lipolytica*

Received: 19 September 2005 / Revised: 14 November 2005 / Accepted: 16 November 2005 / Published online: 10 January 2006

Abstract There is currently an increasing number of cytochrome P450 (CYP450) monooxygenase encoding genes becoming available from various genome-sequencing projects. These enzymes require association with cytochrome P450 reductase (CPR) to achieve optimal activities. In this study, the *CYP53B1* gene, which encodes a benzoate *para*-hydroxylase, was successfully cloned from *Rhodotorula minuta* and overexpressed in *Yarrowia lipolytica* E150. Multiple copies of the *CYP53B1* cDNA were cloned under the *POX2* promoter, while the *Y. lipolytica CPR* was cloned under the isocitrate lyase promoter. Whole cell biotransformation of benzoic acid to *para*-hydroxybenzoic acid (pHBA) was used to analyse the hydroxylase activity of the recombinant *Y. lipolytica* UOFS Y-2366. Different induction conditions were tested in shake flask cultures. The highest concentration of pHBA produced by UOFS Y-2366 was 1.6 g l^{-1} after 200 h when stearic acid was repeatedly added to the media. *R. minuta* accumulated up to 1.8 g l^{-1} of pHBA within only 24 h. Thus, the specific hydroxylase activity of *Y. lipolytica* UOFS Y-2366 [approximately 0.07 U (g dry wt.)$^{-1}$] was about 30 times lower than the specific hydroxylase activity of *R. minuta* [2.62 U (g dry wt.)$^{-1}$]. However, the hydroxylation activity obtained with *Y. lipolytica* was one of the highest hydroxylation activities thus far reported for whole cell biotransformation studies carried out with yeasts expressing foreign CYP450s.

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Introduction

The cytochrome P450 (CYP450) protein family comprises a superfamily of ubiquitous heme-thiolate proteins that incorporate oxygen into a wide range of substrates (Werck-Reichhart and Feyereinsen 2000). These monooxygenases contribute to vital processes in the cell, such as carbon source assimilation in microorganisms, biosynthesis of hormones and detoxification of xenobiotics in animals and plants. In prokaryotes, the CYP450s are soluble proteins, while in eukaryotes, they are usually bound to the endoplasmic reticulum or inner mitochondrial membranes (Werck-Reichhart and Feyereinsen 2000).

In most cases, a separate reduced nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 reductase (CPR) transfers electrons from NADPH to the CYP450 (Werck-Reichhart and Feyereinsen 2000). Heterologous expression of CYP450s in foreign hosts thus usually requires the coexpression of a compatible CPR. In order for the heterologous expression system to be functional, the P450 and CPR should be in the correct ratio (Dong and Porter 1996; Backes and Kelley 2003).

Yarrowia lipolytica is a non-conventional yeast that has also been explored as an alternative host for heterologous expression of CYP450s (Fickers et al. 2005). Functional expression of cDNAs encoding two mammalian CYP450s, namely bovine P450 17α (*CYP17A* cDNA; Juretzek et al. 2000a) and human P450 1A1 (*CYP1A1* cDNA; Nthangeni et al. 2004), has been achieved in recent years. The highest activities were observed when multiple copies of *CYP1A1* were coexpressed with the *Y. lipolytica* NADPH-P450 reductase (*YlCPR*). Multiple copies of *CYP* genes were introduced by using vectors that rely on the zeta sequences of the retrotransposon Ylt1 to accomplish non-homologous integration into the genomes of *Y. lipolytica* strains H222 and Po1d as well as a defective *ura3d4* marker to ensure multiple integrations (Fickers et al. 2005). In the first study, both genes were under control of the strong *Y. lipolytica* isocitrate lyase (*ICL*) promoter (*pICL*), while in the second study, the *CYP450* gene was under control of the *Y. lipolytica* acyl-CoA oxidase 2 (*POX2*) promoter (*pPOX2*)

while both *pICL* and *pPOX2* were tested for controlling the *YlCPR* gene. *pICL* is induced by acetate, ethanol and fatty acids, while *pPOX2* is induced by alkanes and fatty acids (Juretzek et al. 2000b; Pignède et al. 2000). In the first study, diploid strains containing multiple cassettes for both *CYP17A* and *YlCPR* were constructed from haploid multicopy transformants (Juretzek et al. 2000a). In the second study, *CYP1A1* and *YlCPR* were integrated into the genome of strain Po1d independently of one another (Nthangeni et al. 2004). The JMP62 vector with the non-defective *ura3d1* marker was used for single integration, and the JMP64 vector with the defective *ura3d4* marker for multicopy integration (Juretzek et al. 2001) of *CYP1A1*, while the JMP 21 vector with the selective *LEU2* marker was used for integration of one or two copies of *YlCPR*.

There is currently an increasing number of CYP450 encoding genes, particularly from plants and fungi, becoming available from various genome sequencing projects. The functions and substrate specificities of these CYP450s are in most cases not known. We are interested in the expression of other fungal CYP450s in *Y. lipolytica*. The *CYP53 B1* gene of *Rhodotorula minuta*, a basidiomycetous yeast, encodes a benzoate *para*-hydroxylase (Fujii et al. 1997). In this study, the JMP64 vector (Nthangeni et al. 2004) was used for the cloning of *CYP53B1* cDNA under *pPOX2* control and its insertion in multiple copies into *Y. lipolytica* E150, a strain containing zeta sequences for homologous recombination (Juretzek et al. 2001). The JMP21 vector (Nthangeni et al. 2004) was used to introduce an additional copy of the *YlCPR* gene under control of *pICL*. Different induction conditions were tested for optimal biotransformation of benzoic acid (BA) to *para*-hydroxy benzoic acid (pHBA) in shake flask cultures.

Materials and methods

Cloning of *CYP53B1* cDNA into *Y. lipolytica* E150

Strains, vectors and media

R. minuta CBS 2177 was obtained from the MIRCEN Yeast Culture Collection of the University of the Free State (UFS), South Africa. *Y. lipolytica* strain E150 (*MatB*, *his1*, *ura3-302*, *leu2-270*, *xpr2-322*, *XPR2^{P}::SUC2*) and the expression vectors JMP64 and JMP21-pICL-CPR were obtained from the Laboratoire Microbiologie et Génétique Moléculaire, Institute National Agronomique Paris-Grignon, France. *Y. lipolytica* UOFS Y-2366 was deposited in the MIRCEN Yeast Culture Collection of the UFS.

Y. lipolytica was grown in YPD (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} glucose). Minimal media YNBD [1.7 g l^{-1} YNB Base (Difco), 4 g l^{-1} NH_4Cl, 10 g l^{-1} glucose, 50 mM phosphate buffer] and YNBcasa (YNBD supplemented with 2 g l^{-1} casamino acid) were used as transformation selection media (biotransformation media are described later).

Escherichia coli TOP 10 (Invitrogen) was used for plasmid preparations. Bacterial cells were grown in Luria–Bertani media supplemented with ampicillin (100 μg ml^{-1}) or kanamycin (50 μg ml^{-1}). The cloning of polymerase chain reaction (PCR) products was performed using pGEM-T Easy vector (Promega).

Isolation of total RNA from R. minuta grown in chemically defined medium supplemented with l-phenylalanine

R. minuta CBS 2177 was pre-grown in YPD broth for 16 h. The cells were washed with physiological solution (9 g l^{-1} NaCl) and transferred to chemically defined (CD) medium (Fujii et al. 1987). The CD medium contained (per litre of deionized water) 20 g glucose, 5 g $(NH_4)_2SO_4$, 5.74 g KH_2PO_4, 9.26 g Na_2HPO_4, 1 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.H_2O$, 0.1 g NaCl, 0.5 mg *p*-aminobenzoic acid, 0.5 g thiamine–HCl, 50 mg Na-citrate, 30 mg $MnCl_2.4H_2O$, 20 mg $ZnCl_2$, 20 mg $FeCl_2.6H_2O$, 2 mg $CuSO_4.5H_2O$, 2 mg $CoCl_2.6H_2O$, 1 mg $Na_2MoO_4.2H_2O$ and 1 mg $K_2B_4O_7.xH_2O$, pH 7. For CYP53 induction, glucose was replaced with L-phenylalanine (1 g l^{-1}).

Total RNA was isolated using TRIzol (Invitrogen). RNA samples were dried under vacuum and dissolved in formamide (50 μl).

RT-PCR amplification of CYP53B1 cDNA from RNA isolated from R. minuta grown in CD medium supplemented with l-phenylalanine

Primers were designed to amplify the full-length coding sequence of the *CYP53B1* gene. The primers used were MbF 5′ggcctaggATGGGCATAGTCCAAGAAG3′ and MbR 5′ggcctaggTAGGCATCAATGGATCTG3′. The sequences in lower case letters contain the *Avr*II recognition sites to facilitate cloning into the JMP vectors.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the Access RT-PCR Introductory System (Promega). The following cycle profiles were used: 48°C for 45 min, 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 59°C for 1 min and 68°C for 1 min, with a final step at 68°C for 7 min.

The PCR product was gel purified and ligated into a pGEM-T Easy vector. The plasmid DNA was digested with *Avr*II, and the released insert was ligated into the JMP64 vector that had been digested with *Avr*II (Biolabs) and dephosphorylated with alkaline phosphatase (Promega).

Transformation of Y. lipolytica with vectors containing cDNA

Competent cells of *Y. lipolytica* were prepared as described by Barth and Gaillardin (1996). Vector DNA (JMP64; 5 μg)

containing the *CYP53B1* cDNA insert was linearized with *Not*I (Roche) and transformed into strain E150. Clones that showed the highest benzoate *para*-hydroxylase activity were also transformed with the JMP21-pICL-CPR vector (Nthangeni et al. 2004).

Southern hybridization

Southern hybridization was performed using standard protocols (Sambrook and Russel 2001) and supplier's instructions (Roche). Total genomic DNA (5 μg) isolated from strains transformed with JMP64 *CYP53B1* was double-digested overnight with *Pst*I and *Xba*I (Roche) at 37°C. The digested DNA was electrophoresed on 1% agarose gel, blotted onto 0.22-μm nylon membrane (Separations) and probed using the digoxigenin-labeled 1.5 kb RT-PCR product from *CYP53B1*. Hybridization was performed at 65°C for 24 h. High stringency post-hybridization washes were performed as follows: 2×5 min in 2×SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature, followed by washing 2×15 min in 0.1% SSC, 0.1% SDS at 65°C.

Screening transformants for benzoate para-hydroxylase activity

Transformants were streaked four times on YPD agar. The recombinant cells were pre-cultured in YPD broth (10 ml) in shake flasks (100 ml) and incubated at 28°C with shaking for 24 h. YPS medium [10 g l^{-1} yeast extract, 20 g l^{-1} peptone and 20 g l^{-1} stearic acid (SA)] (5 ml in 100-ml flasks) was inoculated with pre-cultures (500 μl) and incubated at 28°C for 24 h before BA (2 g l^{-1}) was added. Samples (200 μl) were taken at intervals. The samples were acidified to pH 3 with 3% (v/v) formic acid (100 μl) and extracted with ethyl acetate (200 μl). Organic layer was transferred to a new tube and dried under vacuum. Dried samples were resuspended in ethyl acetate (50 μl), and aliquots (10 μl) were spotted on Alugram Sil G/UV_{245} thin layer chromatography (TLC) plates (Machery-Nagel). Plates were developed in the top layer of a mixture of di-*n*-butyl ether (Merck), formic acid (Merck) and H_2O (90:7:3). Dried plates were viewed under short wavelength ultraviolet (UV) light.

Optimization of hydroxylase activity in shake flasks

Turbidimetric measurements

Culture samples (500 μl) in 1.5 ml microcentrifuge tubes were vortexed for 5 min and centrifuged at 10 000×*g* for 10 min. Supernatants were discarded, and the pellets washed and resuspended in physiological saline (500 μl). Turbidity of samples (200 μl), suitably diluted before transfer to a microtitre plate, was measured at 620 nm using a Labsystems iEMS microtitre plate reader MF (Thermo BioAnalysis Company, Helsinki, Finland).

Dry weight measurements

Cyclohexane (2 ml) and 5 M NaOH (400 μl) were added to 4 ml broth samples in test tubes, vortexed for 5 min and then filtered under vacuum through preweighed glass fibre filters (GF52 47MMBX200; Schuell). The biomass on the filter was washed with a mixture of distilled water (4 ml), cyclohexane (2 ml) and 5 M NaOH (400 μl) followed by washing with 26 ml of distilled water. Filters were oven-dried (110°C) and cooled in a desiccator.

Biotransformation experiments

For the biotransformation experiments, precultures were grown in YPD broth until an OD_{620} of ~5–10 was reached. The main cultures (50 ml YPD broth buffered with 50 mM phosphate buffer pH 8 in 500-ml Erlenmeyer flasks) were inoculated to a final OD_{620} of 0.3. The cultures were incubated on a rotary shaker at 180 rpm and 25°C.

Stock solutions of SA, oleic acid (OA), BA and pHBA (100 g l^{-1}supplemented with 1 ml l^{-1} Tween 80) were prepared by adjusting the pH and by continuously stirring until the substrates either dissolved or formed homogenous suspensions. Olive oil and ethanol were added directly.

Inducing substrates (20 g l^{-1}) were added after 36 h of growth followed by BA (5 g l^{-1}) 18 h later (see graphs for details). Product inhibition was tested by adding pHBA (0.5 g l^{-1}) 5 h prior to substrate addition. In the case of *R. minuta*, BA (5 g l^{-1}) was added after 36 h of growth.

Extraction and analysis

Samples (500 μl) were taken at intervals, acidified to pH 3 with 3 M HCl (50 μl) and extracted twice with *tert*-butylmethyl ether (Fluka) (300 μl) containing myristic acid (1 g l^{-1}; The British Drug Houses) as internal standard.

Extracts (50 μl) were methylated with trimethylsulfonium hydroxide (50 μl) before gas chromatographic (GC) analysis. Concentrations were determined from predetermined standard curves. GC analysis was carried out using a Hewlett-Packard 5890 series II gas chromatograph with a Chrompack CP wax polar column, 30 m×0.53 mm×1 μm. The GC conditions were H_2 as carrier gas at 5 ml min^{-1}, split ratio 1:40. Oven was initially at 120°C for 5 min, increased at 10°C min^{-1} to 300°C, held for 7 min, Flame Ionization Detector 350°C.

Glucose analysis was done on a Water Breeze HPLC with Differential Refractive Index Detector; Waters SUGARPACK I column, 300×7.8 mm at 84°C; mobile phase deionized water at 84°C and 0.5 ml min^{-1}; sample volumes 20 μl.

Results

RT-PCR was performed to amplify a cDNA fragment of 1.5 kb from mRNA isolated from *R. minuta* CBS 2177

grown in CD medium supplemented with L-phenylalanine as only carbon source. Sequencing confirmed that the amplified product was *CYP53B1* cDNA (Fujii et al. 1997). The cDNA was cloned into *Y. lipolytica* E150 by using the shuttle vector JMP64 (Nthangeni et al. 2004). Transformants appeared on minimal medium without uracil after 10 days. Transformants were streaked four times on a rich medium (YPD) to stabilise, since transformants with multiple integrations are unstable after growth on minimal media (Pignède et al. 2000).

To test for benzoate *para*-hydroxylase activity, ten clones were grown in YP broth supplemented with SA, and BA was added after 24 h. The transformants produced within 24 h sufficient product to detect with TLC analysis (Fig. 1a). The level and nature of *CYP53B1* integration into *Y. lipolytica* were compared by Southern hybridization. A major band with a size of approximately 3.5 kb was observed for all the clones except for clone 4 (Fig. 2). This common major band is an indication that the integration was in tandem, since the zeta sequences are tandemly arranged in the genome of *Y. lipolytica* E150 (Juretzek et al. 2001). The intensity of this major band correlated with the biotransformation results (Fig. 1a). The band observed for clones 3, 7 and 8 had the highest intensity, and these clones also gave the highest concentration of pHBA in the bioconversion experiments. In some clones, several other bands were also observed (Fig. 2). This is an indication that *CYP53B1* was also integrated in other places in the genome.

Since previous studies had shown that additional copies of *YlCPR* are necessary for the optimal activity of heterologously expressed CYP450s, clones 3, 7 and 8 were subsequently transformed with JMP21-pICL-YlCPR

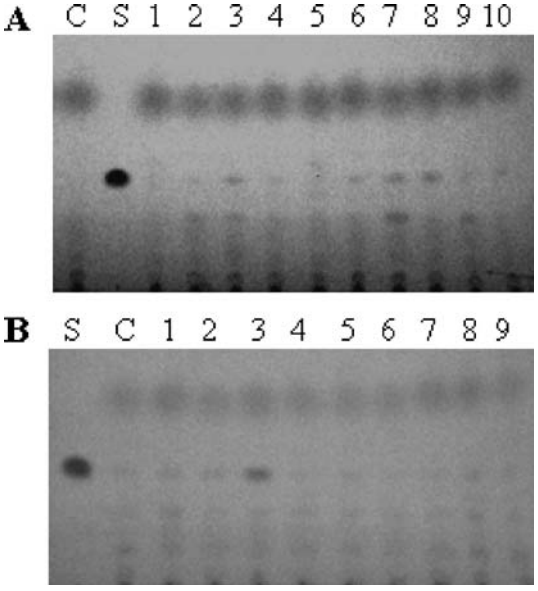

Fig. 1 Biotransformation of BA by *Y. lipolytica* E150 strains. **a** E150 transformed with JMP64-CYP53B1. **b** Clone 7 from **a** transformed with JMP21-pICL-YlCPR. Lanes: *S para*-hydroxybenzoic acid, *C* clone transformed with void vector, *1–10* different transformants. Samples were taken 24 (**a**) and 5 h (**b**) after addition of BA. The samples were extracted with ethyl acetate, dried, resuspended in ethyl acetate and spotted on TLC plates

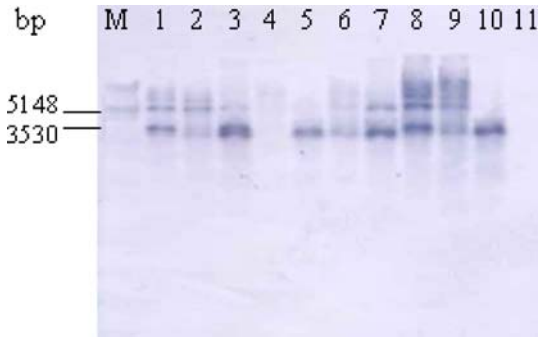

Fig. 2 Southern blot analysis of transformants obtained after transformation of *Y. lipolytica* E150 with JMP64-CYP53B1. DNA was double-digested with *Xba*I and *Pst*I and probed with *CYP53B1* cDNA. Lanes: *1–10* clones 1–10, *11* E150 transformed with void vector, *M* λDNA digested with *Hin*dIII/*Eco*RI. Hybridization was performed at 65°C, and all washes were of high stringency

(Nthangeni et al. 2004) containing *YlCPR* under control of *pICL*. Transformation was only successful with clone 7. The clones that were transformed with JMP21-pICL-YlCPR showed a significant improvement in the benzoate *para*-hydroxylase activity (Fig. 1b). The product was detected within 5 h after the addition of BA. Clone 3, subsequently labeled UOFS Y-2366, showed the highest activity and was used for further biotransformation experiments. Nthangeni et al. (2004) also observed that, occasionally, clones that were with transformed with JMP21-pICL-YlCPR or JMP21-pPOX2-YlCPR had double the activity of the majority of clones and showed that these clones had two additional copies of the cloned *YlCPR*.

When biotransformations were carried out in the conditions used for identification of the best transformants and extracts were subjected to GC analysis, product formation leveled off after 72 h, and concentrations never exceeded 0.5 g l^{-1}. To improve pHBA production, we tested several induction conditions (Fig. 3). Nthangeni et al. (2004) used YPDH medium containing both glucose (10 g l^{-1}) and olive oil (2.5% v/v) to monitor induction of CYP1A1 activity driven by *pPOX2*. Therefore, UOFS Y-2366 was subsequently grown in YPD broth with olive oil added after 36 h and BA 18 h later. Production of pHBA started to level off within 50 h after BA addition, and production stopped within 96 h. The pHBA concentration then remained constant at approximately 0.4 g l^{-1} for more than 100 h (Fig. 3a). Similar results were obtained when OA was used as inducer (Fig. 3b).

The possibility of product inhibition, which has been reported to be a problem with P450-based hydroxylations (Duetz et al. 2001), was investigated by adding pHBA (0.5 g l^{-1}) to an OA-induced culture 5 h prior to BA addition. The amounts of additional pHBA formed were similar to those formed by control cultures to which no pHBA had been added (Fig. 3b,c). Product inhibition was thus not the reason why hydroxylation activities could not be maintained.

Differences in optical density (OD) measurements of 36-h samples can be ascribed to the fact that samples were taken shortly after the addition of the inducers, which

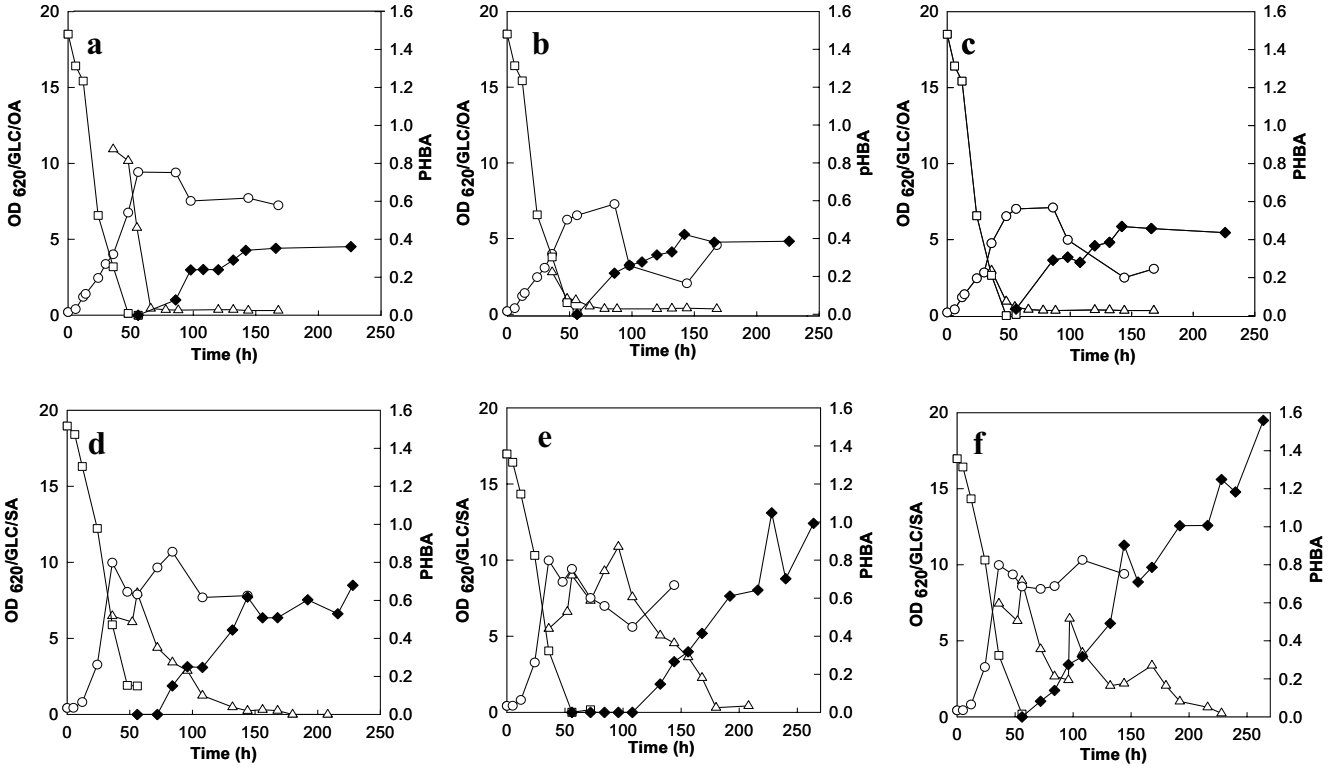

Fig. 3 Biotransformation of BA (0.5% w/v added after 54 h) by *Y. lipolytica* UOFS Y-2366 grown in YPD broth with different additions made after 36 h. **a** 20 ml l^{-1} olive oil. **b** 20 g l^{-1} OA. **c** 20 g l^{-1} OA and 0.5 g l^{-1} pHBA added after 49 h. **d** 20 g l^{-1} SA. **e** 20 g l^{-1} SA and 20 ml l^{-1} ethanol. **f** 20 g l^{-1} SA added followed by 5 g l^{-1} every 56 h. Glc-glucose (g l^{-1}; *open squares*), OD_{620nm} (*open circles*), fatty acid (g l^{-1}; *open triangles*), pHBA (g l^{-1}; *closed diamonds*). Note that in **c** only additional pHBA formed is shown, not total pHBA

affected pelleting of the samples. Dry weights of samples taken immediately before the addition of inducers were approximately 7 g l^{-1}. After 56 h of growth (i.e. 20 h after addition of inducers), dry biomass was 11 g l^{-1} for cultures that had received olive oil and 9 g l^{-1} for samples that had received OA. Although the concentration of the inducers added was 20 g l^{-1}, only 11 g l^{-1} OA was recovered immediately after olive oil addition and less than 3 g l^{-1} after OA addition. This can probably be ascribed to a combination of poor mixing/sampling (an unavoidable problem with such hydrophobic substrates) and rapid accumulation of OA into the cells (Mlickova et al. 2004). Within 12 h after OA addition, the OA concentration had dropped to 1 g l^{-1}.

In these three experiments, an average volumetric production rate of approximately 6 mg l^{-1} h^{-1} was obtained during the first 42 h after substrate addition. The hydroxylation rate was apparently not constant, but rapid during the first 30 h and slower later. This may be due to the fact that the inducer (OA) was consumed so rapidly. We therefore tested SA as an inducer (Fig. 3d–f). SA as inducer increased volumetric hydroxylation activity to 7 mg l^{-1} h^{-1} (Fig. 3d). This hydroxylation activity seemed to be more constant and appeared to correlate with the slower utilization of SA, which is a solid and is much less soluble than OA. Indeed, bioconversion stopped 88 h after SA addition, when there was no more SA in the media. Addition of a cosubstrate, ethanol, together with SA delayed the onset of the hydroxylation activity by 36 h, probably until all ethanol had been utilized (Fig. 3e). The level of hydroxylation activity (7 mg l^{-1} h^{-1}) remained the same as with SA alone. When SA was added repeatedly, the same hydroxylation activity (7 mg l^{-1} h^{-1}) was maintained for 200 h, and a final product concentration of almost 1.6 g l^{-1} (32% conversion) was reached (Fig. 3f).

When SA was added as inducer, the dry biomass was approximately 20 g l^{-1} after 56 h of growth (i.e. 20 h after addition of inducers). A volumetric activity of 7 mg l^{-1} h^{-1} then translates into a specific activity of 0.04 U (g dry wt.)$^{-1}$ (where 1 U is defined as 1 μmol of product formed per minute). In the case of the cultures induced with olive oil and OA, less dry biomass was obtained so that specific activities were higher, i.e. 0.07 and 0.08 U (g dry wt.)$^{-1}$, respectively. It is possible that the dry biomass was overestimated and specific activity thus underestimated in the case of cultures to which SA had been added, because SA is very insoluble and very difficult to wash from the filters despite washing the filters with cyclohexane and 0.5 M NaOH.

When the hydroxylation of BA by the recombinant *Y. lipolytica* UOFS Y-2366 was compared with the biotransformation by the wild-type *R. minuta* (Fig. 4), it was evident that the wild-type organism performs much better, although we had used strong promoters and a system which introduced multiple copies of *CYP53B1*. Although pHBA was completely degraded by *R. minuta*, it still accumulated within 24 h up to 1.8 g l^{-1} (13 mM) pHBA, which was

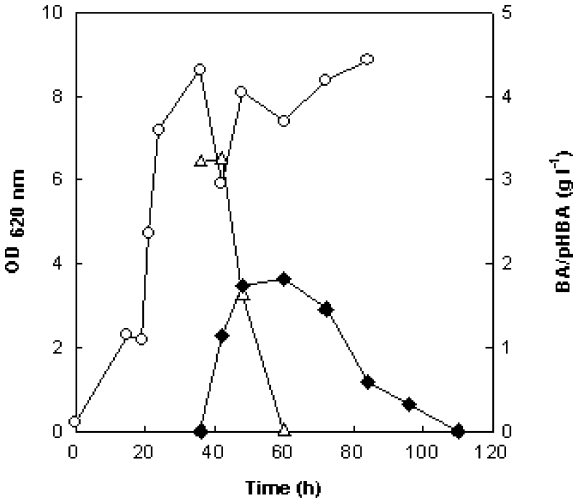

Fig. 4 Biotransformation of BA using *R. minuta* grown on YPD with 5 g l^{-1} BA added after 36 h. OD_{620nm} (*open circles*), BA (*open triangles*), pHBA (*closed diamonds*)

more than the maximum concentration of 1.6 g l^{-1} (11 mM) accumulated by *Y. lipolytica* UOFS Y-2366 after 214 h. If the sum of the rates of pHBA accumulation (144 mg l^{-1} h^{-1}) and pHBA consumption (36 mg l^{-1} h^{-1}) is taken as a measure of hydroxylase activity, the benzoate *para*-hydroxylase activity in *R. minuta* was in the order of 178 mg l^{-1} h^{-1}. Dry biomass of *R. minuta* at the time of BA addition (36 h of growth) was 8.2 g l^{-1}, thus giving a specific hydroxylase activity of 2.62 U (g dry wt.)$^{-1}$. The maximum specific hydroxylation activities of *Y. lipolytica* UOFS Y-2366 [0.07–0.08 U (g dry wt.)$^{-1}$] were thus at least 30 times lower than the specific hydroxylase activity of the wild-type *R. minuta*.

Discussion

Although comparison with the wild-type strain made the results obtained with *Y. lipolytica* UOFS Y-2366 seem disappointing, it should be kept in mind that the heterologous expression of bacterial hydroxylases in *E. coli* often resulted in lower hydroxylase activities than those observed with the wild-type bacteria from which the genes had been cloned (Duetz et al. 2001). The hydroxylation activity obtained with *Y. lipolytica* UOFS Y-2366 was higher than the hydroxylation activities [1.3–1.8 μU (g dry wt.)$^{-1}$] previously reported by Nthangeni et al. (2004) for the biotransformation of ethoxyresorufin by *Y. lipolytica* Po1d overexpressing human *CYP1A1* together with *YlipCPR*. It should be noted that these rates were obtained under analytical activity assay conditions (1-ml reactions were carried out in UV cuvettes). Attempts to use these strains for larger-scale biotransformations of acetanilide, an alternative cheaper substrate of CYP1A1 (Liu et al. 1998), were unsuccessful, because even control strains degraded acetanilide (Obiero, unpublished results).

The heterologous expression of eukaryotic CYP450s in *Y. lipolytica* is giving promising results, but questions such as why it was necessary to continuously add SA and why activities are lower than in the wild-type organism still remain. A possible explanation for the lower activity might be that the cloned CYP450 has to compete with endogenous P450s for the available CPR. It has been shown that a fatty acid (myristic acid) induces at least 4 of the 12 *CYP52* genes present in *Y. lipolytica* (Iida et al. 2000; Fickers et al. 2005). Therefore, it will probably be better to use another promoter such as the *ICL* promoter, rather than the *POX2* promoter, to drive expression of cloned *CYP* genes.

Expression of *CYP53* genes is a useful tool for studying fundamental questions relating to CYP450 expression in *Y. lipolytica* and for optimization of whole cell hydroxylations, because (i) wild-type *Y. lipolytica* has no CYP53 genes or benzoate *para*-hydroxylase activity; (ii) pHBA is not further oxidized or degraded by *Y. lipolytica* (this work and Obiero, unpublished results); (iii) BA and pHBA are not toxic to *Y. lipolytica* at pH >7 (Obiero, unpublished results); (iv) BA is a relatively cheap substrate for larger scale process optimization; (v) BA and pHBA are easily detected on TLC, which facilitates screening of transformants for activity; (vi) BA and pHBA are relatively soluble in water, thus facilitating sampling from shake flasks. The results obtained in this study thus open the way for further development and optimization of *Y. lipolytica* expression systems for heterologous expression of fungal (and other eukaryotic) CYP450s.

Acknowledgements This research was funded by financial support from the National Research Foundation in South Africa and the Institut National de la Recherche Agronomique and the Centre National de la Recherche Agronomique Scientifique in France. The authors are grateful to Mr. P.J. Botes for assistance with GC analysis.

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