Appl Microbiol Biotechnol (2009) 83:849–857
DOI 10.1007/s00253-009-1917-8

$P450_{BM-3}$-catalyzed whole-cell biotransformation of α-pinene with recombinant *Escherichia coli* in an aqueous–organic two-phase system

Hendrik Schewe · Dirk Holtmann · Jens Schrader

Received: 30 June 2008 / Revised: 11 February 2009 / Accepted: 12 February 2009 / Published online: 6 March 2009

Abstract A recombinant *Escherichia coli* BL21 (DE3) strain overexpressing a variant of $P450_{BM-3}$ (V26T/R47F/A74G/F87V/L188K; abbreviated: BL21 ($P450_{BM-3}$ QM)) oxyfunctionalizes the bicyclic monoterpene α-pinene to α-pinene oxide, verbenol, and myrtenol. To address the low water solubility and the toxicity of terpenoids, an aqueous–organic two-phase bioprocess was developed. Diisononyl phthalate was selected as a biocompatible organic carrier solvent capable of masking the toxic effects mediated by α-pinene and of efficiently extracting the products enabling scale-up to the bioreactor. With an aqueous to organic phase ratio of 3:2 and 30% (*v*/*v*) of α-pinene in the organic phase, a biocatalytic product formation period of more than 4 h was achieved. A comparison of the biotransformation performance of BL21 ($P450_{BM-3}$ QM) and a strain with an additional heterologous NADPH regeneration system comprising glucose facilitator and dehydrogenase, but only expressing half the amount of $P450_{BM-3}$ QM, shows comparable product concentrations of 1,020±144 and 800±61 mg l_{Aq}^{-1}, respectively. The total product yields $Y_{P/P450}$ ($\mu mol\,\mu mol_{P450}^{-1}$) were 80% higher when the strain with the cofactor regeneration system was used. A total product concentration of over 1 g l_{Aq}^{-1}, corresponding to the highest value reported for microbial α-pinene oxyfunctionalization so far, marks a promising step forward toward a future application of recombinant microorganisms for the selective oxidation of terpenoids to value-added products.

H. Schewe · D. Holtmann · J. Schrader (✉)
Biochemical Engineering Group, DECHEMA e.V.,
Karl-Winnacker-Institut,
Theodor-Heuss-Allee 25,
60486 Frankfurt, Germany
e-mail: schrader@dechema.de

Keywords Aqueous–organic two-phase bioprocess · $P450_{BM-3}$ · Whole-cell · Biotransformation · Cofactor regeneration · Pinene · Phthalate

Introduction

Cytochrome P450 monooxygenases (P450) are high-potential biocatalysts because of their ability to regio- and stereoselectively insert an oxygen atom from molecular oxygen into allylic positions or unactivated C–H bonds under mild conditions, which is difficult to obtain by conventional organic synthesis (Urlacher et al. 2004). We coupled the quintuple variant V26T/R47F/A74G/F87V/L188K of the cytosolic P450 monooxygenase CYP102A1 from *Bacillus megaterium* ($P450_{BM-3}$ QM; Lentz et al. 2001) with a recombinant intracellular glucose-driven NAD(P)H regeneration system based on co-expression of a glucose facilitator (GLF) from *Zymomonas mobilis* for uptake of unphosphorylated glucose via facilitated diffusion and an $NADP^+$-dependent glucose dehydrogenase from *B. megaterium* (GlcDH) that oxidizes glucose to gluconolactone in *Escherichia coli* BL21 (DE3; abbreviated: BL21 $P450_{BM-3}$ QM/GlcDH/GLF). $P450_{BM-3}$ is a catalytically self-sufficient P450 monooxygenase containing both the P450 domain and the corresponding reductase in a single polypeptide chain (Narhi and Fulco 1986). By this means, the increased NADPH demand of the cell was successfully addressed (Schewe et al. 2008). $P450_{BM-3}$ QM catalyzes the oxyfunctionalization of α-pinene to α-pinene oxide, verbenol, and myrtenol in a ratio of 5:2:1. We chose this reaction as model system despite its only moderate regioselectivity because the precursor α-pinene is an inexpensive byproduct of the wood processing industry, and the products are valuable natural flavor compounds or precursors thereof.

Furthermore, $P450_{BM-3}$ is known for being susceptible to rational and evolutionary enzyme engineering approaches (Urlacher et al. 2004), which will probably lead to further improved substrate specificities and regioselectivities in the future. These results served as a first proof-of-concept for an improved α-pinene biooxidation with "cofactor-enhanced" *E. coli* overexpressing an engineered $P450_{BM-3}$. However, because pure α-pinene was used as second phase, the biocatalytic performance was severely limited, and a scale-up to the bioreactor was not possibe. Thus, though enzymatic oxidation of terpene hydrocarbons may be of superior regio- and stereoselectivity than chemical oxidation, the low water solubility and the toxicity of terpenoids still form the main hurdles for the development of a biocatalytic system (Schrader 2007).

A technical solution can be the development of biphasic systems, which rank among the four most important technological advances in replacement of classical chemical processes by biocatalyst-mediated transformations (Lilly 1994). Biotransformations in aqueous–organic two-phase systems have already been successfully employed in whole-cell biocatalysis and have often been found to effectively increase the yields compared with conventional bioprocess set-ups (for reviews, refer to Léon et al. (1998), Lye and Woodley (2001), and Malinowski (2001)). The organic phase serves as reservoir for hydrophobic substrates and as product sink, while the aqueous concentration stays low thereby reducing inhibitory or toxic effects on the whole-cell biocatalyst (Woodley and Lilly 1990; Wubbolts et al. 1994). This approach allows integration of bioconversion and product removal (in situ product recovery, ISPR), thus improving overall process productivity. A few two-phase bioprocesses have already been developed to process scale (Lye and Woodley 2001). The selection of a biocompatible organic carrier solvent with favorable partition coefficients is crucial for the implementation of an effective bioconversion in an aqueous–organic biphasic system (Cruz et al. 2004; León et al. 1998). A number of other desirable carrier solvent characteristics such as low emulsion-forming tendency, chemical, thermal, and biological stability, favorable properties for product recovery, and low price govern the choice of a suitable organic carrier solvent for an aqueous–organic two-phase process. In order to maintain prolonged cell activity, a very important criterion is the biocompatibility as the organic carrier solvent will be in direct contact with the whole-cell biocatalyst (Bruce and Daugulis 1991). Laane et al. (1987) developed a system to assess biocompatibility by relating solvent toxicity to its $\log P_{Oct}$ value (logarithm of the ratio of equilibrium concentrations of an organic compound in a two-phase system composed of *n*-octanol and water). Depending on the microorganism and the molecular structure of the organic solvent used, as a rule of thumb, organic solvents with $\log P_{Oct}$ values higher than 4–5 are generally recognized as biocompatible (Laane et al. 1987; Vermuë et al. 1993).

In order to prolong the product formation period in the biotransformation of α-pinene ($\log P_{Oct}$=4.5) with *E. coli* overexpressing $P450_{BM-3}$ (QM), which already stopped after 1 h under conventional conditions (Schewe et al. 2008), and thereby further enhance product yield, an aqueous–organic two-phase process based on a biocompatible organic carrier solvent was developed in the present work. By this means, scale-up to the bioreactor was possible leading to total product concentrations in the gram per liter scale, which are the highest values reported for a microbial α-pinene oxyfunctionalization up to now.

Materials and methods

All measurements were conducted in duplicate at least.

Chemicals

All chemicals used were purchased from Sigma/Aldrich (Schnelldorf, Germany) or Carl Roth (Karlsruhe, Germany) in puriss. p.a. quality, except diisononyl phthalate and butyl stearate, which were technical grade mixtures of esters.

Media

Luria–Bertani (LB) and Terrific Broth (TB) media were prepared like previously described (Schewe et al. 2008). SOC medium used for regeneration of transformed cells was purchased from Invitrogen (Karlsruhe, Germany).

M9 minimal medium was employed during optimization of the bioprocess and was prepared as previously described (Sambrook and Russell 2001). Glucose was used as carbon source at 10 g l^{-1}. Furthermore, 7.5 mg l^{-1} manganese chloride tetrahydrate, 5.25 mg l^{-1} zinc sulfate, 1.5 mg l^{-1} boric acid, 1.25 mg l^{-1} sodium molybdate dihydrate, 0.7 mg l^{-1} copper sulfate, 4.2 mg l^{-1} EDTA, 24.4 mg l^{-1} ferrous sulfate heptahydrate, and 0.9 mg l^{-1} cobalt chloride were added in the form of a trace element solution.

Strains, plasmids, preparation and transformation of competent cells, and determination of enzymatic activities and P450 concentration

Strains and plasmids used in this work as well as preparation and transformation of competent cells, plasmid selection and determination of recombinant enzymatic activities, and determination of P450 concentration (CO difference spectra) were identical to those published in Schewe et al. (2008).

In vitro measurement of NADPH consumption of $P450_{BM-3}$ QM

Measurements were carried out as previously described (Schewe et al. 2008). To investigate the stability of the organic solvents, a 30 mM solution of the respective organic solvent in DMSO was mixed with cell-free extract containing 14 μM $P450_{BM-3}$ QM in 50 mM potassium phosphate buffer, pH7.5, to obtain a final concentration of 0.6 mM organic solvent. Reaction was started by adding 0.5 mM NADPH in 50 potassium phosphate buffer, pH7.5. Measurements were conducted at room temperature.

Characterization of DINP as component of an aqueous–organic two-phase system

For measurement of specific glucose consumption rates, increasing volumes of α-pinene or diisononyl phthalate (DINP) of 5% to 50% (*v*/*v*), respectively, were added to 1 g l^{-1} of *E. coli* BL21 (DE3) suspended in a constant volume of 40 ml M9 medium containing 8 g l^{-1} glucose. In order to account for the variation of total volume and to adjust energy input, different dimensions of Erlenmeyer flasks were chosen to obtain a constant ratio of fluid volume to flask volume of 0.16–0.18. The flasks were incubated at 300 rpm for 4 h on a Certomat R shaking incubator (B. Braun Biotech International, Melsungen, Germany) and 37°C. The positive control consisted of a cell suspension without any addition of an organic phase. Specific glucose consumption rate (g h^{-1} g^{-1} cell dry weight) was measured in duplicate by linear regression of specific glucose consumption (g g^{-1} cell dry weight), which was measured hourly over 4 h. Samples were taken from the aqueous phase, and after centrifugation, glucose concentration was measured using an YSI 2700 glucose analyser (YSI, Yellow Springs, USA).

Product analysis and determination of partition coefficients

Emulsions were either extracted with an equal volume of *n*-hexane followed by phase separation by centrifugation, or the aqueous–organic emulsion was broken directly by centrifugation. Samples were dehydrated on sodium sulfate and analyzed by GC (GC17A with FID analyzer, Shimadzu, Kyoto, Japan) or GC-MS (GC17A with Q5050 mass spectrometer, Shimadzu, Kyoto, Japan). Chromatographic standards and programs used were the same as published before (Schewe et al. 2008), and only the split applied was 10:1 in this work. The specific initial product formation rate Q was determined by plotting the specific product concentration (μmol product per μmol of P450) against time (0–60 min) and linear regression. The product concentration related to aqueous phase volume c_{Aq} (mg l_{Aq}^{-1}) was calculated from product concentrations in the organic phase and the phase ratio of the aqueous to the organic phase.

For determination of partition coefficients, 4 ml of aqueous solutions of verbenol and myrtenol, respectively, in a concentration range from 0.25 to 2 mM, was covered with an equal volume of organic solvent. The two-phase system was allowed to equilibrate under vigorous shaking at 25°C overnight. After phase separation by centrifugation, a sample from the organic phase was diluted 1:10 in *n*-hexane. Concentration in the organic phase was analyzed by gas chromatography. Aqueous concentrations were determined by GC (150°C isotherm for 9 min; carrier gas, helium; column flow, 0.55 ml min^{-1}; split injection, 10:1) using a DB-WAXetr column (J&W Scientific, 30 m × 0.25 mm × 0.25 μm). Pinene oxide undergoes autoxidation and rearrangement reactions in the presence of water (Boontawan and Stuckey 2005; Buffon and Schuchardt 2003). Hence, an alternative approach to characterize its partition behavior was established. Different concentrations of α-pinene oxide were dissolved in 2 ml of organic solvent and added to 4 ml of deionized water. The two-phase system was vortexed for 10 min, and the phases were separated by centrifugation. The aqueous phase was extracted with an equal volume of hexane and analyzed using a GC-MS. The same approach was used for determination of partition coefficients of α-pinene.

Pre- and main cultivation and aqueous–organic two-phase biotransformation

To increase reproducibility of recombinant protein expression, colonies of freshly transformed *E. coli* BL21 (DE3) cells served as inoculum for the preparation of precultures in TB medium. During analysis of the impact of the organic phase composition, cell growth and recombinant protein expression were carried out in the bioreactors (FedBatchPro, DASGIP, Juelich, Germany) themselves. A volume of 150 ml M9 medium was transferred into each bioreactor featuring a total capacity of 400 ml and inoculated with 2% (*v*/*v*) preculture. Cells were grown overnight in fed-batch mode at 37°C and constant stirrer speed of 500 rpm under carbon source limitation by automatically feeding glucose from a 500 g l^{-1} stock solution using the DO-stat feeding strategy. The pH was kept at 7.2 using a mixture of 1 M sodium hydroxide and 20% (*v*/*v*) ammonium hydroxide, which served as base and N-source. Recombinant protein expression was started at OD_{600}=30 by manual addition of 0.5 mM IPTG and was carried out at 37°C and 700 rpm for 3 h.

Alternatively, as modified recombinant protein expression system during comparison of biotransformations with BL21 ($P450_{BM-3}$ QM) and BL21 ($P450_{BM-3}$ QM/GlcDH/GLF), main cultures with the appropriate amount of antibiotics were inoculated with 2% (*v*/*v*) preculture and

grown in TB medium in Erlenmeyer flasks without baffles at 37°C and 300 rpm. The cultures were induced at OD_{600}= 1 with 0.1 mM IPTG and were, after addition of 0.12 mg l^{-1} ferric chloride, incubated at 37°C. After several hours, the cultures were centrifuged and resuspended in 150 ml TB medium and transferred into the bioreactors of a FedBatchPro parallel fermentation system.

In order to start the biotransformation, 100 ml of different mixtures of α-pinene and diisononyl phthalate was added to 150 ml cell suspension. Data collection and process control was carried out with the DASGIP Control software (version 3.10). During biotransformation, aeration and agitation by a magnetic stir bar were kept constant at 5 lh^{-1} of compressed air and 1,100 rpm, respectively. Maintenance of pH at 7.2 was accomplished with 20% (*v*/*v*) ammonium hydroxide. During biotransformation glucose was added from a 500 g l^{-1} stock solution. Samples were taken directly from the emulsion. Negative controls of α-pinene oxidation were performed under comparable reaction conditions with plasmid-free *E. coli* BL21 (DE3) cells.

Results

Screening and analysis of suitable organic carrier solvents

A screening of several organic carrier solvents suitable for application in an aqueous–organic two-phase bioprocess was carried out. *n*-Decane, dodecane, corn oil, olive oil, oleic acid, diisononyl phthalate, and butyl stearate, which all met criteria such as low water solubility, chemical and thermal stability, and high flashpoints, were chosen for further experimental investigations. Specific growth rates μ after addition of 17% (*v*/*v*) of the organic solvents to submerged cultures of *E. coli* BL21 (DE3) were compared to the maximal specific growth rate μ_{max} of a positive control without addition of an organic phase. Values of 0.8–1 for $\mu\ \mu_{max}^{-1}$ proved that all seven organic solvents met the obligatory criterion of being biocompatible with *E. coli*. All organic solvents except oleic acid showed low emulsion forming tendency after vigorous shaking for several hours in a two-phase system (data not shown).

Unwanted side reactions of $P450_{BM-3}$ QM with the organic carrier solvent were investigated by spectrophotometric in vitro analysis of NADPH consumption rates in cell-free extracts of BL21 ($P450_{BM-3}$ QM). Lauric acid (12:0), a well-known substrate for $P450_{BM-3}$ wild-type and variants thereof (Lentz et al. 2001), was used as positive control (Table 1).

Since it showed the lowest NADPH consumption rate of all tested organic solvents, DINP was used for the further development of the aqueous–organic two-phase biotransformation system.

Determination of partition coefficients

Verbenol and myrtenol showed comparable partition coefficients of 77.5±12.2 and 74.3±0.3 in a DINP/water system, respectively. After dissolving α-pinene oxide in DINP and adding deionized water, no α-pinene oxide and only trace amounts of α-pinene oxide derived byproducts were detected by GC-MS analysis of an organic extract of the aqueous phase (data not shown). This result indicates that virtually no partition of α-pinene oxide from the organic to the aqueous phase occurred. Because only negligible accumulation of α-pinene oxide derived byproducts were monitored in the course of whole-cell biotransformation of α-pinene, an immediate and almost quantitative extraction of α-pinene oxide into DINP once it is biocatalytically generated can therefore be assumed. Finally, partition of the precursor α-pinene to the DINP phase was even more pronounced because its concentration in the aqueous phase could not be reliably determined due to restrictions by the lower detection limit of the analysis method used.

Characterization of DINP as component of an aqueous–organic two-phase system

The toxic effects mediated by DINP and α-pinene as organic solvent of an aqueous–organic two-phase system

Table 1 $LogP_{Oct}$ of several organic solvents and corresponding NADPH consumption rates of $P450_{BM-3}$ QM measured at 340 nm at pH 7.5 and room temperature

Organic solvent	$LogP_{Oct}$	NADPH consumption rate ($\mu molmin^{-1}\,\mu mol_{P450}^{-1}$)
Lauric acid[a]	4.6	640±1
Oleic acid	7.7	192±2
n-Decane	5.6	105±8
Dodecane	6.6	46±3
Butyl stearate	9.7	12±0.9
Olive oil	7.5	12±0.4
Corn oil	7.4	7±0.5
DINP	9.4	0±0.7

Mean values of duplicate experiments are given

[a] Positive control for NADPH consumption

were characterized by measuring the specific glucose consumption rate as indicator for vitality of an *E. coli* BL21 (DE3) cell suspension in the presence of increasing volumetric contents α-pinene and DINP, respectively (Fig. 1). In order to form an organic phase being able to supply adequate amounts of substrate and a sufficient product sink, a volumetric content of 5% (*v*/*v*) was chosen as lower limit, while 50% (*v*/*v*) marks the upper limit for an aqueous–organic two-phase system before inversion of the homogenous phase occurs. Decreasing glucose consumption rates were interpreted as loss of cell vitality.

The fact, that 95% of the cell vitality was already lost at only 5% (*v*/*v*) α-pinene indicated that cell inactivation started at even lower α-pinene concentrations. In contrast, after addition of DINP, cell cultures showed three to four times higher relative glucose consumption rates even at volumetric contents of up to 50% (*v*/*v*), revealing a less pronounced detrimental effect by the organic phase.

Two-phase biotransformation of α-pinene in lab-scale bioreactors

Since mixing conditions in Erlenmeyer flasks and bioreactors differ considerably, DINP was tested for its biocompatibility under biotransformation conditions in a stirred bioreactor environment. Indeed, *E. coli* cells showed metabolic activity while suspended in a stirred and aerated bioreactor containing a DINP-medium two-phase system. Both, BL21 ($P450_{BM-3}$ QM) and BL21 ($P450_{BM-3}$ QM/GlcDH/GLF) were metabolically active, indicated by the typical alternation of the dissolved oxygen (DO) time course during DO-stat controlled feeding, i.e., decrease in dissolved oxygen concentration when glucose was added and subsequently metabolized, followed by an increase after consumption of the glucose (data not shown). In contrast, no metabolic activity was observed with α-pinene as organic solvent, which emphasizes that using a carrier solvent is a mandatory prerequisite for the development of an α-pinene biotransformation process.

DINP proved to be nonbiodegradable by BL21 ($P450_{BM-3}$ QM). GC-MS data of the organic phase revealed no accumulation of alkanols, such as isononyl alcohol, as it would be the case after enzymatic cleavage of the ester bonds of DINP and other esters contained in the technical grade solvent.

Figure 2 shows specific initial product formation rates Q (μmol oxidized products h^{-1} $\mu mol_{P450}{}^{-1}$) and total product concentrations related to the aqueous phase volume c_{Aq} (mg total oxidized products $l_{Aq}{}^{-1}$) after 4 h of whole-cell biotransformation with BL21 ($P450_{BM-3}$ QM) in bioreactors operated in parallel with different DINP/α-pinene mixtures as organic phase but under otherwise identical reaction conditions. c_{Aq} was chosen as key parameter to identify optimal organic phase compositions that yielded maximal product accumulation under given process conditions. To account for different intracellular P450 expression levels, which were in the range of 0.1–0.2 μmol/g of cell dry weight, Q was calculated as well. Considering the partition coefficients of the products, a sufficient capacity of the organic phase, acting as product sink, has to be ensured. Furthermore, the hydrophobic substrate, which preferably dissolves in the organic phase, must be efficiently delivered to the aqueous phase. Two-phase bioreactor experiments with DINP contents of 5–15% (*v*/*v*), which are physiolog-

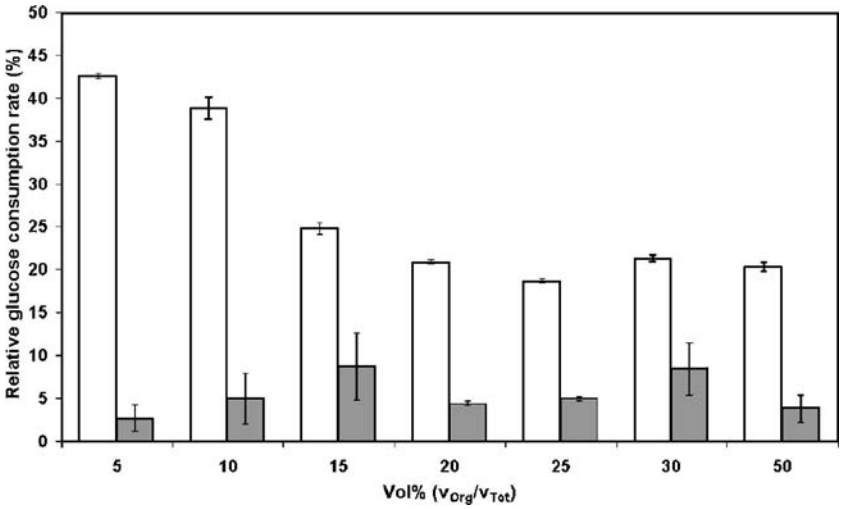

Fig. 1 Relative specific glucose consumption rate of *E. coli* BL21 (DE3) with increasing volumetric contents of (-)-α-pinene (*gray bars*) and DINP (*white bars*) in an aqueous–organic two-phase system with a constant aqueous volume of 40 ml. The positive control, i.e., no organic solvent (100% value), showed a specific glucose consumption rate of 2 g h^{-1} g^{-1} of cell dry weight at 37°C over 4 h. Relative specific glucose consumption rate was calculated by relating specific glucose consumption from two-phase cultures to the specific glucose consumption of the positive control. Mean values of duplicate measurements are shown

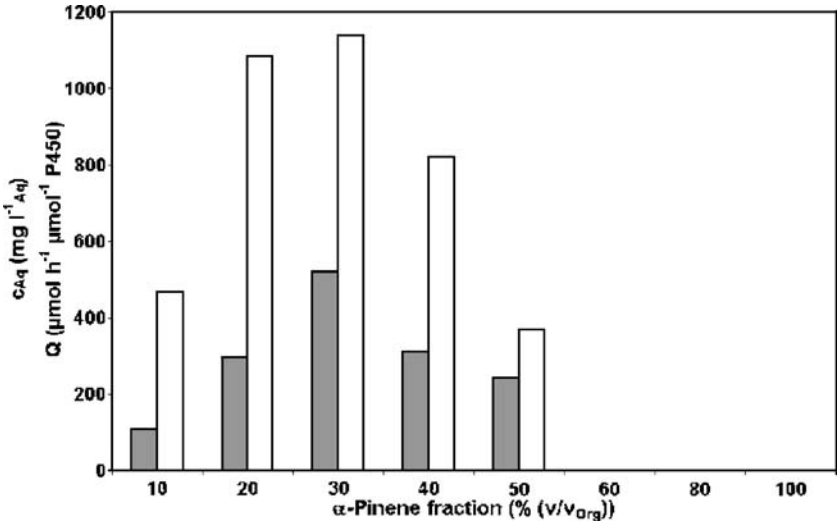

Fig. 2 Influence of different volumetric α-pinene fractions (% (*v*/v_{Org})) in the organic phase on total product concentration related to aqueous phase volume c_{Aq} (*gray columns*) and specific initial product formation rate *Q* (*white columns*) of oxidized products produced by BL21 ($P450_{BM-3}$ QM) in an aqueous–organic two-phase biotransformation after a process time of 4 h. Each column pair represents one bioreactor experiment. The total volume of the aqueous and organic phase was 150 and 100 ml, respectively. Applying an α-pinene fraction of 60%, 80%, and 100% (*v*/v_{Org}) did not result in any product formation

ically advantageous (according to Fig. 1), showed low product concentrations (data not shown). Obviously, the availability of α-pinene, which was dissolved in volumetric contents of 5–50% (*v*/v_{Org}) in the organic phase, was too low under these conditions. Thus, an organic solvent content of 40% (*v*/*v*) was chosen to assure both substrate availability and biocatalyst activity (cf. Fig. 1).

Figure 2 illustrates that product formation was not only lacking when pure α-pinene was applied as organic solvent but also with α-pinene contents of 80% and 60% (*v*/v_{Org}) in DINP. Additionally, no metabolic activity (glucose or oxygen consumption) was measured in these experiments (data not shown). However, when the volumetric content of α-pinene was set to 50% (*v*/v_{Org}), a significant increase in c_{Aq} and *Q* was monitored. Further shifting the ratio toward the biocompatible DINP led to an increase in both c_{Aq} and *Q*. Maximum values of c_{Aq} and *Q* of 520 mg l_{Aq}^{-1} and 1,140 µmol h^{-1} μmol_{P450}^{-1}, respectively, were obtained with a mixture of 70% (*v*/v_{Org}) DINP and 30% (*v*/v_{Org}) α-pinene. The improved α-pinene oxidation resulted from a combination of an increased specific initial product formation rate and prolonged product formation period of BL21 ($P450_{BM-3}$ QM). No increase in cell dry weight concentration was monitored after addition of organic phase mixtures of DINP and α-pinene.

Varying the stirrer speed and measuring both c_{Aq} and *Q*, increasing values were monitored at stirrer speeds of 800, 900, and 1,000 rpm. *Q* reached a maximum of 1,490 µmol h^{-1} μmol_{P450}^{-1} at 1,100 rpm where 465 mg l_{Aq}^{-1} total products were accumulated. Further increasing stirrer speed to 1,500 and 2,000 rpm led to decreasing amounts of accumulated products (data not shown).

With these optimized bioprocess conditions, i.e., an aqueous to organic phase ratio of 3:2 with DINP/α-pinene 70:30 (*v*/*v*) and a stirrer speed of 1,100 rpm, a direct comparison of the biotransformation performance of BL21 ($P450_{BM-3}$ QM) and BL21 ($P450_{BM-3}$ QM/GlcDH/GLF) was carried out (Fig. 3). Due to a modified recombinant protein expression method, higher intracellular expression titers of $P450_{BM-3}$ QM of 0.5 and 0.25 µmol/g of cell dry weight in BL21 ($P450_{BM-3}$ QM) and BL21 ($P450_{BM-3}$ QM/GlcDH/GLF), respectively, were reached. Normalized to the actual P450 concentration in the cells, BL21 ($P450_{BM-3}$ QM/GlcDH/GLF) showed a superior specific α-pinene oxidation rate ($Y_{P/P450}$), especially in the initial phase of the biotransformation (Fig. 3a). On average, BL21 ($P450_{BM-3}$ QM/GlcDH/GLF) showed a twofold higher $Y_{P/P450}$ over 5 h of biotransformation, indicating that the recombinant cofactor regeneration system allowed for a more effective use of intracellular $P450_{BM-3}$ QM for α-pinene oxidation.

The combination of optimized process conditions and recombinant cofactor regeneration in biocatalyst BL21 ($P450_{BM-3}$ QM/GlcDH/GLF) resulted in yields of over 0.8 g l_{Aq}^{-1} of oxidized products. Due to lower expression levels of $P450_{BM-3}$ QM in BL21 ($P450_{BM-3}$ QM/GlcDH/GLF), the total product concentration was lower than that produced by BL21 ($P450_{BM-3}$ QM; Table 2). Nevertheless, BL21 ($P450_{BM-3}$ QM/GlcDH/GLF) was on par with BL21 ($P450_{BM-3}$ QM) in the initial phase of the biotransformation (Fig. 3b).

Decrease in dissolved oxygen concentration after glucose addition was monitored even after cessation of product formation after 5 h, indicating continuing metabolic

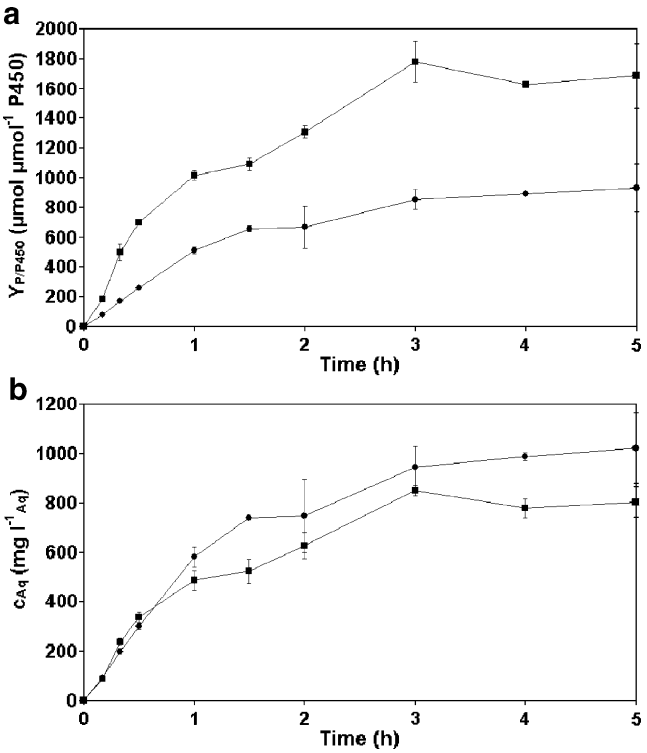

Fig. 3 Total product formation kinetics of α-pinene biotransformation with BL21 ($P450_{BM-3}$ QM; *closed circles*) and BL21 ($P450_{BM-3}$ QM/GlcDH/GLF; *closed squares*) in an aqueous–organic two-phase bioreactor. **a** P450 specific total product yield $Y_{P/P450}$ (µmol µmol$^{-1}_{P450}$). **b** Total product concentration related to aqueous phase volume c_{Aq} (mg l$^{-1}_{Aq}$). The organic phase consisted of 70% (*v*/*v*) DINP and 30% (*v*/*v*) α-pinene. The volume ratio of the aqueous phase to the organic phase was 3:2 at a total volume of 250 ml. Mean values of duplicate experiments and measurements are given

activity. P450 concentration or in vitro activity after product formation period could not be determined by the methods applied due to protein denaturation in cell-free extracts caused by residual non-removable trace amounts of organic solvent. Chemical autoxidation of α-pinene could be excluded since no product formation was observed when biotransformation was performed with *E. coli* BL21 (DE3) without recombinant expression of $P450_{BM-3}$ QM as negative control.

Discussion

Out of seven organic solvents tested for suitability as organic carrier solvent in an aqueous–organic two-phase biotransformation, DINP ($\log P_{Oct}$=9.4) turned out to best fulfil the desirable characteristics, such as biocompatibility and non-biodegradability with respect to the *E. coli* strain and P450 monooxygenase used. Oleic acid, *n*-decane, dodecane, butyl stearate, olive oil, and corn oil promoted unwanted diversion of catalytic activity of $P450_{BM-3}$ QM from the desired biotransformation as revealed by causing partly considerable NADPH consumption rates in in vitro assays (cf. Table 1). Since no further analysis for oxidized products was performed, no statement on whether these molecules served as substrate for $P450_{BM-3}$ QM, which was initially generated by rational evolution to achieve hydroxylation activity toward short-chain fatty acids and other hydrophobic substrates (Lentz et al. 2001) or promoted uncoupling of NADPH− from substrate oxidation, could be made. In contrast, $P450_{BM-3}$ QM exhibited almost no NADPH consumption when DINP was present in the in vitro assay.

DINP was identified as suitable carrier solvent significantly less toxic than α-pinene (cf. Fig. 1), enabling us the development of a tailored aqueous–organic two-phase bioprocess on bioreactor scale with a prolonged product formation period. However, during bioreactor operation, the organic phase consisting of α-pinene and DINP, still caused biostatic effects, as indicated by cessation of cell growth after addition of the organic phase (α-pinene/DINP ratio of 30:70 (*v*/v_{Org})). In contrast, two-phase bioprocesses using *bis*(2-ethylhexyl)phthalate, another phthalic acid ester successfully employed in *E. coli* based aqueous–organic two-phase biotransformations (Bühler et al. 2003; Cruz et al. 2004; de Carvalho and da Fonseca 2004) as organic carrier solvent, showed cell growth of recombinant *E. coli* cultures after addition of the organic phase (Panke et al. 2000; Park et al. 2006). Presumably, the absence of cell growth in the present study was caused by a five- to tenfold higher concentration of α-pinene in the phthalate carrier phase than the

Table 2 Process performance of BL21 ($P450_{BM-3}$ QM) and BL21 ($P450_{BM-3}$ QM/GlcDH/GLF) in an aqueous–organic two-phase system with a phase ratio of 3:2

	BL21 ($P450_{BM-3}$ QM)	BL21 ($P450_{BM-3}$ QM/GlcDH/GLF)
Total product concentration related to aqueous phase volume c_{Aq} (mg l_{Aq}^{-1})	1,020±144	800±61
Total product yield $Y_{P/X}$ (mg g^{-1} cdw)	96±15	64±8.3
Total product yield $Y_{P/P450}$ (µmol µmol$_{P450}^{-1}$)	930±160	1,680±220
Specific initial product formation rate *Q* (µmol h^{-1} µmol$_{P450}^{-1}$)	720±15	1,020±12
Intracellular expression titer of $P450_{BM-3}$ QM (µmol g^{-1} cdw)	0.5±0.03[a]	0.25±0.04[a]

The organic phase consisted of 70% (*v*/v_{Org}) DINP and 30% (*v*/v_{Org}) α-pinene. Cell dry weight concentration was adjusted to 12 g l^{-1} in all experiments. Mean values of duplicate bioreactor experiments are given. Biotransformation time was 5 h

[a] Mean values of fourfold CO difference spectrum measurement

concentration of similarly toxic styrene ($\log P_{Oct}$=3) used in the bioprocesses mentioned above.

The use of pure α-pinene ($\log P_{Oct}$=4.5) in the two-phase biotransformation led to total loss of product formation (Fig. 2) as well as glucose and oxygen consumption. In contrast, we previously found products of α-pinene biotransformation when using a neat α-pinene phase as organic phase in two-phase Erlenmeyer flask experiments (Schewe et al. 2008). This effect may be ascribed to enhanced stress in which the cells are exposed to in a two-phase bioreactor. As a result of a higher energy input in a stirred bioreactor associated with an improved phase mixing and an enhanced mass transfer of toxic α-pinene from the organic to the aqueous phase as well as an increased interfacial area (Bar 1988; Hocknull and Lilly 1987), *E. coli* faces elevated molecular toxicity mediated by the accumulation of α-pinene in its cell membranes. These data emphasize that in this case, identifying a biocompatible organic carrier solvent was a mandatory prerequisite for successful scale-up and bioprocess development. By empirically adjusting system parameters such as mixing intensity, organic phase composition, and phase ratio accordingly, a good compromise between maximum interfacial surface resulting in optimal substrate availability and product recovery and minimum loss of biological activity due to toxic effects was identified.

Consistent with previously published data (Schewe et al. 2008), where we reported the positive effect of a recombinant intracellular cofactor regeneration system on the efficiency of $P450_{BM-3}$-based α-pinene oxidation in Erlenmeyer flasks, the same effect was observed during α-pinene biotransformations in bioreactors as well. BL21 ($P450_{BM-3}$ QM/GlcDH/GLF) achieved a more efficient use of $P450_{BM-3}$ QM in a controlled bioreactor environment than BL21 ($P450_{BM-3}$ QM), especially in the initial phase of α-pinene oxidation, illustrated by a 100% increase in P450 specific initial product formation rate and specific product yield (cf. Fig. 3a). By further optimization of $P450_{BM-3}$ QM expression titers in BL21 ($P450_{BM-3}$ QM/GlcDH/GLF), which were only half the amount as in BL21 ($P450_{BM-3}$ QM), product concentrations can be escalated to even higher values.

To further improve process productivity, adverse effects mediated by the organic phase should be reduced. Obviously, direct interfacial contact of whole-cell biocatalysts to the organic phase containing DINP, α-pinene, and biotransformation products, which additionally competitively inhibit $P450_{BM-3}$ QM activity (Schewe et al. 2008), negatively influenced biocatalytic activity, cell integrity, and vitality, resulting in total cessation of product formation after 4–5 h (cf. Fig. 3). Adverse effects may be avoided by optimizing contact between substrate and product and the whole-cell biocatalyst, e.g., by using solid polymer beads as partitioning phase (Morrish and Daugulis 2008) or by shielding the whole-cell biocatalyst with a protective polymer (Kawakami et al. 1990).

Microbial conversion of pinenes has been investigated since the early 1960s (Shukla and Bhattacharyya 1968; Trudgill 1994). Due to many diverse degradation pathways, there are many interesting products resulting from oxyfunctionalization of pinenes. However, bioconversion yields have usually been in the milligrams per liter range and thus rather low compared to other terpene biooxidations, possibly due to the more complex bicyclic structure of the pinenes (Schrader 2007). One noteworthy exception was an α-pinene biotransformation with *Hormonema* sp. leading to 0.3 g l^{-1} verbenone and 0.4 g l^{-1} verbenol after 96 h (van Dyk et al. 1998), but biotransformation results were not easily reproducible. In this context, total product concentrations of over 1 g l_{Aq}^{-1} after only 4 h of biotransformation as presented in this work mark a very promising step forward toward a future industrial application of recombinant microorganisms for the oxidative refinement of the abundantly available natural monoterpene α-pinene.

Acknowledgments This research was financially supported by the German Federal Ministry of Economics and Technology via the AiF (projects no. 119 ZN and no. 15089 N). We thank H. Sahm, Research Centre Juelich for plasmid pET24*glcdh* and pZY507*glf*, U. Schwaneberg, Jacobs University Bremen for the supply with 12-*p*NCA, and R.D. Schmid, Institute of Technical Biochemistry, Stuttgart for providing pET28*bm-3 qm*.

References

Bar R (1988) Effect of interphase mixing on a water-organic solvent two-liquid phase microbial system: ethanol fermentation. J Chem Tech Biotechnol 43:49–62

Boontawan A, Stuckey DC (2005) Mass transfer of terpenes through a silicone rubber membrane in a liquid-liquid contacting system. Biotechnol Prog 21:1680–1687

Bruce LJ, Daugulis AJ (1991) Solvent selection strategies for extractive biocatalysis. Biotechnol Prog 7:116–124

Buffon R, Schuchardt U (2003) Heterogenization of alkene epoxidation catalysts. J Braz Chem Soc 14:347–353

Bühler B, Bollhalder I, Hauer B, Witholt B, Schmid A (2003) Chemical biotechnology for the specific oxyfunctionalization of hydrocarbons on a technical scale. Biotechnol Bioeng 82:833–842

Cruz A, Fernandes P, Cabral JMS, Pinheiro HM (2004) Solvent partitioning and whole-cell sitosterol bioconversion activity in aqueous-organic two-phase systems. Enzyme Microb Technol 34:342–353

de Carvalho CC, da Fonseca MM (2004) Solvent toxicity in organic-aqueous systems analysed by multivariate analysis. Bioprocess Biosyst Eng 26:361–375

Hocknull MD, Lilly MD (1987) The δ-1-dehydrogenation of hydrocortisone by *Arthrobacter simplex* in organic aqueous two-liquid phase environments. In: Laane C, Tramper J, Lilly MD (eds) Biocatalysis in organic media. Elsevier, Amsterdam, pp 669–674

Kawakami K, Tsuruda S, Miyagi K (1990) Immobilization of microbial cells in a mixed matrix of silicone polymer and calcium alginate gel: epoxidation of 1-octene by *Nocardia corallina* B-276 in organic media. Biotechnol Prog 6:357–361

Laane C, Boeren S, Vos K, Veeger C (1987) Rules for optimization of biocatalysis in organic solvents. Biotechnol Bioeng 30:81–87

Lentz O, Li Q-S, Schwaneberg U, Lutz-Wahl S, Fischer P, Schmid RD (2001) Modification of the fatty acid specificity of cytochrome $P450_{BM-3}$ from *Bacillus megaterium* by directed evolution: a validated assay. J Mol Catal B: Enzym 15:123–133

León R, Fernandes P, Pinheiro HM, Cabral JMS (1998) Whole-cell biocatalysis in organic media. Enzyme Microb Technol 23:483–500

Lilly MD (1994) Advances in biotransformation processes. Trans I Chem Eng 72:27–34

Lye GJ, Woodley JM (2001) Advances in the selection and design of two-liquid phase biocatalytic reactors. In: Cabral JMS, Mota M, Tramper J (eds) Multiphase bioreactor design. Taylor & Francis, London, pp 115–134

Malinowski JJ (2001) Two-phase partitioning bioreactors in fermentation technology. Biotechnol Adv 19:525–538

Morrish JL, Daugulis AJ (2008) Improved reactor performance and operability in the biotransformation of carveol to carvone using a solid-liquid two-phase partitioning bioreactor. Biotechnol Bioeng 101:946–956

Narhi LO, Fulco AJ (1986) Characterization of a catalytically self-sufficient 119, 000 dalton cytochrome P-450 monooxygenase induced by barbiturates in bacillus megaterium. J Biol Chem 261:7160–7169

Panke S, Wubbolts MG, Schmid A, Witholt B (2000) Production of enantiopure styrene oxide by recombinant *Escherichia coli* synthesizing a two-component styrene monooxygenase. Biotechnol Bioeng 69:91–100

Park JB, Bühler B, Habicher T, Hauer B, Panke S, Witholt B, Schmid A (2006) The efficiency of recombinant *Escherichia coli* as biocatalyst for stereospecific epoxidation. Biotechnol Bioeng 95:501–512

Sambrook J, Russell DW (2001) Molecular cloning—a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Schewe H, Kaup BA, Schrader J (2008) Improvement of P450(BM-3) whole-cell biocatalysis by integrating heterologous cofactor regeneration combining glucose facilitator and dehydrogenase in *E. coli*. Appl Microbiol Biotechnol 78:55–65

Schrader J (2007) Microbial flavour production. In: Berger RG (ed) Flavours and fragrances chemistry, bioprocessing and sustainability. Springer, Berlin Heidelberg, pp 509–573

Shukla OP, Bhattacharyya PK (1968) Microbial transformation of terpenes: part XI—pathways of degradation of α- and β-Pinenes in a soil *Pseudomonad* (PL-Strain). Indian J Biochem 5:92–101

Trudgill PW (1994) Microbial metabolism and transformation of selected monoterpenes. In: Ratledge C (ed) Biochemistry of microbial degradation. Kluwer, London, pp 33–61

Urlacher VB, Lutz-Wahl S, Schmid RD (2004) Microbial P450 enzymes in biotechnology. Appl Microbiol Biotechnol 64:317–325

van Dyk MS, van Rensburg E, Moleleki N (1998) Hydroxylation of (+)-limonene, (−) α-pinene and (−) β-pinene by a Hormonema sp. Biotechnol Lett 20:431–436

Vermuë M, Sikkema J, Verheul A, Bakker R, Tramper J (1993) Toxicity of homologous series of organic solvents for the gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the gram-negative bacteria *Acinetobacter* and *Pseudomonas* Sp. Biotechnol Bioeng 42:747–758

Woodley J, Lilly M (1990) Extractive biocatalysis: the use of two-liquid phase biocatalytic reactors to assist product recovery. Chem Eng Sci 45:2391–2396

Wubbolts MG, Hoven J, Melgert B, Witholt B (1994) Efficient production of optically active styrene epoxides in two-liquid phase cultures. Enzyme Microb Technol 16:887–893