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FERMENTATION, CELL CULTURE AND BIOENGINEERING

Integrated organic–aqueous biocatalysis and product recovery for quinaldine hydroxylation catalyzed by living recombinant *Pseudomonas putida*

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Abstract In an earlier study, biocatalytic carbon oxyfunctionalization with water serving as oxygen donor, e.g., the bioconversion of quinaldine to 4-hydroxyquinaldine, was successfully achieved using resting cells of recombinant *Pseudomonas putida*, containing the molybdenum-enzyme quinaldine 4-oxidase, in a two-liquid phase (2LP) system (Ütkür et al. J Ind Microbiol Biotechnol 38:1067–1077, 2011). In the study reported here, key parameters determining process performance were investigated and an efficient and easy method for product recovery was established. The performance of the whole-cell biocatalyst was shown not to be limited by the availability of the inducer benzoate (also serving as growth substrate) during the growth of recombinant *P. putida* cells. Furthermore, catalyst performance during 2LP biotransformations was not limited by the availability of glucose, the energy source to maintain metabolic activity in resting cells, and molecular oxygen, a possible final electron acceptor during quinaldine oxidation. The product and the organic solvent (1-dodecanol) were identified as the most critical factors affecting biocatalyst performance, to a large extent on the enzyme level (inhibition), whereas substrate effects were negligible. However, none of the 13 alternative solvents tested surpassed 1-dodecanol in terms of toxicity, substrate/product solubility, and partitioning. The use of supercritical carbon dioxide for phase separation and an easy and efficient liquid–liquid extraction step enabled 4-hydroxyquinaldine to be isolated at a purity of >99.9% with recoveries of 57 and 84%, respectively. This study constitutes the first proof of concept on an integrated process for the oxyfunctionalization of toxic substrates with a water-incorporating hydroxylase.

Keywords Biocatalysis · Oxyfunctionalization · Molybdenum-containing hydroxylase · Two-liquid phase · Downstream processing

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Introduction

Industrial interest in chemical reactions catalyzed by enzymes and microorganisms is growing continuously [5, 15, 30]. An important and potentially interesting type of bioconversion is the regio- and stereoselective oxyfunctionalization of aliphatic and aromatic compounds, which are poorly water-soluble and toxic to microorganisms. In order to avoid solubility- and toxicity-related complications and to enable in situ product removal, a second organic phase can be used for the biotransformations of organic compounds of commercial interest [20, 42, 43].

The use of two-liquid phase (2LP) systems, consisting of a water-immiscible organic solvent and an aqueous phase, was shown to enable high overall concentrations of toxic and/or inhibitory substrates and products, the shift of reversible reactions into a desired direction, the exploitation of kinetics to control multistep reactions, enhanced enantiomeric excess, and easy product recovery [5, 20, 23, 42, 43]. In 2LP bioprocesses, partitioning and mass transfer of the substrate and the product between the phases,

oxygen mass transfer, properties of the microorganisms, and the reaction conditions have to be considered to evaluate and avoid mass transfer limitation. The appropriate choice of the organic phase may, depending on the spreading coefficient of the chosen solvent, S_{OW}, even enhance the oxygen-transfer rate, which is critical to sustain oxygen-dependent reactions and energy metabolism [38, 41]. Downstream processing in general is a key part of bioprocess development. In 2LP processes, this includes phase separation and product isolation [34, 43]. Phase separation in particular can be critical when stable emulsions are formed [4].

We recently described quinaldine 4-oxidase (Qox)-containing *Pseudomonas putida* as a biocatalyst for regioselective hydroxylation of quinaldine to 4-hydroxyquinaldine, both of which are poorly soluble in water and toxic to cells even at low concentrations [37]. This study constituted the first biotechnological in vivo application of Qox, highlighting the potential of hydroxylating dehydrogenases for achieving carbon oxyfunctionalizations without active aeration and reducing limitations encountered with other oxidoreductases (e.g., cofactor regeneration) in technical applications. The use of 1-dodecanol as the organic carrier solvent allowed solubility and toxicity/inhibition problems to be overcome and higher overall product concentrations to be obtained. However, in comparison to the single aqueous phase system, resting cells showed reduced Qox activities in this 2LP system.

In the study reported here, possible factors limiting the activities achieved with Qox-containing resting cells were investigated, including intrinsic microbial activity (level of induction during growth and metabolic state of the cells), substrate and oxygen mass transfer, availability of oxygen (as the final electron acceptor during substrate hydroxylation), and glucose (as the energy source to maintain metabolically active cells), as well as toxic and/or inhibitory effects of the substrate, product, and organic solvent. As a proof of concept, the Qox-based process was combined with an efficient downstream processing.

Materials and methods

Bacterial strains, plasmid, and chemicals

Pseudomonas putida KT2440 [2] and plasmid pKP1 [26] were used as the recombinant host strain and the expression vector, respectively, for the functional expression of *qoxLMS* genes. Media components and other chemicals were obtained from Merck (quinaldine, ≥98%; Hohenbrunn, Germany), Sigma-Aldrich (4-hydroxyquinaldine, ≥98%; Steinheim, Germany), or Fluka (1-dodecanol, 98.5%; Buchs, Switzerland).

Media and growth conditions

Cells were either grown on Luria–Bertani broth or M9 minimal medium [29] supplemented with 0.5% (w/v) of benzoate in baffled Erlenmeyer flasks in horizontal shakers at 200 rpm and 30°C. Media contained 500 µg mL^{-1} ampicillin for selection of pKP1-containing cells. Induction of *qoxLMS* expression from plasmid pKP1 was achieved by benzoate, which served as the growth substrate.

Fed-batch cultivation and 2LP biotransformation

A 3-L bioreactor (KLF, Bioengineering, Wald, Switzerland) was used for batch and fed-batch cultivation of *P. putida* KT2440 (pKP1) as described previously [37]. To investigate benzoate metabolism and induction with benzoate, three different exponential feeding regimes for benzoate and ammonium, targeted at growth rates of 0.20, 0.35, and 0.50 h^{-1}, were applied during fed-batch cultivation. For 2LP biotransformations, cells grown at a growth rate of 0.35 h^{-1} were harvested, resuspended, and used with 1-dodecanol as the organic carrier solvent at an organic:aqueous phase ratio of 1:5, as described previously [37]. Samples from the organic and aqueous phases were prepared for gas chromatography (GC) analysis using known protocols [6].

Determination of specific hydroxylation activity in resting cell assays

Cells from batch and fed-batch cultivation were harvested by centrifugation (Multifuge 1 S-R; Kendro GmbH, Langenselbold, Germany) at 4°C and 4,600 *g* for 10 min. The cell pellet was then washed twice with 50 mM potassium phosphate buffer (pH 7.4) and resuspended in the same buffer supplemented with 1% (w/v) glucose as the energy source. Resting cell assays were performed with a cell dry weight (CDW) concentration of 0.5 g_{CDW} L^{-1} as described previously [37].

Phase separation and product isolation

After completion of biotransformation, the stable emulsion (approx. 210 mL), containing the *P. putida* cells, was treated in portions (approx. 52 mL in each run) with supercritical carbon dioxide ($scCO_2$) in a high-pressure vessel (Büchi, Uster, Switzerland). The conditions applied during the $scCO_2$ treatment were as follows: pressure (p) = 250 bar, temperature (T) = 45°C, CO_2 mass fraction (w_{CO_2}) = 0.75, and time (t) = 60 min [4]. After $scCO_2$ treatment, the organic phase (15 mL) was separated by centrifugation at 4,500 *g* for 40 min and placed into a pre-warmed separatory funnel. 4-hydroxyquinaldine was

extracted from 1-dodecanol under basic conditions using 1 M NaOH (4 × 3 mL). During extraction, the separatory funnel was heated using a heat gun to prevent solidification of the organic phase [melting point (T_m) of 1-dodecanol is 24°C]. The aqueous extracts were pooled and washed with diethyl ether (2 mL) to remove traces of organic solvent, and the pH was adjusted to 7 by the addition of 1 M HCl. The volume was reduced by heating until crystallization started to occur. At this point, the mixture was allowed to cool down slowly to room temperature. The resulting slurry was filtered and washed with ice-cold redistilled water (2 × 1 ml), and the mother liquor was collected and concentrated to yield a second crop of crystals. The combined crystals were dried overnight under vacuum to obtain a pure isolate with constant mass. A melting point apparatus (Büchi Melting Point B-540; Büchi Labortechnik AG, Flawil, Switzerland) was used to determine the melting temperature. Yields for phase separation and isolation were calculated using corresponding concentrations determined by GC.

Analytics

Samples from the single aqueous and 2LP biotransformations were analyzed by high-performance liquid chromatography (HPLC) and GC, respectively, for quinaldine and 4-hydroxyquinaldine quantification as described previously [37]. Benzoate, catechol, and muconic acid were identified and quantified using a CC Nucleosil 100-5 HD column (pore size 100 Å, particle size 5 µm, length 25 cm, inner diameter 4 mm; Macherey–Nagel, Oensingen, Switzerland) on an HPLC apparatus (Elite LaChrom, Merck–Hitachi, Darmstadt, Germany) with a mobile phase containing 0.1% (v/v) H_3PO_4 and 10% (v/v) methanol in water at a flow rate of 0.7 ml min^{-1}; detection was by UV at 230, 276, and 262 nm, respectively.

Glucose concentrations were determined by a spectrophotometric method using the Enzytec D-glucose kit following the instructions of the manufacturer (Scil Diagnostics GmbH, Viernheim, Germany). Protein concentrations were determined by the colorimetric Bio-Rad DC protein assay (Bio-Rad Laboratories, CA, US).

Results

Qox expression levels with different benzoate-based cultivation regimes

Qox activities of resting cells in a 2LP system [6.9 U g_{CDW}^{-1} with 1-dodecanol at 30°C, pH 7.4, 1500 rpm, without active aeration, organic:aqueous phase ratio of 1:5, biomass concentration of 9 g_{CDW} L^{-1}, 1% (w/v) glucose] were considerably lower than those of resting cells in a single aqueous phase system (20.7 ± 4.5 U g_{CDW}^{-1} at subtoxic substrate concentrations using the same aqueous medium) [37]. Achieved activities decreased even further in the course of 2LP biotransformations. During cell growth, benzoate functioned as both growth substrate and effector of XylS, the activator of P_m promoter-mediated *qoxLMS* expression from plasmid pKP1. Reduced induction due to low benzoate availability during benzoate-limited fed-batch growth, in contrast to non-limited batch growth, was a possible reason for the reduced initial activities during biotransformation. To investigate the effect of cultivation mode (batch and fed-batch) on Qox activity and the availability of benzoate to fully induce cells, we determined the concentrations of benzoate and the metabolites accumulating in the course of growth on benzoate as well as Qox activities as a measure for Qox expression levels (resting cell assays).

After batch growth for 8–9 h ($\mu = 0.51$ h^{-1}), at which time benzoate was completely consumed, a biomass concentration of 2.1 g_{CDW} L^{-1} was obtained with yields on benzoate ($Y_{X/B}$) and ammonium ($Y_{X/N}$) of 0.48 ± 0.01 and 4.88 ± 0.03 g_{CDW} g^{-1}, respectively. Cell growth was allowed to continue until 6.9, 9.4, and 4.9 g_{CDW} L^{-1} were reached via fed-batch cultivation applying three different feeding regimes aiming at growth rates (μ) of 0.2, 0.35, and 0.5 h^{-1}, respectively (Fig. 1). For the composition of the feed during fed-batch cultivation, the C:N ratio of substrate consumption during batch growth was considered in all cases. However, both benzoate and ammonium apparently became limiting when $\mu_{fed\text{-}batch}$ was 0.2 and 0.35 h^{-1}, respectively. No limitation of benzoate and ammonium was observed at a targeted μ of 0.5 h^{-1}. In all cases, the intended $\mu_{fed\text{-}batch}$ was achieved, at least initially. During batch cultivation, accumulation of the *ortho* pathway intermediates *cis*, *cis*-muconic acid (up to a concentration of 0.17 g L^{-1}) and benzoate *cis*-dihydrodiol (in trace amounts, not shown) was observed, whereas catechol did not accumulate throughout batch and fed-batch cultivation. The *cis*, *cis*-muconic acid produced during batch growth was consumed, when benzoate was limiting during fed-batch cultivation.

Qox activities determined in resting cell assays with cells harvested from batch and fed-batch cultures followed the same trend, independently of the benzoate feeding strategy. During batch cultivation, lower and even decreasing Qox activities (from 10.5 ± 0.3 to 8.5 ± 0.2 U g_{CDW}^{-1}) were observed as compared to the average Qox activities (F2–F4) during fed-batch cultivation (16.1 ± 0.4, 14.7 ± 0.6, and 15.2 ± 0.5 U g_{CDW}^{-1} at growth rates of 0.2, 0.35, and 0.5 h^{-1}, respectively) (Fig. 2). The Qox activities achieved during fed-batch cultivation are at the lower bound, but comparable to the activities reached in an earlier study with shake flask cultures (20.7 ± 4.5 U g_{CDW}^{-1}) [37]. These

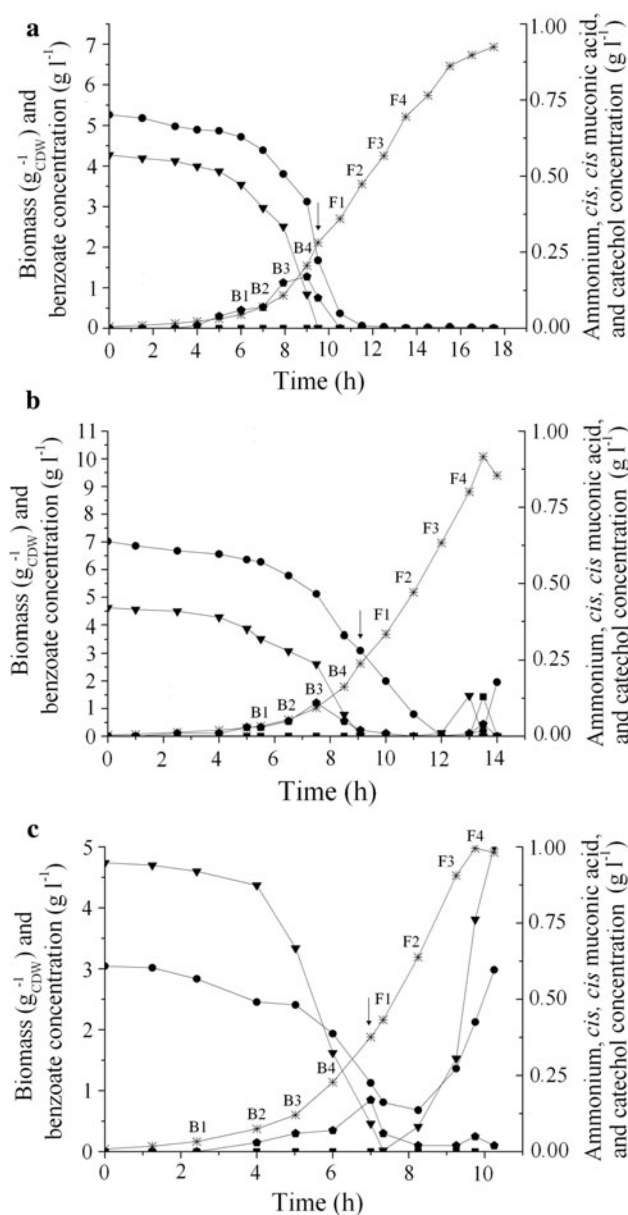

Fig. 1 Consumption of benzoate (*black inverted triangle*) and ammonium (*black circle*), production of *cis*, *cis*-muconic acid (*black pentagon*) and catechol (*black square*), and biomass (*asterisk*) during batch (*B*) and fed-batch (*F*) cultivation of *Pseudomonas putida* KT2440 (pKP1) followed by fed-batch cultivation at specific growth rates of 0.2 (**a**), 0.35 (**b**), and 0.5 h^{-1} (**c**). *Arrow* Start of fed-batch cultivation

results reveal that the reduced activities are not due to limited benzoate availability and thus reduced induction during fed-batch cultivation.

Mass transfer effects

The biotransformation rate not only depends on the intrinsic activity of microbial cells but also on the rate of substrate mass transfer to the microorganisms. Thus, the

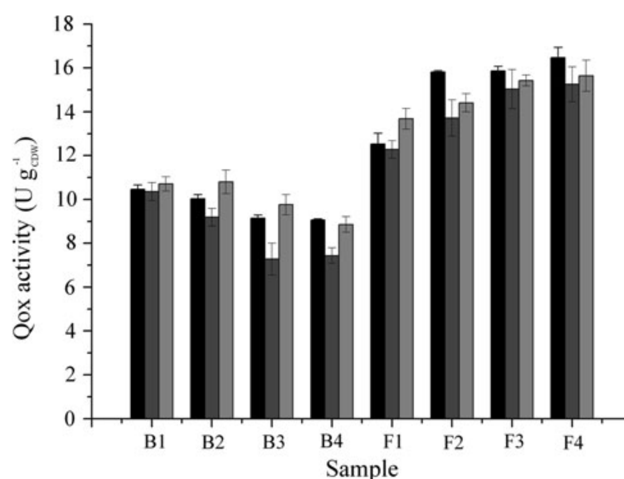

Fig. 2 Quinaldine 4-oxidase (*Qox*) activities of cells harvested from batch (*B*) and fed-batch (*F*) cultivation with a limiting benzoate feed for specific growth rates of 0.2 (low; *black bar*), 0.35 (medium; *dark-gray bar*), and 0.5 h^{-1} (high; *light-gray bar*). Activities were determined by resting cell assays as described previously [37]. Samples (*B1–B4*, *F1–F4*) were taken every hour (see Fig. 1 for details)

relative concentration of both available substrate and biocatalyst are critical parameters that possibly determine bioconversion efficiency. To investigate whether substrate mass transfer from the organic phase to the cells is limiting or not, the bioconversion rates at various substrate and cell concentrations and organic:aqueous phase ratios were determined in separate biotransformation experiments (Table 1).

At organic:aqueous phase ratios of 1:5 and 1:3, the initial Qox activities were almost identical, indicating that the interfacial area was not limiting the reaction rates. At a constant substrate concentration of 200 mM in the organic phase, the volumetric biotransformation rate increased linearly with increasing biomass concentration and became limited at high biomass concentrations (>8.7 $g_{CDW}\ L^{-1}$) (Table 1, Fig. 3a). At a constant biomass concentration of 8.7 $g_{CDW}\ L^{-1}$ in the aqueous phase, the initial Qox activity did not vary significantly at substrate concentrations between 100 and 300 mM (Table 1, Fig. 3b). These results reveal that substrate concentrations down to 100 mM and substrate mass transfer to the cells were not limiting the hydroxylation rates up to a biomass concentration of 8.7 $g_{CDW}\ L^{-1}$. Above 8.7 $g_{CDW}\ L^{-1}$, substrate mass transfer to the cells became limiting (Fig. 3a).

Glucose and oxygen availability during biotransformation

Glucose, which is potentially required as the energy source for the maintenance of biocatalyst integrity, may limit the catalytic activity in the later stages of biotransformation.

Table 1 Effect of organic:aqueous phase ratio and substrate and biomass concentration on initial specific activity of quinaldine 4-oxidase-containing *Pseudomonas putida* KT2440 (pKP1)

Organic:aqueous phase ratio	Substrate concentration (mM)	Biomass concentration (g_{CDW} L^{-1})	Initial Qox activity (U $g_{CDW}{}^{-1}$)
1:3	200	8.7	6.8 ± 0.3[a]
1:5	200	8.7	6.9 ± 0.8[a]
1:5	100	8.7	6.6 ± 0.6
1:5	300	8.7	5.4 ± 0.6
1:5	200	3.4	6.2 ± 0.2
1:5	200	6.2	5.6 ± 0.4
1:5	200	8.7	6.0 ± 0.1
1:5	200	14.3	3.6 ± 0.2
1:5	300	17.2	4.4 ± 0.4

Qox, Quinaldine 4-oxidase; CDW, cell dry weight

[a] Adapted from [37]

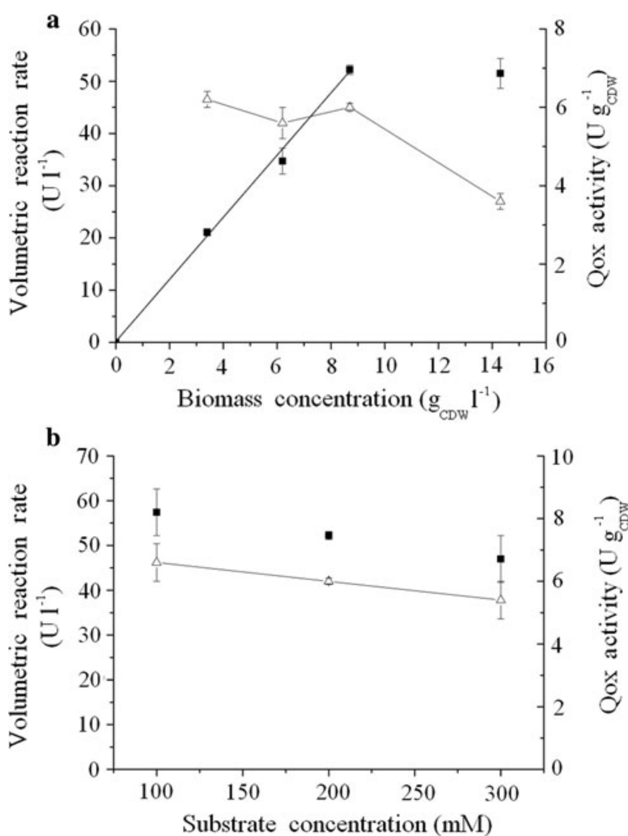

Fig. 3 Effect of biomass (**a**) and substrate concentration (**a**) on volumetric reaction rate (*black square*) and Qox activity (*open triangle*)

Thus, glucose concentration and consumption were investigated during biotransformation. Resting cells initially consumed glucose at a rate of 1.3 mmol $g_{CDW}{}^{-1}$ h^{-1}, which decreased to 0.3 mmol $g_{CDW}{}^{-1}$ h^{-1} after 3 h of biotransformation, and 1.6 g L^{-1} glucose was still present after 16 h. This result indicates that the biotransformation rate was not limited by the amount of available glucose for maintaining metabolically active cells.

Another factor, which may affect the achievable hydroxylation activity is the availability of molecular oxygen to which electrons generated by quinaldine hydroxylation are directly or indirectly (via electron transfer chain) transferred. Qox activities of 5.9 ± 0.2 and 6.0 ± 0.6 U $g_{CDW}{}^{-1}$ were obtained with 0.7 and 1.4 vvm (volume per volume per minute) aeration, respectively. Compared to the Qox activity of 6.0 ± 0.3 U $g_{CDW}{}^{-1}$ achieved without active aeration (oxygen transfer only from the headspace via stirring) [37], active aeration did not enhance the hydroxylation rate. In all cases, the dissolved oxygen tension (DOT) remained above 80%, indicating that oxygen availability did not limit biocatalyst activity.

Effect of product accumulation and organic phase on biocatalyst performance

The lower Qox activities in the 2LP system compared to the single aqueous phase system and the activity decrease during biotransformation may be caused by toxic and/or inhibitory effects of substrate, product, and/or organic phase. The toxic effect of substrate and product on cell physiology has been investigated in single aqueous phase systems by determining the substrate and product concentrations at which half maximal specific growth rates (2.5 and 1 mM, respectively) and complete growth inhibition (4 and 1.7 mM, respectively) were observed [37]. In the course of 2LP biotransformations, the maximum aqueous substrate and product concentrations of 0.74 and 0.43 mM, respectively, are below these toxicity limits. However, the presence of 1-dodecanol, with a relatively low log P_{oct} value of 5, may enhance the stress on cells. This was investigated by adding 1-dodecanol phases (organic:aqueous phase ratio: 1:5) containing different product concentrations to the growing cells.

The decrease in growth rate from 0.75 to 0.32 h^{-1} following the addition of pure 1-dodecanol to the growing

cells points to a toxic effect of this solvent. The addition of increasing product concentrations into the 1-dodecanol phases reduced the growth rate still further to 0.03 h^{-1} at a 4-hydroxyquinaldine concentration of 100 mM in 1-dodecanol (corresponding to an aqueous concentration of approx. 0.77 mM). These results reveal that 1-dodecanol and 4-hydroxyquinaldine together exert a considerable effect on cell physiology, which may in turn affect the hydroxylation activities during 2LP biotransformations.

Taking into consideration the possibility that substrate- and/or product-inhibition may also play a prominent role on enzyme level, we determined the Qox activities of resting cells in the presence of subtoxic and toxic substrate and product concentrations. At subtoxic (2 mM, the standard assay condition) and toxic (5 mM) substrate concentrations, the achieved Qox activities were 18.9 ± 0.2 and 17.5 ± 0.4 U $g_{CDW}{}^{-1}$, respectively (Fig. 4), indicating that the substrate, even at toxic concentrations, did not affect the achieved Qox activity. However, the addition of product to the reaction medium, even at a subtoxic concentration (1 mM), reduced the Qox activity by 25% compared to the standard assay conditions. At toxic concentrations (3 mM), the achieved Qox activity dropped to 43% of that observed at standard assay conditions (Fig. 4). These results indicate that there is a prominent inhibitory effect of the product, but not the substrate, at both subtoxic and toxic concentrations, and that both the inhibition of growth and catalytic activity follow different kinetics.

The effect of the organic solvent on Qox activity was tested with resting cells incubated with 1-dodecanol prior to quinaldine bioconversion and also with various concentrations of 1-dodecanol present during quinaldine bioconversion (Fig. 5). Pre-incubation of resting cells with 1-dodecanol [20% (v/v) of the culture volume] for 20 min before the start of the hydroxylation reaction (1-dodecanol was absent during the reaction) did not affect the achieved Qox activity, whereas the presence of 1-dodecanol during quinaldine bioconversion decreased the Qox activity by 42 and 46%, even at low concentrations of 21.5 µM (the saturation concentration of 1-dodecanol in water) and 1% (v/v), respectively. This indicates an inhibition of Qox activity by 1-dodecanol when the latter is present in the reaction medium.

To determine whether the presence of the organic phase and product accumulation in the course of the 2LP biotransformation cause cell toxification and cell lysis, the protein content in the reaction medium was analyzed at regular time intervals (see Materials and methods) and compared to cells which were incubated in the bioreactor under biotransformation conditions, but in the absence of substrate and organic phase. A 68% increase in protein concentration was observed within the first 30 min following the addition of the organic phase. However, no

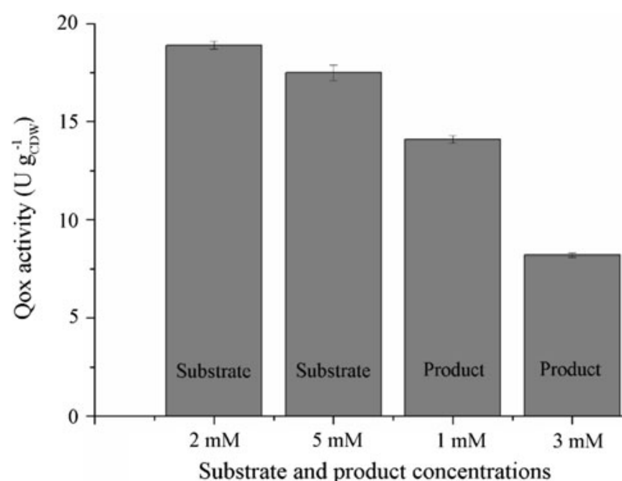

Fig. 4 Effect of substrate and product on Qox activity of resting *P. putida* KT2440 (pKP1)

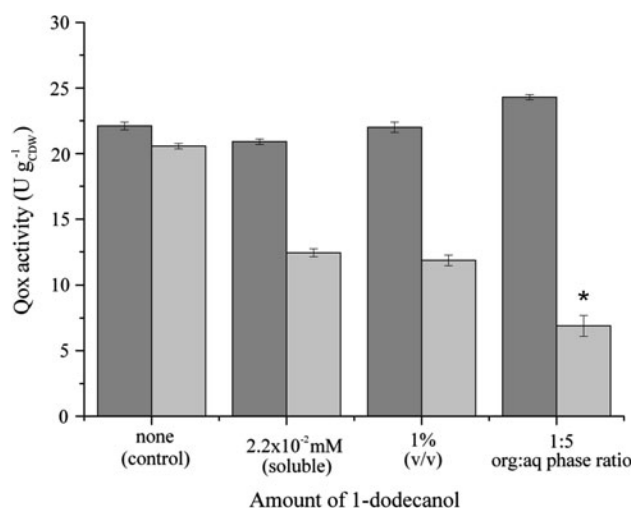

Fig. 5 Effect of 1-dodecanol on Qox activity. In the absence of 1-dodecanol {cells pre-incubated with 1-dodecanol [20% (v/v) of the culture volume] prior to resting cell assay} (*dark-gray bar*) and in the presence of 1-dodecanol during the resting cell assay (*light-gray bar*). *Asterisk* Data from bioreactor

further increase in protein concentration was observed with product accumulation during the biotransformation (results not shown). In contrast, the concentration of protein in the reaction buffer in which the cells were incubated without the organic phase and biotransformation increased gradually over time and, after 18 h, reached the same level as in the presence of the organic phase (86% more than the initial protein concentration of approx. 0.9 g L^{-1}). Clearly, addition of the organic phase initially accelerated cell lysis, but it did not promote protein release in the later stages of the biotransformation.

Process integration with simple and efficient downstream processing

Downstream processing in biocatalytic 2LP processes includes phase (emulsion) separation and product isolation from the organic phase, both of which are key parts of integrated process development. The emulsion formed during biotransformation was very stable, and the separation of organic and aqueous phases was not possible by centrifugation, even at high centrifugal force (13,000 *g*) and after prolonged centrifugation time (1 h). As an alternative, we tested treatment with $scCO_2$ for phase separation [4] followed by product isolation from the organic phase by liquid–liquid extraction. The separation of the organic phase by the $scCO_2$ treatment allowed the recovery of 15 ml of the organic phase (out of an initial volume of approx. 35 ml in the emulsion), corresponding to a volumetric organic phase recovery of 43%. Since the separated organic phase was enriched in product content after $scCO_2$ treatment, the overall product recovery based on the product amount in the emulsion before $scCO_2$ treatment was higher (57%). Product enrichment was caused by organic phase extraction into the $scCO_2$-phase and non-uniform product and 1-dodecanol loss due to the rapid release of CO_2, as discussed below (Table 2).

Following phase separation, 4-hydroxyquinaldine was extracted from the organic phase by liquid–liquid extraction under basic conditions (using 1 M NaOH as extractant), which allowed for the extraction of the product but not the substrate. Experiments with a model system in which quinaldine and 4-hydroxyquinaldine were dissolved in 1-dodecanol showed that, after two extraction steps, the product was quantitatively extracted into 1 M NaOH, whereas the substrate remained in the organic phase. After extraction (4-hydroxyquinaldine in the form of an ionic salt with Na^+), the solution was neutralized by the addition of HCl. Following crystallization from boiling water and drying overnight under vacuum to obtain a constant mass, a pure white isolate was obtained [$T_m = 233.1$–233.8°C (uncorrected); literature value: 232°C [1]). GC analysis of this white isolate, obtained from the emulsion by $scCO_2$ treatment, extraction, and crystallization, showed only a single peak with a retention time corresponding to that of a commercially available 4-hydroxyquinaldine standard. The product recovery achieved by liquid–liquid extraction was 84%, yielding 138 mg of product with a purity of >99.9%. This successful coupling of biotransformation and downstream processing illustrates the feasibility of Qox-based integrated bioprocessing on technical scale.

Discussion

Benzoate metabolism and induction during biocatalyst production

Growth inhibition by benzoate at concentrations above 10 mM due to reduced catechol 1,2-dioxygenase activity and benzoate-dependent elongation of lag phases has been reported previously for *P. putida* HL06-3 [3]. Such inhibition by benzoate also may explain the long lag phase of 6–7 h which we observed in our study during batch growth with an initial benzoate concentration of 35 mM (Fig. 1). Comes and Beelman [9] reported growth inhibition by sodium benzoate, a widely used food preservative, for *Escherichia coli* O157:H7. In that study, cells were shown to be severely damaged and killed due to acidification by benzoate, which enters cells as uncharged benzoic acid [9]. For *P. putida* KT2440, complete growth inhibition was observed at benzoate concentrations above 75 mM, whereas concentrations up to 45 mM did not show any effect on the growth rate [27]. Thus, the benzoate concentrations used in our experiments (35 mM) were not expected to cause any reduction in growth rate.

Dual benzoate and ammonium limitation during fed-batch growth at specific rates of 0.2 and 0.35 h^{-1} indicates a change in the C:N ratio of substrate consumption, as the feed composition was adjusted taking the C:N ratio calculated from batch cultures ($\mu_{max} = 0.5\ h^{-1}$) into account (Fig. 1a, b). However, when the feed was initiated upon complete consumption of benzoate, the *ortho* pathway intermediates that accumulated during batch growth were available as additional substrates for growth. This, together with an overestimated C:N ratio (calculated from batch cultivation), explains the increased relative consumption of the nitrogen source, resulting in the observed N-limitation. Under N-limited conditions, *P. putida* is known to produce polyhydroxyalkanoates (PHAs) from fatty acids and sugars

Table 2 The amounts of 1-dodecanol, quinaldine, and 4-hydroxyquinaldine before and after supercritical carbon dioxide treatment

Product	In the emulsion after biotransformation (g)	In the recovered organic phase after $scCO_2$ treatment (g)	Loss due to CO_2 release (g)
1-Dodecanol	29.08	12.46	16.62
Quinaldine	0.03	0.02	0.01
4-Hydroxyquinaldine	0.29	0.16	0.13

$scCO_2$, Supercritical carbon dioxide

[16]. PHA accumulation may be the reason for the decrease in bacterial growth, as previously reported for *P. putida* KT2442 [7].

The low and slightly decreasing activities during batch cultivation considerably increased with the initiation of the feed and during fed-batch cultivation with a controlled DOT of 30% (Fig. 2). The oxygen level has previously been reported to influence the transcription of genes encoding molybdenum-containing enzymes or proteins involved in the biosynthesis or regulation of these enzymes [32]. For example, XdhC is a protein involved in molybdenum cofactor (Moco) insertion into and the maturation of xanthine dehydrogenase (XDH), a molybdenum-containing hydroxylase like Qox [24]. It has been shown that the *xdhC* expression level, and thus XDH activity, decreased with increasing oxygen concentration in the medium [13]. Similarly, we found that reduction of the DOT level from 30 to 20% led to a 17% increase in Qox activity (data not shown). Such sensitivity to high DOT levels may explain the higher activities during fed-batch cultivation where the DOT level was controlled at 30%, whereas during batch cultivation, high initial DOT levels of 95–100% decreased to minimum values of 30–50% at the end of batch growth. This sensitivity also explains the lower activities achieved during batch cultivation in reactors as compared to shake flasks, where dissolved oxygen levels are typically lower.

The Qox activities of cells taken from fed-batch cultivation (Fig. 2) are comparable to those of cells taken from shake flask cultures which were not limited by benzoate (20.7 ± 4.5 U g_{CDW}^{-1}) [37], illustrating that the cells are fully induced and that limited benzoate availability was not the reason for the reduced activities during 2LP biotransformations.

Mass transfer effects

Substrate mass transfer is a key parameter determining the bioconversion rates achievable in a 2LP system, where substrate uptake may be accomplished by three different mechanisms [11, 31]. Microbial cells may assimilate the substrate either from the aqueous phase in dissolved form (the first uptake mechanism), via solubilization by surface-active compounds produced by the microorganisms (the second uptake mechanism), and/or directly from the organic phase (the third uptake mechanism). Thus, mass transfer from the organic to the aqueous phase and properties of both the microorganisms and the substrate (hydrophobicity and solubility in aqueous solution), as well as reaction conditions (phase ratio, mixing, cell concentration, and substrate concentration) have to be considered to evaluate mass transfer limitation. However, it is difficult to distinguish experimentally between uptake mechanisms that may apply simultaneously [40].

Aromatic hydrocarbons are poorly soluble in water, and the dissolution of such compounds is critical in terms of bioavailability [33]. Considering the partition coefficient of 180 ± 10 for quinaldine in the 1-dodecanol-based 2LP system [37], the maximal aqueous quinaldine concentrations (in equilibrium) are approximately 1.1, 0.7, and 0.5 mM at the start of biotransformation, at the time point when reduced Qox activities were observed, and after 6 h, respectively. Assuming that only quinaldine dissolved in the aqueous phase serves as the reaction substrate (the first uptake mechanism) and considering the kinetics [V_{max} (maximum rate) $= 19.04 \pm 0.29$ U g_{CDW}^{-1} and $K_s = 64 \pm 4$ μM] [37], Qox activities are expected to be in the range of V_{max} in the case of mass transfer over the phase boundary not being limiting. Such a mass transfer limitation, for the first uptake mechanism, would depend on substrate and biocatalyst concentrations as well as on surface area (and thus phase ratio and stirring speed).

Hydrocarbon-degrading microorganisms, including *Pseudomonas* species, are capable of synthesizing biosurfactants which enhance hydrocarbon solubility in water by microemulsion formation [10]. However, in samples taken during 2LP biotransformations, aqueous quinaldine concentrations were in the range of values estimated using the partition coefficient. This indicates that the substrate solubility was not enhanced by surface-active compounds. Thus, the second mechanism seems not to be involved in substrate uptake.

For water-immiscible or poorly water soluble substrates, substrate uptake via the direct contact of cells with the organic phase is assumed [40]. In this case, microbial activity highly depends on the interfacial area and thus on organic phase fraction and stirring speed, but also on substrate and biomass concentrations.

Based on these considerations, uptake most probably occurred via the first and/or the third mechanism and was not limited under all conditions tested except for cell densities clearly above 8.7 g_{CDW} L^{-1} (Table 1).

Metabolic activity and biocatalyst performance

Pseudomonas putida KT2440 (pKP1) was used as a non-growing whole-cell biocatalyst with glucose supplied in the reaction medium as the energy source for cell maintenance. The presence of glucose has been found to enhance the biocatalyst stability, especially in long-term experiments (approx. 42 h), but not to be necessary to sustain initial activities [37]. The decrease in glucose uptake rate during biotransformation (from 1.3 to 0.3 mmol g_{CDW}^{-1} h^{-1}) points to a decrease in the metabolic activity of the resting cells during the first 2–3 h. A similar decrease in glucose uptake rate (from 1.2 to 0.5 mmol g_{CDW}^{-1} h^{-1}) was

observed with resting cells under the same conditions without 1-dodecanol and biotransformation. This result indicates that the decrease in glucose uptake rate, which can be linked to a decrease in metabolic activity, was not due to the presence of 1-dodecanol and biotransformation, but was rather an adaptation to resting cell conditions, as has been proposed for resting *E. coli* cells [19]. However, in contrast to Qox catalysis, styrene monooxygenase-catalyzed styrene epoxidation by resting recombinant *E. coli* was found to depend directly on metabolic activity (for NADH regeneration), as exemplified by increased glucose uptake rates during oxygenase catalysis [19].

Oxygen mass transfer

Molecular oxygen is not required as the oxygen donor for quinaldine hydroxylation, but rather as a respiration substrate functioning directly or indirectly (via the respiratory chain) as electron acceptor for quinaldine hydroxylation. Since oxygen intake was achieved only by stirring and by utilizing the head-space oxygen, the Qox-based bioprocess described here may be limited by oxygen transfer. However, the DOT level remaining above 80% during the course of the biotransformation and the fact that active aeration did not enhance biocatalyst activities indicate that the low oxygen demand can be accomplished without active aeration. Interestingly, the DOT level in the presence of resting cells and in the absence of 1-dodecanol dropped to 10%. Linek and Benes studied the effect of solvents in 2LP systems on oxygen transfer and reported a correlation of oxygen transfer with the spreading coefficient S_{OW} of organic solvents [21]. Organic solvents with a negative S_{OW} show no influence on the mass transfer coefficient, but a negative influence on the specific exchange area, whereas organic solvents with a positive S_{OW} have a positive effect on both the mass transfer coefficient and the specific exchange area. Clarke and colleagues explained the k_La (mass transfer coefficient) enhancement by the decrease in surface tension in the presence of organic phases, which additionally act as an oxygen reservoir [8]. The strategy of adding small droplets of organic solvent to a culture medium to improve oxygen transfer has been reported previously [17, 18, 28]. The S_{OW} of 1-dodecanol was calculated to be negative (-11.9 mN m^{-1}) using previously reported interfacial tension data at 30°C [35, 39]. This contradicts the observed enhancement of oxygen transfer by 1-dodecanol which, however, still functions as an oxygen reservoir. Such effects of organic phases with a negative spreading coefficient are still under discussion [12]. Our results indicate that 1-dodecanol, despite its negative S_{OW}, enhanced oxygen transfer during biotransformation.

Effect of substrate, product, and organic solvent on biocatalyst performance

Substrate, product, and organic solvent may affect biocatalyst performance both on the cell level (toxicity) and the enzyme level (inhibition). The toxic effects of quinaldine (log $P_{oct} = 2.59$) and 4-hydroxyquinaldine (log $P_{oct} = 1.65$) have been investigated and discussed previously [37], whereas a possible inhibition of biocatalyst performance is discussed herein. Quinaldine hydroxylation was found not to be affected by toxic quinaldine concentrations. Thus, the reduced activities in the beginning and the decrease in activity during the course of 2LP biotransformations cannot be related to substrate toxicity or inhibition. However, subtoxic (1 mM) and toxic (3 mM) 4-hydroxyquinaldine concentrations reduced Qox activities, indicating the occurrence of product inhibition on the enzyme level.

The low initial activity of 6.9 ± 0.8 U g_{CDW}^{-1} may be due to the presence of the organic phase. In addition to revealing the toxicity of 1-dodecanol (growth rate reduction by 64% in its presence), the results presented in Fig. 5 reveal that 1-dodecanol has a prominent inhibitory effect on hydroxylation activity (Fig. 5, light-gray bars). The unaffected Qox activities of cells incubated with 1-dodecanol prior to biotransformation (Fig. 5, dark-gray bars) indicate that 1-dodecanol did not have a deactivating effect on cells (cell toxification or disruption of electron transport chain) and did not cause irreversible Qox deactivation.

Organic solvents and toxic aromatic compounds are known to cause significant changes in membrane structure and function due to the disruption of the membrane potential, increase in membrane fluidity, permeabilization, and change in the lipid-to-protein ratio [33]. These changes cause a significant decrease in cell viability and even destructive openings in the cell envelope [25]. Membrane disruption and cell lysis would result in protein release into the reaction medium. Our results on the time-course of protein concentrations in the reaction medium in the presence of the organic phase indicate that the introduction of 1-dodecanol affected membrane integrity and led to partial cell lysis. However, the glucose consumption rates of cells which were incubated with 1-dodecanol were only slightly reduced, indicating that cells incubated in the presence of 1-dodecanol remained metabolically active. Overall, the obtained results suggest that inhibition of the electron transfer chain and/or Qox by 1-dodecanol reduced the initial Qox activities, which subsequently dropped further due to the accumulation of inhibiting product during the course of biotransformation. To improve the achieved productivities, an alternative organic phase would be a solution. However, among the 13 solvents tested, alkanols, such as 1-decanol and 1-dodecanol, were the only

feasible organic solvents in terms of substrate and product solubility [37]. In terms of biotransformation performance, 1-dodecanol was preferable to 1-decanol. Shorter and longer chain alcohols are not suitable due to toxicity (log $P_{oct} < 4$) and solidification problems (melting temperatures above room temperature), respectively. For in situ product removal, solid phase extraction (SPE) and 2LP approaches are the best studied and most applied methods [23]. Consequently, SPE and the use of neoteric solvents such as liquid extractants [22] should be tested as possible alternatives to the integrated 2LP approach described in this study.

Downstream processing

For whole-cell 2LP biocatalysis, the formation of stable emulsions makes phase separation difficult on a technical scale. $scCO_2$ has recently been shown to destabilize such emulsions efficiently and irreversibly [4]. This technique was successfully used for the separation of the stable 1-dodecanol-aqueous emulsion, which was presumably formed due to the emulsifying effect of microbial cells, as shown for *E. coli* in a 2LP system with (bis)-ethylhexyl phthalate as organic phase. Erickson and Nakahara attributed this phenomenon to the prevention of coalescence due to the presence of cells [14]. The low recovery of the organic phase (15 out of 35 ml) can be explained by the dissolution of 1-dodecanol in $scCO_2$ during phase separation followed by 1-dodecanol loss during fast CO_2 release. The latter involved a pressure decrease, which in turn caused a temperature decrease and 1-dodecanol solidification. The lower solubility of the product in $scCO_2$ explains the increase of product concentration in the remaining organic phase (Table 2). The recovery can be improved significantly by optimizing the setup (tubings, etc.), keeping the system temperature above the T_m of 1-dodecanol, and introducing the emulsion in a continuous way. The application of $scCO_2$ for phase separation in the described emulsion (containing 1-dodecanol and *P. putida* KT2440) broadens the scope of $scCO_2$-based phase separation in terms of microbial strain and solvent used.

Following treatment with $scCO_2$, 4-hydroxyquinaldine was isolated from the organic phase via liquid–liquid extraction. As both 4-hydroxyquinaldine (product) and quinaldine (starting material) readily form HCl salts, extraction under acidic conditions was disregarded in favor of extraction under basic conditions. The pK_a of the hydroxyl group in 4-hydroxyquinoline, a compound analogous to 4-hydroxyquinaldine, has been experimentally determined ($pK_a = 11.06$) [36]. Because of the acidity of the 4-hydroxyl group, the 4-hydroxyquinaldine was selectively extracted from the organic phase using 1 M NaOH solution as basic aqueous extractant, allowing the separation of product from unreacted substrate. After neutralization, 4-hydroxyquinaldine could be crystallized directly from the resulting aqueous phase. The ease of and high product recovery (84%) achieved by liquid–liquid extraction coupled with a preceding phase separation by means of $scCO_2$ emphasizes the technical feasibility and efficiency of integrated Qox-based 2LP bioprocessing.

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