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ORIGINAL PAPER

Regioselective aromatic hydroxylation of quinaldine by water using quinaldine 4-oxidase in recombinant *Pseudomonas putida*

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Abstract Biocatalytic hydrocarbon oxyfunctionalizations are typically accomplished using oxygenases in living bacteria as biocatalysts. These processes are often limited by either oxygen mass transfer, cofactor regeneration, and/or enzyme instabilities due to the formation of reactive oxygen species. Here, we discuss an alternative approach based on molybdenum (Mo)-containing dehydrogenases, which produce, rather than consume, reducing equivalents in the course of substrate hydroxylation and use water as the oxygen donor. Mo-containing dehydrogenases have a high potential for overcoming limitations encountered with oxygenases. In order to evaluate the suitability and efficiency of a Mo-containing dehydrogenase-based biocatalyst, we investigated quinaldine 4-oxidase (Qox)-containing *Pseudomonas* strains and the conversion of quinaldine to 4-hydroxyquinaldine. Host strain and carbon source selection proved to be crucial factors influencing biocatalyst efficiency. Resting *P. putida* KT2440 (pKP1) cells, grown on and induced with benzoate, showed the highest Qox activity and were used for process development. To circumvent substrate and product toxicity/inhibition, a two-liquid phase approach was chosen. Without active aeration and with 1-dodecanol as organic carrier solvent a productivity of 0.4 g l_{tot}^{-1} h^{-1} was achieved, leading to the accumulation of 2.1 g l_{tot}^{-1} 4-hydroxyquinaldine in 6 h. The process efficiency compares well with values reported for academic and industrially applied biocatalytic oxyfunctionalization processes emphasizing the potential and feasibility of the Qox-containing recombinant cells for heteroaromatic carbon oxyfunctionalizations without the necessity for active aeration.

Keywords Biocatalysis · *Pseudomonas putida* · Molybdenum-containing dehydrogenase · Two-liquid phase · Resting cell biotransformation

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Introduction

Quinaldine 4-oxidase (Qox), a molybdenum-containing enzyme using water as hydroxylating agent, is interesting for overcoming intrinsic limitations encountered with other oxidoreductases such as oxygenases, peroxidases, and oxidases for carbon oxyfunctionalizations. For instance, oxygenases, the most applied enzyme class for C–H oxyfunctionalization, derive the oxygen atom introduced into the product from molecular oxygen and typically depend on an additional electron donor like NAD(P)H to completely reduce oxygen. In such molecular oxygen-requiring reactions, the oxygen supply has to be well controlled to avoid oxygen transfer limitations from gas to liquid phases and/or enhanced biocatalyst inactivation by reactive oxygen species [29]. Oxygen mass transfer, poor enzyme stabilities, and often low catalytic rates are key limitations for the development of an efficient biocatalytic process based on oxygenases [9, 11]. In addition, the necessity for the regeneration of redox cofactors such as NADH or NADPH leads to increased costs and complexity, independent of the

biocatalyst form used, i.e., whole cells or isolated enzymes [57]. In contrast to oxygenases, Mo-containing dehydrogenases use water as oxygen donor and produce reducing equivalents during hydroxylation [15, 21]. Thus, these enzymes have the potential to overcome the limitations encountered with other oxidoreductases, e.g., oxygen supply, reactive oxygen species, and cofactor regeneration, for efficient in vivo oxyfunctionalization reactions.

Qox originates from the Gram-positive bacterium *Arthrobacter nitroguajacolicus* Rü61a, which utilizes quinaldine as the sole source of carbon, nitrogen, and energy via the anthranilate pathway (Fig. 1) [24, 39]. Qox has a broad substrate spectrum including a variety of N-heterocyclic substrates [50]. Remarkably, quinoline, 8-chloroquinaldine, 2- and 8-monochloroquinoline, isoquinoline, 1,2-benzodiazine, quinazoline, and phthalazine are converted at a higher rate than quinaldine, the prototype substrate serving as model substrate in this study. Furthermore, Qox oxidizes aromatic aldehydes such as benzaldehyde, salicylaldehyde, vanillin, and cinnamaldehyde to the corresponding acids [15, 50]. Short-chain aliphatic aldehydes are, however, not converted. Qox is a molybdo-iron/sulfur flavoprotein with a hexameric $(LMS)_2$ structure and contains a molybdopterin cytosine dinucleotide cofactor (Mo-MCD) in its large subunit *L*, FAD in its medium subunit *M*, and two distinct [2Fe–2S] clusters in its small subunit *S* [13]. The Mo-center catalyzes substrate hydroxylation upon which two electrons are transferred via the [2Fe–2S] clusters and FAD to the final electron acceptor, which is not known for many Mo-containing dehydrogenases [16]. For Qox, electrons may be directly transferred to molecular oxygen [50] or indirectly via the respiratory chain. As molecular oxygen does not function as oxygen donor, but rather as respiration substrate, the demand for molecular oxygen is reduced in technical applications. Furthermore, hydroxylating dehydrogenases may even be applied in alternative respiratory systems, i.e., under anaerobic conditions where electrons generated during substrate hydroxylation are directed to an alternative electron acceptor such as sulphate or nitrate, rather than molecular oxygen.

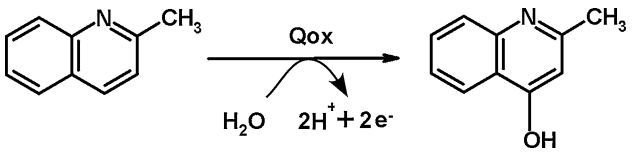

Fig. 1 First step in the *Anthranilate pathway* of quinaldine degradation. Quinaldine 4-oxidase hydroxylates quinaldine in *ortho* position to 4-hydroxyquinaldine, introducing the oxygen atom from water. Under the assay and analysis conditions, only the enol tautomer of the product was observed

Until recently, studies on Qox [12, 16, 50] and other Mo-containing dehydrogenases [12, 31, 52] focused mainly on structural and biochemical investigations. This study constitutes the first biotechnological in vivo application of Qox-containing recombinant *P. putida* strains for productive biocatalytic C–H oxyfunctionalizations and represents the potential of Qox to achieve carbon oxyfunctionalizations without active aeration, reducing the process complexicity, costs, and limitations in technical applications.

Materials and methods

Bacterial strains, plasmid

Recombinant *qoxLMS* gene expression in *Pseudomonas* strains was achieved by means of the plasmid pKP1 [38], a *qoxLMS*-containing derivative of the broad-host-range cloning vector pJB653. The expression of *qoxLMS* is regulated by the plasmid-encoded XylS protein which activates the P_m promoter-mediated expression in the presence of XylS effectors such as benzoate or 2-methylbenzoate. The strains, plasmid, and their relevant properties are summarized in Table 1. *Pseudomonas* strains were transformed with plasmid pKP1 by electroporation (EasyjecT, EQUIBIO, Thermo Electron, USA).

Media and growth conditions

Bacteria were either grown on Luria–Bertani (LB) broth or M9 minimal medium [45] supplemented with 0.5% (w/v)

Table 1 Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant properties	Reference or source
P. putida KT2440	TOL plasmid-cured derivative of *P. putida* mt-2	[1]
Pseudomonas sp. strain VLB120	Wild-type, styrene prototroph, solvent-tolerant *Pseudomonas* strain	[35]
P. putida GPo12	OCT plasmid-cured derivative of *P. putida* GPo1	[48]
P. putida S12	Wild-type, styrene prototroph, solvent-tolerant *Pseudomonas* strain	[20]
pJB653	Broad-host-range cloning vector; P_m promoter, *xylS*, Amp^r	[7]
pKP1	*qoxLMS* genes inserted into pJB653	[38]

of carbon source (glucose, glycerol, citrate, succinate, or benzoate) and 500 μg ml^{-1} ampicillin to select for plasmid pKP1. Cultures were routinely incubated in baffled Erlenmeyer flasks in horizontal shakers at 200 rpm and 30°C. To investigate quinaldine and 4-hydroxyquinaldine toxicity, growth inhibition of *P. putida* KT2440 was studied by determining growth rates after addition of various inhibitor concentrations as described elsewhere [10].

Chemicals

Quinaldine (≥98%) and 4-hydroxyquinaldine (≥98%) were obtained from Merck (Hohenbrunn, Germany), and Sigma-Aldrich (Steinheim, Germany), respectively. 1-Dodecanol (98.5%) and 1-decanol (≥95%) were obtained from Fluka (Buchs, Switzerland).

Determination of enzyme activity in resting cell assays

After precultivation in LB medium, cells harboring pKP1 were grown on M9 medium with different carbon sources and induced with 2 mM 2-methylbenzoate when an optical density at 450 nm (OD_{450}) of 0.6–0.7 was reached. Three hours after induction, cells were harvested by centrifugation (Multifuge 1 S-R, Kendro GmBH, Germany) at 4°C and 4,600 × *g* for 10 min. The cell pellet was washed twice and resuspended in 50 mM potassium phosphate buffer (pH 7.4) supplemented with 1% (w/v) energy source to an OD_{450} of 2.3 corresponding to a cell dry weight concentration of 0.5 g_{CDW} l^{-1}. The correlation factor (0.223) for OD_{450} to cell dry weight (CDW) was determined as described before [4]. The cell suspension was then distributed into Pyrex tubes in 2-ml aliquots and adapted to the reaction conditions in a water bath (Aquatron, Infors AG, Switzerland) at 30°C and 250 rpm for 5 min. The reaction was initiated by adding 4 μl of a 1 M quinaldine stock solution in ethanol and stopped by adding 2 ml ice-cold methanol (reaction time as indicated in the text). Cells were removed by centrifugation (4,600 × *g*, 4°C, 10 min) and the supernatant was analyzed for quinaldine and 4-hydroxyquinaldine by HPLC. The specific activity was calculated as enzyme activity per g_{CDW} (U g_{CDW}^{-1}), where one unit (U) is the activity which produces 1 μmol of product per min.

Determination of partition coefficients

The partition coefficients of quinaldine and 4-hydroxyquinaldine in a two-liquid phase system consisting of M9 medium and 1-decanol or 1-dodecanol were determined by adding different concentrations of the compounds (50, 100, 150, and 200 mM) into a mixture of equal volumes (1 ml) of the two phases. Phase equilibration, phase separation, and sample preparation for GC analysis were performed as described elsewhere [10]. The partition coefficient (*P*) is defined as the ratio of organic to aqueous phase concentrations of the compound of interest.

Two-liquid phase biotransformations

A preculture on LB medium was first diluted 100-fold in 100 ml of M9 medium supplemented with 5 g l^{-1} benzoate, which was then used to inoculate 2 l of the same medium in a 3-l bioreactor (KLF, Bioengineering, Wald, Switzerland). The pH was kept constant at 7.4 by using 5 M NaOH and 30% (v/v) H_3PO_4. After complete benzoate consumption during batch cultivation (cell concentration: 1.9 g_{CDW} l^{-1}), an exponential feed of 25% (w/v) benzoate and 4% (w/v) ammonium was started. During this fed-batch phase, the aeration rate was set at 1.8 vvm and the dissolved oxygen tension (DOT) was maintained above 30% of saturation by adjusting the stirring speed. Cells were grown at a predetermined growth rate of 0.5 h^{-1} until a biomass concentration of 6.7 g_{CDW} l^{-1} was obtained, then harvested by centrifugation (4,600 × *g*, 4°C, 10 min) and resuspended to a cell density of 9 g_{CDW} l^{-1} in 50 mM potassium phosphate buffer containing 1% (w/v) glucose. Biotransformations were performed in 300-ml RALF reactors (Bioengineering AG, Wald, Switzerland) at a stirring speed of 1,500 rpm. 1-Decanol or 1-dodecanol, containing quinaldine, was added to the resting cell suspensions in varying phase ratios, and samples were taken at regular time intervals. Organic and aqueous phases were separated by centrifugation (Centrifuge 5403, Dr. Vaudaux AG, Switzerland) at 13,000 × *g* for 10 min and prepared for GC analysis as described elsewhere [10].

Analytical procedures

Identification and quantification of quinaldine and 4-hydroxyquinaldine were performed using HPLC and GC. A CC Nucleosil 100-5 C18 HD column (100-Å pore size, 5-μm particle size, 25 cm × 4 mm inner diameter; Macherey–Nagel, Oensingen, Switzerland) was used to separate the analytes on an HPLC apparatus (Elite LaChrom, Merck-Hitachi, Germany) at a flow rate of 0.7 ml min^{-1} with a mobile phase of 70% water and 30% methanol (with a gradient to obtain 100% methanol in 15 min) and DAD detection at 211 nm. Benzoate was quantified by using the same column with a mobile phase of 30% acetonitrile and 70% water containing 0.1% H_3PO_4 at a flow rate of 0.7 ml min^{-1} and UV detection at 210 nm. Samples from the two-liquid phase biotransformation and partitioning experiments were analyzed by GC (Trace GC Ultra, Thermo Fisher Scientific, Waltham, Massachusetts equipped with a 30-m factor-four capillary column VF-5ms (Varian, Middelburg, the Netherlands)) with N_2 as the

carrier gas and FID detection. A temperature profile was applied as follows: from 50 to 100°C at a rate of 15°C min^{-1}, from 100 to 300°C at 50°C min^{-1}, and 300°C for 3 min.

Results

P. putida KT2440: the most promising host for recombinant *qoxLMS* expression

Host strain selection is a key parameter effecting biocatalyst performance. For recombinant *qoxLMS* expression and Qox-based biocatalysis, host selection was restricted to *Pseudomonas* strains, which, in contrast to *E. coli*, are able to produce the Mo-MCD cofactor [38]. The use of wild-type *A. nitroguajacolicus* Rü 61a was not suitable, since its growth on chemically defined media was slow. The strain also further degraded the product of interest via the anthranilate pathway. Two solvent-tolerant strains (*P. putida* S12 and *Pseudomonas* sp. strain VLB120) and two strains generally suitable for recombinant gene expression (*P. putida* GPo12 and *P. putida* KT2440) were first tested for their ability to metabolize quinaldine (see Table 1 for strain characteristics). None of the strains showed significant growth on quinaldine within 50 h. Subsequently, these strains were transformed with plasmid pKP1 and evaluated for Qox activity in resting cell assays after cultivation on various growth substrates (benzoate, glucose, citrate, glycerol, and succinate) and induction with 2-methylbenzoate. Qox activity also was taken as a measure for gene expression, since none of the three components of QoxLMS was distinguishable from proteins in the cell extract on standard SDS-PAGE gels. *P. putida* S12 and *P. putida* KT2440 showed the highest activities (14.7 ± 1.1 and 25.8 ± 0.7 U g_{CDW}^{-1}, respectively) when grown on benzoate (Fig. 2), whereas *P. putida* GPo12 and *Pseudomonas sp.* strain VLB120 showed no or low Qox activities, respectively, with all growth substrates used. Growth on glycerol was very slow for all strains and no activity was observed. The results indicate that the achieved Qox activity is highly dependent on the host strain and the growth substrate used. In conclusion, *P. putida* KT2440 was the most promising host and thus was further used for the development of a Qox-based biocatalytic process.

Careful carbon and energy source selection enhances the Qox activity

As the growth substrate considerably affected the Qox activity achieved with different *Pseudomonas* strains, the effect of using different carbon and energy sources during both growth and the following bioconversion with resting cells was evaluated in more detail for *P. putida* KT2440

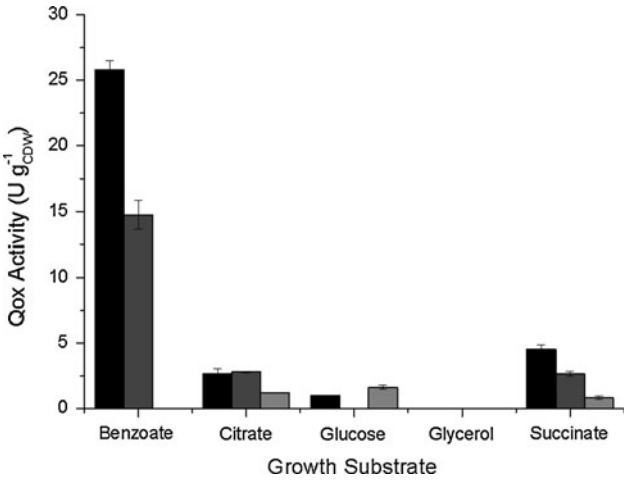

Fig. 2 Quinaldine 4-oxidase activities of various recombinant *Pseudomonas* strains grown on various growth substrates and induced with 2-methylbenzoate. Resting cell assays were carried out for 10 min with 1% (w/v) glucose as energy source and a cell concentration of 0.5 g l^{-1}. *Black bar P. putida* KT2440 (pKP1), *dark grey bar P. putida* S12 (pKP1), *grey bar Pseudomonas* sp. strain VLB120 (pKP1)

(pKP1). Thereby, growth rates and cell yields also were considered as these parameters are important for an efficient biocatalyst production.

With succinate, the highest growth rate was achieved in the absence of induction (0.83 h^{-1}), but the obtained Qox activities after induction were rather low (≤8.6 U g_{CDW}^{-1}). Growth on benzoate, being itself an inducer of *qoxLMS* expression, enabled a high Qox activity of up to 27.2 U g_{CDW}^{-1} and still a reasonable growth rate of 0.60 h^{-1}. Compared to cells grown on glucose, citrate, and succinate, benzoate-grown cells showed at least threefold higher activities with all energy sources applied during the bioconversion and irrespective of induction with 2-methylbenzoate (Fig. 3). Achieved Qox activities were similar with all energy sources applied during resting cell bioconversions except for benzoate, which reduced activities, e.g., by half for benzoate-grown cells. Even without energy source, activities of benzoate-grown cells were unaffected (and twice as high as in the case of benzoate addition), pointing out that in the tested period of 10 min, there was no requirement for an energy source. A potential inhibition by benzoate was further investigated for benzoate-grown cells by varying the amount of benzoate in resting cell assays. Indeed, the Qox activity decreased with increasing benzoate concentrations (data not shown). Thus, benzoate inhibits Qox activity, although it is the most suitable carbon and energy source for *qoxLMS* expression during growth. This inhibition by benzoate hinders the use of growing cells for biotransformation. In general, no by-product formation was observed, i.e., 4-hydroxyquinaldine was the sole product. In conclusion, the use of resting *P. putida* KT2440 (pKP1) grown on benzoate was considered

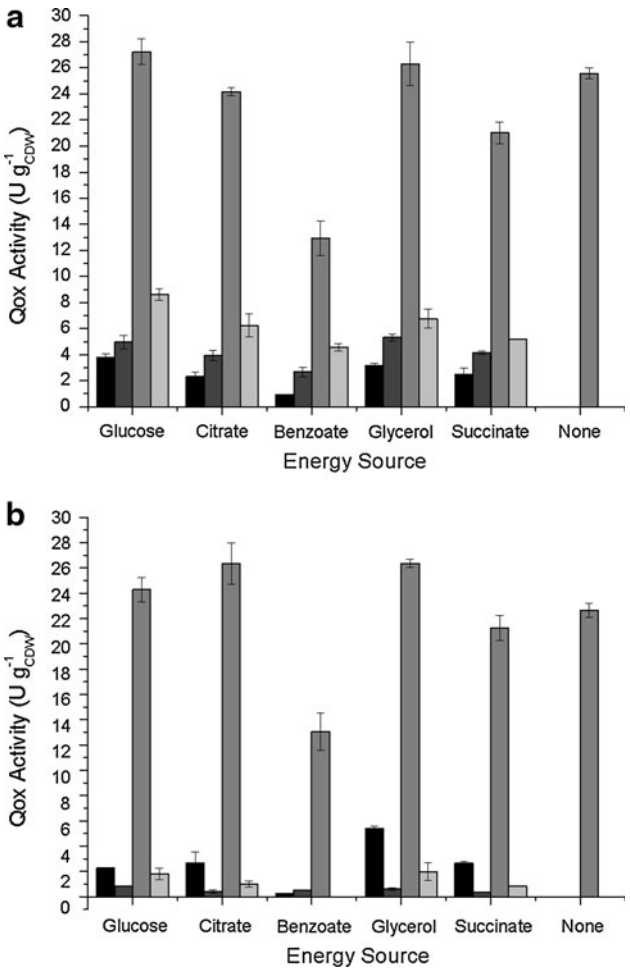

Fig. 3 Quinaldine 4-oxidase activities of *P. putida* KT2440 (pKP1) utilizing various growth substrates and energy sources with (**a**) and without (**b**) induction by 2-methylbenzoate. Resting cell assay without an energy source was only carried out with cells grown on benzoate. Growth substrates: *Black bar* glucose, *dark grey bar* citrate, *grey bar* benzoate, *light grey bar* succinate

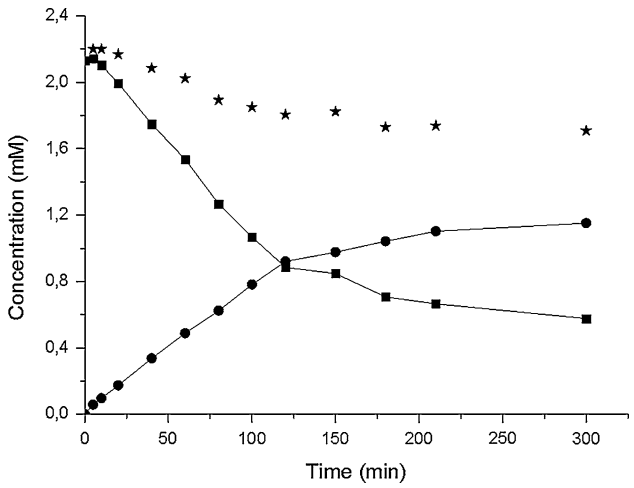

Fig. 4 Substrate consumption and product formation by resting *P. putida* KT2440 (pKP1). Resting cell assay was performed with a cell concentration of 0.47 g l^{-1}. *Filled square* quinaldine, *filled circle* 4-hydroxyquinaldine, *filled star* total

to be suitable for the development of a Qox-based biocatalytic process.

Qox-based whole-cell biocatalysts show a high stability, moderate biological variance, and Michaelis–Menten type kinetics

In a previous study, different colonies of freshly transformed *Pseudomonas* strains showed variations in recombinant oxygenase activities. Variations were lower for solvent-sensitive *P. putida* KT2440 as compared to solvent-tolerant *P. putida* DOT-T1E and *P. putida* S12 (Meyer, D., personal communication). To investigate the reproducibility of Qox activity, nine different colonies of recombinant *P. putida* KT2440 (pKP1) grown on agar plates were tested in parallel for quinaldine hydroxylation with glucose as the energy source and after growth on benzoate. The specific activity of the nine cell preparations (20.7 ± 4.5 U g^{-1}_{CDW}) showed a variation of 21.7%. The experimental error in the activity measurements was determined to be 2.3% considering 30 independent experiments. These results point to moderate biological variance.

To investigate the stability of the microbial biocatalyst under reaction conditions, the time course of substrate depletion and product formation was followed in resting-cell reactions. Both substrate depletion and product formation followed a linear trend for 2 h, whereupon the hydroxylation rate decreased (Fig. 4). The long-term stability in the absence of hydroxylation was investigated by incubating the cells in buffer with and without glucose at 30°C. After 42 h of incubation with glucose, the resting cells still showed 64% of the initial activity, whereas this value was 11% without glucose. These results indicate a high stability of metabolically active cells (consuming glucose), which is compromised when product accumulation occurs.

The kinetics of in vivo quinaldine hydroxylation by benzoate-grown cells was analyzed by varying quinaldine concentrations from 2.8 μM to 4 mM. The cells showed Michaelis–Menten type kinetics (Fig. 5) with apparent values for the maximal reaction rate V_{max} and the substrate uptake constant K_s of 19.04 ± 0.29 U g^{-1}_{CDW} and 64 ± 4 μM, respectively (Table 2). Since the kinetic parameters were determined for whole cells, V_{max} and K_s are referred to as *apparent*.

Biocatalyst performance is affected by product inhibition and toxicity

Solvents having a logP_{oct} (the logarithm of the partition coefficient of a solvent in an octanol–water system) between 1 and 4 are considered to be toxic for microbial cells [28, 42].

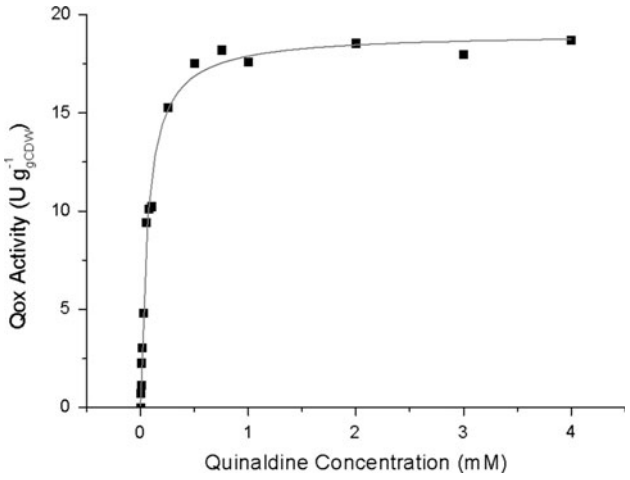

Fig. 5 Kinetics of quinaldine 4-oxidase catalysis in whole-cells of recombinant *P. putida* KT2440. Activities were determined by resting cell assays as described in the Materials and methods. The solid line shows a weighed non-linear regression for enzyme kinetic data by means of OriginPro 7.5

Quinaldine and 4-hydroxyquinaldine, with logP_{oct} values of 2.59 and 1.65, respectively [46], are expected to be toxic to microbial cells. Since product accumulation compromised biocatalyst stability and viability may affect electron transfer, substrate and product toxicities were investigated in more detail. Growth was monitored in cultures of *P. putida* KT2440 incubated with different substrate and product concentrations. Both quinaldine and 4-hydroxyquinaldine affected growth, i.e., half maximal specific growth rates ($\mu_{max}/2$) were observed at concentrations of 2.5 and 1 mM and complete inhibition above 4 and 1.75 mM, respectively. At toxic product concentrations, we observed reduced initial Qox activities, which were unaffected at toxic substrate concentrations (results not shown), indicating product inhibition at the enzyme level. To overcome substrate and product toxicity/inhibition, a two-liquid phase system, which allows maintaining low concentrations in the aqueous phase, was applied.

Biocatalyst performance in a two-liquid phase system

Organic phase selection is crucial for the development of a suitable two-liquid phase bioprocess setup. Thus, 12 commonly used organic solvents with a log$P_{oct} \geq 4$, namely 1-decanol, 1-dodecanol, octane, decane, dodecane, hexadecane, ethyldecanoate, methyllaureate, ethyloleate, dibutylphthalate, dihexylphthalate, and dioctylphthalate, were evaluated. 4-hydroxyquinaldine was either not or hardly soluble in organic solvents other than 1-decanol (log $P_{oct} = 4$) and 1-dodecanol (log $P_{oct} = 5$). A bulk second phase of 1-decanol present at an organic:aqueous phase ratio of 1:3 reduced the growth rate of *P. putida* KT2440 by 23%, whereas 1-dodecanol did not affect the growth rate at all. The partition coefficients for quinaldine and 4-hydroxyquinaldine were determined to be 229 ± 33 and 237 ± 11 in the 1-decanol-M9 system and 182 ± 10 and 130 ± 12 in the 1-dodecanol-M9 system. Based on these solubility, toxicity, and partitioning aspects, 1-decanol and 1-dodecanol were considered suitable for two-liquid phase bioprocessing.

To investigate the performance of *P. putida* KT2440 (pKP1) in a two-liquid phase process setup, resting cells grown on benzoate in batch and fed-batch mode were applied in bioreactors at two organic:aqueous phase ratios (1:3 and 1:5). With both solvents, specific product formation rates were constant for 3–6 h, but then decreased dramatically (Fig. 6). Since 4-hydroxyquinaldine was the sole product and was not further degraded as shown in separate experiments (results not shown), the decrease in product formation rate during the two-liquid phase biotransformation was due to loss of biocatalyst activity, indicating a limitation or inhibition. Oxygen was not limiting as the DOT level remained above 90%. Oxygen intake only by stirring (no active aeration) sufficed to sustain such high dissolved oxygen concentrations indicating a rather low oxygen demand. Initial biocatalyst activities did not depend on the phase ratio (Table 3). Thus, decreasing the ratio led to faster product accumulation in the organic phase but lower total product concentrations after 18 h. The maximal specific activity with 1-dodecanol was higher than with 1-decanol. However, the average productivities achieved over the first 6 h were similar with the two solvents. Although activities were lower than in the single aqueous phase system, the two-liquid phase system allowed the formation of substantial product amounts at reasonable productivities.

Table 2 Kinetic parameters for Qox

Biocatalyst	K_m app [μM]	k_{cat} app [s^{-1}]	Reference
Qox isolated from *A. ilicis* Rü61a	34	16.7	[38]
Qox isolated from *P. putida* KT2440 (pKP1)	35	29.4	[38]
Qox isolated from *P. putida* KT2440 (pKP1)	38	19.3	[26]
Whole cells of *P. putida* KT2440 (pKP1) (benzoate grown)	64	9.4[a]	This study

[a] Estimated for intracellular Qox based on Qox content

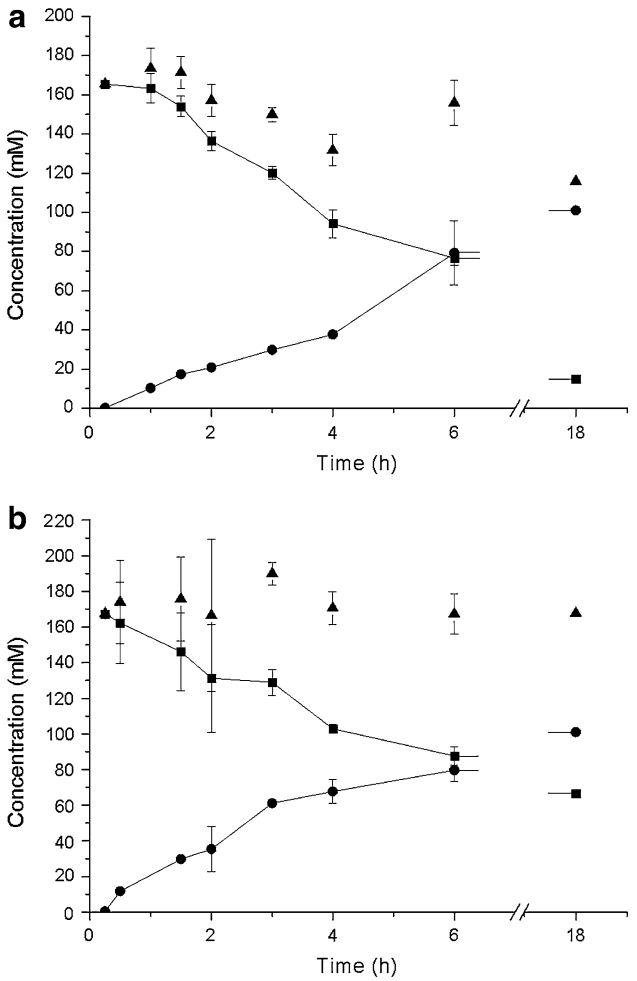

Fig. 6 Biotransformation of quinaldine to 4-hydroxyquinaldine by resting *P. putida* KT2440 (pKP1) with **a** 1-decanol, **b** 1-dodecanol as the organic phase present at an organic:water ratio of 1:5 (*filled square* quinaldine, *filled circle* 4-hydroxyquinaldine, *filled triangle* total)

Discussion

Biocatalyst characterization

To date, research on Qox focused on biochemical and structural aspects of the isolated enzyme involving the addition of a suitable artificial electron acceptor such as *p*-iodonitrotetrazoliumviolet (INT) [12, 13, 26, 38, 50]. Here, recombinant whole cells containing Qox were used to overcome the requirement for an artificial electron acceptor. For this purpose, an appropriate host strain had to be identified.

Attempts for functional expression of genes encoding Qox and other Mo-MCD-containing enzymes in *E. coli* failed [3, 6, 18, 38]. Obviously, *E. coli* is unable to synthesize Mo-MCD or integrate it into the apoprotein. The different *Pseudomonas* strains tested in this study showed large variations in the achieved Qox activity, presumably due to the variation in their regulatory background. The P_m promoter and the positive regulator XylS originate from the TOL plasmid of *P. putida* mt-2 [2, 17]. *Pseudomonas putida* KT2440 is a TOL plasmid-cured derivative of *P. putida* mt-2 [1]. This may explain the higher activities achieved with *P. putida* KT2440 as compared to the other *Pseudomonas* strains tested.

The achieved Qox activity was not only dependent on the host strain, but also on the energy source used in resting cell biotransformations and the growth substrate. The carbon source-dependent repression of the P_m promoter-dependent *xyl* regulatory system has already been reported for *P. putida* mt-2 growing on glucose [22] and succinate at non-limiting concentrations [14]. The lack of Qox activity in glycerol-grown *P. putida* KT2440 (pKP1) may be explained by slow expression kinetics [55]. It was also reported that the expression profile from P_m was considerably higher when succinate was used as the carbon source as compared to glycerol and glucose [22, 55]. A two- to fivefold-higher Qox activity with succinate-grown cells compared to glucose-grown cells confirms these findings (Fig. 2). When benzoate was used as the carbon source, Qox activities were fivefold and 25-fold higher as compared to succinate-, and glucose-grown cells, respectively (Fig. 2), which can be ascribed to relieved catabolite repression. Ramos et al. reported that XylS-mediated induction of P_m depends on the type of inducer with 2-methylbenzoate showing a 1.5-fold higher induction ratio (ratio of enzyme level in induced cells compared to uninduced cells) than benzoate [40, 41]. However, growth on and long-term induction with benzoate was adequate for achieving high Qox activities and additional induction with 2-methylbenzoate did not improve activities of benzoate-grown cells (Fig. 3).

Interestingly, we observed that the Qox activity was reduced by half when benzoate was present during the hydroxylation reaction catalyzed by resting cells. This inhibitory effect of benzoate can not be on the DNA level, but may be ascribed to competitive inhibition on the enzyme level. A steady-state kinetic study is necessary to understand the type of inhibition. As a consequence, growth and reaction phases should be separated to achieve efficient hydroxylation.

The estimated apparent K_s for whole cells was in the same range but twice as high as the K_m of purified Qox (Table 2), which can be due to the cell membrane forming a diffusional barrier for the transport of substrate and product. Based on the calculated V_{max} (19.04 ± 0.29 U g_{CDW}^{-1}) and assuming that 1.15% of the total protein content was Qox [38], k_{cat} was estimated to be 9.4 s^{-1}, which again is in the same range but somewhat lower than the values reported for isolated Qox [26, 38]. Factors potentially limiting Qox

Table 3 Comparison of two-liquid phase biotransformations with resting cells of *P. putida* KT2440 (pKP1) as the biocatalyst and 1-decanol and 1-dodecanol as carrier solvents

Parameter	1-decanol		1-dodecanol	
	1:3	1:5	1:3	1:5
Initial specific activity [U g_{CDW}^{-1}]	4.8 ± 0.4 (0–3 h)	4.8 ± 0.8 (0–6 h)	6.8 ± 0.3 (0–3 h)	6.9 ± 0.8 (0–4 h)
Product concentration in organic phase [mM] (6 h)	31.8	79.2	43.1	79.6
Total product concentration [mM] (6 h)	8.1	13.5	11.0	13.7
Total product concentration [mM] (18 h)	19.5	17.2	19.5	17.3
Productivity [g l_{tot}^{-1} h^{-1}] (6 h)	0.2	0.4	0.3	0.4

Values in brackets give time ranges or time points, for which the parameters given are valid

activity in whole cells include substrate uptake and the availability of the unknown electron acceptor.

Biocatalyst performance and possible limiting factors

Toxic/inhibitory effects of quinaldine and 4-hydroxyquinaldine were overcome by the use of 1-decanol (log $P_{oct} = 4$) or 1-dodecanol (log $P_{oct} = 5$) as carrier solvents. Both solvents are on the toxicity border for solvent sensitive cells. However, solvent-sensitive *P. putida* KT2440 was able to endure bulk amounts of 1-decanol and 1-dodecanol. An RND-type efflux pump (PP1386-PP1385) [43] and an ABC transporter similar to efflux pumps for organic solvents (PP0960-PP0958) [19] were reported to be active in *P. putida* KT2440. Such efflux systems presumably enable the use of solvents with a moderately low log P_{oct}.

We observed that Qox activities of resting cells in a two-liquid phase system (6.9 and 4.9 U g_{CDW}^{-1} with 1-dodecanol and 1-decanol, respectively) were considerably lower than the activity of resting cells in single aqueous phase systems (20.7 ± 4.5 U g_{CDW}^{-1} with substrate concentrations below the toxicity limit). The substrate concentrations in the organic phase decreased from 170 to 90 mM and from 165 to 80 mM in the course of the two-liquid phase biotransformations with 1-dodecanol and 1-decanol, respectively. These concentrations correspond to aqueous substrate concentrations of 0.74–0.50 and 0.92–0.62 mM in the respective two-liquid phase systems. Considering the kinetic studies, Qox activities in the range of V_{max} would be expected for these substrate concentrations (Fig. 5). Possible reasons for the reduced activities in the two-liquid phase system include a limitation in benzoate availability and thus reduced induction during growth, substrate mass transfer limitations, and toxic effects of substrate, product, and solvent. The low Qox activities achieved in the two-liquid phase system further decreased in the course of the biotransformations after 6 and 4 h with 1-decanol and 1-dodecanol, respectively. Possible reasons for this activity decrease include substrate mass transfer limitation and toxification of the whole-cell biocatalyst due to product accumulation.

Oxygen limitation could be excluded as a possible reason for lower or decreasing hydroxylation rates, since the DOT level remained high, although oxygen intake was accomplished by stirring only. Obviously, no active aeration via a sparger was necessary to meet the oxygen demand during biotransformations, emphasizing the potential of Qox to overcome a prominent limitation, i.e., oxygen supply and transfer in technical applications.

If substrate mass transfer is limiting in two-liquid phase systems, biotransformation rates correlate with the interfacial area, which depends on organic phase fraction, stirring speed, and amount of biomass [54]. In order to increase the surface area and thus mass transfer [49], a high agitation speed was applied during the two-liquid phase biotransformations. Initial specific and volumetric rates did not depend on the organic:aqueous phase ratio (Table 3), which indicates that substrate mass transfer over the phase boundary was not limiting during the two-liquid phase biotransformation. It is clear that toxic aqueous substrate and product concentrations should be avoided for high biocatalyst activities [32]. During the two-liquid phase biotransformations, the substrate and product concentrations in the aqueous phase were below toxic levels. When the accumulated 4-hydroxyquinaldine concentration was above 80 and 70 mM (corresponding to aqueous concentrations of 0.3 and 0.5 mM) with 1-decanol and 1-dodecanol as the organic phase, respectively, Qox activity decreased remarkably. In the single aqueous phase system, the Qox activity decreased when both substrate and product concentrations amounted to 0.9 mM (Fig. 4). These results indicate that the hydroxylation activity of Qox might be affected by a combined effect of phase and/or molecular toxicity of substrate, product, and solvent during the two-liquid phase biotransformations. Furthermore, product inhibition might occur and this remains to be investigated.

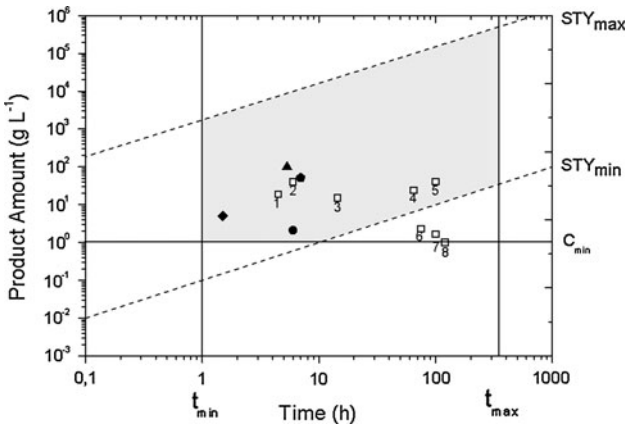

Fig. 7 Operational window for bioprocesses catalyzed by Mo-containing dehydrogenases. The *grey area* represents the window for feasible bioprocesses. t_{min}: minimum time required for a bioprocess, t_{max}: the longest time reported for a dehydrogenase-based process, STY_{min}: minimum required space–time yield for a bioprocess in the fine-chemical industry, STY_{max}: maximum space–time yield reported for quinoline 2-oxidoreductase, C_{min}: minimum product concentration required for a bioprocess in the fine-chemical industry (see text for details). Process 1 by Lonza (*filled hexagon*): *Achromobacter xylosoxydans* DSM 2783–based bioprocess with a product concentration of 51.1 g l^{-1} within 7 h. Process 2 by Lonza (*filled diamond*): *P. putida* NCIP 10521-based bioprocess with a product concentration of 5.1 g l^{-1} within 1.5 h. Process 3 by Lonza (*filled triangle*): *P. putida* NCIP 8176-based bioprocess with a product concentration of 99.7 g L^{-1} within 5.3 h. Qox-based Process (*filled circle*) (this study). Processes 1–5 (*open square*): Non-heme oxygenase-based bioprocesses with a product concentration of 18.9 (1; [36]), 39.9 (2; [33]), 15 (3; [8]), 24 (4; [27]), and 41 g l^{-1} (5; [47]) within 4.5, 6, 14.5, 65, and 100 h, respectively. Processes 6–8 (*open square*): P450-based bioprocesses with a product concentration of 2.3 (6; [53]), 1.67 (7; [34]), and 1 (8; [37]) g l^{-1} within, 75, 100, and 120 h, respectively

Current status and feasibility of the Qox-based hydroxylation process

To evaluate the efficiency of the Qox-based bioprocess among the industrially applied processes for carbon oxyfunctionalization, an operational window was defined as proposed previously [44, 56] (Fig. 7). For the lower boundaries of the operational window, the minimum space–time yield (STY_{min}), the minimum product concentration (c_{min}), and the minimum reasonable process time (t_{min}) were set at 0.1 g l^{-1} h^{-1}, 1 g l^{-1}, and 1 h, respectively [25, 51]. The maximum space–time yield (STY_{max}) was theoretically estimated to be 1529.4 g l^{-1} h^{-1} based on activity data for quinoline 2-oxidoreductase [18]. The maximum process time (t_{max}) of 350 h was adapted from L-phenylalanine dehydrogenase-based L-phenylalanine production [23]. This operational window was used to evaluate the Qox-based bioprocess and to compare it with dehydrogenase-based processes operated by Lonza Ltd. (Switzerland) for nicotinic acid hydroxylation (closed symbols, see legend of Fig. 7 for details) [30] and oxygenase-based processes (open symbols, processes 1–8) [8, 25, 27, 33, 34, 36, 37, 47, 53]. All processes except three P450-based bioprocesses lie within the area representing potentially feasible and industrially applicable bioprocesses. The Qox-based process with a productivity of 0.4 g l^{-1} h^{-1} for 6 h is comparable to the dehydrogenase-based processes by Lonza and various oxygenase-based processes (processes 1–5 in Fig. 7) in terms of process time and productivity, whereas the achieved product concentration is rather low. It is important to emphasize that the Qox-based process has a high potential for improvement of all three parameters by eliminating the limitations encountered during the two-liquid phase biotransformation and increasing cell concentrations. Considering the application point of view, the ability of Qox to catalyze carbon oxyfunctionalization with low requirements for molecular oxygen makes it a very interesting enzyme for industrial oxyfunctionalization processes.

The class of pterin-dependent hydroxylating dehydrogenases comprises a broad range of industrially interesting enzymes for the hydroxylation of highly activated carbon (e.g., xanthine, nicotine-, 6-hydroxynicotinate-, isonicotinate-, and nicotinate dehydrogenases), moderately activated heteroaromatic ring carbon (e.g., Qox, quinoline 2-, isoquinoline 1-, quinoline 4-carboxylate 2-, and quinaldic acid 4-oxidoreductases), and even rather unactivated benzylic carbon (e.g., ethylbenzene dehydrogenase) [5, 15]. Together with the high process efficiency achieved in this study (with Qox) and by Lonza (with nicotinate dehydrogenase), this emphasizes the broad scope of these water incorporating enzymes.

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