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Bioprocess design guided by *in situ* substrate supply and product removal: Process intensification for synthesis of (*S*)-1-(2-chlorophenyl)ethanol

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ABSTRACT

We report herein on bioprocess development guided by the hydrophobicities of substrate and product. Bioreductions of *o*-chloroacetophenone are severely limited by instability of the catalyst in the presence of aromatic substrate and (*S*)-1-(2-chlorophenyl)ethanol. *In situ* substrate supply and product removal was used to protect the utilized *Escherichia coli* whole cell catalyst based on *Candida tenuis* xylose reductase during the reaction. Further engineering at the levels of the catalyst and the reaction media was matched to low substrate concentrations in the aqueous phase. Productivities obtained in aqueous batch reductions were 21-fold improved by addition of 20% (v/v) hexane, NAD^+, expression engineering, cell permeabilization and pH optimization. Reduction of 300 mM substrate was accomplished in 97% yield and use of the co-solvent hexane in subsequent extraction steps led to 88% recovery. Product loss due to high catalyst loading was minimized by using the same extractant in bioreduction and product isolation.

1. Introduction

The number of potentially useful enzymes for synthetic purposes is vast but only few have made commercial success. The main hindrance for application of biocatalysts in industrial synthesis is the poor performance of enzymes under process conditions given that industrial enzymes will always operate far away from their natural conditions. A central task of biocatalysis is the conversion of sparingly water soluble substrates that are toxic to enzymes at millimolar levels. High catalyst loading and dilution of toxic substrates are often used strategies for coping with fast catalyst inactivation (Pollard and Woodley, 2007). However, this presents only a partial solution that has clear practical limits. In whole-cell biocatalysis, one main problem caused by high cell concentrations is in downstream processing where the efficiency of solids separation from the liquid product phase and product loss to the solid phase are central issues. Now, because volumetric productivity in the reaction and product yield in downstream processing are affected differently by changes in the catalyst loading, optimization of process conditions for unstable biocatalysts is a challenging task that necessitates an integrated solution. The design cycle of whole cell biocatalysis comprises enzyme selection, cell and reaction engineering, product recovery and scale-up. Limitations are generally overcome in a stepwise manner and to date no fixed methods for bioprocess design have been established (Schmid et al., 2001; Tufvesson et al., 2010). Here, we report on process development guided by the properties of substrate and product. We have previously identified *Candida tenuis* xylose reductase (*Ct*XR) as highly selective catalyst for the reduction of *o*-chloroacetophenone (Kratzer et al., 2011). *Ct*XR is among the very few bio- and chemo-catalysts capable of (*S*)-1-(2-chlorophenyl)ethanol production in absolute stereoselectivity (>99% ee) and high yields (for methods other than *Ct*XR-catalyzed reduction, see Baratta et al., 2008; Evans et al., 2003; Gröger et al., 2006; Itoh et al., 1999; Yang et al., 2007; Zeror et al., 2008; Zhu et al., 2006). For biocatalytic synthesis, we coupled NADH-dependent reduction of *o*-chloroacetophenone to NAD^+-dependent oxidation of formate, catalyzed by formate dehydrogenase from *Candida boidinii* (*Cb*FDH). The sparingly water soluble *o*-chloroacetophenone and 1-(2-chlorophenyl)ethanol turned out to be unexpectedly toxic and deactivated the isolated enzyme within <3 min. Co-expression of *Ct*XR and *Cb*FDH in *Escherichia coli* provided a simple and low-cost catalyst with the enzymes 'immobilized' in the cellular environment (Kratzer et al., 2011). However, the whole cell biocatalyst was disintegrated within hours in the presence of 100 mM substrate, which seriously limited product concentration and catalyst total turnover number. In typical batch reactions that used 40 g *E. coli* cell dry weight (CDW)/L, the concentration of (*S*)-1-(2-chlorophenyl)ethanol did not exceed 14 mM, resulting in a modest turnover number of 0.055 $g_{product}/g_{CDW}$. In the present work, we report on the second process design cycle starting from substrate supply to overcome limitations due to catalyst instability. The

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general reactor operation modes (batch, fed-batch) and substrate delivery options (feed, *in situ* supply) are summarized in Table 1 (reviewed in Kim et al., 2007). Higher toxicity of the product as compared to the substrate ruled out substrate feeding as a method to protect the catalyst. Separation of the catalyst-containing, aqueous phase from product and substrate was achieved by addition of a hydrophobic, second phase. The most common auxiliary phases, organic solvents, ionic liquids and hydrophobic resins, were tested with respect to percentage (volume ratio) and substrate loading. Our work provides, to the best of our knowledge, the most comprehensive study on substrate supply in the field of biocatalysis. Integration of the operational units 'substrate supply' and 'product isolation' was achieved using *in situ* supply and removal of the hydrophobic substrate and product, respectively. The subsequent engineering at the levels of the whole-cell catalyst and the reaction media was matched to altered reaction conditions. Even though the whole cell-catalyzed reduction of *o*-chloroacetophenone presents an extreme case of catalyst instability, the problem of catalyst inactivation by hydrophobic substrates is common to a large number of biotransformations in organic synthesis (Burton et al., 2002; Doig et al., 2002; Kizaki et al., 2001; Kratzer et al., 2011; Pfruender et al., 2006; Schmid et al., 2001).

2. Methods

2.1. Chemicals, materials and strains

NADH (sodium salt; ⩾98% pure), NAD^+ (free acid; ⩾97.5% pure), D-xylose, and ampicillin were purchased at Roth (Karlsruhe, Germany). Sodium formate, polymyxin B sulfate, kanamycin, chloramphenicol, *o*-chloroacetophenone and the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ($BMIMPF_6$; product number 18122) were obtained from Sigma–Aldrich (Vienna, Austria). B-Per® Reagent was from Pierce (Rockford, IL, USA) and 1-(2-chlorophenyl)ethanol from Alfa Aesar (Karlsruhe, Germany). The resin Amberlite™ XAD7HP (free of charge sample) was from Rohm and Haas (Coventry, UK). All other chemicals were from Sigma–Aldrich/Fluka or Roth, and were of the highest purity available. 15 and 2 mL tubes were purchased at Sarstedt (Wr. Neudorf, Austria) and Eppendorf (Wien, Austria), respectively.

The microorganisms used were *E. coli* BL21 (DE3) harboring *Ct*XR and *Cb*FDH genes on a pETDuet-1 vector (pETDuet_XR_FDH; BL21_XR_FDH; Kratzer et al., 2008) and *E. coli* Rosetta2 carrying pETDuet_XR_FDH and an additional pRSF-1b plasmid encoding *Cb*FDH (Rosetta_XR_2FDH; Mädje et al., 2012).

2.2. Cultivation of strains

Strains were grown in 1000 mL baffled shaken flasks containing 200 mL of LB media supplemented with 115 mg/mL ampicillin. LB media for strain *E. coli* Rosetta_XR_2FDH additionally contained 34 mg/L chloramphenicol and 50 mg/L kanamycin. Recombinant protein production used a standard procedure in which cultures were cooled from 37 to 18 °C when an optical density of 1.1 (±10%) was reached and protein production was induced by addition of 250 μM isopropyl-β-D-thiogalactopyranosid (IPTG). After 20 h of cultivation, cells were harvested by centrifugation. Samples were taken and the B-Per® cell lysis reagent was used for protein extraction prior to enzyme activity measurements.

2.3. Enzyme activity measurements in the cell-free extracts

Reductase and dehydrogenase activities were assayed spectrophotometrically by monitoring the reduction or oxidation of NAD(H) at 340 nm. Typically, rates of 0.05–0.10 ΔA/min were measured over a time period of 5 min. One unit of enzyme activity refers to 1 μmol of NADH consumed or formed per minute. All measurements were performed with a Beckman DU-800 spectrophotometer thermostated at 25 °C. The assay for *Ct*XR contained 10 mM *o*-chloroacetophenone and 300 μM NADH; that for *Cb*FDH contained 200 mM sodium formate and 2 mM NAD^+. *o*-Chloroacetophenone was dissolved in ethanol prior to dilution into buffer to give a final ethanol concentration of 5% (w/w). Activity measurements were performed in 100 mM potassium phosphate buffer with pH values adjusted to 7.5, 7.0, 6.5, and 6.2. Reactions were always started by the addition of coenzyme. Measured rates were corrected for appropriate blank readings accounting for non-specific oxidation or reduction of NAD(H) by the cell extracts.

2.4. Evaluation of substrate and product "toxicity"

E. coli BL21_XR_FDH cells were diluted to a concentration of 40 g_{CDW}/L in 100 mM potassium phosphate buffer, pH 7.5. 10 mL of cells were filled into 15 mL Sarstedt tubes and *o*-chloroacetophenone or (*S*)-1-(2-chlorophenyl)ethanol were added at concentrations between 0 and 50 mM. Experiments were carried out on an end-over-end rotator (SB3 from Stuart) at 30 rpm, thermostated at 30 (±1) °C. 100 μL samples were taken after 0.25, 0.5, 1, 1.5, 2, 3, 4 and 5 h. The reaction mixture was diluted 20-fold with buffer such that no organic phase (from insoluble substrate) remained, and cells were then collected by centrifugation. After cell lysis using B-Per, enzyme activities were assayed as described above.

2.5. Whole-cell bioreduction of o-chloroacetophenone

E. coli cells were diluted in 100 mM potassium phosphate buffer (pH 7.5, 7.0, 6.5 or 6.2) to concentrations between 30 and 80 g_{CDW}/L. The liquid substrate *o*-chloroacetophenone was used in concentrations between 3 and 300 mM. It was pre-dissolved in ethanol to give a final ethanol concentration of maximally 5% (w/w). This level of ethanol does neither interfere with the activity of isolated *Ct*XR nor with whole cell bioreductions based on *Ct*XR. The solubility of *o*-chloroacetophenone is 10 mM under the conditions used; reactions with higher substrate concentrations took place in an aqueous-organic two-phase system (micro-emulsion; Xie et al., 2006). The concentration of the co-substrate sodium formate always exceeded that of the ketone substrate by 50 mM (minimum 80 mM). Batch reductions were performed in a total volume of 1 mL incubated in 2-mL Eppendorf tubes. Fed batch reductions were likewise performed in 15 mL Sarstedt tubes with final volumes of 10 mL. The substrate was added intermittently. The frequency is denoted as (amount (mM)/time interval (min)) in Table 2. Experiments were carried out at 30 (±1) °C incubated on an end-over-end rotator (SB3 from Stuart) at 30 rpm.

2.5.1. Resin-based conversions

The polymeric absorbent Amberlite™ XAD7HP was rinsed with distilled water and equilibrated in 100 mM potassium phosphate

Table 1
General bioprocess operation modes using a conventional tank reactor.

Reactor operation	Substrate delivery	
	Aqueous system	Auxiliary phase (*in situ* substrate supply)
Batch	Traditional one-pot	Water immiscible 2nd phase or solid resin
Fed-batch	Substrate feed neat or dissolved in aqueous media	Substrate feed neat, dissolved in 2nd phase or adsorbed on resin

Table 2
Effect of substrate concentration and feeding rate on product concentration obtained in batch and fed-batch reductions of *o*-chloroacetophenone.

Cells (g_{CDW}/L)	Substrate (mM)	Substrate addition (amount/interval)[a]	Product (mM)	Product (%)	Total turnover ($g_{product}/g_{CDW}$)
42	30	Batch-mode	14	47	0.052
42	30	Fed-batch 3 mM/40 min	17	57	0.063
31	50	Batch-mode	9	18	0.045
31	50	Fed-batch 10 mM/60 min	25	50	0.126
43	60	Batch-mode	11	18	0.040
43	60	Fed-batch 3 mM/20 min	18	30	0.070

[a] Fed-batch: substrate was predissolved in ethanol and added intermittently to a final ethanol concentration of 5% (w/w). Reaction conditions: pH 7.5, reaction time 300–400 min. Substrate and product concentrations were determined, losses in the mass balance were ⩽20%.

buffer, pH 6.2. Substrate was loaded by gentle mixing of substrate and resin in buffer on an end-over-end rotator for 2 h at 30 °C and 30 rpm (0.5 mL). The reaction was started by addition of 0.5 mL *E. coli* cell-suspension (80 g_{CDW}/L) and co-substrate. After specified reaction times, product and unconverted substrate were desorbed with ethanol (1:1 v/v ratio) at 50 °C for 2 h (950 rpm).

Preparative synthesis of (*S*)-1-(2-chlorophenyl)ethanol was performed with 30% (v/v) polymeric adsorbent Amberlite™ XAD7HP in a total volume of 15 mL. The reaction mixture contained 0.6 g_{CDW} of *E. coli* Rosetta_XR_2FDH, 1.5 mmol *o*-chloroacetophenone loaded on 30% (v/v) resin and 2.25 mmol sodium formate in a 100 mM potassium phosphate buffer, pH 6.2. After 24 h of incubation, product and remaining substrate were extracted twice by ethyl acetate addition in a 1:1 ratio. Substrate and remaining product were desorbed twice by 15 mL ethyl acetate on an end-over-end rotator (SB3 from Stuart) at 30 rpm (30 °C) for 1 h. Phase separation was done by centrifugation at 5000*g* for 20 min, the organic phases were united, dried with sodium sulfate and ethyl acetate was evaporated under reduced pressure.

2.5.2. Water immiscible co-solvents

The substrate was dissolved in the co-solvent (hexane, heptane, dodecane, ionic liquid ($BMIMPF_6$)) and added to the cell containing aqueous phase in 20% or 50% (v/v) ratios and total volumes of 1 mL. After specified reaction times, product and unconverted substrate concentrations were measured in the aqueous and the organic phases.

Preparative synthesis of (*S*)-1-(2-chlorophenyl)ethanol was performed with 20% (v/v) hexane in a total volume of 15 mL. 0.6 g_{CDW} of *E. coli* Rosetta_XR_2FDH and 5.25 mmol sodium formate were diluted into 12 mL potassium phosphate buffer (100 mM, pH 6.2) supplemented with 500 μM NAD^+ and 36 μM polymyxin B sulfate. The reaction was started by addition of 4.5 mmol *o*-chloroacetophenone dissolved in 3 mL hexane. After 24 h of incubation, product and remaining substrate were extracted thrice by hexane addition in a 1:1 ratio. Extraction was performed on an end-over-end rotator (SB3 from Stuart) at 30 rpm for 2 h (room temperature). Phase separation was done by centrifugation at 5000*g* for 20 min, the organic phases were united and hexane was evaporated under reduced pressure.

2.6. Analytical methods

2.6.1. Aqueous batch and fed-batch reductions

Aqueous samples were diluted with ethanol as required to obtain a homogeneous liquid phase.

2.6.2. Batch reductions with hydrophobic resin

Samples were diluted with ethanol (1:10 v/v) and incubated for 120 min at 30 °C and 950 rpm to desorb substrate and product from the solid resin.

2.6.3. Batch reductions with water immiscible co-solvents

Biomass, aqueous and second phase were separated by centrifugation.

All samples were diluted into the mobile phase (20% (v/v) acetonitrile) to concentrations of <50 mM analyte. Aqueous and second phases were separately analyzed.

Chiral HPLC was performed on a LaChrom HPLC system (Merck–Hitachi) equipped with an L-7400 UV-detector and a thermostated column oven. A reversed phase CHIRALPAK AD-RH column from Daicel (purchased at VWR International, Vienna, Austria) was used and detection was at 210 nm absorbance. Baseline separation of the *R*- and *S*-alcohol was obtained with 20% (v/v) acetonitrile as eluent at a flow rate of 0.5 mL/min and a temperature of 40 °C (Kratzer et al., 2011). Authentic standards were used for peak identification, and quantification was based on peak area that was suitably calibrated with standards of known concentration. Detection limits of *o*-chloroacetophenone and 1-(*o*-chlorophenyl)-ethanols were below 0.005 mM.

3. Results and discussion

3.1. Reaction engineering to optimize substrate supply

The supply method of non-natural substrates that are toxic to biocatalysts is a key consideration in process optimization. However, little has been published that addresses the incompatibility of sparingly water soluble substrates with biocatalysts. In a conventional batch reactor, the substrate is either added at the beginning or, to prevent catalyst inactivation, fed at a low concentration to the reactor. Application of an auxiliary phase that is water immiscible allows the separation of the toxic substrate from the catalyst-containing, aqueous phase. The substrate concentration in the aqueous phase is low and converted substrate is replenished by partition from the second phase (Kim et al., 2007). The *in situ* substrate supply enables at the same time product removal into the second phase and thereby simplifies product recovery. Feeding of substrate that is predissolved in the second phase provides a highly concentrated substrate stream and constitutes a combination of fed-batch operation and *in situ* substrate supply. An outline of the general reaction operation modes and substrate delivery options is depicted in Table 1 (reviewed in Kim et al., 2007). Here, we provide a comprehensive study on *o*-chloroacetophenone supply for bioreduction by *Ct*XR. Differential toxicities of substrate and product guided the process development and reduced the number of possibilities summarized in Table 1. The most common auxiliary phases, organic solvents, ionic liquids and hydrophobic resins, were tested with respect to percentage (volume ratio) and substrate loading. The product concentration, obtainable with a certain amount of catalyst, served as selection criterion.

3.1.1. Batch and fed-batch reductions of o-chloroacetophenone

The most evident strategy to reduce the effect of toxic substrate is to use less substrate in bioreductions. We decreased substrate concentrations in batch reductions from 100 mM to 30, 50 and

60 mM and determined product concentrations and total turnover numbers ($g_{product}/g_{CDW}$) (Table 2). The highest product concentration in batch conversions was determined to 14 mM and was obtained with a substrate concentration of 30 mM. Higher substrate concentrations led to lower product concentrations and conversions. The decline in total turnover number from 0.052 to 0.040 $g_{product}/g_{CDW}$ in response to an increase in substrate concentration from 30 to 60 mM suggests substrate toxicity (Table 2). Doig et al. (2002) have previously shown that the lactone substrate bicycle[3.3.0]hept-2-en-6-one is inhibitory in whole cell Baeyer–Villiger oxidations at concentrations 10-fold lower as compared to the regioisomeric products. The inhibitory effect of the lactone substrate was overcome by substrate feeding and *in situ* substrate supply. We used controlled supply of the ketone substrate to circumvent toxic effect of *o*-chloroacetophenone on whole cell catalyst BL21_XR_FDH. *o*-Chloroacetophenone was added stepwise to final concentrations of 30, 50 and 60 mM. Slow addition of substrate increased total turnover numbers 1.2–2.8-fold as compared to batch reductions with identical catalyst loading and substrate (end)-concentration (Table 2). A feeding rate of 10 mM substrate added each 60 min led to a highest product concentration of 25 mM. Although fed-batch addition of substrate improved product titre, low final product concentrations in fed-batch experiments indicated toxic or inhibiting effects of substrate and product on the whole cell biocatalyst. We determined catalyst stabilities in the presence of *o*-chloroacetophenone or (*S*)-1-(2-chlorophenyl)ethanol to separate substrate effects form product effects.

3.1.2. Toxicity of o-chloroacetophenone and 1-(2-chlorophenyl) ethanol on BL21_XR_FDH

BL21_XR_FDH was incubated with substrate or product under conditions similar to batch conversions. Loss of intracellular *Ct*XR and *Cb*FDH activities was measured in dependence of incubation time. Table 3 shows calculated half-life times of *Ct*XR and *Cb*FDH activities in the presence of 0–50 mM *o*-chloroacetophenone or 1-(2-chlorophenyl)ethanol. Reference values of half-life times for *Ct*XR and *Cb*FDH incubated in buffer were determined to 5 and 8 d, respectively. At substrate and product concentrations of 30 mM half-life times of *Ct*XR and *Cb*FDH decreased to $\leqslant$1%, equal to $\sim$1 h. The presence of 50 mM 1-(2-chlorophenyl)ethanol had an especially negative impact on *Ct*XR and *Cb*FDH activities and shrunk the whole cell catalyst half-life time to 10 min. Accumulation of the highly toxic product provides the explanation for the only moderate increases in process yield in fed-batch compared to batch conversions. Solubilities of *o*-chloroacetophenone and 1-(2-chlorophenyl)ethanol are 10 and 50 mM, respectively, under the conditions used. Substrate and product concentrated above solubility limits lead to aqueous-organic two-phase systems in the form of micro-emulsions (Xie et al., 2006). The observation that only hydrophobic molecules which are dissolved in the aqueous phase accumulate in the cell membrane (de Bont, 1998) might explain the higher toxicity of better soluble 1-(2-chlorophenyl)ethanol. Solid or liquid second phases are repulsed by the hydrophilic parts of the cell wall and are therefore of minor toxicity to cells (Weber and de Bont, 1996). Results from toxicity studies were used to guide *in situ* substrate supply and product removal strategies.

3.1.3. In situ substrate supply and product removal

The biocatalyst retained the half-life time of 5 d, determined in plain buffer, in the presence of $\leqslant$3 mM *o*-chloroacetophenone (Table 3). Toxicities of *o*-chloroacetophenonen and corresponding alcohol(s) in higher concentrations require the separation of product(s) and substrate from whole cell catalysts during reductions. A convenient method to protect biocatalysts in the aqueous phase is the *in situ* extraction of hydrophobic compounds onto or into nonpolar, second phases (Schmid et al., 2001). We chose immobilization via adsorption onto a polymeric matrix and extraction into a second organic phase in the present study.

3.1.3.1. Hydrophobic resin as second phase. Advantages of solid resins as compared to traditional organic solvents are insolubility in the aqueous phase, no volatilization and hence high biocompatibility and reduced environmental hazard (Guo et al., 2010). We used white beads of the acrylic polymer Amberlite™ XAD7HP as adsorbent and tested its loading capacity for *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethanol. An amount of $\geqslant$20% (v/v) resin kept the effective substrate concentration in a suspension of 100 mM substrate in buffer below 3 mM. A mixture of 50 mM substrate, 50 mM product and 20% (v/v) resin resulted in substrate and product concentrations of 2 and 3 mM, respectively. We therefore used resin concentrations of 20%, 30%, 35%, 40% and 60% (v/v) in bioreductions of 100 mM *o*-chloroacetophenone (Table 4). The highest analytical product concentration of 86 mM was obtained with 30% (v/v) resin and corresponds to 6 and 3.4-fold increases as compared to aqueous batch and fed-batch reductions (Table 2). Doubling of substrate and resin concentrations to 200 mM *o*-chloroacetophenone and 60% (v/v) resin led to only 54 mM product (Table 4). The generally observed reduced yields in experiments with resin concentrations >30% (v/v) suggest low substrate concentrations in the aqueous phase and hence decreased reduction rates. Insufficient stirring of the highly viscous reaction mixtures might furthermore lead to limitations in interfacial mass transfer. The highest obtained product concentration of 13 g/L is comparable to resin-based whole cell biotransformations described in literature. Product concentrations of 21 and 17 g/L were reported for a

Table 3
Half-life times ($T_{1/2}$)[a] of intracelluar *Ct*XR and *Cb*FDH in the presence of different substrate and product concentrations.

Concentration (mM)	*o*-Chloroacetophenone		(*S*)-1-(2-chlorophenyl)ethanol	
	$T_{1/2}$ XR (min)	$T_{1/2}$ FDH (min)	$T_{1/2}$ XR (min)	$T_{1/2}$ FDH (min)
0	6931	11552	6931	11552
3	6931	7702	n.d.	n.d.
6	3466	6931	n.d.	n.d.
10	770	495	330	990
20	112	173	248	239
30	71	67	49	53
40	n.d.	n.d.	21	16
50	51	65	10	10

[a] Enzyme deactivation followed exponential decay. n.d. not determined. Reaction conditions: (BL21_XR_FDH) = 40 g_{CDW}/L, pH 7.5.

Table 4
Effect of *in situ* substrate supply and product removal by a hydrophobic resin on bioreductions of *o*-chloroacetophenone.

Resin (v/v)	Substrate addition (mM)	Product[c] (mM)	Product[c] (mM) NAD^+[,a]	Product[c] (mM) NAD^+[,a] Polymyxin[b]
–	100	16	38	48
Resin 10%	100	29	n.d.	n.d.
Resin 20%	100	66	n.d.	n.d.
Resin 30%	100	86	85	67
Resin 35%	100	83	n.d.	n.d.
Resin 40%	100	72	n.d.	n.d.
–	200	7	n.d.	20
Resin 60%	200	27	n.d.	n.d.

n.d. not determined.
[a] (NAD^+) = 500 μM.
[b] (Polymyxin B sulfate) = 36 μM. Reaction conditions: (BL21_XR_FDH) = 40 g_{CDW}/L, pH 7.5, reaction time 24 h. Losses in the mass balance were $\leqslant$20%.
[c] Standard deviations were $\leqslant$10% (n = 2; n = 3).

methyl benzoylformate bioreduction (Guo et al., 2010) and the Baeyer–Villiger oxidation of bicycle[3.3.0]hept-2-en-6-one, respectively (Alaphand et al., 2003).

3.1.3.2. Water immiscible solvents as second phases. A widely held notion is that the toxicity of organic solvents on whole cells decreases as their log*P* value increases. The main mechanism of solvent toxicity is the accumulation of hydrophobic compounds in cellular membranes with concomitant cell permeabilization. Lipophilic solvents with log*P* values >4 do not reach a high membrane concentration due to their low water solubility whereas solvents with log*P* values of 1–4 are more water soluble and dissolve well in the membrane (de Bont, 1998). We have previously shown that relatively polar organic solvents with log*P* < 4 are not well suited as second phases in *E. coli* BL21_XR_FDH-catalyzed reductions (Kratzer et al., 2008, 2011). Therefore three common solvents covering a log*P* from 4.11 to 6.8 and one ionic liquid were selected. Dodecane (log*P* 6.8), heptane (log*P* 4.66), hexane (log*P* 4.11) and $BMIMPF_6$ (1-butyl-3-methylimidazolium hexafluorophosphate) were used in 20% and 50% (v/v) as second phases. Results from two-phase whole cell reductions of 100 mM *o*-chloroacetophenone are shown in Table 5. The product (*S*)-1-(2-chlorophenyl)ethanol was obtained in 84% reduction yield with 20% (v/v) hexane compared to 66% obtained with heptane, 27% with dodecane and 31% with the ionic liquid and 16% lacking a second phase. Similar experiments with 50% (v/v) co-solvents resulted in significantly lower yields. To increase product concentrations we performed reductions of 200 mM *o*-chloroacetophenone with varying concentrations of hexane. Table 5 shows the results of whole cell bioreductions with 20% (v/v), 30% (v/v) and 40% (v/v) hexane. A highest product concentration of 120 mM was obtained with 20% hexane.

We have measured substrate and product concentrations in the aqueous and water immiscible second phases during the reactions (Table 5). Substrate concentrations measured in different aqueous phases were between <1 and 2 mM, product concentrations varied from 0.5 to 14 mM. Product concentrations determined in the aqueous phases were highest using 20% (v/v) hexane or heptane (Table 5). Higher reduction yields obtained with hexane and heptane as compared to yields from dodecane or ionic liquid conversions suggest a decrease in mass transfer resistance over the cell wall by cell permeabilization. The co-solvents hexane and heptane show furthermore higher toxicities to the cell wall as compared to dodecane and ionic liquid (de Bont, 1998; Pfruender et al., 2006). Moreover, low substrate concentrations in the aqueous phase decrease the intracellular substrate availability by slowing down the enzymatic reaction given that enzymatic rate is linearly dependent on substrate concentration ($K_{o\text{-chloroacetophenone}}$ > 5 mM). Lower yields in reactions containing ionic liquid or a higher percentage of the second phase (50% (v/v)) are therefore explicable by lower substrate concentrations in the aqueous phase (Table 5). The differential effect of a high substrate concentration in the aqueous phase on cell activity and stability requires subtle balancing of substrate supply. The following activity engineering is matched to the changed reaction conditions i.e. relatively low substrate concentrations in the aqueous phase.

3.2. Activity engineering of the catalyst under process conditions

The duet vector pETDuet-1b was used for co-expression of *Ct*XR and *Cb*FDH in *E. coli* BL21 (DE3) (BL21_XR_FDH) as previously reported. Specific activities of *Ct*XR and *Cb*FDH in the crude cell extract were determined to 515 and 110 U/g_{CDW}, respectively. The alcohol synthesis activity was measured with an *o*-chloroacetophenone concentration of 10 mM. This represents the upper solubility limit of *o*-chloroacetophenone in the buffered solution. The concentration of *o*-chloroacetophenone in the aqueous phase of the 20/80 (v/v) hexane/buffer system is, however, only ⩽1 mM. The reductase activity under these conditions is hence in the range of the cofactor recycling activity of 110 U/g_{CDW}. Therefore, to increase the catalytic activity of the whole cell catalyst under process conditions, both activities must be improved. One strategy to boost the activities of both enzymes was to optimize cofactor availability by supplementation with NAD^+. The external addition of $NAD(P)^+$ has been shown to increase yields in whole cell reductions previously (Ema et al., 2008; Kataoka et al., 2003; Kratzer et al., 2008, 2011). However, cofactor supplementation of the reaction requires cell permeabilization as the charged molecules $NAD(P)^+$ can not enter intact cells.

3.2.1. Reaction engineering to improve cofactor and (co)-substrate availability

We added 500 μM NAD^+ to whole cell reductions of 100 mM *o*-chloroacetophenone (Tables 4 and 5). Yields of bioreductions in plain buffer increased from 16% to 38% upon supplementation with NAD^+. The substrate was nearly completely converted under conditions in which NAD^+ (0.5 mM) was added to reaction systems containing 20% (v/v) hexane- or heptane. Addition of NAD^+ to reaction

Table 5
Effect of *in situ* substrate supply and product removal by immiscible co-solvents on bioreductions of *o*-chloroacetophenone.

Co-solvent (v/v)	Added substrate (mM)	Total product[c] (mM)	Total product[c] (mM) NAD^+[,a]	Total product[c] (mM) NAD^+[,a] Polymyxin[b]	Product in the aqueous phase (mM)	Substrate in the aqueous phase (mM)
–	100	16	38	48	–	–
Hexane 20%	100	84	94	98	13.5	⩽1
Hexane 50%	100	36	44	48	7	⩽1
Heptane 20%	100	66	85	97	12	⩽1
Heptane 50%	100	41	43	45	5.5	⩽1
Dodecane 20%	100	27	25	29	5.5	2
Dodecane 50%	100	33	36	35	4.5	2
Ionic liquid 20%	100	31	28	22	2	<1
Ionic liquid 50%	100	23	23	19	0.5	<1
–	200	7	n.d.	20	–	–
Hexane 20%	200	60	n.d.	82	14	1
Hexane 30%	200	58	n.d.	79	12	1
Hexane 40%	200	31	n.d.	57	11	1

n.d. not determined.
[a] (NAD^+) = 500 μM.
[b] (Polymyxin B sulfate) = 36 μM. Reaction conditions: (BL21_XR_FDH) = 40 g_{CDW}/L, pH 7.5, reaction time 24 h. Substrate and product concentrations were determined in both phases, losses in the mass balance were ⩽20%.
[c] Standard deviations were ⩽10% (n = 2; n = 3).

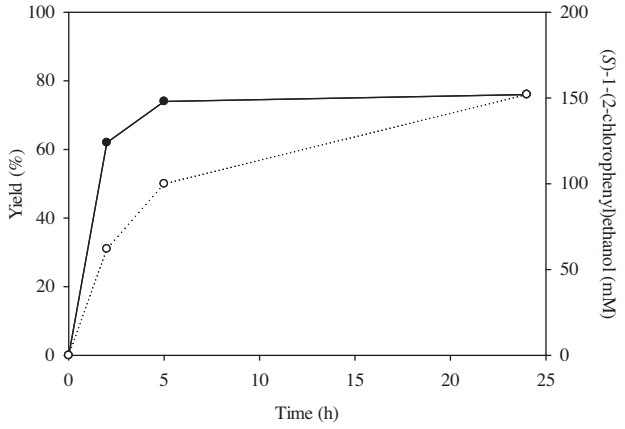

Fig. 1. Time courses of 200 mM *o*-chloroacetophenone reduction catalyzed by BL21_XR_FDH (○) and Rosetta_XR_2FDH (●). (Cells) = 40 g_{CDW}/L, (hexane) = 20% (v/v); (NAD^+) = 500 μM, (polymyxin B sulfate) = 36 μM, pH 7.5.

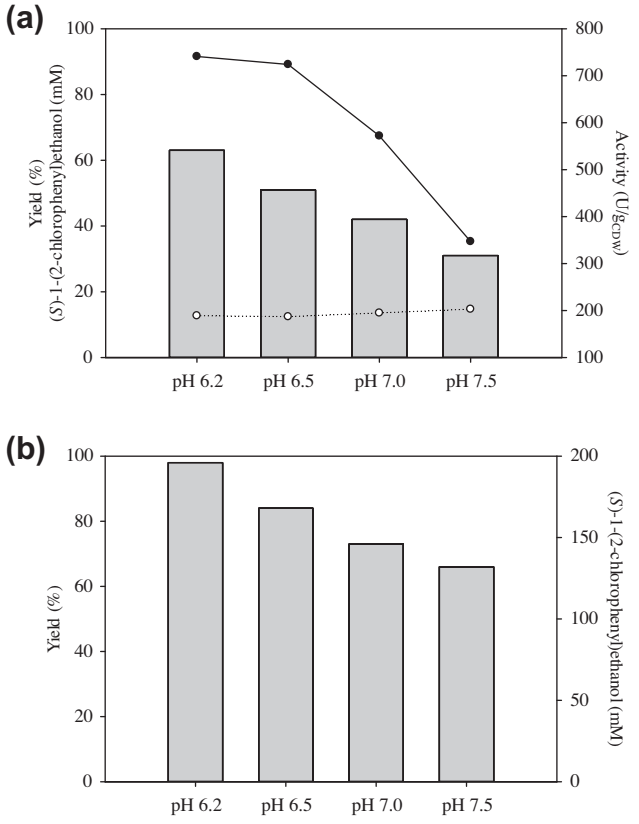

Fig. 2. Effect of pH-value on *Ct*XR- and *Cb*FDH-activities and corresponding yields in whole cell bioreductions of *o*-chloroacteophenone. (a) *Ct*XR- and *Cb*FDH-activities measured in the cell-free extract at different pH-values and corresponding reduction yields of 100 mM *o*-chloroacetophenone (● = *Ct*XR, ○ = *Cb*FDH; gray bars = reduction yields). (b) Reduction yields of a reaction mixture containing 200 mM *o*-chloroacetophenone, 20% (v/v) hexane, 500 μM NAD^+, 36 μM and potassium phosphate buffer at different pH-values (gray bars = reduction yields). (Rosetta_XR_2FDH) = 40 g_{CDW}/L, reaction times 24 h.

mixtures with dodecane, ionic liquid or resin as second phase did not increase yields. Our results indicate that dodecane, ionic liquid or resin are not by themselves permeabilizing the cells and that furthermore the concentration of *o*-chloroacetophenone and the corresponding alcohol are below a cell-permeabilizing concentration. Improved yields by the addition of NAD^+ in reduction

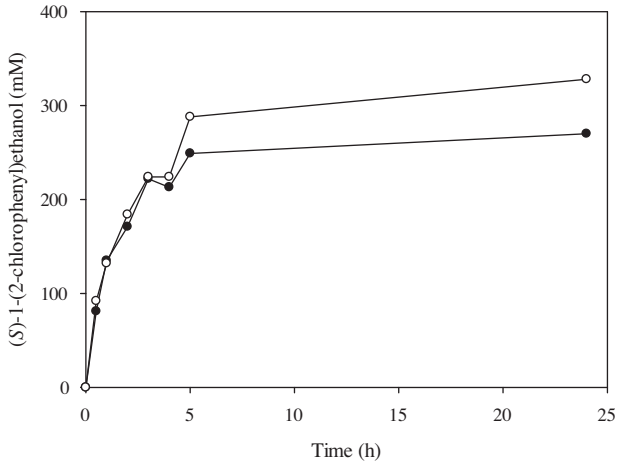

Fig. 3. Time courses of 300 and 400 mM *o*-chloroacetophenone reduction catalyzed by Rosetta_XR_2FDH. ● = 300 mM *o*-chloroacetophenone; ○ = 400 mM *o*-chloroacetophenone; (Rosetta_XR_2FDH) = 40 g_{CDW}/L, (hexane) = 20%, (NAD^+) = 500 μM, (polymyxin B sulfate) = 36 μM, pH 6.2.

mixtures containing 20% (v/v) hexane or heptane suggest either solvent or substrate permeabilization of the cell wall.

We have previously used the antibiotic polymyxin B sulfate to make the cell wall more easily permeable for externally added NAD^+, co-substrate and co-product. Polymyxin B sulfate shows a locally disruptive effect on cell wall integrity of gram-negative bacteria and induces pore formation in the membrane. Addition of polymyxin B sulfate and NAD^+ led to complete reduction (⩾97%) of 100 mM *o*-chloroacetophenone containing 20% hexane or heptane as co-solvents (Table 5). Highest product concentrations of ~160 mM were obtained using 200 mM substrate and 20% hexane or heptane (Table 5). Reductions of 100 and 200 mM substrate in plain buffer were improved ~3-fold by externally added NAD^+ and polymyxin B sulfate induced pore formation. The absence of yield improvement by polymyxin B sulfate when resin, dodecane or $BMIMPF_6$ were used as second phase suggests either deactivation of polymyxin B sulfate or low substrate concentrations in the aqueous phase (Tables 4 and 5).

3.2.2. Host engineering

We co-transformed *E. coli* Rosetta 2 with pETDuet_XR_FDH (Kratzer et al., 2008) and a pRSF plasmid carrying the FDH gene (strain Rosetta_XR_2FDH; Mädje et al., 2012). Enzyme expression in the rare codon host Rosetta 2 and the increase in gene copy number led to a 2-fold higher intracellular formate dehydrogenase activity as compared to BL21_XR_FDH. Rosetta_XR_2FDH and BL21_XR_FDH were used in reaction systems containing 100 mM substrate and 30% (v/v) resin or 200 mM substrate and 20% (v/v) hexane. Experiments with resin showed no detectable difference between the two strains (data not shown) indicating again mass transfer over the cell wall and substrate availability as main obstacles in resin-based bioreductions. Reduction of 200 mM substrate in 20% (v/v) hexane supplemented with NAD^+ and polymyxin B sulfate yielded 76% product with both strains, the initial reduction rate, however, was doubled with Rosetta_XR_2FDH (Fig. 1).

The improvement of a balanced redox-system needs the increase of both enzyme activities to take effect. It was previously shown that the xylose reductase activity decreases upon a pH change from 6 to 8 (Kratzer et al., 2004). We therefore optimized Rosetta_XR_2FDH activity by a pH change in the reaction buffer. The *o*-chloroacetophenone reductase activity measured in the cell-free extract doubled from 347 to 741 U/g_{CDW} in response to a pH shift from 7.5 to 6.2. The *Cb*FDH activity determined by pH

Table 6
Mass balance analysis of preparative-scale product isolation from resin-based and two-phasic *o*-chloroacetophenone bioreductions[a].

Step	Yield (%)	Step	Yield (%)
Bioreduction (100 mM substrate, 30% (v/v) resin)[b]	91	Bioreduction (300 mM substrate, 20% (v/v) hexane)[a]	97
1st Extraction (ethyl acetate)	76	1st Extraction (hexane)	74
2nd Extraction (ethyl acetate)	21	2nd Extraction (hexane)	11
Dewatering (Na_2SO_4)	94	3rd Extraction (hexane)	3
Isolated yield	91	Isolated yield	88
Final yield	83	Final yield	85

[a] Total volume = 15 mL.
[b] pH 7.5.
[c] (NAD^+) = 500 μM, (polymyxin B sulfate) = 36 μM, pH 6.2. Reaction conditions: (Rosetta_XR_2FDH) = 40 g_{CDW}/L, reaction time 24 h.

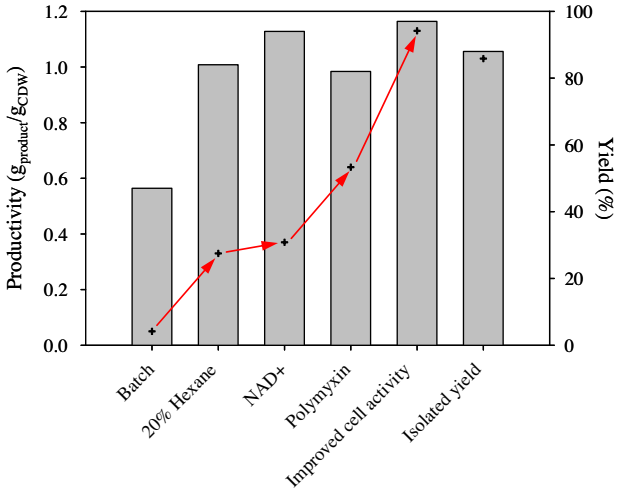

Fig. 4. Stepwise improvement of yields and productivities in bioreductions by process, reaction and host engineering. To an aqueous batch reduction 20% (v/v) hexane, 500 μM NAD^+ and 36 μM polymyxin B sulfate were added, the pH of the aqueous phase was shifted from 7.5 to 6.2 and BL21_XR_FDH was replaced by Rosetta_XR_2FDH. + = Productivities ($g_{product}/g_{CDW}$); gray bars = yields (%).

6.2 decreased by 25% as compared to the value obtained at pH 7.5 (Fig. 2(a)). We compared whole cell reductions at pH values of 6.2, 6.5, 7.0, and 7.5. Yields in aqueous batch reductions of 100 mM substrate increased 2-fold upon a pH shift from 7.5 to 6.2 (Fig. 2(a)). The reflexion of *Ct*XR-activity in reduction yields suggests equal extra- and intracellular pH values and hence a high perforation level of the cell.

The benefit from 2-fold increases in xylose reductase and FDH activities was best seen in the reductions of 300 and 400 mM substrate in two-phasic systems using 20% hexane supplemented with NAD^+ and polymyxin B sulfate. We obtained product concentrations of 285 and 323 mM with the catalyst Rosetta_XR_2FDH in reaction mixtures buffered to 6.2 (Fig. 3). Initial reduction rates (r_s) of 68 and 77 U/g_{CDW} were calculated from the amount of product formed after 30 min for reactions using 300 and 400 mM substrate, respectively. These values reflect most probably the reduction velocity limited by substrate availability in the cell. We determined substrate concentrations below 1 mM in the cell-containing, aqueous phases after a reaction time of 30 min.

3.3. Scale-up and product isolation

Workup issues are often neglected in whole cell biocatalysis and hardly found in literature even though they have a huge impact on final yields. Product isolation considerably contributes to the overall production cost and economization of product isolation is a key tool to maximize productivities. We investigated the isolation of (*S*)-1-(2-chlorophenyl)ethanol obtained from 15 mL-scale resin-based and two-phasic whole cell bioreductions. Analytical yields of 91% and 97% were obtained in whole cell reductions containing resin and 100 mM substrate or hexane, additives and 300 mM substrate (Table 6). Easy scalability was demonstrated by analytical yields that were, within the experimental error, equal to yields from 1 mL bioreductions. The product from the resin-based reduction was extracted twofold with ethyl acetate in a total yield of 97%. No product or substrate was found in the aqueous phase indicating a loss of 3% product in the biomass. The ethyl acetate-phase was dried with sodium sulfate prior to evaporation at reduced pressure. The product (*S*)-1-(2-chlorophenyl)ethanol was isolated in a final yield of 83% (0.19 g) as colorless liquid (Table 6). The reaction mixture containing 20% hexane and 291 mM product was extracted thrice with hexane. The overall yield of the extraction steps was 88%. No product was detected in the aqueous phase. We omitted drying of the hexane phase due to low solubility of water in hexane (<50 mg/L). A final yield of 85% (0.60 g) was determined after hexane evaporation at reduced pressure. We have previously reported on a final yield of 67% from an aqueous bioreduction of 100 mM *o*-chloroacetophenone by 40 g_{CDW}/L *E. coli* BL21_XR_FDH cells (Kratzer et al., 2011). A product solution of 97 mM was obtained with product concentrated above its solubility limit of 80 mM. The product emulsion was dissolved by the addition of ethanol and cell debris was centrifuged. However, cellular residues coagulated at the organic-aqueous interface during extraction with CH_2Cl_2. The present method using hexane as extraction media and as co-solvent in bioreductions has several advantages. First, use of the same organic solvent in two-phasic bioreductions and in downstream processing reduces process complexity and number of steps, second, hexane is nontoxic and product loss in the cell debris is reduced as compared to extraction with CH_2Cl_2. The relatively easy separation of cell debris from the hexane phase furthermore renders additional filtration steps obsolete (Gröger et al., 2006).

4. Conclusion

High toxicities of *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethanol to biocatalysts require the (1) use of whole cell catalysts and (2) separation of substrate and product from the catalyst-containing phase. Hexane used as co-solvent increased volumetric productivity and allowed for further catalyst and reaction engineering. Integration of the operational units 'bioreduction' and 'product isolation' led to simplified product recovery. Process development steps are briefly summarized in Fig. 4. The presented improvements in product concentration correspond to process intensification in almost its original definition, that the efficacy of the process is enhanced by two orders of magnitude (Ramshaw, 1999).

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