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Pushing the limits: Cyclodextrin-based intensification of bioreductions

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ABSTRACT

The asymmetric reduction of ketones is a frequently used synthesis route towards chiral alcohols. Amongst available chemo- and biocatalysts the latter stand out in terms of product enantiopurity. Their application is, however, restricted by low reaction output, often rooted in limited enzyme stability under operational conditions. Here, addition of 2-hydroxypropyl-β-cyclodextrin to bioreductions of *o*-chloroacetophenone enabled product concentrations of up to 29 % w/v at full conversion and 99.97 % e.e. The catalyst was an *E. coli* strain co-expressing NADH-dependent *Candida tenuis* xylose reductase and a yeast formate dehydrogenase for coenzyme recycling. Analysis of the lyophilized biocatalyst showed that *E. coli* cells were leaky with catalytic activity found as free-floating enzymes and associated with the biomass. The biocatalyst was stabilized and activated in the reaction mixture by 2-hydroxypropyl-β-cyclodextrin. Substitution of the wild-type xylose reductase by a D51A mutant further improved bioreductions. In previous optimization strategies, hexane was added as second phase to protect the labile catalyst from adverse effects of hydrophobic substrate and product. The addition of 2-hydroxypropyl-β-cyclodextrin (11 % w/v) instead of hexane (20 % v/v) increased the yield on biocatalyst 6.3-fold. A literature survey suggests that bioreduction enhancement by addition of cyclodextrins is not restricted to specific enzyme classes, catalyst forms or substrates.

1. Introduction

Cyclodextrins (CDs) are macrocyclic oligosaccharides composed of six to more than 100 α-(1,4) linked D-glucopyranose units. These naturally occurring products of starch degradation most commonly consist of 6, 7 or 8 glucose units that form a truncated cone with a hydrophilic exterior surface and a more hydrophobic interior cavity. CDs are able to accommodate appropriately sized, hydrophobic molecules in host·guest complexes (S·CD). Their ability to form inclusion compounds is widely exploited to enhance aqueous solubility and stability of hydrophobic molecules (Del Valle, 2004; Jambhekar and Breen, 2016). Less known are specific interactions between CDs and proteins: CDs host aromatic surface residues in host·guest complexes and 'cap' solvent exposed amino acids. The most often reported effect is protein stabilization by hydrophobic surface reduction and masking of protease-attack sites (Aachmann et al., 2011, 2004; Serno et al., 2011). Application of CDs in biocatalysis has hence potential to overcome two frequently encountered problems: low solubilities (and stabilities) of substrates and products and deterioration of biocatalysts under process conditions. However, the application of CDs to promote biotransformations is still limited to a relatively low number of examples. The majority of them relate to reaction enhancement by solubilization of sparingly water-soluble substrates (Bungaruang et al., 2016; Schmölzer et al., 2018; Yuan et al., 2019) or by inhibitor blocking in product- or substrate-inhibited reactions (Bonnet et al., 2010; Gubica et al., 2011). Improved reaction rates and product concentrations upon addition of CDs have been also assigned to stabilization and activation of catalytic enzyme(s) under process conditions (Cao et al., 2017; Wang et al., 2019; Zhang et al., 2016). Enhanced enantioselectivity by addition of CDs has been reported for lipases (Bonnet et al., 2010; Mine et al., 2003). Cell permeabilization was identified as an additional effect of CDs in whole-cell biocatalysis (He et al., 2016; Giorgi et al., 2019).

Here, we studied the addition of CDs to bioreductions of *o*-chloroacetophenone to (*S*)-1-(2-chlorophenyl)ethanol. The product constitutes a chiral building block for L-clorprenaline, polo-like kinase 1 inhibitors, lysophosphatidic acid receptor agonists and cannabinoid receptor 2 agonists (Nettekoven et al., 2016; Sato et al., 2012). The used enzyme was xylose reductase from *Candida tenuis* (*Ct*XR), an enzyme well known for its ability to convert a broad spectrum of ketones with high enantioselectivities (Kratzer et al., 2006; Krump et al., 2014).

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However, *Ct*XR showed moderate activities and low stabilities under reaction conditions. Therefore, a more stable *E. coli* whole-cell catalyst based on *Ct*XR and *Candida boidinii* formate dehydrogenase (*Cb*FDH) for NADH recycling was designed. in situ extraction of hydrophobic substrates and products into hydrophobic solvents was used to further separate the biocatalyst from toxic substrates and products. A catalyst loading of ~40 g_{CDW}/L *E. coli* whole-cell catalyst led to full conversion of maximally 300 mM *o*-chloroacetophenone (total turnover number 1.1 $g_{product}/g_{CDW}$). The reaction was still limited by catalyst lifetime in the presence of hydrophobic *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethanol (Schmölzer et al., 2012). In the present study, the replacement of organic solvents by a CD resulted in substantial improvement of product concentrations: First, we selected the most suitable CD for reaction intensification. Then, effects of the CD on substrate solubilization and catalyst performance (activity and stability) were dissected and bioreductions were optimized aiming at full conversion at high substrate loading. Finally, a survey amongst literature reports on bioreduction enhancement by cyclodextrins was carried out.

2. Material and methods

2.1. Chemicals and strains

2-Hydroxypropyl-β-cyclodextrin (HBC, degree of substitution 2–9, product code OH05393, batch number OH053931501) and γ-cyclodextrin were purchased at Carbosynth (Berkshire, UK), β-cyclodextrin and *o*-chloroacetophenone were from Sigma-Aldrich (Vienna, Austria), 1-(2-chlorophenyl)ethanol was from Alfa Aesar (Kandel, Germany), NADH-disodium salt, NAD^+ and Pierce™ BCA Protein Assay Kit were from Roth (Karlsruhe, Germany), B-PER© cell lysis buffer was from Thermo Scientific (Vienna, Austria). 2-mL reaction tubes were from Eppendorf (Vienna, Austria). Other chemicals were from Sigma-Aldrich/Fluka or Roth, and were of the highest purity available. Two *E. coli* Rosetta2 strains co-expressing *Ct*XR variants (GenBank ID wild-type *Ct*XR AF074484) and *Cb*FDH (GenBank ID AJ011046) were used: XR_wild-type strain based on *Ct*XR wild-type (Schmölzer et al., 2012) and XR_D51A strain based on the *Ct*XR mutant D51A. *Ct*XR D51A was previously described (Kratzer et al., 2006). Co-transformation of *E. coli* Rosetta2 with pET11a carrying the *Ct*XR D51A gene and pRSF-1b carrying the *Cb*FDH gene was accomplished by electroporation. Whole-cell biocatalyst production was previously described by Leis et al. (2017) and is summarized in the Supplementary data.

2.2. Intrinsic solubilities (S_0), complex stoichiometries (S•CD) and binding constants ($K_{S\cdot CD}$)

Phase solubility studies were carried out according to the method described by Higuchi and Connors (1965). HBC stock solutions were prepared in potassium phosphate buffer (100 mM, pH 6.2) and diluted to final volumes of 1 mL. An average molecular weight of 1453 g/mol was assumed for HBC (degree of 2-hydroxypropyl substitution 2–9 per β-CD molecule, Carbosynth information). Guest molecules (*o*-chloroacetophenone or (*S*)-1-(2-chlorophenyl)ethanol) were added in excess amounts to HBC solutions of defined concentrations. Mixtures of HBC and guest molecules were equilibrated for 24 h on an end-over-end rotator at 30 rpm and room temperature. Subsequent to equilibration, samples were centrifuged for 10 min at 13.2k rpm. Defined aliquots were taken from the HBC and guest molecule containing aqueous phase, diluted into isopropanol/anhydrous ethanol (1:9) and mixed on a vortex mixer. The decreased reaction medium polarity favoured decomplexation. Thus, HPLC measurements allowed determination of the apparently dissolved guest molecule concentration (S_{tot}), i.e. concentration of the guest molecule's inherent solubility (S_0) plus concentration of the formed complex (S•CD). A plot of S_{tot} versus HBC concentration reveals S_0 as intersect. The obtained slope (k) details complex stoichiometry: $k \leq 1$ suggests a 1:1 complex, $k > 1$ a 2:1 complex. Binding constants (K) can be calculated by rearranging the chemical equilibrium for cyclodextrin and guest molecules where solely the slope (k) and the intrinsic solubility are needed for determination. Equilibrium constants were calculated using Eq. 1 for 1:1 complexes and Eq. 2 for 2:1 complexes (Del Valle, 2004; Loftsson et al., 2004).

$$K_{1:1} = \frac{k}{S_0 \cdot (1-k)} \quad (1)$$

$$K_{2:1} = \frac{k}{(S_0^2 \cdot (2-k))} \quad (2)$$

Quantification of *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl) ethanol) was accomplished by HPLC as described previously (Schmölzer et al., 2012).

2.2.1. HBC•*o*-chloroacetophenone complex preparation

As the complex stoichiometry for (*S*)-1-(2-chlorophenyl)ethanol) with HBC resulted in two product molecules being enclosed at once, a two-fold substrate excess with regard to HBC was applied for complexation. HBC•*o*-chloroacetophenone complex formation was prepared by liquid complexation: HBC was dissolved in potassium phosphate buffer followed by stoichiometric substrate addition. The mixture was vortexed for 10 s. Alternatively, in case of a substrate loading of 2 M, cyclodextrins were directly mixed with the stoichiometric amounts of substrate along with 50 µL potassium phosphate buffer (100 mM, pH 6.2).

2.3. Catalyst preparations, bioreductions and product analysis

2.3.1. Catalyst preparations for bioreductions

All catalyst preparations started from lyophilized whole-cell catalyst.

Whole biomass (as suspended and rehydrated). Lyophilized biomass (40 mg or 80 mg) was rehydrated in 1 mL potassium phosphate buffer (100 mM, pH 6.2) containing NAD^+ (0.5−6 mM) and sodium formate (50 mM excess on [substrate]). Used 2-mL Eppendorf tubes were placed on a thermomixer for 30 min at 25 °C and 1400 rpm.

Pelleted biomass. The pellet was obtained by centrifugation (3220 *g*, 25 °C, 10 min) of the respective reaction mixture (1 mL) subsequently to the bioreduction.

Supernatant (cell-free catalyst). Lyophilized and rehydrated whole-cell catalyst (40 or 80 mg/mL in 1 mL) was centrifuged at 3220 *g* and 25 °C for 10 min.

2.3.2. Bioreductions

With whole biomass (as suspended and rehydrated). Rehydrated cells were combined with HBC and *o*-chloroacetophenone and filled up with potassium phosphate buffer (100 mM, pH 6.2) to a total working volume of 1 mL (biomass concentrations 1.5–40 mg_{CDW}/mL). Reaction mixtures with 300 mM substrate were incubated for 24 h, mixtures with higher substrate concentrations for 48 h. Incubation took place on an end-over-end rotator (30 rpm) at room temperature.

With pelleted biomass. Rebatch bioreductions were prepared with the pelleted biomass instead of the whole biomass under otherwise identical conditions.

With the supernatant (cell-free catalyst). Bioreductions with the cell-free catalyst were prepared with the supernatant instead of the whole biomass under otherwise identical conditions.

2.3.3. Bioreductions of 1.9 M *o*-chloroacetophenone and product isolation

Rehydrated whole-cell catalyst (40 mg), *o*-chloroacetophenone (1.9 M), sodium formate (1.95 M), HBC (75 mM) and NAD^+ (6 mM) were adjusted to 1 mL with potassium phosphate buffer (100 mM, pH 6.2) and placed on an end-over-end rotator (30 rpm) for 48 h. Subsequent to the reaction, the product was extracted thrice by adding 1.5 (v/v) ethyl acetate followed by centrifugal phase separation at 3220 g, 25 °C for 10 min. The product (*S*)-1-(2-chlorophenyl)ethanol was concentrated under reduced pressure.

2.4. Determination of catalyst activity and effect of HBC on the catalyst

2.4.1. Catalyst activity measured prior to bioreductions

Total enzyme activities of the whole biomass (extracellular and intracellular enzymes). The suspension was directly subjected to cell lysis and protein extraction with B-PER© cell lysis reagent. Enzyme activities were assayed subsequently to the protein extraction.

Enzyme activities of the supernatant (extracellular enzymes). The rehydrated biomass was centrifuged and the activities of xylose reductase and formate dehydrogenase that had leaked out of the cells were determined in the supernatant.

Spectrophotometric determination of enzyme activities. Reductase and dehydrogenase activities were assayed by monitoring the reduction or oxidation of NAD(H) at 340 nm (Beckman Coulter DU 800® spectrophotometer). Rates of 0.05−0.10 ΔA/min were measured over a time period of 5 min. 1 μmol of NADH formed or consumed per minute equals one unit of enzyme activity. The assay for *Ct*XR wild-type contained 700 mM D-xylose and 300 μM NADH; that for *Cb*FDH contained 200 mM sodium formate and 2 mM NAD^+. Assays were performed in 100 mM potassium phosphate buffer (pH 6.2) and started by adding NADH or NAD + . Non-specific background oxidation/reduction was considered by measuring blank activities.

2.4.2. Effect of HBC on the catalyst (whole biomass as suspended and rehydrated)

Rehydrated cells (40 mg in 500 μL) were added to 2-mL Eppendorf tubes containing pre-dissolved HBC (0, 38, 75, 150, 310 mM) and were filled up to 1 mL (potassium phosphate buffer, 100 mM, pH 6.2). The mixtures were placed on an end-over-end rotator (30 rpm). Samples (10 μL) were taken over time and directly subjected to spectrophotometric determination of enzyme activities.

2.5. Effect of HBC on enzyme stabilities in the presence of heptane

Experiments were performed with *Ct*XR (wild-type) and *Cb*FDH double-purified from the whole-cell catalyst by affinity chromatography. The protocol followed the standard protocol for *Ct*XR purification using a Red 31 dye ligand column (6 × 2.6 cm, Mayr et al., 2000) (Supplementary data). The specific activity for *Ct*XR was calculated to 12.4 U/mg, while partially purified FDH was determined to 0.64 U/mg. Enzymes were stored at −20 °C. 0.5 mL of heptane was added to 2-mL Eppendorf tubes containing *Ct*XR (0.1 mg/mL) or *Cb*FDH (0.1 mg/mL) and HBC (0, 38, 75, 150, 310 mM) in potassium phosphate buffer (100 mM, pH 6.2). Samples were placed on an end-over-end rotator at 30 rpm and room temperature for 24 h. Enzyme activities were measured over time. All samples were prepared in triplicates.

2.6. HPLC analysis

Subsequent to the reaction, mixtures were vortexed vigorously. Aliquots (100–500 μL) were taken, diluted stepwise in 7:3 ethanol/dH_2O and vortexed. Alternatively, the work up was performed by transferring the whole reaction mixture (1 mL) into 15 mL Sarstedt tubes followed by addition of 4 mL isopropanol. The mixture was vortexed and centrifuged for 10 min, 25 °C and 3220 *g*. The resulting supernatant was isolated, syringe-filtered and properly diluted using isopropanol. Quantification of *o*-chloroacetophenone, (*S*)-1-(2-chlorophenyl) ethanol) and (*R*)-1-(2-chlorophenyl)ethanol) was accomplished by chiral HPLC as described previously (Schmölzer et al., 2012). Conversions were calculated as Eq. (3):

$$Conversion = \frac{[Product]}{[Product] + [Substrate]} \quad (3)$$

Total recoveries of substrate and product in small-scale bioreductions were between 66 and 99 %. Analyte loss suggested that parts of hydrophobic substrates and products remained in the cell sludge and/or adsorbed to plastics (tubes, tips). Generally, higher recoveries were obtained after harsh reaction conditions, i.e. higher substrate concentrations and longer reaction times (Leis et al., 2017). CD concentrations played a minor role in analyte recovery.

3. Results and discussion

3.1. Cyclodextrin selection

The initial intention of the present study was to protect the catalyst in the reaction mixture by complexing hydrophobic substrate and product. Therefore, we searched for CDs capable of hosting *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethanol. The main determinant for the formation of a tight S•CD complex is the cavity size of the cyclodextrin. In most applications, CDs with 6, 7 or 8 α-(1,4) linked D-glucopyranose units, referred to as α-, β- or γ-CDs, are used. α-CD is able to complex low molecular weight molecules or aliphatic compounds. β-CD accommodates aromatics and heterocycles and is mostly used to complex drugs, flavors, cosmetic ingredients and pesticides. Main drawback of β-CD is its low water-solubility (18 g/L) caused by H-bond aided self-aggregation in aqueous solution. Derivatization of the 2-, 3-, and 6-OH groups disturbs crystallization and leads to amorphous structures favoring gel-formation over precipitation. Etherification of the glucose C2-OH groups with 2-hydroxypropyl groups increased the solubility of β-CD to ~45 % (w/v) (Caligur, 2008; Szejtli, 2004). γ-CD can accommodate even larger molecules. However, γ-CD has weaker complex-forming ability than β-CDs (Del Valle, 2004). We tested 2-hydroxypropyl β-CD (abbreviated HBC), unmodified β-CD and γ-CD as additives for bioreductions of 300 mM *o*-chloroacetophenone. The addition of substrate to an aqueous solution of 75 mM HBC resulted in a visible increase in apparent solubility (S_{tot}) of *o*-chloroacetophenone. Loading of 4 g_{CDW}/L lyophilized and rehydrated XR_wild-type cells to mixtures of HBC and 300 mM substrate yielded 89 % product (Fig. 1A). Addition of substrate to solutions of 75 mM β-CD or γ-CD led to visible precipitation. Conversions obtained with 4 g_{CDW}/L cells and 300 mM substrate in the presence of β-CD or γ-CD were 10 and 9 %, respectively, similar to reaction mixtures without CDs (data with β-CD or γ-CD not shown). Therefore, we focused on complex-formation of *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl)ethanol with HBC.

3.2. Complex characterization

Formation of host•guest complexes leads to an apparent solubility (S_{tot}) increase of the hydrophobic guest molecule. We observed linear increases in S_{tot} for *o*-chloroacetophenone and (*S*)-1-(2-chlorophenyl) ethanol at stepwise elevated HBC concentrations (Fig. 2). It has been previously shown that slopes with $k \leq 1$ indicate 1:1 complexes, slopes with $k > 1$ imply 2:1 complexes, respectively. In the latter case, two guest molecules bind to one cyclodextrin molecule (Del Valle, 2004; Loftsson et al., 2004). The obtained slope of k 0.92 for *o*-chloroacetophenone suggested hence a 1:1 complex, while a slope of k 1.29 for (*S*)-1-(2-chlorophenyl)ethanol a 2:1 complex. Intersects reflect the intrinsic solubilities of guest molecules (S_0), which were 5.8 mM for *o*-chloroacetophenone and 19.0 mM for (*S*)-1-(2-chlorophenyl)ethanol. Binding constants ($K_{[S\cdot CD]}$) can be calculated by rearranging the chemical equilibrium for S•CD formation where solely the slope (k) and the intrinsic solubility are needed for determination (equs. 1, 2; Del Valle, 2004). For *o*-chloroacetophenone $K_{[S\cdot CD]}$ was calculated to 2.0 mM^{-1}, while $K_{[S\cdot CD]}$ for (*S*)-1-(2-chlorophenyl)ethanol equals 5.0 mM^{-1}. The higher the constants are, the more effective complexation is.

3.3. Bioreductions of 300 mM o-chloroacetophenone

3.3.1. Bioreductions using the whole biomass as catalyst

Previously, 300 mM *o*-chloroacetophenone represented the highest

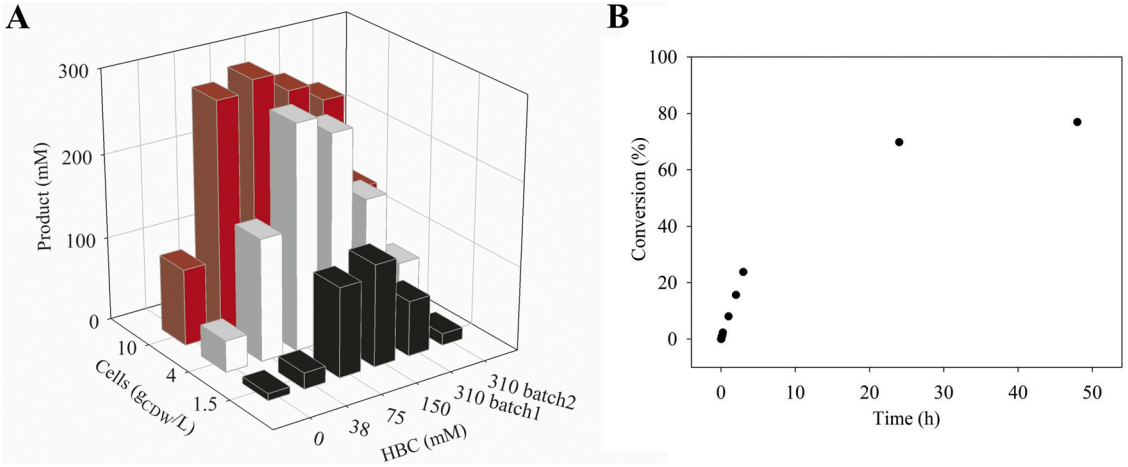

Fig. 1. (A) Bioreductions of 300 mM *o*-chloroacetophenone with lyophilized and rehydrated biomass (XR_wild-type strain). Product concentrations obtained at different cell and HBC concentrations are shown. (NAD^+ 0.5 mM, reaction time 24 h. Recoveries were 65 – 88 %, maximal std. dev. ± 8.6, each experiment was done in triplicates. Data with std. dev. summarized in the Supplementary data.) (B) Time course of the reaction with a catalyst loading of 4 g_{CDW}/L and 75 mM HBC.

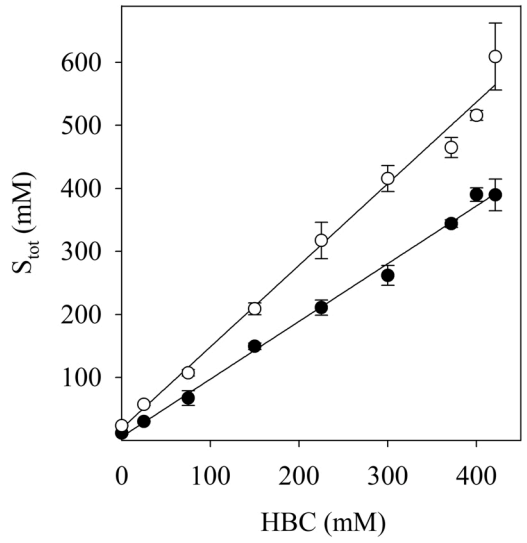

Fig. 2. Phase solubility relationships. Apparent guest molecule solubility ($S_{tot}=S_0+S\cdot CD$) related to HBC concentrations (● *o*-chloroacetophenone, ○ (*S*)-1-(2-chlorophenyl)ethanol, linear fit for *o*-chloroacetophenone y = 0.92x+5.84, for (*S*)-1-(2-chlorophenyl)ethanol y = 1.29x+19.04).

substrate concentration that was fully reduced by 40 g_{CDW}/L XR_wild-type cells (conversion 97 %). A sufficiently long catalyst lifetime was ensured by in situ substrate supply and product removal with hexane as second solvent (Schmölzer et al., 2012). Here, we replaced the second solvent by HBC and reduced the amount of catalyst. Concentrations of XR_wild-type cells were varied from 1.5–10 g_{CDW}/L and HBC from 0–310 mM (Fig. 1A). 310 mM HBC corresponded to the amount of HBC necessary to fully dissolve 300 mM *o*-chloroacetophenone (Fig. 2). In each case, addition of HBC increased conversions compared to unmodified mixtures. 10 g_{CDW}/L catalyst and addition of 75 mM HBC resulted in full conversion (97 %). At higher cyclodextrin concentrations, conversions dropped again. Time course analysis of the reaction mixture with 4 g_{CDW}/L and 75 mM HBC showed high catalyst activity over 24 h. The conversion increased upon prolongation of the bioreduction from 24 to 48 h further 7 % (Fig. 1B). Conversions with 0, 75- and 150-mM HBC were investigated for prolonged reaction times. The increase in reaction time from 24 to 48 h resulted in up to 25 % higher conversions without HBC and up to 69 % with HBC (summarized in the Supplementary data).

3.3.2. *Rebatch using the pelleted biomass as catalyst*

High catalyst activity over several hours indicated reusability of the whole-cell catalyst. For this purpose, the catalyst (whole cells, cell debris) was separated from the first reaction mixtures by centrifugation and used as catalyst in further, identical batch reactions. However, conversions in second batches were between 22 and 53 % of the first batches (batch1, batch2, Fig. 1A, Table 1). Therefore, the supernatant of rehydrated cells was investigated to quantify catalyst loss by centrifugal catalyst recovery.

3.3.3. *Bioreductions with the supernatant*

Conversions with the supernatants of 1.5 and 4 g_{CDW}/L resulted in 48 and 62 % of the conversions obtained with the whole biomass (both in the presence of 75 mM HBC). The supernatant of 10 and 40 g_{CDW}/L led to full conversions (≥98 %) (Table 1).

3.3.4. *Biocatalyst activity*

Activities of the whole biomass (extracellular and intracellular enzymes), measured after cell lysis and protein extraction for xylose reductase and formate dehydrogenase were 1590 U/g_{CDW} and 154 U/g_{CDW}, respectively. The rehydrated biomass was centrifuged and the activities of xylose reductase and formate dehydrogenase that had leaked out of the cells were determined to 512 U/g_{CDW} and 72 U/g_{CDW}, respectively, in the supernatant. Hence, approximately 40 % of the catalytic activities were free-floating enzymes and not associated with the biomass.

Catalyst activities as measured prior to bioreductions in initial rate measurements and total turnover numbers obtained in bioreductions of 300 mM *o*-chloroacetophenone were summarized in Table 1. The supernatants of 1.5 g_{CDW}/L achieved approximately half of the total turnover number of the whole biomass in the presence of 75 mM HBC (expressed as g product per g_{CDW}, referring to the used biomass, Table 1). The supernatants of 10 and 40 g_{CDW}/L fully reduced 300 mM of substrate with total turnover numbers similar to the whole biomass. Table 1 shows furthermore conversions of 300 mM *o*-chloroacetophenone in the presence of the highest amount of HBC used (310 mM). The first batch (batch 1) was performed with the whole biomass (resuspended cells comprising extracellular and intracellular enzymes). The second batch (batch 2) was performed with the pelletized catalyst from batch 1 i.e. with the enzymes attached to the biomass (intracellular enzymes). In the present case, catalyst activity is divided into free-floating activity (extracellular enzymes) and activity attached to the biomass after centrifugation (intracellular enzymes). Addition of HBC stabilizes extracellular enzymes seen as high activity of the

Table 1
Comparison of different catalyst preparations in the whole cell reduction of 300 mM *o*-chloroacetophenone based on conversions and yields on biocatalysts[a]. (Product concentrations are shown. Experiments with whole biomass and pelleted biomass were done in triplicates; mean values with std. dev. are shown. Experiments with supernatant and 40g_{CDW}/L were done in duplicates; mean values are shown).

Catalyst preparation[b]	Biomass amount			
	1.5g_{CDW}/L	4g_{CDW}/L	10g_{CDW}/L	40g_{CDW}/L
Whole biomass as rehydrated (total activity XR 1590 U/g_{CDW}, FDH 154 U/g_{CDW} i.e. extracellular and intracellular enzymes) Conversion (mM) (with 75 mM HBC) Yield on biocatalyst ($g_{product}/g_{CDW}$)	107 ± 18 11.2	268 ± 15 10.5	292 ± 3.6 4.6	297 1.2
Supernatant (centrifugation after rehydration, XR 510 U/g_{CDW}, FDH 72 U/g_{CDW} i.e. extracellular enzymes) Conversion (mM) (with 75 mM HBC) Yield on biocatalyst ($g_{product}/g_{CDW}$)	51 5.3	166 6.5	293 4.6	300 1.2
Whole biomass as rehydrated (total activity XR 1590 U/g_{CDW}, FDH 154 U/g_{CDW} i.e. extracellular and intracellular enzymes) Conversion (mM) (with 310 mM HBC, batch 1) Yield on biocatalyst ($g_{product}/g_{CDW}$)	66 ± 10 6.9	158 ± 10 6.2	248 ± 8 3.9	n.d.
Pelleted biomass from batch 1 (intracellular enzymes) Conversion (mM) (with 310 mM HBC, batch 2) Yield on biocatalyst ($g_{product}/g_{CDW}$)	14 ± 5 1.5	69 ± 2 2.7	133 ± 6 2.1	n.d.

n.d. not determined. [a]NAD^+ 0.5 mM, reaction time 24 h. [b]XR activities measured in initial rate measurements using xylose.

supernatant and intracellular enzymes seen as activity in pelletized biomass from reaction mixtures.

Note that the reductase activities were measured with xylose and not *o*-chloroacetophenone for the following reasons: *o*-Chloroacetophenone and 1-(2-chlorophenyl)ethanol have deactivating effects on free xylose reductase (Schmölzer et al., 2012). The low solubility of *o*-chloroacetophenone impedes full saturation of the biocatalyst. *o*-Chloroacetophenone forms microemulsions (Xie et al., 2006), the true concentration of a ≥5.8 mM *o*-chloroacetophenone solution is hence flawed (5.8 mM represents the solubility limit S0, Fig. 2). The measured xylose reductase activity of 1590 U/g_{CDW} on xylose corresponds to approximately 350 U/g_{CDW} on *o*-chloroacetophenone.

3.4. Optimization of the bioreduction

3.4.1. Catalyst activity limitation

The time course of a typical bioreduction using the whole biomass (suspended and rehydrated) as catalyst in the presence of 75 mM HBC and 0.5 M NAD^+ (Fig. 1B) showed an initial rate of 102 U/g_{CDW} at a substrate concentration of 300 mM. The value was 66 % of the totally applied *Cb*FDH activity with 154 U/g_{CDW}. The corresponding *Ct*XR activity on *o*-chloroacetophenone was 350 U/g_{CDW}. However, the activity of the wild-type *Ct*XR was diminished by low *o*-chloroacetophenone and NADH concentrations: The S_0 of *o*-chloroacetophenone was 5.8 mM at a $K_{m,o\text{-chloroacetophenone}}$ of 5.1 mM, the one of NADH was maximally 0.5 mM at a $K_{m,NADH}$ of 0.17 mM (Krump et al., 2014; Kratzer et al., 2011). The *Ct*XR activity (as measured under substrate-saturated conditions) corrected for low coenzyme and *o*-chloroacetophenone concentrations led to a rest activity of ~70 % (using $v = v_{max}[NADH][o\text{-chloroacetophenone}]/(K_{i,NADH}K_{m,o\text{-chloroacetophenone}} + K_{m,o\text{-chloroacetophenone}}[NADH] + K_{m,NADH}[o\text{-chloroacetophenone}] + [NADH][o\text{-chloroacetophenone}])$;

($K_{i,NADH}$ is an apparent dissociation constant for NADH, a value of $K_{m,NADH}$ was estimated). Furthermore, formate exerted non-competitive inhibition on the wild-type *Ct*XR ($K_{i,formate}$ 182 mM; Neuhauser et al., 1998). Inclusion of inhibition by formate further decreased rest activities to ~25 % equal to 80 U/g_{CDW}. This value was in the order of the initial rate and well below the FDH activity with 154 U/g_{CDW}. The wild-type reductase displayed a k_{cat}/K_m value of ~430 $M^{-1}s^{-1}$ for *o*-chloroacetophenone (no full saturation achieved within limits of solubility, (Kratzer et al., 2011). An Asp51 to Ala replacement (D51A mutant) resulted in a ~13-fold higher k_{cat}/K_m value (5750 $M^{-1}s^{-1}$). In order to improve the bioreduction, the catalytic reductase was replaced by the D51A mutant (XR_ D51A strain). Additionally, a high catalyst loading of 40 g_{CDW}/L was applied. The XR_ D51A strain accomplished 97.6 % conversion in a reaction mixture containing 1.5 M substrate, 75 mM HBC and 6 mM NAD^+. In comparison, 62 % were obtained with the original strain under otherwise identical conditions. Comparison of XR_wild-type and XR_D51A strains is summarized in Table 2.

3.4.2. NAD^+ and substrate concentrations

Effects of increased NAD^+ and substrate concentrations are summarized in Table 2. NAD^+ added at concentrations of 0.5 and 3 mM led to conversions of 20 and 95 % for 1.5 M substrate with the XR_D51A strain. The 4.6-fold higher conversion of 1.5 M *o*-chloroacetophenone by an increase of NAD^+ concentration from 0.5 to 3 mM suggested a ~3–5-fold higher $K_{m,NADH}$ of the D51A *Ct*XR compared to the wild-type *Ct*XR (0.17 mM, Krump et al., 2014). Higher substrate concentrations of 2 M, 2.5 M and 3.2 M led to conversions of 92 %, 33 % and 3.1 %, respectively, in the presence of 75 mM HBC. We experienced an increase in reaction mixture viscosity and a decrease in miscibility at higher substrate concentrations. Reaction mixtures started to lose fluidity at substrate concentrations ≥ 2 M.

3.4.3. Concentration of HBC

HBC was varied between 38 and 310 mM in reaction mixtures with 2 M substrate and high loading of the XR_ D51A strain (Fig. 3). Conversions in reactions with HBC followed the trend observed for reductions of 300 mM substrate: Positive effects on conversions were observed up to 75 mM HBC while conversions dropped at higher HBC concentrations. The expected correlation of HBC and substrate concentration on conversion was not experienced. Only low amounts of substrate and product were complexed at HBC-to-substrate ratios of 0.25 to

Table 2
Comparison of XR_wild-type and XR_D51A strains in bioreductions of *o*-chloroacetophenone. Effects of increasing NAD^+ (0.5 – 6 mM) and *o*-chloroacetophenone (1500 – 3200 mM) concentrations. (Product concentrations with std. dev. are shown. Each experiment was done in triplicates.).

Strain	NAD^+ (mM)	Substrate (mM)	Product (mM)
XR_D51A	0.5	1500	308 ± 14
XR_D51A	1.5	1000	840 ± 95
XR_wild-type	1.5	1500	740 ± 42
XR_D51A	3	1500	1430 ± *n.d.*
XR_D51A	6	1500	1464 ± 20
XR_wild-type	6	1500	930 ± 36
XR_D51A	6	2000	1840 ± 42
XR_D51A	6	2500	833 ± 70
XR_D51A	6	3200	99 ± 45

n.d. not determined. Catalyst loading was 40 g_{CDW}/L whole biomass as suspended and rehydrated, HBC concentration was 75 mM, reaction time 48 h. Recoveries 65–88%.

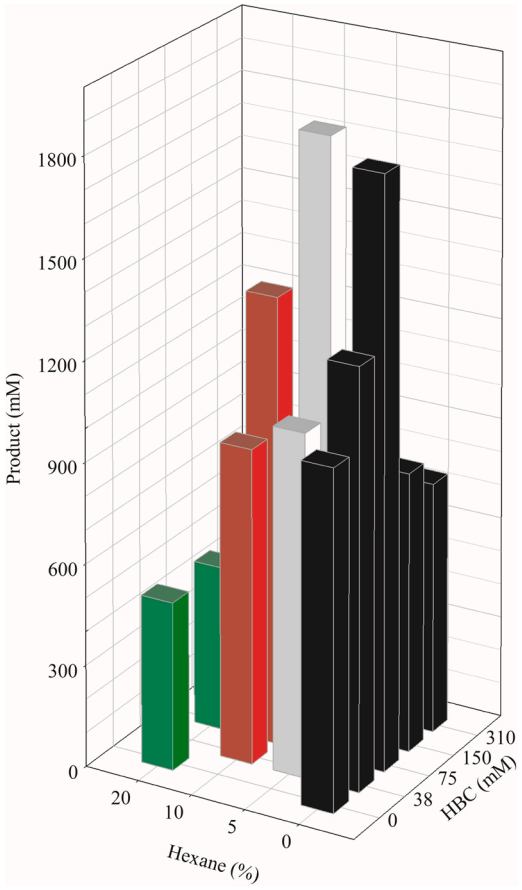

Fig. 3. Bioreductions of 2 M *o*-chloroacetophenone with 40 g_{CDW}/L lyophilized and rehydrated XR_D51A strain. Product concentrations obtained at different HBC and hexane concentrations are shown. (Catalyst loading 40 g_{CDW}/L, NAD^+ 6 mM, reaction time 48 h. Recoveries 93-99 %, maximal std. dev. ± 15 %, each experiment was done in triplicates.) (Data with std. dev. summarized in the Supplementary data.).

0.02 (Figs. 1A, 3). Complexation of catalyst-deactivating substrate and product with HBC was therefore ruled out as major reason for bioprocess-intensification. Obviously, HBC exerted another effect on the reaction.

3.4.4. Addition of water-immiscible organic solvents

CDs were used in previous studies as 'shuttles' to increase substrate/product transfer from the organic to the aqueous phase and vice versa (Bricout et al., 2009). Here, we added low amounts of hexane and heptane to reaction mixtures with 2 M substrate and 0–75 mM HBC (Fig. 3). No improvement was experienced upon addition of 5–20 % hexane or heptane to the reaction mixture. Addition of 5 % hexane had no effect on conversions, higher concentrations of hexane had a negative effect. Addition of heptane had a similar effect as hexane (summarized in the Supplementary data.) A study of the effect of heptane on the isolated, free-floating enzymes showed residual activities of 86 and 50 % of *Ct*XR wild-type and *Cb*FDH in plain buffer after 24 h (0 mM HBC). In the presence of 50 % v/v heptane residual activities decreased to 1 and 2 % within 24 h (0 mM HBC) The additional presence of 75 mM HBC stabilized free reductase and dehydrogenase tremendously. Residual activities of 98 and 80 % were determined for *Ct*XR wild-type and *Cb*FDH, respectively (summarized in the Supplementary data).

3.4.5. Additives to alter reaction medium polarity

The polarity of the reaction medium was increased with potassium chloride in order to strengthen hydrophobic interactions in host·guest complexes (Loftsson and Brewster, 2012). Vice versa, isopropanol was used to decrease reaction media polarity and thereby increase solubilities of substrate and product. Supplementation of reaction mixtures containing 150 mM HBC with 50 and 100 mM potassium chloride or 0.5–5 % v/v isopropanol to reactions had no effect on conversions. Addition of 10 % isopropanol led to 10 % lower conversions. No positive effects by altered reaction medium polarity were observed compared to unmodified reaction mixtures (summarized in the Supplementary data.)

3.4.6. Full conversion of 1.9 M o-chloroacetophenone and product isolation

We aimed at the maximally achievable product concentration at full conversion. Main variables tested were concentrations of substrate (300 mM to 3.2 M), HBC (0–310 mM), catalyst (1.5–40 g_{CDW}/L) and NAD^+(0.5–6 mM). Highest conversions were always obtained with 75 mM HBC, irrespective of the substrate, catalyst and NAD^+ concentrations (Figs. 1A, 3). A substrate concentration of 1.9 M was converted in 98.1 ± 2.3 % yield and 99.97 ± 0.05 % e.e. with 40 g_{CDW}/L XR_D51A cells (whole biomass as suspended and rehydrated), 75 mM HBC and 6 mM NAD^+. Extractive work-up of the product led to an isolated yield of 84 %. (Note that the high amount of product loss during work-up is partly due to a small reaction volume of 1 mL).

3.5. HBC effect on the catalyst

Reusability of the pelleted biomass from bioreduction mixtures containing HBC (Fig. 1A, Table 1) prompted studying catalyst activity and stability in the presence of HBC. We therefore determined *Ct*XR and *Cb*FDH activities in the presence of 0–310 mM HBC (Table 3). HBC addition after cell rehydration and sampling directly from suspensions of lyophilized cells showed up to 300 % activity for *Ct*XR and 180 % activity for *Cb*FDH compared to initial values without HBC (no cell lysis and protein extraction was performed.) The highest activities were obtained after 6 h with 75 mM HBC (activities in samples without HBC also showed highest values after 6 h). After 24 h, *Ct*XR rest activities in the presence of HBC were 40–190 % of the starting activity without HBC. The *Ct*XR rest activity was only 5 % without HBC. *Cb*FDH rest activity after 24 h was 90 % in the absence of HBC, while in the presence 120–170 % of the starting *Cb*FDH activity without HBC was found.

3.5.1. Catalyst stability

Isolated and free-floatig *Ct*XR and *Cb*FDH were efficiently stabilized by HBC against adverse effects of the water-immiscible heptane or hexane and water-miscible co-solvent isopropanol (section 3.4.4; summarized in the Supplementary data). In previous studies we experienced deactivation of the free enzymes within minutes and of whole-cell catalysts within maximally 5 h in the presence of substrate and product above their solubility limits (Schmölzer et al., 2012).

3.5.2. Catalyst form and activity

In the present case, cell integrity was severely impaired by biomass lyophilization: Approximately 40 % of total *Ct*XR and *Cb*FDH activities were found in the supernatant of lyophilized, rehydrated and centrifuged cells. The addition of HBC to suspended catalyst (whole biomass) led to an increase in enzyme activities over time (measured as initial rates from cell suspension samples and hence mainly covering free-floating enzymes, Table 3). The measured activities were up to 170 % and 108 % of the totally applied *Ct*XR and *Cb*FDH units, respectively (measured as initial rates after cell lysis and protein extraction, Table 3). Increasing activities upon addition of HBC might indicate catalyst activation and further protein extraction from the biomass. Therefore, high conversions and product concentrations were possible in a scenario of (1) abolished mass transfer by free-floating enzymes, (2) substantial stabilization of the free enzymes by HBC and (3) an up to 1.7-fold activation of *Ct*XR by HBC.

Table 3
Effect of HBC on specific enzyme activities[a]. The totally applied activities were 1590 and 154 U/g_{CDW} for *Ct*XR[b] and *Cb*FDH, respectively[c] (extracellular and intracellular enzymes). Data with std. dev., each experiment was done in triplicates.

	*Ct*XR activity (WT) (U/g_{CDW})[b]			*Cb*FDH activity (U/g_{CDW})		
HBC (mM)	0 h	6 h	24 h	0 h	6 h	24 h
0	874 ± 173	959 ± 264	40 ± 16	92 ± 6	151 ± 12	84 ± 15
38	1097 ± 144	913 ± 121	611 ± 72	116 ± 12	123 ± 15	124 ± 26
75	1679 ± 98	2668 ± 84	1620 ± 117	102 ± 5	166 ± 8	107 ± 6
150	1778 ± 221	1006 ± 68	349 ± 22	136 ± 13	154 ± 9	157 ± 18
310	1660 ± 119	1636 ± 324	576 ± 127	64 ± 20	142 ± 18	108 ± 3

[a]Samples were taken directly from suspensions of lyophilized cells. [b]XR activities measured in initial rate measurements using xylose. [c]The totally applied activities were measured after cell lysis and protein extraction.

3.6. Literature survey

Cyclodextrin-promoted bioreductions have been previously reported. In the present study, an *E. coli* whole-cell catalyst co-expressing an aldo-keto reductase superfamily member (*Ct*XR) and a member of the D-specific dehydrogenases of 2-hydroxy acids (*Cb*FDH) was used. Enhancement of bioreductions by addition of cyclodextrins is not restricted to specific enzyme classes. Stabilizing and activating effects on coupled oxidoreductase systems (free enzymes or whole-cell catalysts) were observed for all main oxidoreductase superfamilies. Zelinski et al. (1999) reported on bioreductions of hydrophobic ketones by a coupled oxidoreductase system. The used carbonyl reductase was a Zn-dependent alcohol dehydrogenase from *Candida parapsilosis* and the coenzyme regenerating enzyme *Cb*FDH. The continuous reduction of 2-acetylnaphthalene was performed in a membrane reactor in the presence of 50 mM heptakis-(2,6-di-O-methyl)-β-cyclodextrin. Only 1.2 % enzyme deactivation was experienced in 24 h in comparison to 85 % in control experiments without cyclodextrin. Improvement of bioreduction rates upon addition of β-CD have been reported for an *E. coli* whole-cell catalyst based on *Candida magnoliae* carbonyl reductase and *Bacillus megaterium* glucose dehydrogenase, both short-chain dehydrogenase/reductases. Addition of 0.2 and 0.4 mol/mol β-CD to reductions of 5-hydroxymethylfurfural and ethyl 4-chloro-3-oxobutanoate led to up to 6-fold reaction rate improvements (He et al., 2018, 2015). Similarly, addition of 0.06 mol/mol β-CD to bioreductions of ethyl 4-chloro-3-oxobutanoate increased reduction rates of an *E. coli* whole-cell catalyst co-expressing *Sporidiobolus salmonicolor* β-carbonylcarboxylic ester reductase (an aldo-keto reductase) and *Bacillus megaterium* glucose dehydrogenase 1.8-fold (He et al., 2016). It was previously demonstrated that the interaction of CDs takes place at specific sites on the protein surface. A hydrophilic 'cap' (the CD) is placed on solvent exposed aromatic residues. Thereby, aggregation is suppressed if hosted residues are responsible for aggregation and degradation is prevented in cases where the point of protease-attack is sterically 'masked' by the CD (Aachmann et al., 2011, 2004). Further effects such as altered stereoselectivities, reduction of mass transport limitations and changes in enzyme dynamics were also reported (Fasoli et al., 2006).

4. Conclusions

The bioreduction of *o*-chloroacetophenone is limited by catalyst instability. Application of hexane (20 %) as in situ substrate reservoir and product sink with 20 % hexane as co-solvent increased product concentrations to maximally 328 mM (total turnover 1.3 $g_{product}/g_{CDW}$) (Schmölzer et al., 2012). Unexpectedly high product concentrations of up to 1.5 M were obtained with the same catalyst upon replacement of hexane by HBC. Main effects of HBC were enzyme stabilization and cell permeabilization (He et al., 2016, 2015). Minor effects were enzyme activation, substrate and product complexation. Use of a more active catalytic reductase (D51A *Ct*XR) led to 1.9 M product and total turnover numbers of 7.3 $g_{product}/g_{CDW}$ (Table 4). Through the use of HBC, product concentrations obtained with the moderately active and labile *Ct*XR became comparable to the most positive bioreduction examples using highly active and stable oxidoreductases (Chamouleau et al., 2007; Chen et al., 2016; Ema et al., 2008; Jakoblinnert et al., 2011; Kataoka et al., 1999). The previously experienced low catalyst stability under process conditions was overcome by HBC addition. Thereby the main limiting factor was shifted from catalyst stability towards reaction mixture miscibility and viscosity (section 3.4.2). Literature reports suggest that bioreduction enhancement by addition of cyclodextrins is a universal method that is neither restricted to specific enzyme classes nor to catalyst form (whole-cell, free-floating enzymes) (section 3.6. Literature survey).

Table 4
Effect of additives on chemistry metrics of *o*-chloroacetophenone bioreductions.

	Aqueous, no additive[a]	20 % Hexane[b]	75 mM HBC[c]	75 mM HBC, high NAD^+, red. mutant[d]
Product concentration (g/L) (Conversion (%))	10.6 (68)	51.4 (82)	45.6 (97)	292 (98.1)
Yield on biocatalyst ($g_{product}/g_{CDW}$)	0.27	1.28	4.56	7.30

[a]NAD^+ 0.5 mM, substrate 100 mM, 40 g_{CDW}/L XR_wild-type strain (data from Kratzer et al., 2011).
[b]NAD^+ 0.5 mM, substrate 400 mM, 40 g_{CDW}/L XR_wild-type strain (data from Schmölzer et al., 2012).
[c]NAD^+ 0.5 mM, substrate 300 mM, 10 g_{CDW}/L XR_wild-type strain.
[d]NAD^+ 6 mM, substrate 1.9 M, 40 g_{CDW}/L XR_D51A strain.

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Appendix A. Supplementary data

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