Enzymatic Dynamic Reductive Kinetic Resolution Towards 115 g/L (S)-2-Phenylpropanol

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Abstract

Background

Published biocatalytic routes towards chiral 2-phenylpropanol by oxidoreductases showed product concentrations of maximally 80 mM. Enzyme deactivation turned out as one major limitation and was attributed to adduct formation of the aldehyde substrate with the catalytic reductase.

Results

A Candida tenuis xylose reductase single-point mutant (CtXR D51A) with very high catalytic efficiency (43·10^3 s^-1 M^-1) for (S)-2-phenylpropanal was identified. The enzyme showed high enantioselectivity for the (S)-enantiomer but was deactivated by 0.5 mM substrate within 2 h. A whole-cell biocatalyst based on the engineered reductase and a yeast formate dehydrogenase for NADH-recycling provided substantial stabilization of the reductase. The relatively slow in situ racemization of 2-phenylpropanal and the still limited biocatalyst stability required a subtle adjustment of the substrate-to-catalyst ratio. A value of 3.4 g_{substrate}/g_{cell-dry-weight} turned out as compromise between product enantiopurity and conversion. A catalyst loading of 40 g_{cell-dry-weight} was used to convert 1 M racemic 2-phenylpropanal to (S)-phenylpropanol in 93.1% e.e.

Conclusion

Mainly hydrolases have been exploited for the production of profenols at industrial scale so far. The herein established bioreduction presents an alternative route towards profenols that is competitive to hydrolase-catalyzed kinetic resolutions.

Highlights

- A Candida tenuis xylose reductase D51A mutant showed a 270-fold higher enzymatic activity and improved enantioselectivity for (S)-2-phenylpropanol compared to the wild-type enzyme.
- Use of a whole-cell catalyst stabilized the enzyme >1000-fold under reaction conditions.
- Efficient kinetic resolution of racemic 2-phenylpropanal by a whole-cell catalyst was demonstrated.
- (S)-2-phenylpropanol was produced in 843 mM and 93.1% e.e.
- The substrate-to-biocatalyst ratio was the main factor determining product enantiopurity and concentration.

Background

2-Aryl-1-propanols are key core synthons of profen-type non-steroidal anti-inflammatory drugs (NSAIDs) (1). The simplest 2-aryl-1-propanol, 2-phenylpropanol (Lilac-Hyacinth odor), is a fragrance ingredient of personal care products itself but is also used as precursor in the synthesis of further fragrances (2, 3). Differing biological activities of R- and S-profens as well as enantioselectivity in odor perception have drawn attention towards production of optically pure 2-aryl-1-propanols. Over the last decades, a number of biocatalytic routes have been proposed including kinetic resolutions by hydrolases, nitrile-converting enzymes or oxidases, asymmetrization of prochiral precursors by enzymatic decarboxylation and isomerization by styrene oxide isomerase. Kinetic resolutions by means of hydrolases and oxidoreductases are the two most advanced strategies (reviewed in 4). The economic attractiveness of kinetic resolutions, that are restricted to maximally 50% yield, are increased by in situ racemization of the unused antipode in dynamic kinetic resolution (DKR). DKR strategies exploiting hydrolases and oxidoreductases make use of the relatively fast racemization of 2-aryl-1-propanoic acids/esters and 2-aryl-1-propanals (Scheme 1). Hydrolase-catalyzed kinetic resolutions were the first biocatalytic routes towards enantiopure profens and numerous lipases and esterases have been tested for their enantioselectivities towards several profens (5). Product concentrations of 0.5 M and enantiopurities of up to 99% e.e. were reported and hydrolases have been exploited at industrial scale (6, 7). Hydrolases are still attractive, though with less IP opportunities (4). Oxidoreductases are an interesting alternative, however, less investigated. Previously published biocatalytic routes towards chiral profens by oxidoreductases show markedly lower product concentrations of maximally 80 mM 2-aryl-1-propanols (4, 8, 9, 10, 11, 12). However, oxidoreductases generally outperform hydrolases in terms of enantioselectivity. Here, we report on an enzymatic dynamic reductive kinetic resolution towards (S)-2-phenylpropanol. A single point mutant of the xylose reductase from Candida tenuis (CtXR, superfamily of aldo-keto reductases) with high catalytic activity and excellent enantioselectivity for (S)-2-phenylpropanol was used in E. coli whole-cell reductions. Reaction optimization with the aim to achieve high enantioselectivity and product concentration at full conversion was accomplished. The established enzymatic dynamic reductive kinetic resolution (DYRKR) is compatible with lipase-based processes in terms of product concentration and optical purity.

Results

Motivated by a basal activity of wild-type CtXR on rac-2-phenylpropanal (k_{cat}/K_{m, rac} 130 s^-1 M^-1, k_{cat} 0.05 s^-1), we tested a number of substrate binding mutants for activity and enantioselectivity in the reduction of 2-phenylpropanal. The used enzyme is a member of the aldo-keto reductase superfamily (AKRs) and converts xylose to xylitol in the central sugar metabolism of its native host Candida tenuis. CtXR shows, like many other AKRs, broad substrate specificity. Its biocatalytic applicability was, however, limited by moderate catalytic activity and low stability of the wild-type enzyme. Several substrate-binding site mutants with improved specificity for aromatic ketones were used in the synthesis of (R)-ethyl mandelates and (S)-phenylethanol (13, 14).
The substrate-binding cavity of aldo-keto reductases is mainly formed by residues from three large and flexible loops (15, 16). Loop flexibilities provide the structural basis for relaxed substrate specificities but complicate rational engineering (17). A binding mode of the natural substrate D-xylose (open chain form) was previously modelled with C-1 of xylose within hydride-transfer distance above the nicotinamide C-4 and the carbonyl oxygen hydrogen-bonded to the general acid catalyst Tyr-S2. Therein, the aldehyde hydrogen pointed towards the indole ring of Trp-24, the C2 hydroxyl interacted with Asn-310 and C4 and C5 hydroxyls with Asp-51 (18). Here, we probed CτXR wild-type and single-point mutants of the main substrate recognition residues Trp-24, Asn-310 and Asp-51 as catalysts for 2-phenylpropanal reduction. The replacement of Trp-24 by smaller phenylalanine and tyrosine increased activities on bulky ketone-substrates (13, 19). Asp-51 contributes the most to the relative polarity of the binding site; substitution by alanine led to improved selectivity for the aromatic ketone o-chloroacetophenone (14).

Kinetic constants of 2-phenylpropanal reduction by CτXR variants

Table 1 summarizes results of a steady-state kinetic analysis of NADH-dependent reduction of racemic and (S)-2-phenylpropanal by CτXR wild-type and mutants.

(S)-2-phenylpropanal. Kinetic parameters obtained with the racemic substrate and the (S)-2-phenylpropanal were compared and the ratio of the specificity constants formed \( (k_{cat}/K_m)^{rac}/(k_{cat}/K_m)^{S} \) (Table 1). For the extreme case of sole activity with the Senantiomer, a ratio of ~2, for equal acceptance of S and Renantiomer a ratio of 1 and for preference of the Renantiomer ratios < 1 were expected. The wild-type showed a ratio of 1.23 and preference for the S-enantiomer. The D51A mutant showed a ratio of 1.54 and hence a stronger preference for the S-enantiomer. The W24F, W24Y and N310A mutants, however, displayed ratios < 1 and therefore preference for the R-enantiomer.

**Table 1**

|                                  | CτXR          | (S)-2-phenylpropanal | Ratio |
|----------------------------------|---------------|----------------------|-------|
|                                  | rac-2-phenylpropanal | (S)-2-phenylpropanal | specificity constants |
| CτXR                             | \( k_{cat}/K_{m,rac} \) (s^{-1}M^{-1}) | \( K_{m,rac} \) (µM) | \( k_{cat}/K_{m,S} \) (s^{-1}M^{-1}) | \( K_{m,S} \) (µM) |
| wild-type                        | 130           | 350                  | 160   | 450   | 1.23      |
| D51A                             | 28·10³        | 170                  | 43·10³ | 120   | 1.54      |
| W24F                             | 13^a          | n.a.                 | 12    | n.a.  | 0.92      |
| W24Y                             | 10^a          | n.a.                 | 9     | n.a.  | 0.90      |
| N310A                            | 88            | 280                  | 68    | 330   | 0.77      |
| N310D                            | no activity   | no activity          | no activity | no activity |

^aLimited substrate solubility prevented saturation of the enzyme; \( k_{cat}/K_m \) was calculated from the slope of the Michaelis–Menten plot where the rate is linearly dependent on the substrate concentration.

Reduction of rac-2-phenylpropanal by isolated D51A CτXR

We used isolated CτXR D51A in bioreductions of 0.5 mM rac-2-phenylpropanal. The substrate (logP 2.11, https://scifinder.cas.org/) displayed a maximal solubility of 0.5 mM in buffer. Product concentrations and e.e. values obtained at enzyme concentrations between 240 and 0.6 U/mL are listed in Table 2. As expected, the product e.e. values increased with decreasing amounts of enzyme. Unexpectedly, reactions stopped after 2 h and maximal product concentrations of 120 µM were achieved (for a time course see the Supplementary data). We suspected enzyme deactivation as cause of low conversions and used a whole-cell biocatalyst based on D51A CτXR in further experiments.
Table 2
Conversions and product e.e.-values of rac-2-phenylpropanal reduction by isolated D51A CxR. *

| CxR (U/mL) | (S)-Phenylpropanol (µM) | e.e. (S)-Phenylpropanol (%) |
|------------|--------------------------|-----------------------------|
| D51A (240) | 25                       | 23                          |
| D51A (20)  | 16                       | 38                          |
| D51A (3.4) | 11                       | ≥ 99                        |
| D51A (0.6) | 4                        | ≥ 99                        |

*aReaction time 2 h. *bMeasured with 0.5 mM rac-2-phenylpropanal.

Reduction of rac-2-phenylpropanal by E. coli co-expressing CxR D51A and a yeast formate dehydrogenase

Bioreductions were accomplished by lyophilized and rehydrated biomass or cell-free supernatant of engineered E. coli. The highly hydrophobic substrate formed a second phase in the reaction buffer above its solubility limit. Stirring led to droplet formation that were stabilized against coalescence in the presence of lyophilized biomass or supernatant.

100 mM rac-2-phenylpropanal

Conversions and product e.e. values for the reduction of 100 mM 2-phenylpropanal are summarized in Fig. 1. The amount of catalyst was varied between 4 and 40 gCDW/L. Comparison of lyophilized cells to cell-free supernatant used as catalyst was facilitated by using an equal amount of biomass for catalyst preparation in both cases. It has been previously shown that > 40 % of the total biomass activity is found as extracellular enzymes and hence in the supernatant (14). Conversions were similar for biomass and supematant reflecting the severely impaired cell integrity caused by biomass lyophilization. Only at a low biocatalyst concentration of 4 gCDW/L, the supernatant led to significantly lower conversion (30 % lower). Supernatant and whole biomass showed equally high e.e. values of 95.3 % at 4 gCDW/L. Product enantiopurity and conversions increased gradually with increasing biocatalyst load. Notably, the e.e. values obtained with the supernatant decreased more strongly than the e.e. values from reductions with the whole biomass. At a catalyst load equal to 40 gCDW/L the e.e. values were 27 and 46 % for supernatant and whole biomass, respectively.

Optimization of 2-phenylpropanal bioreduction

Substrate concentration. Product concentration is the most important metric for bioprocesses to become compatible to chemical processes. Therefore, we increased the substrate concentration to 1 M at varying catalyst concentrations (lyophilized, rehydrated biomass) (Fig. 2, Table 3). The effects of increased catalyst loading on conversion and e.e.-values showed a similar but less pronounced trend compared to bioreductions with 100 mM substrate. A catalyst loading of 20 gCDW/L led to an e.e. value of 95 % compromised by a conversion of 28 %. Increase of the catalyst loading to 40 gCDW/L decreased the e.e. value to 93 % at 56 % conversion. A substrate concentration of 2 M turned out as too high, only low conversions of ~15 % were reached (Table 3).

NAD+ concentration. We added higher concentrations of the co-enzyme NAD+ to further push the reaction towards full conversion. At 12 and 14 mM of NAD+, conversions up to 99 % were reached, again at e.e. values of 92–93 % (Fig. 2, Table 3).

Cyclodextrin addition. The addition of 75 mM 2-hydroxypropyl-β-cyclodextrin has previously shown to boost bioreductions based on the used catalyst (14). Here, the addition of 38, 75 or 115 mM 2-hydroxypropyl-β-cyclodextrin had no significant effect on bioreductions of 1 and 2 M 2-phenylpropanal (Table 3).

Fed-batch. The step-wise addition of substrate at 0, 2 and 4 h to a total substrate concentration of 1 M led to a 10 % increase of conversion (Table 3).

Recovery, isolated yield, reproducibility and by-products

Leis et al. (20) previously suggested that hydrophobic substrates and products remain in the cell sludge of the used biocatalyst. Here, a high excess of ethyl acetate was required for product extraction prior to analysis. Obtained product concentrations in bioreductions of 1 and 2 M were between 27 and 84 % as shown in Table 3.

Recovery. Substrate/product loss in the biomass was found to be <15% under the conditions used.

Reproducibility. Reaction replicates (N = 7) of bioreductions with 40 gCDW/L and 6 mM NAD+ showed high reproducibility with a mean value of 62 % product and a standard deviation of 4 %. The enantiomeric excess was 93.3 ± 1.1 % e.e. The formation of broad peaks prevented quantification of the aldehyde substrate by chiral, reversed-phase HPLC. We therefore analyzed bioreduction samples additionally by chiral GC-FID.
Table 3
Conversions and product e.e.-values of rac-2-phenylpropanal reduction by lyophilized whole-cell catalyst. Effects of catalyst loading, substrate concentration, co-enzyme concentration and HBC-addition. a

| Catalyst loading (gCDW/L) | rac-2-Phenylpropanal (M) | NAD⁺ (mM) | HBC (mM) | (S)-Phenylpropanol (mM) | e.e. (S)-Phenylpropanol (%) |
|--------------------------|-------------------------|----------|----------|------------------------|----------------------------|
| 20                       | 1                       | 3        | 0        | 276                    | 95.1                       |
| 20                       | 1                       | 3        | 38       | 456                    | 94.1                       |
| 20                       | 1                       | 3        | 75       | 494                    | 93.3                       |
| 30                       | 1                       | 3        | 0        | 425                    | 95.0                       |
| 30                       | 1                       | 3        | 38       | 496                    | 94.2                       |
| 30                       | 1                       | 3        | 75       | 455                    | 94.2                       |
| 40                       | 1                       | 3        | 0        | 564                    | 93.4                       |
| 40                       | 1                       | 6        | 0        | 619                    | 93.3                       |
| 40                       | 1 M fed-batch           | 6        | 0        | 679                    | 94.0                       |
| 40                       | 1                       | 8        | 0        | 732                    | 94.1                       |
| 40                       | 1                       | 10       | 0        | 843                    | 93.1                       |
| 40                       | 1                       | 12       | 0        | 839                    | 92.9                       |
| 40                       | 1                       | 14       | 0        | 765                    | 92.3                       |
| 40                       | 1                       | 6        | 38       | 634                    | 94.3                       |
| 40                       | 1                       | 6        | 75       | 598                    | 92.9                       |
| 40                       | 1                       | 6        | 115      | 592                    | 92.5                       |
| 40                       | 2                       | 6        | 0        | 279                    | 95.4                       |
| 40                       | 2                       | 6        | 38       | 306                    | 93.4                       |
| 40                       | 2                       | 6        | 75       | 306                    | 92.2                       |

aReaction time 48 h.

By-products. The high reactivity of the substrate 2-phenylpropanal prompted investigation of possible by-products from chemical or bio-chemical reactions. It has been previously shown that acetophenone forms by oxygen-catalyzed degradation of rac-2-phenylpropanal (21). We found 7% of acetophenone to be formed maximally and only trace amounts of its enzymatic reduction product in bioconversions of 1 M phenylpropanal, using 40 gCDW/L cells and 6 mM NAD⁺ (22). The substrate is also in a chemical equilibrium between rac-2-phenylpropanal and its corresponding hydrates. The previously reported enzymatic oxidation of 2-phenylpropanal hydrates to the corresponding carboxylic acids was not observed (12, 23). No substrate-related enol or aldol was found in detectable amounts (for data of NMR analyses see the Supplementary data).

Isolated yield. To confirm the identity of the obtained product, hydrophobic compounds were extracted from two reaction mixtures containing 1 M 2-phenylpropanol (reaction volume 2 mL, 40 gCDW/L catalyst, 6 mM NAD⁺). An analytical yield of 78 % was determined by HPLC (product concentration). The solvent and unreacted 2-phenylpropanol (bp 92 – 94°) were removed under reduced pressure. Product composition was 86 % 2-phenylpropanol, 7 % acetonilide and 7 % ethyl acetate (extractant) by 1H-NMR. No 2-phenylpropanol was found. An isolated yield of 64 % was obtained (203 mg with a product content of 86 %). (For data of HPLC, GC and NMR analyses see the Supplementary data).

Literature survey
Reported enzymatic reductive kinetic resolution of rac-2-phenylpropanal studies are summarized in Table 4. Bioreductions of rac-2-phenylpropanal have been accomplished with free-floating enzymes (Table 4, entries 1–5, 8–10,12) and immobilized enzymes (entries 6,7,11). Previous studies aimed at probing bioreduction catalysts (free and immobilized oxidoreductases) in the kinetic resolution of rac-2-phenylpropanal (entries 4–11). Most enantioselective enzymes preferred the (S)-aldehyde (entries 1–8, 10). Rocha-Martín et al. (24) reported on an anti-prelog specific ADH from Thermus thermophilus HB27 (entry 11). Dong et al. (8) accomplished the evolution of ADHs for the formation of (S)- and (R)-alcohols by directed evolution of an ADH from Thermoanaerobacter brockii that displayed moderate prelog-type selectivity (entries 8,9). The used enzymes had to display not only high enantioselectivities but also sufficient stabilities in the presence of the substrate that can form adducts with groups on the enzymes (25). HLADH was used in most studies as it turned out to be enantioselective, stable in the presence of substrate up to a concentration of 165 mM, and useful in coupled substrate strategies (oxidation of cheap alcohols for NADH-recycling). All other examples of selective ADHs stem from thermophilic organisms and display intrinsically high stabilities towards adverse effects of the reaction media. Remarkably, the often-used host E. coli shows native activity towards
2-phenylpropanal (entry 13). Buffered solutions containing water-soluble co-solvents (also used as sacrificial substrate for NADH-recycling) were used frequently. The aqueous phase was required for the racemisation of the substrate. Grunwald et al. (11) tested HLADH in organic solvent and used isopropylether with 0.5 % buffer as reaction medium. A product concentration of 46 mM with 95 % e.e. was obtained, however at a conversion of 15 % (entry 7). Others used bi-phasic solvents (entries 4 and 6). The highest published product concentration of 82 mM was achieved in a buffer/isopropylether mixture (47:63) (entry 4).
| Entry | Bioreduction catalyst, NAD(P)H-recycling strategy | rac'-2-phenylpropanal | Medium, (auxiliary substrate) | Product (Conversion) | Enantiopurity | Aim of the study | Ref. |
|-------|--------------------------------------------------|-----------------------|------------------------------|---------------------|--------------|----------------|------|
| 1     | Free enzyme, coupled substrate 1,4-butanediol     | 5 mM                  | Buffer pH 7.5, 1 % v/v CH₃CN, (2.5 mM 1,4-butanediol) | 5 mM (99 %)         | 95 % e.e. S | Probing the enzyme's co-enzyme recycling ability using 1,4-butanediol oxidation to lactone | 26   |
| 2     | Free enzyme, coupled substrate ethanol            | 0.5 mM                | Buffer pH 7.5, (0.5 M ethanol) | 0.38 mM (75 %)      | 98 % e.e. S | Investigation of DKR including substrate racemization velocity | 27, 10 |
| 3     | Free enzyme, phenylpropanal oxidation for NADH-recycling | 75 mM                | Buffer pH 7.5; 4 % v/v MTBE | 23 mM (31 %)        | 97 % e.e. S | Investigation of the biocatalytic asymmetric disproportionation (biocatalytic Cannizzaro reaction) | 12, 28 |
| 4     | Free enzyme, coupled substrate ethanol            | 165 mM                | Buffer, 63 % v/v isopropylether, (0.6 M ethanol) | 82 mM (50 %)        | 96 % e.e. S | Probing the enzyme's enantioselectivity and co-enzyme recycling ability | 29   |
| 5     | Free enzyme, coupled substrate ethanol            | 30 mM                 | Buffer pH 8, (5% v/v ethanol) | 28 mM (93 %)        | 93 % e.e. S | One-pot, two-step reaction: rac'-2-phenyl-1-propanol oxidation to rac'-2-phenylpropanal followed by enantioselective bioreduction under dynamic conditions. | 1    |
| 6     | Immobilized enzyme, coupled substrate ethanol     | 5 mM                  | Buffer pH 7.5, 50 % v/v hexane, (0.5 M ethanol) | 4.2 mM (84 %)       | >98 % e.e. S | Characterization of the immobilized catalyst | 30   |
| 7     | Immobilized enzyme, coupled substrate ethanol     | 300 mM                | Isopropyl ether (saturated with buffer), 0.5 % buffer pH 7.0, (1 M ethanol) | 46 mM (15 %)        | 95 % e.e. S | Probing the enzyme's substrate scope and enantioselectivity in organic solvent | 11   |
|       | **Thermostable ADHs** (enzyme superfamily)        |                       |                              |                     |              |                |      |
| 8     | Free *Thermoanaerobacter brockii* LG296 ADH mutant (Zn-containing ADH), coupled substrate isopropanol | 30 mM                | Buffer pH 7.4, (20 % v/v isopropanol) | 23 mM (75 %)       | 95 % e.e. S | Development of enantioselective mutants | 8    |
| 9     | Free *Thermoanaerobacter brockii* LG277 ADH mutant (Zn-containing ADH), coupled substrate isopropanol | 10 mM                | Buffer pH 7.4, (20 % v/v isopropanol) | 7.5 mM (75 %)      | 92 % e.e. R | Development of enantioselective mutants | 8    |
| 10    | Free *Sulfolobus solfataricus* ADH-10 (Zn-containing ADH), coupled substrate ethanol | 5 mM                  | Buffer pH 9, (5 % ethanol) | 3.7 mM (74 %)       | 98 % e.e. S | Probing the enzyme's substrate scope and enantioselectivity | 9    |
Discussion

Catalyst development

Activities of CtXR variants

Wild-type CtXR showed a catalytic efficiency of 130 s\(^{-1}\)M\(^{-1}\) with a low \(K_m\) of 350 µM and corresponding \(k_{cat}\) of 0.05 s\(^{-1}\) for the reduction of rac-2-phenylpropanal.

Asp-51: Comparison of CtXR variants in the reduction of 2-phenylpropanal (Table 1) showed that replacement of the charged Asp-51 by alanine improved the enzyme's activity substantially. The catalytic efficiency increased 215-fold for racemic 2-phenylpropanal and 270-fold for the (S)-aldehyde compared to the wild-type. Likewise, the catalytic efficiency for reduction of o-chloroacetophenone was improved 13-times by the D51A mutation (14) whereas a 50-fold decrease was observed for the conversion of xylose (19). Our results stressed the notion made by Kavanagh et al. (18) that Asp-51 in CtXR contributes the most to the relative polarity of the binding site and hence specificity for sugar substrates. Replacement of aspartate by alanine turned out as general strategy to improve CtXR's efficiency towards hydrophobic substrates.

Trp-24: Reduced enzyme activity towards 2-phenylpropanal by removal of the bulky Trp24 confirmed that the presence of Trp-24 is important for the efficient conversion of aldehydes (13, 19).

Low solubilities of hydrophobic substrates generally impede the determination of kinetic parameters. 2-Phenylpropanal showed extremely low solubility (0.5 mM) in buffered medium. Small \(K_m\) values obtained with CtXR variants enabled the comparison of CtXR variants (especially for the D51A mutant with \(K_m\) values of below 200 µM for the racemic aldehyde and the (S)-aldehyde). The comparability to other oxidoreductases that display higher \(K_m\) values remained, however, problematic. D51A CtXR had a ~ 40-fold higher \(k_{cat}\) value and a ~ 30-fold smaller \(K_m\) value for the racemic substrate compared to published values for Thermoanaerobacter brockii ADH variants (8).

Enantioselectivities of CtXR variants

The enantioselectivity of an enzyme is defined by the ratio of catalytic efficiencies for the two enantiomers (\(E = (k_{cat}/K_{m,S})/(k_{cat}/K_{m,R})\), 32). In the present case, the determination of the catalytic efficiencies of enantiopure (S) and (R)-aldehydes was compromised by \textit{in situ} substrate racemization. Published racemization velocities of 2-phenylpropanal were 75·10\(^{-6}\) s\(^{-1}\) (\(k_{rac}\)), equal to half-lives of ~ 2 h (\(t_{1/2}\)) (27). The relatively slow racemization should generally enable determination of \(k_{cat}/K_{m,\text{enantiomer}}\) with enzymatic assays lasting 5 minutes. We have, however, experienced slow racemization of the pure enantiomers during freeze-storage (-18°C). Hence, enantioselectivities expressed as \((k_{cat}/K_{m,S})/(k_{cat}/K_{m,R})\) in Table 1 show approximate values that are still useful to guide enzyme selection and reaction optimization. The wild-type showed preference for the (S)-aldehyde (ratio of 1.23).
**Asp-51, Asn-310.** The D51A mutant showed a stronger preference for the Senantiomer (a ratio of 1.54) and the N310A for the R-enantiomer (ratio of 0.77). Asp-51 and Asn-310 are on opposite sides of the substrate binding pocket (Fig. 3). Asp-51 is suggested to interact with the natural substrate xylose at C3, C4 and C5 hydroxyls, Asn-310 with the C2 hydroxyl (1B). A docking of wild-type CrXR (in complex with NAD+; 33) with xylose and (S)- and (R)-2-phenylpropanal was made (Fig. 3AB). Replacement of Asp-51 by alanine might facilitate interaction between the alanine and the phenyl-ring of (S)-2-phenylpropanal (Fig. 3C). After replacement of Asn-310 by alanine, interaction between alanine and the phenyl-ring chain of (R)-2-phenylpropanal becomes plausible for N310A (Fig. 3D). Docking results supported findings from the kinetic studies: D51A mutation improved transition state stabilization of the (S)-aldehyde while N310A mutation improved stabilization of the (R)-aldehyde, respectively.

**Catalyst stabilization**

CrXR is generally known as a relative labile enzyme and half-lives in the presence of 5 to 10 mM o-chloroacetophenone and 1-(2-chlorophenyl)ethanol shrank to < 3 min. In the case of o-chloroacetophenone and its reaction product the unfavourable log P-values (~ 2) were held responsible for fast enzyme deactivation. Use of the enzyme as catalytic oxidoreductase in whole-cell catalysts (E. coli, S. cerevisiae, C. tenuis) had previously shown to stabilize the enzyme substantially and product concentrations were improved > 10-fold (34). Reactive aldehydes are known to form adducts with proteins at the lysine, histidine and cysteine side chains (25). Enzyme deactivation by 2-phenylpropanal might hence follow different mechanisms compared to o-chloroacetophenone. Use of the whole cells provided an extreme case of catalyst stabilization in the presence of 2-phenylpropanal: The isolated enzyme was deactivated by 0.5 mM aldehyde whereas the whole-cell catalyst and the supernatant thereof were able to tolerate and convert 1 M of the substrate. Stabilization of the catalytic enzyme by whole-cells and cell debris was previously reported for the synthesis of (R)-phenylacetylcarbinol from benzaldehyde and pyruvate by a Candida utilis pyruvate decarboxylase (35). The stabilization was ascribed to membrane components that form a microenvironment around the enzyme and thereby decrease aldehyde transfer to the enzyme and protect the enzyme from deactivation at the aqueous/organic interphase.

**Product-enantiopurity and concentration**

In the case of an enzyme that converts one enantiomer much faster than the other, the first percentages of product will show high enantiopurity, whereas a decrease is expected in the course of the reaction (expressed in the Chen equation, 32). A dependence of product enantiopurity on reaction progress, especially at high enzyme loading, was experienced by us and others despite DKR conditions. Substrate racemization velocity was identified as main limitation towards high product enantiopurity (12, 28). Here, substrate-to-catalyst ratio turned out as main factor determining product enantiopurity. The e.e. value of the product showed a strong dependence on substrate-to-catalyst ratio below 3.4 g substrate/g CDW. At 3.4 g substrate/g CDW product enantiopurities of ~ 94 % were obtained at 100 mM and 1 M 2-phenylpropanal concentrations and e.e. values were only slightly increasing at higher substrate-to-catalyst ratios (Fig. 4).

We previously stated a maximal catalytic loading of 40 to 50 g CDW/L in bioreductions to minimize product loss of hydrophobic substances in the biomass fraction during downstream processing (36). The minimally applicable substrate-to-biocatalyst ratio of 3.4 g substrate/g CDW gives a substrate loading of 1 to 1.25 M. Higher substrate-to-biocatalyst ratios (2 M substrate at 40 g CDW/L, or 1 M substrate at 20–30 g CDW/L) led to lower product formation. The catalyst was fully deactivated during the reaction time of 48 h (14). Elevated NAD+ concentrations of 6–14 mM were used to fully exploit the catalytic activity of the coupled oxidoreductase system. In the present case, a compromise between enantiopurity and conversion had to be found. Limiting factors are substrate racemization velocity, catalyst loading and catalyst stability.

**Conclusion**

Optimization of an enzymatic dynamic reductive kinetic resolution of racemic 2-phenylpropanal towards 843 mM (S)-2-phenylpropanal was accomplished. The multilevel engineering included engineering of the wild-type CrXR for improved enzyme activity and enantioselectivity, use of an E. coli whole-cell catalyst for enzyme stabilization and coenzyme recycling and optimization of the substrate-to-catalyst ratio.

**Enzyme level.** Several substrate-binding mutants based on a xylose-binding model (18) were investigated. The D51A CrXR variant showed high selectivity and catalytic activity towards (S)-2-phenylpropanal in initial rate measurements. However, fast enzyme deactivation was experienced in the bioreduction of 0.5 mM racemic 2-phenylpropanal. A literature survey revealed 9 examples of bioreductions catalyzed by free enzymes (and 3 examples of immobilized enzymes), all of which were either HLADH or enzymes from thermophilic hosts. The low number of reported enzymes and the low concentrations of obtained product stressed a general deactivating effect of the reactive aldehyde on enzymes.

**Whole-cell level.** The whole-cell catalyst based on D51A CrXR showed, much to our delight, high stability in the presence of 1 M phenylpropanal. Our results indicate a strong protecting effect by the whole-cell catalyst (and the supernatant thereof). We used lyophilized and rehydrated E. coli co-expressing D51A CrXR and yeast formate dehydrogenase as catalyst.
Reaction level. The most important factor to obtain high enantiopurities and product concentrations was the ratio of substrate to catalyst (Fig. 1, Fig. 4, Table 3). The amount of NAD⁺ was the second most important factor. Catalyst and co-enzyme concentration directly affected reduction rate. High reduction velocity led to high conversions but also lower product enantiopurities. The conversion with a catalyst loading of 40 g CDW, 10 mM NAD⁺ and 1 M substrate represented a compromise to obtain (S)-phenylpropanol in ~ 93 % e.e. enantiopurity and 843 mM product concentration (Table 4, entry 14).

We explain higher e.e. -values obtained with the whole biomass as compared to the supernatant by the presence of cells and cell debris (Fig. 1). The substrate was added in concentrations far above its solubility limit of 0.5 mM and hence formed a second phase. The presence of cellular components led to a very fine dispersion of the rac-2-phenylpropanal in form of an oil-in-water emulsion (37). Organic/aqueous phase mass transfer was facilitated and led to a scenario where used substrate (mainly (S)-form) was replenished fast by the racemic substrate. As a result, the (S)-aldehyde was constantly supplied and consumed, the (R)-aldehyde supplied and (slowly) racemized. Use of a stable and selective enzyme (D51A CtXR) enabled the synthesis of a highly enantiopure product under these conditions.

Materials And Methods

Chemicals, enzymes and strains

Chemicals

Racemic 2-phenylpropanal (98 %), racemic 2-phenylpropanol (97%) and racemic 1-phenylethanol (≥ 98 %) were purchased from Sigma-Aldrich/Fluka (Vienna, Austria), (S)-2-phenylpropanal (95 %) and (R)-2-phenylpropanol (95 %) were from Accela (Prien – Chiemsee, Germany), 2-hydroxypropyl-ß-cyclodextrin (HBC, batch number OH053931501) was from Carbosynth (Berkshire, UK). NAD⁺ (98 %), acetonitrile (≥ 99) and ethyl acetate (≥ 99,9 %) were from Roth (Karlsruhe, Germany). Other chemicals were from Sigma-Aldrich/Fluka or Roth, and were of the highest purity available. Materials for genetic experiments were reported elsewhere (19).

Enzymes

The used reductases were wild-type and single-point mutants of Candida tenuis xylose reductase (CtXR wild-type GenBank ID AF074484). Site-directed mutagenesis and amino acid insertion for CtXR mutants D51A, W24F, W24Y, N310A, N310D were carried out by using inverse PCR as described elsewhere (19). Production of CtXR (wild-type and mutants D51A, W24F, W24Y, N310A, N310D) was previously described (19).

Whole-cell biocatalyst

An E. coli Rosetta2 strain co-expressing CtXR D51A and CbFDH (GenBank ID AJ011046) was used. Whole-cell biocatalyst production was previously described by Rapp et al. (14). The biomass was frozen at -20°C, lyophilized (Christ α 1–4 lyophilizer from Braun Biotech International) and stored at -20°C.

Total enzyme activities of the biomass (extracellular and intracellular enzymes). 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 (14).

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 to 512 U/g CDW and 72 U/g CDW, respectively, in the supernatant. The procedures for activity determination were described earlier (14).

Enzyme-substrate docking

PyMOL Molecular Graphics System (Open-Source, Schrödinger, LLC) was used for enzyme/ligand structure depictions. Ligand docking experiments were performed in YASARA (YASARA Biosciences GmbH, Vienna, Austria) using Autodock Vina (38) with standard parameters.

Enzyme kinetics

Steady-state enzyme kinetics

Kinetic parameters of NADH-dependent 2-phenylpropanal reduction by CtXR variants were determined spectrophotometrically as described earlier (19). Solubility of 2-phenylpropanal in water was elevated to 0.5 mM by addition of 25 % v/v DMSO. Substrate solutions were freshly prepared and immediately used to avoid non-enzymatic decomposition and, with (S)-2-phenylpropanal, racemization in aqueous solution. A typical measurement period was 5 minutes. Non-specific background activity was considered by measuring blank activities. The added DMSO had no effect on the enzyme's activity with the natural substrate D-xylose.

Bioreductions of racemic 2-phenylpropanal

Reduction by the isolated D51A CtXR
Racemic 2-phenylpropanal was dissolved in DMSO prior to dilution into 50 mM potassium phosphate buffer, pH 7.0 to a final DMSO concentration of 25 %. The substrate (final concentration 0.5 mM) was incubated at 25°C in the presence of 0.1 mM excess [NADH] and D51A C2KR for 2 hours. For time-course analysis, samples (100 µL) were taken from the reaction mixtures (final reaction volume 1.5 mL) at specified timepoints. All samples were diluted 1:1 with acetonitrile and centrifuged prior to analysis by chiral HPLC.

Whole-cell bioreduction

Lyophilized cells were re-hydrated in potassium phosphate buffer (100 mM, pH 6.2, re-hydration volume ≤ 50 % v/v of the total bioreduction mixture) in the presence of NAD⁺ (3–14 mM) and sodium formate (50 mM excess on [substrate]) using 2 mL Eppendorf tubes. The tubes were placed on a thermomixer for 30 min at 25°C and 1400 rpm. In case of HBC-aided conversions, cyclodextrins were weighed out separately in Eppendorf tubes followed by adding rac-2-phenylpropanal and 50 µL buffer. Tubes were vortexed vigorously for complexation of substrate and HBC. Afterwards, re-hydrated cells were combined with HBC/substrate complexes and filled up to a total working volume of 1 mL. Alternatively, substrate was added directly to the re-hydrated cells if no HBC was applied. Eppendorf tubes were sealed with parafilm and vortexed until emulsification was reached. The mixtures were reacted for 24 or 48 h at room temperature using an end-over-end rotator (30 rpm). All samples were prepared in duplicates.

Fed-batch. The fed-batch bioreductions started as batch reactions with 330 mM substrate, 6 mM NAD⁺ and 40 g<sub>CDW</sub>/L. 50 µL rac-2-phenylpropanal were fed after 2 and 4 h to a total substrate concentration of 1 M. The reaction was carried out for 48 h. Fed-batch experiments were performed in duplicates.

Product isolation. Two bioreduction mixtures (1 mL reaction volume each) of 1 M 2-phenylpropanal, 40 g<sub>CDW</sub>/L and 6 mM NAD⁺ were pooled after incubation for 48 h. The hydrophobic reaction compounds were extracted by four consecutive extraction steps using 1 v/v ethyl acetate per step. The pooled organic phase was evaporated under reduced pressure (Heidolph Laborota 4001, Schwabach, Germany) at 40°C. The final weight of the sample was determined.

Substrate/product recovery. For recovery experiments at substrate/product concentrations of 1 M, reaction mixtures containing 20 or 40 g<sub>CDW</sub>/L catalyst were prepared without adding NAD⁺. The samples were incubated applying conditions used for biotransformations and extracted following the procedure described in Sect. 2.4.1. Substrate/product recovery was performed in duplicates.

Cell-free bioreduction

Lyophilized cells (40 mg) were re-hydrated for 30 min in 1 mL potassium phosphate buffer (100 mM, pH 6.2) at 1400 rpm and 25°C on a thermomixer followed by centrifugation (13.2k rpm; 10 min; 25°C). The supematant was isolated and either applied directly or diluted 1/2, 1/4 or 1/10 corresponding to the activity of 40, 20, 10 or 4 g<sub>CDW</sub>/L. rac-2-phenylpropanal (100 mM), sodium formate (150 mM) and NAD⁺ (6 mM) were added afterwards. All mixtures were adjusted to 1 mL and placed on an end-over-end rotator (30 rpm) for 24 h. All samples were prepared in duplicates.

Analytical methods

Sample preparation from whole-cell or cell-free bioreductions

For HPLC and GC analyses, ethyl acetate (1 mL) was added to 1 mL of a reaction performed in 2 mL Eppendorf tubes. The tubes were vortexed followed by transferring the mixture into 15 mL Sarstedt tubes. Tubes were filled up to 10 mL with ethyl acetate, vortexed and centrifuged for 15 min, 25°C and 3220 g for extraction. Final dilutions in ethyl acetate contained 5 mM substrate/product. For NMR analyses, substrate/product present in ethyl acetate after extraction was transferred into round-asks and evaporated under reduced pressure. The isolated substrate/product was directly diluted in deuterated methanol (20 µL substrate/product + 680 µL solvent).

Chiral HPLC analysis

HPLC analysis was performed using a Merck Hitachi LaChrom HPLC system equipped with a Merck L-7490 RI detector, an L-7400 UV-detector, a reversed phase Chiralpak® AD-RH column (from Daicel, obtained at Sigma Aldrich, Austria, Vienna) and a thermo-stated column oven (40°C). The mobile phase used contained 25 % acetonitrile in ddH₂O. A flowrate of 30 mL/min was applied. HPLC standards were prepared deploying racemic product in a range of 0.1, 0.5, 1, 5, 10 mM. Peak areas at corresponding retention times were used to calculate concentrations. The enantiomeric excess of the major product (S)-2-phenylpropanol was calculated by e.e. = (S-alkanol – R-alkanol)/(S-alkanol + R-alkanol). Retention times and chromatograms of authentic standards (main products (S)-2-phenylpropanol, (R)-2-phenylpropanol), by-products (acetophenone, phenylethanol) were summarized in the Supplementary data. The aldehydes gave broad peaks and were quantified by GC-FID.

Chiral GC-FID analysis

GC analysis was performed on an Agilent 7890A GC with FID detection (12). Carrier gas was H₂, the chiral column was a Hydrodex®-β-TBDAc column with 25 m length and an inner diameter of 0.25 mm (from Macherey-Nagel obtained from FisherScientific, Austria, Vienna). The injection volume was 5 µL, the inlet temperature 230°C, the split ratio 50:1 and the flow 1 mL/min, the detector temperature 250°C. The following temperature program was used for separation of analytes: 110°C/hold 10 min; 2°C per min to 123°C/hold 3 min; 10°C per min to 200°C/hold 1 min. Retention times and chromatograms of authentic standards (main products ((S)-2-phenylpropanol, (R)-2-phenylpropanol), substrate ((S)-2-phenylpropanol, (R)-2-phenylpropanol) by-products (acetophenone, phenylethanol) are summarized in the Supplementary data.
NMR analysis

$^1$H-NMR spectra of isolated substrate/product from biotransformations (78 % conversion) were recorded using a 300 MHz Bruker NMR unit (300 MHz for $^1$H) at 300 K. Chemical shifts (δ) were depicted in ppm relative to the resonance of the solvent (MeOD or DMSO-d$_6$).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available in the Supplementary data and from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

CR performed/analyzed bioreduction experiments and was involved in experimental design and manuscript preparation. SP-M performed experiments with isolated enzymes (kinetics, bioreductions). ET was involved in experimental design and bioreduction analyses. BN made contributions to study-conception, interpretation of data and revised the manuscript. RK concepted the study, contributed to the design of experiments, interpreted the data and drafted the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Conversions (% bars) and product e.e.-values (% crosses) of 100 mM rac-2-phenylpropanal reduction by lyophilized whole-cell catalyst (blue bars) and cell-free catalyst (orange bars). Effects of catalyst form and loading were studied. NAD+ concentration 6 mM. Reaction time 24 h. Data are based on HPLC measurements, conversions are product concentrations. (For data see the Supplementary data.)
Figure 2

Conversions (bars) and product e.e.-values (crosses) of 1 M rac-2-phenylpropanal reduction by lyophilized whole-cell catalyst (grey bars 20gCDW/L, blue bars 30gCDW/L, green bars 40gCDW/L). Effects of catalyst loading and co-enzyme concentration (NAD+ 3-14 mM) are shown. Reaction time 48 h. Data are based on HPLC measurements, conversions are product concentrations. (For data see Table 3.)
Figure 3

Wild-type CtXR active site with NAD+ (PDB 1MI3, 33) and modelled substrates. (A) Xylose (blue carbons, red oxygens), (B) (S)- and (R)-2-phenylpropanal (S-enantiomer yellow carbons, R-enantiomer brown carbons, red oxygens), (C) (S)-2-phenylpropanal (yellow carbons, red oxygen), (D) (R)-2-phenylpropanal (brown carbons, red oxygen). Possible hydrogen bonds between substrates and the enzyme are shown as dashed lines, distances in Å.
Figure 4

The effect of substrate-to-biocatalyst (whole-cell biocatalyst) ratio on product enantiopurity. Blue diamonds show conversions with 100 mM rac-2-phenylpropanal (3 mM NAD+, reaction time 24 h, data Figure 1), green circles conversions with 1 M substrate (3 mM NAD+, reaction time 48 h, data Figure 2), black cross conversion with 2 M substrate (6 mM NAD+, reaction time 48 h, data Table 3).

Supplementary Files

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