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Application of robust ketoreductase from *Hansenula polymorpha* for the reduction of carbonyl compounds

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ABSTRACT

Enzyme-catalysed asymmetric reduction of ketones is an attractive tool for the production of chiral building blocks or precursors for the synthesis of bioactive compounds. Expression of robust ketoreductase (KRED) from *Hansenula polymorpha* was upscaled and applied for the asymmetric reduction of 31 prochiral carbonyl compounds (aliphatic and aromatic ketones, diketones and β-keto esters) to the corresponding optically pure hydroxy compounds. Biotransformations were performed with the purified recombinant KRED together with $NADP^+$ recycling glucose dehydrogenase (GDH, *Bacillus megaterium*), both overexpressed in *Escherichia coli* BL21(DE3). Maximum activity of KRED for biotransformation of ethyl-2-methylacetoacetate achieved by the high cell density cultivation was 2499.7 ± 234 U g^{-1}_{DCW} and 8.47 ± 0.40 U·mg^{-1}_{E}, respectively. The KRED from *Hansenula polymorpha* is a very versatile enzyme with broad substrate specificity and high activity towards carbonyl substrates with various structural features. Among the 36 carbonyl substrates screened in this study, the KRED showed activity with 31, with high enantioselectivity in most cases. With several ketones, the *Hansenula polymorpha* KRED catalysed preferentially the formation of the (*R*)-secondary alcohols, which is highly valued.

Introduction

Biocatalysis has long been known as a green technology, capable of delivering highly stereo-, chemo- and regioselective transformations that usually allow the number of steps in a synthetic route to be reduced [1]. Recent contributions developed by numerous research groups demonstrate the broad diversity of impressive opportunities for chemoenzymatic processes and also underline their potential as valuable solutions in chemical synthesis [2–7].

Biocatalytic reductions are commonly used for the asymmetric synthesis of a broad range of chiral alcohols in high chemical yields and optical purities [6,8,9]. Ketoreductases (KREDs), also described as alcohol dehydrogenases (ADH) or carbonyl reductases (CR), catalyze efficiently the reduction of carbonyl groups in a great variety of carbonyl compounds. In contrast with other enzymes, KREDs are very promiscuous, which provides huge advantage in biocatalysis. They have been recognized as the best catalysts for the synthesis of optically pure secondary alcohols [10–13], which are very important compounds as chiral intermediates for the synthesis of bioactive compounds especially in the synthesis of pharmaceuticals [14–17] and in fragrance industry [18–22]. Engineered KREDs have been successfully used in pharmaceutical industry including the production of chiral hydroxy-intermediates of statins, such as cholersterol-lowering drug atorvastatin (Lipitor®) [20], production of montelukast,the active pharmaceutical ingredient (API) of the antiasthmatic drug Singulair® [23], and atazanavir (Reyetaz®), which is antiretroviral medication used to treat HIV/AIDS [20]. The large–scale production of the antibiotic sulopenem with KRED-catalysed step, has been also reported [24]. The key chiral intermediate for all these synthetic processes was produced through stereoselective enzymatic reduction. KREDs are also efficient catalysts for the reduction of aromatic ketones [25,26], diaryl ketones [27], diketones [28], as well as β-ketoesters [29–32], showing high enantioselectivity. Particularly, substituted β-ketoesters [29–31], are starting material for the production of optically active β- hydroxy carboxylic acids, key building blocks and precursors of many bioactive compounds [33–35]. However, the reduction of sterically hindered aromatic substrates remains a great challenge. Only a few specialized commercially available or engineered KREDs have shown activity

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towards different aromatic bulky substrates [36], such as from *Rhodococcus ruber* ADH-A [37], *Ralstonia* sp. [36], and *Lactobacillus brevis* (LBADH) [14,38]. There are a few studies aiming at the improvement of the biocatalytic activity of enzymes that utilize bulky substrates bearing aryl [36], diaryl [27,39], or aliphatic moieties [40].

One of the important application of KRED is the production of chiral alcohols such as (*R*)-1-phenyl 1,2-ethanediol, which can be used for the synthesis of the antidepressant drug fluoxetine [41], or 1-phenylethanol, while its derivatives can be used as starting material for various drugs such as (*S*)-clorprenaline, (*S*)-fluoxetine, (*R*)-atomoxetine, and (*R*)-salbutamol [42], and therefore these substrates were amongst others used in screening of KRED (*Hansenula polymorpha*) specificity in this work.

In terms of stereoselectivity, most of KREDs follow Prelog's rule [43], where the (*S*) enantiomer is considered to be Prelog product whereas the (*R*) is the anti-Prelog product. Both stereoisomers of alcohols (*R* or *S*) are often required in asymmetric synthesis to be used for pharma and fine products industry, thus, ketoreductases following Prelog or anti-Prelog's rule are equally desired. Since natural occurrence of ketoreductases with anti-Prelog stereoselectivity is rarer [44], there is currently active interest toward the discovery of new ketoreductases or engineering Prelog ketoreductases showing anti-Prelog enantioselectivity [45]. The principles of stereoselectivity were investigated recently for mutants of *Lactobacillus kefir* short-chain alcohol dehydrogenase using quantum mechanical calculations and molecular dynamics simulations correlated with the enzymes x-ray crystal structures [46].

KRED (*Hansenula polymorpha*) [47], which is produced and researched in this work, belongs to the superfamily of short chain dehydrogenases/ reductases (SDRs), for which their structure is containing Rossmann fold- motif for coenzyme binding, with a central beta sheet of 6–7 strands sandwiched between three alpha helices on each side. However, crystal structure for this particular enzyme is not available. The classical SDR enzymes have a chain length of about 250 amino acid residues and a catalytic tetrad of Tyr, Lys, Ser, and Asn in most of the characterized SDRs [48–50]. Its natural substrate is L-sorbose, which is reduced to D-glucitol by KRED. *Hansenula polymorpha* ketoreductase was previously used for the synthesis of (*S*)-2-chloro-1-(3-chloro-4--fluorophenyl)-ethanol, a chiral key intermediate for an anticancer candidate compound, in high yield and excellent enantioselectivity [47].

In our previous study the successful co-immobilization of ketoreductase (KRED) from *Hansenula polymorpha* together with glucose dehydrogenase (*Bacillus megaterium*) to PVA particles was developed [51], in order to achieve reusability of this two-enzyme system.

In this study, we present recombinant KRED (*Hansenula polymorpha*) upscale production by high cell density cultivation, its purification and substrate specificity using thirty-six diverse carbonyl compounds with the newly prepared enzyme. The size, type, and location of substituents on the substrate's carbonyl moiety was varied followed by the proper substrate screening.

Experimental

Bacterial strain and plasmids

Escherichia coli BL21(DE3) was obtained from Novagen (EMD Biosciences, Madison, WI, USA). Plasmids pET28b(+) containing genes for KRED and GDH were described in a previous study [51]. Restriction maps of plasmids are available in Fig. A.11 and A.12 (Supplementary material, Appendix A).

Chemicals and media

Kanamycin was purchased from Gibco® (Life Technologies, Glasgow, UK). Nicotinamide adenine dinucleotide phosphate ($NADP^+$ and NADPH) were purchased from Prozomix Ltd. (Haltwhistle, UK).

All other chemicals were of analytical grade and commercially available. Luria-Bertani (LB) medium contained 10 g L^{-1} tryptone, 5 g L^{-1} of yeast extract and 10 g L^{-1} of NaCl, and plates additionally contained 2 % (w/v) agar. Fermentation medium contained 30 g L^{-1} glycerol, 10 g L^{-1} tryptone, 5 g L^{-1} $(NH_4)_2SO_4$, 3.64 g L^{-1} $NaH_2PO_4{\cdot}2H_2O$, 3.87 g L^{-1} KCl, 4 g L^{-1} citric acid, 2.4 g L^{-1} $MgSO_4{\cdot}7H_2O$, and 10 % (v/v) trace elements solution. Trace elements solution contained 109 g L^{-1} citric acid monohydrate, 3.4 g L^{-1} $CaCl_2{\cdot}2H_2O$, 2.46 g L^{-1} $ZnSO_4{\cdot}7H_2O$, 1.52 g L^{-1} $MnSO_4{\cdot}H_2O$, 0.5 g L^{-1} $CuSO_4{\cdot}5H_2O$, 0.43 g L^{-1} $CoSO_4{\cdot}7H_2O$, 9.67 g L^{-1} $FeCl_3{\cdot}6H_2O$, 0.03 g L^{-1} H_3BO_3, and 0.024 g L^{-1} $Na_2(MoO_4).2H_2O$ and was sterilised via a syringe filter with a polyethersulfone (PES) membrane (Filtropur S, 0.2 μm; Sarstedt, Germany). All other media were autoclaved (121 °C, 120 kPa, 20 min) and then supplemented with 30 μg·mL^{-1} of kanamycin. Feeding medium contained 630 g L^{-1} glycerol, 170 g L^{-1} $MgSO_4{\cdot}7H_2O$, and 114.4 g L^{-1} $NaH_2PO_4{\cdot}2H_2O$. Components of feeding media were autoclaved (121 °C, 120 kPa, 20 min) separately and then mixed aseptically.

Biomass preparation

A colony of *E. coli* BL21(DE3) pET28b+ *kred* was transferred into a glass tube with 3 mL of LB medium (2.2) and incubated overnight at 37 °C and 200 rpm. Subsequently, 1 mL of culture was transferred to 100 mL LB media (in 500 mL flask) and cultivated under the same conditions until reached an OD_{600} of ~0.5–0.6. This served as an inoculum for the production processes, which were carried out in 3 L bioreactor (BioFlo® 115, New Brunswick Scientific, Edison, NJ, USA) containing 1 L (A) or 1.5 L (B) of fermentation media (2.2), under the following controlled conditions: fermentation at 30 °C (A) or 37 °C (B), with a change to 30 °C prior to induction; pH 7 (maintained with NH_3); 1 vvm; and DO (dissolved oxygen) = 30 % in fed-batch mode. The DO level was maintained by an agitation cascade at 200–1200 rpm. Cultivation was initiated by adding 1% (v/v) of inoculum culture. Cells were induced at the end of the exponential growth phase by adding isopropyl β-D-thiogalactopyranosid (IPTG) to a final concentration 0.5 mM. Feeding media (2.2) was pumped in pulses of 50 mL (A) or 75 mL (B) when the substrate was depleted. Samples taken from the bioreactor were analysed for optical density (OD_{600}), glycerol concentration and protein activity. Cells were harvested by centrifugation (7197*g*, 15 min, 4 °C) after approximately 8 h of induction. The supernatant was discarded, and biomass stored at –80 °C.

Protein purification

The cell pellet (from Cultivation A) was resuspended in 0.1 M potassium phosphate buffer (pH 6.5) to final biomass concentration 18 *g* L^{-1} for purification experiments and 2.8 *g* L^{-1} for activity assays. Cells were disrupted by continual disruptor (CF Range, Constant Systems Ltd., Daventry, UK) in two cycles at 4 °C and 20 kPSI. The cells debris were removed by centrifugation (7197*g*, 30 min, 4 °C).

Cell extract was filtered by vacuum filtration with a mixed cellulose ester (MCE) filter membrane (0.45 μm, Fisher Scientific, Pittsburgh, PA, USA) and then 400 mL was loaded onto a Ni Sepharose™ 6 Fast Flow (GE Healthcare, Chicago, IL, USA) affinity resin (100 mL) in a PALL LRC50 × 080–200V01 column (diameter 50 mm, Pall Life Sciences, Port Washington, NY, USA). Purification on a lower scale (5 mL HisTrap™FF column) was described in a previous study [51]. We applied the same conditions in the scaled-up purification. Elution of enzymes was monitored by UV (280 nm), and fractions were analysed by SDS-PAGE. Selected fractions with enzyme were pooled, concentrated 10 times, and desalted against 10-times (v:v) buffer change with 0.1 M potassium phosphate buffer (pH 6.5) using an ÄKTA flux system (GE Healthcare, Chicago, IL, USA) with an Kvick™ Start 30 kDa ultrafiltration cassette (GE Healthcare, Chicago, IL, USA). The flow rate of solution was 50 mL·min^{-1}; trans-membrane pressure was kept at 0.14 MPa. The concentrations of purified enzymes were measured spectrophotometrically at 280 nm using an Eppendorf μCuvette® G1.0 (Eppendorf, Hauppauge,

NY, USA).

Enzyme activity assay

The NADPH-dependent KRED activity was assayed by measuring the change in absorbance at 340 nm corresponding to the oxidation of NADPH ($\varepsilon_{340} = 6220\ M^{-1}{\cdot}cm^{-1}$) using an 8453 UV–vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The reaction solution for the enzyme activity assay used a total volume of 1 mL containing 35 mM of (±)-ethyl-2-methylacetoacetate and 0.2 mM of NADPH in 100 mM phosphate buffer (pH 6.5) and 20 μL of enzyme/cell extract of appropriate concentration (from Cultivation A). One unit of KRED activity is defined as the amount of enzyme that consumed 1 μmol of NADPH per min at pH 6.5 and 37 °C.

Biotransformations with purified KRED

Small-scale screening reactions were carried out in 1.5 mL plastic test tubes with a total volume of 1 mL of reaction mixture containing carbonyl substrate (20–46 mM, Table 2), glucose (0.1 M), $NADP^+$ (1 mM), KRED (0.06–4.8 mg mL^{-1}), GDH (0.03–0.072 mg mL^{-1}) and potassium phosphate buffer (0.1 M, pH 6.5). Reductions were performed at 37 °C and 550 rpm in a thermomixer (Eppendorf, Germany) for 24 h. Reaction aliquots (50 μL) were taken during biotransformations at various time intervals and extracted with ethyl acetate (200 μL) by 10 s of vortexing, followed by centrifugation (13,300*g*, 1 min), the extracts were analysed by gas chromatography (GC).

Scaled-up biotransformations were performed in a glass, double-jacketed, tempered 25 mL reactor, monitored online with a pH probe (Mettler Toledo, Columbus, Ohio, USA) connected to a BioFlo115® unit (New Brunswick Scientific, Edison, NJ, USA) with selected substrates **1a**, **1b**, **1e**; **2b, 2d**; **3a**, **3b** and **10a** (Table 3, Entries 1–8) and automatically adjusted with 0.5 M KOH to pH 6.5 or 5.5 (**2d**, Table 3, Entry 5). Substrate concentration was 20 mM, and total volume of reaction mixture was 15 mL. Reactions were performed in parallel batches if a larger amount of product was necessary for further analysis. After 24 h, samples were centrifuged at room temperature for 15 min and the supernatant extracted with ethyl acetate (3 × 25 mL) and washed with brine and hexane. The organic layer was dried with sodium sulfate, evaporated under vacuum and the isolated products analysed by NMR. The ^{1}H NMR and ^{13}C NMR spectra (Fig. A.4–A.10; Supplementary material, Appendix A) were recorded on a 500 MHz instrument. Spectra were referenced to residual chloroform (δ 7.26 ppm, ^{1}H; δ 77.16 ppm, ^{13}C). Chemical shifts are reported in ppm. In cases when the emulsion was very difficult to eliminate, a continuous extraction process was followed for the isolation of the desired product. The absolute configuration of **2d** and **3d** were determined by GC analysis using a chiral column and after comparison of their retention time with the corresponding data for the commercially available optically active (*R*)-1-phenylethanol and (*R*)-3-octanol (Sigma Aldrich, Germany).

Determination of the absolute configuration of selected reduced products

The absolute configuration of the reduced products from substrates **1a, 1b, 1e** (Table 3, Entries 1–3), **2b** (Table 3, Entry 4), **3a–b** (Table 3, Entries 6 and 7), **10a** (Table 3, Entry 8) was determined by the modified

Table 1
Process parameters of two fed-batch cultivations A and B of *E. coli* BL21(DE3) + pET28b*kred*.

Cultivation	c_0 glycerol ($g{\cdot}L^{-1}$)	c_{DCW} ($g{\cdot}L^{-1}$)	Y [a] ($g\ g^{-1}$)	$a_{SP\ max}$ [b] ($U\ g^{-1}_{DCW}$)	a_{SP} [c] ($U\ g^{-1}_{DCW}$)	a_V [d] ($U{\cdot}L^{-1}$)
A	30	38.7	0.298	1799.1 ± 65.6	1799.1 ± 65.6	68,120 ± 2490
B	90	49.7	0.429	2499.7 ± 234	2220.1 ± 16.1	97,220 ± 5760

DCW = dry cell weight.
[a] yield = g of cells per g of glycerol.
[b] maximal achieved specific enzyme activity per g of dry cell weight.
[c] specific enzyme activity per g of dry cell weight at the end of cultivation.
[d] total enzyme volume activity per L of fermentation media at the end of cultivation.

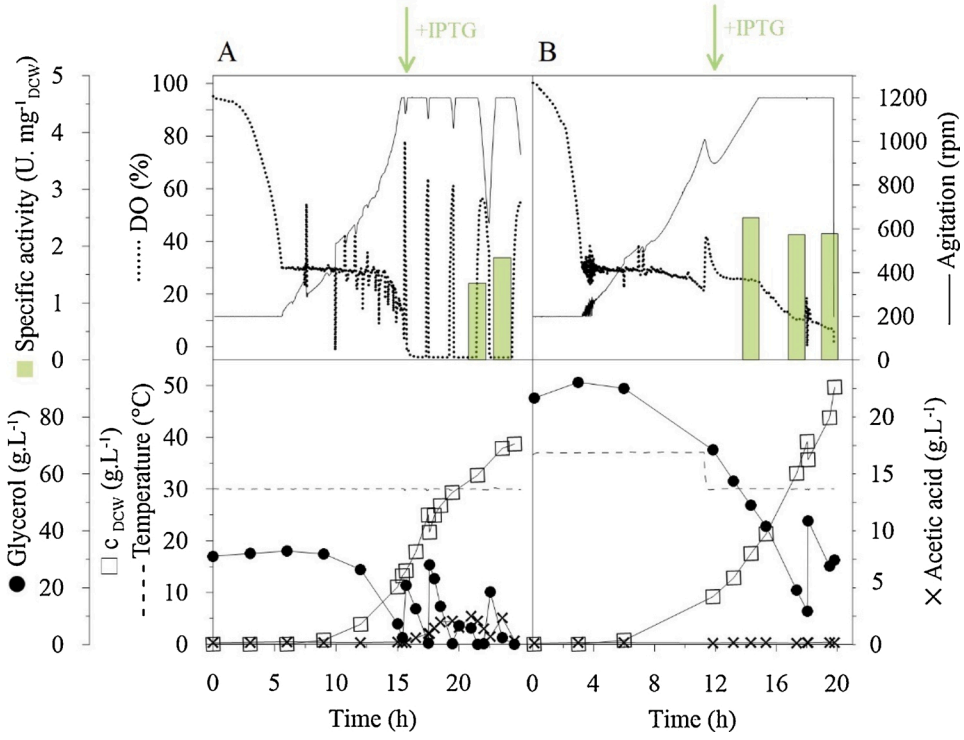


Fig. 1. Fed-batch cultivations A and B of *E. coli* BL21(DE3) with expression of KRED.

Mosher NMR method [52–54]. The enantiomerically enriched hydroxy compound was treated separately with the two enantiomers of methoxyphenyl acetic acid (MPA). A 1.1 eq. of *N, N′*-dicyclohexylcarbodiimide (DCC) (0.11 mmol) and a 1.1 eq. of the corresponding *(R)* or *(S)* MPA (0.11 mmol) were added to a solution of the corresponding alcohol (0.1 mmol) in dry CH_2Cl_2. Finally, a catalytic amount of 4-dimethylaminopyridine (DMAP) was added to the reaction mixture, which was then stirred at 0 °C for 4–6 h. After completion of the reaction, the urea product was filtered, the filtrate evaporated and then chromatographed with 20/1 (v/v) Hex/EtOAc, producing the corresponding MPA-ester. The resulting pair of diastereomeric ester derivatives was analysed by ^{1}H NMR in $CDCl_3$ (Bruker 500 MHz, Billerica, Massachusetts, USA). The absolute configuration of the alcohol moiety was assigned based on the difference in the chemical shifts of corresponding resonances of the paired diastereomers, based on the literature [52,55–58]. Chemical shift differences for at least one set of enantiotopic protons were large enough and sufficiently separated from other signals that the enantiomeric compositions could be determined directly from relative peak areas. All chemical shifts and the $\Delta\delta^{syn\ anti}$ were measured from the ^{1}H NMR spectra of the corresponding chemically formed *syn-anti* mixtures in $CDCl_3$ solution (Tables A.2–A.8; Supplementary material, Appendix A). In order to confirm the absolute configuration, optical rotation was measured for each sample by a polarimeter (A. Krüss Optronic GmbH, Hamburg, Germany) and compared with the corresponding literature data for each reduced compound. The measurements were taken under the same conditions (concentration, solvent) in order to compare reliably the calculated optical rotation with the literature data. When the available amount of the secondary alcohol was limited, the optical rotation was measured in the closest concentration to the corresponding literature concentration and found to be in agreement with the literature data (see Supplementary material, Appendix A).

Determination of the relative configuration

The products from the bioreduction of α-substituted β-keto esters **1a** and **1b** (Table 3, Entries 1 and 2), which bear two stereogenic centres, were analysed as follows: For the assignment of their absolute configuration, the relative configuration was determined first by using ^{1}H NMR spectroscopy. The assignment was achieved by recording the ^{1}H NMR spectrum of the *syn-anti* mixture. According to the literature [59], the upfield carbinol hydrogen signal corresponds to the *anti*, and the downfield signal to the *syn*, stereoisomer [52–54]. After recording the ^{1}H NMR spectra of the chemically formed *syn-anti* mixtures, which were obtained easily by a chemical reduction, we proceeded to a direct chemical shift comparison with the ^{1}H NMR spectra of the corresponding optically pure hydroxy compounds.

Analytics

The bioconversion of the carbonyl compounds was monitored by GC using a flame ionisation detector (GC-FID; Agilent Technologies 7890 N, Santa Clara, CA, USA) with an HP-FFAP capillary column (Agilent J&W, 30 m ×0.25 mm ×0.25 μm) using H_2 as the carrier gas at a flow of 1.5 mL·min^{-1}. The volume of injection was 1 μL with a 100:1 split. The oven temperature was initially 90 °C, increasing to 250 °C at a gradient of 5 °C·min^{-1} followed by a hold at 250 °C for 2 min. The percentage conversion was evaluated as 100 times the peak area of alcohol product divided by the sum of the peak area of the ketone substrate and the peak area of the alcohol product. The optical purity of products was determined by GC analysis using a CP-Chirasil-DEX CB chiral column (Agilent J&W, 25 m ×0.25 mm ×0.25 μm) with H_2 flow at 1.15 mL·min^{-1}. The volume of injection was 1 μL with a 50:1 split. The oven temperature was initially 80 °C, increasing to 200 °C at gradient of 5 °C/min and held for 2 min. Enantiomeric excess (*ee* %) was evaluated as follows: (peak area percentage of major product – peak area percentage of second product)/(peak area percentage of major product + peak area percentage of second product) × 100.

Results and discussion

Gene cloning and enzyme expression

The KRED from *Hansenula polymorpha* was cloned to *E. coli* BL21 (DE3) for the reduction of carbonyl substrates to optically active hydroxy compounds, while the GDH from *Bacillus megaterium* was applied to regenerate the NADPH cofactor. Cloning and flask enzyme expression has been previously reported [51].

High cell density biomass production

Substrate screenings require significant amounts of KRED, and therefore a high cell density cultivation (HCDC) protocol for *E. coli* BL21 (DE3) expressing this enzyme was optimised. The goal was to achieve as high a biomass concentration as possible with an active recombinant protein. Glycerol was applied as a carbon source to eliminate acetic acid production, since it has an inhibitory effect on both cell growth and protein production [60,61]. To our knowledge, this is the first report of an HCDC process of recombinant cells expressing a KRED. There are data in the literature for many other enzymes, such as lyases or nitrilases, produced by *E. coli* HCDC from glycerol [62,63].

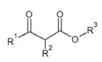
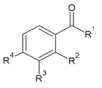
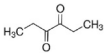
Cultivation and induction temperature profile is one of the most important parameters for successful protein production in *E. coli*. Likewise, the starting concentration of the carbon source affects the lag phase of microorganism growth and thus also protein production time. That is why these two parameters need to be optimised for each protein that is produced by this system. There were two different sets of fed-batch conditions tested (Fig. 1). The first set included a lower cultivation temperature (30 °C) with lower starting glycerol (30 g L^{-1}) and substrate feeding up to 30 g L^{-1} (Fig. 1A). The second set used a higher cultivation temperature (37 °C) before induction and starting glycerol concentration of 90 g L^{-1} with fed-batch feeding to 30 g L^{-1} (Fig. 1B).

According to Fig. 1 and summarised parameters displayed in Table 1, Cultivation A (lower initial glycerol and temperature) was almost 5 h elongated and resulted in lower biomass and cell productivity compared to Cultivation B. Enzyme produced by Cultivation B showed higher specific activity and increased total activity per L of cultivation medium. The highest published biomass density of *E. coli* was about 220 g L^{-1} [64],however no protein expression was made in the process. During recombinant protein production in *E. coli* is probably impossible to achieve similar biomass densities, due to using of lower temperatures and the enormous load on the cellular apparatus by protein production. Biomass density in Cultivation B (49.7 g_{DCW}·L^{-1}) is the highest reported for *E. coli* with functional KRED expression. The biomass yield (grams of cells per gram of glycerol) showed more effective utilisation of glycerol in Cultivation B. Expression of KRED was detected not only by activity, but was also obvious in SDS-PAGE analysis (Fig. A.1, Supplementary material, Appendix A), where the increase in KRED (32 kDa) was observed [47,51] by the longer IPTG induction. Acetic acid in both Cultivations A and B did not exceed 2.5 g L^{-1}, although cells were limited by oxygen according to the zero DO value after 16 h for Cultivation A, which could support acetic acid production [60]. The higher activity in Cultivation B may be a result of several factors acting together, such as the metabolic state of cells, cell concentration at induction point or different anti-foaming agent. Thus, we can conclude that a set of condition B is more suitable for this enzyme. According to the results, HCDC cultivation is a very efficient tool for the rapid and efficient production of recombinant KREDs, and thus it is possible to reduce the volume of culture media and costs for applications in organic syntheses, whether using whole cells or purified enzyme.

The same KRED was produced by expression in vector pBMS2000 with glucose-6-phosphate dehydrogenase in host cells *E. coli* BL21Star

(DE3) with a concentration of wet cells of ~19 g.L^{-1}, which represents approximately 4.36 g_{DCW}.L^{-1} according to conversion relationship [65] and its 217-fold lower biomass concentration than that reported in this study (49.7 g_{DCW}.L^{-1}, Table 1, Cultivation B). Activity of the recombinant KRED produced by *E. coli* BL21(DE3) in this work was measured in cell extract by using (±)-ethyl-2-methylacetoacetate (**1a**) as substrate, and, at the end of cultivations, its values were 1799.1 ± 65.6 U. g^{-1} $_{DCW}$ (A) and 2220.1 ± 16.1 U. g^{-1} $_{DCW}$ (B) (Table 1). It must be

Table 2
Enzymatic reduction of β-keto esters (**1a–h**), aryl alkyl ketones (**2a–j**), aliphatic ketones (**3a–e**), cyclic, bicyclic or other ketones (**4a**, **4b**, **8a–15a**) and diketones (**5a–7a**) by using purified KRED from *Hansenula polymorpha*.

Entry	Substrate	Compound	R^1	R^2	R^3	R^4	Conversion [a] (%)	*ee* [a] (%)	*de* [a] (%)
1		1a[c]	-Me	-Me	-Et	–	>99	86	>99
2		1b[c]	-Me	-Et	-Et	–	>99	80	96
3		1c	-Me	-H	-Et	–	>99	0	–
4		1d[c]	-Me	-Bn	-Et	–	74	67	n.d.[b]
5		1e	-Me	-H	-Me	–	>99	>99	–
6		1f	-Me	-H	-*t*-Bu	–	>99	92	–
7		1g[c]	-Me	-CH_2CN	-*t*-Bu	–	>99	96	n.d.
8		1h	-*sec*-Bu	-H	-Me	–	89	46	n.d.
9		2a	-Me	-H	-OH	-H	96	>99	–
10		2b	-Me	-H	-H	-Me	>99	90	–
11		2c	-Me	-H	-H	-OMe	96	98	–
12		2d	-Me	-H	-H	-H	85	60	–
13		2e	-Me	-H	-H	-Br	74	95	–
14		2f	-Me	-Me	-H	-H	0	–	–
15		2g	-Me	-OH	-H	-H	0	–	–
16		2h	–Et	-H	-H	-H	>99	74	–
17		2i	-CH_2OH	–H	-H	-H	>99	>99	–
18		2j	-CH_2OAc	-H	-H	-H	>99	50	–
19		3a	-Me	-$(CH_2)_4CH_3$	–	–	>99	>99	–
20		3b	-Me	-$(CH_2)_5CH_3$	–	–	>99	>99	–
21		3c	-Me	-$CH(OH)CH_3$	–	–	>99	66	–
22		3d	-Et	-$(CH_2)_4CH_3$	–	–	97	>99	–
23		3e	-Me	-$(CH_2)_{10}CH_3$	–	–	0	–	–
24		4a[c]	-Me	-H	–	–	18	n.d.	n.d.
25		4b[c]	-H	-Me	–	–	99	n.d.	n.d.
26		5a	–	–	–	–	>99	60	–
27		6a	–	–	–	–	>99	>99	–
28		7a[c]	–	–	–	–	>99	51	91
29		8a	–	–	–	–	>99	n.d.	–
30		9a	–	–	–	–	>99	88.	–
31		10a	–	–	–	–	>99	n.d	–
32		11a[c]	–	–	–	–	62	n.d.	n.d.
33		12a	–	–	–	–	64	80	–
34		13a	–	–	–	–	>99	>99	–
35		14a	–	–	–	–	0	–	–
36		15a	–	–	–	–	0	–	–

[a] Determined by GC analysis using chiral column after 24 h reaction. In each case, the racemic product was prepared for calibration. [b] insufficient GC separation, [c] racemic mixture.

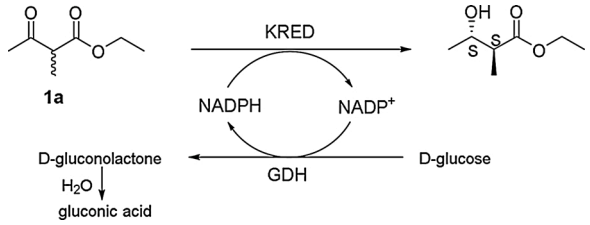


Fig. 2. Reduction of (±)-ethyl-2-methyl acetoacetate (**1a**) to (2*S*,3*S*)-ethyl 2-methyl-3-hydroxybutanoate using KRED from *Hansenula polymorpha* and glucose/GDH cofactor regeneration system.

Table 3
Determination of the absolute configuration of products from scaled-up enzymatic reductions using KRED from *Hansenula polymorpha*.

Entry	Substrate	c_S (mM)	Conversion [a] (%)	*ee* [a] (%)	*de* [b] (%)	Abs. Config.[d]
1	1a[c]	20	97	86	>99, *anti*	2*S*,3*S*
2	1b[c]	20	97 ± 1.2	82.4 ± 2.3	>99, *anti*	2*S*,3*S*
3	1e	20	>99	>99	–	*S*
4	2b	20	82.3 ± 0.7	93.6 ± 2.4	–	*R*
5	2d	20	85	60	–	*R* [a]
6	3a	20	95.3 ± 0.3	>99	–	*R*
7	3b	20	97.7 ± 1.9	>99	–	*R*
8	3d	20	97	>99		*R*
9	9a	20	94.5 ± 4.5	96 ± 2	–	*R*

[a]Determined by GC analysis using chiral column after 24 h reaction. In each case, the racemic product was prepared for calibration, [b] determined by ^{1}H NMR spectroscopy; [c] racemic mixture, [d] determined by ^{1}H NMR analysis of the corresponding MPA esters using modified Mosher's NMR method [52].

noted here that there are no published activity data for this substrate for comparison.

Protein purification and activity assay

Purification of His-tagged recombinant KRED was optimised using affinity chromatography (FPLC) with Ni^{2+} resins. The small-scale purification (5 mL HisTrap™FF column) was recently reported [51] and the same conditions were applied in a scaled-up purification using 100 mL of resin (Ni Sepharose™ 6 Fast Flow). Elution of enzymes was monitored by UV (280 nm, Fig. A.2, Supplementary material, Appendix A). The presence of pure protein in eluted fractions was confirmed by SDS-PAGE analysis (32 kDa; line 910; Fig. A.3; Supplementary material, Appendix A). From one purification batch, 460 mg of pure protein was achieved from crude extract (400 mL, c_{DCW} =18 g·L^{-1}), representing yields of 4.60 mg mL$^{-1}{}_{resin}$ and 64 mg g$^{-1}{}_{DCW}$. With a 5-mL column [51], an efficiency of 7.8 mg mL$^{-1}{}_{resin}$ and 72 mg g$^{-1}{}_{DCW}$ was reported for this KRED, suggesting that the maximum isolation capacity of 100 mL resin was not reached. The amount of protein obtained from 1 g of cells was slightly lower, probably due to the losses during concentration and desalting caused by upscaling of downstream processes.

Results of purification are difficult to compare with other reported isolations because most of the KREDs used are commercial products [23,66]; if they are purified using the same resin, authors frequently do not publish any relevant data or yields [39,40]. Romano et al. purified 70 mg of recombinant KRED1-Pglu from 4 g of wet cell paste in one batch (17.5 mg g$^{-1}{}_{DCW}$) using an HIS-Select® Nickel Affinity Gel [26], approximately 3.7-fold lower yield per g of biomass than that reported here. This may be due to lower resin efficiency or lower expression of recombinant protein.

Activity of the purified enzyme was determined spectrophotometrically using ethyl-2-methylacetoacetate as a substrate. Initial specific activity was 8.47 ± 0.40 U·mg$^{-1}{}_{E}$, which is lower than the previously reported 13.06 U·mg$^{-1}{}_{E}$ from a 5-mL column purification [51]. This may be due to a decrease in enzyme activity during the upscaling of downstream processes.

Substrate screening

Enzymatic reduction of ketones with yeast, bacteria or isolated enzymes, including NADPH recycling by GDH, has provided numerous optically pure alcohols [67–72] which are high-added-value compounds frequently used in the pharma or fine products industries [1,5]. Ketoreductases are the catalysts of choice for these transformations and can give either enantiomer of the alcohol, and in general they follow Prelog's rule with most substrates. Therefore, investigation of enzymes showing anti-Prelog preference remains a great challenge. In order to demonstrate the wide range of substrates tolerated by the purified KRED from *Hansenula polymorpha*, as well as its activity and enantioselectivity, an extensive portfolio of various carbonyl substrates was investigated, and the results are shown in Table 2. Other various isolated or engineered KREDs have been reported in the literature to reduce the same substrates, occasionally with opposite enantio-preference, which is quite desirable.

A total of 36 carbonyl compounds with broad range of structural features (**1a–15a**, Entries 1–36, Table 2) were screened. The substrate range included eight β-keto esters, (Entries 1–8, Table 2) four of which were α-substituted (**1a**, **1b**, **1d**, **1 g**, Entries 1, 2, 4, 7, Table 2), acetophenone and its derivatives (**2 a–g,** Entries 9–15), aryl alkyl ketones (**2h–j**, Entries 16–18), aliphatic ketones (acyclic ketones **3a–e**, Entries 19–23), cyclic ketones (**4a, 4b**, Entries 24, 25), methyl ketones **8a** and **10a** (Entries 29, 31), diketones (**5a-7a**, Entries 26–28), bicyclic ketones (**9a**, **11a**, Entries 30, 32) and various ketones containing thiophene or cyclopropyl rings amongst others (**12a**, **13a**, Entries 33, 34),. Regeneration of NADPH cofactor was accomplished by using the well-established GDH/glucose recycling system [73]. The reaction conditions used for the screening were the same as those previously optimised using (±)-ethyl-2-methylacetoacetate (**1a**) as the model substrate (Fig. 2) [51]. Before conducting the screening with KRED, we tested the stability of each substrate under the standard enzymatic reaction conditions without addition of the enzyme (0.1 M potassium phosphate buffer, 0.1 M glucose, 1 mM $NADP^+$, GDH 0.03–0.072 mg mL^{-1}, 20–46 mM of substrate, pH 6.5, 37 °C). No reaction was detected after 24 h.

All the results of Table 2, including conversions and stereoselectivities, were repeated in duplicate and are reproducible. The KRED showed excellent activity with most of the β-keto esters (compounds **1a–c**, **1e–g**), with >99 % conversion. The size of the ester group Me, Et or *t*-Bu had no significant effect on conversions or enantioselectivity. Conversion was decreased when benzyl group (Bn) was the α-substituent (**1d**, Entry 4, Table 2), while the introduction of an electron-withdrawing substituent (−CH_2CN) at the stereogenic centre, enhancing the acidity and therefore the mobility of the proton at the α-position (**1 g**, Table 2, Entry 7), resulted in excellent conversion (>99 %) and increased KRED affinity. It is important to note here that the reduction of the four α-substituted β-keto esters **1a**, **1b**, **1d**, **1 g** (Table 2, Entries 1, 2, 4, 7) was quantitative, with conversions of >99 % due to a dynamic kinetic resolution (DKR) process [74–80] through in situ racemisation of these substrates via enolisation. Among the eight β-ketoesters tested (**1a–h**, Table 2, Entries 1–8), the highest enantioselectivity (>99 % *ee*) and conversion (conv. > 99 %) was achieved in the case of methyl acetoacetate (**1e**, Table 2, Entry 5) where methyl (*S*)-3-hydroxybutyrate was formed exclusively. The KRED showed high diastereoselectivity with α-substituted β-keto esters **1a** and **1b** (*de* >96 %, Table 2, Entries 1, 2); only the *anti*-isomers were observed and their absolute configuration was determined to be (2*S*,3*S*), as shown in Table 3 (Entries 1 and 2).

As has been previously reported, most commercially available

recombinant KREDs produce the (2*R*,3*S*) or the (2*R*,3*R*) diastereomer (KREDs 102, 103, 106 and 117 and KREDs 107and 121 respectively) for the same keto esters **1a** and **1b** [29]. In particular, in the reduction of ethyl-2-methyl acetoacetate (**1a**, Table 2, Entry 1), the optically active (2*S*,3*S*)-hydroxy ester was produced and KRED *Hansenula polymorpha* showed higher enantioselectivity (86 % *ee*) compared to the previously reported commercially available KRED107 (70 % *ee*) [30]. Recently, Wei et al. reported the reduction of methyl acetoacetate for the production of methyl (*R*)-3-hydroxybutyrate with 87 % conversion by using an anti-Prelog stereospecific carbonyl reductase [25].

The structure of the screened aryl alkyl ketones varied in terms of the position of substituents on the aromatic ring: *ortho* (**2f**, **2 g**, Table 2, Entries 14, 15) *meta* (**2a**, Table 2, Entry 9) and *para* (**2b**, **2c**, **2e**, Table 2, Entries 10, 11, 13) position. The enzyme showed no activity towards *ortho*-substituted acetophenones (*o*-methyl, **2f**, Entry 14 and *o*−OH, **2 g**, Entry 15, Table 2), most probably due to steric hindrance [25,81]. The enzyme showed increased activity, with conversions between 74 % and >99 % and high enantioselectivity (90 %–>99 % *ee*), for the *meta*- and *para*-substituted acetophenones (**2a**, **2b**, **2c**, **2e**, Table 2, Entries 9, 10, 11, 13). The lowest enantioselectivity between the group of acetophenone and its derivatives was observed for acetophenone **2d** (60 % *ee*, Table 2, Entry 12). Previous reports had shown increased enzymatic activity with bromine and other halogens or electron-withdrawing groups as *para* substituents [11,25]. In our case, the KRED showed slightly decreased activity with *para*-Br-acetophenone (**2e**; 74 % conversion, 95 % *ee*, Table 2, Entry 13). Excellent conversion was derived with ketones **2h–j** (conv. > 99 %, Table 2, Entries 16–18), which contained –Et, $-CH_2OH$ and $-CH_2OAc$ next to the carbonyl group. The highest enantioselectivities were observed with hydroxy ketone **2i** (>99 % *ee*, Table 2, Entry 17) and *meta*−OH substituted acetophenone (**2a)**, (>99 % *ee*, Table 2, Entry 9). This confirms the positive effect of electron-withdrawing groups if there are suitable spatial arrangements in the active centre of the enzyme. According to previously published data with recombinant protein (KRED1-Pglu) overexpressed in *E. coli* [26], longer chains connected to the carbonyl group resulted in decreased enzymatic activity. Stereoselective reduction of aliphatic ketones with substituents longer than four carbon atoms on both sides of the carbonyl group or remote aliphatic keto esters still remains challenging [82,83]. Among the aliphatic ketones, the highest conversion (>99 %) and excellent enantioselectivities up to >99 % *ee* were achieved for acyclic ketones **3a, 3b, 3d** (Table 2, Entries 19, 20, 22), and good enantioselectivity was observed with hydroxy ketone **3c** (66 % *ee*, Table 2, Entry 21). Moreover, with 4-phenylbutan-2-one **9a** and methyl ketones **8a** and **13a**, the KRED showed excellent activity and enantioselectivity (conv. > 99 % and 88– >99 % *ee*, Table 2, Entries 29, 30, 34). This result, compared to the obtained data for acetophenone (**2d**; 80 % conv., 60 % *ee*, Table 2, Entry 12), shows that the KRED can potentially reduce more efficiently those methyl ketones bearing longer or bulkier aryl substituents next to the carbonyl group. The enzyme activity decreased dramatically in the case of the methyl ketone **3e** (tridodecan-2-one, Table 2, Entry 23), where there is a long nonpolar carbon chain of 11 carbon atoms, assuming that spatial limits in the active site of the enzyme were involved [84]. The position of the methyl group on the cyclohexanone ring in the cases of 2- and 3-methyl cyclohexanones (**4a** and **4b**, respectively) was an important factor in their conversion (Table 2, Entries 24, 25). Conversion of 3-methyl-cyclohexanone (**4b**) was 5-fold higher than that of 2-methyl-cyclohexanone (**4a)** after 24 h (conv. 99 % compared to conv. 18 %), most probably due to the increased steric hindrance of the methyl group in **4a**. Our further substrate specificity investigations with the KRED from *Hansenula polumorpha* showed high activity with most of the tested substrates (**4b**, **5a–8a**, Table 2, Entries 25–29). The enantioselectivity was excellent with diketone **6a** and ketone **13a** (>99 % *ee*, Table 2, Entries 27, 34). The diastereoselectivity for the reduced products of the chiral bicyclic ketone **11a** (Table 2, Entry 32) could not be determined because of insufficient GC separation. For the chiral diketone **7a**, the observed stereoselectivity was *de* 91 % and *ee* 51 %; however, the reduced products were not analysed further. As shown in Table 2, no activity was observed with ketones **14a** and **15a** (Entries 35, 36, Table 2). Only two enzymes (KRED 101 and 132) have been reported to show activity towards **14a** (1.48 and 2.20 U. mg^{-1}, respectively), but *ee* was not determined [84]. In the cases where the KRED showed low substrate conversions, the absolute configuration of the products was not determined and therefore it was not possible to compare with literature data. In order to further determine the absolute configuration of the reduced products, scale-up of reactions (15 mL) was performed for nine selected substrates from every group (keto esters, aryl ketones, aliphatic ketones) with which KRED showed good conversion and enantioselectivity during substrate screening. Determination of the absolute configuration of the optically pure hydroxy products was performed by a modified Mosher's method [52–58], and the results are summarised in Table 3.

Interestingly, the KRED showed *R*-enantiopreference with the following ketones: *para*-Me-acetophenone (**2b**), acetophenone (**2d**), 2-heptanone (**3a**), 2-octanone (**3b**) and 3-octanone (**3d**) as well as with 4-phenylbutan-2-one (**9a**) (Table 3, Entries 4–9). In contrast, in the case of β-keto ester **1e** (Table 3, Entry 3), the KRED showed *S*-enantiopreference and the methyl (*S*)-3-hydroxybutanoate was produced exclusively.

These results suggest anti-Prelog selectivity for the enzyme (Table 3), which is quite rare with these types of substrates [85]. We would like to emphasise here that previous attempts to reduce 2-octanone (substrate **3b**) with commercial ketoreductases [84] and genetically engineered KREDs [40] showed that most of these enzymes did not display significant activities. The successful production of the *R*-enantiomer was reported for whole-cell biocatalyst *Acetobacter pasteurianus* GIM1.158 (95 % yield; >99 % *ee*) [85] and *Candida rugosa* (92 % yield; 95 % *ee*) using ionic liquids as solvents to increase the solubility of the substrate [86]. There are few reports for the enzymatic production of 3-octanol, of which the most successful was when *Lactobacillus brevis* ADH was used, showing 84.6 % conversion and >99 % *ee* for (*R*)-3-octanol [87] and when *E. coli* cells with ADH from *Rastolnia sp.* were used to produce (*S*)-3-octanol (98 % *ee*) [36]. 3-octanone (**3d)** was successfully reduced using the KRED from *Hansenula polymorpha* with high conversion and excellent enantioselectivity (conv. 97 %, >99 % *ee*, Table 3, Entry 8) and the 3-octanol produced had *R* configuration, as determined by chiral GC analysis and after comparison of the retention time with the corresponding data for the commercially available optically active (*R*)-3-octanol.

(*R*)/(*S*)-3-octanol and other aliphatic chiral alcohols are bioaroma compounds and could be used in the fragrance industry. Also, (*R*)-3-octanol is a known pheromone that acts as sex attractant for *Myrmica scabrinodis* [88].

All the above results indicated that the KRED from *Hansenula polymorpha* is a very versatile enzyme with a broad substrate specificity and high activity towards carbonyl substrates with various structural features (Table 2). Among the 36 carbonyl substrates which were screened in this study (Table 2), the KRED showed activity with 31 and no activity was found with only five substrates.

Conclusions

Ketoreductase from *Hansenula polymorpha* was successfully overexpressed in *Escherichia coli* BL21(DE3) in a high cell density process, with biomass concentration 49.7 g L^{-1} and high specific activity (2220.1 ± 16.1 U g^{-1}_{DCW}). The purified enzyme showed activity towards 31 substrates, including aliphatic and aromatic ketones, acetophenone and substituted acetophenones, aryl alkyl ketones, diketones and β-keto esters. Interestingly, the KRED from *Hansenula polymporpha* catalysed the enantioselective reduction of 2-heptanone, 2-octanone, 3-octanone, *p*-Me-acetophenone and 4-phenyl-2-butanone for the production of the optically active corresponding alcohols, with remarkable conversions (>99 %) and excellent enantioselectivities (>99 % *ee*). In these

bioreductions the *R*-enantiomer was formed, suggesting anti-Prelog preference for the KRED. This result is particularly important since there are very few known ketoreductases in the literature that show anti-Prelog selectivity during ketone reduction, especially for aliphatic ketones such as 2-octanone and 3-octanone.

CRediT authorship contribution statement

Tatiana Petrovičová: Investigation, Validation, Writing - original draft. **Dominika Gyuranová:** Investigation, Validation. **Michal Plž:** Investigation. **Kamela Myrtollari:** Investigation, Data curation. **Ioulia Smonou:** Methodology, Writing - review & editing. **Martin Rebroš:** Methodology, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.mcat.2020.111364.

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