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Stereoselective reduction of aromatic ketones by a new ketoreductase from *Pichia glucozyma*

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Abstract A new NADPH-dependent benzil reductase (KRED1-Pglu) was identified from the genome of the non-conventional yeast *Pichia glucozyma* CBS 5766 and overexpressed in *E. coli*. The new protein was characterised and reaction parameters were optimised for the enantioselective reduction of benzil to (*S*)-benzoin. A thorough study of the substrate range of KRED1-Pglu was conducted; in contrast to most other known ketoreductases, KRED1-Pglu prefers space-demanding substrates, which are often converted with high stereoselectivity. A molecular modelling study was carried out for understanding the structural determinants involved in the stereorecognition experimentally observed and unpredictable on the basis of steric properties of the substrates. As a result, a new useful catalyst was identified, enabling the enantioselective preparation of different aromatic alcohols and hydroxyketones.

Keywords Ketoreductase · Carbonyl reductase · *Pichia glucozyma* · Enantioselective reduction · Biocatalysis · Stereoselective

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Introduction

The use of ketoreductases (KREDs, often also described as carbonyl reductases (CR) or alcohol dehydrogenases (ADH)) is a well-recognised method for the enantioselective reduction of prochiral ketones into stereodefined chiral alcohols (Hall and Bommarius 2011; Hollmann et al. 2011). Reduction of aromatic ketones has been studied with different reductases, with particular focus on the reduction of sterically hindered (bulky) substrates, not easily recognised by most of the available KREDs (Lavandera et al. 2008). Commercially available or engineered KREDs can reduce structurally different (including bulky) ketones and their use has been also scaled-up to industrial processes (Huisman et al. 2010; Liang et al. 2010). Other alcohol dehydrogenases, such as the ones from *Rhodococcus ruber* (ADH-A; Stampfer et al. 2004), *Ralstonia* sp. (RasADH; Lavandera et al. 2008), and *Lactobacillus brevis* (LBADH; Haberland et al. 2002) have been identified as suitable biocatalysts for the reduction of different aromatic bulky substrates. Steric effects of the substrates on the activity of LBADH towards aromatic ketones (acetophenone derivatives) were accurately studied. In most cases, a tendency for higher activity with smaller substrates could be detected, but noticeable deviations from this general trend were observed due to stereoelectronic effects (Rodríguez et al. 2014). Understanding how substrate-binding residues of the protein affect substrate binding orientation and thus stereoecognition is necessary not only for predicting enantioselectivity, but also for designing rational or semirational mutations, aimed at increasing or changing the stereobias of a given reduction (Baerga-Ortiz et al. 2006; O'Hare et al. 2006). This approach was employed for mutating a carbonyl reductase from *Sporobolomyces salmonicolor* AKU4429 (SSCR) and switching its enantiopreference in the reduction of *para*-substituted acetophenones (Zhu et al. 2008). This SSCR

variant has been recently used for the enantioselective reduction of asymmetric benzophenone derivatives (Li et al. 2009).

Whole cells of the non-conventional yeast *Pichia glucozyma* CBS 5766 (now reclassified as *Ogataea glucozyma*) have been used for the reductive biotransformation of various aromatic ketones and ketoesters (including bulky ones), often showing interesting stereoselectivity (Forzato et al. 2001; Gandolfi et al. 2009; Rimoldi et al. 2011a; Husain et al. 2011; Rimoldi et al. 2011b; Contente et al. 2015). The reduction of these substrates frequently occurred with activity and enantioselectivity strongly dependent on the conditions of growth and biotransformation, suggesting the possible occurrence of different ketoreductases acting on aromatic ketones. Notably, whole cells of *P. glucozyma* carried out the selective monoreduction of different 1,2-diarylethanediones (benzils) (Hoyos et al. 2008; Fragnelli et al. 2012). Enzymes with benzil reductase activity, belonging to the short-chain dehydrogenase/reductase (SDR) family, have been found in bacteria (Maruyama et al. 2002; Pennacchio et al. 2013) and yeasts (Johanson et al. 2005). Here, we report the identification of a benzil reductase from *P. glucozyma* and a study of its activity/enantioselectivity towards different aromatic ketones using the recombinant protein (KRED1-Pglu) overexpressed in *Escherichia coli*.

Materials and methods

General

Phusion DNA polymerase was from New England Biolabs (EuroClone S.p.A., Pero (MI), Italy). pET26b(+) vector was from Novagen (Merck Millipore, Vimodrone (MI), Italy).

HIS-Select® Nickel Affinity Gel used for protein purification was purchased from Sigma Aldrich (Milano, Italy).

Pichia glucozyma CBS 5766 was from CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands). Stocks of the strain were stored at −20 °C in 15 % *v/v* glycerol and revitalized liquid YPD (yeast extract 5 g/L, peptone 5 g/L, glucose 20 g/L, pH 6.0) medium prior use. The bacterial strain *E. coli* XL1 blue was used for the propagation of the recombinant plasmid. *E. coli* BL21(DE3)Star (Merck Millipore, Vimodrone, Italy) was used for expression of KRED1-Pglu. Both were used according to the instruction of the suppliers.

All the DNA manipulations and bacterial transformations were carried out according to standard protocols (Sambrook and Russell 2001) or manufacturers' instructions, unless otherwise stated.

Protein concentration was determined with the Bradford method on a Pharmacia Biotech Ultrospec 1000 using bovine serum albumin as standard (Bradford 1976).

Production of the recombinant protein

Identification of KRED1-pglu gene

Genome of *P. glucozyma* CBS 5766 was sequenced by Baseclear (BaseClear B.V., Leiden, The Netherlands) using Illumina Casava pipeline version 1.8.0; assembly has been performed using the "de novo assembly" option of the CLC Genomics Workbench version 5.1. Annotation has been made on the assembled contig or scaffold sequences using the GAPS Annotation Pipeline from Progenus (Gembloux, Belgium). Upon analysis, 311 oxidoreductases, 517 transferases, 512 hydrolases, 71 lyases, 53 isomerases and 109 ligases were annotated and, among the oxidoreductases, a unique benzil reductase gene (named KRED1-Pglu) was found after BLAST analysis.

Cloning

KRED1-Pglu gene, coding for a putative benzil reductase, was amplified from the genomic DNA of *P. glucozyma* by PCR using the following primers, carrying *Nde*I and *Hin*dIII restriction sites: forward 5′-ATAC**CATATG**ACGAAGGTGACTGTTGTGAC-3′; reverse 5′-AGAG**AAGCTT**GGCGTACTCCTTCAACTCTG-3′.

The amplified gene was then cloned into a pET26b(+) vector using the *Nde*I and *Hin*dIII restriction sites. With this cloning strategy, the resulting protein is expressed with an N-terminal His_6 tag. The correct construction of the expression plasmid was confirmed by direct sequencing.

Protein expression

Cultures of *E. coli* BL21(DE3)Star transformed with the resulting plasmid were grown overnight at 37 °C in LB medium supplemented with 25 μg/mL kanamycin. The seed culture was then diluted into a stirred bioreactor (Biolafitte & Moritz bioreactor, Pierre Guerin Technologies) containing 4 L of cultivation medium (Terrific Broth, 12 g/L bacto-tryptone, 24 g/L yeast extract, 4 g/L glycerol, 2.3 g/L KH_2PO_4, 9.4 g/L K_2HPO_4, pH 7.2) to an initial OD_{600nm} of 0.05. Cultivation was carried out in batch-mode at 37 °C, 250 rpm stirring and 250 L/h aeration rate. Cells were grown until OD_{600nm} reached the value of 0.8. The cultures were induced for 20 h with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Cells were then harvested by centrifugation at 4500 rpm for 30 min, washed once with 20 mM phosphate buffer at pH 7.0 and stored at −20 °C.

Protein purification

Cells were suspended in 50 mM Tris–HCl, 100 mM NaCl, 6 mM imidazole, pH 8.0 buffer. Proteins were extracted by sonication (5 cycles of 30 s each, in ice, with 1 min interval) and cell debris were harvested by centrifugation at 15,000 rpm for 30 min at 4 °C. The enzyme was purified by affinity chromatography with HIS-Select® Nickel Affinity Gel. Briefly, the column was equilibrated with 50 mM Tris–HCl, 100 mM NaCl, 6 mM imidazole, pH 8.0 and the crude extract loaded; column was then washed with 50 mM Tris–HCl, 100 mM NaCl, 6 mM imidazole, pH 8.0; finally, the adsorbed enzyme was eluted with 50 mM Tris–HCl, 100 mM NaCl, 250 mM imidazole, pH 8.0.

Pellets, crude extracts and collected fractions were analysed by SDS-PAGE. The fractions showing the presence of a band of the expected size (27 kDa) were pooled, dialysed against 50 mM Tris–HCl buffer pH 8.0 and stored at −20 °C. Typically, starting from 4 g of wet cell paste, it was possible to obtain 70 mg of pure protein.

Benzil-reductase activity test

Activity measurements were performed spectrophotometrically at 340 nm by determining the consumption of NAD(P)H at 25 °C in a half-microcuvette (total volume 1 mL) for 5 min. One unit (U) of activity is defined as the amount of enzyme which catalyses the consumption of 1 μmol of NAD(P)H per minute under reference conditions, namely with 0.25 mM NAD(P)H and 0.47 mM benzil as substrate (added as concentrated DMSO solution; final DMSO concentration in cuvette amounts to 0.1 %), in 50 mM Tris–HCl buffer, pH 8.0.

Temperature and pH activity and stability studies

Temperature and pH optima were studied conducting the benzil-reductase test at temperatures between 15 and 50 °C and at pH values between 4 and 12 (using suitable buffers). To investigate its temperature-dependent stability, the enzyme was incubated at different temperatures (15, 28, 37, 50 and 60 °C) in 50 mM Tris–HCl buffer pH 8.0; residual activity was assessed conducting the benzil reductase test under reference conditions at different time points between 0 and 24 h of incubation. pH-dependent stability was investigated preincubating the enzyme in different buffers at pH between 5 and 11 at 25 °C. Residual activity was measured at pH 8.0, 25 °C, at different time points between 0 and 24 h, and compared to the value before incubation.

Substrate range

KRED1-Pglu substrate range was studied carrying out the reduction assay under reference conditions (0.25 mM NADPH, 50 mM Tris–HCl buffer pH 8.0, 25 °C), using 0.5 mM of each compound (in final 0.1 % DMSO); relative activities were expressed as per cent values versus benzil.

Determination of kinetic parameters

Kinetic parameters for the reduction of benzil were determined using the reduction assay (0.07 g/L of KRED1-Pglu, 0.25 mM NAD(P)H, 50 mM Tris–HCl buffer pH 8.0, 30 °C). Data were fitted to suitable kinetic models with KaleidaGraph software (Synergy Software Inc., USA); kinetic parameters were calculated with the same program.

Preparative reductions

Molar conversion and enantioselectivity towards different aromatic ketones and ketoesters were determined by performing biotransformations at 50-mg scale, using an enzyme-coupled system (glucose-glucose dehydrogenase (GDH) from *Bacillus megaterium*; Bechtold et al. 2012) for cofactor recycling. Reductions were carried out in 10-mL screw-capped test tubes with a reaction volume of 5 mL with KRED1-Pglu (20 mU/mL), GDH (1 U/mL), $NADP^+$ (0.1 mM), substrate (1 g/L), glucose (4× mmol of substrate) suspended in 50 mM Tris/HCl buffer pH 8.0. GDH activity and stability in our reaction system were checked before its use (data not shown).

Analyses ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm, and coupling constants (J) are expressed in Hz.

HPLC analyses were performed with a Jasco Pu-980 equipped with a UV–vis detector Jasco UV-975. The following HPLC columns were used: LichroCART (250×4.6 mm), Chiralcel OD (250×4.6 mm, Daicel), Chiralcel OD-H (250×4.6 mm, Daicel), Chiralcel OJ (250×4.6 mm, Daicel), Lux cellulose 2 (250×4.6 Phenomenex) Lux cellulose 3 (250×4.6 Phenomenex). (*R*) and (*S*) isomers were identified by comparison of their retention times with those of synthetic standards.

Analytical data of product of biotransformations are as follows:

Benzoin (**2a**)

$[\alpha]_D^{25}$=+37.1° (*c* 0.8 Acetone) lit.[1] $[\alpha]_D^{25}$=+44.2° (*c* 1.0 acetone)

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.88–7.93 (m, 2H), 7.48–7.53 (m, 1H), 7.23–7.41 (m, 7H), 5.95 (d, J=6 Hz, 1H), 4.55 (d, J=6 Hz, 1H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 199.1, 139.2, 134.0, 133.7, 129.3, 129.2, 128.8, 128.7, 127.9, 76.4 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD column (n-hexane/*i*-PrOH 90:10, 0.7 mL/

min, 254 nm): t_r (*S*)-benzoin 14.7 min, t_r (*R*)-benzoin 21.6 min).

Diphenylmethanol (**2b**)

^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.19 (d, *J*=4.0 Hz, 1H), 5.86 (d, *J*=4.0 Hz, 1H), 7.25–7.28, (m, 2H), 7.32–7.35 (m, 4H), 7.38–7.40 (m, 4H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 143.8 128.5, 127.6, 126.5, 76.2 ppm.

The molar conversion was determined by HPLC using LichroCART 250–4,6 MeOH/H_2O 65:35, 0.7 mL/min, 220 nm: t_r diphenylmethanol 12.5 min, t_r benzophenone 20.6 min.

Ethyl mandelate (**2d**)

$[\alpha]_D^{25}$=+88.7° (*c* 0.7 EtOH) lit.[2] $[\alpha]_D^{22}$=+97.33° (*c* 1.1 EtOH).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.35–7.42 (m, 5H), 5.15 (s, 1H), 4.15–4.25 (m, 2H), 1.20 (m, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 173.7, 138.5, 128.7, 128.5, 126.6, 73.0, 62.3, 14.2 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD-H column (n-hexane/*i*-PrOH 90:10, 1.0 mL/min, 210 nm): t_r (*S*)-ethyl mandelate 6.0 min, t_r (*R*)-ethyl mandelate 10.0 min).

1-Phenylethanol (**2e**)

$[\alpha]_D^{25}$=+40.0° (*c* 0.5 chloroform) lit.[3] $[\alpha]_D^{25}$=+36.0° (*c* 1.0 chloroform).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.20–7.35 (m, 5H), 4.85 (q, *J*=6.4 Hz, 1H), 1.46 (d, *J*=6.4 Hz, 3H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ 145.9, 128.5, 127.5, 125.4, 70.4, 25.2 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD column (n-hexane/*i*-PrOH 95:5, 0.7 mL/min, 254 nm): t_r (*R*)-1-phenylethanol 14.2 min, t_r (*S*)-1-phenylethanol 18.0 min.

1-Phenylpropanol (**2f**)

$[\alpha]_D^{25}$=+20.5° (*c* 0.2 chloroform) lit.[4] $[\alpha]_D^{20}$=+22.2° (*c* 1.0 chloroform).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.35–7.22 (m, 5H), 4.54 (t, *J*=6.5 Hz, 3H), 2.20 (br s, 1H, OH), 1.83–1.68 (m, 2H), 0.89 (t, *J*=7.5 Hz, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 144.6, 128.4, 127.5, 126.0, 76.0, 31.9, 10.1 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD-H column (n-hexane/*i*-PrOH 98:2, 1.0 mL/min, 254 nm): t_r (*R*)-1-phenylpropanol 16.5 min, t_r (*S*)-1-phenylpropanol 19.5 min.

1-Phenylbutanol (**2g**)

$[\alpha]_D^{25}$=−40.5° (*c* 0.2 chloroform) lit.[7] $[\alpha]_D^{25}$=−43.5° (*c* 1.0 chloroform).

^{1}H-NMR (300 MHz, CDCl3): δ 7.34–7.32 (m, 4H), 7.27–7.24 (m, 1H), 4.66 (t, *J*=6.5 Hz, 1H), 1.93 (br s, 1H, OH), 1.80–1.63 (m, 2H), 1.46–1.27 (m, 2H), 0.92 (t, *J*=7.5 Hz, 3H) ppm.

^{13}C-NMR (75 MHz, CDCl3): δ 145.0, 128.4, 127.5, 125.9, 74.4, 41.2, 19.0, 13.9 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD column (n-hexane/*i*-PrOH 99:1, 0.5 mL/min, 254 nm): t_r (*R*)-1-phenylbutanol 19.4 min, t_r (*S*)-1-phenylpbutanol 23.2 min.

1-Pyridin-3yl-ethanol (**2i**)

$[\alpha]_D^{25}$=−38.04° (*c* 0.4 ethanol) lit.[4] $[\alpha]_D^{20}$=−39.0° (*c* 0.93 ethanol).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.46–8.33 (m, 2H), 7.75–7.70 (m, 1H), 7.24 (dd, J^1=8.0 Hz, J^2=5.0 Hz, 1H), 4.90 (q, *J*=6.5 Hz, 1H), 4.5 (br s, 1H, OH), 1.49 (d, *J*=6.5 Hz, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 148.0, 147.0, 141.8, 133.5, 123.5, 67.5 25.1 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Phenomenex Lux cellulose 2 column (n-hexane/*i*-PrOH 90:10, 0.7 mL/min, 254 nm): t_r (*R*)-(1-pyridin-3yl)ethanol 20.46 min, t_r (*S*)-(1-pyridin-3yl)ethanol 25.30 min.

1-Pyridin-3yl-propanol (**2j**)

$[\alpha]_D^{25}$=−40.0° (*c* 0.2 methanol) lit.[6] $[\alpha]_D^{20}$=−41.4° (*c* 2.1 methanol).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.31 (m, 1H), 8.25 (m, 1 H), 7.63 (m, 1 H), 7.15 (m, 1 H), 4.90 (s, 1 H), 4.51 (t, *J*= 6.6 Hz, 1 H), 1.68 (m, 2 H), 0.82 (t, *J*=7.41 Hz, 3 H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$,): δ 147.9, 147.4, 140.6, 133.9, 123.3, 72.8, 31.9, 9.8 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Phenomenex Lux cellulose 2 column (n-hexane/*i*-PrOH 90:10, 0.7 mL/min, 254 nm): t_r (*R*)-(1-pyridin-3yl)propanol 18.2 min, t_r (*S*)-(1-pyridin-3yl)propanol 21.1 min.

(1-Thiophen-2-yl)ethanol (**2k**)

$[\alpha]_D^{25}$=−20.80° (*c* 0.2 chloroform) lit.[4] $[\alpha]_D^{20}$=−23.2° (*c* 0.79 chloroform).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.25–7.18 (m, 1H), 6.95–6.92 (m, 2H), 5.07 (q, *J*=6.5 Hz, 1H), 2.54 (br s, 1H, OH), 1.56 (d, *J*=6.5 Hz, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 150.0, 126.6, 124.4, 123.2, 66.2, 25.2 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Phenomenex Lux cellulose 3 column (n-hexane/*i*-PrOH 90:10, 0.5 mL/min, 254 nm): t_r (*S*)-(1-thiophen-2-yl)ethanol 13.5 min, t_r (*R*)-(1-thiophen-2-yl)ethanol 15.90 min.

(1-Furan-2-yl)ethanol (**2l**)

$[\alpha]_D^{25}$=−20.5° (*c* 0.2 chloroform) lit.[4] $[\alpha]_D^{20}$=−13.6° (*c* 0.55 chloroform).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.36 (dd, J^1=2.0 HzJ^2= 1.0 Hz, 1H,), 6.32 (dd, J^1=3.0 HzJ^2=2.0 Hz, 1H,), 6.22 (d, *J*=3.0 Hz, 1H,), 4.86 (q, *J*=6.5 Hz, 1H,), 2.35 (br s, 1H, OH), 1.52 (d, *J*=6.5 Hz, 3H,) ppm.

^{13}C NMR (100 MHz, $CDCl_3$): δ 157.7, 141.8, 110.1, 105.1, 63.6, 21.2 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Phenomenex Lux cellulose 3 column (n-hexane/*i*-PrOH 98:2, 0.5 mL/min, 220 nm): t_r (*S*)-(1-furan-2-yl)ethanol 19.3 min, t_r (*R*)-(1-furan-2-yl)ethanol 21.3 min.

3-Hydroxy-3-phenylpropanenitrile (**2m**)

$[\alpha]_D^{25}$=−40.5° (*c* 0.2 ethanol) lit.[8] $[\alpha]_D^{20}$=−57.7° (*c* 2.6 ethanol).

^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.30–7.45(m, 5H), 5.1 (t, *J*=6.0,1H), 2.79 (d, *J*=6 Hz, 2H), 2.36 (br s, 1H, OH) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$) δ 140.1, 128.9, 128.8, 125.5, 117.6, 70.2, 28.1.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OJ column (n-hexane/*i*-PrOH 90:10, 1 mL/min, 216 nm): t_r (*S*)-3-hydroxy-3-phenylpropanenitrile 21.4 min, t_r (*R*)-3-hydroxy-3-phenylpropanenitrile 25.0 min.

2-Hydroxy-1-phenylpropan-1-one (**2v**)

$[\alpha]_D^{25}$=−100° (*c* 0.2 chloroform) lit.[8] $[\alpha]_D^{27}$=−91.1° (*c* 0.3 chloroform).

^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.87 (d, *J*=8.2 Hz, 2H), 7.57 (t, *J*=7.42 Hz, 1H), 7.46 (t, *J*=7.7 Hz, 2H), 5.17 (m, 1H), 3.5 (br s, 1H, OH), 1.45 (d, *J*=7.0 Hz, 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$) δ 202.3, 133.9, 133.4, 128.7, 128.6, 69.3, 22.2.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD column (n-hexane/*i*-PrOH 95:5, 0.4 mL/min, 254 nm): t_r (*R*)-2-hydroxy-1-phenylpropan-1-one 17.2 min, t_r (*S*)-2-hydroxy-1-phenylpropan-1-one 21.9 min.

3-Hydroxy-1-phenyl-butan-1-one (**2w**)

$[\alpha]_D^{25}$=+54.2° (*c* 0.1 chloroform) lit.[10] $[\alpha]_D^{27}$=+67.5° (*c* 1.2 chloroform).

^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.97 (dd, J^1=8.5, J^2= 2.0 Hz, 2H), 7.50 (m, 3H), 4.42 (m, 1H), 3.24 (br s, 1H, OH), 3.10 (d, *J*=6.0 Hz, 2H) 1.30 (d, *J*=6.4 Hz 3H) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$) δ 200.95, 136.8, 133.6, 128.8, 128.1, 64.1, 46.5, 22.5 ppm.

The enantiomeric excess (ee %) was determined by HPLC using Chiralcel OD-H column (n-hexane/*i*-PrOH 95:5, 0.8 mL/min, 254 nm): t_r (*R*)-3-hydroxy-1-phenyl-butan-1-one 12.1 min, t_r (*S*)-3-hydroxy-1-phenyl-butan-1-one 13.6 min.

Accession numbers

GenBank accession number of *KRED1-Pglu* gene is KR080472.

Results

Analysis of the genome of *Pichia glucozyma* CBS 5766 revealed the occurrence of a sequence (called KRED1-Pglu) with high homology with known benzil reductases. The corresponding His-tagged protein was successfully expressed in *E. coli*, yielding an active enzyme accounting for about 40 % of the total protein content of the cell extract.

Activity and enantioselectivity

The activity of KRED1-Pglu was first investigated for reduction of benzil, observing that the enzyme is NADPH-dependent. The enzyme exhibited the highest activity at pH between 7.0 and 8.0, being stable at pH between 6.0 and 8.0 (residual activity of 90 % and 80 %, respectively). KRED1-Pglu showed the highest activity between 30 °C and 35 °C and was quite stable up to a temperature of 30 °C, above which its activity started to decrease. As a compromise between activity and stability, the following studies have been carried out at 30 °C in 50 mM Tris/HCl buffer pH 8.0. Kinetic parameters for monoreduction of benzil by KRED1-Pglu were determined using 0.07 mg/ml of protein, NADPH as cofactor, at 30 °C and pH 8.0 (Table 1).

The initial rates of the biotransformation of different aromatic ketones and ketoesters were determined using a substrate-independent assay (Table 2, column 5); relative activities were expressed as per cent values versus benzil. Molar conversion and enantioselectivity were determined by performing the reduction of the substrates on a 50 mg scale, using an "enzyme-coupled" system (glucose/glucose dehydrogenase, GDH) (Table 2).

Preparative reduction of benzil (**1a**) resulted in total conversion into (*S*)-benzoin (ee >98 %) in 2 h; bulky benzophenone (**1b**) (which is transformed at low rates) was reduced to diphenylmethanol in 24 h with good yields (66 %), showing that a sluggish initial activity does not necessarily imply low conversion, as already observed for the reduction of aromatic ketones using LBADH (Rodríguez et al. 2014). Ethyl 2-oxo-2-phenylacetate (**1d**) was totally reduced within 30 min, albeit with mediocre *S*-enantioselectivity (ee 40 %). No consistent trend could be detected between bulkiness of the substrates and initial rates; for instance, the series **1e-h** showed an erratic tendency of activities with respect to the increasing bulkiness of the substrate, similar to what observed with LBADH (Rodríguez et al. 2014). KRED1-Pglu gave pronounced anti-Prelog stereorecognition in the reduction of acetophenone (**1e**) and propiophenone (**1f**) to yield the corresponding (*R*)-1-aryl-2-ethanol, whereas other aromatic ketones (**1g** and **1i-1m**) provided the corresponding (*S*)-alcohol (Prelog rule) with high enantioselectivity. This remarkable switch of enantioselectivity between similar substrates is not common among ketoreductases able to reduce aromatic ketones (Lavandera et al. 2008; Zhu et al. 2006). Finally, KRED1-Pglu showed no activity towards much hindered substrates like β-γ-ketoesters (**1n-1u**).

Table 1 Kinetic parameters for monoreduction of benzil by KRED1-Pglu

k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
0.14±0.02	0.64±0.10	0.22

Table 2 Investigation of the substrate range of KRED-Pglu concerning the reduction of different aromatic ketones and ketoesters

Entry	Substrate	Ar	R	Activity (%)[a]	Yield (%)	ee (%)	Time (h)[b]
1	**1a**	Phenyl	COPh	100	>95	>98 (*S*)	2
2	**1b**	Phenyl	Phenyl	15	66	–	24
3	**1c**	Phenyl	CH_2Ph	<5	<5	<5	24
4	**1d**	Phenyl	COOEt	115	>95	40 (*S*)	0.5
5	**1e**	Phenyl	CH_3	21	40	95 (*R*)	24
6	**1f**	Phenyl	CH_2CH_3	25	60	97 (*R*)	24
7	**1g**	Phenyl	$CH_2CH_2CH_3$	18	56	97 (*S*)	24
8	**1h**	Phenyl	$CH_2CH_2CH_2CH_3$	<5	<5	<5	24
9	**1i**	3-Pyridyl	CH_3	58	>95	>98 (*S*)	24
10	**1j**	3-Pyridyl	CH_2CH_3	60	>95	>98 (*S*)	24
11	**1k**	2-Thienyl	CH_3	51	>95	>98 (*S*)	24
12	**1l**	2-Furanyl	CH_3	48	>95	>98 (*S*)	24
13	**1m**	Phenyl	CH_2CN	55	>95	95 (*S*)	24
14	**1n**	Phenyl	CH_2COOEt	<5	<5	<5	24
15	**1o**	Phenyl	$CH_2COOtBu$	<5	<5	<5	24
16	**1p**	Phenyl	$CH(CH_3)COOEt$	<5	<5	<5	24
17	**1q**	Phenyl	$CH(CH_3)COOtBu$	<5	<5	<5	24
18	**1r**	Phenyl	CH_2CH_2COOEt	<5	<5	<5	24
19	**1s**	Phenyl	$CH_2CH_2COOtBu$	<5	<5	<5	24
20	**1t**	Phenyl	$CH_2CH(CH_3)COOEt$	<5	<5	<5	24
21	**1u**	Phenyl	$CH_2CH(CH_3)COOtBu$	<5	<5	<5	24

[a] Relative activities measured using the benzil-reductase activity test and expressed as percent values referred to benzil

[b] Referred to the maximum yield

1-Phenylpropane-1,2-dione (**1v**) and 1-phenylbutane-1,3-dione (**1w**) were also tested; both diketones were exclusively reduced at the less hindered carbonyl group with formation of (*S*)-2-hydroxy-1-phenylpropan-1-one (**2v**) and (*S*)-3-hydroxy-1-phenylbutan-1-one (**2w**) as the only reaction products, with complete enantioselectivity (Fig. 1).

The observed activities and enantioselectivity cannot be explained by simply assuming a model of the active site with two hydrophobic pockets differing in size, where the large pocket binds the phenyl moiety, while the small one defines whether the other substituent can be properly accommodated and thus controls reaction rates and enantioselectivity. Since the experimental results were unpredictable on the basis of just the steric properties of the substrates, a molecular modelling study was carried out for understanding the structural determinants for the observed stereorecognition.

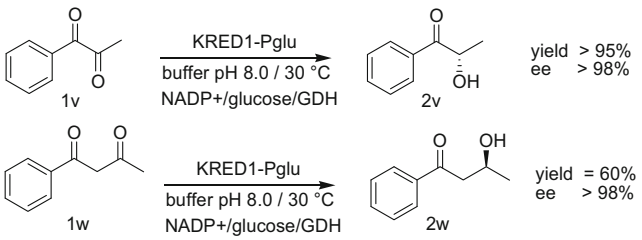

Fig. 1 Reduction of 1-phenylpropane-1,2-dione (**1v**) and 1-phenylbutane-1,3-dione (**1w**) with KRED1-Pglu

As the starting point for homology modelling, the PDB database (http://www.rcsb.org/pdb/) was scanned with BLAST against the target sequence of the KRED1-Pglu while setting the maximal E-threshold=100; the search retrieved several items. The top-scoring identified template, an uncharacterized oxidoreductase YIR035C from *Saccharomyces cerevisiae* (UniProt entry P40579), and the target share 46 % identity; YIR035C has been crystallized in its *apo* form (X-ray structure as PDB entry 3KZV). The two sequences were automatically aligned and manually adjusted, and a model for the target protein was built by comparative modelling. The structure so obtained did not allow, however, for any effective docking of NAD(P)H to it. The second best match, *cis*-2,3-dihydrobiphenyl-2,3-diol dehydrogenase from *Comamonas testosteroni* (UniProt entry Q46381), shares 31 % identity with the target and has been crystallized in a complex with NAD (PDB entry 2Y99). After alignment of the sequences with *T-coffee* and superposition of the cognate structures, NAD was imported from the template to the model and the complex was energy-minimized, which resulted in minor RMSD overall and a negligible displacement of the amino acids predicted to be involved in the catalytic process (annotations to UniProt entry P40579). However, once more,

no effective docking of the test substrates could be obtained to the model•NAD complex.

Various PDB entries of *holo* enzymes, crystallized as to contain both NAD and a substrate/substrate analog/inhibitor (2JAP, 1NAS, 4NBU), were thus inspected from the previous BLAST output; clavulanic acid dehydrogenase from *Streptomyces clavuligerus* (UniProt entry Q9LCV7; PDB entry 2JAP; 30 % identity with the target) was selected as further template because of the comparable size of its substrate to our test substrates. After superposition of the structures, substrate clavulanic acid was imported from 2JAP to our model•NAD. Effective docking was possible to the ternary complex model•NAD•clavulanic acid after energy minimization; we will refer to the outcome of this procedure as KRED1-Pglu model.

Docking to KRED1-Pglu model was carried out using different *protocols* (rigid receptor, induced fit), various *forcefields* (MMF94x, Amber12:EHT) and different *electrostatics setups* (distance-dependent dielectric, reaction field); comparing the position of the redox centre in the top scoring poses for the test substrates in each procedure with that of the redox centre of clavulanic acid taken as reference suggested that the combination: *rigid receptor*, *Amber12:EHT*, and *distance-dependent dielectric* performs best. This protocol was then used to dock to the KRED1-Pglu model all selected substrates.

The procedures of molecular modelling provided insight into the ways of enzyme-substrate interaction and allowed computing the affinities (dissociation constant values, K_ds) for different substrates, with an accuracy within one order of magnitude, as discussed by Eberini et al. (2006). Table 3 lists the in silico affinities of the enzyme for selected substrates alongside the in vitro activities (same as in Table 2). Binding of a substrate to the enzyme active site is necessary, but not sufficient for catalysis to take place: once this prerequisite is met, the rate of the process is controlled by the stereoelectronic property of the substrate, a feature not directly addressed by the classical docking procedure. Within these boundaries, the relative order of the computational data is in agreement with the experimental results and the enzyme is most active on benzil (entry 1), the substrate with the highest affinity for the catalytic site.

When the series of aryl aliphatic ketones is docked into the enzyme active site, the orientation of the top-scoring placements changes depending on the length of the aliphatic chain, which defines the stereochemistry of the reductive reaction. Figure 2 shows the relative placement of acetophenone (substrate **1e**; colour = green) and butyrophenone (substrate **1h**; colour = yellow), and their position with respect to the reductive hydride (arrow) of NAD(P)H (CPK colours). Lengthening the aliphatic chain produces steric clashes with amino acids in the enzyme catalytic site (specifically, W147 and L208) and results in an inversion of the predominant orientation during the interaction. As a result, the stereochemistry predicted for the stereocenter of the alcohol products is different in either case and identical to what is obtained in vitro.

Table 3 In silico affinities of KRED-Pglu for selected substrates

Entry	Substrate	Ar	R	Activity (%)	K_i
1	**1a**	Phenyl	COPh	100	7.94×10^{-6}
2	**1b**	Phenyl	Phenyl	5	1.21×10^{-5}
5	**1e**	Phenyl	CH_3	11	6.15×10^{-5}
6	**1f**	Phenyl	CH_2CH_3	35	3.36×10^{-5}
7	**1g**	Phenyl	$CH_2CH_2CH_3$	10	2.32×10^{-5}
8	**1h**	Phenyl	$CH_2CH_2CH_2CH_3$	<5	1.38×10^{-5}

Discussion

Ketoreductases (KREDs) stereoselectively reduce prochiral ketones giving access to optically pure secondary alcohols (Hall and Bommarius 2011; Hollmann et al. 2011). Bulky aromatic ketones can be reduced by specialised KREDs (Haberland et al. 2002; Stampfer et al. 2004; Lavandera et al. 2008; Huisman et al. 2009). Benzil reductases (Maruyama et al. 2002; Johanson et al. 2005; Pennacchio et al. 2013) are specific KREDs with high affinity towards very hindered 1,2-diphenyl-ethanediones. Sequence comparison among the benzil reductases from bacteria, yeasts and mammals showed that conserved amino acid residues are essential for benzil reductase activity and can be useful for fishing out their genes from genomes. Whole cells of wild-type *Pichia glucozyma* CBS 5766 have been used for a number of reductions of aromatic ketones, including symmetric and asymmetric 1,2-diaryl-ethanediones (benzils); the genome of *P. glucozyma* CBS 5766 was annotated and a putative gene for benzil reductase was identified based on sequence homology with known benzil reductases and cloned in *E. coli*. The His-tagged protein (named as KRED1-Pglu) was purified and used for the reduction of different aromatic ketones; the enzyme showed a marked preference for NADPH and was used on

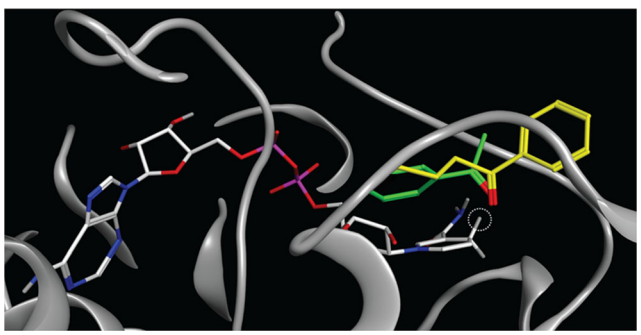

Fig. 2 Close-up to the catalytic site in the model of KRED1-Pglu. NADH is rendered with CPK colors; the hydride atom involved in the reduction process is marked with a dotted circle. Two substrates, acetophenone (**1e**) and butyrophenone (**1 h**) are rendered in green and yellow. Their orientation in the enzyme pocket is opposite, leading to inverted configuration in the alcohol products

preparative scale coupled with a glucose dehydrogenase (GDH) for cofactor recycling. Benzil was reduced giving (*S*)-benzoin as the sole observable product, whereas the use of whole cells yielded lower enantiomeric excesses and further reduction to hydrobenzoin (Fragnelli et al. 2012), indicating the occurrence in *P. glucozyma* of other KR(s) active not only on benzil but also on benzoin. Very hindered benzophenone was also reduced, as previously observed with selected KRs (Truppo et al. 2007). Aromatic ketoesters were poorly reduced by KRED1-Pglu, whereas whole cells of *P. glucozyma* CBS 5766 were very effective and sometimes highly enantioselective on these substrates, indicating the occurrence of other enantioselective KRs in this unconventional yeast (Contente et al. 2014). Acetophenone and propiophenone were reduced to the corresponding (*R*)-alcohols (anti-Prelog's rule), whereas butyrophenone was converted into the corresponding alcohol with different stereobias, giving (*S*)-phenylbutanol with high enantioselectivity. This shift of stereopreference was explained by modelling the interaction between substrate and protein and observing that the length of the aliphatic chain governs the stereochemistry of the biotransformation, determining which prostereogenic face of the ketone can be favourably attacked by the hydride. The key role of the aliphatic chain can also be seen in the case of valerophenone, where the longer chain determines steric clashes with amino acids in the enzyme catalytic site and the ketone is not reduced. KRED1-Pglu was also employed for the reduction of 1-phenylpropane-1,2-dione and 1-phenylbutane-1,3-dione leading to the enantioselective formation of the α-methyl alcohols as the only product, suggesting that KRED1-Pglu has potential as catalyst for the preparation of hydroxyketones with high chemical and optical purity.

In conclusion, a new (bio)catalyst for the preparation of bulky aromatic chiral alcohols and hydroxyketones with high enantioselectivity by reduction of the corresponding ketones has been identified and produced from an unconventional yeast. The different and erratic stereopreferences towards simple aromatic ketones were explained on the basis of modelling studies. Further studies could be useful/help to identify, generate and analyse KRED1-Pglu mutants, in which the stereoselectivity of the reductive reaction can be carefully tuned in order to customize and scale-up specific reactions with industrial relevance.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest All the authors declare they have no conflict of interest.

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