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

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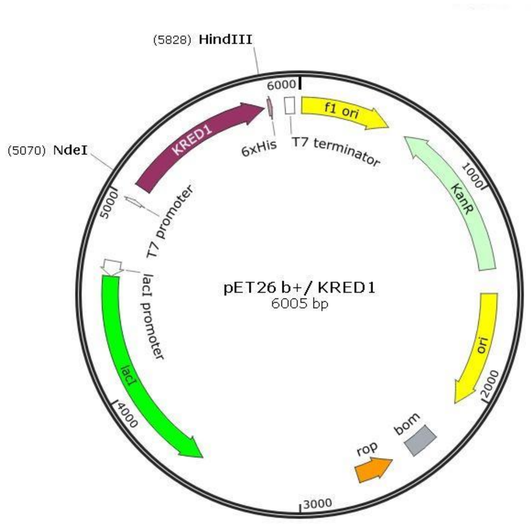
A new NADPH-dependent benzil reductase (KRED1-Pglu) was identified from the genome of the non-conventional yeast *Pichia glucozyma* CBS 5766 and overexpressed in *E. coli*. The new protein was characterised and reaction parameters were optimised for the enantioselective reduction of benzil to (*S*)-benzoin. A thorough study of the substrate range of KRED1-Pglu was conducted; 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.

Protein cloning, expression and purification

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* BL21(DE3)Star, yielding an active enzyme accounting for about 40% of the total protein content of the cell extract (Fig.1).



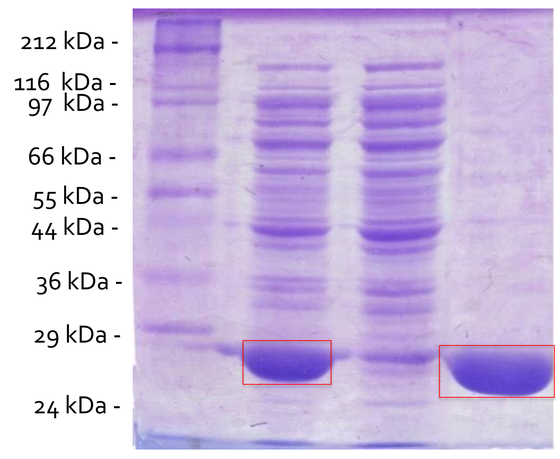
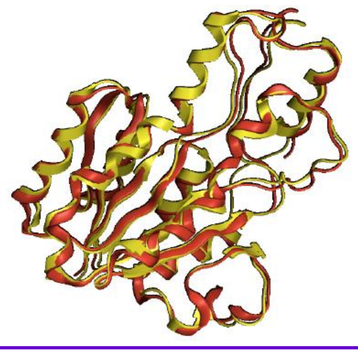


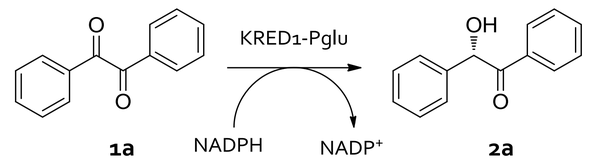
Fig.1



15 mg/g cell of pure proteine were obtained

pH, temperature activity and stability

Temperature and pH optima were studied conducting the benzil-reductase test at temperatures between 15 ° C and 50 ° C and at pH values in 4-12 range (using suitable buffers). Activity measurements were performed spectrophotometrically at 340 nm by determining the consumption of NAD(P)H at 25 ° C in a half-micro cuvette (total volume: 1 mL) for 5 min.



KRED1-Pglu showed

highest <u>activity</u> between 30 ° C and 35 ° C

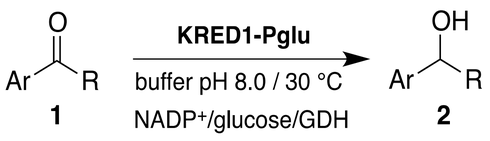
<u>stability</u> up to a temperature of 30 ° C

highest <u>activity</u> at pH between 7.0 and 8.0

<u>stability</u> at pH between 6.0 and 8.0

As a compromise between activity and stability, biotransformations have been carried out at 30 ° C in 50 mM Tris/HCl buffer pH 8.0.

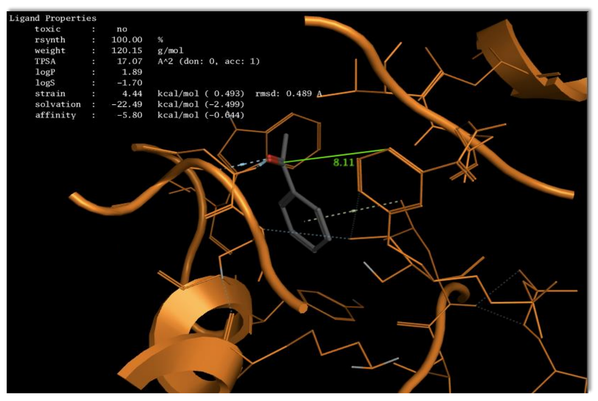
Biotransformations and modelling



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

Molar conversion and enantioselectivity towards different ketones and ketoesters were determined by performing biotransformations at 50 mg-scale , using an enzyme-coupled system (glucose-glucose dehydrogenase- GDH- from *Bacillus megaterium*).

Acetophenone



Butyrophenone

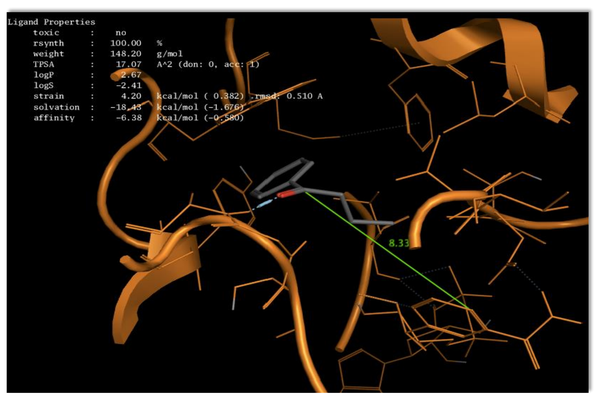
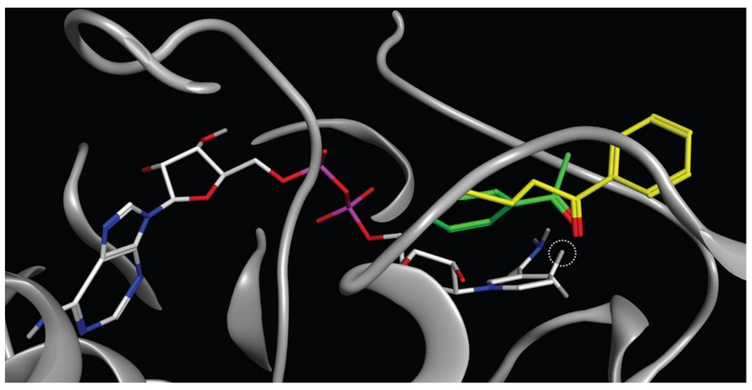


Fig. 2



When the series of aryl ketones is docked into enzyme active site, the orientation of the top-scoring poses changes depending on the length of aliphatic chain which defines the stereochemistry of the reductive reaction. Figure 2 shows the relative placement of acetophenone and butyrophenone, and their position with respect to the reductive hydride. Lengthening the aliphatic chain produces steric with amino acids in the enzyme catalytic site and results in an inversion of prevailing 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.*

A new (bio)catalyst for the preparation of bulky aromatic chiral alcohols with high enantioselectivity by reduction of the corrisponding 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.

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