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Wickerhamomyces subpelliculosus as whole-cell biocatalyst for stereoselective bioreduction of ketones

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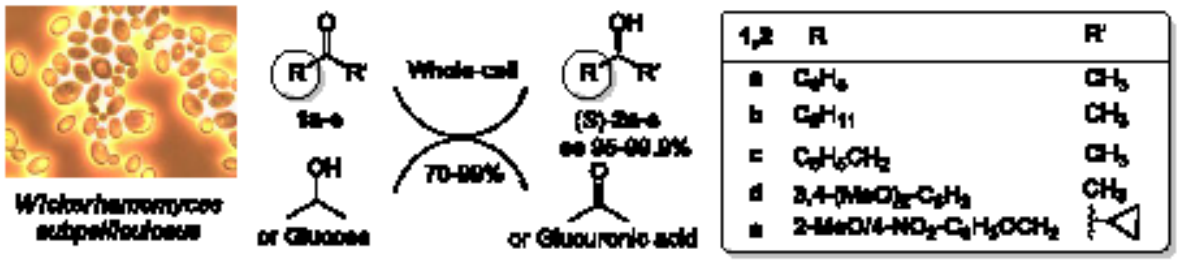
Graphical abstract



Highlights

- *Wickerhamomyces subpelliculosus* was recognized as excellent whole-cell biocatalyst for bioreduction of ketones.
- Glucose and 2-propanol were useful as cosubstrates in the bioreductions.
- Strains of *W. subpelliculosus* showed diverse characteristics, including optimal pH, temperature and organic solvent tolerance.

Abstract

Newly isolated strains of *Wickerhamomyces subpelliculosus* were recognized as excellent whole-cell biocatalyst for bioreduction of various ketones. The biocatalytic properties of the new strains were demonstrated in this study by stereoselective bioreduction of acetophenone **1a**, 2-heptanone **1b**, phenylacetone **1c**, 3,4-dimethoxyphenylacetone **1d** and 1-cyclopropyl-2-(2-methoxy-4-nitrophenoxy)ethanone **1e**. Our study is the first report on application of *W. subpelliculosus* as whole-cell biocatalyst for stereoselective bioreduction of prochiral ketones. In these processes, both the freshly harvested cell paste and the lyophilized cell powder were tested as biocatalyst using glucose or 2-propanol at various concentrations as cosubstrates for cofactor regeneration. The newly isolated strains of *W. subpelliculosus* showed diverse characteristics, including optimal pH, temperature and organic solvent tolerance. Bioreductions of phenylacetone **1c** applying glucose as cosubstrate under various mild conditions resulted (*S*)-

1-phenylpropanol [(*S*)-**2c**] in good to excellent conversion (c = 63.4% – 99.9%) with excellent enantiomeric excess [$ee_{(S)\text{-}\mathbf{2c}}$= 98.7% – 99.8%].

Keywords: stereoselective bioreduction, *Wickerhamomyces subpelliculosus*, whole-cell biotransformation, ketone, ketoreductase, yeast

1. Introduction

Nature is rife with ketoreductases. Researchers successfully isolated and characterized members of this enzyme group from microbes as well as human samples [1]. In the past two decades the development of ketoreductases has been sharply increasing, mainly due to the fact that yeast-mediated reduction of prochiral ketones results in high stereoselectivity and still acceptable yield[2,3]. The number of syntheses carried out with stereoselective reduction of prochiral ketones by ketoreductases increased significantly. A number of chiral alcohols produced by yeasts from prochiral ketones are already well known as chiral building blocks in the synthesis of various pharmaceuticals[4-,5,6,7,8].

The use of isolated enzymes in industrial synthesis to produce chiral synthons and enantiomerically pure products is, however, often hindered by the low stability of enzymes under process conditions[9]. A convenient way to stabilize enzymes is the use of the whole microbial cell as a biocatalyst[10,11]. Production of recombinant cells containing ketoreductases with a proper cofactor regeneration system takes months for a team of skilled scientists [12]. Applications with native hosts allows deferral of recombinant biocatalyst production [13] or may compete with recombinant whole-cell biocatalysts.

Ketoreductase activity of various yeast strains aiming industrial applications was studied earlier (Scheme 1) [5,15]. Ketones **1a**, **1c** and **1d** were successfully reduced to the

corresponding secondary chiral alcohols (*S*)-**2a,c,d** using lyophilized whole cells of *Zygosaccharomyces rouxii* and *Debaryomyces hansenii* [5]. Reductions of **2c** with yeast-cell-based biocatalysts of *Debaryomyces carsonii, Candida guilliermondii, Pichia kijperi* and *Saccharomyces bayanus* resulted in modest yields of (*S*)-**2c**. The produced alcohols (*S*)-**2a-c** were useful in the syntheses of various enantiomerically pure chemicals [16-,17,18], (*S*)-**2d** was a key intermediate in the synthesis of benzodiazepines [19]. Furthermore, **1e** and both enantiomers of **2e** were important building blocks of antagonists for melanin concentrating hormone receptor-1 (MCHR-1)[20,21].

The yeast *Wickerhamomyces subpelliculosus* was first described and classified by Bedford[22] as *Hansenula subpelliculosus*, which was revised and reclassified by Kurtzman [23]. As a result of morphological, physiological and genetic tests, the genus *Hansenula* including *Hansenula subpelliculosus* was transferred to the genus *Pichia* [24]. Recently, genera *Issatchenkia*, *Starmera*, *Williopsis* and *Pichia* were reclassified and species *Pichia subpelliculosus* proposed to belong genus *Wickerhamomyces* based on the phylogenetic analysis of the genes coding the large-subunit (LSU) and the small-subunit (SSU) rRNA and the elongation factor 1α (EF-1α)[25].

Herein we report the taxonomic identification of several novel yeast isolates as *Wickerhamomyces subpelliculosus* and their application as whole-cell biocatalysts in highly stereoselective bioreduction of three simple prochiral ketones **1a-c** and two further ketones **1d,e** leading to secondary alcohols of pharmacological importance.

2. Materials and methods

2.1. Reagents, solvents

All chemicals and starting materials were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fluka (Milwaukee, WI, USA) and Alfa Aesar Europe (Karlsruhe, Germany) and were purified when it was necessary. Prior to use, solvents from Merck KGDA (Darmstadt, Germany) were dried and/or freshly distilled.

2.2. Analytical methods

The NMR spectra of compounds **1a-d** and **2a-d** were recorded in $CDCl_3$ on a Bruker DRX-300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. The NMR spectra of compounds **1e** and **2e** were recorded in $CDCl_3$ on Varian spectrometer operating at 400 MHz, 20 °C for ^{1}H and 100.5 MHz for ^{13}C nuclei. NMR signals are given in ppm on the δ scale. Infrared spectra were recorded on a Bruker ALPHA FT-IR spectrometer and wavenumbers of bands are listed in cm^{-1}. Optical rotation was measured on PerkineElmer 241 polarimeter at the D-line of sodium. The polarimeter was calibrated with measurements of both enantiomers of menthol.

Bioreductions of **1a-d** were analyzed by GC on Agilent 4890 or Agilent 5890 equipment with FID detector and Hydrodex β-6TBDM column (25 m × 0.25 mm × 0.25 µm film with heptakis-(2,3-di-O-methyl-6-O-t-butyldimethylsilyl)-β-cyclodextrine; Macherey &Nagel) or Hydrodex β-TBDAc column (25 m × 0.25 mm × 0.25 µm film with heptakis-(2,3-di-O-acetyl-6-O-t-butyldimethylsilyl)-β-cyclodextrine; Macherey&Nagel), using H_2 carrier gas (injector: 250°C, FID detector: 250 °C, head pressure: 12 psi, 50:1 split ratio). GC data [(oven program); t_r (min)]: for **1a** and **2a** [(8 min at 120 °C); **1a**: 2.7; (*R*)-**2a**: 4.8; (*S*)-**2a**: 5.0], for **1b** and **2b** [(50 min at 40°C); **1b**: 15.7; (*S*)-**2b**: 39.5; (*R*)-**2b**: 39.7] for **1c** and **2c** [(5 min at 80 °C, 80-120°C, 2 °C min^{-1}); **1c**: 15.7; (*S*)-**2c**: 20.2; (*R*)-**2c**: 20.7] on Agilent GC 4890. For **1d** and **2d** [(20 min at 60 °C,

60-180 °C, 4 °C min^{-1}); **1d**: 46.9; (*S*)-**2d**: 47.1; (*R*)-**2d**: 47.4] on Agilent GC 5890. Conversion and the enantiomeric excess was determined by GC.

Enantiomeric excess and conversion of **2e** was analyzed by HPLC on a Agilent 1100 equipment using Lux Amylose-1 [4.6 mm × 150 mm × 5 µm,]; eluents: A (H_2O), B (acetonitrile/2-propanol 80/20); flow rate: 0.5 mL min^{-1}; method: A= 50% (0-10 min), A= 50–65 % (10-13 min); A: 50 %, 13-16 min; column temperature: 20 °C, λ = 240 nm. Retention times, t_r (min): (*R*)-**2e**: 10.73; **1e**: 11.41; (*S*)-**2e**: 12.15. A comparative measurement to assign the elution order of the enantiomers of **2e** was carried out on CHIRALPAK® IC, [4.6 mm x 250 mm x 5 um] column; eluents: A (0.05% TFA in water/methanol 80/20), B (0.05% TFA in acetonitrile/methanol 80/20); flow rate: 0.5 mL min^{-1}; method: B= 30% (start), B= 55% (0-25 min), B= 100% (25-30 min), B= 100% (30-40 min), at room temperature, UV detection 240 and 340 nm. Retention times, t_r (min): (*R*)-**2e**: 24.9; (*S*)-**2e**: 26.7; **1e**: 32.7 [20].

2.3. Biocatalysts

Wickerhamomyces subpelliculosus CBS 5767 was purchased from Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. *Wickerhamomyces subpelliculosus* WY3 and *Wickerhamomyces subpelliculosus* WY13 were isolated, identified and deposited in Witaria Yeast collection (Witaria LLC, Budapest, Hungary).

2.3.1. Production of biocatalysts

Strains are stored in lyophilized ampules in Witaria's strain culture collection. Yeasts are maintained and cultivated on Yeast Malt Agar (HiMedia M424) and Yeast Malt Broth (HiMedia M425). Cultivation was carried out as described earlier [5] in 10 liter fermenter.

2.3.2. Lyophilization of the yeast cells

Following the cultivation, the cells were sedimented by centrifugation at 4000 g (Sigma 6K, rotor 12500H, 6000 rpm) at 8 °C for 15 min. The freeze-dried cell paste (as 150 g aliquots) supplemented with skim milk (10 w/w%, Scharlau 06-019) was placed onto sterilized and pre-cooled (-35 °C) shelves of a freeze-dryer (Christ Epsilon 2-6D LSCplus Pilot Freeze). Freeze-drying was carried out applying a constant vacuum of 0.850 mbar and the temperature was gradually increased until the temperature reached 15 °C (1.66 °C h^{-1}, 33 h). The final drying was performed at 25 °C and 100 mbar for 1 h. Batches of the lyophilized powder (moisture content between 5 and 8 m/m%) were stored in hermetically closed plastic bags at RT.

2.4. DNA isolation, PCR amplification and phylogenetic analysis

In order to extract the genomic DNA, the strains were suspended in 25 µL of 0.5 M NaOH solutions. Following an incubation for 15 min at room temperature, 25 µL of 1M TRIS-buffer (pH 8) and 300 µL of diethyl pyrocarbonate (DEPC)-treated water was added to the suspension and the slurry was centrifuged at 10,000 g for 3 min. The 18S-25-28S ribosomal DNA internal transcribed spacer (ITS) region was amplified by PCR using ITS1F (5'- TCC GTA GGT GAA CCT GCG G-3') and ITS4R (5'-TCC TCC GCT TAT TGA TAT -3') primers [25] in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the following temperature profile: initial denaturation at 98 °C for 5 min, followed by 32 amplification cycles of 30 s at 94 °C, 45 s at 55 °C and 1 min at 70 °C, followed by a final extension at 70 °C for 10 min. The PCR reaction mixture contained 1U of LC *Taq* DNA Polymerase (Fermentas, Vilnius, Lithuania), 200 mM of each deoxynucleoside triphosphate, 1× *Taq* buffer with $(NH_4)_2SO_4$ (Fermentas, Vilnius, Lithuania), 2 mM of $MgCl_2$, 0.65 mM of each primer, 1 µL of DNA template in a final volume of 50 µL. Amplification was performed by GeneAmp PCR System (Model 2400, Applied Biosystems, Foster City, USA). PCR products were visualized by UV light in 1% agarose gel stained with ethidium-bromide. To determine the nucleotide sequences of the PCR products purified by EZ-10 Spin Column PCR Products Purification Kit (Bio Basic,

Amherst, USA), Sanger sequencing reaction was applied. Sequences were aligned by Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nih.gov/BLAST) using the NCBI GenBank nucleotide database.

2.5. Synthetic procedures

*2.5.1. Synthesis of 1-cyclopropyl-2-(2-methoxy-4-nitrophenoxy)ethanone **1e***

1-Cyclopropyl-2-(2-methoxy-4-nitrophenoxy)ethanone **1e** was prepared according to Zhao et al. [20] with slight modifications. Briefly, 1-cyclopropylethanone was brominated and the resulted 2-bromo-l-cyclopropylethanone was coupled with potassium 2-methoxy-nitrophenolate in DMF.

1e: yellow powder; mp: 117-118 °C {lit. mp: 112-113 °C [20]}; IR (KBr): 3096, 2958, 1717, 1590, 1516, 1498, 1334, 1285, 1088, 1013, 863 and 747 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 1.0 (m, 2H, CH_2), 1.2 (m, 2H, CH_2), 2.2 (m, 1H, CH), 4.0 (s, 3H, OCH_3), 4.9 (s, 2H, OCH_2), 6.8 (d, *J* = 8.9 Hz, 1H, Ar-H), 7.8 (d, *J* = 2.6 Hz, 1H, Ar-H), 7.9 (dd, *J* = 8.9 Hz, *J* = 2.6 Hz, 1H, Ar-H); ^{13}C NMR (100.5 MHz, $CDCl_3$) δ ppm: 12.2 (CH_2), 17.2 (CH), 56.4 (OCH_3), 73.6 (OCH_2), 107.1 (Ph), 111.8 (Ph), 117.5 (Ph), 142.3 (Ph), 149.2 (Ph), 152.8 (Ph), 205.4 (OC). ^{1}H NMR and ^{13}C NMR spectra were in accordance with literature data [20].

*2.5.2. Synthesis of racemic 1-cyclopropyl-2-(2-methoxy-4-nitrophenoxy)ethanol **2e***

Reduction of 1-cyclopropyl-2(2-methoxy-4-nitrophenoxy)ethanone **1e** (3.50 g 13.9 mmol), according to the published method [20], provided racemic alcohol (**2e**, 3.22g).

rac-**2e**: yield: 92.8%; yellow powder; mp: 78-79 °C; IR (KBr): 3566, 3444, 3010, 1589, 1525, 1336, 1280, 1093, 1019, 865 and 747 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.3 – 0.4 (m, 1H, CH_2), 0.4 – 0.5 (m, 2H, CH_2), 0.5 – 0.7 (m, 2H, CH_2), 0.9 – 1.7 (m, 1H, CH), 2.4 – 2.5 (br.s, 1H, OH), 3.4 (ddd, *J* = 8.1 Hz, *J* = 8.1 Hz, *J* = 2.9 Hz, 1H, CH), 3.9 (s, 3H, OCH_3), 4.1 (dd, *J* = 9.6 Hz, *J* = 7.9 Hz, 1H, OCH_2), 4.3 (dd, *J* = 9.6 Hz, *J* = 2.9 Hz, 1H, OCH_2), 6.9 (d, *J* =

8.9 Hz, 1H, Ar-H), 7.7 (d, *J* = 2.6 Hz, 1H, Ar-H), 7.9 (dd, *J* = 8.9 Hz, *J* = 2.6 Hz, 1H, Ar-H); ^{13}C NMR (100.5 MHz, $CDCl_3$) δ ppm: 2.1 (CH_2), 2.8 (CH_2), 13.5 (CH), 56.2 (OCH_3), 73.6 (OCH_2), 74.4 (OCH_2), 106.7 (Ph), 111.6 (Ph), 117.7 (Ph), 141.8 (Ph), 149.2 (Ph), 153.8 (Ph). ^{1}H NMR and ^{13}C NMR spectra were in accordance with literature data [20].

2.6. Bioreduction of ketones **1a-d**

2.6.1. Comparison of bioreductions of **1c** *with freshly harvested and lyophilized yeast cells*

Freshly harvested and sedimented cell paste (100 mg) or lyophilized cells (300 mg) as biocatalyst was added to a mixture of ketone **1c** (20 mg), glucose (6 w/v%) in phosphate buffer (5 mL, 100 mM, pH 7.5) in 20 ml Erlenmeyer-flasks. The mixtures were shaken on an orbital shaker (150 rpm) at 30 °C for 24 h. Then 0.5 mL from the mixture was extracted with ethyl acetate (1 mL) dried over Na_2SO_4 and analyzed by GC.

2.6.2. Bioreduction of ketones **1a-d** *with lyophilized cells in the presence of various cosubstrates*

Lyophilized cell powder (300 mg) was suspended in phosphate buffer (5 mL, 100 mM, pH 7.5) containing one of the ketones **1a**, **1b**, **1c** or **1d** (20 mg) and glucose (1, 2, 4, 8 or 16 w/v%) or 2-propanol (1, 2, 4, 8 or16 v/v%) or without cosubstrate in 20 mL Erlenmeyer-flasks. The mixture was shaken on an orbital shaker (150 rpm) at 30 °C for 24 h. Then 0.5 ml from the mixture was extracted with ethyl acetate (1 mL) dried over Na_2SO_4 and analyzed by GC.

2.6.3. Bioreduction of ketones **1a-d** *on preparative scale*

Lyophilized cell powder (1500 mg) was suspended in phosphate buffer (25 mL, 100 mM, pH 7.5) containing one of the ketones **1a**, **1b**, **1c** or **1d** (100 mg) and glucose (4 w/v%) in 20 mL Erlenmeyer-flasks. The mixture was shaken on an orbital shaker (150 rpm) at 30 °C for 24 h. Then the mixture was extracted with ethyl acetate (50 mL) dried over Na_2SO_4 and

concentrated under reduced pressure. The residue was purified by plate chromatography (silica gel, eluent: hexane – ethyl acetate 10:4) to yield the corresponding secondary alcohol (*S*)-**2a-d**.

*(S)-**2a***: yield: 20.3%; ee: 99.1% (by GC); colorless oil; Rf: 0.65; $[\alpha]_D^{25}$= -64.2 (c 1.0, $CHCl_3$); {lit.: $[\alpha]_D^{22}$= -62.8 (c 1.0, $CHCl_3$), for ee= 98.5%[26], $[\alpha]_D^{22}$= -57.7 (c 1.0, $CHCl_3$), for ee= 89%[27]}; IR (film): 3331, 2972, 1449, 1075, 1009, 897, 759 and 697 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 1.5 (d, *J*= 6.4 Hz, 3H, CH_3), 2.0 (s, H, OH), 4.9 (m, H, CH) 7.3-7.4 (m, 5H, Ar-H); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ ppm: 25.4 (CH_3), 70.6 (CH), 125.6 (Ph), 127.6 (Ph), 128.7 (Ph), 146.6 (Ph). ^{1}H and ^{13}C NMR data agreed with the reported spectra [27,28].

*(S)-**2b***: yield: 76.8%; ee: 99.9% (by GC); colorless liquid; Rf: 0.71; $[\alpha]_D^{25}$: +11.6 (c 1.0, $CHCl_3$); {lit.: $[\alpha]_D^{25}$= +12.1 (c 0.66, ethanol)[29], $[\alpha]_D^{25}$= +10.4 (c 0.52, $CHCl_3$)[30]}; IR (film): 3332, 2959, 2927, 2859, 1459, 1375, 1112 and 950 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 0.9 (t, *J*= 6.4 Hz, CH_3); 1.2 (d, *J*= 6.4 Hz, CH_3); 1.3-1.5 (m, 6H, C_3H_6); 1.6 (s, 1H, OH); 3.8 (m, 1H, CH); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ ppm: 14.2 (CH_3), 22.8 (CH_2), 23.6 (CH_3), 25.6 (CH_2), 32.0 (CH_2), 39.5 (CH_2), 69.4 (CH). IR and ^{1}H-NMR data agreed with the reported spectra[31].

*(S)-**2c***: yield: 58.4%; ee: 99.9% (by GC); colorless oil; Rf: 0.53; $[\alpha]_D^{25}$= +38.2 (c 1.0, $CHCl_3$); {lit.: $[\alpha]_D^{22}$= +4.9 (c 1.0, $CHCl_3$), for ee= 56.4% [26] $[\alpha]_D^{25}$= +48.1 (c 1.0, benzene)[32]}; IR (film): 3356, 2986, 1453, 1116, 1078, 936, 740, 697 and 504 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 1.3 (d, *J*= 6.1 Hz, 3H CH_3), 1.8 (s, 1H, OH), 2.7-2.8 (m, 2H, CH_2), 4.1 (m, 1H, CH), 7.3-7.4 (m, 5H, Ar-H); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ ppm: 22.8 (CH3), 45.8 (CH), 98.9 (CH), 126.1 (Ph), 128.59 (Ph), 129.5 (Ph), 130.6 (Ph). ^{1}H and ^{13}C NMR data agreed with the reported spectra [26].

*(S)-**2d***: yield: 63.9% ee: >99.9% (by GC); colorless oil; Rf: 0.20; $[\alpha]_D^{25}$: +25.4 (c 1.0, $CHCl_3$); {lit.: $[\alpha]_D^{25}$= +26.4 (c 1.85, $CHCl_3$) [19]}; IR (film): 3319, 2962, 2837, 1519, 1540,

1263, 1238, 1159, 1028 and 806 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 1.3 (d, *J*= 5.7 Hz, 3H, CH_3), 1.6 (s, 1H, OH), 2.6-2.8 (m, 2H, CH_2), 3.6 (s, 3H, O-CH_3), 3.9 (s, 3H, O-CH_3), 4.0 (m, 1H, CH), 6.7-6.8 (m, 3H, Ar-H); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ ppm: 22.9 (CH_3), 45.5 (CH_2), 56.1 (CH_3), 56.2 (CH_3), 69.1 (CH), 111.6 (Ph), 112.7 (Ph), 121.5 (Ph), 131.2 (Ph), 147.9 (Ph), 149.2 (Ph). IR, ^{1}H and ^{13}C NMR data agreed with the reported spectra [19].

2.6.4. Temperature effects on bioreductions with Wickerhamomyces subpelliculosus strains

Lyophilized cell powder (300 mg) was suspended in phosphate buffer (5 mL, 100 mM, pH 7.5) containing one of the ketones **1a**, **1b**, **1c** or **1d** (20 mg) and glucose (4 w/v%) in 25 mL Erlenmeyer-flasks. The mixture was shaken on an orbital shaker (150 rpm) at a given temperature (30 °C; 35 °C; 40 °C; 45 °C; 50 °C; 55 °C or 60 °C) for 24 h. Then a 0.5 mL portion of the mixture was extracted with ethyl acetate (1 mL), and the extract was dried over Na_2SO_4 and analyzed by GC.

2.6.5. pH effects on bioreductions with Wickerhamomyces subpelliculosus strains

The effect of pH on ketoreductase activity was determined in various pH ranges using appropriate buffers (pH 3.0 – 5.0: 100 mM sodium citrate buffer, pH 6.0 – 8.0: 100 mM sodium phosphate buffer, pH 9.0 – 11.0: 100 mM sodium carbonate – sodium bicarbonate buffer). Lyophilized cell powder (300 mg) was suspended in the buffer (5 mL) containing the ketone (20 mg of **1a**, **1b**, **1c** or **1d**) and glucose (4 w/v%) in 25 mL Erlenmeyer-flasks. The mixture was shaken on an orbital shaker (150 rpm) at 30 °C for 24 h. Then a 0.5 mL portion of the mixture was extracted with ethyl acetate (1 mL), and the extract was dried over Na_2SO_4 and analyzed by GC.

2.6.6. Effect of organic solvents on ketoreductase activity of Wickerhamomyces subpelliculosus strains

Lyophilized cell powder (300 mg) was suspended in phosphate buffer (5 mL, 100 mM, pH 7.5) containing ketone **1c** (20 mg), glucose (4 w/v%) and various organic solvents (2.5 or 5 v/v%) in 25 mL Erlenmeyer-flasks. The mixture was shaken on an orbital shaker (150 rpm) at 30 °C for 6 h. Then a 0.5 mL portion of the mixture was extracted with ethyl acetate (1 mL), and the extract was dried over Na_2SO_4 and analyzed by GC.

2.6.7. Bioreduction of 1-cyclopropyl-2-(2-methoxy-4-nitrophenoxy)ethanone **1e**

Lyophilized cell powder (300 mg) was suspended in phosphate buffer (5 mL, 100 mM, pH 7.5) containing of ketone **1e** (10 mg), glucose (4 w/v%) and DMSO (2.5 or 5 v/v%) in 20 mL Erlenmeyer-flasks. The mixture was shaken on an orbital shaker (150 rpm) at 30°C for 24 h. Then a 0.5 mL portion of the mixture was extracted with ethyl acetate (1 mL), and the extract was dried over Na_2SO_4 and analyzed by HPLC. The residue was purified by plate chromatography (silica gel, eluent: hexane – ethyl acetate 1:1) to yield the corresponding secondary alcohol (*S*)-**2e**.

(*S*)-**2e**: yield: 51.5%; ee: 94.2 % (by HPLC); yellow powder; Rf: 0.25; $[\alpha]_D^{25}$: +13.6 (c 1.0, $CHCl_3$); The IR, ^1H and ^{13}C-NMR spectra of (*S*)-**2e** were indistinguishable from that of *rac*-**2e**.

3. Results and discussion

3.1. Strain selection and identification of Wickerhamomyces subpelliculosus strains as potential biocatalysts for bioreductions

In preliminary experiments, more than 300 different bacterium and yeast strains of Witaria Strain Collection (WY) were isolated and screened for ketoreductase activity. After preliminary activity tests 10 candidates were selected for more detailed investigations and for selectivity screenings. Two yeast strains with high ketoreductase activity were taxonomically identified as *Wickerhamomyces subpelliculosus* (Table 1). To our best knowledge, there were only three

examples published on bioreductions of various ketones with strain *Hansenula subpelliculosa* MY 1552 [33,34] and *Pichia subpelliculosa* CBS 5767 [35] (which are considered as synonyms of *Wickerhamomyces subpelliculosus*) but *Wickerhamomyces subpelliculosus* has never been mentioned as biocatalyst in any stereoselective bioreduction.

3.2. Bioreduction of prochiral ketones **1a-e**

This study describes the application of *W. subpelliculosus* strains (WY3, WY13) in stereoselective bioreduction of ketones **1a-e** (Scheme 1). Five structurally different ketones, acetophenone **1a** (aryl methyl), 2-heptanone **1b** (alkyl methyl), phenylacetone **1c**, 3,4-dimethoxy-phenylacetone **1d** (aralkyl methyl) and 1-cyclopropyl-2-(2-methoxy-4-nitrophenoxy)-ethanon **1e** (aryl cyclopropyl) were chosen to characterize the ketoreductase activity. The *W. subpelliculosus* strains were used first for screening the bioreduction on analytical scale and later on preparative scale as well.

Ample information is available about the bioreduction of these ketones, hence we can easily compare *W. subpelliculosus* with other strains published earlier [16-18]

First, the newly isolated strains *W. subpelliculosus* WY3 and WY13 were compared with the *W. subpelliculosus* CBS 5767 in the bioreduction of phenylacetone **1c** (Table 2). In the preliminary tests freshly harvested and lyophilized cells were compared under the reactions conditions which were applied for bioreductions of benzofuranyl ketones by freshly harvested and lyophilized *Saccharomyces cerevisiae* cells [36]. Bioreduction of phenylacetone **1c** either by the freshly harvested or the lyophilized cells of the control yeast CBS 5767 resulted in low yield (conversion under 10%). Contrarily, the bioreduction of phenylacetone **1c** either by the fresh or the lyophilized WY3 and WY13 cells resulted in (*S*)-**2c** in good conversion and in excellent selectivity (*ee* >99%). Based on this result *W. subpelliculosus* WY3 and WY13 strains

were further investigated. In the subsequent experiments we used only lyophilized cells, since they are easy to store, use and transport [5].

*3.3. Effect of cosubstrates on bioreductions of ketones **1a-d***

Next, the efficacy of *W. subpelliculosus* WY3 and WY13 were examined in the bioreductions of **1a-d** in the presence of glucose and 2-propanol as proper cosubstrates for cofactor regeneration in yeast-mediated bioreductions [5]. It was found that the nature of cosubstrates significantly influenced the conversion. Reductions of the ketones **1a-d** in the presence of glucose (Table 3) gave significantly higher conversions than their counterparts supplemented with 2-propanol (Table 4).

The moderate conversion observed in bioreductions of ketones **1a-d** in the presence of 1 and 2 w/v% glucose almost doubled in most cases in the presence of 4 w/v% glucose. The 8 w/v% glucose concentration proved to be the optimal cosubstrate amount for the reduction of **1c** resulting in (*S*)-**2c** in excellent conversions and enantiomeric excess ($c_{(S)\text{-}\mathbf{2c}}$ = 99.8% and $ee_{(S)\text{-}\mathbf{2c}}$= 99.6% with strain WY3; and $c_{(S)\text{-}\mathbf{2c}}$ = 99.9%, $ee_{(S)\text{-}\mathbf{2c}}$= 99.8% with strain WY13). Worth mentioning that the reductions of **1b** and **1d** by the newly isolated strains WY3 and WY13 yielded the products in extremely high enantiomeric excess ($ee_{(S)\text{-}\mathbf{2b,d}}$ >99.9%) but were only moderately productive. The lowest conversion and enantiomeric excess were observed in reductions of **1a** ($c_{(S)\text{-}\mathbf{2a}}$ = 8.2–40.6 %, $ee_{(S)\text{-}\mathbf{2a}}$ = 92.4–97.8 %, respectively).

In the next series of experiments 2-propanol was tested as cosubstrate at different concentrations in the yeast-mediated reductions of **1a-d**. Unfortunately, only low cosolvent tolerance of the strains *W. subpelliculosus* WY3 and WY13 was observed as moderate to negligible ketoreductase activities were found at 2-propanol concentrations over 4 v/v% (Table 4). The high content of this cosolvent might be harmful for these strains. The optimal 2-propanol concentrations were in WY3 mediated bioreductions 2 v/v% for **1a** and **1d** and 4 v/v%

for **1b** and **1c**. In case of WY13 the highest productivity and enantiomer selectivity were observed in reductions of **1a,b**,**d** with 2 v/v% 2-propanol and **1c** was used with 4 v/v% 2-propanol. The highest productivity was observed in the bioreduction of **1a** by the strain *W. subpelliculosus* WY13 in presence of 2 v/v% 2-propanol ($c_{(S)\text{-}\mathbf{2a}}$ = 72.4% and $ee_{(S)\text{-}\mathbf{2a}}$= 94.9%). The fact, that optima of the bioreductions of **1a-d** were found at 2 or 4% amounts of 2-propanol (Table 4) indicated that lower efficiency of 2-propanol as cosubstrate compared to glucose (Table 3) is only partially due to the deleterious effect of 2-propanol as organic solvent (see Section 3.6).

The results of bioreductions of ketone **1c** with the newly isolated strains *W. subpelliculosus* WY3 ($c_{\mathbf{2c}}$= 98.9%, $ee_{(S)\text{-}\mathbf{2c}}$= 98.7%) and WY13 ($c_{\mathbf{2c}}$= 99.3%, $ee_{(S)\text{-}\mathbf{2c}}$= 99.8%) at 2 w/v% glucose concentration (Table 3) surpassed the outcome of bioreductions of **1c** with lyophilized cells of *Zygosaccharomyces rouxii*; ($c_{\mathbf{2c}}$= 99%, $ee_{(S)\text{-}\mathbf{2c}}$= 82%) or *Debaryomyces hansenii* ($c_{\mathbf{2c}}$= 78%, $ee_{(S)\text{-}\mathbf{2c}}$= 77%)[5]. The enantiotope selectivity of *W. subpelliculosus* WY3 and WY13 was excellent in bioreductions of ketone **1d** at 2 w/v% glucose concentration ($c_{\mathbf{2d}}$= 21.4%, $ee_{(S)\text{-}\mathbf{2d}}$ >99.9% with WY3 and $c_{\mathbf{2d}}$= 48.5%, $ee_{(S)\text{-}\mathbf{2d}}$ >99.9% with WY13) and higher than with *Z. rouxii* ($c_{\mathbf{2d}}$= 17%, $ee_{(S)\text{-}\mathbf{2d}}$= 80%) or *D. hansenii* ($c_{\mathbf{2d}}$= 58%, $ee_{(S)\text{-}\mathbf{2d}}$= 99%)[5].

*3.4. Effect of temperature on bioreductions of ketones **1a-d***

Ketoreductase activity of the two isolates (WY3 and WY13) was investigated in bioreductions of four ketones **1a-d** at various temperatures (Figure 1). Expectedly, both isolates showed modest thermal tolerance in bioreductions being the most active at around 30 °C. Temperature over 40 °C proved to be detrimental for the ketoreductase activity of the strains. Interestingly, 35 °C was the most suitable temperature for the bioreduction of **1c** with WY13 strain ($c_{(S)\text{-}\mathbf{2c}}$ = 73.1%, $ee_{(S)\text{-}\mathbf{2c}}$ >99.9%).

*3.5. Optimal pH in bioreductions of ketones **1a-d***

The effect of pH on ketoreductase activity and selectivity of the WY3 and WY13 strains was studied using appropriate buffers with pH values between 3.0 and 11.0 (Figure 2). The optimal pH was determined in bioreductions of four ketones **1a-d** with both yeast strains separately. Overall, it could be concluded that strain WY13 was active in a wider range of pH than strain WY3 (mostly due to the higher activity in the alkaline pH range between 8–10) but the enantiotope selectivity of the reduction was usually higher with strain WY3 than with WY13. In addition, the pH optima for activity with strains WY3 and WY13 were different towards the various ketones **1a-d**. As the optima for activity and selectivity were often different in the bioreductions of the ketones **1a-d** the optimal operating pH was determined by taking the selectivity seriously into account. In the bioreduction of **1a** the optimal operating pH was 6 with strain WY13 ($c_{(S)\text{-}\mathbf{2a}}$ =49.0%, $ee_{(S)\text{-}\mathbf{2a}}$ = 99.5%) but 7 with strain WY3 ($c_{(S)\text{-}\mathbf{2a}}$ =27.6%, $ee_{(S)\text{-}\mathbf{2a}}$ = 99.5%). In biotransformations of **1b** the optimal operating pH shifted one pH unit lower for both strains to 5 with strain WY13 ($c_{(S)\text{-}\mathbf{2b}}$ =56.1%, $ee_{(S)\text{-}\mathbf{2b}}$ = 99.9%) and to 6 with strain WY3 ($c_{(S)\text{-}\mathbf{2b}}$ =35.6%, $ee_{(S)\text{-}\mathbf{2b}}$ = 99.9%). With both strains the most robust bioreductions could be performed from ketone **1c** in wide operating pH range 3–10 for WY13 ($c_{(S)\text{-}\mathbf{2c}}$ >99%, $ee_{(S)\text{-}\mathbf{2c}}$ >98%) or 5–8 for WY3 ($c_{(S)\text{-}\mathbf{2c}}$ >99%, $ee_{(S)\text{-}\mathbf{2c}}$ >99%). Although with lower conversions ($c_{(S)\text{-}\mathbf{2d}}$ 21–62% with WY13 and 9–49% with WY3), ketone **1c** could be reduced by excellent enantiotope selectivity ($ee_{(S)\text{-}\mathbf{2d}}$ >99.5%) in a wide pH range of 3–11.

Because these isolates (WY3 and WY13) of *W. subpelliculosus* are whole-cell biocatalysts, it is obvious that they contain a number of different ketoreductases with various properties. Thus, the variations in optimal pH/operation condition for bioreductions of the various ketones **1a-d** are understandable by assuming slightly different expression levels/concentrations of these enzymes within the two different strains.

3.6. Effect of organic solvents on ketoreductase activity in bioreductions of phenylacetone **1c**

The significant influence of organic solvents on ketoreductase activity and selectivity is well-known in case of many ketoreductase-producing strains [37,38]. Strains from *Chryseobacterium* sp. could tolerate a wide selection of organic solvents [39], furthermore in bioreductions with baker's yeasts several organic solvents had a positive impact on activity and/or selectivity [29,40]. This prompted us to study the effect of several organic solvents on ketoreductase activity and selectivity of the strains WY3 and WY13 in the bioreduction of **1c** as an appropriate substrate (Table 5). Because in the absence of organic solvents the bioreductions of **1c** resulted in 99% conversion after 24 h, the reaction time in the organic solvent tolerance tests has been shortened to 6 h to enable observation of the positive effects.

Although with some variations, the organic solvent tolerance of the strains WY3 and WY13 were similar. The best tolerated solvents by the two *W. subpelliculosus* isolates were dimethyl sulfoxide and *i*-octane with >50% residual conversions and *ee* >94% up to 5 v/v% concentration level compared to the bioreduction without cosolvent. *n*-Hexane was somewhat less tolerated up to 5 v/v% level (>37% residual conversions and *ee* >94%). Methanol could be tolerated only up to 5 v/v% level (>52% residual conversions and *ee* >96%). The worst tolerated solvents by the strains WY3 and WY13 were acetonitrile and toluene casing significantly decreased ketoreductase activity and selectivity even at 2.5 v/v% level (<10% residual conversions and *ee* <80%). Thus, apparently no correlation was observed between solvent inactivation and solvent logP values [-1.3 (dimethyl sulfoxide), -0.85 (methanol), 3.1 (toluene), 3.5 (*n*-hexane) and 4.5 (*iso*-octane)]. Interestingly, when 2-propanol was added to the system in the presence of 4% glucose, the conversions were significantly lower (e.g $c_{(S)\text{-}\mathbf{2c}}$ = 7.6% and 3.9% at 2.5% 2-propanol content with WY3 and WY13, respectively in Table 5) than found in the presence of 2-propanol as sole cosubstrate (e.g $c_{(S)\text{-}\mathbf{2c}}$ = 45.6% and 46.0% at 4% 2-propanol content with WY3 and WY13, respectively in Table 4). The solvent tolerance results can be best explained by assuming at least two ketoreductases acting on substrate **1c**, the (*S*)-selective one(s) with

lower organic substrate tolerance as major biocatalyst and another (*R*)-selective one(s) with better organic solvent tolerance as minor contributor to the bioreduction.

In addition to the role organic solvents as modifiers of activity and/or selectivity, they can act as co-solvents improving the solubility of not-well-soluble substrates. This advantageous effect was clearly demonstrated by this study in the biotransformations of **1e** (Table 6). When bioreduction of ketone **1e** was tried under the "standard" conditions (4 w/v% of glucose as a cosubstrate in phosphate buffer at pH 7.5 and 30 °C) with either of the strains WY3 or WY13, apparently no product formation was observed in 24 h. Successful bioreductions of **1e** with modest conversions and good enantiotope selectivities could be performed in presence of 2.5 v/v% DMSO as co-solvent ($c_{(S)\text{-}\mathbf{2e}}$ = 51.5%, $ee_{(S)\text{-}\mathbf{2e}}$ = 94.2% with WY3 and $c_{(S)\text{-}\mathbf{2e}}$ = 47.0%, $ee_{(S)\text{-}\mathbf{2e}}$ = 92.8% with WY13). The bioreductions performed at presence of 5 v/v% DMSO indicated that larger amounts of DMSO are harmful for the whole-cell bioreduction ($c_{(S)\text{-}\mathbf{2e}}$ = 11.3% with WY3 and $c_{(S)\text{-}\mathbf{2e}}$ = 1.5% with WY13). The fact that DMSO tolerance profiles were different for bioreductions of ketones **1c** (Table 5) and **1e** (Table 6) further supported the hypothesis that in bioreductions of ketones **1a-e** with these *W. subpelliculosus* isolates at least two ketoreductases were involved with different solvent tolerance.

4. Conclusion

Our study – related to our program aiming discovery of novel strains as whole-cell biocatalysts with significant ketoreductase activity – revealed two novel strains of *Wickerhamomyces subpelliculosus* WY3 and WY13 applicable as efficient biocatalyst for bioreduction of ketones. To our best knowledge, this is the first study exploring the excellent ketoreductase activity and selectivity of *W. subpelliculosus* strains. Not only the freshly harvested cells of the two newly isolated *W. subpelliculosus* WY3 and WY13 strains but also their lyophilized cells – even after long-term storage – proved to be excellent biocatalysts in

bioreduction of various ketones **1a-e**. Both 2-propanol and glucose were investigated as cosubstrate in the bioreductions. As expected, *g*lucose was tolerated at higher concentration (up to 16 w/v%) than 2-propanol (up to 4 v/v%). The excellent results achieved in bioreduction of phenylacetone **1c** even at high concentration of glucose (8 w/v %) as cosubstrate [*c*= 99.8%, $ee_{(S)\text{-}\mathbf{2c}}$= 99.6% with WY3; and *c*= 99.9%, $ee_{(S)\text{-}\mathbf{2c}}$= 99.8%] indicated the usefulness of these novel whole-cell biocatalysts. Both strains showed moderate tolerance of temperature (up to 40 °C) stability in wide pH range (3–10) and variable tolerance of a few organic solvents up to 5 w/v%. Based on our results, *W. subpelliculosus* can be recommended as an excellent extension to the toolbox of whole-cell biocatalyst with ketoreductase activity for production of chiral alcohols in high enantiopurity.

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Figure 1. Effect of temperature on bioreduction of **1a** (A), **1b** (B), **1c** (C) and **1d** (D) with strains WY3 (conversion: ■, enantiomeric excess: ●) and WY13 (conversion: □, enantiomeric excess: ○); substrate to dry cell ratio: 1:15; glucose: 4 w/v%; pH 7.5; 24 h. Due to the large uncertainty of enantiomeric excess for reactions of low conversion, no *ee* values are shown when conversion remained under 2%.

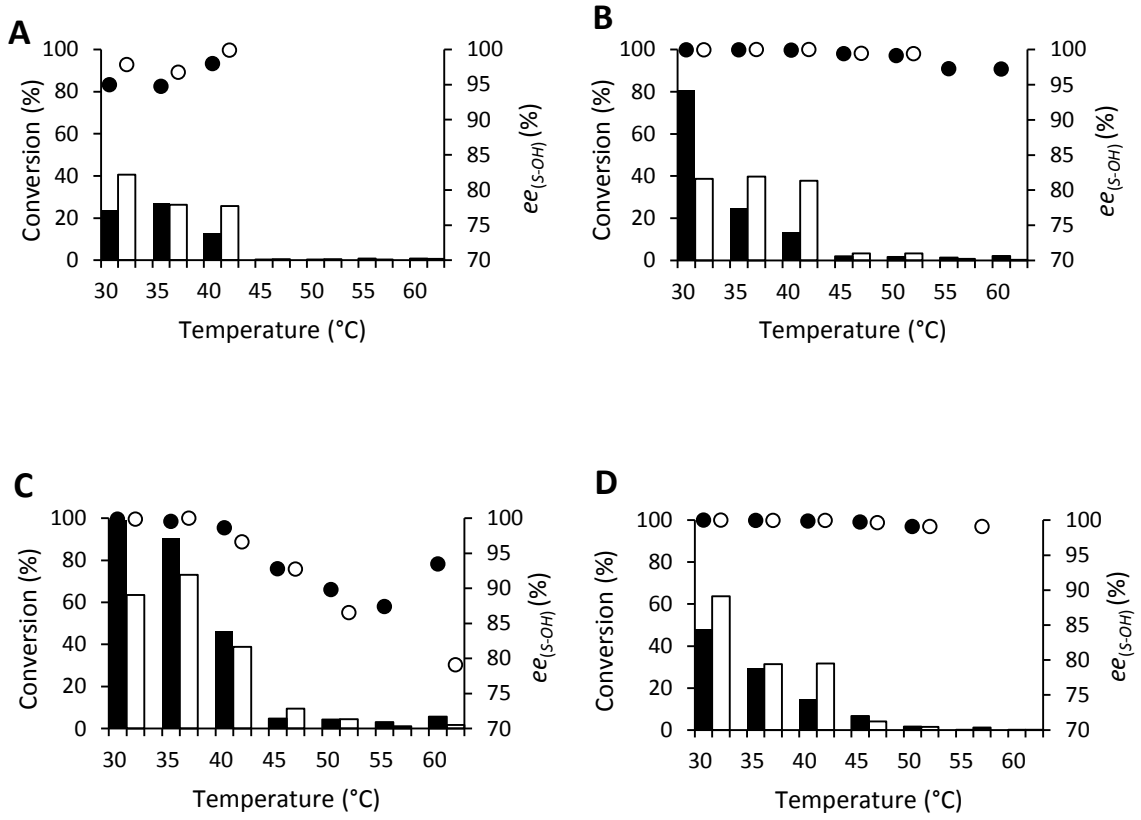
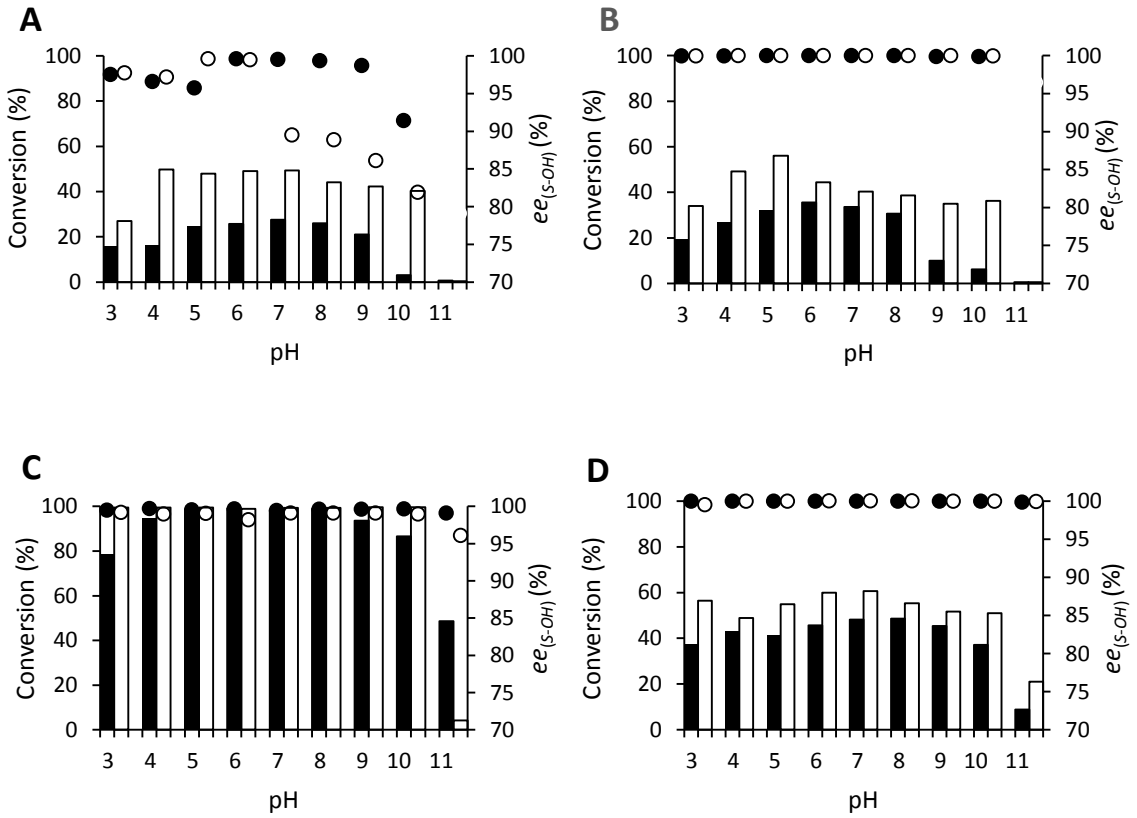
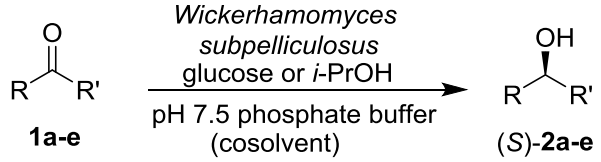


Figure 2. Effect of pH on bioreduction of **1a** (A), **1b** (B), **1c** (C) and **1d** (D) with strains WY3 (conversion: ■, enantiomeric excess: ●) and WY13 (conversion: □, enantiomeric excess: ○). Buffers: 100 mM sodium citrate (pH 3.0 – 5.0), 100 mM sodium phosphate (pH 6.0 – 8.0) and 100 mM sodium carbonate – sodium bicarbonate (pH 9.0 – 11.0); substrate to dry cell ratio: 1:15; glucose: 4 w/v%; 30 °C; 24 h. Due to the large uncertainty of enantiomeric excess for reactions of low conversion, no *ee* values are shown when conversion remained under 2%.



Scheme 1. Bioreduction of prochiral ketones **1a-e** by *Wickerhamomyces subpelliculosus* cells



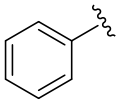
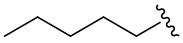
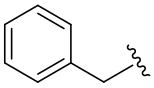
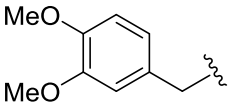
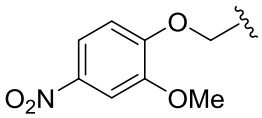
1,2	R	R'
a		CH_3
b		CH_3
c		CH_3
d		CH_3
e		

Table 1. The phylogenetic affiliation of the fungal strains based on their 18S-28S ribosomal DNA internal transcribed spacer (ITS) region sequences compared to that of *Wickerhamomyces subpelliculosus* NR 111336 as closest phylogenetic relative

Strain ID	Similarity (sequenced/target DNA)
WY3	99.8% (494/495)
WY13	99.5% (559/561)
CBS5767	100.0% (574/574)

Table 2. Reduction of **1c** with freshly harvested and lyophilized *W. subpelliculosus* cells (substrate to dry cell ratio: 1:15; glucose: 6 w/v%; pH 7.5; 30 °C; 24 h)

Strain ID	Form	*c* (%)	$ee_{(S)\text{-}\mathbf{2c}}$ (%)
WY3	wet paste	83.1	99.6
WY3	lyophilized	95.8	99.1
WY13	wet paste	99.0	99.9
WY13	lyophilized	99.0	99.7
CBS5767	wet paste	8.1	99.9
CBS5767	lyophilized	5.2	99.8

Table 3. Effect of glucose as cosubstrate on the reductase activity and selectivity of *W. subpelliculosus* WY3 and WY13 in reductions of ketones **1a-d** (substrate to dry cell ratio: 1:15; pH 7.5; 30 °C; 24 h)

WY3								
Glucose (%)	$c_{\mathbf{2a}}$ (%)	$ee_{(S)\text{-}\mathbf{2a}}$ (%)	$c_{\mathbf{2b}}$ (%)	$ee_{(S)\text{-}\mathbf{2b}}$ (%)	$c_{\mathbf{2c}}$ (%)	$ee_{(S)\text{-}\mathbf{2c}}$ (%)	$c_{\mathbf{2d}}$ (%)	$ee_{(S)\text{-}\mathbf{2d}}$ (%)
0	1.3	93.6	3.2	98.1	3.4	91.3	1.4	99.1
1	7.0	95.2	14.6	99.5	99.1	99.7	20.8	> 99.9
2	8.4	92.4	15.8	99.7	98.9	98.7	21.4	> 99.9
4	23.9	95.0	80.8	99.9	99.1	99.8	48.0	> 99.9
8	15.0	94.9	50.3	> 99.9	99.8	99.6	30.3	> 99.9
16	8.2	95.6	55.3	> 99.9	99.7	99.5	49.0	> 99.9
WY13								
Glucose (%)	$c_{\mathbf{2a}}$ (%)	$ee_{(S)\text{-}\mathbf{2a}}$ (%)	$c_{\mathbf{2b}}$ (%)	$ee_{(S)\text{-}\mathbf{2b}}$ (%)	$c_{\mathbf{2c}}$ (%)	$ee_{(S)\text{-}\mathbf{2c}}$ (%)	$c_{\mathbf{2d}}$ (%)	$ee_{(S)\text{-}\mathbf{2d}}$ (%)
0	1.8	93.6	4.8	98.7	3.5	95.2	2.8	99.7
1	9.4	97.6	17.5	99.6	98.3	99.6	35.9	> 99.9
2	9.4	96.7	23.3	> 99.9	99.3	99.8	48.5	> 99.9
4	40.6	97.8	38.7	> 99.9	63.4	99.9	63.7	> 99.9
8	20.7	97.4	56.8	> 99.9	99.9	99.8	53.4	> 99.9
16	34.8	97.5	69.3	> 99.9	97.1	99.9	69.9	> 99.9

Table 4. Effect of 2-propanol as cosubstrate on the ketoreductase activity and selectivity of *W. subpelliculosus* WY3 and WY13 in reductions of ketones **1a-d** (substrate to dry cell ratio: 1:15; pH 7.5; 30 °C; 24 h, due to the large uncertainty of enantiomeric excess for reactions of low conversion, no ee values are shown when conversion remained under 2%)

WY3								
2-Propanol (%)	$c_{\mathbf{2a}}$ (%)	$ee_{(S)\text{-}\mathbf{2a}}$ (%)	$c_{\mathbf{2b}}$ (%)	$ee_{(S)\text{-}\mathbf{2b}}$ (%)	$c_{\mathbf{2c}}$ (%)	$ee_{(S)\text{-}\mathbf{2c}}$ (%)	$c_{\mathbf{2d}}$ (%)	$ee_{(S)\text{-}\mathbf{2d}}$ (%)
0	1.3	-	3.2	98.1	3.4	91.3	1.4	-
1	3.7	98.0	15.2	99.6	21.1	99.0	4.8	99.9
2	20.3	99.1	19.4	99.9	22.9	99.5	17.4	99.9
4	5.2	97.0	24.9	99.9	45.6	99.4	6.9	99.8
8	0.4	-	3.1	99.7	0.8	-	<1	-
16	0.3	-	3.3	99.5	1.9	-	<1	-
WY13								
2-Propanol (%)	$c_{\mathbf{2a}}$ (%)	$ee_{(S)\text{-}\mathbf{2a}}$ (%)	$c_{\mathbf{2b}}$ (%)	$ee_{(S)\text{-}\mathbf{2b}}$ (%)	$c_{\mathbf{2c}}$ (%)	$ee_{(S)\text{-}\mathbf{2c}}$ (%)	$c_{\mathbf{2d}}$ (%)	$ee_{(S)\text{-}\mathbf{2d}}$ (%)
0	1.8	-	4.8	98.7	3.5	95.2	2.8	99.7
1	10.0	94.2	7.2	99.3	10.2	96.8	8.4	99.9
2	72.4	94.9	16.0	99.9	23.8	99.4	15.7	99.9
4	6.2	98.0	2.2	99.4	46.0	99.4	8.1	99.9
8	1.1	-	0.9	-	4.0	94.4	<1	-
16	0.3	-	5.5	99.7	1.7	-	<1	-

Table 5. Effect of organic solvents on ketoreductase activity and selectivity of *W. subpelliculosus* strains WY3 and WY13 in reduction of ketone **1c** (**1c** to dry cell ratio: 1:15; glucose: 4 w/v%; pH 7.5; 30 °C; 6 h; due to the large uncertainty of enantiomeric excess for reactions of low conversion, no *ee* values are shown when conversion remained under 2%).

Organic solvent	Concentration	WY3		WY13	
	(v/v %)	*c* (%)	$ee_{(S)\text{-}\mathbf{2c}}$ (%)	*c* (%)	$ee_{(S)\text{-}\mathbf{2c}}$ (%)
-	-	43.2	97.0	52.4	98.6
DMSO	2.5	36.4	98.4	29.8	96.3
DMSO	5	36.0	97.4	29.2	94.4
Methanol	2.5	30.4	96.2	22.5	97.4
Methanol	5	9.8	90.5	6.2	92.5
i-Propanol	2.5	7.6	88.3	3.9	84.7
i-Propanol	5	2.7	81.3	2.3	85.6
Acetonitrile	2.5	3.5	76.1	3.1	79.9
Acetonitrile	5	1.7	-	0.5	-
Toluene	2.5	3.1	69.8	3.1	80.3
Toluene	5	2.3	69.8	2.5	73.4
n-Hexane	2.5	27.7	95.9	20.2	91.7
n-Hexane	5	26.8	94.5	19.5	95.1
i-Octane	2.5	37.2	96.3	38.2	96.4
i-Octane	5	35.0	96.0	21.6	96.0

Table 6. Bioreduction of **1e** by *W. subpelliculosus* strains WY3 and WY13 (**1e** to dry cell ratio: 1:30, glucose: 4 w/v%, pH 7.5, 30 °C, 24 h; due to the large uncertainty of enantiomeric excess for reactions of low conversion, no *ee* values are shown when conversion remained under 2%).

Cosolvent	Concentration	WY3		WY13	
	(v/v %)	*c* (%)	$ee_{(S)\text{-}\mathbf{2e}}$ (%)	*c* (%)	$ee_{(S)\text{-}\mathbf{2e}}$ (%)
-	-	<1	-	<1	-
DMSO	2.5	51.5	94.2	47.0	92.8
DMSO	5	11.3	> 99.9	1.5	-