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# Combined Microbial Oxidation and Reduction: a New Approach to the High-yield Synthesis of Homochiral Unsaturated Secondary Alcohols from Racemates

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*Key Words*: Microbial oxidation, Microbial reduction, *Bacillus stearothermophilus*, *Yarrowia lipolytica*, Unsaturated secondary R-alcohol

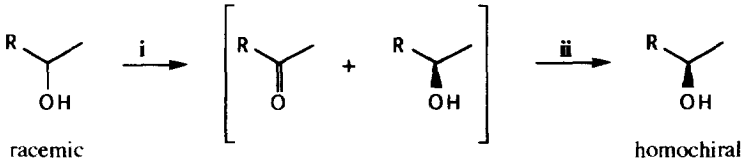
***Abstract:*** The oxidation of racemic secondary alcohols with *Bacillus stearothermophilus* followed by reduction of the mixture with *Yarrowia lipolytica* to afford high yields of the enantiomerically pure R-alcohols **1a,b,d** is described. Comparisons with *Yarrowia lipolytica* reduction, *Bacillus stearothermophilus* oxidation, and the contemporary use of the two microrganisms have been made.

Enantiomerically pure secondary alcohols are very common as pheromones,[1] aroma, flavour enhancing compounds, intermediates and chiral auxiliaries in organic synthesis.[2] The methodologies to obtain these chiral compounds are the microbial or enzymatic reduction of the corresponding ketones and the kinetic resolution of the racemic alcohols *via* esterification or hydrolysis of the corresponding esters with lipases. A broad range of ketones are reduced with Baker's yeast[3] or using purified alcohol dehydrogenases.[4] During the course of the reaction with Baker's yeast the S-alcohol was almost exclusively obtained, while the enzyme delivers the hydride either from the *si*-side (i.e. *Lactobacillus kefir* alcohol dehydrogenase[5]) or *re*-side (i.e. *Thermoanaerobium brockii* alcohol dehydrogenase[6]) of the ketone to give (R)- or (S)-alcohols, respectively. For most cases, the stereochemical course of the reaction, which is mainly dependent on the steric requirements of the substrate, may be predicted from a simple model which is generally referred to as "Prelog's rule."[7] The majority of the commercially available dehydrogenases used for the stereospecific reduction of ketones and the majority of microrganisms follow the Prelog's rule giving the S-alcohols. However, the enzymatic resolution of the racemic alcohols with lipases,[8] when a high enantioselectivity is exhibited, stops at 50% conversion stage.[9]

In this field our previous work described an alternative method for the kinetic resolution of secondary alcohols *via* oxidation with Baker's yeast[10] and *Bacillus stearothermophilus*[11,12] affording R-enantiomer in high enantiomeric excess and only recently an "anti-Prelog" behaviour (formation of the R-alcohol) is reported for the microbial reduction of various carbonyl compounds with *Yarrowia lipolytica* strains, a recent yeast species.[13]

In this paper we describe a new approach to the high-yield synthesis of homochiral secondary R-alcohols starting from the corresponding racemates which consists in the following pathway: i) kinetic resolution *via*

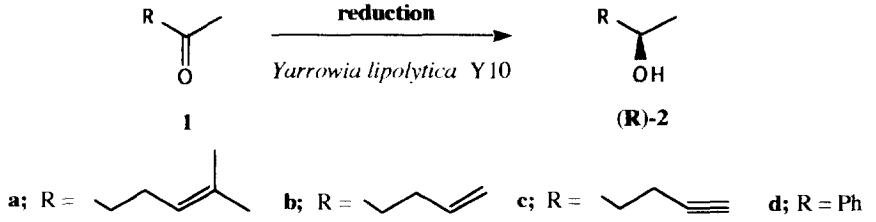
oxidation with *Bacillus stearothermophilus*; ii) reduction of the obtained oxidation mixture with *Yarrowia lipolytica* (Scheme 1).




**Scheme 1**

This methodology is compared with the other classical approaches: i) reduction with *Yarrowia lipolytica* of the corresponding ketones; ii) kinetic resolution via oxidation with *Bacillus stearothermophilus* of the racemic alcohols. On the whole the combination of the oxidation followed by the reduction is a choice when the reduction is not so efficient and considering that the kinetic resolution stops at 50% conversion.

The results of the reduction of the ketones **1a-d** (Scheme 2) with *Yarrowia lipolytica* Y10[14] are summarized in Table 1.




**Scheme 2**

**Table 1.** Reduction of the ketones **1a-d** with *Yarrowia lipolytica* Y10.

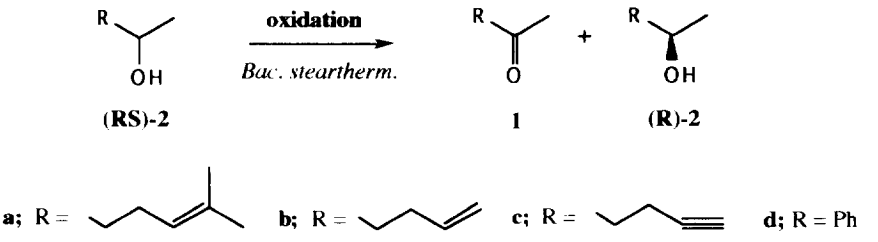
| ketone | culture media (time h) | alcohol (yield %) | ee % [a](abs. conf.)[b] |
|---|---|---|---|
| **1a** | synthetic (24) | **2a** (75) | 95 (R) |
| **1b** | synthetic (24) | **2b** (26) | 68 (R) |
| **1b** | saboraud (24) | **2b** (30) | 48 (R) |
| **1c** | synthetic (24) | **2c** (83) | 94 (S) |
| **1d** | synthetic (24) | **2d** (26) | 62 (R) |
| **1d** | saboraud (24) | **2d** (25) | 100 (R) |

[a] Determined by GLC on chiral column containing *n*-pentyl-dimethyl-β-cyclodextrin. b Determined comparing the sign of the optical rotation with that of the literature (ref. 15).

The substrate was added to a growing culture of *Yarrowia lipolytica* as a concentrated solution in DMSO and the incubation was prolonged for 24 h at 28°C. 5-Methyl-5-hepten-2-one **1a** and 5-hexyn-2-one **1c** are reduced

withgood yields and enantiomeric excesses to R-sulcatol **2a** (75%, ee 95%) and S-hexynol **2c** (83%, ee 94%), respectively. The anomalous behaviour of **1c** in respect to a series of nonconjugated unsaturated ketones is also observed in the reduction of **1c** with *Thermoanaerobium brockii* alcohol dehydrogenase where the ketone gives the R- instead of the S-enantiomer.[15] The reductions of the 5-hexen-2-one **1b** and of the acetophenone **1d** produce the corresponding R-alcohols **2b** and **2d** with quite good ee (48-100%) but with low yields (25-30%) also varying the culture media.

The R-alcohols are also obtained by the kinetic resolution of the racemic unsaturated secondary alcohols **1a-d** *via* oxidation with *Bacillus stearothermophilus* (Scheme 3) and the results are summarized in Table 2.




**Scheme 3**

**Table 2.** Kinetic resolution of unsaturated racemic alcohols **1a-d** with *Bacillus stearothermophilus*.

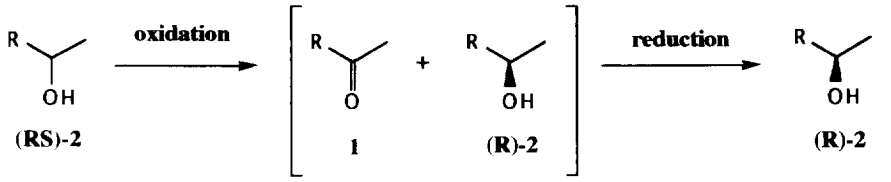
| Alcohol | Products [a] | % ratio[b] | Time (h) | % ee[b] (abs. conf.)[c] |
|---|---|---|---|---|
| **2a** | **1a** | 52 | 3 | |
| | **2a** | **48** | | **98 (R)** |
| **2b** | **1b** | 51 | 4 | |
| | **2b** | **49** | | **100 (R)** |
| **2c** | **1c** | 51 | 20 | |
| | **2c** | **49** | | **99 (R)** |
| **2d** | **1d** | 53 | 6 | |
| | **2d** | **47** | | **85 (R)** |

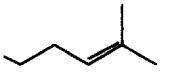
[a] The mixture of the products **1** an **2** was purified on a short column of silica gel (recovered material 90%). [b] Determined by GLC on chiral column containing *n*-pentyl-dimethyl-β-cylclodextrin. [c] Determined comparing the sign of the specific rotation with that of the literature (ref. 15).

The substrate was added to a growing culture as a concentrated solution in DMSO and the incubation was prolonged for the appropriate time at 39 °C. All the reactions afforded the ketones **1a-d** leaving the unreacted R-enantiomers of the corresponding alcohols **2a-d** (47-49%) with good enantiomeric excesses (85-100%).

On the basis of these results considering that in some cases the microbial reduction of the ketones is not so efficient and the oxidation stops at 50% conversion, and since both the reactions bring to the R-enantiomer, we combined the oxidation and the reduction in order to obtain higher yields of homochiral R-alcohols starting from the corresponding racemates. The methodology consists in carrying out the kinetic resolution of the racemic

alcohol by oxidation. For the growth of *Bac. stearotherm.* a poorer nutrient broth was used. In one case the same nutrient broth has been utilized for the oxidation of the alcohols **2** (method A), in another one (method B) the cells of *Bac. stearotherm.* were centrifuged and suspended in phosphate buffer. The oxidation reactions, in both cases, are carried out at 39°C for 5 h and then the cells were centrifuged and the surnatant, containing the mixture of ketone **1** and the unreacted alcohol **2**, was added to grown up (see experimental: method A) and centrifuged cells of *Yarrowia lipolytica*. The reduction was continued for a further 24 h at 28°C (Scheme 4).




**a**; R =  **b**; R =  **d**; R = Ph

**Scheme 4**

**Table 3.** Combined oxidation and reduction of the racemic alcohols **1a,b,d**.

| (R,S)-2 | Method[a] | Oxidation[b] -Reduction[c] | | |
|---|---|---|---|---|
| | | **1** (yield %) | **2**(yield %) | ee%[d](abs. conf.) |
| **2a** | A | **1a** (13) | **2a** (87) | **100** (R) |
| **2a** | B | **1a** (9) | **2a** (91) | **100** (R) |
| **2a** | C[e] | **1a** (18) | **2a** (82) | **90** (R) |
| **2b** | A | **1b** (0) | **2b** (100) | **100** (R) |
| **2b** | B | **1b** (9) | **2b** (91) | **100** (R) |
| **2b** | C[e] | **1b** (15) | **2b** (85) | **40** (R) |
| **2d** | A | **1d** (12) | **2d** (88) | **40** (R) |
| **2d** | B | **1d** (20) | **2d** (80) | **85** (R) |
| **2d** | C[e] | **1d** (0) | **2d** (100) | **0** |

[a] The reaction is carried out: in the nutrient broth of *Bacillus stearothermophilus* (method A); in phosphate buffer pH 7.2 (method B); in the mixing of the nutrient broth of *Bacillus stearothermophilus* and *Yarrowia lipolytica* (method C). [b] The crude reaction mixture of the oxidation with *Bacillus stearothermophilus* is centrifuged and the surnatant is added to the grown cells of *Yarrowia lipolytica*. [c] The *Yarrowia lipolytica* cells, grown up in a synthetic medium (see experimental), are centrifuged and the reduction is carried out with the surnatant obtained from the oxidation. [d] Enantiomeric excesses are calculated from glc analysis on chiral column containing *n*-pentyl-dimethyl-β-cylclodextrin. [e] The reaction is carried out with mixed grown up cells of *Bacillus stearothermophilus* and *Yarrowia lipolytica*.

The results of the combined oxidation and the reduction are reported in Table 3.

The oxidation of 6-methyl-5-hepten-2-ol (sulcatol) **2a** followed by reduction afforded in very high yield (87-91%) the pure R-enantiomer (method A and B). Moreover, pure (R)-5-hexen-2-ol **2b** is obtained in quantitative

yield (method A). It is worth mentioning that in the case of the R-sulcatol **2a** the combination of the two reactions affords only a small improvement, while for the hexenol **2b** this methodology is more efficient than the single reactions.

High yield (80%) and good enantiomeric excess (85%) are also obtained with 1-phenylethanol **2d** carrying out the reactions in phosphate buffer while the method A (nutrient broth of *Bac. stearotherm.*) affords poorer enantiomeric excess (40%). Obviously this approach is not used when the reduction affords the S-enantiomer (i.e. 5-hexyn-2-one **1c**).

As shown in Table 3 (redox), a parallel experiment was made mixing *Bacillus stearothermophilus* and *Yarrowia lipolytica*, grown up in the appropriate culture media, and adding the alcohols **2a,b,d**. The incubation was continued for 24 h at 28°C. Worst results are obtained for the racemic alcohols **2a** (85% yield, ee 90%) and **2b** (85% yield, ee 40%), while the alcohol **2d** is completely not resolved.

In conclusion, matching oxidation with *Bacillus stearothermophilus* and reduction with *Yarrowia lipolytica*, it is possible to obtain homochiral R-alcohols starting from the racemate in high yields. This methodology is a third pathway and is a choice when the reduction with *Yarrowia lipoltyca* is not so efficient and/or because the oxidation stops at 50% conversion.

## Experimental

Optical rotations were measured on a Perkin Elmer Model 241 polarimeter. Gas chromatographic analyses were performed on a Carlo Erba GC 6000 Vega series 2. The ketones **1a** (Fluka) and **1d** (Fluka) and the alcohols **2a** (Aldrich) and **2d** (Fluka) are commercially available. The alcohols **2b**[16] and **2c** were obtained by reduction with sodium borohydride of the corresponding ketones **1b**[17] and **1c**.[18]

5-Hexyn-2-ol **2c** showed the following: oil; $^1$H NMR (200 MHz) δ 1.18 (d, 3 H, $J$ = 5.9 Hz), 1.62 (q, 2 H, $J$ = 6.7 Hz), 1.94 (t, 1 H, $J$ = 2.5 Hz), 2.17 (br s, 1 H), 2.28 (dt, 2 H, $J$ = 2.5 and 6.7 Hz), 3.91 (m, 1 H).

**Separation by GLC.** Enantiomer separation was obtained on a Megadex 5 column (25 m X 0.25 mm) containing *n*-pentyl dimethyl β-cylodextrin in OV 1701 from Mega s.n.c. For compound **1a**: helium 0.8 atm; temp. 80-200°C (1.5°C/min); retention time in min (after acetylation with acetic anhydride and pyridine) 3.88 **1a**, 5.96 (**S**)-**2a**, 6.72 (**R**)-**2a**. For compound **1b**: helium 0.9 atm; temp. 80°C; retention time in min 4.76 **1b**, 8.7 (**S**)-**2b**, 8.95 (**R**)-**2b**. For compound **1c**: helium 0.9 atm; temp 100-200°C (2°C/min); retention time in min 3.15 **1c**, 4.41 (**S**)-**2c**, 4.58 (**R**)-**2c**. For compound **1d**: helium 0.8 atm; temp. 100-200°C (2°C/min); retention time in min 10.21 **1d**, 14.84 (**R**)-**2d** 15.43 (**S**)-**2d**. The absolute configurations of the compounds were determined to be R comparing the sign of their specific rotation with those of the literature: (R)-6-methyl-5-hepten-2-ol **2a**[15] $[\alpha]_D$ = -14.5 (c 1.3, EtOH); (R)-5-hexen-2-ol **2b**[15] $[\alpha]_D$ = -12.1 (c 4.6, $CHCl_3$); (R)-5-hexyn-2-ol **2c**[15] $[\alpha]_D$ = -25.9 (c 3.5, $CHCl_3$); (R)-1-phenylethanol **2d**[11] $[\alpha]_D$ = 41 (c 5.1, $CHCl_3$).

**Microbial Reduction with *Yarrowia lipolytica* Y10 of the Ketones 1a-d. General Procedure.** A synthetic culture medium is prepared adding to 1 L of water glucose (50 g), $(NH_4)_2SO_4$ (5g), $KH_2PO_4$ (2 g), $CaCl_2$ (0.25 g), $MgSO_4.7H_2O$ (0.25 g), inositol (25 mg), $H_3BO_3$ (1 mg), $ZnSO_4$ (1 mg), $MnCl_2$ (1 mg), $FeCl_2$ (0.5 mg), $CuSO_4$ (0.1 mg), tiamine (0.3 mg), biotine (0.025 mg), calcium pantothenate (0.3 mg), pyridoxine (0.3 mg) and nicotinic acid (0.3 mg). The culture of *Yarrowia lipolytica* Y10 was set up in 500 mL conical flask containing the synthetic medium (100 mL) and stirred at 28°C for 48 h.

To the resulting suspension of grown cells the ketone **1** (0.2 g) in DMSO (1 mL) was added. After 24 h the reaction mixture was extracted with diethyl ether (100 mL) with a continous liquid-liquid extractor, dried over anhydrous $Na_2SO_4$ and concentrated under vacuum. The alcohols were purified by column chromatography (silica gel, petroleum ether/diethyl ether 80:20). The yields are reported in Table 1 and the enantiomeric excesses are calculated by GLC analysis on chiral column.

**Microbial oxidations with *Bacillus stearothermophilus* of the Alcohols 2a-d. General procedure.** The nutrient broth was prepared dissolving bactotryptone (2 g), yeast extract (1 g) and sodium oxalate (0.3 g) in distilled water (100 mL). The culture of *Bacillus stearothermophilus* ATCC2027 was set up in 500 mL conical flask containing the nutrient broth (100 mL) and stirred at 39°C for 48 h. To the resulting suspension of grown cells the racemic alcohol **2** (0.3 g) in DMSO (1 mL) was added. After the appropriate time (see Table 2), the reaction mixture was extracted with diethyl ether (100 mL) with a continous liquid-liquid extractor, dried over anhydrous $Na_2SO_4$ and concentrated under vacuum. The products were purified by column chromatography (silica gel, petroleum ether/diethyl ether 80:20) to give the ketones and the unreacted alcohols (total yields 90%). The **2:1** molar ratios and the enantiomeric excesses from GLC are given in Table 2.

**Combined oxidations and reductions of Alcohol 2a,b,d. General procedure.**

**Method A.** The nutrient broth for the growth of *Bacillus stearothermophilus* was prepared dissolving glucose (1 g), bactotryptone (5 g), yeast extract (1 g) in water (1 L). A sterilized nutrient broth (50 mL) was inoculated with a loopful of the *Bacillus stearothermophilus* and incubated for 48 h at 39°C under stirring. To the resulting suspension of grown cells the racemic alcohol **2** (0.1 g) in DMSO (1 mL) was added. After stirring for a further 5 h at 39°C, the cells were centrifuged. The surnatant was added to the centrifuged cells of *Yarrowia lipolytica* grown for 48 h at 28°C in 50 mL of a stirred synthetic medium (see above for reduction).
The reduction with *Yarrowia lipolytica* was continued for 24 h at 28°C under stirring. The reaction mixture was centrifuged and the surnatant was extracted with diethyl ether (50 mL) with a continous liquid-liquid extractor, dried over anhydrous $Na_2SO_4$ and concentrated under vacuum. The products were purified by column cromatography (silica gel, petroleum ether/diethyl ether 80:20) to obtain the alcohols and the unreacted ketones. The yields and the enantiomeric excesses (from GLC) are listed in Table 3.

**Method B.** The oxidation and the subsequent reduction were carried as above (method A) but the grown up cells of *Bacillus stearothermophilus*, after centrifugation, were suspended in phosphate buffer at pH 7.2 (50 mL) and the racemic alcohol **2** (0.1 g) in DMSO (1 mL) was added. The results are summarized in Table 3.

**Method C.** The suspesion of the grown up cells (method A) of *Bacillus stearothermophilus* (50 mL) and the suspension of grown up cells (in synthetic medium) of *Yarrowia lipolytica* (50 mL) were mixed and the racemic alcohol **2** (0.1 g) was added. After 24 h stirring at 27°C the reaction mixture was centrifuged and the surnatant was extracted with diethyl ether (50 mL) with a continous liquid-liquid extractor, dried over anhydrous $Na_2SO_4$ and concentrated under vacuum. The products were purified by column cromatography (silica gel, petroleum ether/diethyl ether 80:20) to obtain the alcohols and the unreacted ketones (Table 3 ).

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