# Enantioselective Hydrolysis of D,L-Menthyl Benzoate to L-(−)-Menthol by Recombinant *Candida rugosa* Lipase LIP1

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Dedicated to Roger Sheldon on the occasion of his 60th birthday.

**Abstract:** The stereoselective synthesis of L-menthol is an attractive process in the flavor and fragrance industry. One promising way to obtain optically pure menthol is the enantioselective hydrolysis of menthol esters under enzymatic catalysis. We developed an effective and highly enantioselective method for the synthesis of L-(−)-menthol (>99% EE) by hydrolyzing the key industrial starting compound, D, L-menthyl benzoate. The enzyme of choice was the lipase from *Candida rugosa* (CRL). While commercially available preparations of this lipase showed only minor selectivity ($E = 15$), excellent enantiomeric purity ($E > 100$) was achieved using the heterologously expressed isoenzyme LIP1.

**Keywords:** enantioselectivity; kinetic resolution; lipase; menthol

## Introduction

The use of enzymes in stereoselective synthesis is meanwhile a common procedure. Several dozen processes are currently running on an industrial scale producing up to hundreds of tons of optically pure key intermediates for pharmaceutical uses and agrochemistry.[1] Among the most frequently used biocatalysts are lipases (E.C. 3.1.1.3) and esterases (E.C. 3.1.1.1) as they are widely available, stable, do not require cofactors and show a remarkable enantioselectivity towards a broad range of substrates.[2,3]

D,L-Menthyl benzoate is the starting compound to produce L-menthol in the thousand tons per year scale through an industrial process involving preferential crystallization of the D-enantiomer, which is subsequently chemically re-racemized, while the L-enantiomer is subjected to acidic hydrolysis leading to pure L-menthol.[4] Alternatively, asymmetric hydrogenation using a Rh-BINAP-catalyst can yield optically pure L- or D-menthol as described for the so-called Takasago process.[4]

The key industrial starting compound **1** has so far escaped any lipase-catalyzed kinetic resolution, although lipases of different origins have been successfully investigated as biocatalysts for the enantioselective hydrolysis of D,L-menthyl esters or the acylation of D,L-menthol.[5] This is most likely due to the low activity of lipases towards D,L-menthyl benzoate, which can be attributed to the generally low acceptance of bulky aromatic residues in the acidic moiety.[3]

Starting from the results of a preliminary screening of commercially available lipases and esterases we decided to focus on the heterologously expressed *Candida rugosa* lipase. The possibility to obtain this enzyme in a pure form – non-contaminated with other hydrolytic enzymes – enabled the production of L-menthol at an optical purity not achievable with the commercial preparations.

## Results and Discussion

Whilst screening around 30 commercially available hydrolases we found a number of enzymes which showed good to high enantioselectivity in the hydrolysis of D,L-menthyl acetate as well as in the acylation of D,L-menthol (data not shown). However, we found that only one lipase preparation from *Candida rugosa* (Lipase AY "Amano" 30) was able to hydrolyze the key industrial substrate, menthyl benzoate **1** (Figure 1). Unfortunately, the enantioselectivity of Lipase AY was very ($E = 15$) low and not sufficient for an industrial application (Table 1). As the *Candida rugosa* genome encodes for more than six lipase isoenzymes and at least

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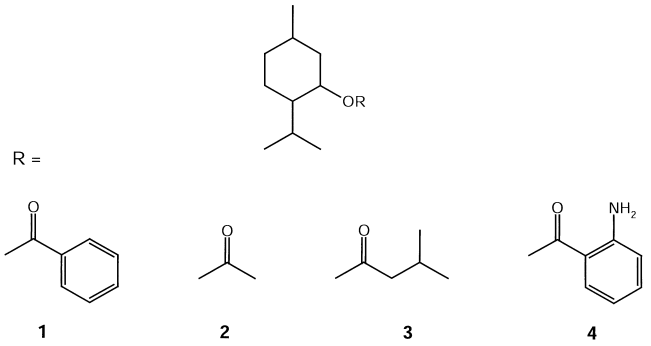


**Figure 1.** D,L-Menthyl esters **1**–**4** used for kinetic resolution.

**Table 1.** Enantioselectivity of commercial CRL (Lipase AY "Amano" 30) in the kinetic resolution of D,L-menthyl benzoate **1**.

| Time [h] | Enantiomeric excess | | Conversion [%] | $E$[a] |
|---|---|---|---|---|
| | [% $ee_S$][b] | [% $ee_P$][c] | | |
| 2 | 14 | 86 | 14 | 14.9 |
| 4 | 17 | 86 | 16 | 15.5 |
| 6 | 31 | 82 | 27 | 13.4 |
| 8 | 56 | 78 | 42 | 14.3 |

[a] Enantioselectivity $E$ was calculated according to Chen et al.[10]
[b] % ee of remaining substrate.
[c] %ee of produced L-(–)-menthol.

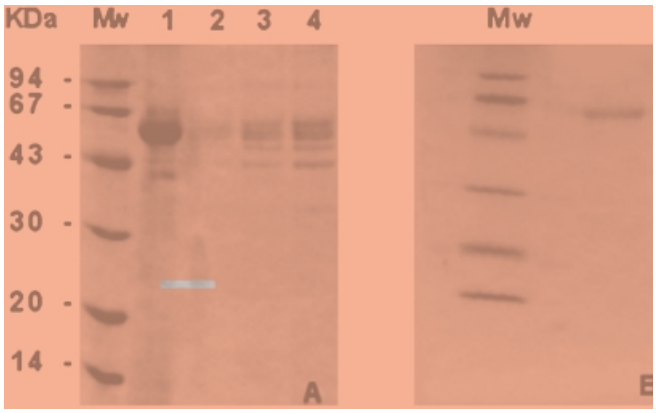


**Figure 2.** SDS-PAGE analysis of commercial and recombinant CRLs. **A** lanes 1–4: commercial CRL preparation (Lipase AY "Amano" 30) at different dilutions. **B** recombinant CRL isoenzyme LIP1. Mw: molecular weight standard.

**Table 2.** Enantioselectivity of recombinant CRL in the kinetic resolution of D,L-menthyl benzoate **1**.

| Time [h] | Enantiomeric excess | | Conversion [%] | E[a] |
|---|---|---|---|---|
| | [% $ee_S$] | [% $ee_P$] | | |
| 2 | 2 | > 99 | 2 | > 100 |
| 4 | 11 | > 99 | 10 | > 100 |
| 6 | 28 | > 99 | 22 | > 100 |
| 8 | 82 | > 99 | 45 | > 100 |

[a] see Table 1.

two other carboxyl esterases,[6] we suspected that the lack in enantiorecognition was due to opposite selectivity of the various biocatalysts present in the commercial enzyme preparation.

Thus, we performed the functional expression of the most prominent *Candida rugosa* isoenzyme LIP1 in the methylotrophic yeast *Pichia pastoris* after creating a synthetic gene.[7] This was necessary, as the non-canonical CUG codon encodes for serine in *Candida rugosa*, but leads to translation into leucine in *Pichia pastoris*.[8] After this modification, we were able to produce recombinant CRL in large amounts and high purity as confirmed by SDS-PAGE analysis (Figure 2). This also confirms that the commercial CRL preparation (Lipase AY "Amano" 30) contains several proteins, whereas the recombinant preparation is composed of only one protein band, the CRL LIP1 isoenzyme.

The recombinant CRL isoenzyme LIP1 exhibits remarkably high enantioselectivity ($E > 100$) in the kinetic resolution of **1** (Table 2). After 8 h at 40 °C this led to nearly 50% L-(–)-menthol formation of excellent optical purity (> 99.9% ee) as confirmed by gas chromatography, polarimetry and NMR-spectroscopy.

This high selectivity is not restricted to menthyl benzoate, but also allowed efficient resolution of other D,L-menthyl esters (Figure 1, Table 3). Recombinant CRL also exhibits high enantioselectivity ($E > 100$) in the resolution of menthyl acetate **2** and menthyl isovalerate **3.** Towards **2** again a notably lower enantioselectivity of lipase AY ($E = 13$) could be confirmed. A clear reduction in activity of recombinant CRL was found when changing the starting material. The branched substrate **3** is converted significantly more slowly than **1** and highest conversion rates were achieved with **2**.

No conversion was found for menthyl anthranilate **4**, although this compound is very similar to **1**, which undergoes facile conversion. One reason might be that the presence of the amino functionality in an *ortho*

**Table 3.** Enantioselectivity of recombinant and commercial CRL towards different menthyl esters after 8 h at 40 °C.

| CRL | Substrate | Enantiomeric excess | | Conversion [%] | E[a] |
|---|---|---|---|---|---|
| | | [% $ee_S$] | [% $ee_P$] | | |
| recombinant | D,L-3 | 31 | > 99 | 24 | > 100 |
| recombinant | D,L-4 | No conversion | | | |
| recombinant | D,L-2 | 93 | > 99 | 48 | > 100 |
| Lipase AY | D,L-2 | 49 | 78 | 38 | 13 |

[a] see Table 1.

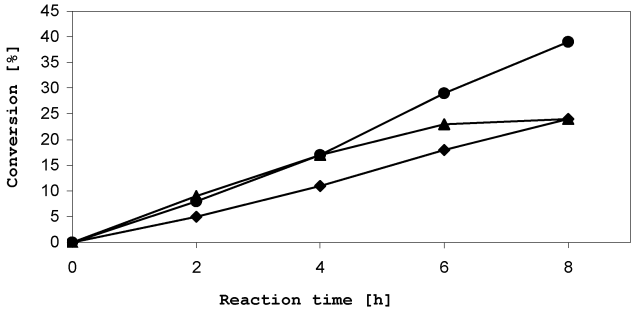


**Figure 3.** Time courses of the hydrolysis of D,L-menthyl isovalerate by recombinant CRL at different temperatures; (◆) 40 °C, (●) 50 °C, (▲) 60 °C.

position to the carboxylic group causes steric hindrance or negative polar effects.

Resolutions of **3** at different temperatures revealed that recombinant CRL considerably lost activity at 60 °C whereas at 40 °C initial rates where much lower; highest conversions were achieved at 50 °C (Figure 3). Nevertheless enantioselectivity was not significantly effected by the temperature changes, as in all cases *E*-values $>100$ were determined.

## Conclusion

By using heterologously expressed recombinant CRL, we have found an effective and highly enantioselective method for the kinetic resolution of D,L-menthol starting from the readily available industrial benzoate derivative. On the basis of these results, biotransformations using commercial CRL preparations should be carefully reinvestigated with recombinant enzymes. The presence of several competing enzymes with opposite stereoselectivity, which may be found in commercial enzyme preparations, can considerably decrease the optical purity of the desired product. This observation is also in accordance with previous findings for pig liver esterase, where it could also been shown that the recombinant and thus pure enzyme provides significantly better enantioselectivities towards several tested chiral substrates.[9]

As we suggest that the lack of enantiorecognition of the commercial crude CRL preparation is due to an opposite enantioselectivity of at least one of the isoenzymes it would be interesting to identify and characterize this "antagonist(s)" of LIP1.

## Experimental Section

### General Remarks

All chemicals were purchased from Fluka (Buchs, Switzerland) and Sigma-Aldrich (Deisenhofen, Germany) at highest purity available. Menthyl derivatives were a gift from Haarmann & Reimer Holzminden, Germany. NMR data were recorded on a 500 MHz Bruker spectrometer. All pH-Stat experiments were performed on a device from Metrohm (Herisau, Switzerland). Recombinant CRL LIP1 was expressed in P. pastoris and purified as described before.[7]

### Lipase-Catalyzed Resolution of D,L-Menthyl Esters

Unless stated otherwise, reactions were carried out in capped vials placed in a thermostatted bath at 40 °C. The reaction mixture for the enantioselective hydrolysis was composed of 0.5 mM substrate and 0.2% (w/v) gum arabic, which was homogenized in sodium phosphate buffer (pH 7.2, 100 mM). The kinetic resolution was started by adding 400 U lipase. Samples were extracted with ethyl acetate and determination of enantiomeric purity and conversion was performed by gas chromatography (column: FS-Cyclodex beta-I/P, carrier gas: $H_2$, flame ionization detector).

### Resolution of D,L-Menthyl Benzoate on a Preparative Scale

D,L-Menthyl benzoate (2.1 g) was suspended in 250 mL sodium phosphate buffer (pH 7.2, 100 mM). Bioconversion was initiated by adding 5000 U recombinant CRL. The reaction was carried out in a 1-L round-bottom flask at 40 °C. The reaction was stopped after 20 h at 50% conversion (related to the total amount of D,L-menthyl benzoate). After removing the enzyme from the reaction solution by filtration and extraction of the reaction mixture with ethyl acetate, substrate and reaction product were separated by column chromatography on silica gel using petroleum ether/ethyl acetate (10:1, v/v) as eluent. Substrate and reaction product were characterized by NMR spectroscopy and the enantiomeric purity of both was determined by gas chromatography and polarimetry.

### Determination of Lipase Activity

Lipase activity was measured with tributyrin as substrate in a pH-stat assay at 30 °C and pH 7.2. One unit was defined as the amount of enzyme required to release 1 μmol of fatty acid per minute under assay conditions.

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