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Biocatalytic enantiomeric resolution of L-menthol from an eight isomeric menthol mixture through transesterification

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ABSTRACT

The four diastereomers of menthol and their enantiomers, namely DL-menthol, DL-neomenthol, DL-neoisomenthol and DL-isomenthol, were synthesised by the hydrogenation of thymol to yield an eight isomer liquid menthol. A suitably selective lipase was sought to preferentially esterify L-menthol in organic solvent, hence simplifying separation from the diasteromeric mix through distillation.

From an initial screen a commercial *Pseudomonas fluorescens* lipase (Amano AK) was selected, and vinyl acetate was chosen as a suitable irreversible acyl donor for transesterification. The reaction reagent ratios were optimised, and an enantiomeric excess (ee) of L-menthol of greater than 95% was reproducibly achievable at a conversion of 30% DL-menthol (0.68 M) at ≤50 °C. On the basis of the composition of liquid menthol the reaction had a diastereomeric excess (de) of 82%. The enzyme was recycled 150 times in 5 ml batch reactions using liquid menthol and achieving an overall yield of 184.3 g DL-menthol/g commercial enzyme preparation. The by-product acetic acid, formed by hydrolysis of menthyl acetate, was found to cause a high degree of enzyme inactivation.

The resolution reaction was scaled up 400 fold to 2 L and the enzyme recycled 38 times with an average conversion of the available L-menthol of 59% and a volumetric productivity of 1.2 g/L/h.

1. Introduction

Menthol is a compound utilised in a wide range of consumer products for its cooling effect and refreshing flavour. The demand for L-menthol in flavour, fragrance, pharmaceutical, tobacco and oral hygiene industries was estimated at 12,000 metric tons in 2000 [1]. The majority of the world's supply of natural L-menthol is derived from distillations from the leaves of the numerous subspecies of mint (*Mentha arvensis* or *piperita*). However, menthol is also obtained synthetically via a number of routes. One of these is the Takasago process starting from myrcene [2], while another is the Haarmann & Reimer/Symrise process via hydrogenation of thymol. Unfortunately this latter reaction provides a mixture of cyclic compounds of which L-menthol is only one. The hydrogenation of thymol generates three stereogenic centres, and hence yields 8 isomers, consisting of a racemic mixture of each of menthol, isomenthol, neomenthol and neoisomenthol (Scheme 1) in non-stoichiometric ratios. Hence a method for isolation of L-menthol from this mixture is required.

Lipases are capable of resolving alcohol racemates through hydrolysis or condensation of the corresponding esters. Such lipase catalysed reactions have become particularly popular because lipases are readily available from commercial sources, are relatively inexpensive, have no co-factor requirement, and are widely used in industry [3]. In enantiomeric kinetic resolution reactions lipases have been demonstrated to effectively transform L-menthol without converting the corresponding D-enantiomer. For instance, researchers were able to isolate L-menthol from DL-menthol by esterification with vinyl acetate or vinyl propionate in the presence of an organic solvent [4,5], or by using tributyrin or triacetin as acyl donors [6]. Physical isolation of L-menthyl ester from the unesterified D-menthol (e.g. by distillation) followed by chemical hydrolysis yields the desired pure L-menthol. However, the technique was not extended to the more complex mixture of diastereomers.

Herein we describe the selective acylation of L-menthol from the 8 isomer liquid menthol mixture by means of lipase catalysed transesterification in organic solvent (Scheme 2). During this reaction, acetaldehyde was generated from the keto-enol

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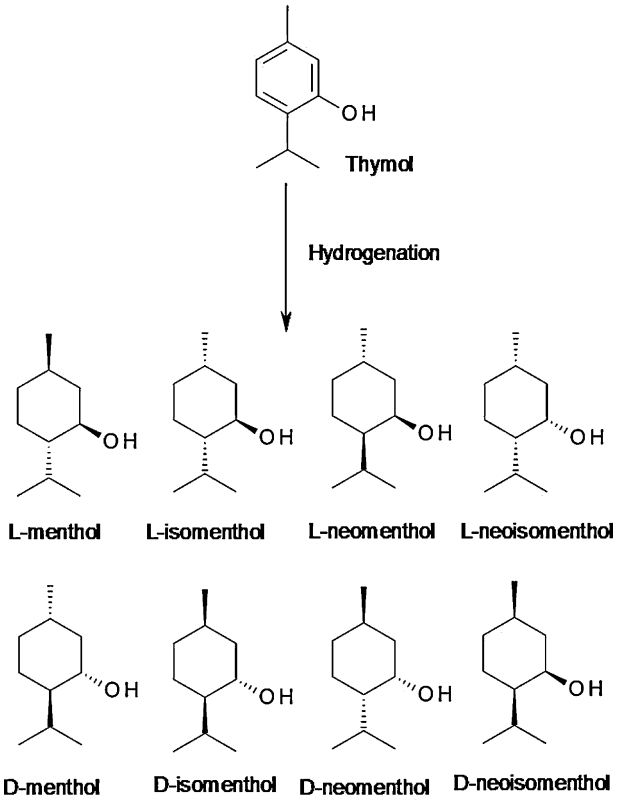


Scheme 1. Menthol diastereomers generated by hydrogenation of thymol.

tautomerisation of vinyl alcohol which is released from the vinyl acetate. The equilibrium for this reaction is far towards the acetaldehyde (K_{eq} 3×10^{-7} at 25 °C) [7], making the overall reaction practically irreversible and shifting the reaction equilibrium to completion.

2. Experimental

Chemicals. Bulk L-menthol (99.7%), DL-menthol (98%) and L-menthyl acetate (99.7%) were obtained from Haarman and Reimer (Symrise), Germany. Vinyl acetate (99%) was obtained from Makeean Polymers, SA; heptane (98%) from Servochem, SA. Isooctane, hexane, cyclohexane, and benzene were all of analytical grade (Servochem, SA). Other chemicals were supplied by Sigma–Aldrich or Fluka.

Liquid menthol was generated in-house by hydrogenation of thymol [8,9] and contained four diastereomeric pairs of menthols, namely DL-menthol (51%), DL-isomenthol (14%), DL-neomenthol (29%) and DL-neoisomenthol (2%), as well as 4% menthones.

Stock solutions of 40% (v/v) liquid menthol were prepared from 400 ml liquid menthol mixed with variable amounts of vinyl acetate (depending on the experiment), which was made up to a litre with heptane.

Lipase preparations. *Pseudomonas fluorescens* lipase was purchased from Biocatalysis (UK) or Fluka. Horse liver esterase and *Pseudomonas cepacia* lipase were obtained from Fluka. The preparations lipase AS, lipase G, lipase AYS, lipase FA15, lipase PS (*P. cepacia*), and lipase AK (*P. fluorescens*) were all sourced from Amano Enzyme Inc. (Japan). Wheat germ lipase, Chirazyme L4 and L6 were purchased from Boehringer Mannheim (Germany), ESL-001-01 was from Diversa (USA), and Carboxylesterase NP was kindly provided by DSM (Netherlands). Other enzymes used are listed in the text or as published previously [10].

2.1. Analytical methods

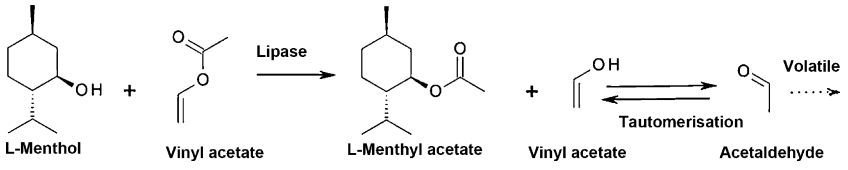
Quantitative Gas Chromatography (GC). A Stabilwax-DA wax column 30 m × 0.25 mm internal diameter (Restek, South Africa) was mounted in a Hewlett Packard 5890A gas chromatograph. The temperature profile was set as hold for 1 min at 70 °C, an increase of 5 °C per min for 8 min to 145 °C holding for 1 min. Injection port and flame ionisation detector were set at 250 °C. Detector gases: air at 250 kPa, H_2 at 110 kPa. Auxiliary gas: N_2 at 28 cm^3/min. With each set of samples a standard of L-menthyl acetate and DL-menthol was run. Run time was 17 min. Elution times (min) vinyl acetate 1.83; acetic acid 9.8; menthyl acetate 12.5; DL-menthol 14.2. Results are reported as percentage conversion of available DL-menthol:

$$\frac{\text{menthyl acetate}}{\text{menthol} + \text{menthyl acetate}} \times 100 = \%\text{Conversion} \quad \text{(mass/mass)}$$

Chiral GC method. Chrompack WCOT Fused Silica Column 25 m × 0.25 mm i.d. coating CP Chirasil-Dex CB, 0.25 μm film thickness. Carrier gas H_2 at 8 psi back pressure. Injection port and flame ionisation detector were set at 250 °C. Detector gases: air at 250 kPa, H_2 at 110 kPa. Auxiliary gas: N_2 at 28 cm^3/min. Oven 100 °C (isothermal). Split vent flow: 100 cm^3/min, septum purge: 3 cm^3/min. Run time: 30 min. The difference in concentration of the two menthyl acetate enantiomers as a percentage of the total menthyl acetate provided the enantiomeric excess (%ee_p), of the product menthyl acetate. This, in combination with the conversion, was used to calculate the enantiomeric ratio of the reaction. Elution times were (in min): L-neomenthyl acetate 12.5; L-isomenthyl acetate 13.5; D-neomenthyl acetate 13.8; D-isomenthyl acetate 14; L-menthyl acetate 14.5; DL-neoisomenthyl acetate 16.5 and 17; D-menthol 18; D-neomenthol 18.8; L-neomenthol 19.5; D-menthol 21.5; L-menthol 22.5; DL-neoisomenthol 22.5; L-isomenthol; D-isomenthol. The contribution by neoisomenthol to the peak at 22.5 min was subtracted from the L-menthol peak using the data from the non-chiral GC method.

$$\frac{(\text{L-menthyl acetate} - \text{D-menthyl acetate})}{(\text{L-menthyl acetate} + \text{D-menthyl acetate})} \times 100 = \%ee_p$$

Analytical standards included L-menthyl acetate (98% ex Aldrich Cat. No. 44, 105-8); L-menthol (99% Fluka Cat. No. 63660); D-menthol (>99% ex Fluka Cat. No. 63658); D-isomenthol (100% Aldrich Cat. no. 1-7757).



Scheme 2. Transesterification of L-menthol.

2.2. Determination of enzyme activity using the 1-phenylethanol transesterification assay (Amano standard assay).

Enzyme, 100 mg, was weighed out in quadruplicate into glass vials, to which 3 ml of a stock solution (20% (v/v) 1-phenylethanol and 80% (v/v) vinyl acetate) was added. The test tubes were incubated at 30 °C for 20 min with stirring. The reaction was stopped by placing the test tubes in ice water. The reaction products were then separated from the enzyme by centrifugation at 3000 rpm for 15 min. A sample of 0.2 ml of the supernatant was added to 0.8 ml acetone in a vial and then analysed for 1-phenylethanol and 1-phenethyl acetate by quantitative GC.

$$\frac{\text{1-phenethyl acetate}}{\text{1-phenylethanol} + \text{1-phenethyl acetate}} \times 100 = \%\text{Conversion}$$

Amano AK P. fluorescens lipase. A comparison of lipase batches using the *p*-nitrophenyl palmitate (*p*-NPP) assay, the Amano 1-phenylethanol assay and the menthol transesterification reaction showed variation in enzyme activity between batches. Hence adjustments in reaction enzyme load were made in the various reactions to compensate for this. The *p*-nitrophenyl palmitate hydrolysis assay was performed according to [11]. Lot LAKY05515 achieved 6.13% conversion (%*C*) by the 1-phenylethanol assay and exhibited 27,152 U/g by the Japanese Industrial Standard (JIS) method); Lot LAKY0950502 4.82%*C* (25,800 U/g by JIS); Lot LAKV07510 10.3%*C* (29,000 U/g by JIS); Lot LAKX09510 5.5%*C*.

2.3. Screening for lipase catalysed esterification of L-menthol

In 2 ml vials 1–10 mg lipase was incubated at 30 °C in 2 ml organic solvent (toluene, cyclohexane, hexane, pentane, decane, 2-propanol or heptane) containing 0.075 M L-menthol and 0.075 M of each acyl donor (vinyl acetate, butyric acid, octanoic acid or lauric acid). The reaction was stopped by centrifuging the enzyme out of suspension (3000 rpm for 15 min) and a sample from the supernatant spotted onto TLC plates for analysis. TLC analysis was performed on silica-60 plates using 90:10 hexane:ethyl acetate as the mobile phase. The plates were dipped in 0.5% $KMnO_4$ in acetone, and heat dried for spot colour development.

2.4. Comparison of Amano AK and PS lipases

Reactions (20 ml) were performed at 40 °C in the carousel reaction system (Radleys Starfish, Radleys, UK). Samples were taken after 24 h and analysed for menthol (% mass/mass) and for the ratio of D-menthol to L-menthol.

2.5. Reaction kinetic studies

To determine the influence of vinyl acetate concentration on reaction performance, duplicate reactions containing increasing concentrations of vinyl acetate (0–2.6 M) were conducted. An L-menthol concentration of 3.2 M (50%, m/v) was used with heptane as solvent. Amano AK enzyme, 151 mg, was added to each 5 ml reaction. All reactions were performed for 5 h at 50 °C by incubation in stirred silicon oil baths on magnetic stirrer hot plates (750 rpm).

A second set of reactions were performed under the same conditions with increasing concentrations of L-menthol 0–3.2 M (0–50%, m/v) and a fixed vinyl acetate concentration of 3.2 M.

2.6. Enzyme inactivation by reaction components

The enzyme was exposed to reactants or products, washed three times with heptane to remove the compound (with recovery by centrifugation at 3000 rpm for 15 min), and the residual activity of the enzyme determined by the Amano assay.

To reduce volatility of acetaldehyde or vinyl acetate during preparation, tubes were placed in ice water.

(i) *Menthyl acetate*. Duplicate reactions containing 20 mg/ml Amano AK, 0.7 M vinyl acetate and 0.32 M L-menthol were performed. The DL-menthyl acetate concentration in the 6 h reaction at 25 °C was varied from 0 to 0.64 M. Control experiments were prepared as above but without enzyme addition. The effect of this constituent was also evaluated using the Amano assay, where 1-phenylethanol (20%, v/v) was mixed with the menthyl acetate (0–0.64 M) and vinyl acetate (1.6–1.32 ml).

(ii) *Vinyl acetate*. Lipase (151 mg) was incubated with heptane and 0–3.5 M vinyl acetate (both dried over anhydrous magnesium sulphate) at 50 °C for 5 h.

(iii) *Acetaldehyde*. Lipase (100 mg) was added to glass reaction vials. The 1.6 M acetaldehyde stock solution consisted of 45.5 ml acetaldehyde made up to 500 ml with heptane. Acetaldehyde has a boiling point of 21 °C, and so to maintain the acetaldehyde in solution during the incubation period, all test tubes were held at 15 °C with agitation over an extended period of 2 weeks.

(iv) *Acetic acid*. Lipase (20 mg) was incubated with 0–0.4 M acetic acid in heptane at 25 °C.

2.7. Reaction enantioselectivity

The effect of the presence of the other 6 isomers (DL-isomenthol, DL-neomenthol and DL-neoisomenthol) was determined by comparing 40% (m/v) of the 8 isomer mix as starting material to reactions using 10% synthetic DL-menthol. After addition of vinyl acetate (2.65 ml) the reaction was topped up with heptane to 10 ml. Enzyme loads of 0.3 and 0.6 were compared at 40 °C with stirring over four reaction cycles in Multi-Reactor™ (Robo Synthon, CA, USA). These consisted of jacketed reactor vessels that housed up to 16 × 50 ml test tubes, and was fitted with a temperature control unit and a magnetic stirrer. The test tubes were closed with a Teflon cap.

Isomenthol enrichment was achieved using a DL-isomenthol and DL-menthol mixture distilled from liquid menthol, and then adding DL-menthol to achieve various ratios of DL-isomenthol and DL-menthol.

The influence of the ratio of D-menthol to L-menthol was investigated by blending DL-menthol and L-menthol. The reactions were performed with 26% (m/m) total menthol, 337.5 mg Amano AK, and 1 equiv. of vinyl acetate with respect to total menthols present.

2.8. Reaction optimisation

A statistically designed set of experiments was drawn up using the Design Ease software package (Stat-Ease Inc., MN, USA) in order to study the effect of variables on the conversion and enantioselectivity of the lipase catalysed esterification of DL-menthol. A 2^4 (half factorial) design was drawn up with ranges for four variables: temperature, DL-menthol and vinyl acetate concentration, and enzyme loading (mg of enzyme per g of DL-menthol). Each of the reactions of the statistical design was carried out on a 250–300 g scale in the Maxi-Reactor™ (Robo Synthon, CA, USA) that consisted of 6 × 450 ml jacketed reactor vessels, with individually controlled temperature and agitation units. The agitation was provided by bi-convex-shaped magnetic stirrer bars.

2.9. Optimum molar ratio of vinyl acetate to L-menthol

Amano AK lipase (151 mg) was added to glass reaction vials. For each of the ratios evaluated, the DL-menthol concentration in

Table 1
Screening of selected enzymes for esterification of menthol with mixed acyl donors.

Enzyme	Solvent						
	Acyl donor	Toluene	Cyclohexane	Hexane	Pentane	Decane	2-Propanol	Heptane
Hog pancreas lipase	Vinyl acetate	–	ND	+	ND	–	ND	–
Fluka	Butyric acid	++	ND	–	ND	+	ND	+
Cat No. 62300	Octanoic acid	++	ND	–	ND	+	ND	–
	Lauric acid	++	ND	–	ND	–	ND	–
Hog liver Esterase	Vinyl acetate	ND	ND	–	–	–	ND	
Boehringer	Butyric acid	ND	ND	–	ND	–	ND	
	Octanoic acid	ND	ND	++	ND	+	ND	
	Lauric acid	ND	ND	–	–	–	ND	
Porcine pancreas	Vinyl acetate	ND	ND	ND	–	–	–	ND
Chirazyme L6	Butyric acid	ND	ND	ND	–	+	–	ND
Boehringer	Octanoic acid	ND	ND	ND	++	++	+	ND
	Lauric acid	ND	ND	ND	+	–	++	ND
Aspegillus oryzae lipase	Vinyl acetate	–	ND	+	ND	–	ND	–
Fluka	Butyric acid	–	ND	–	ND	+	ND	–
Cat No. 62285	Octanoic acid	+	ND	+	ND	+	ND	–
	Lauric acid	++	ND	–	ND	+	ND	+
Rhizomucor miehei lipase	Vinyl acetate	+	++	–		–	–	+
Fluka	Butyric acid	–	–	–	–	++	–	ND
Cat No. 62291	Octanoic acid	+	++	–	–	+	–	ND
	Lauric acid	+	+	–	+	–	ND	ND
Rhizopus japonicus	Vinyl acetate	–	++	–	+	ND	ND	ND
Nagase enzyme lipase A-10FG	Butyric acid	–	–	–	++	ND	ND	ND
	Octanoic acid	++	+	+	–	ND	ND	ND
	Lauric acid	++	++	–	++	ND	ND	ND
Candida antarctica lipase	Vinyl acetate	+	ND	ND	ND	ND	ND	ND
Fluka	Butyric acid	++	ND	ND	ND	ND	ND	ND
Cat No. 62299	Octanoic acid	–	ND	ND	ND	ND	ND	ND
	Lauric acid	–	ND	ND	ND	ND	ND	ND
Candida antarctica fraction A	Vinyl acetate	++	++	ND	ND	+	–	ND
Chirazyme L5	Butyric acid	–	–	ND	ND	–	–	ND
Boehringer	Octanoic acid	+	++	ND	ND	–	+	ND
	Lauric acid	–	–	ND	ND	+	–	ND
Candida cylindracea lipase	Vinyl acetate	–	+	+	+	–	–	ND
Fluka	Butyric acid	–	–	–	–	+	–	ND
Cat No. 62316	Octanoic acid	++	++	–	+	+	–	ND
	Lauric acid	–	–	–	+	+	+	ND
Chromobacterium viscosum	Vinyl acetate	++	ND	ND	ND	ND	ND	ND
lipoprotein lipase	Butyric acid	++	ND	ND	ND	ND	ND	ND
Fluka	Octanoic acid	–	ND	ND	ND	ND	ND	ND
Cat No. 62333	Lauric acid	+	ND	ND	ND	ND	ND	ND
Burkholderia Chirazyme L1	Vinyl acetate	ND	+	+	++	+	–	ND
Boehringer	Butyric acid	++	–	–	–	+	+	ND
	Octanoic acid	+	++	–	+	–	+	ND
	Lauric acid	+	–	–	+	–	++	ND
Pseudomonas cepacia	Vinyl acetate	++	ND	–	+	–	ND	ND
lipoprotein lipase	Butyric acid	–	ND	–	+	–	–	ND
Fluka	Octanoic acid	++	ND	–	–	++	+	ND
Cat No. 62309	Lauric acid	–	ND	–	–	–	–	ND
Pseudomonas sp. Chirazyme L4	Vinyl acetate	–	–	ND	+	ND	–	+
Boehringer	Butyric acid	–	++	ND	–	ND	+	–
	Octanoic acid	–	+	ND	+	ND	+	++
	Lauric acid	+	–	ND	–	ND	++	–
Pseudomonas. fluorescens lipase	Vinyl acetate	–	++	ND	ND	–	–	+
Fluka	Butyric acid	–	–	ND	ND	+	–	+
Cat No. 62312	Octanoic acid	+	–	ND	ND	+	–	+
	Lauric acid	++	–	ND	ND	++	–	+
Recombinant ex *Bacillus thai*	Vinyl acetate	–	ND	ND	ND	ND	ND	–
carboxylesterase NP	Butyric acid	–	ND	ND	ND	ND	ND	+
DSM	Octanoic acid	ND	ND	ND	ND	ND	ND	–
	Lauric acid	–	ND	ND	ND	ND	ND	–
Metagenome	Vinyl acetate	+	+	+	+	+	++	+
ESL-001-01	Butyric acid	–	–	ND	–	ND	–	ND
Recomb. Biocat.	Octanoic acid	+	++	++	–	++	–	++
	Lauric acid	+	–	ND	–	ND	+	ND

"–", "+" and "++" represent no activity, some activity, and strong activity, respectively.
ND = Not Determined.

Table 2
A comparison between the performance of Amano lipases AK and PS on the esterification of DL-menthol at 24 h.

Reaction cycle	Enzyme	Substrate concentration (%)	Conversion (%)	ee (%)	*E*	Menthol mol balance (%)
	None	40	0.1	0	1.0	100
1	PS	10	0.8	74.2	6.8	104
1	PS	25	1.3	79.4	8.8	108
1	PS	40	1.5	78.4	8.4	111
2	PS	10	1.0	75.4	7.2	104
2	PS	25	1.5	79.6	8.9	104
2	PS	40	1.5	79.8	9.0	106
1	AK	10	5.5	93.2	30.0	101
1	AK	25	13.1	95.6	51.2	106
1	AK	40	15.2	95.6	52.6	109
2	AK	10	10.3	95	43.4	102
2	AK	25	20.5	96	62.4	104
2	AK	40	21.3	96.1	64.8	101

heptane was maintained at 2.28 M (made up to 20 ml) and the vinyl acetate to L-menthol molar ratio was varied from 0.25:1 to 3:1. The test tubes were incubated at 50 °C. Each resolution was performed in quadruplicate.

2.10. Small scale enzyme recycle

Glass test tubes (10 ml) with solvent resistant Teflon caps and containing magnetic stirrer bars were used for small scale batch recycles. To the tubes were added 5 ml of a 20% menthol stock solution and 100 mg of the enzyme. The initial vinyl acetate to L-menthol ratio for the transesterification reaction was 2:1. Incubation was at 50 °C for 24 h.

2.11. Batch stirred tank reactions

Thermally insulated glass vessels (2.2 L) with water heating jackets and glass baffles (Glasstek, South Africa) were connected to circulating water baths set at 50 ± 0.5 °C. The reactors were charged with 2 L of reaction mixture, which consisted of vinyl acetate 5.6% (v/v) (2:1 molar ratio to L-menthol), liquid menthol 20% (v/v), heptane (to volume) 74.4% (v/v), plus 25 g/L enzyme (Amano AK, Lot LAX09510). Agitation at 450 rpm for 23 h was achieved with magnetic stirrer bars or Heidolph R2R 2021 overhead stirrers fitted with 2 radial flow impellers, thereby keeping the enzyme in suspension.

Filtration was more practical than centrifugation at large scale, and hence the stirrers were stopped to let the enzyme settle from suspension, and a sintered filter (porosity 3: 16–40 μm pore size, 6 cm diameter) was suspended in the organic phase. This was connected to a vacuum pump (at 60–80 kPa) and used to remove the liquid. The enzyme was washed with 100 ml heptane and then re-charged with reaction mixture for the next reaction cycle. The heptane from the wash step was combined with the product mixture.

3. Results and discussion

3.1. Screening for lipase catalysed esterification of L-menthol

Enzymes were evaluated for the formation of menthol esters in the presence of a mixture of acyl donors (Table 1). The promising thermostable enzyme ESL-001-01 was evaluated at the 1 ml scale (with either vinyl acetate or octanoic acid as an acyl donor) in a range of solvents and at three temperatures (30, 50 or 70 °C), but could not provide an *E* above 7 (data not shown). When vinyl acetate in combination with toluene was used at 30 °C, an ee of 74.6% was achieved but at a conversion of only 1.4% and an *E* of 6.9. The same enzyme with octanoic acid in decane gave, at best, 26.2% conversion and an ee of only 28.6% (an *E* of 1.9). Hence investigation into this enzyme was discontinued. Animal extract derived enzymes were not investigated further due to considerations relating to use for food products.

Three of the positive reactions were catalysed by enzymes from *Pseudomonas* species, which are known to be selective for L-menthol [4,5], while two of the preparations were from *Candida* species, which are also known to be selective for this substrate [12]. Comparison of *Pseudomonas* sp. lipases from different suppliers during the esterification of menthol by vinyl acetate was performed. *P. cepacia* lipase from Fluka and Amano showed differences in the esterification of menthol (12 and 17%, respectively). *P. fluorescens* lipase from Biocatalysis did not esterify menthol, while *P. fluorescens* lipase preparation from Fluka did (19%). The *Pseudomonas* lipase L4 from Boehringer was less effective (12%). Such differences could be due to the strains employed by various manufacturers. Due to their availability in industrial quantities, crude preparations of *P. fluorescens* lipase (Amano AK) and *P. cepacia* (Amano PS) were evaluated further.

3.2. Comparison of Amano AK and PS lipases

The *P. cepacia* (PS) lipase from Amano has previously been demonstrated to have a high enantioselectivity towards L-menthol in this and similar reactions [5,13]. However, in 20 ml reactions involving DL-menthol and vinyl acetate, the Amano AK enzyme resulted in a higher enantiomeric excess of L-menthol (Table 2), and was therefore selected for all further studies. Heptane was selected as the solvent of choice due to low neurotoxic properties [14] and a suitable partition coefficient [15].

The optimum Amano AK lipase concentration required to produce almost 50% conversion of 12% (v/v) DL-menthol was established in 5 ml reactions. The enzyme concentration of 1.2 g per % liquid menthol in a litre reaction (Fig. 1) provided near complete conversion and was chosen for future experiments.

3.3. Reaction kinetic studies

For vinyl acetate (the co-substrate), the enzyme K_M was determined to be 0.33 M (2.8%, m/v). Therefore to avoid acyl donor based kinetic limitations, enough vinyl acetate should be included in the initial reaction mixture to leave a residual of 0.7 M (5.6%) upon complete conversion of the L-menthol.

The K_M value was 1.13 M for L-menthol (17.6%, m/v) (Fig. 2). This indicates that the enzyme activity would be limited by the L-menthol concentration throughout the transesterification reaction of 40% (m/v) liquid menthol. The maximum specific activity of the

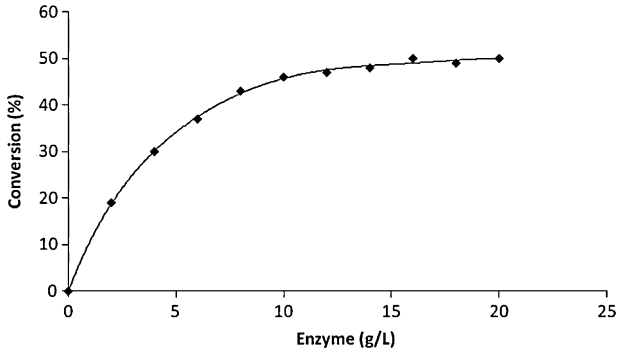


Fig. 1. Transesterifcation of 12% (v/v) liquid menthol with varying enzyme concentrations.

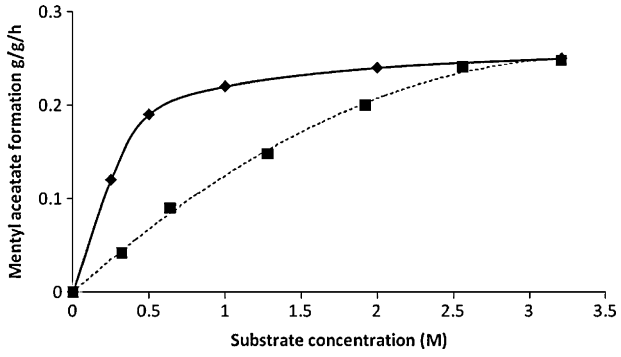


Fig. 2. Specific activity of Amano AK with vinyl acetate (♦) and L-menthol (■) concentration determined in excess of the co-substrate.

biocatalyst was 0.25 g menthyl acetate/g enzyme preparation/h on L-menthol.

A comparison of reaction rates on L-menthol, DL-menthol and liquid menthol up to L-menthol levels of 1.3 M was also conducted. The reaction rates with L- and DL-menthol were similar at comparative L-menthol concentrations, indicating no significant interference by the D-enantiomer on the initial rate of reaction. However, the study with liquid menthol indicated that the apparent specific activity on liquid menthol was at least 40% lower than was observed with L-menthol (data not shown). This suggests that while the other menthol isomers were poor substrates for this enzyme, some competition for binding at the active site may occur.

3.4. Enzyme inactivation by reaction components

The enzyme was incubated with reaction matrix components, washed with heptane, and the residual enzyme activity measured by the Amano assay.

(i) *Menthyl acetate*. There appeared to be a 72% increase in activity on addition of 0.64 M DL-menthyl acetate to the reaction. This effect was confirmed using the Amano assay with a 30% increase in activity at the same concentration.
(ii) *Vinyl acetate* up to concentrations of 3.5 M did not appear to be toxic to the enzyme.
(iii) *Acetaldehyde*. A 20% loss in residual enzyme activity was observed after 48 h incubation in 0.3 M acetaldehyde at 15 °C. Although the denaturing effect would probably increase with temperature, so would the removal of acetaldehyde due to volatility (in a ventilated reactor).

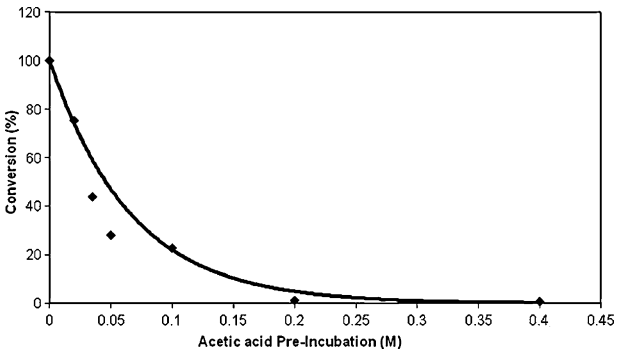


Fig. 3. Residual activity of Amano AK (as measured by the Amano assay) after exposure to increasing acetic acid concentrations in heptane at 25 °C.

(iv) *Acetic acid*. Acetic acid formation was previously detected in the transesterification reactions. This experiment indicated that addition of acetic acid had a significant inactivating effect on the enzyme (Fig. 3). Over 20% of enzyme activity was lost after incubation for 20 h in 0.02 M acetic acid. No residual enzyme activity was displayed after exposure to 0.2 M acetic acid under these conditions. As this dramatic loss of activity was not observed to occur with incubation with vinyl acetate, it can be assumed that acetic acid formation occurs through the water-mediated hydrolysis of menthyl acetate. Available sources of water for the reaction are the water associated with the biocatalyst (up to 10% (m/m) in spray- and freeze-dried enzyme preparation) and a low level of water in the reaction components and bulk solvent. Due to the severity of the denaturing effect of acetic acid on the activity of the enzyme, the production of acetic acid, and therefore water addition, must be limited under process conditions. At a reaction temperature of 50 °C the rate of denaturation in the presence of acetic acid could be anticipated to completely denature the enzyme. However, the biocatalyst exhibits significantly greater stability under process conditions than would be expected from this experiment, indicating that other factors are involved.

3.5. Reaction enantioselectivity

To evaluate whether the other menthol isomers influenced the enantioselectivity of the reaction, two experiments were performed. Firstly, the isomenthol component was artificially enhanced, but this did not influence reaction enantiomeric excess (Table 3). Having eliminated the influence of isomenthol, it was possible to determine the effect of neomenthol by comparison of DL-menthol with liquid menthol (which contained a significant proportion of neomenthol (29%) as well as isomenthol and traces of isoneomenthol). As can be seen (Table 4), the presence of the other 6 isomers had little effect on the enantioselectivity.

Increasing the amount of D-menthol present reduced the ee of the L-menthyl acetate formed (Table 5), falling below 95% ee when the starting L-menthol concentration was only 25% of the total DL-menthol. In a reaction this would occur at 30% conversion of the DL-menthol.

3.6. Reaction optimisation

Analysis of the results of the statistically designed set of experiments (Table 6) indicates that increased temperature had the largest negative effect on the enantiomeric ratio (Fig. 4), resulting from the decrease in the enantiomeric ratio at higher conversion. This effect has been previously observed [5,16]. By comparison, vinyl acetate concentration (or molar ratio of vinyl acetate to

Table 3
The effect of isomenthol concentration on the enantiomeric excess.

DL-Menthol (%, m/m)	Isomenthol (%, m/m)	Isomenthol (% liquid menthol)	DL-Menthol conversion (%)	ee (%)	*E*
40.0	0.0	0.0	20.3	96.7	75.8
49.8	2.8	5.2	17.3	96.9	77.5
49.5	4.6	8.6	17.5	96.8	75.2
46.9	9.1	16.2	17.4	97.0	80.1
47.3	10.3	17.8	20.0	97.0	83.2
22.4	19.6	47.0	17.7	97.2	86.4

Table 4
Enantioselective L-menthylacetate synthesis.

Substrate	Enzyme mass (mg)	Cycle 1		Cycle 2		Cycle 3		Cycle 4		Average %*C*	Average %ee	Average *E*
		%C	%ee	%C	%ee	%C	%ee	%C	%ee			
10% DL-menthol	302	21.6	96.7	41.2	95.7	38.6	95.6	39.1	95.4	35.1	96	83
10% DL-menthol	604	22.4	97.1	48.4	94.6	50.8	93.4	49.0	93.6	42.7	96	81
40% liquid menthol	302	17.8	97.2	31.7	96.3	32.8	96.1	30.9	96.1	28.3	95	102
40% liquid menthol	604	23.8	97.1	46.2	94.8	47.4	94.0	47.4	94.0	41.2	95	90

%C: conversion of L-menthol to L-menthyl acetate.

Table 5
The effect of menthol enantiomeric ratio on the conversion and enantioselectivity of the Amano AK lipase catalysed acetylation of menthol.

Initial L-menthol as % of total DL-menthol	% Menthyl acetate formed (40 °C)					
	8 h			24 h		
	L-Menthyl acetate	D-Menthyl acetate	%ee	L-Menthyl acetate	D-Menthyl acetate	%ee
100	15.7	0	100	48.15	0	100
75	17.5	0.09	99	54.5	0.33	99
60	18.5	0.2	98	57.4	0.75	97
50	19.7	0.3	97	59.75	1.165	96
25	23.7	1.07	91	68.4	4.35	88
0	0	0.83	0	0	2.85	0

menthol) only had a small positive effect on reaction enantioselectivity. The data also showed that the enzyme reaction could be performed at 50 °C and provide a 35% conversion of DL-menthol, while maintaining an ee of 95%, and an *E* of 64.

3.7. Optimum molar ratio of vinyl acetate to L-menthol

Similar to the reaction optimisation study, Fig. 5 shows that ratios of 1:1 to 3:1 vinyl acetate to menthol demonstrated similar initial results during the transesterification of DL-menthol to menthyl acetate, but the final product increased about 25% at ratios of 2.5:1 and 3:1, giving about 80% conversion of the L-menthol, and providing a kinetic advantage towards the end of the reaction. Although the kinetic data showed that the enzyme had a higher affinity for vinyl acetate than for menthol, excess vinyl acetate is required to optimise the reaction rate and to compensate for volatilisation into the reactor headspace.

3.8. Small scale enzyme recycle

To determine the recyclability of the enzyme, the Amano AK lipase was reused 150 times (Fig. 6) achieving an average concentration of 171 mM menthyl acetate and an overall yield of 184.3 g

Table 6
Statistically designed experiments for reaction optimisation.

Run	DL-Menthol concentration (%, m/m)	Temperature (°C)	Enzyme (mg/g)	Vinyl acetate:menthol ratio	DL-Menthol conversion (%)	ee_p%	*E*
1	60	35	100	1:1	25.9	96.3	73.7
2	20	35	50	1:1	12.9	93.0	31.6
3	60	50	50	1:1	32.9	94.3	53.9
4	60	35	50	2:1	16.6	96.1	60.6
5	20	50	100	1:1	34.3	94.7	60.1
6	20	35	100	2:1	18.4	97.9	117.0
7	20	50	50	2:1	24.4	96.1	68.1
8	60	50	100	2:1	42.8	93.8	65.8
9	40	42.5	75	1.5:1	30.2	96.6	87.3
10	40	42.5	75	1.5:1	29.2	96.7	88.2
11	20	35	50	1:1	13.8	97.8	104.8
12	20	35	50	1:1	14	97.8	105.1
13	20	35	100	1:1	20	97.8	114.2
14	60	50	100	1:1	42.8	93.8	65.8
15	20	50	100	2:1	31.9	95.6	69.2
16	60	35	100	2:1	26	97.4	106.1

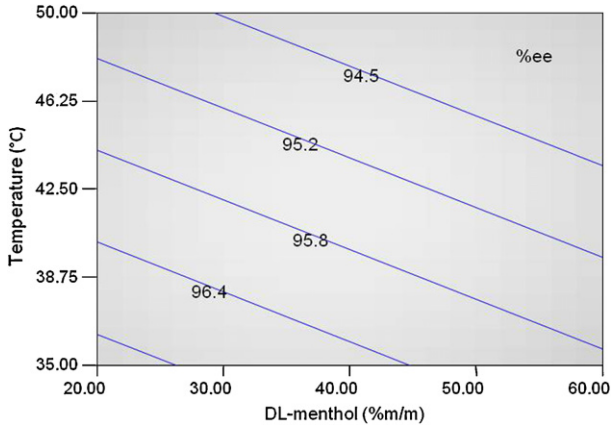


Fig. 4. Interaction between temperature, DL-menthol concentration, and enantiomeric excess. Enzyme loading at 100 mg/g.

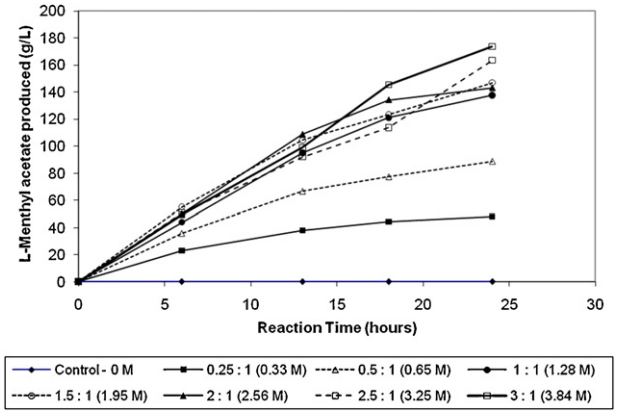


Fig. 5. The effect of varying the molar ratio of vinyl acetate to L-menthol during transesterification in the presence of 2.6 M (40%, m/v) DL-menthol.

L-menthol/g enzyme preparation. This sustained activity was possible as lipases tend to be more stable in organic solvents than in aqueous media [17]. The conversion for the first cycle was lower than the initial recycle, a phenomenon that was frequently observed, and possibly due to menthyl acetate hydrolysis caused by unbound water associated with the enzyme [16]. This resulted in the formation of an average of 54 mM acetic acid in the reaction mixture.

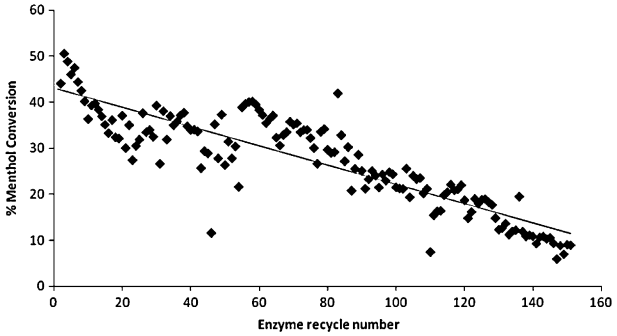


Fig. 6. Batch recycles of Amano AK lipase enzyme at 50 °C. Low conversion for recycle 45 was due to malfunction of temperature control.

Table 7
Bench scale batch stirred tank enzyme recycle experiments.

Reaction recycle	Conversion (%, m/m)					Std. dev.
	BSTR 1	BSTR 2	BSTR 3	BSTR 4	Mean	
1	25.67	26.02	24.80	24.53	25.25	0.71
2	30.64	31.44	31.96	31.94	31.50	0.62
3	34.45	35.61	33.37	33.50	34.23	1.03
4	38.30	35.84	33.56	33.84	35.39	2.19
5	24.49	28.71	28.48	28.40	27.52	2.03
6	33.45	27.49	33.67	33.39	32.00	3.01
7	34.37	29.14	27.58	28.70	29.95	3.02
8	34.37	30.27	29.69	29.63	30.99	2.27
9	ND	31.12	29.75	30.47	30.44	0.69
10	33.57	29.46	ND	29.49	30.84	2.36
11	37.52	34.97	28.53	35.33	34.09	3.87
12	38.04	39.05	38.74	36.78	38.15	1.01
13	38.40	34.88	39.25	38.14	37.67	1.92
14	22.54	22.67	23.68	23.90	23.20	0.69
15	30.18	22.23	24.04	26.00	25.61	3.41
16	30.18	27.73	28.88	30.77	29.39	1.36
17	34.4	32.11	31.91	30.13	32.14	1.75
18	32.67	30.2	29.8	30.74	30.85	1.27
19	33.76	31.2	32.42	32.47	32.46	1.05
20	33.59	33.65	30.25	20.65	29.54	6.13
21	30.75	28.42	30.27	30.78	30.06	1.11
22	30.68	29.04	29.2	29.15	29.52	0.78
23	31.61	29.7	29.65	31.24	30.55	1.02
24	ND	ND	29.28	32.09	30.69	1.99
25	29.23	27.61	28.41	29.04	28.57	0.73
26	29.53	28.18	28.62	29.19	28.88	0.60
27	ND	28.37	26.01	29.43	27.94	1.75
28	27.63	24.62	25.97	25.73	25.99	1.24
29	27.78	30.14	29.5	29.81	29.31	1.05
30	29.55	28.75	29.01	29.92	29.31	0.53
31	29.96	28.71	28.2	28.8	28.92	0.74
32	27.52	28.19	27.8	28.31	27.96	0.36
33	25.01	24.7	28.67	25.31	25.92	1.85
34	26.52	27.28	27.45	27.94	27.30	0.59
35	23.59	23.02	24.84	24.96	24.10	0.95
36	23.44	20.97	21.12	27.43	23.24	3.01
37	21.58	21.91	21.38	28.46	23.33	3.43
38	19.99	20.4	20.08	26.99	21.87	3.42
Average	30.14	28.75	28.81	29.56	29.33	1.72

3.9. Bench scale batch stirred tank reactions

Having determined that the ee was >95% at 30% conversion of DL-menthol in the liquid menthol (i.e. 15.3% of the total menthols) during reaction optimisation (above) we then ran a set of four reactors in batch mode with enzyme recycle, wherein the target was limited to 30% conversion of DL-menthol. To limit the conversion to an average of 30% the enzyme load was reduced to 25 g/L, as calculated from the data (Fig. 1). Although there is some variability in the conversion (Table 7), this correlated with variations in the batches of the liquid menthol and agitation. The average L-menthyl acetate volumetric productivity was 1.2 g/L/h.

Enzyme losses occurred due to formation of airborne dust by the enzyme powder during manipulations of the enzyme between reaction cycles, and adhesion on the sintered glass filters during filtration. These losses were in the order of 25 mg (0.1%) per recycle. In addition a proportion of the enzyme activity was lost due to thermal and physical denaturation. In spite of these losses, the activity remained relatively constant over extended periods, with an average conversion of L-menthol of 59%.

This reaction provided an enantiomeric ratio (E) of >50. Importantly when 95% of the L-menthol in the liquid menthol was esterified, the bulk of the other menthol isomers remained unesterfied. Only 4% of the D-menthol, 2.5% of the DL-neomenthol, and 3.5% of the DL-isomenthol were esterified. A much higher portion of the DL-neoisomenthol (18.5%) was esterified, although this was

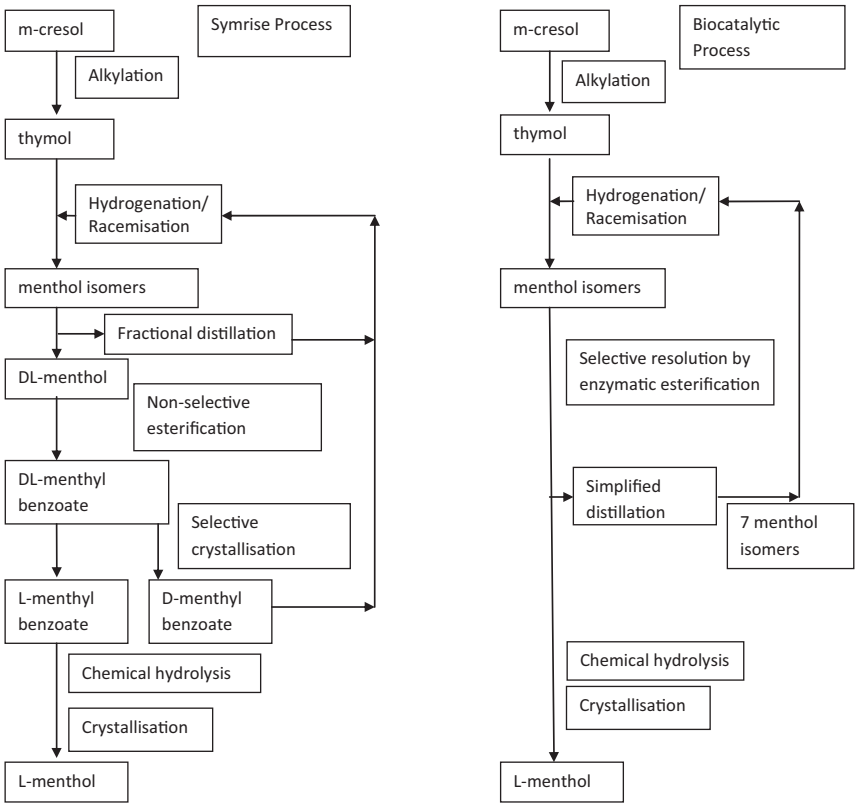


Fig. 7. Comparison of the Symrise process and the process described here.

insignificant as DL-neoisomenthol typically comprised only 2–3% of the liquid menthol. This yielded a diastereomeric excess (de) of 82% of L-menthol per total menthol.

After the reaction, the mixture can be distilled to remove the L-menthyl acetate which can be hydrolysed in a 70:30 ethanol:water mixture with 1.1 equiv. of sodium hydroxide and the resultant L-menthol isolated by crystallisation. The toxic acetaldehyde generated is vacuum distilled and combusted. The other 7 menthol isomers (and residual L-menthol) can be recycled back to liquid menthol through a racemisation reaction, ensuring complete use of the material. The menthol isomers were isomerised and racemised, over the same nickel catalyst used for thymol hydrogenation, in a slurry reactor at 200 °C and 0.6 MPa H_2 [9,18].

This process contains fewer and simpler unit operations than the original Symrise process on which it is based (Fig. 7). Another interesting biocatalytic variation of the Symrise process involves highly selective *Candida rugosa* lipase based hydrolysis of the L-enantiomer of DL-menthyl benozoate [19].

4. Conclusion

In this research we demonstrated the selective esterification of L-menthol from an 8 isomer mixture. Amano AK in heptane catalysed the transesterification of L-menthol with vinyl acetate at a significantly higher rate than D-menthol resulting in menthyl acetate enriched in the L-enantiomer. The presence of isomenthol or neomenthol had no significant effect on either the rate of menthol conversion or the final ee of the menthyl acetate. Although the presence of the other isomers reduced conversion, this could be compensated by increasing the enzyme load. The reaction by-products acetaldehyde and, particularly, acetic acid were shown to inactivate the enzyme. Interestingly, the strength of this effect seems to be reduced during operational studies, suggesting that the substrate may stabilise or protect the enzyme. Menthyl acetate hydrolysis can also reduce overall reaction yields on vinyl acetate and the reaction rate, and hence the water available in the reaction mixture must be kept to a minimum.

The reaction was successfully scaled up by a factor of 400 (from 5 ml to 2 L) in batch stirred tank reactors at 50 °C and the enzyme was recycled a total of 38 times at this scale. The free enzyme can convert 30% of the DL-menthol with an enantiomeric ratio of 65 and a diastereomeric excess (de) of 82%.

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