Biocatalytic enantiomeric resolution of *l*-menthol from an eight isomeric menthol mixture

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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 hexane, hence simplifying separation from this diasteromeric mix through distillation.

From an initial screen of over 70 enzyme preparations, a commercial *Pseudomonas fluorescens* lipase (Amano AK) was selected, and vinyl acetate was chosen as a suitable irreversible acyl donor for transesterification. The enzyme was recycled a total of 150 times in 5 ml batch reactions using liquid menthol and achieving an overall yield of 184.31 g *dl*-menthol/g enzyme.

An enantiomeric excess 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 ratio of 81%. The resolution reaction was scaled

up 400 fold to 2 L and the ezyme recycled 38 times with an average conversion of the available *l*-menthol of 59%.

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 (McCoy, 2010). 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*) grown around the world. However, menthol is also obtained synthetically via a number of routes, one of which is via hydrogenation of thymol. Unfortunately this 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 (Kirk et al 2002). In kinetic enantiomeric resolution reactions lipases have been demonstrated to effectively transform *l*-menthol without converting the corresponding *d*-enantiomer, for instance Wu et al (1996, 1997) were able to isolate *l*-menthol from *dl*-menthol by esterification with vinyl acetate or vinyl propionate in the presence of an organic solvent, while Gray et al (1990) used tributyrin or triacetin as acyl donors. Physical isolation of *l*-menthyl ester from other menthol isomers (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 diastereomer liquid menthol mixture by means of lipase catalysed transesterification in organic solvent (Scheme 2). During this reaction, acetaldehyde was produced as a by-product from the

tautomerisation of vinyl alcohol which is formed from the vinyl acetate. The volatile acetaldehyde is lost from the reaction mixture, which makes the reaction irreversible and hence shifts the reaction equilibrium towards reaction completion.

2. Methods

Chemicals: Bulk *l*-menthol (99.7%), *dl*-menthol (98%) and *l*-menthyl acetate (99.7%) were obtained from Haarman and Reimer, Germany. Vinyl acetate (99%) was obtained from Makeean polymers, SA, heptane (98 %, *n*-heptane content of 37.5%) from Servochem, SA. Isooctane, hexane, cyclohexane, benzene were all of analytical grade (Servochem, SA). Other chemicals were supplied by Sigma-Aldrich and Fluka.

Liquid menthol was generated in-house by hydrogenation of thymol (Dudas and Hanika, 2009; Dudas et al 2005) 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 and 109.2 ml vinyl acetate, which was made up to a litre with heptane. Lower percentages were prepared in the same way maintaining the molar ratio of vinyl acetate to *l*-menthol at 2:1 and increasing the percentage of heptane added.

Lipase preparations: *Pseudomonas fluorescen*s lipase was purchased from Biocatalysis (UK). Horse liver esterase, *P. cepacia* and *P. fluorescens* lipases were obtained from Fluka. Lipase AS, lipase G, lipase AYS, lipase FA15, lipase PS (*P. cepacia* lipase), and lipase AK (*P. fluorescen*s) were all sourced from Amano Enzymes (Japan). Wheat germ lipase, Chirazyme L4 and L6 were purchased from Boehringer (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 in Steenkamp and Brady (2003).

Analytical methods

Quantitative Gas Chromatography (GC): A Stabilwax-DA wax column 30 metre x 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, and hence giving a run time of 11 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 menthol was run. Run time was 17 min. Elution times (min) vinyl acetate 1.83; acetic acid 9.8; menthyl acetate 12.5; menthol 14.2. Results are reported as percentage conversion of available *dl*-menthol. Conversions were calculated as such:

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

Analytical standards: *l*-menthyl acetate (98% ex Aldrich Cat. no. 44, 105-8); *l*-menthol (99% ex Fluka Cat. no. 63660); *d*-menthol (>99% ex Fluka Cat. no. 63658); *d*-isomenthol (100% ex Aldrich Cat. no. 1-7757).

Chiral GC Method (%ee): Chrompack WCOT Fused Silica Column 25 m x 0.25 mm i.d. coating CP Chirasil-Dex CB, 0.25 µm film thickness. Carrier gas Hydrogen 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 minutes. The difference in concentration of the two menthyl actate 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*-mentyl 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.

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

Determination of enzyme activity using the 1-phenethyl alcohol transesterification assay (Amano Assay).

Enzyme, 100 mg, was weighed out in quadruplicate into glass vials, to which 3 ml of a stock solution (20% v/v 1-phenethyl alcohol, 80% v/v vinyl acetate) was added. The test tubes were incubated at 30°C for 20 minutes 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 minutes. 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-phenyl acetate by quantitative GC.

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

Amano AK *P. fluorescen*s lipase: A comparison of batches of lipase by the *p*-nitrophenyl palmitate (*p*-NPP) assay, the Amano 1-phenylethanol assay and the menthol transesterification reaction at the 5 ml scale 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 Vorderwülbecke, *et al* 1992. Lot LAKY05515 achieved 6.13 % conversion (%C) by the 1-Phenyl ethyl ethanol 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 000U/g by JIS); Lot LAKX09510 5.5% C.

Esterification Screening of Lipases

In 2 ml vials 10 - 100 mg lipase was weighed. To this 962 µl of an organic solvent (pentane, hexane, heptane, isooctane, decane, cyclohexane or benzene) and 23 µl liquid menthol was added. The acyl donor (14 µl of vinyl acetate, butyric, valeric or octanoic acid was added) to make the final reaction volume up to 1 ml. These vials were incubated near the enzyme thermal optimum (23 30, 37 or 70°C, depending on the enzyme) for 48 hours. The reaction was stopped by centrifuging the enzyme out of suspension (3000 rpm for 15 minutes) 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.

Enzyme recycle

Glass test tubes (10 ml) with solvent resistant Teflon caps containing magnetic stirrer bars were used for the small scale batch recycles. To the tubes was added 5 ml of a 20% menthol stock solution and 100 mg of the enzyme. These glass test tubes were incubated in a bath containing silicon oil maintained at 50°C by heating stirrer plates. Batch time, unless otherwise stated, was 24 h. The initial vinyl acetate to *l*-menthol ratio for the transesterification reaction was 2:1, and heptane was the solvent.

Reaction Enantioselectivity

Multi Reactors: The Multi-Reactor™ (Robo Synthon, CA, USA) consisted of jacketed reactor vessel that housed up to 16 x 50 ml test tubes, and was fitted with a temperature control unit and a magnetic stirrer. The reaction mix was agitated using small magnetic stirrer bars. The test tubes were closed with a Teflon cap.

A typical experimental procedure was as follows: heptane (10.52 g), synthetic *l*-menthol (ex Aldrich, 4.49 g) and vinyl acetate (2.65 ml) were transferred to the reactor. Enzyme (first recycle free Amano AK, 337.5 mg) was added when the temperature was at about 25ºC, and the reactors were then heated to 40ºC with stirring.

Reaction Optimisation

Maxi Reactors: The Maxi-Reactor™ (Robo Synthon, CA, USA) consisted 6 x 450 ml jacketed reactor vessel, with individually controlled temperature and agitation units. The agitation was provided by bi-convex-shaped magnetic stirrer bars.

Batch stirred tank reactions

Glass vessels (2.2 L) with water heating jackets (at the base and sides of the vessel) were constructed by Glasstek (Gauteng, South Africa). Glass baffles (4) extend the height of the vessel and form part of the interior wall. The vessels were connected to circulating water baths set at 50°C ± 0.5, and thermally insulated with cotton wool-filled heavy foil jackets. 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 was achieved with magnetic stirrer bars or Heidolph R2R 2021 overhead stirrers fitted with 2 radial flow impellers mixed the reactions at 450 rpm for 24 hours, keeping the enzyme in suspension.

At large scale filtration was more practical than centrifugation, 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) suspended in the organic phase and connected to a vacuum pump (at 60 – 80 kPa) was used to remove the liquid. The heptane/menthol mix was stored in 2 L Schott bottles until analysis and subsequently pooled in 25 litre steel drums. 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.

Results and Discussion

Screening for lipase catalysed esterification of *l*-menthol

From a selection of commercial lipases and esterases, a total of 74 commercial enzyme preparations were evaluated in a pre-screen for the synthesis of butanol vinyl acetate esters. Only 16 enzymes showed transesterification activity, and these were evaluated for the formation of menthol esters (Table 1). .

We evaluated the reaction (again at the 1 ml scale) catalysed by the thermostable enzyme ESL-001-01 with either vinyl acetate or toluene in a range of solvents and three temperatures (30, 50 or 70ºC), but did not achieve an E above 7 (data not shown). When vinyl acetate in combination with toluene was used with ESL-001-01 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. Similarly the animal enzymes were not considered further due to concerns about use in food products.

Three of the positive reactions were catalysed by enzymes from *Pseudomonas*, which are known to be selective for *l*-menthol (Wu et al 2006, 2007), while two of the preparations were from *Candida* species, which are also known to be selective for this substrate (Othman

et al 2008). 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 (Biocatalysis) did not esterify menthol, although another *P. fluorescens* lipase preparation (Fluka) did (19%), while 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.

Comparison of AK and PS lipases

The *P. cepacia* lipase from Amano has previously been demonstrated to have a high enantioselectivity towards *l*-menthol in this and similar reactions (Cernia et al 1998; Wu et al 1997). Reactions (20 ml) were performed at 40°C in the carousel reaction system (Radleys Starfish, Radleys, UK). Stock solutions were made up containing 10, 25 and 40% *dl*-menthol, vinyl acetate in a ratio of 1:1 with respect to the racemic menthol and heptane (which was selected as the bulk solvent due to low neurotoxic properties (Impurities: Guideline for Residual Solvents)) and suitable partition coefficient (Laane et al 1987). The amounts of enzyme used for the different reactions were 151 mg free AK per 20 ml for the 10% substrate and 378 mg and 604 mg respectively for the 25 and 40% substrate concentrations. The Amano PS enzyme was used as 233 mg, 583 mg and 932 mg respectively for the 10, 25 and 40% substrate concentrations. The control reactions contained no enzyme. Samples were taken after 24 h and analysed for menthol (%m/m) and for the ratio of *d*-menthol:*l*-menthol using a chiral GC method. Based on the results (Table 2), the Amano AK enzyme was selected for all further studies as it resulted in a higher enantiomeric excess of *l*-menthol.

Enzyme recycle

To determine the recyclability of the enzyme, the Amano AK lipase was reused 150 times (Fig 1) achieving an overall yield of 184.3 g *l*-menthol/g enzyme. This sustained activity was possible as lipases tend to be more stable in organic solvents than in aqueous media (Zaks and Klibanov, 1988). The conversion for the first cycle was lower than the initial recycle (a phenomenon that was frequently observed), after which, the conversion decreased steeply to

below 40% conversion within seven recycles, and then stabilised, converting 30 - 40% of the available *dl*-menthol for 74 recycles. The enzyme retained above 50% activity for about 100 recycles. It was observed that, when the remaining vinyl acetate concentration was low in a particular recycle, the acetic acid produced in the reaction was high. This could be due to the unbound water associated with the enzyme or water in the solvent permitting the enzymatic hydrolysis of *l*-menthyl acetate to release acetic acid. Decrease in yields due to hydrolysis has been observed elsewhere (Bai et al 2006).

Establishing the optimum enzyme concentration

The optimum enzyme 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 Amano AK (Fig. 2) provided near complete conversion and was chosen for future experiments.

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 was not seen to 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. Reactions were performed on 10 ml scale (Multi-Reactors) to compare the conversions and enantiomeric excess of reactions performed with either 10% synthetic *dl*-menthol or 40% liquid menthol (a mixture of 8 menthol isomers). Reactions were performed at 40°C and samples were taken over 24 h for concentration and chiral analysis. As can be seen (Table 4), the presence of the other 6 isomers had little effect on the enantioselectivity.

The effect of initial menthol enantiomeric ratio: A set of experiments was carried out using different ratios of *d*-menthol and *l*-menthol, with all other variables kept constant. Each of the experiments was carried out at 40°C, with 26% (m/m) total menthol concentration, and with one equivalent of vinyl acetate with respect to total menthols present. Each of the reactions was carried out on a 15 - 20 g scale in Multi-Reactors. 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.

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 esterification of *dl*-menthol catalysed by Amano AK. A 2^4 (half factorial) design was drawn up with ranges for four variables (Table 6): temperature, *dl*-menthol concentration, vinyl acetate 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 Maxi Reactors.

Statistical analysis of these results indicates that increased temperature had the largest negative effect on the enantiomeric ratio (Fig 3), resulting from the decrease in the enantiomeric ratio at higher conversion. This effect has been previously observed by Wu et al (1997) and Bai et al (2006). By comparison, vinyl acetate concentration (or molar ratio of vinyl acetate to menthol ratio) only had a small positive effect on reaction enantioselectivity. The data also showed that the enzyme reaction could be performed at 50ºC and provide a 35% conversion, while maintaining an ee of 95%, and an E of 64. This E was sufficient to provide material for further process steps.

Optimum molar ratio of vinyl acetate to *l*-menthol

To glass reaction vials 151 mg of Amano AK was added. For each of the ratios evaluated, the menthol substrate concentration was maintained at 2.28 M *dl*-menthol in heptane (made up to 20 ml) and the VA molar ratio to *l*-menthol was varied from 0.25:1 to 3:1. The test tubes were incubated at 50°C in heated silicon oil baths on stirrer plates and agitated by means of magnetic bars. Each resolution was performed in quadruplicate. Figure 4 shows the effect of the different vinyl acetate ratios on the rate of product formation. Ratios of 1:1 to 3:1 demonstrated similar initial results during the transesterification of menthol to menthyl

acetate, but above 1:1 the final product increased by about 25% at ratios of 2.5:1 and 3:1, giving about 80% conversion of the *l*-menthol.

Reaction kinetic studies

Influence of vinyl acetate concentration: Duplicate reactions containing increasing concentrations of vinyl acetate (0-2.6 M) were performed. An *l*-menthol concentration of 3.2 M (50% m/v) was added with heptane as solvent. Amano AK enzyme, 151 mg, was added to each 5 ml reaction. The test tubes were incubated at 50°C for 5 h. All reactions were performed at a 2 ml scale and 50°C by incubation in silicon oil baths on magnetic stirrer hot plates, 750 rpm and ambient pressure.

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.

A second set of reactions were performed under the same conditions with increasing concentrations of *l*-menthol 0-3.21 M (0-50% m/v) and a fixed vinyl acetate concentration of 3.2 M. Using a non-linear regression model V_{max} was determined to occur at 2.6 M *l*-menthol (Fig. 5), approximately equivalent to 40% m/m *l*-menthol. The K_m value was 1.13 M *l*-menthol (17.6% m/v). 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 biocatalyst was 0.25 g menthyl acetate/g enzyme/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 levels indicating no significant interference on the initial rate of reaction, either kinetically or physically, by the presence of the *d*-enantiomer. 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 are known to be poor substrates for this enzyme, some competition for the active site may occur.

Enzyme inactivation by reaction components

In these experiments the enzyme was exposed to reaction matrix components and then washed three times with heptane by centrifugation to remove the compound, and the residual activity of the enzyme was subsequently measured by the Amano assay.

Menthyl acetate: 20 mg/mL Amano AK enzyme (of Lot LAKV 07510) was pre-incubated in 0, 0.47 M and 0.93 M *dl*-menthyl acetate (Sigma-Aldrich) and heptane for 20 h at room temperature (approximately 25°C) with agitation. The results indicated that this did not negatively affect subsequent performance of the enzyme in but rather seemed to enhance the performance of the enzyme almost 2 fold at 0.5 M racemic menthyl acetate.

Subsequently, further experiments were carried out on the effect of *dl*-menthyl acetate during the menthol transesterification reaction. Duplicate reactions containing 20 mg/mL Amano AK, 0.7 M vinyl acetate and 0.32 M (5% m/v) *l*-menthol substrate concentrations were performed. The *dl*-menthyl acetate (Sigma-Aldrich) concentration in the 6 h reaction was varied from 0 to 0.64 M. Control experiments were prepared as above but without enzyme addition. There appeared to be a 72% increase in activity on addition of 0.64 M menthyl acetate to the reaction.

This effect was confirmed using the Amano assay, where 1-phenethyl alcohol (20% v/v) was mixed with the menthyl acetate (0 – 0.64 M) and vinyl acetate (1.6 – 1.32 ml). All tubes were placed in ice water during preparation and the reaction started by adding 2 ml of the reactant liquid to the enzyme and bringing the reactants to incubation temperature. There appeared to be no product inhibition effect on the enzyme activity in the presence of increasing menthyl acetate concentrations on the phenyl ethanol reaction with a 30% increase in activity at 0.64 M menthyl acetate.

Vinyl acetate: A set of reactions was set up by incubating the enzyme in dried vinyl acetate and dried heptane (dried over anhydrous magnesium sulphate). Lipase, 151 mg (Amano AK; Lot LAKY0950502) was incubated with heptane and 0 - 3.5 M vinyl acetate. The test tubes were incubated in silicon oil baths heated to 50°C with stirring for 5 h . The enzyme was

then washed three times with dried heptane. Pre-incubating the enzyme in dried vinyl acetate up to concentrations of 3.5 M did not appear to be toxic to the enzyme.

Acetaldehyde: Lipase, 100 mg (Amano AK; Lot LAKY0950502) was added to glass reaction vials. A 1.6 M acetaldehyde stock solution was prepared by adding 45.5 ml acetaldehyde to a 500 ml volumetric flask and making up to the mark with heptane.

During the preparation, the stock solution, heptane and test tubes were kept in ice water as acetaldehyde has a boiling point of 21°C. To maintain the acetaldehyde in solution during the pre-incubation period, all test tubes were held at 15°C with agitation. To assess the toxicity effects of acetaldehyde at this low temperature, a pre-incubation period of 2 weeks was chosen. Test tubes from each concentration were sacrificed at various times. 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).

Acetic acid: Acetic acid formation was previously observed in the transesterfication reaction (Fig 1.). The effect of acetic acid on the activity of the enzyme was assessed. Amano AK (20 mg of Lot LAKV 07510) was pre-incubated in 0 - 400 mM acetic acid and heptane at approximately 25°C with agitation. The enzyme was separated from the pre-incubation mixture by centrifugation (3000 rpm, 15 minutes).

The study indicated that addition of acetic acid had a significant denaturing effect on the enzyme (Fig. 6). Over 20% of enzyme activity was lost after pre-incubation for 20 h in 20 mM acetic acid. No residual enzyme activity was displayed after exposure to 200 mM acetic acid under these conditions.

As this dramatic loss of activity was not observed in the presence of vinyl acetate, it can be assumed that acetic acid was not formed by the enzyme, but that it occurs through the water-mediated hydrolysis of the menthyl acetate reaction product. 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 (addition with each recycle). As could be seen in Fig 1 the acetic acid level

generated in the first reaction was 120 mM, decreasing in the next reaction to about 70 mM and then decreasing to < 50 mM with extended recycles as the water associated with the enzyme is depleted. 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 study, and hence enzyme recycle was possible.

Bench Scale Batch Stirred Tank Reactions

Having determined that the ee was >95% at 30% conversion of *dl*-menthol in the liquid menthol 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 30% the enzyme load was reduced 25 g/L, as calculated from the data (Fig 2). Although there is some variability in the conversion (Table 7), this correlated with the changes in the batches of the liquid menthol and agitation.

Enzyme losses occur 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 are in the order of ~ 25 mg per reaction, and hence about 0.1 g of enzyme was lost 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. In a 95% conversion of *l*-menthol, the other isomers in liquid menthol remained largely unreacted. The *dl*-menthol contributed 51% of the total menthol. Only 4% of the *d*-menthol, 2.5% of the 29% *dl*-neomenthol, and 3.5% of the 14% *dl*-isomenthol were esterified. A much higher portion of the *dl*-neoisomenthol 18.5% was esterified, although this was insignificant as it typically comprised only 2-3% of the liquid menthol. This yielded a diastereomeric excess of 82%.

After the reaction the mixture can be distilled to remove the *l*-menthyl acetate, and 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 (Dudas and Hanika 2009; Chaplin et al 2002).

6. Conclusion

In this research we demonstrated the selective esterification of *l*-menthol from an 8 diasteromer mixture. Amano AK in heptane catalysed the transesterification of *l*-menthol with the acetate from vinyl acetate at a significantly higher rate than *d*-menthol resulting in menthyl acetate enriched in the *l*-enantiomer. The presence of isomenthol had no significant effect on either the rate of *d/l*-menthol conversion or the final ee of the *dl*-menthyl acetate. The reaction by-products acetaldehyde and particularly acetic acid were shown to exert a denaturing effect on the enzyme during pre-incubation studies using artificial media. Interestingly, the strength of this denaturing effect seems to be significantly reduced during operational studies suggesting that artificial studies to investigate single component toxicity are not representative of the process conditions endured by the biocatalyst.

The reaction was successfully scaled up by a factor of 400 (from 5 ml to 2 L) in batch stirred tank reactors and the enzyme was recycled a total of 38 times. The free enzyme appears to be stable under these conditions and converted 30 % of the available *dl*-menthol with an enantiomeric ratio of 65.

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Table 1: Screening of selected enzymes for esterification of menthol with individual acyl donors

Enzyme	Solvent							
	Acyl donor	Toluene	Cyclo-hexane	Hexane	Pentane	Decane	Iso-propanol	Heptane
Hog pancreas lipase Fluka	vinyl acetate	-	ND	+	ND	-	ND	-
	butyric acid	++	ND	-	ND	+	ND	+
	octanoic acid	++	ND	-	ND	+	ND	-
	lauric acid	++	ND	-	ND	-	ND	-
Hog liver Esterase Boehringer	vinyl acetate	ND	ND	-	-	-	ND	
	butyric acid	ND	ND	-	ND	-	ND	
	octanoic acid	ND	ND	++	ND	+	ND	
	lauric acid	ND	ND	-	-	-	ND	
Porcine pancreas Chirazyme L6 Boehringer	vinyl acetate	ND	ND	ND	-	-	-	ND
	butyric acid	ND	ND	ND	-	+	-	ND
	octanoic acid	ND	ND	ND	++	++	+	ND
	lauric acid	ND	ND	ND	+	-	++	ND
Aspegillus oryzae lipase Fluka	vinyl acetate	-	ND	+	ND	-	ND	-
	butyric acid	-	ND	-	ND	+	ND	-
	octanoic acid	+	ND	+	ND	+	ND	-
	lauric acid	++	ND	-	ND	+	ND	+
Rhizomucor miehei lipase Fluka.	vinyl acetate	+	++	-		-	-	+
	butyric acid	-	-	-	-	++	-	ND
	octanoic acid	+	++	-	-	+	-	ND
	lauric acid	+	+	-	+	-	ND	ND
Rhizopus japonicus Nagase Enz. Lilipase A-10FG	vinyl acetate	-	++	-	+	ND	ND	ND
	butyric acid	-	-	-	++	ND	ND	ND
	octanoic acid	++	+	+	-	ND	ND	ND
	lauric acid	++	++	-	++	ND	ND	ND
Candida antarctica lipase Fluka	vinyl acetate	+	ND	ND	ND	ND	ND	ND
	butyric acid	++	ND	ND	ND	ND	ND	ND
	octanoic acid	-	ND	ND	ND	ND	ND	ND
	lauric acid	-	ND	ND	ND	ND	ND	ND
Candida antarctica fraction A Chirazyme L5 Boehringer	vinyl acetate	++	++	ND	ND	+	-	ND
	butyric acid	-	-	ND	ND	-	-	ND
	octanoic acid	+	++	ND	ND	-	+	ND
	lauric acid	-	-	ND	ND	+	-	ND
Candida cylindracea lipase Fluka	vinyl acetate	-	+	+	+	-	-	ND
	butyric acid	-	-	-	-	+	-	ND
	octanoic acid	++	++	-	+	+	-	ND
	lauric acid	-	-	-	+	+	+	ND
Chromobacterium viscosum lipoprotein lipase Fluka	vinyl acetate	++	ND	ND	ND	ND	ND	ND
	butyric acid	++	ND	ND	ND	ND	ND	ND
	octanoic acid	-	ND	ND	ND	ND	ND	ND
	lauric acid	+	ND	ND	ND	ND	ND	ND
Burkholderia Chirazyme L1 Boehringer	vinyl acetate	ND	+	+	++	+	-	ND
	butyric acid	++	-	-	-	+	+	ND
	octanoic acid	+	++	-	+	-	+	ND
	lauric acid	+	-	-	+	-	++	ND
Pseudomonas cepacia lipoprotein lipase Fluka	vinyl acetate	++	ND	-	+	-	ND	ND
	butyric acid	-	ND	-	+	-	-	ND
	octanoic acid	++	ND	-	-	++	+	ND
	lauric acid	-	ND	-	-	-	-	ND
Pseudomonas sp. Chirazyme L4	vinyl acetate	-	-	ND	+	ND	-	+
	butyric acid	-	++	ND	-	ND	+	-

Enzyme	**Solvent**							
Boehringer	octanoic acid	-	+	ND	+	ND	+	++
	lauric acid	+	-	ND	-	ND	++	-
Pseudomonas. fluorescens	vinyl acetate	-	++	ND	ND	-	-	+
Lipase	butyric acid	-	-	ND	ND	+	-	+
Fluka	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	-
Boehringer	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

Where "-", "+" and "++" represent no activity, some activity, and strong activity respectively.

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

Reaction cycle	Enzyme	Substrate conc (%)	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

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	Ave
		%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	8
10% *dl*-menthol	604	22.4	97.1	48.4	94.6	50.8	93.4	49.0	93.6	42.7	96	8
40% Liquid menthol	302	17.8	97.2	31.7	96.3	32.8	96.1	30.9	96.1	28.3	95	1
40% Liquid menthol	604	23.8	97.1	46.2	94.8	47.4	94.0	47.4	94.0	41.2	95	9

%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

Table 6: Statistically designed experiments for reaction optimisation.

Run	*dl*-Menthol Conc (%m/m).	Temp/ °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

Table 7: Bench scale batch stirred tank enzyme recycle experiments.

Reaction recycle	Conversion (% m/m)					Std dev.
	BSTR Rxn 1 % C	BSTR Rxn 2 % C	BSTR Rxn 3 % C	BSTR Rxn 4 % C	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

Reaction recycle	Conversion (% m/m)					Std dev.
	BSTR Rxn 1 % C	BSTR Rxn 2 % C	BSTR Rxn 3 % C	BSTR Rxn 4 % C	Mean	
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

Figure Legends

Scheme 1: Menthol diasteromers generated by hydrogenation of thymol.

Scheme 2: Transesterification of *l*-menthol.

Figure 1. Batch recycles of Amano AK lipase enzyme at 50°C. ■ Vinyl acetate consumed, ◊ menthyl acetate formed, ▲ acetic acid present. Low conversion for recycle 45 was due to malfunction of temperature control.

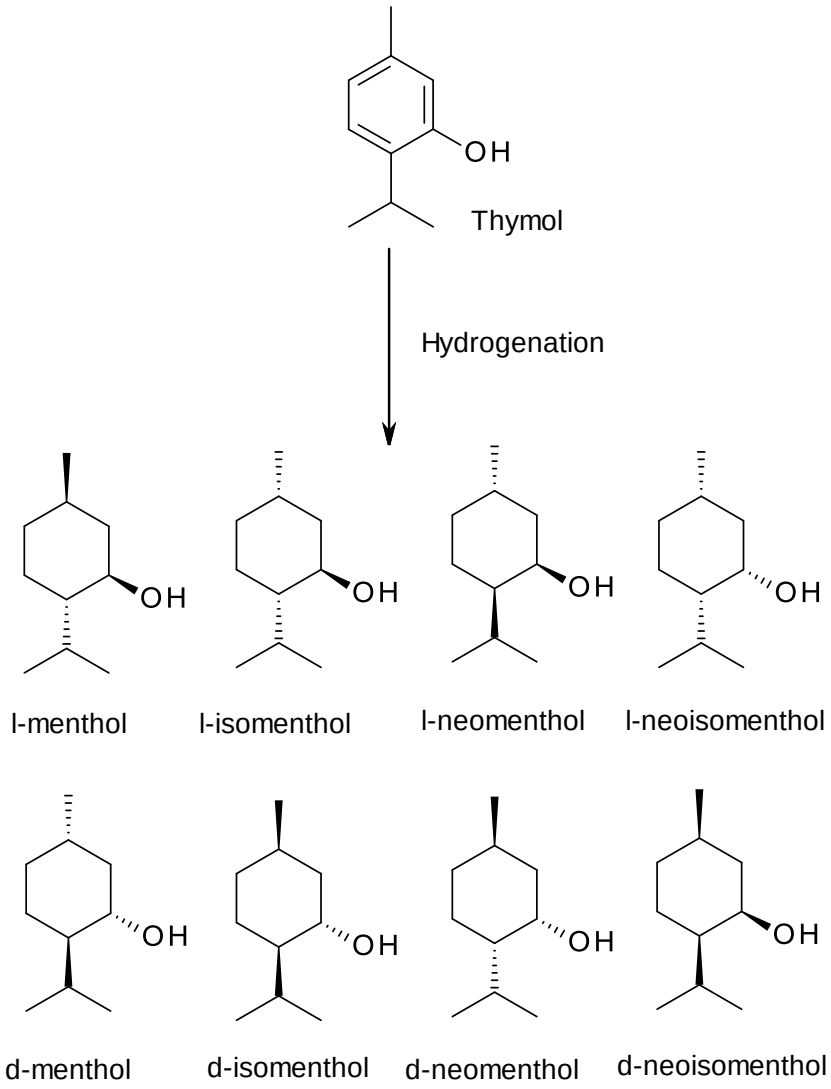
Figure 2. Resolution of 12% v/v liquid menthol with varying enzyme concentrations.

Figure 3. 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.

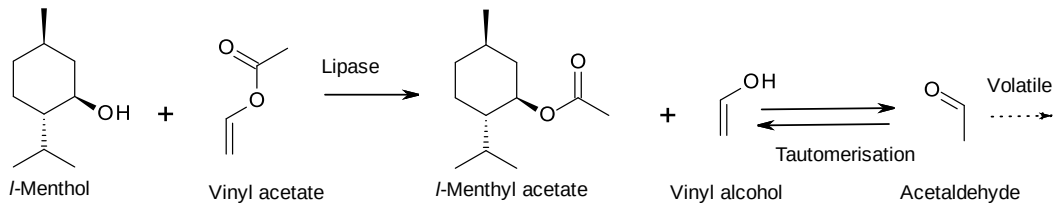
Figure 4: Interaction between temperature, *dl*-menthol concentration, and enantiomeric excess. Enzyme loading at 100 mg/g.

Figure 5. Specific activity of Amano AK with vinyl acetate (■) and *l*-menthol (▲) concentration determined in excess of the co-substrate.

Figure 6. Residual activity of Amano AK (as measured by the Amano assay) after exposure to increasing acetic acid concentrations in heptane at 25°C.



Scheme 1



Scheme 2

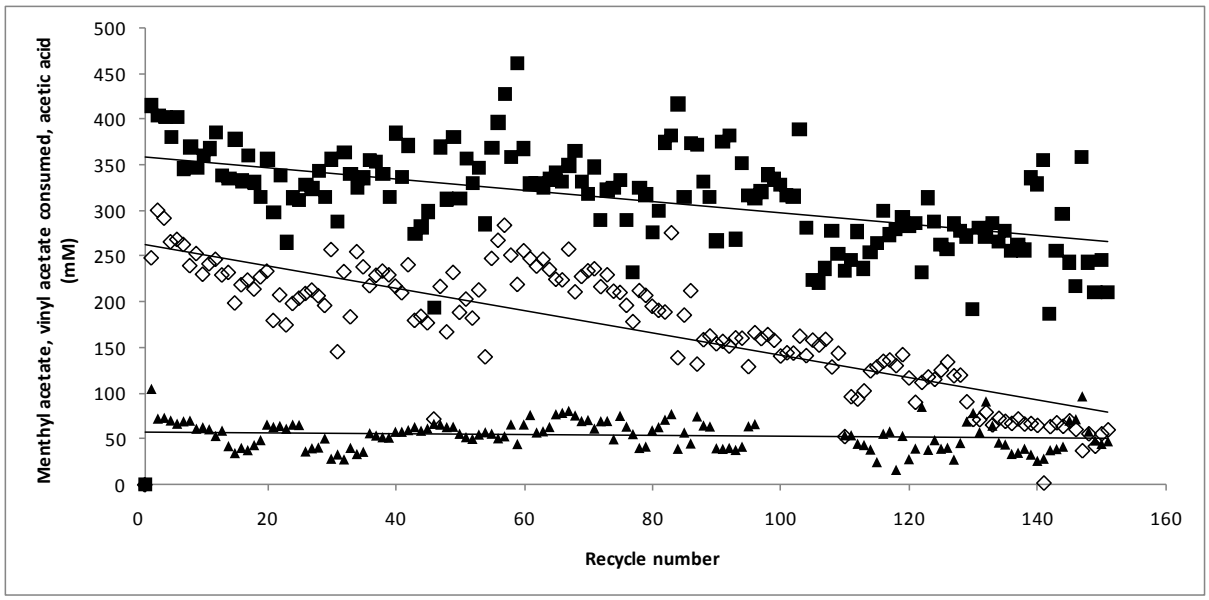

Menthyl acetate, vinyl acetate consumed, acetic acid (mM)
500
450
400
350
300
250
200
150
100
50
0
0
20
40
60
80
100
120
140
160
Recycle number

Figure 1

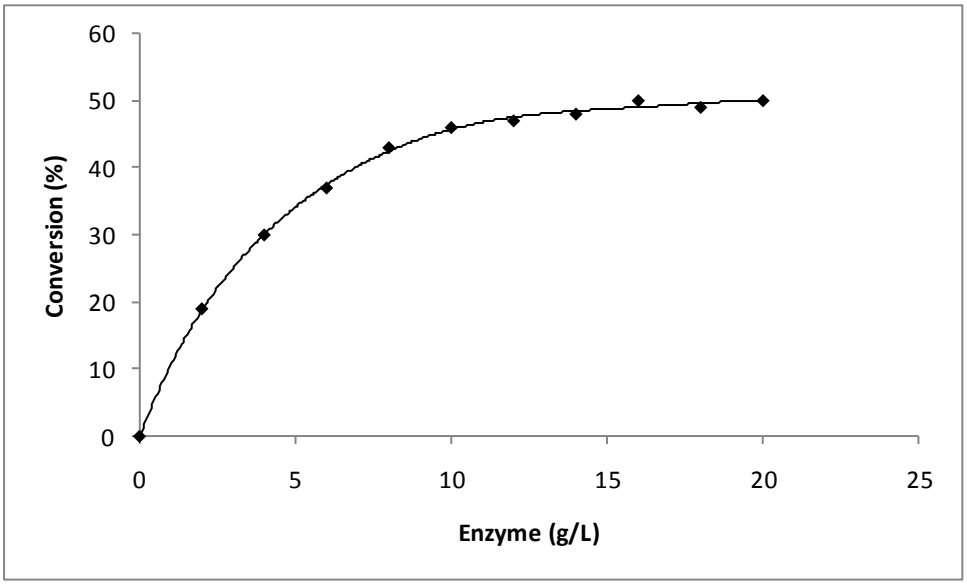

Conversion (%)
60
50
40
30
20
10
0
0
5
10
15
20
25
Enzyme (g/L)

Figure 2

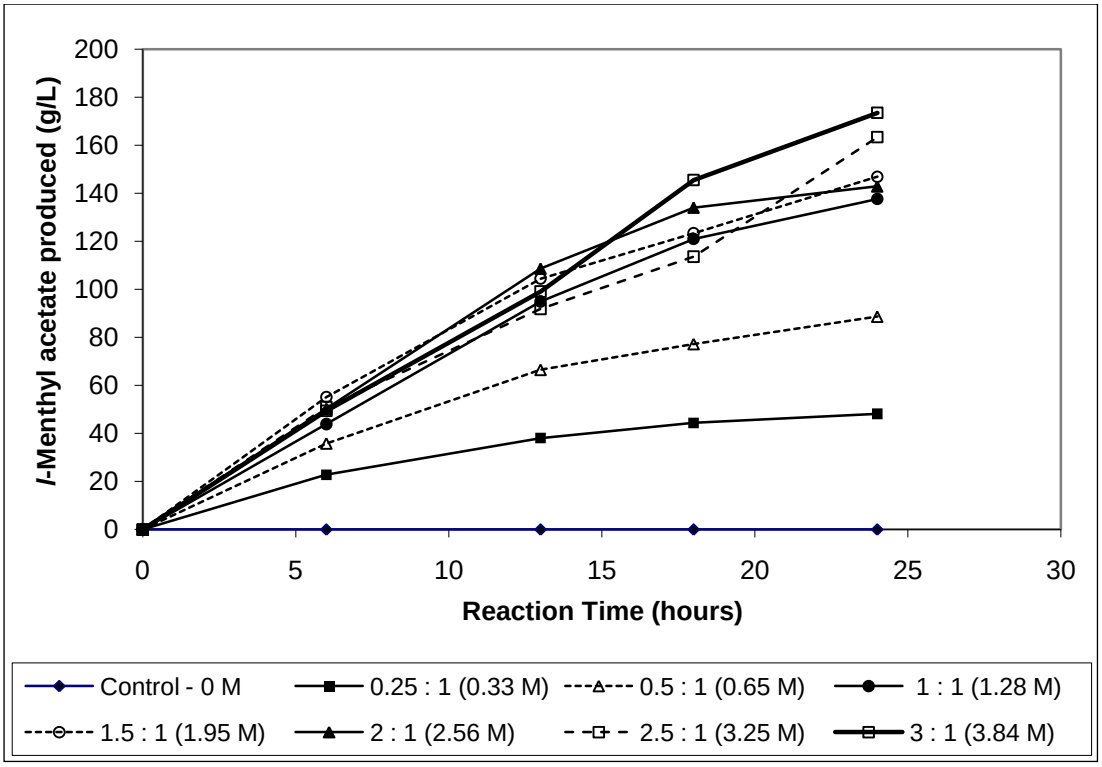

200
180
160
140
120
100
80
60
40
20
0
l-Menthyl acetate produced (g/L)
0
5
10
15
20
25
30
Reaction Time (hours)
Control - 0 M
0.25 : 1 (0.33 M)
0.5 : 1 (0.65 M)
1 : 1 (1.28 M)
1.5 : 1 (1.95 M)
2 : 1 (2.56 M)
2.5 : 1 (3.25 M)
3 : 1 (3.84 M)

Figure 3

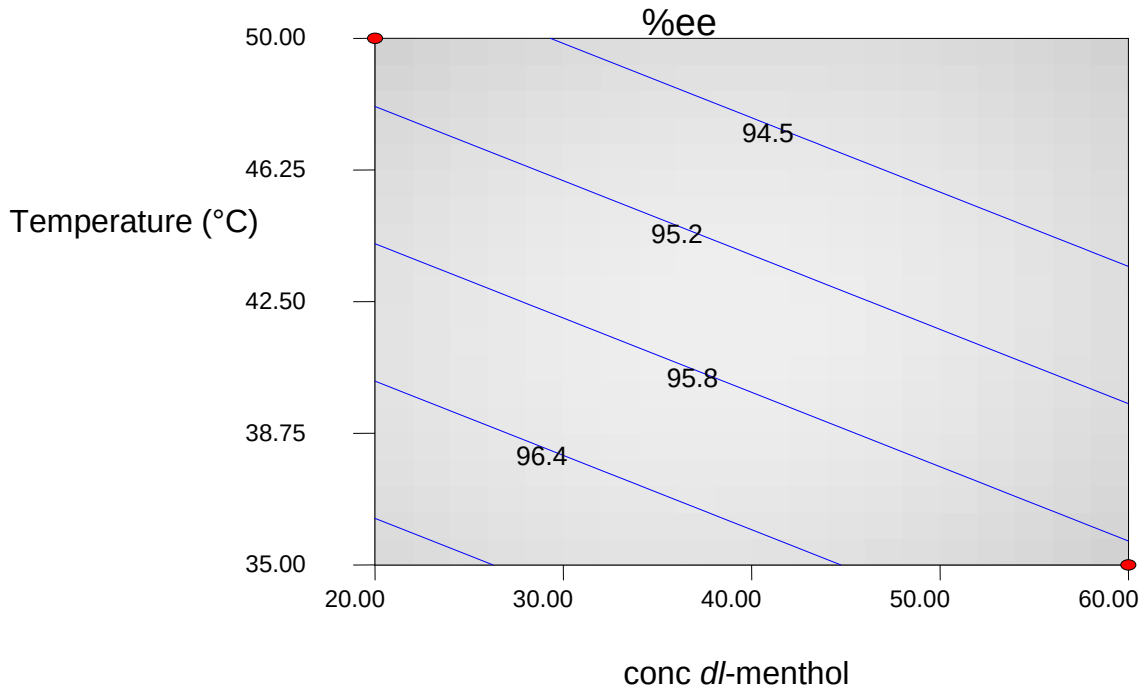

%ee
50.00
46.25
42.50
38.75
35.00
Temperature (°C)
94.5
95.2
95.8
96.4
20.00
30.00
40.00
50.00
60.00
conc dl-menthol

Figure 4

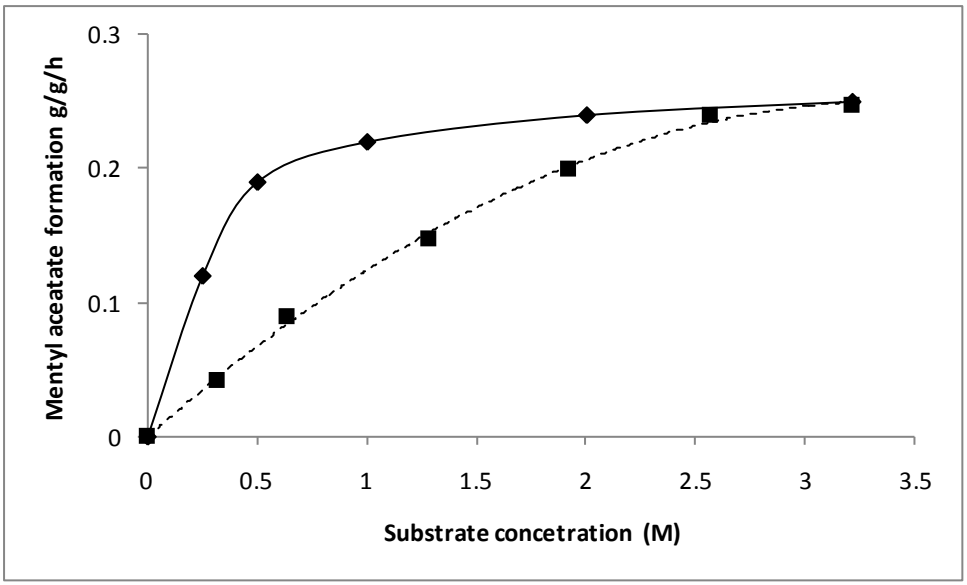

Mentyl aceatate formation g/g/h
0.3
0.2
0.1
0
0
0.5
1
1.5
2
2.5
3
3.5
Substrate concetration (M)

Figure 5

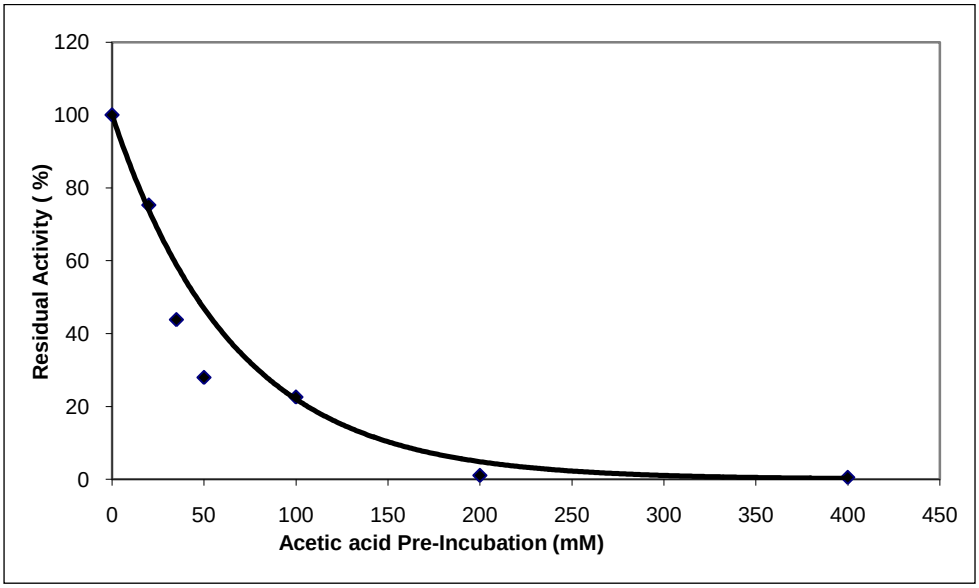

120
100
80
60
40
20
0
Residual Activity (%)
0
50
100
150
200
250
300
350
400
450
Acetic acid Pre-Incubation (mM)

Figure 6