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Enantioselective synthesis of (*R*)-citronellal from geraniol with an immobilised copper alcohol oxidase and ene reductase†

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(*R*)-Citronellal is one of the key chiral intermediates in the synthesis of the isomer (–)-menthol, one of the most commercialised terpenoid flavours worldwide. Enzymatic approaches could represent a less energy-demanding alternative for its synthesis, such as a previously reported bienzymatic cascade starting from inexpensive, commercially available geraniol. A copper radical oxidase (*CgrAlcOx*) followed by a flavin-dependent ene reductase (*OYE2*) were used to obtain (*R*)-citronellal. Here, we used a metal-affinity immobilisation strategy on the His-tagged enzymes for the cascade and studied enzyme recovery and reusability as well as increased solvent tolerance. After screening a panel of resins for enzyme immobilisation and water-immiscible co-solvents, we successfully obtained 95% conversion to (*R*)-citronellal with 96.9% enantiomeric excess (ee) in a concurrent cascade after 7 h of reaction time, starting from 10 mM of geraniol.

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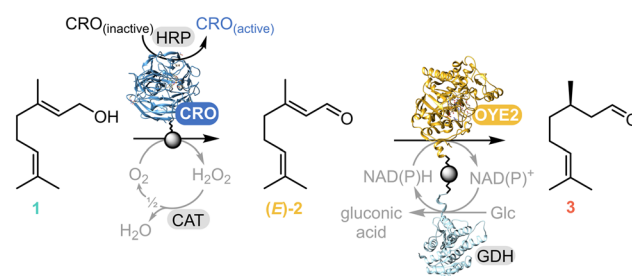
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Introduction

The cyclic terpene (–)-menthol is one of the primary constituents of essential oils from peppermint (*Mentha x piperita* L.) and corn mint (*Mentha arvensis*).^{1,2} Thanks to its minty aroma, refreshing flavour, and cooling and analgesic properties,³ this compound has a wide range of applications, spanning from the food and fragrance industries⁴ to the cosmetic⁵ and pharmaceutical sectors.⁶ Synthetic menthol accounts for 60% of the 34 000 metric tons produced per year, with three main chemical routes used for its industrial production (ESI† Scheme S1).^{6,7} (*R*)-Citronellal is a key chiral intermediate in two of these processes to obtain the enantiomer associated with the pleasant minty aroma, (1*R*,2*S*,5*R*)-(–)-menthol.^{6–8} Among these, the process developed by BASF starts from citral, a mixture of *E*- and *Z*-isomers, referred to as geranial and neral, respectively (Scheme S1C†). In order to obtain the desired enantiomer of citronellal, an energy intensive distillation of citral to isolated

neral is required. This step is followed by asymmetric reduction with a rhodium complex with chiral ligands, achieving only ~87% ee of (*R*)-citronellal.⁶ An enzymatic process offers a promising alternative, providing higher enantioselectivity under mild conditions.^{9–12} Recently, an *in vitro* enzymatic cascade for the production of (*R*)-citronellal has been reported by our group,¹³ starting from the relatively inexpensive substrate geraniol. In the first step, geraniol **1** is oxidised to geranial (*E*)-**2** by a copper radical alcohol oxidase (*CRO*) from *Colletotrichum graminicola* (*CgrAlcOx*). This is followed by a reduction step to (*R*)-citronellal **3** catalysed by an ene reductase of the old yellow enzyme family (*OYE2* from



Scheme 1 CRO–OYE2 enzymatic cascade for the synthesis of (*R*)-citronellal **3** starting from geraniol **1**. The first step is the oxidation of geraniol **1** to geranial (*E*)-**2**, which is further reduced in the second step to (*R*)-citronellal **3**. CRO = copper radical alcohol oxidase (*CgrAlcOx*); HRP = horseradish peroxidase, CAT = catalase, GDH = glucose dehydrogenase, NAD(P)⁺/NAD(P)H = nicotinamide adenine dinucleotide (phosphate), oxidised and reduced form.

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Fig. 1 Resin screening: monitoring of product formation for the reaction catalysed by the immobilised enzymes; each enzymatic system evaluated separately (system a: *CgrAlcOx*, system b: OYE2 + GDH). The relative amount of geranial obtained from geraniol oxidation and the amount of (*R,S*)-citronellal obtained from citral reduction were used to select the carrier. Conditions a (CRO-catalysed geraniol oxidation): 50 mg_{dry resin} immobilised *CgrAlcOx*, 10 mM geraniol (as 1% v/v in acetone), 360 U mL⁻¹ catalase, 12 μM HRP in 50 mM NaPi buffer pH 8.0 and 20% v/v heptane, 30 min, 23 °C, 200 rpm. Conditions b (OYE2-catalysed citral reduction): 50 mg_{dry resin} co-immobilised OYE2 and GDH, 40 mM glucose, 1 mM NADP⁺, 20 mM citral in acetone (as 1% v/v in acetone), 100 mM KPi buffer pH 8.0 and 30% v/v MTBE, 5 h, 25 °C, 150 rpm.



Fig. 2 Influence of solvents. (A) Solvent screening for the OYE2-catalysed citral reduction. Reaction conditions: 50 mg_{dry resin} co-immobilised OYE2 and GDH, 40 mM glucose, 1 mM NADP⁺, 20 mM citral (as 1% v/v in acetone), 100 mM KPi buffer pH 8.0 and 30% v/v organic solvent, 5 h, 25 °C, 150 rpm. (B) Reusability of OYE2 and GDH immobilised on Chromalite/Co with different heptane concentrations. Reaction conditions: 50 mg_{dry resin} co-immobilised OYE2 and GDH, 100 mM glucose, 1 mM NADP⁺, 20 mM citral (as 1% v/v in acetone), 100 mM KPi buffer pH 8.0 and 20, 30 or 50% v/v heptane, 5 h, 25 °C, 150 rpm.

evaluated (Fig. 2B). Using a lower concentration of 20% v/v heptane (Fig. 2B), the relative amount of citronellal formed was consistently higher for three runs carried out with the same batch of immobilised enzymes.

Enzyme recovery and reuse is one of the main advantages of using immobilisation techniques, making the process more cost-effective and appealing for industrial applications.²⁷ With the same amount of solvent, the reusability of the immobilised *CgrAlcOx* on SepLife/Ni was also tested, but proved to be lower, with the conversion to geranial decreasing from 80% to 37% after the second run (II) and to 32% after three runs (III) (Fig. 3).

Immobilising enzymes using their His-tag allows both purification and immobilisation in a single step,²⁸ avoiding chromatography steps for enzyme purification that can increase the overall process costs of around one order of magnitude.²⁹ After measuring the enzyme specific activity of the cell-free extracts (CFEs) of OYE2 and GDH and comparing them to those of the purified enzymes (Table S1†), we approximated that around 10% of the proteins in the CFE

were the protein of interest. To reproduce the ratio used for co-immobilisation of purified enzymes (*i.e.*, 10 mg g_{resin}⁻¹ OYE2, 3 mg g_{resin}⁻¹ GDH), we performed immobilisation with 100 mg of CFE containing OYE2 and 30 mg of CFE containing GDH. Using this resin, we achieved the same relative amount of product in a 5 h reaction starting from citral (Fig. S5†): 57% conversion to citronellal with 85% ee with immobilised CFEs compared to 58% of citronellal with 80% ee with the purified enzymes. The successful one-step purification and co-immobilisation of OYE2 and GDH demonstrated the possibility of using the immobilised CFE for the cascade.

When combining the two steps with *CgrAlcOx* immobilised on SepLife/Ni and OYE2-GDH immobilised on Chromalite/Co, we obtained 95% of citronellal with 96.9% ee in a 7 h one-pot concurrent cascade (Fig. 4). This result suggests that the previously reported *CgrAlcOx* inhibition⁸ can be avoided using immobilised enzymes in a biphasic



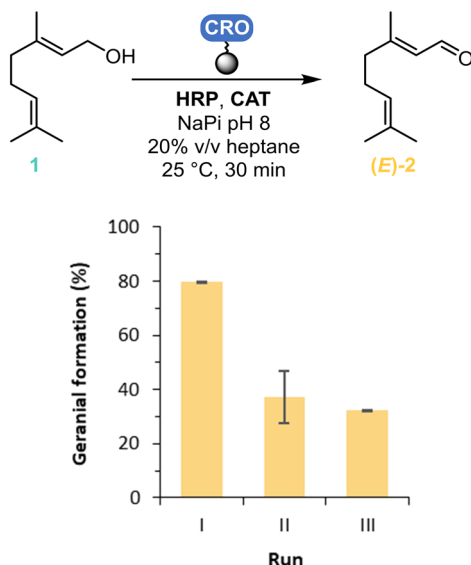


Fig. 3 Reusability of *CgrAlcOx* immobilised on SepLife/Ni. Reaction conditions: 50 mg_{dry resin} of immobilised *CgrAlcOx*, 10 mM of geraniol (as 1% v/v in acetone), 360 U mL⁻¹ of catalase, 3 μM of HRP, 100 mM KPi buffer pH 8.0 and 20% v/v heptane, 1 mL volume, 25 °C, 30 min, 200 rpm.

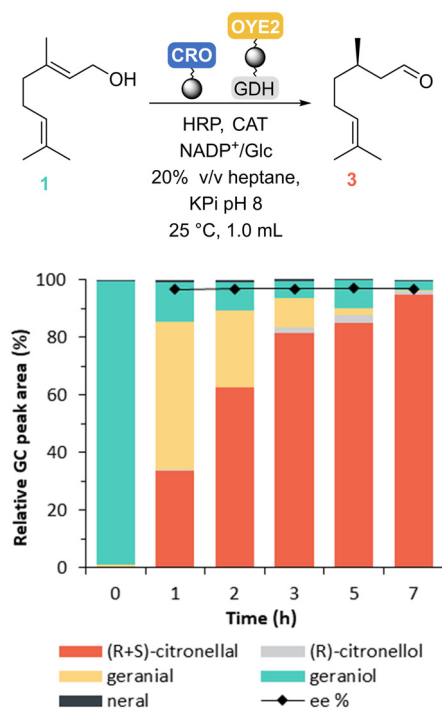


Fig. 4 One-pot enzymatic CRO-OYE2 cascade time course. Reaction conditions: 50 mg_{dry resin} immobilised *CgrAlcOx* and 50 mg_{dry resin} co-immobilised OYE2_{CFE} and GDH_{CFE}, 360 U mL⁻¹ catalase, 3 μM HRP, 10 mM geraniol (as 1% v/v in acetone), 100 mM glucose, 1 mM NADP⁺, 100 mM KPi buffer pH 8.0 and 20% v/v heptane, 25 °C, 180 rpm.

system with 20% v/v heptane. This improvement might be explained by a protecting effect of heptane in which citronellal preferentially resides. In this hydrophobic solvent, the hydration rate of citronellal is likely to be lower, limiting

the formation of the geminal-diol that we thought caused *CgrAlcOx* inhibition.^{13,30} Over time, minor (*R*)-citronellol formation was observed, which appears to be catalysed by the GDH (see GC chromatogram Fig. S16†).

In addition, we evaluated the reusability of the immobilised enzymes for the full cascade (Fig. 5A). After the second run (II), the relative amount of (*R*+*S*)-citronellal dropped to 35%, and a further decrease to 12% and 9% after three (III) and four (IV) runs, respectively. It should also be noted that the reusability of *CgrAlcOx* was lower than that of OYE2-GDH (Fig. 2B and 3), indicating that *CgrAlcOx* is likely contributing more to the lack of enzyme re-usability for the full cascade. The poor reusability of the system could be due to the reversible nature of metal affinity immobilisation, rendering this type of immobilisation more prone to leaching compared to other methods, such as covalent immobilisation.¹² We suspected that leaching of the enzymes from the solid support is responsible for this decrease in performance; however we were unable to detect the enzyme in the reaction supernatant by SDS-PAGE (Fig. S7†). Another reason may be a lack of enzyme stability (due to enzyme inactivation or copper leaching) during the reaction.

Lastly, we successfully doubled the substrate concentration to 20 mM of geraniol, achieving 76% conversion to citronellal with 96.7% ee after 7 h. Extending the reaction time to 18 h (overnight) further increased the relative amount of citronellal to 86% with 96.6% ee (Fig. 5B and S6†). These findings confirm that *CgrAlcOx* is not fully inhibited by the presence of (*R*)-citronellal. A small amount of neral was consistently observed throughout the reaction, likely due to its presence as an impurity in the geraniol substrate, contributing to the formation of (*S*)-citronellal. Additionally, geranial may undergo isomerisation to neral during the course of the reaction,¹⁴ which could further explain the slight decrease in ee values over time.

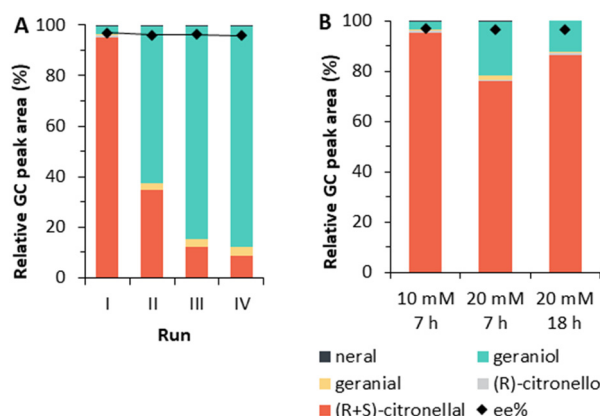


Fig. 5 Evaluation of (A) reusability of the immobilised enzymatic system and (B) increased substrate concentration, in the one-pot cascade. Reaction conditions: 50 mg_{dry resin} immobilised *CgrAlcOx* and 50 mg_{dry resin} co-immobilised OYE2_{CFE} and GDH_{CFE}, 360 U mL⁻¹ catalase, 3 μM HRP, 10 or 20 mM geraniol in acetone (final concentration, 1% v/v), 100 mM glucose, 1 mM NADP⁺, 100 mM KPi buffer pH 8.0 and 20% v/v heptane, 7 or 18 h, 25 °C, 180 rpm.

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