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Tunable Production of (*R*)- or (*S*)-Citronellal from Geraniol via a Bienzymatic Cascade Using a Copper Radical Alcohol Oxidase and Old Yellow Enzyme

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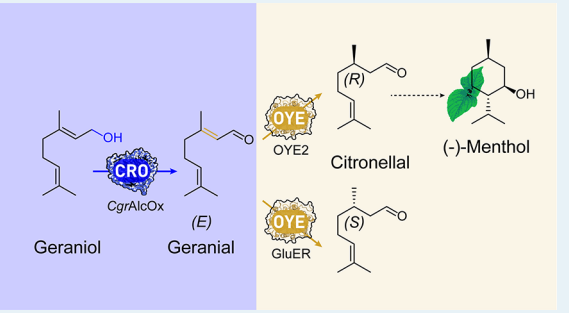
ABSTRACT: Biocatalytic pathways for the synthesis of (−)-menthol, the most sold flavor worldwide, are highly sought-after. To access the key intermediate (*R*)-citronellal used in current major industrial production routes, we established a one-pot bienzymatic cascade from inexpensive geraniol, overcoming the problematic biocatalytic reduction of the mixture of (*E*/*Z*)-isomers in citral by harnessing a copper radical oxidase (*Cgr*AlcOx) and an old yellow enzyme (OYE). The cascade using OYE2 delivered 95.1% conversion to (*R*)-citronellal with 95.9% *ee*, a 62 mg scale-up affording high yield and similar optical purity. An alternative OYE, GluER, gave (*S*)-citronellal from geraniol with 95.3% conversion and 99.2% *ee*.



KEYWORDS: *biocatalysis, biocascade, citronellal, citral, geranial, enzymatic oxidation, enzymatic reduction*

The acyclic terpene citronellal—which gives off an intense lemon-, citronella-, and rose-type odor[1]—is a valuable molecule for its use in flavors and fragrances[2] and is also of utmost importance as a precursor for the industrial synthesis of (−)-menthol, one of the chiral compounds with the largest commercial importance[3] and one of the most sold flavors.[4] Among the eight stereoisomers of menthol, only (−)-menthol holds the characteristic "cooling" effect and the peppermint minty odor, clean of off-flavor.[5] Two of the three main industrial chemical synthesis routes to (−)-menthol (Supporting Information (SI) Scheme S1) employ (*R*)-citronellal (Scheme S2A) as an intermediate.[6,7] In order to improve process sustainability and to provide access to alternative feedstock, alleviating the dependency on fossil or unstable natural resources,[8] (*R*)-citronellal could be advantageously produced via biocatalytic approaches. Alternative routes harnessing inexpensive achiral substrates are especially sought-after.[6] An ideal biocatalytic route would be the production of (*R*)-citronellal from the available, industrially relevant citral.[9] This reduction reaction can be carried out using flavin mononucleotide (FMN)-containing ene-reductases of the old yellow enzyme (OYE; EC 1.6.99.1) family.[10−12] Ubiquitous in Nature, OYEs are found in bacteria, fungi, plants, cyanobacteria, and recently algae[13] and catalyze the asymmetric reduction reaction of a wide variety of α,β-unsaturated compounds.[14−16] However, such a biocatalytic route remains challenging,[17] since citral is found as a mixture of two isomers (geranial or (*E*)-isomer and neral or (*Z*)-isomer) (Scheme S2), which greatly influences the enantioselectivity of available OYEs.[18] So far, no OYE has been able to achieve efficient conversion of citral and yield enantiopure (*R*)-citronellal with >95% enantiomeric excess (*ee*),[19] as the enzymes tested were hampered by the presence of both citral isomers and despite enzyme engineering attempts,[17,20,21] only OYE2p could reach 88.8% *ee* starting from an *E*/*Z* citral mixture of 10:9.[22] To avoid the energetic-costly separation of citral isomers by distillation and prevent their isomerization,[23] a direct approach would be to supply *in situ* the OYE with the appropriate *E*-isomer (i.e., geranial). To this end, we envisioned that a subfamily of copper radical oxidases (CROs), so-called CRO-AlcOx, able to oxidize a wide range of primary activated and unactivated alcohols to the corresponding aldehydes,[24,25] could fulfill this role.

CRO-AlcOx (EC 1.1.3.13; AA5_2[26,27]) are organic cofactor-free enzymes that recently emerged from the exploration of the fungal CROs family.[24,28,29] CROs are better known through the archetypal galactose 6-oxidase from *Fusarium graminearum* (*Fgr*GalOx; EC 1.1.3.9; AA5_2), extensively studied,[30−34] engineered,[35−41] and broadly applied[42−47] since their initial discovery more than 60 years ago.[48] Only recently a few

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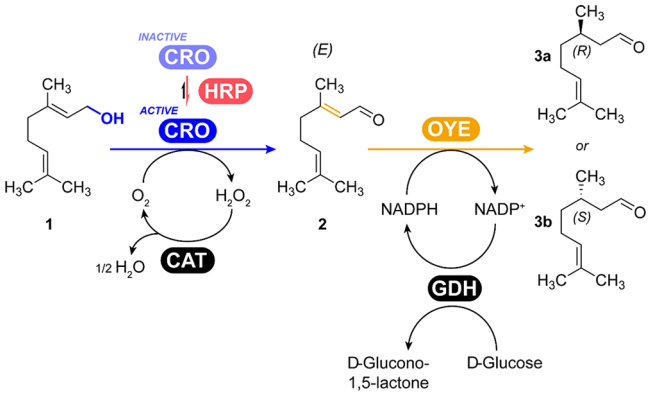
studies have started to investigate the characteristics and application potential of CRO-AlcOx.[25,49−51] A better understanding of these enzymes is needed to foster their use as biocatalysts. To date, CRO-AlcOx have never been evaluated for application in multistep enzymatic reactions, while alcohol oxidation is a key step in the synthesis route of many valuable chemicals.[52] Similarly, OYEs, despite being known for decades, have been only marginally used in cascade reactions until recently.[53] Coupling these two enzymatic systems together is therefore of interest to apprehend their potential in more complex environments and to probe their robustness and relevance for biotechnological applications.

In this study, we developed a bienzymatic cascade composed of the CRO-AlcOx-catalyzed oxidation of the widely available terpene geraniol,[54] to yield specifically geranial further hydrogenated by an OYE into either (*R*)-citronellal or (*S*)-citronellal (Scheme 1). This work unlocks access to (*R*)-citronellal with high optical purity using a wild-type OYE and establishes for the first time the use of a CRO-AlcOx in a multienzymatic cascade, contributing to a better understanding and control of these promising enzymes.

Scheme 1. Geraniol Oxidation by a CRO-AlcOx (Here *Cgr*AlcOx) and Subsequent Geranial Reduction to (*R*)- or (*S*)-Citronellal by an OYE[a]



[a]Compounds are (**1**) geraniol, (**2**) geranial, (**3a**) (*R*)-citronellal, and (**3b**) (*S*)-citronellal.

The initial step of the cascade was first considered. While geraniol had already been described as a good substrate of *Cgr*AlcOx in a previous study,[24] no conversion assay or product analysis was performed. We therefore evaluated the ability of *Cgr*AlcOx to convert geraniol (10 mM), starting with previously established conditions on octan-1-ol,[25] which include catalase (CAT) for *in situ* H_2O_2 dismutation, and horseradish peroxidase (HRP) for *Cgr*AlcOx activation.[49] We observed the facile conversion of geraniol (>99%, turnover number TON 10,000) in only 15 min (turnover frequency TOF 11.1 s^{-1}), at mild temperature (23 °C), and the formation of one isomer of citral (Figure 1, Figures S12 and S13). This citral isomer was further identified as geranial by ^{1}H NMR analysis (Figure S17) based on the study of Zeng et al.[55] The concentrations of accessory enzymes CAT and HRP were then further investigated. As expected, both accessory enzymes are required to sustain the *Cgr*AlcOx activity. A minimum of 0.5 μM HRP (Figure 1A) and 0.5 μM CAT (Figure 1B) were required to reach the maximum conversion efficiency. At least 1 μM *Cgr*AlcOx was required for total conversion of geraniol in 15 min (Figure 1C). Interestingly, the HRP requirement was much lower here compared with that for the conversion of octan-1-ol in our previous study,[25] which could be due to the activated nature of the substrate in this study, rendering its oxidation easier. While HRP has been used as a CRO activator for a long time,[56] the underlying mechanism remains unclear. A direct protein−protein interaction between the peroxidase and the AlcOx could be involved.[49]

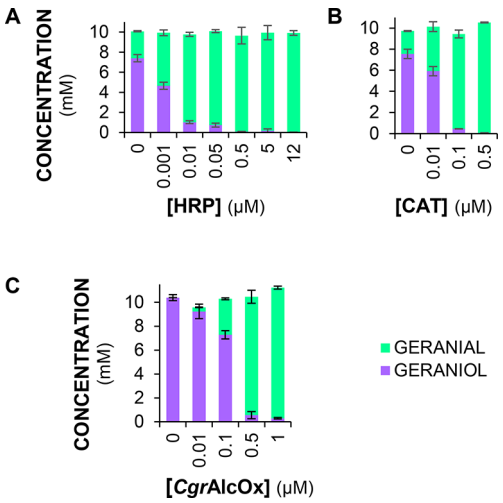


Figure 1. *Cgr*AlcOx-catalyzed oxidation of geraniol with (A) varying concentrations of HRP ([CAT] = 0.5 μM), (B) varying concentrations of CAT ([HRP] = 0.5 μM), and (C) varying concentrations of *Cgr*AlcOx ([HRP] = 0.5 μM, [CAT] = 0.5 μM). For panels B and C, *Cgr*AlcOx was used at 1 μM. Error bars represent standard deviation (s.d., independent experiments, n = 3). The legend in panel C applies also for panels A and B. All reactions were incubated for 15 min at 23 °C, under shaking (190 rpm).

We then investigated the second part of the cascade (Scheme 1) to establish suitable conditions for the OYE-catalyzed reduction step, preferably resulting in enantiopure (*R*)-citronellal. Given the exceptionally fast formation of geranial by *Cgr*AlcOx (TOF 11.1 s^{-1}; Figure 1), it was desirable to identify conditions for a fast reduction by an OYE. The reduction step was investigated using citral (commercial mixture of neral and geranial). The supply of redox equivalents to the OYE was ensured by a NADPH regeneration system promoted by a glucose dehydrogenase from *Bacillus subtilis* (*Bs*GDH). Initially, we investigated the influence of the concentration of OYE2 from *Saccharomyces cerevisiae* on the reduction of 20 mM citral over 5 h (Figure S4). As expected, increased enzyme concentrations resulted in higher conversions, reaching 94.6% in 5 h with 10.67 μM OYE2, giving a TON of 1,773 and a TOF of 0.10 s^{-1}. However, we observed that, with higher conversions, the *ee* of the product (*R*)-citronellal decreased (Figure S5). To investigate this decline in *ee*, we carried out a time-course monitoring of conversion and *ee* values over a 6 h reaction (Figure 2). As previously observed, with increased conversion over time, the *ee* decreased. We expected the OYE-catalyzed reduction of geranial to occur faster than that of neral,[18] changing the ratio between geranial and neral over time. The consumption of neral eventually leads to (*S*)-citronellal, explaining the decreased optical purity of (*R*)-citronellal over time, although we currently lack an explanation why the ratio between the remaining geranial and neral showed only a small change in a nonlinear manner (Table S1). Finally, we explored the

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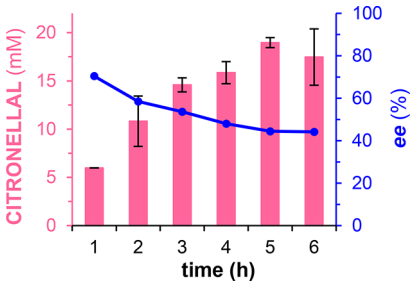

Figure 2. OYE2-catalyzed reduction of citral to citronellal over 6 h. The pink bars correspond to the concentration of the citronellal product (*R* + *S* enantiomers). The blue plot corresponds to the enantiomeric excess of (*R*)-citronellal versus (*S*)-citronellal. Reaction conditions: 20 mM citral, 10.67 μM OYE2, 1 mM $NADP^+$, 40 mM glucose, 6 U/mL *Bs*GDH, 100 mM KPi buffer pH 8.0, incubated at 25 °C and 300 rpm. Products were analyzed on a chiral GC-FID. Error bars represent standard deviation (s.d., independent experiments, n = 2).

influence of the $NADP^+$ concentration on conversion and observed that increased concentrations resulted in higher conversions with 1 mM and 2 mM $NAPD^+$, compared with 0.1 and 0.5 mM (Figure S6).

Based on the parameters we had determined for each individual enzymatic step, we then carried out the one-pot bienzymatic (*Cgr*AlcOx and OYE2) cascade, starting from geraniol as substrate. By providing only geranial to the OYE2 thanks to the oxidation of geraniol by *Cgr*AlcOx, we anticipated that the OYE2-catalyzed reduction should yield preferentially the (*R*)-citronellal.[18] Accordingly, we observed the formation of (*R*)-citronellal with an *ee* ≥ 95% in 2.5 h (Figures 3A and S8). Parallel cascade experiments coupling *Cgr*AlcOx with OYEs from *Thermus scotoductus* (*Ts*OYE)[57] or *Gluconobacter oxydans* (GluER)[58] yielded the alternative (*S*)-citronellal product, with ≥99% *ee* (Figure S16) and respective conversion yields of 37% and 95.3%. Extending the reaction time from 16 to 24 h for *Ts*OYE did not allow further improvement of the conversion yield (Figure S9), probably due to poor substrate affinity of *Ts*OYE toward this β-substituted substrate.[59,60] The use of higher temperature (i.e., 40 °C) for the conversion of citral by the *Ts*OYE only brought a minor enhancement (Figure S7). A *Ts*OYE double mutant with wider substrate specificity, *Ts*OYE-C25D/I67T,[61] only showed a 2-fold increase in conversions compared with the *Ts*OYE wild type with ≥99% *ee* (Figure S7); therefore, GluER remained the best OYE to achieve high yield.

When performing the full cascade in a concurrent one-pot system, we observed a proportion of geraniol that was not oxidized (Figures 3A and S14A). We conjectured that in the conditions we applied, *Cgr*AlcOx could be partly inhibited by the final citronellal product. Indeed, conversions of geraniol by *Cgr*AlcOx performed in the presence of exogenously added citronellal resulted in an incomplete reaction (Figure S10). Such observation is consistent with a hypothesis formulated previously on the possible inhibition of *Cgr*AlcOx by hydrated alkyl-aldehydes.[25,49] In the case of geranial, the conjugation effect stabilizes the molecule in its aldehyde form and disfavors its hydration, whereas citronellal does not benefit from this conjugation effect and would partly form *geminal*-diols upon hydration of the aldehyde,[62,63] likely inhibiting *Cgr*AlcOx.

To avoid initial *Cgr*AlcOx inhibition with citronellal, we performed a sequential one-pot conversion (with OYE2) by running first a 15 min reaction with all reagents except *Bs*GDH and leaving an additional 2.5 h of reaction after addition of

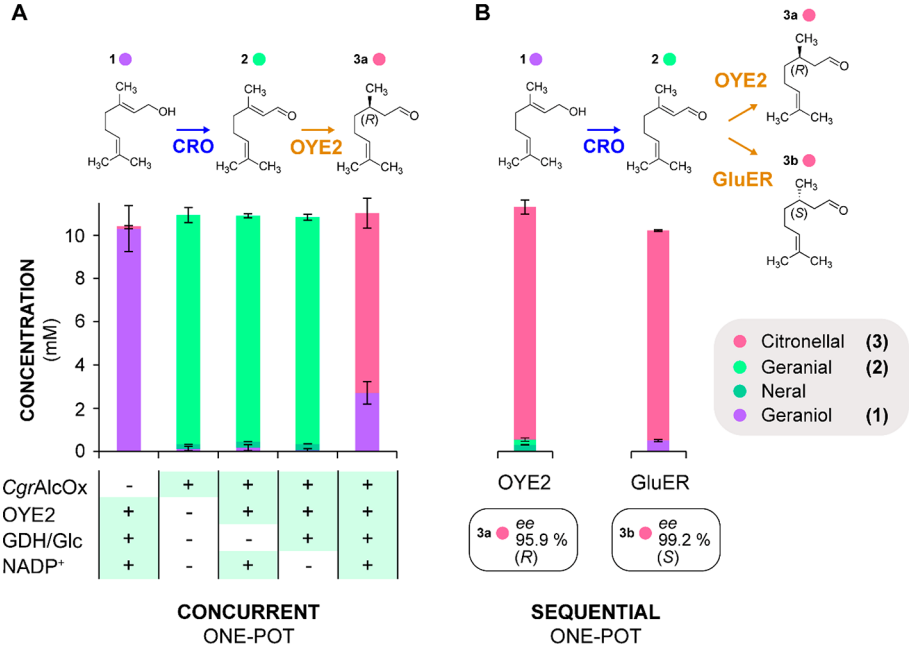

Figure 3. Bienzymatic conversion of geraniol to citronellal by *Cgr*AlcOx and OYEs. (A) Concurrent one-pot cascade reaction in 2.5 h with OYE2 to (*R*)-citronellal. (B) Sequential one-pot cascade reaction using either OYE2 (to (*R*)-citronellal) or GluER (to (*S*)-citronellal): first step (*Cgr*AlcOx conversion of geraniol to geranial) performed in 15 min; second step (OYE conversion of geranial to citronellal) performed in 2.5 h. Analysis by GC-FID (error bars show s.d. independent experiments, n = 3). Note: the y axis displayed in panel A applies for panel B. Reaction conditions: 1 μM *Cgr*AlcOx, 0.5 μM catalase, 0.5 μM HRP, 10.67 μM OYE2 or 8 μM GluER, 6 U/mL *Bs*GDH, 40 mM glucose, 1 mM $NADP^+$, pH 8.0 (50 mM NaPi buffer), 1% v/v acetone. Reactions were incubated at 23 °C, under shaking (200 rpm). For the reactions displayed in panel B, all reagents except for *Bs*GDH were present at the first step; the second step was initiated by the addition of *Bs*GDH to the reaction mixture.

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*Bs*GDH. Under these conditions, >99% of geraniol was converted and 95.1% of the intermediate geranial was converted to (*R*)-citronellal with 95.9% *ee* (Figures 3B and S14B).

Encouraged by the enantioenriched (*R*)-citronellal obtained with *Cgr*AlcOx and OYE2, we carried out the bienzymatic cascade reaction at a larger scale, i.e. in a 20 mL reaction volume, with a starting concentration of geraniol of 20 mM (corresponding to 62 mg). To ensure the completion of the cascade, the reaction times were increased to 1 h for the alcohol oxidation step (catalyzed by *Cgr*AlcOx), followed by 5 h for the conjugated alkene reduction step (catalyzed by OYE2). Additionally, prior to starting the reaction, the headspace and reaction media were saturated with pure oxygen to circumvent potential oxygen limitation in the first step. The resulting (*R*)-citronellal was simply extracted with ethyl acetate without further purification and characterized by chiral GC (Figure S15) and NMR spectroscopy (Figures S18 and S19). Conversion of the geraniol was 98% with a final isolated yield of 72% with 44.3 mg of (*R*)-citronellal with 95.1% *ee*. ^{1}H NMR showed a highly pure product after extraction with ethyl acetate (Figure S18). Comparison of the catalytic efficiencies of the enzymes showed a TON of 17,458 (TOF 4.85 s^{-1}) for *Cgr*AlcOx and 1,636 (TOF 0.09 s^{-1}) for OYE2. Considering that class III OYEs such as *Ts*OYE afford the (*S*)-enantiomer exclusively,[14] it is possible that the incomplete enantioselectivity observed with OYE2 (class II) may be due to kinetic differentiation.

To increase the catalytic efficiency of our system, small-scale experiments were carried out at higher substrate concentrations. Under the same reaction conditions as above, *Cgr*AlcOx was able to convert 91% (±6.7%) of 50 mM geraniol in 2.5 h. The conversion was most likely hampered by lack of oxygen in the medium. Further upscaling of the reaction would require another reactor design to ensure sufficient oxygen supply to the *Cgr*AlcOx. A possible solution to overcome the oxygen limitation would be the use of a segmented flow reactor that has recently been implemented in biocatalysis.[64,65] We have previously demonstrated higher substrate concentrations for the OYE-catalyzed reaction along with others,[66,67] and we do not foresee any limitations for further scale-up.

In conclusion, we established a one-pot bienzymatic cascade starting from inexpensive geraniol to specifically yield (*R*)-citronellal in high optical purity ≥95% *ee*, overcoming the problematic reduction of the mixture of (*E*/*Z*)-isomers in citral by OYEs.[20] This cascade is tunable, by switching the OYE to produce the alternative enantiomer, and scalable, retaining the high optical purity. Together these results provide a biocatalytic method for the production of the key intermediate (*R*)-citronellal in the synthesis of (−)-menthol, the most sold flavor worldwide.[4] We anticipate our biocatalytic cascade to provide an alternative route to achieve enantiopure (*R*)-citronellal and to expand the use of CRO-AlcOx as platform enzymes for multienzymatic reactions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.1c05334.

Details on enzyme production and purification, experimental procedures, extended conversion data, GC chromatograms, and NMR spectra (PDF)

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Author Contributions

‡D.R. and G.T.H. contributed equally.

Author Contributions

D.R. and G.T.H. carried out most of the experimental work, interpreted the data, and wrote the manuscript; B.B. provided guidance in the experimental work. M.Y. drove the NMR experiments and the corresponding result interpretations and was involved in the manuscript writing; C.E.P., B.B., J.-G.B., M.L., and F.L. were involved in the study design and manuscript writing; C.E.P. conceptualized the study, supervised the work, and finalized the manuscript. All authors approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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