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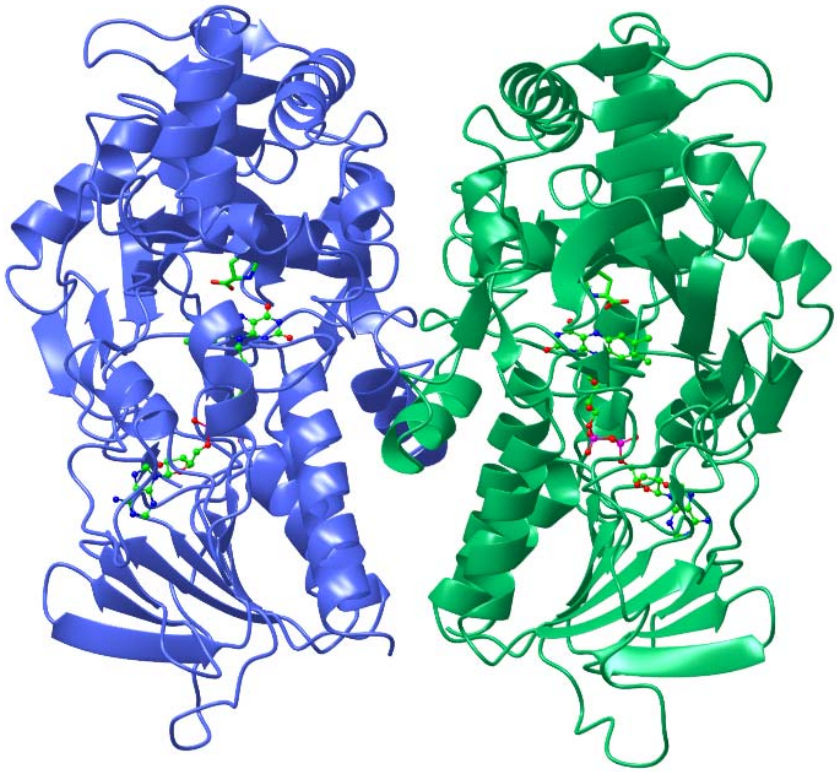

Towards preparative-scale, biocatalytic alkene reductions†‡

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Simple strategies for using alkene reductase enzymes to produce gram-scale quantities of both (*R*)- and (*S*)-citronellal have been developed. The methodology is easily accessible to non-specialist laboratories, allowing alkene reductases to be added to the toolbox of routine synthetic transformations.

Enzyme-mediated alkene reductions have recently emerged as a new approach to asymmetric C=C bond reductions.[1–13] These catalysts have proven particularly useful for electron-deficient olefins conjugated to weakly basic functional groups such as aldehydes, ketones and nitro moieties. Past efforts have focused mainly on constructing and characterizing libraries of alkene reductases. The state of these collections has now matured to the point where applications to organic synthesis can be contemplated. To date, however, there are relatively few examples of reaction strategies that allow the promising results uncovered by small-scale screening studies to be easily scaled up to gram quantities (or greater).[2] To address this problem, we focused on biocatalytic routes to both enantiomers of citronellal **2**, a valuable building block in asymmetric synthesis (Scheme 1). The (*R*)-enantiomer is particularly useful as the starting material for the Takasago route to menthol.[3]

The low cost of citral (an approximately 3 : 2 mixture of geometric isomers **1** and **4**) makes it an attractive precursor for both (*R*)- and (*S*)-**2**. Both whole-cell[4] and purified alkene reductases[5] have been tested as catalysts for citral reduction on small scales, both with respect to reaction volume and substrate concentration. Here, we used citral reduction as a testbed to address two major issues associated with synthetic applications of alkene reductases. First, could we identify a pair of biocatalysts that delivered both citronellal enantiomers with $\geq 98\%$ ee while avoiding unwanted side-reactions such as carbonyl reduction? Second, could these biocatalysts be used to supply gram-scale quantities of the target compounds by employing simple techniques accessible to non-specialist laboratories? Our results show that both questions can be answered in the affirmative.

Based on our analysis of alkene reductases in sequence databases,[2c] we constructed a library of 17 biocatalysts that were overexpressed in *Escherichia coli* as fusion proteins with glutathione *S*-transferase (GST).[6] The affinity tag allowed a one-step purification so that screening reactions could be carried out with pure proteins, thereby minimizing side-reactions such as carbonyl reduction. Screening this collection against citral uncovered several enzymes with modest (*R*)-[7] and some with very good (*S*)-selectivity.[8] To a first approximation, yeast alkene reductases of the old yellow enzyme (OYE) family produced mainly (*R*)-**2** while bacterial and plant OYEs showed the opposite stereochemical preference. The best choices were *Pichia stipitis* OYE 2.6 and *E. coli* NemA for (*R*)- and (*S*)-**2**, respectively. We also selected glucose dehydrogenase (GDH) for NADPH regeneration.

Citral presents several challenges as a starting material. Net *trans*-addition of H_2 to the geometric isomers geranial **1** and neral **4** yields enantiomeric products.[5a] This reduces the reaction's enantioselectivity since all the alkene reductases currently in our collection show only a modest preference for **1** *vs.* **4**. Under some conditions, the geometric isomers **1** and **4** interconvert,[9] which further complicates the process. Finally, both the enal substrates and the aldehyde products are reactive electrophiles that could deactivate alkene reductases and other enzymes by covalent modification.[1b] For all of these reasons, the identification of stereoselective alkene reductases solves only the first part of the synthetic problem. Making these biocatalysts useful for preparative purposes requires careful attention to process design, and this became our next focus. Since (*R*)-**2** is commercially more important, we concentrated initially on this enantiomer, then demonstrated the utility of the optimized conditions by substituting the (*S*)-selective reductase with no additional modifications. The reaction conditions developed here have also been applied successfully to other activated alkenes in our laboratory, further underscoring their utility.

Because earlier studies suggested that the substrate and/or product irreversibly harmed the biocatalysts,[4a] our first strategy for producing (*R*)-citronellal **2** used biphasic conditions (1 : 1 hexanes and 100 mM KP_i, pH 7.5) to protect purified GST-OYE 2.6 and GDH from deactivation by the citral feedstock (Fig. 1). Under these conditions, glucose consumption was tightly coupled to alkene reduction, indicating that side-reactions such as hydrogen peroxide formation were not significant under the reaction conditions. The data also revealed that OYE 2.6 has only a slight preference for geranial over neral and that alkene isomerization is relatively slow under our reaction conditions. Unfortunately, the product concentration reached only *ca.* 30 mM after 51 h. A variety of other water-immiscible solvents were screened, but no significant improvement was observed.

Cross-linked enzyme aggregates (CLEAs) have been successfully employed for a wide variety of enzyme-catalyzed conversions.[10] Often, CLEA incorporation stabilizes biocatalysts significantly against inactivation. Using the procedure of

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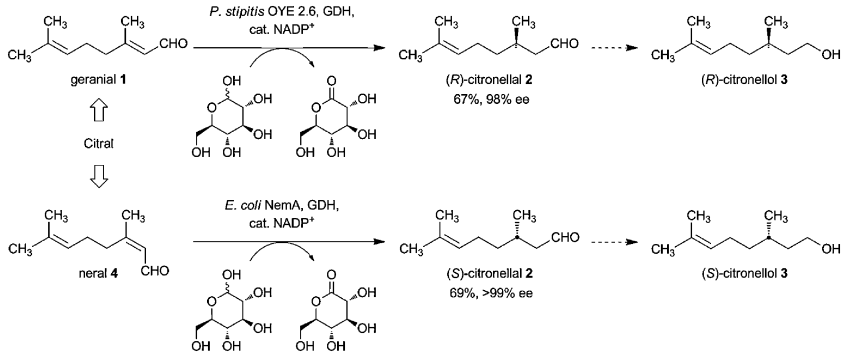

Scheme 1

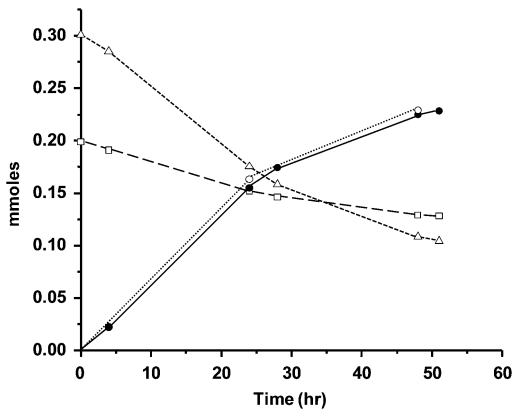

Fig. 1 Time course for OYE 2.6-mediated reduction of citral under biphasic conditions. Quantities determined by GC are plotted as mmoles present (geranial **1**, △; neral **4**, □; citronellal **2**, ●) or mmoles consumed (glucose, ○). The reduction was carried out in a 1 : 1 mixture of hexanes and 100 mM KP_i, pH 7.5 using purified GST-OYE 2.6 and GDH at room temp.

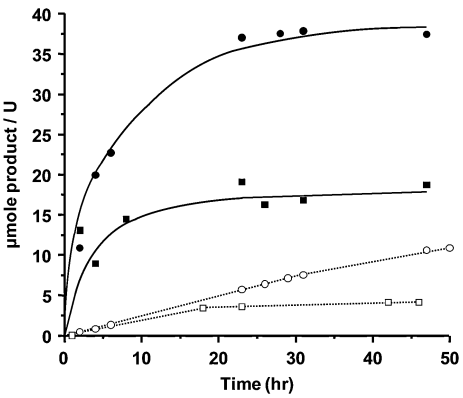

Fig. 2 Comparison between strategies for the OYE 2.6-mediated reduction of geranial **1**. Quantities determined by GC are plotted as μmoles of citronellal **2** formed per unit of GST-OYE 2.6 activity. The enzyme was employed as free protein in buffer (●) or 1 : 1 mixture of hexanes and buffer (○) or as a CLEA in buffer (■) or a 1 : 1 mixture of hexanes and buffer (□).

Scism and Bachmann,[11] we prepared aggregates from mixtures of crude extracts containing OYE 2.6 and GDH along with added neutral protein (BSA or lysozyme) using $(NH_4)_2SO_4$ precipitation followed by glutaraldehyde treatment. We hoped that the glutaraldehyde crosslinking process would involve surface lysine side-chains that might otherwise react with citral or citronellal and thereby inactivate the enzymes. Despite varying the quantity of additional inert protein, its identity (BSA or lysozyme) or the level of cross-linker, the CLEA form of the enzymes consistently yielded lower activities when compared to the free counterparts in aqueous or biphasic conditions (Fig. 2). Surprisingly, the longevities of the bioconversions were also not improved by CLEA formation. We also attempted to mimic the possible protection afforded by CLEA formation (capping of surface lysine residues) without the mass transfer limitations of aggregate formation by treating GST-OYE 2.6 with acetic anhydride.[12] Unfortunately, this treatment diminished catalytic efficiency without prolonging the productive reaction time (data not shown). Taking all these results into consideration, we decided to use free enzymes in a single-phase system. While these conditions led to OYE inactivation after *ca.* 20 h, this disadvantage was more than offset by the higher volumetric productivity and final product titer.

To maximize enantioselectivity and reaction rate, geometrically pure (>98%) geranial **1** was used in place of citral. A variety of geranial feed strategies were investigated; the simplest involved monitoring base demand (which arises from coupled glucose oxidation) to show when the available geranial had been consumed and more was required. Under these conditions, an 86% conversion was observed after 12 h and (*R*)-**2** was produced with 95% ee. For best results, it was important to keep the total added substrate and EtOH below 200 mM and 10%, respectively.

The optimization studies described above used affinity-purified GST-OYE 2.6 to avoid competing carbonyl reduction by *E. coli* dehydrogenases. This side-reaction has also been noted in other whole-cell citral reductions.[4b] Since the *E. coli* YahK protein is known to reduce citronellal **2**,[13] we incubated the corresponding knockout strain[14] with racemic **2**. After 20 h, 89% had been reduced to alcohol **3**, showing that at least one more *E. coli* alcohol dehydrogenase is involved in the undesired over-reduction. Rather than pursuing additional gene knockouts, we found that fractionating the GST-OYE 2.6 crude extract with ammonium sulfate virtually eliminated carbonyl reduction and made it possible to prepare large quantities of the biocatalyst with minimal effort.

We reduced freshly-prepared geranial **1** on a 15 mmole scale using an ammonium sulfate-treated crude extract containing GST-OYE 2.6 in a total volume of 100 mL.[15] Conversion reached 95% after 5.75 h when the reaction was terminated and the product isolated by solvent extraction followed by column chromatography. (*R*)-Citronellal **2** was isolated in 67% yield with 98% ee. No citronellol was observed. To test the generality of the process design, we applied analogous conditions to neral **4**. A crude extract was prepared from cells overexpressing *E. coli* GST-NemA. Because this preparation showed very high specific activity toward neral, competing carbonyl reduction was not significant and ammonium sulfate fraction was omitted. Using the methodology described above, (*S*)-citronellal **2** was obtained in 69% yield with 99% ee after a 4 h reaction (>98% conversion).[16]

The conditions developed here open the possibility of employing alkene reductases in preparative-scale reactions. We focused mainly on volumetric productivity since scaling up reaction volumes provides a straightforward means to converting larger substrate quantities. The lack of carbonyl reduction is an important advantage of our methodology. No special equipment is required and the reactions are carried out in standard organic glassware. The methodology is currently being used successfully in our laboratory for additional substrates and we anticipate that it will be broadly applicable, making the ever-increasing number of available alkene reductases useful in synthetic planning and execution.

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7 (*R*)-selective enzymes (accession number in parentheses): *Saccharomyces pastorianus* OYE1 (Q02899), 61% ee; *Saccharomyces cerevisiae* OYE2 (Q03558), 87% ee; *S. cerevisiae* OYE3 (P41816), 21% ee; *Schizosaccharomyces pombe* OYEA (Q09670), 45% ee; *Kluyveri marxianus* OYE (Q6I7B7), 44% ee; *Pseudomonas putida* NemA (Q88I29), 17%; *P. stipitis* OYE 2.6 (XP_001384055.1), 90%.

8 (*S*)-selective enzymes (accession number in parentheses): *Escherichia coli* NemA (P77258), 79%; *Pseudomonas putida* OYE (Q88K07), 55%; *Synechococcus elongatus* OYE (Q31R14), 60% ee; *Arabidopsis thaliana* OPR1 (Q8LAH7), 99%; *A. thaliana* OPR3 (Q9FUP0), 99%; *Solanum lycopersicon* OPR (Q9XG54), 78%; Rat Ltb_4 dehydrogenase (P97584), 19%.

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15 An 85 mL sample of 100 mM KP_i, pH 7.5 containing glucose (44.4 mmoles, 8.0 g) was degassed for 1 h, then transferred to a 500 mL round bottom flask under Ar. GDH 102 (100 U, 1 mg), NADPH (12 μmoles, 10 mg) and ammonium sulfate-purified GST-OYE 2.6 (100 U, 13 mL) were added. After stirring at 300 rpm for 15 min at room temp., geranial (5.2 mmoles, 0.79 g) dissolved in 0.83 mL of EtOH was added. The pH was maintained at 7.5 using a pH stat (1 M KOH titrant) and additional portions of geranial were added after 1.5 and 3.0 h. After 5.75 h, GC/MS analysis showed 95% conversion. The mixture was acidified to pH 4 with 1 M HCl and stirred overnight with CH_2Cl_2 (100 mL). The aqueous portion was extracted with additional CH_2Cl_2 (2 × 50 mL), then the organic layers were combined, filtered over Celite, washed with brine (3 × 50 mL), dried over Na_2SO_4 and passed over a small bed of silica gel. After concentration under vacuum, the crude product (2.12 g) was adsorbed onto silica gel deactivated with 10% H_2O (5 g) and then added to a silica column (60 g) equilibrated with hexanes. The desired product was eluted with 1 : 9 Et_2O/hexanes to yield 1.59 g of a pale yellow liquid (99% purity by GC) whose spectral data matched those of (*R*)-citronellal. $[\alpha]_D^{24} = +18.22$ (c = 7.30, $CHCl_3$).

16 GDH 102 (100 U, 1 mg), NADPH (12 μmoles, 10 mg) and a crude extract containing *E. coli* GST-NemA (480 U, 11 mL) were stirred for 15 min in 85 mL of degassed KP_i, pH 7.5, containing glucose (44.4 mmoles, 8.0 g). Neral was added in three equal portions (total 15.5 mmoles, 2.38 g) as an EtOH solution (total 2.5 mL) at 0, 1.5 and 2.5 h. The reaction was maintained at pH 7.5 and GC/MS indicated complete consumption of neral after 3.5 h. Product isolation as described above yielded 1.62 g of a pale yellow oil (98% purity by GC) whose spectral data matched those of (*S*)-citronellal. $[\alpha]_D^{24} = -13.71$ (c = 5.55, $CHCl_3$).

