

Article

Cascading Old Yellow Enzyme, Alcohol Dehydrogenase and Glucose Dehydrogenase for Selective Reduction of (*E/Z*)-Citral to (*S*)-Citronellol

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Abstract: Citronellol is a kind of unsaturated alcohol with rose-like smell and its (*S*)-enantiomer serves as an important intermediate for organic synthesis of (−)-*cis*-rose oxide. Chemical methods are commonly used for the synthesis of citronellol and its (*S*)-enantiomer, which suffers from severe reaction conditions and poor selectivity. Here, the first one-pot double reduction of (*E/Z*)-citral to (*S*)-citronellol was achieved in a multi-enzymatic cascade system: N-ethylmaleimide reductase from *Providencia stuartii* (NemR-PS) was selected to catalyze the selective reduction of (*E/Z*)-citral to (*S*)-citronellal, alcohol dehydrogenase from *Yokenella* sp. WZY002 (YsADH) performed the further reduction of (*S*)-citronellal to (*S*)-citronellol, meanwhile a variant of glucose dehydrogenase from *Bacillus megaterium* (BmGDH_{M6}), together with glucose, drove efficient NADPH regeneration. The *Escherichia coli* strain co-expressing NemR-PS, YsADH, and BmGDH_{M6} was successfully constructed and used as the whole-cell catalyst. Various factors were investigated for achieving high conversion and reducing the accumulation of the intermediate (*S*)-citronellal and by-products. 0.4 mM NADP⁺ was essential for maintaining high catalytic activity, while the feeding of the cells expressing BmGDH_{M6} effectively eliminated the intermediate and by-products and shortened the reaction time. Under optimized conditions, the bio-transformation of 400 mM citral caused nearly complete conversion (>99.5%) to enantio-pure (*S*)-citronellol within 36 h, demonstrating promise for industrial application.

Keywords: old yellow enzyme; alcohol dehydrogenase; glucose dehydrogenase; (*E/Z*)-citral; (*S*)-citronellol; selective reduction; cascade biocatalysis

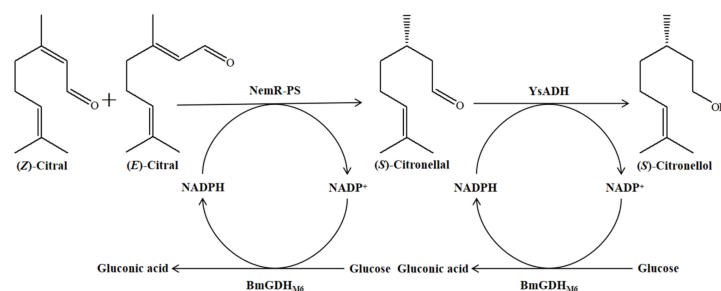
1. Introduction

Citronellol is one of essential components in various kinds of perfumes and a promising molecule with pharmacological activities [1,2]. The (*S*)-enantiomer of citronellol serves as an important intermediate for the synthesis of optically active chemicals, e.g., (−)-*cis*-rose oxide, (*S*)-pulegone, and 3(*S*)-methyl-heptanoic acid [3–5]. The flavor of (*S*)-citronellol is also different from that of (*R*)-citronellol [6]. Hence, the selective synthesis of both citronellol and its (*S*)-enantiomer would be considerably valuable. The synthesis of citronellol commonly relies on chemical methods starting from citral, geraniol, or citronellal [7–9], but rarely on biotransformation [10,11]. Since citral is a kind of bulky chemical and readily available, the manufacture of citronellol from citral could be more profitable. However, chemical hydrogenation of citral remains a challenge because it possesses an isolated C=C bond, as well as the conjugated C=O and C=C bonds. As a result, chemical hydrogenation

could result in various products: citronellal, citronellol, nerol, geraniol, dihydrocitronellal, and 3,7-dimethyloctanol [7,9]. An enzymatic approach offers a promising alternative to chemical approach because the enzymes retain superior chemoselectivity and enantioselectivity [12]. Old yellow enzymes are known to reduce the conjugated C=C bond of α,β -unsaturated aldehydes and ketones [13,14], meanwhile alcohol dehydrogenases catalyze the reversible reduction of the C=O bond [15–17]. The FMN-containing old yellow enzymes catalyze the asymmetric hydrogenation of citral at the expense of a nicotinamide cofactor, producing citronellal with one stereocenter. In addition, old yellow enzymes require the substrate bearing electron-withdrawing group and do not function on geraniol. Hence, citral instead of geraniol and racemic citronellal turns out to be the right choice for old yellow enzyme-mediated synthesis of (S)-citronellol.

In the conceptual enzymatic synthesis of (S)-citronellol from citral, (S)-enantioselective old yellow enzyme reduced citral to (S)-citronellal, which was then reduced to (S)-citronellol by alcohol dehydrogenase. The double reduction of citral requires efficient NAD(P)H regeneration, e.g., the glucose dehydrogenase/glucose system [18]. Commercially available citral represents a mixture of *trans*-isomer and *cis*-isomer [19], and the reduction of (*E*)-citral and (*Z*)-citral often leads to the products with complementary optical properties [14,20]. Thus, old yellow enzyme with strict (S)-enantioselectivity is mandatory to obtain enantio-pure (S)-citronellol in the double reduction of (*E/Z*)-citral. The reduction of (S)-citronellal to (S)-citronellol, in which the enantioselectivity is not required, can also benefit from the use of enzymes because enzymatic hydrogenation usually runs at room temperature and ambient pressure and gives high chemo- or regio-selectivity [21]. The use of multiple enzymes also offers the option of a cascade approach instead of sequential approach. One-pot cascade approach could improve catalytic efficiency, reduce the product recovery, enable the reversible reaction for the desired product formation and minimize the use of harmful or unstable compounds [22–24]. Moreover, the use of a whole-cell biocatalyst is considerably attractive since it is no need to purify the enzymes. In the case of hydrophobic substrates and products, the cell envelope offers the protection of intracellular enzymes but also the permeability of hydrophobic substrates to keep the reaction going [25]. In the whole-cell catalyzed multi-enzymatic reaction, the accumulation of intermediate and by-product is often encountered, and the activity orchestra of multiple enzymes is necessary to suppress the formation of undesired compounds and achieve high conversion [21].

In this proof-of-concept study, N-ethylmaleimide reductase from *Providencia stuartii* (NemR-PS, a member of old yellow enzyme family) [26], alcohol dehydrogenase from *Yokenella* sp. WZY002 (YsADH) [15] and a newly developed variant of glucose dehydrogenase from *Bacillus megaterium* (BmGDH_{M6}) [27] were selected to develop double reduction of (*E/Z*)-citral to (S)-citronellol (Scheme 1). All three enzymes were co-expressed in an *Escherichia coli* strain as a whole-cell biocatalyst. Various factors affecting whole-cell catalyzed double reduction of (*E/Z*)-citral were investigated to improve catalytic efficiency and reduce the accumulation of the intermediate (S)-citronellal and by-products, while maintaining strict (S)-enantioselectivity. Taken together, a new enzymatic approach for asymmetric synthesis of (S)-citronellol from (*E/Z*)-citral was provided with high conversion and selectivity.



Scheme 1. Double reduction of (*E/Z*)-citral to (S)-citronellol using the *E. coli* cells co-expressing old yellow enzyme NemR-PS, alcohol dehydrogenase YsADH and glucose dehydrogenase BmGDH_{M6}.

2. Results and Discussion

2.1. Selection of Old Yellow Enzyme with High Activity and Strict (S)-Enantioselectivity

The reduction of (*E/Z*)-citral to (*S*)-citronellal is the key step in the cascade reaction, which requires old yellow enzyme with high activity and strict (*S*)-enantioselectivity. However, the activity and enantioselectivity of old yellow enzyme are usually not desirable for synthetic applications [28,29]. Yeast old yellow enzymes are well characterized for the reduction of α,β -unsaturated aldehydes and ketones [19]. The selection of suitable old yellow enzyme was also inspired by the work on *N*-ethylmaleimide reductase-catalyzed preparative scale reduction of (*Z*)-citral to (*S*)-citronellal [30]. Thus, a small library was constructed including four old yellow enzymes from yeast and six *N*-ethylmaleimide reductase from bacteria (Figure S1), each of which was cloned into a pET28b vector and over-expressed in the *E. coli* BL21 DE(3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed good expression levels for all tested old yellow enzymes (Figure 1). The activities of crude enzymes were compared, and the enantioselectivity in the asymmetric reduction of (*E/Z*)-citral to citronellal was determined by chiral gas chromatography (Table 1). Similar to previous observation [31], old yellow enzymes from yeast showed (*R*)-enantioselectivity. It was notable that all tested *N*-ethylmaleimide reductases possessed strict (*S*)-enantioselectivity in the reduction of (*E/Z*)-citral to citronellal. The activities of *N*-ethylmaleimide reductases were higher than those of old yellow enzymes from yeast. Among them, old yellow enzyme NemR-PS had the highest activity. Thus, NemR-PS was selected for subsequent co-expression with alcohol dehydrogenase and glucose dehydrogenase.

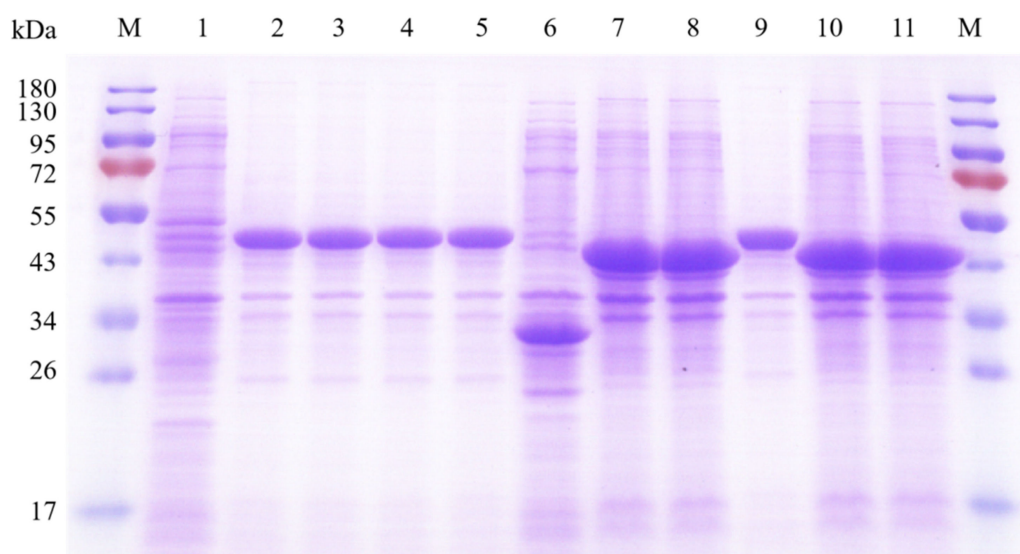


Figure 1. SDS-PAGE analysis of ten old yellow enzymes. Lane M, marker; Lane 1, no induction of old yellow enzyme as the control; lane 2, OYE3 (45 kDa); lane 3, OYE2p (45 kDa); lane 4, OYE2y (45 kDa); lane 5, OYE2y-HG (45 kDa); lane 6, NemR-SG (33 kDa); lane 7, NemR-CK (39 kDa); lane 8, NemR-CR (39 kDa); lane 9, NemR-CT (44 kDa); lane 10, NemR-CS (39 kDa); lane 11, NemR-PS (39 kDa). The percentage of acrylamide in the resolving gel was 12%. The value in the bracket represents the apparent molecular mass of old yellow enzymes.

Table 1. Comparison of activity and enantioselectivity of ten old yellow enzymes in cell-free extracts.

Old Yellow Enzyme	GenBank Accession No.	Strain	Relative Activity (%) ¹	<i>e.e.</i> (%) ²
OYE3	P41816.2	<i>S. cerevisiae</i> S288C	13.2 ± 0.7	23.6 ± 1.6 (<i>R</i>)
OYE2p	AJV32222.1	<i>S. cerevisiae</i> YJM1341	19.8 ± 0.6	93.5 ± 1.9 (<i>R</i>)
OYE2y	QDX15144.1	<i>S. cerevisiae</i> CICC1060	26.1 ± 0.8	47.5 ± 1.5 (<i>R</i>)
OYE2y-HG ³	QDX15144.1	<i>S. cerevisiae</i> CICC1060	33.9 ± 1.3	96.6 ± 2.1 (<i>R</i>)

Table 1. Cont.

Old Yellow Enzyme	GenBank Accession No.	Strain	Relative Activity (%) ¹	e.e. (%) ²
NemR-SG	WP_052967635.1	<i>Shigella sonnei</i> SS046	63.8 ± 2.1	>99 (S)
NemR-CK	ASE84833.1	<i>Citrobacter koseri</i>	70.6 ± 1.5	>99 (S)
NemR-CR	CBG88182.1	<i>Citrobacter rodentium</i> ICC168	77.7 ± 3.0	>99 (S)
NemR-CT	CBA30573.1	<i>Cronobacter turicensis</i> Z3032	82.0 ± 2.7	>99 (S)
NemR-CS	AMH14450.1	<i>Citrobacter</i> sp. FDAARGOS156	93.2 ± 3.3	>99 (S)
NemR-PS	KNZ86848.1	<i>Providencia stuartii</i>	100	>99 (S)

¹ The relative activity of 100% represents the specific activity of 0.17 ± 0.02 U/mg. The results are the averages of triplicate experiments.

² The e.e. value of >99% (S) represents that (R)-citronellal was not detectable in chiral GC analyses. ³ The old yellow enzyme OYE2y-HG represents the variant of OYE2Y with double substitution of R330H and P76G.

2.2. Co-Expression of Old Yellow Enzyme NemR-PS, Alcohol Dehydrogenase YsADH, and Glucose Dehydrogenase BmGDH_{M6}

Alcohol dehydrogenase YsADH (Figure S1) was selected to catalyze the reduction of (S)-citronellal to (S)-citronellol due to its excellent activity and stability [15]. Both NemR-PS and YsADH accepted NADP(H) as coenzyme. Thus, NADP⁺-dependent glucose dehydrogenase BmGDH_{M6} (Figure S1) with improved stability, a newly developed variant of BmGDH with the substitutions of Q252L/E170K/S100P/K166R/V72I/K137R, was used to drive NADPH regeneration [27]. Each gene encoding YsADH, NemR-PS, or BmGDH_{M6} was inserted onto the plasmid pET28b, and the strain expressing YsADH, NemR-PS, or BmGDH_{M6} was constructed and induced (Figure 2a). The activities of crude enzymes NemR-PS, YsADH, and BmGDH_{M6} were 0.17 U/mg, 0.47 U/mg, and 0.34 U/mg (Table 2), respectively, indicating soluble and active expression of those enzymes. In contrast to the strains expressing a single enzyme, the co-expression of NemR-PS, YsADH, and BmGDH_{M6} would simplify the preparation of whole-cell biocatalysts and circumvent diffusion limitations of cell membranes. To achieve co-expression, the gene encoding YsADH was cloned onto one of two multiple cloning sites of the plasmid pACYCDuet-1, offering the strain *E. coli* BL21 DE(3)/pACYCDuet-1-YsADH and the crude YsADH activity of 0.45 U/mg. Then, the gene encoding NemR-PS was inserted into the other multiple cloning sites of pACYCDuet-1, causing the strain *E. coli* BL21 DE(3)/pACYCDuet-1-YsADH-NemR-PS with the co-expression of YsADH and NemR-PS (Figure 2b). The plasmid pET28b-BmGDH_{M6} was further introduced into the cells co-expressing YsADH and NemR-PS, giving rise to a strain *E. coli* BL21 DE(3)/pACYCDuet-1-YsADH-NemR-PS/pET28b-BmGDH_{M6} co-expressing YsADH, NemR-PS, and BmGDH_{M6} (Figure 2c). Cell-free extracts were prepared from the cells co-expressing YsADH, NemR-PS, and BmGDH_{M6}, and the activities of YsADH, NemR-PS and BmGDH_{M6} were determined to be 0.20 U/mg, 0.13 U/mg, and 0.19 U/mg, respectively (Table 2). Due to limited cell capacity, the co-expression of three enzymes resulted in the reduced expression level of each enzyme to some extent [32], although the ratio of YsADH, NemR-PS, and BmGDH_{M6} remained reasonable.

Table 2. The activities of YsADH, NemR-PS, and BmGDH_{M6} in cell-free extracts¹.

Strain	Expression of Enzyme(s)	YsADH (U/mg)	NemR-PS (U/mg)	BmGDH _{M6} (U/mg)
<i>E. coli</i> BL21 DE(3)/pET28b-YsADH	YsADH	0.47 ± 0.02	—	—
<i>E. coli</i> BL21 DE(3)/pET28b-NemR-PS	NemR-PS	—	0.17 ± 0.02	—
<i>E. coli</i> BL21 DE(3)/pET28b-BmGDH _{M6}	BmGDH _{M6}	—	—	0.34 ± 0.03
<i>E. coli</i> BL21 DE(3)/pACYCDuet-1-YsADH	YsADH	0.45 ± 0.03	—	—
<i>E. coli</i> BL21 DE(3)/pACYCDuet-1-YsADH-NemR-PS	YsADH and NemR-PS	0.32 ± 0.01	0.15 ± 0.02	—
<i>E. coli</i> BL21 DE(3)/pACYCDuet-1-YsADH-NemR-PS/pET28b-BmGDH _{M6}	YsADH, NemR-PS and BmGDH _{M6}	0.20 ± 0.01	0.13 ± 0.01	0.19 ± 0.02

¹ The activities of crude enzymes are the averages of triplicate experiments.

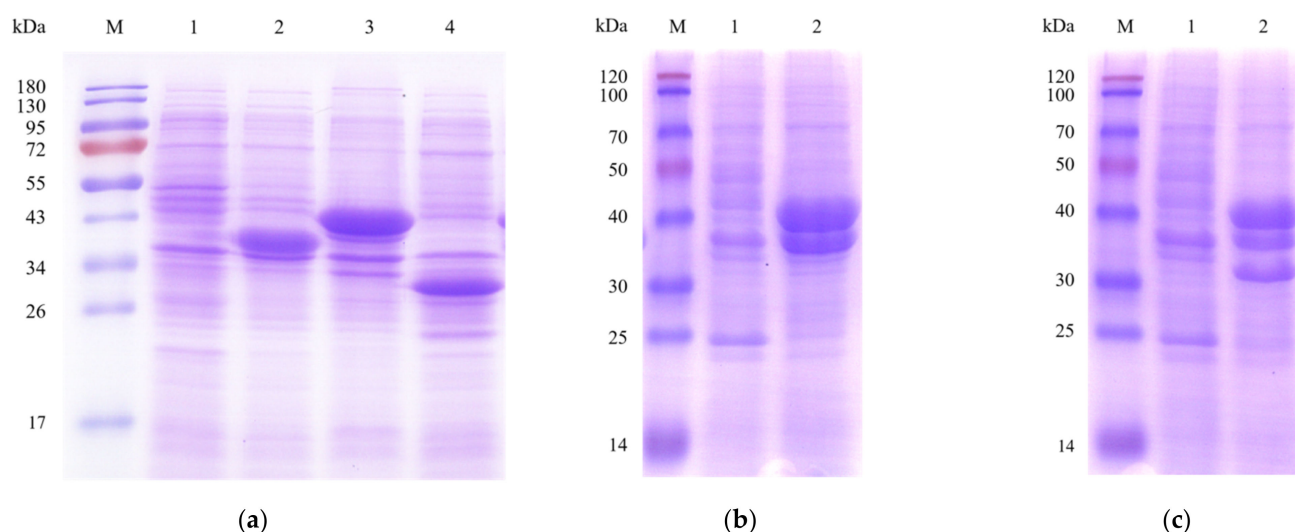


Figure 2. SDS-PAGE analysis of old yellow enzyme, alcohol dehydrogenase and glucose dehydrogenase. (a) Expression of YsADH, NemR-PS, or BmGDH alone. Lane M, marker; lane 1, no induction of YsADH, NemR-PS, or BmGDH as the control; lane 2, YsADH (37 kDa); lane 3, NemR-PS (39 kDa); lane 4, BmGDH_{M6} (31 kDa). (b) Co-expression of YsADH and NemR-PS. Lane M, marker; lane 1, no induction of YsADH and NemR-PS as the control; lane 2, co-expression of YsADH (37 kDa) and NemR-PS (39 kDa). (c) Co-expression of YsADH, NemR-PS, and BmGDH_{M6}. Lane M, marker; lane 1, no induction of YsADH, NemR-PS and BmGDH_{M6} as the control; lane 2, co-expression of YsADH (37 kDa), NemR-PS (39 kDa) and BmGDH_{M6} (31 kDa). The percentage of acrylamide in the resolving gel was 12%. The value in the bracket represents the apparent molecular mass of old yellow enzymes.

2.3. Investigation of Key Factors Affecting Selective Reduction of (E/Z)-Citral to (S)-Citronellol

In the whole-cell catalyzed cascade reaction, the concentrations of substrate, intermediate, and product dynamically vary so that it is hard to sustain the highest activity of each enzyme. Thus, conversion-based assay is more reasonable than enzyme activity assay to evaluate the one-pot double reduction of (E/Z)-citral to (S)-citronellol. The effect of the reaction temperature was determined over a range of 20 °C to 45 °C, and the highest conversion was observed at 30 °C (Figure 3a). When the temperature was greater than 30 °C, the conversion decreased as the temperature rose. To determine the optimal pH, the reaction was carried out at pH levels ranging from 5.0 to 9.0 at 30 °C. The highest conversion was detected at pH 6.5 (Figure 3b). Similarly, the optimal rotation was determined to be 400 rpm (Figure S2). Members of old yellow enzyme family are NAD(P)H-dependent oxido-reductases containing non-covalently bound FMN [33,34]. The conversion increased when NADP⁺ was elevated from 0 to 0.4 mM, and further increase in exogenous NADP⁺ had no significant improvement of conversion (Figure 4a). When the NADP⁺ concentration was set as 0.4 mM, the supplement of exogenous FMN slightly improved the conversion (Figure 4b), implying that the binding of endogenous FMN was tight and sufficient for maintaining the activity. Since the substrate is hydrophobic, the co-solvent addition in the reaction mixture might lead to improved conversion [35]. Consequently, the reactions were tested in the presence of various co-solvents including 1-butanol, acetone, methanol, ethanol, isopropanol, and DMSO (Figure 5a). In contrast to the control without the co-solvent, the supplementing with isopropanol or DMSO increased the conversions from 30% to 80% or 81%, respectively. Considering the convenience of subsequent co-solvent removal, isopropanol was used as co-solvent for further study.

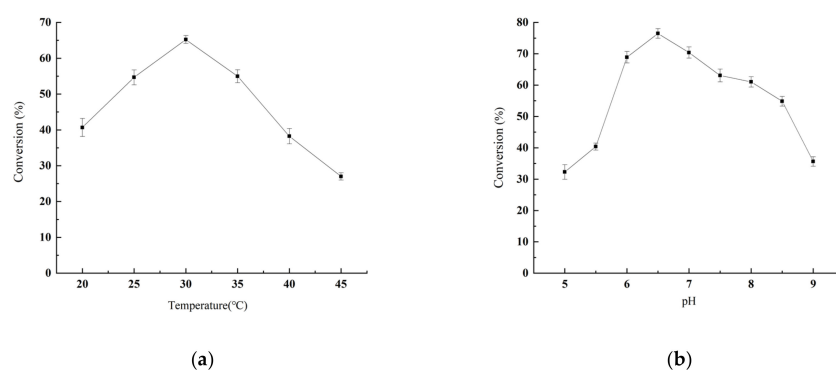


Figure 3. Effect of temperature (a) and pH (b) on enzymatic double reduction of (*E/Z*)-citral to (*S*)-citronellol. The reaction mixture (10 mL) contained 200 mM (*E/Z*)-citral, 600 mM glucose, 1 g wet cells, 0.2 mM NADP⁺, 20% (*v/v*) isopropanol and 50 mM buffer. (a), the reactions in 50 mM Tris-HCl buffer (pH 8.0) were conducted at 20–45 °C and 200 rpm for 12 h. (b), the reactions were conducted at 30 °C, 200 rpm and pH 5–9 for 12 h. Standard deviations are indicated in the diagram (*n* = 3).

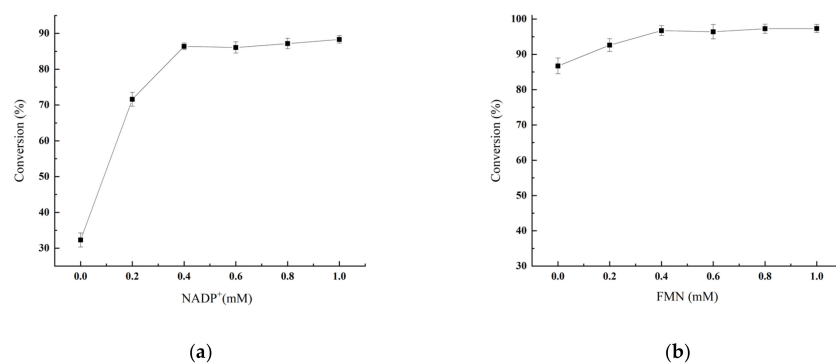


Figure 4. Effect of NADP⁺ (a) and FMN (b) on enzymatic double reduction of (*E/Z*)-citral to (*S*)-citronellol. (a), the reaction mixture (10 mL) contained 200 mM (*E/Z*)-citral, 600 mM glucose, 1 g wet cells, 0–1.0 mM NADP⁺, 20% (*v/v*) isopropanol, and 50 mM PIPES buffer (pH 6.5). (b), the reaction mixture (10 mL) contained 200 mM (*E/Z*)-citral, 600 mM glucose, 1 g wet cells, 0.4 mM NADP⁺, 0–1.0 mM FMN, 20% (*v/v*) isopropanol and 50 mM PIPES buffer (pH 6.5). The reactions were conducted at 30 °C and 400 rpm for 12 h. Standard deviations are indicated in the diagram (*n* = 3).

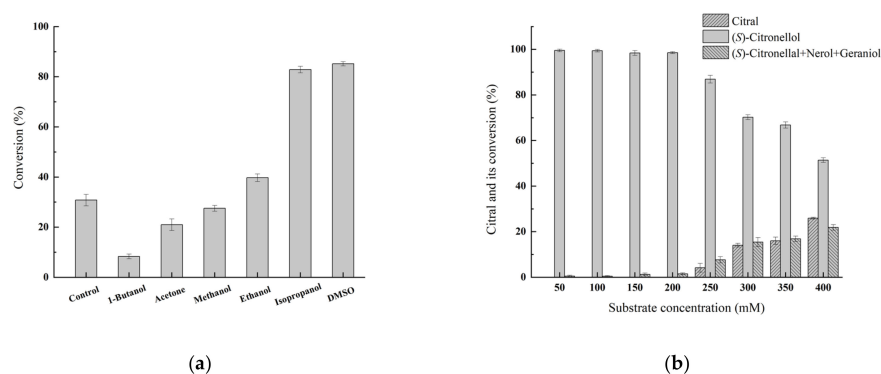


Figure 5. Effect of co-solvent (a) and substrate concentration (b) on enzymatic double reduction of (*E/Z*)-citral to (*S*)-citronellol. (a), the reaction mixture (10 mL) contained 200 mM (*E/Z*)-citral, 600 mM glucose, 1 g wet cells, 0.4 mM NADP⁺, 20% (*v/v*) co-solvent and 50 mM PIPES buffer (pH 6.5). (b), the reaction mixture (10 mL) contained 50–400 mM (*E/Z*)-citral, 150–1200 mM glucose (3 molar equivalents of citral), 1 g wet cells, 0.4 mM NADP⁺, 20% (*v/v*) isopropanol and 50 mM PIPES buffer (pH 7.0). The reactions were conducted at 30 °C and 400 rpm for 12 h. Standard deviations are indicated in the diagram (*n* = 3).

In order to develop an industrially feasible process, it would be desirable to initiate the reaction with high substrate concentration [36]. The substrate concentration was increased in a stepwise manner. The conversions remained high when the substrate concentration was no greater than 200 mM. Further increases of the substrate concentration to 400 mM led to decreased conversions, meanwhile the remaining substrate, the intermediate (S)-citronellal and by-products (nerol and geraniol and) gradually became abundant (Figure 5b).

2.4. Suppression of (S)-Citronellal Accumulation in Selective Reduction of (E/Z)-Citral to (S)-Citronellol

The accumulation of by-product and intermediate would not only reduce the conversion but also aggravate the burden of subsequent product recovery. To elucidate the dynamic change of by-product and intermediate, the time-course of double reduction of 400 mM (E/Z)-citral to (S)-citronellol was depicted in Figure 6a. During the first 6 h, both nerol and geraniol reached the highest accumulation, being attributed to the reduction of C = O bond catalyzed by YsADH (Figure S3). NemR-PS did not function on nerol and geraniol. As the substrate (E/Z)-citral kept being consumed, the YsADH-catalyzed interconversion between (E/Z)-citral and geraniol/nerol shifted the equilibrium to the dehydrogenation of nerol and geraniol, enabling them the re-entry of double reduction of (E/Z)-citral until the complete consumption at 48 h (Figure S3). In contrast to the trends on (E/Z)-citral and geraniol/nerol, the intermediate (S)-citronellal and the product (S)-citronellol kept increasing within 48 h. At the end of cascade reaction, the conversions to (S)-citronellal and (S)-citronellol were 8.8% and 91.2%, respectively, indicating that both (E)-citral and (Z)-citral were reduced to the same intermediate (S)-citronellal (Figure S3). This kind of catalytic performance was tightly associated with the feature of old yellow enzyme. Different from the case catalyzed by NemR-PS, it was observed that enantioselective hydrogenation of C=C bond only occurred for (Z)-isomer rather (E)-isomer in the baker yeast-catalyzed reduction of an unsaturated ketal [37].

According to the accumulation of the intermediate (S)-citronellal, it was reasonable to speculate that the activity of YsADH and BmGDH_{M6} might not be sufficient for fulfilling the complete conversion. To verify the speculation, the attempt to feed 0.5 g wet cells expressing YsADH (*E. coli* BL21 DE(3)/pET28b-YsADH, Table 2) into 10 mL reaction mixture was conducted at 36 h but no improvement was observed (data not shown). However, the other attempt to feed 0.5 g wet cells expressing BmGDH_{M6} (*E. coli* BL21 DE(3)/pET28b-BmGDH_{M6}, Table 2) eliminated the intermediate (S)-citronellal and led to the conversion of >99.5% for the next 12 h (Figure 6b), indicating that BmGDH_{M6} was more fragile in the cascade reaction than YsADH and NemR-PS. It was also interesting to investigate the impact of the timing of BmGDH_{M6} feeding on both conversion and the removal of by-products and the intermediate (S)-citronellal. When the cells expressing BmGDH_{M6} were supplemented at 12 h and 24 h, the complete consumption of by-products and the intermediate (S)-citronellal was observed at 42 h and 36 h, respectively (Figure 6c,d). Based on the trend on the product formation, the BmGDH_{M6} activity at 12 h was still high enough to meet for the requirement of the cascade reaction. In contrast to BmGDH_{M6} feeding at 24 h, the longer exposure of BmGDH_{M6} added at 12 h to the environment with high concentration of organic compounds caused more severe deactivation. Nearly complete conversion (>99.5%) was beneficial to subsequent product recovery and validation. The product in the reaction mixture was extracted with ethyl acetate, then the collected organic phase from extraction was evaporated to remove the solvent, and finally the resulting compound was validated to be (S)-citronellol through a combination of chiral gas chromatography (GC) (Figure S4d), gas chromatography-mass spectrometry (GC-MS) (Figure S5c), ¹H and ¹³C nuclear magnetic resonance (NMR) analyses (Figure S6).

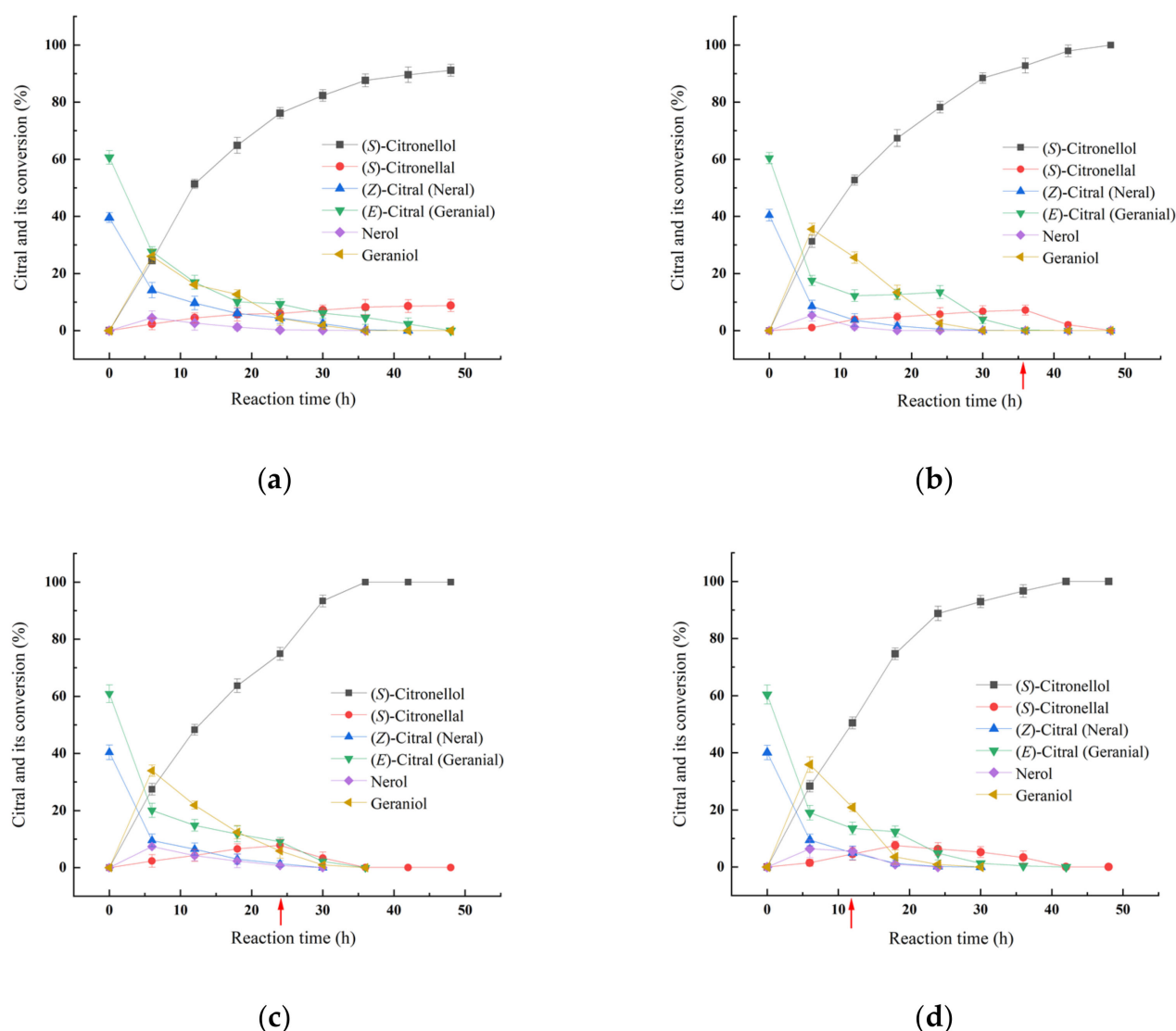


Figure 6. Time-courses of enzymatic double reduction of 400 mM (*E/Z*)-citral to (*S*)-citronellol with/without the feeding of the cells expressing BmGDH_{M6}. The reaction mixture (10 mL) contained 400 mM (*E/Z*)-citral, 1200 mM glucose, 1 g wet cells, 0.4 mM NADP⁺, 20% (*v/v*) isopropanol, and 50 mM PIPES buffer (pH 6.5). The reactions were conducted at 30 °C and 400 rpm for 48 h. (a), no feeding of the cells expressing BmGDH_{M6}. (b), the feeding of 0.5 g wet cells expressing BmGDH_{M6} at 36 h. (c), the feeding of 0.5 g wet cells expressing BmGDH_{M6} at 24 h. (d), the feeding of 0.5 g wet cells expressing BmGDH_{M6} at 12 h. The red arrows represent different timing for feeding the cells expressing BmGDH_{M6}. Standard deviations are indicated in the diagram (*n* = 3).

3. Materials and Methods

3.1. Chemicals, Genes and Organisms

The chemicals (*E/Z*)-citral, geraniol, nerol, (*R*)-citronellal, (*S*)-citronellal, (*R*)-citronellol and (*S*)-citronellol were purchased from Sigma-Aldrich (Shanghai, China). All the other chemicals for medium preparation and strain construction were commercially available. The kits and the enzymes for gene manipulation were purchased from Takara Biomedical Technology Co., Ltd. (Beijing, China). The ClonExpress MultiS One Step Cloning Kit was obtained from Vazyme Biotech Co., Ltd. (Nanjing, China).

The genes encoding old yellow enzyme, alcohol dehydrogenase and glucose dehydrogenase (Figure S1) were codon-optimized and then synthesized by Tsingke Biotechnology Co., Ltd. (Hangzhou, China). The plasmids pET28b and pACYCDuet-1 together with the

strain *E. coli* BL21 (DE3) were used for heterogeneous expression of old yellow enzyme, alcohol dehydrogenase and glucose dehydrogenase.

3.2. Selection of Old Yellow Enzyme with High Activity and (S)-Enantioselectivity

The genes encoding old yellow enzymes (Figure S1) were individually inserted into the sites *Nco* I/*Xho* I of the vector pET28b, and each resulting plasmid was transformed into the host strain *E. coli* BL21(DE3). The recombinant *E. coli* strains were initially grown in LB medium containing 50 µg/mL kanamycin at 37 °C. When the OD₆₀₀ reached 0.6, the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) induced the expression of old yellow enzyme at 23 °C for 13 h. After the induction, cells were washed twice using 50 mM Tris-HCl buffer (pH 8.0) and then harvested by 8000 g centrifugation at 4 °C for 10 min. Following the same procedure, the strain expressing YsADH or BmGDH_{M6}, *E. coli* BL21(DE3)/pET28b-YsADH or *E. coli* BL21(DE3)/pET28b-BmGDH_{M6}, was successfully constructed for the preparation of wet cells.

The collected wet cells were re-suspended in the 50 mM Tris HCl buffer (pH 8.0) and disrupted through ultrasonication for 10 min. After that, the cell-debris pellet and cell-free extract were separated by 10,000× g centrifugation at 4 °C for 10 min. Cell-free extract samples were run by SDS-PAGE analyses [38] and enzyme assays described in Section 3.4.

To determine the enantioselectivity, the reduction of (*E/Z*)-citral to citronellal was conducted using both the strain expressing old yellow enzyme and *E. coli* BL21(DE3)/pET28b-BmGDH_{M6} as biocatalysts. The reaction mixture (10 mL) contained 100 mM (*E/Z*)-citral, 300 mM glucose, 0.5 g wet cells expressing old yellow enzyme, 0.5 g wet cells *E. coli* BL21(DE3)/pET28b-BmGDH_{M6}, 0.2 mM NADP⁺, 50 mM PIPES buffer (pH 7.0). The reaction was carried out at 30 °C and 600 rpm for 2 h. The reaction mixture was extracted with 4 volume equivalents of ethyl acetate. After the extraction, the organic phase was collected after 8000× g centrifugation at room temperature for 10 min and dehydrated by anhydrous sodium sulfate, and 1 µL dehydrated sample was applied for further GC analysis described in Section 3.8.

3.3. Co-Expression of YsADH, NemR-PS and BmGDH_{M6} in *E. coli* BL21 (DE3)

The gene encoding YsADH was amplified from the plasmid pET28b-YsADH using the pair of primers pET28b-YsADH-F and pET28b-YsADH-R (Table S1). The PCR program was composed of the following steps: a 5 min period at 98 °C, 30 cycles of 98 °C (10 s), 58 °C (15 s), and 72 °C (30 s), and a final 5 min extension at 72 °C. Through reverse-PCR, the linear pACYCDuet-1 fragment was amplified from the plasmid pACYCDuet-1 using the pair of primers pACYCDuet-1-F and pACYCDuet-1-R (Table S1). The corresponding PCR program was listed as below: a 5 min period at 98 °C, 30 cycles of 98 °C (10 s), 58 °C (15 s), and 72 °C (90 s), and a final 5 min extension at 72 °C. According to the instruction of the ClonExpress MultiS One Step Cloning Kit, the gene encoding YsADH and the linear pACYCDuet-1 fragment were ligated to form the recombinant plasmid pACYCDuet-1-YsADH through Exnase II-derived homologous recombination. Similarly, the gene encoding NemR-PS was introduced into the plasmid pACYCDuet-1-YsADH to form the recombinant plasmid pACYCDuet-1-YsADH-NemR-PS. The primers used for the construction of the plasmid pACYCDuet-1-YsADH-NemR-PS were listed in Table S2. In the plasmid pACYCDuet-1-YsADH-NemR-PS, the genes encoding YsADH and NemR-PS were located between the sites of *Nco* I/*Hind* III and those of *Nde* I/*Xho* I, respectively. The plasmid pACYCDuet-1-YsADH-NemR-PS was transformed into the host strain *E. coli* BL21(DE3)/pET28b-BmGDH_{M6}, giving the recombinant strain *E. coli* BL21(DE3)/pACYCDuet-1-YsADH-NemR-PS/pET28b-BmGDH_{M6}. According to the procedure described in Section 3.2., the strain *E. coli* BL21(DE3)/pACYCDuet-1-YsADH-NemR-PS/pET28b-BmGDH_{M6} was induced and the cells were collected for further use.

3.4. Enzyme Assays for YsADH, NemR-PS, and BmGDH_{M6} in Cell-Free Extracts

The activities of YsADH, NemR-PS, and BmGDH_{M6} in cell-free extracts were measured at 30 °C by monitoring the change of the absorbance at 340 nm. All the enzyme assays were conducted in triplicate. The 2.5 mL assay mixture for YsADH or NemR-PS was composed of 100 µg crude enzyme, 20 mM substrate (citral for NemR-PS, citronellal for YsADH), 0.4 mM NADPH and 50 mM PIPES buffer (pH 7.0), while that for BmGDH_{M6} contained 100 µg crude enzyme, 20 mM glucose, 0.4 mM NADP⁺, and 50 mM PIPES buffer (pH 7.0). The reaction was started by the addition of the enzyme. One unit of the activity represents the formation or oxidation of 1 µmol NADPH per min. The BCA method was used for the determination of the protein concentrations of all samples along with bovine serum albumin as the standard protein [39].

3.5. Key Factors on Double Reduction of (E/Z)-Citral to (S)-Citronellol

The strain *E. coli* BL21(DE3)/pACYCDuet-1-YsADH-NemR-PS/pET28b-BmGDH_{M6} was used as biocatalyst. The initial reaction mixture (10 mL) contained 200 mM (E/Z)-citral, 600 mM glucose, 1 g wet cells, 0.2 NADP⁺, and 50 mM Tris-HCl buffer (pH 8.0). The initial reaction conditions were 30 °C and 200 rpm for 12 h. The setup of the reactor with hot plate/magnetic stirrer was shown in Figure S7, in which pH was kept constant through auto-titration using 1 M NaOH. The factors were individually varied to test the effect on double reduction of (E/Z)-citral to (S)-citronellol. The optimal temperature was explored by varying the temperatures from 20 °C to 45 °C. The optimal pH was investigated with a pH range from pH 5.0 to 9.0. The buffers used were citrate (pH 5.0 and 5.5), piperazine-1,4-bisethanesulfonic acid (PIPES, pH 6.0, 6.5, and 7.0) and Tris-HCl (pH 7.5, 8.0, 8.5, and 9.0). The rotations were explored within the range of 0 to 600 rpm. The concentrations of NADP⁺ and FMN were tested within the range of 0 to 1.0 mM. The concentrations of substrate were stepwise increased from 50 to 400 mM.

3.6. Suppression of (S)-Citronellal Accumulation through the Feeding of Cells Expressing YsADH or BmGDH_{M6}

To elucidate the dynamic change of by-product and intermediate, the time-courses of double reduction of (E/Z)-citral were explored by sampling every 6 h. The reaction mixture (10 mL) contained 400 mM (E/Z)-citral, 1200 mM glucose, 1 g wet cells, 0.4 mM NADP⁺, 20% (v/v) isopropanol, and 50 mM PIPES buffer (pH 6.5). The reaction was conducted 30 °C and 400 rpm for 48 h. For the whole reaction, pH was kept constant using an auto-titration system (Figure S7). To suppress the by-product formation, 0.5 g wet cells expressing YsADH (*E. coli* BL21 DE(3)/pET28b-YsADH, Table 2) or BmGDH_{M6} (*E. coli* BL21 DE(3)/pET28b-BmGDH_{M6}, Table 2) were supplemented into the reaction mixture after 36 h. After verifying the effectiveness of BmGDH_{M6} feeding, the different feeding timings (12 and 24 h) were further investigated on the conversion and the by-product removal.

3.7. The Optimized Procedure for the Enzymatic Synthesis of (S)-Citronellol

The reaction mixture (10 mL) contained 400 mM (E/Z)-citral, 1200 mM glucose, 1 g wet cells (*E. coli* BL21 DE(3)/pACYCDuet-1-YsADH-NemR-PS/pET28b-BmGDH_{M6}), 0.4 mM NADP⁺, 20% (v/v) isopropanol, and 50 mM PIPES buffer (pH 6.5). The reaction was conducted 30 °C and 400 rpm for 48 h. At the time point of 24 h, 0.5 g wet cells (*E. coli* BL21 DE(3)/pET28b-BmGDH_{M6}) were supplemented into the reaction mixture. For the whole reaction, pH was kept constant using an auto-titration system (Figure S7). (S)-citronellol in the reaction mixture was extracted with 4 volume equivalents of ethyl acetate, and the solvent ethyl acetate was subsequently removed by vacuum evaporation.

3.8. GC, GC-MS, and NMR Analyses

The substrate, intermediate, product and by-products were determined by GC (Agilent 6890N), which parameters were listed as below: detector, FID; chiral capillary column, BGB-174 (BGB Analytik, Böckten, Switzerland, 30 m × 250 µm × 0.25 µm); carrier gas, N₂;

the flow rate, 3 mL/min; the split ratio, 1:19; injection volume, 1 µL; injector and detector, 250 °C. The column temperature program for the quantitation of substrate, intermediate, product and by-products was listed as follows: initial temperature of 90 °C, 20 °C/min ramp to 160 °C for 2 min, and 20 °C/min ramp to 180 °C for 3 min. The retention times were summarized as below: (S)-citronellal, 20.128 min; (R)-citronellal, 21.098 min; nerol, 27.208 min; citronellol, 27.480 min; geraniol, 28.419 min; (E)-citral, 28.842; (Z)-citral, 29.890 min (Figure S4a,b). Specifically, the column temperature program for the determination of (S)-citronellol and (R)-citronellol was listed as follows: initial temperature of 75 °C for 30 min, 0.4 °C/min ramp to 120 °C for 10 min, and 20 °C/min ramp to 180 °C for 3 min. The retention times for (S)-citronellol and (R)-citronellol were 95.481 min and 96.249 min, respectively (Figure S4c).

Citral, citronellal, and citronellol were validated through GC–MS analysis (Agilent7890A/5975C, Agilent Technologies Inc., Santa Clara, CA, USA) using previously-reported parameters (Figure S5) [14]. The organic phase after extraction was evaporated to remove the solvent, and the remaining product (S)-citronellol was further verified through NMR analyses (Avance NEO, Bruker, Switzerland). The NMR spectra was operated at 600 MHz for ¹H and 151 HMz for ¹³C detection (Figure S6).

4. Conclusions

In this work, old yellow enzyme NemR-PS showed high activity and strict (S)-enantioselectivity in the reduction of (E/Z)-citral to (S)-citronellol via (S)-citronellal. The *E. coli* strain co-expressing YsADH, NemR-PS, and BmGDH_{M6} was successfully constructed and induced, offering soluble and active expression of the enzymes. Both YsADH and NemR-PS were NADPH-dependent, and 0.4 mM NADP⁺ was essential for maintaining high catalytic activity. BmGDH_{M6} was more fragile than YsADH and NemR-PS in the cascade reaction, and, thus, the feeding of the cells expressing BmGDH_{M6} was beneficial to eliminate by-products and the intermediate (S)-citronellal and shorten the reaction time. Under the optimized conditions, the double reduction of 400 mM (E/Z)-citral to enantio-pure (S)-citronellal led to nearly complete conversion (>99.5%) with 36 h, demonstrating a new approach for efficient synthesis of (S)-citronellol.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/catal11080931/s1>, Table S1: The primers for the construction of the plasmid pACYCDuet-1-YsADH; Table S2: The primers for the construction of the plasmid pACYCDuet-1-YsADH-NemR-PS; Figure S1: The codon-optimized nucleotide sequences encoding old yellow enzymes, alcohol dehydrogenase and glucose dehydrogenase; Figure S2: The effect of agitation speed on enzymatic double reduction of (E/Z)-citral to (S)-citronellol; Figure S3. Schematic formation and consumption of the intermediate (S)-citronellal and the by-products nerol and geraniol; Figure S4: GC chromatogram for various standards used in the study and the product extracted from the reaction mixture; Figure S5: GC-MS analyses of substrate, intermediate and product; Figure S6: The ¹H NMR (a) and ¹³C NMR (b) analyses of the product (S)-citronellol; Figure S7: The setup of the reactor with constant pH auto-titration system.

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