Supporting Information

Engineering the Activity of Old Yellow Enzyme NemR-PS for Efficient Reduction of (*E*/*Z*)-Citral to (*S*)-Citronellol

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Table S1. The primers for alanine scanning in NemR-PS [1].

Primer	Sequence
W276A-F	5′-GAACCGGAT<u>GCG</u>GCAGGTGGTCAGCCATATAC-3′
W276A-R	5′-ACCACCTGCC<u>CGC</u>ATCCGGTTCGCTCAGAT-3′
D275A-F	5′-AGCGAACCG<u>GCT</u>TGGGCAGGTGGTCAGCC-3′
D275A-R	5′-ACCTGCCCA<u>AGC</u>CGGTTCGCTCAGATGCAG-3′
W103A-F	5′-TACAGCTG<u>GCT</u>CATACGGGTCGTATTAGTCATGTTAGC-3′
W103A-R	5′-CATACGGGT<u>AGC</u>CAGCTGTACTGCAATACGACCACC-3′
R143A-F	5′-TGCCATT<u>GCT</u>GTAGATACCAGCATGCCGCG-3′
R143A-R	5′-GTATCTAC<u>AGC</u>AATGGCACGACCATTTTCATC-3′
F241A-F	5′-TTGGTACC<u>GCT</u>CAGAATACAGATAATGGTCCTAATGAAGTTG-3′
F241A-R	5′-GTATTCTG<u>AGC</u>GGTACCAATCGGACTAATACGAATAC-3′
Q242A-F	5′-TACCTTT<u>CAG</u>AATACAGATAATGGTCCTAATGAAGTTGATG-3′
Q242A-R	5′-CTGTATT<u>CTG</u>AAAGGTACCAATCGGACTAATACGAATAC-3′
S272A-F	5′-GCATCTGG<u>CC</u>GAACCGGATTGGGCAG-3′
S272A-R	5′-CCGGTTC<u>GGC</u>CAGATGCAGATATGCAATATGACG-3′
F351A-F	5′-GAAAGC<u>GCT</u>TATGGTGGTGGTGCCGAAGG-3′
F351A-R	5′-CACCATA<u>AGC</u>GCTTTCCGGACGCTGTG-3′
Y352A-F	5′-AAAGCTTT<u>GCT</u>GGTGGTGGTGCCGAAGG-3′
Y352A-R	5′-CACCACC<u>AGC</u>AAAGCTTTCCGGACGCTGT-3′

[1] The mutation site was underlined.

Table S2. The primers for saturation mutagenesis of the residue D275 in NemR-PS [1].

Primer	Sequence
D275A-F	5′-AGCGAACCG<u>GCT</u>TGGGCAGGTGGTCAGCC-3′
D275A-R	5′-ACCTGCCCA<u>AGC</u>CGGTTCGCTCAGATGCAG-3′
D275I-F	5′-AGCGAACCG<u>ATT</u>TGGGCAGGTGGTCAGCCAT-3′
D275I-R	5′-ACCTGCCCA<u>AAT</u>CGGTTCGCTCAGATGCAGATATG-3′
D275L-F	5′-AGCGAACCG<u>CTG</u>TGGGCAGGTGGTCAGCC-3′
D275L-R	5′-ACCTGCCCA<u>CAG</u>CGGTTCGCTCAGATGCAG-3′
D275M-F	5′-AGCGAACCG<u>ATG</u>TGGGCAGGTGGTCAGCCA-3′
D275M-R	5′-ACCTGCCCA<u>CAT</u>CGGTTCGCTCAGATGCAGATAT-3′
D275F-F	5′-AGCGAACCG<u>TTT</u>TGGGCAGGTGGTCAGCCA-3′
D275F-R	5′-ACCTGCCCA<u>AAA</u>CGGTTCGCTCAGATGCAGATAT-3′
D275W-F	5′-AGCGAACCG<u>TGG</u>TGGGCAGGTGGTCAGCC-3′
D275W-R	5′-ACCTGCCCA<u>CCA</u>CGGTTCGCTCAGATGCAGA-3′
D275Y-F	5′-AGCGAACCG<u>TAT</u>TGGGCAGGTGGTCAGCCA-3′
D275Y-R	5′-ACCTGCCCA<u>ATA</u>CGGTTCGCTCAGATGCAGATAT-3′
D275V-F	5′-AGCGAACCG<u>GTT</u>TGGGCAGGTGGTCAGCCA-3′
D275V-R	5′-ACCTGCCCA<u>AAC</u>CGGTTCGCTCAGATGCAGA-3′
D275S-F	5′-AGCGAACCG<u>AGC</u>TGGGCAGGTGGTCAGCC-3′
D275S-R	5′-ACCTGCCCA<u>GCT</u>CGGTTCGCTCAGATGCAGA-3′
D275T-F	5′-AGCGAACCG<u>ACG</u>TGGGCAGGTGGTCAGCC-3′
D275T-R	5′-ACCTGCCCA<u>CGT</u>CGGTTCGCTCAGATGCAGA-3′

D275N-F	5′-AGCGAACCG<u>AAT</u>TGGGCAGGTGGTCAGCCA-3′
D275N-R	5′-ACCTGCCCA<u>ATT</u>CGGTTCGCTCAGATGCAGATAT-3′
D275Q-F	5′-AGCGAACCG<u>CAG</u>TGGGCAGGTGGTCAGCC-3′
D275Q-R	5′-ACCTGCCCA<u>CTG</u>CGGTTCGCTCAGATGCAGA-3′
D275C-F	5′-AGCGAACCG<u>TGT</u>TGGGCAGGTGGTCAGCCA-3′
D275C-R	5′-ACCTGCCCA<u>ACA</u>CGGTTCGCTCAGATGCAGATAT-3′
D275G-F	5′-AGCGAACCG<u>GGT</u>TGGGCAGGTGGTCAGCC-3′
D275G-R	5′-ACCTGCCCA<u>ACC</u>CGGTTCGCTCAGATGCAGA-3′
D275P-F	5′-AGCGAACCG<u>CCG</u>TGGGCAGGTGGTCAGC-3′
D275P-R	5′-ACCTGCCCA<u>CGG</u>CGGTTCGCTCAGATGC-3′
D275R-F	5′-AGCGAACCG<u>CGT</u>TGGGCAGGTGGTCAGCC-3′
D275R-R	5′-ACCTGCCCA<u>ACG</u>CGGTTCGCTCAGATGCAGA-3′
D275H-F	5′-AGCGAACCG<u>CAT</u>TGGGCAGGTGGTCAGCCA-3′
D275H-R	5′-ACCTGCCCA<u>ATG</u>CGGTTCGCTCAGATGCAGATAT-3′
D275K-F	5′-AGCGAACCG<u>AAG</u>TGGGCAGGTGGTCAGCCA-3′
D275K-R	5′-ACCTGCCCA<u>CTT</u>CGGTTCGCTCAGATGCAGATA-3′
D275E-F	5′-AGCGAACCG<u>GAA</u>TGGGCAGGTGGTCAGCCA-3′
D275E-R	5′-ACCTGCCCA<u>TTC</u>CGGTTCGCTCAGATGCAGATA-3′

[1] The mutation site was underlined.

Table S3. The primers for iterative saturation mutagenesis of the residue F351 in NemR-PS D275G.[1]

Primer	Sequence (5′→3′)
F351I-F	5′-GAAAGC<u>ATT</u>TATGGTGGTGGTGCCGAAGGT-3′
F351I-R	5′-CACCATA<u>AAT</u>GCTTTCCGGACGCTGTGGG-3′
F351L-F	5′-GAAAGC<u>CTG</u>TATGGTGGTGGTGCCGAAGG-3′
F351L-R	5′-CACCATA<u>CAG</u>GCTTTCCGGACGCTGTGG-3′
F351M-F	5′-GAAAGC<u>ATG</u>TATGGTGGTGGTGCCGAAGG-3′
F351M-R	5′-CACCATA<u>CAT</u>GCTTTCCGGACGCTGTGG-3′
F351D-F	5′-GAAAGC<u>GAT</u>TATGGTGGTGGTGCCGAAGG-3′
F351D-R	5′-CACCATA<u>ATC</u>GCTTTCCGGACGCTGTGG-3′
F351W-F	5′-GAAAGC<u>TGG</u>TATGGTGGTGGTGCCGAAG-3′
F351W-R	5′-CACCATA<u>CCA</u>GCTTTCCGGACGCTGTGG-3′
F351Y-F	5′-GAAAGC<u>TAT</u>TATGGTGGTGGTGCCGAAGGTT-3′
F351Y-R	5′-CACCATA<u>ATA</u>GCTTTCCGGACGCTGTGGGT-3′
F351V-F	5′-GAAAGC<u>GTT</u>TATGGTGGTGGTGCCGAAGG-3′
F351V-R	5′-CACCATA<u>AAC</u>GCTTTCCGGACGCTGTGG-3′
F351S-F	5′-GAAAGC<u>AGC</u>TATGGTGGTGGTGCCGAAGG-3′
F351S-R	5′-CACCATA<u>GCT</u>GCTTTCCGGACGCTGTGG-3′
F351T-F	5′-GAAAGC<u>ACG</u>TATGGTGGTGGTGCCGAAGG-3′
F351T-R	5′-CACCATA<u>CGT</u>GCTTTCCGGACGCTGTGG-3′
F351N-F	5′-GAAAGC<u>ATT</u>TATGGTGGTGGTGCCGAAGGTT-3′
F351N-R	5′-CACCATA<u>AAT</u>GCTTTCCGGACGCTGTGGG-3′
F351Q-F	5′-GAAAGC<u>CAG</u>TATGGTGGTGGTGCCGAAGGT-3′
F351Q-R	5′-CACCATA<u>CTG</u>GCTTTCCGGACGCTGTGG-3′

F351C-F	5′-GAAAGC<u>TGT</u>TATGGTGGTGGTGCCGAAGGT-3′
F351C-R	5′-CACCATA<u>ACA</u>GCTTTCCGGACGCTGTGGG-3′
F351G-F	5′-GAAAGC<u>GGT</u>TATGGTGGTGGTGCCGAAGGT-3′
F351G-R	5′-CACCATA<u>ACC</u>GCTTTCCGGACGCTGTG-3′
F351P-F	5′-GAAAGC<u>CCG</u>TATGGTGGTGGTGCCGAAG-3′
F351P-R	5′-CACCATA<u>CGG</u>GCTTTCCGGACGCTGTG-3′
F351R-F	5′-GAAAGC<u>CGT</u>TATGGTGGTGGTGCCGAAGG-3′
F351R-R	5′-CACCATA<u>ACG</u>GCTTTCCGGACGCTGTGG-3′
F351H-F	5′-GAAAGC<u>CAT</u>TATGGTGGTGGTGCCGAAGG-3′
F351H-R	5′-CACCATA<u>ATG</u>GCTTTCCGGACGCTGTGG-3′
F351K-F	5′-GAAAGC<u>AAG</u>TATGGTGGTGGTGCCGAAGGTT-3′
F351K-R	5′-CACCATA<u>CTT</u>GCTTTCCGGACGCTGTGGG-3′
F351E-F	5′-GAAAGC<u>GAA</u>TATGGTGGTGGTGCCGAAGGTT-3′
F351E-R	5′-CACCATA<u>TTC</u>GCTTTCCGGACGCTGTGG-3′
F351A-F	5′-GAAAGC<u>GCT</u>TATGGTGGTGGTGCCGAAGGT-3′
F351A-R	5′-CACCATA<u>AGC</u>GCTTTCCGGACGCTGTGGG-3′

[1] The mutation site was underlined.

Table S4. The primers for constructing the recombinant plasmid pACYCDuet-1-*YsADH***.**

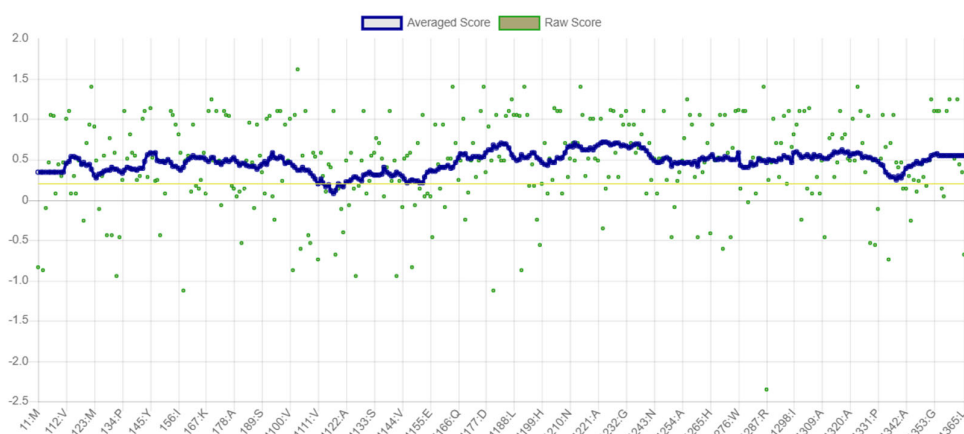| Primer	Sequence
pET28a-*YsADH*-F	5′-GGAGATATACCATGGGCATGTCTATTATAAAAAGCTATGCCGC-3′
pET28a-*YsADH*-R	5′-TTATGCGGCCGCAAGCTTAAAGTCGGCTTGCAGTACCACG-3′
pACYCDuet-1-F	5′-TTTATAATAGACATGCCCATGGTATATCTCCTTATTAAAGTTAAACAAAA-3′
pACYCDuet-1-R	5′-CTGCAAGCCGACTTTAAGCTTGCGGCCGCATAATGC-3′

Table S5. The primers for constructing the recombinant plasmid pACYCDuet-1-*YsADH*-*BmGDH*~M6.~

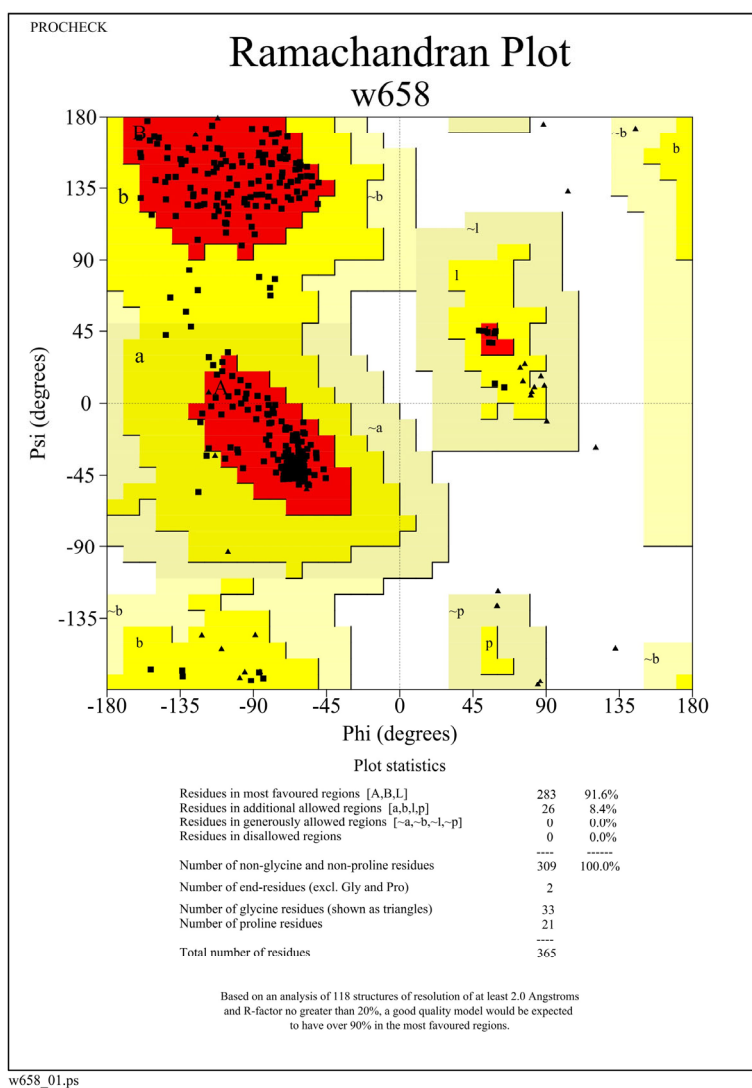| Primer	Sequence
pET28a-*BmGDH*~M6~-F	5′-GTATAAGAAGGAGATATACATATGATGTATAAAGATCTGGAAG-3′
pET28a-*NemR-PS*-R	5′-CGGTTTCTTTACCAGACTCGAGCAGGCTCGGATAATCGGTATAACC-3′
pACYCDuet-1-*YsADH*-F	5′-CTTTTTCTGGCTCATCATATGTATATCTCCTTCTTATACTTAACTAATATACTAAGATGGG-3′
pACYCDuet-1-*YsADH*-R	5′-GATTATCCGAGCCTGCTCGAGTCTGGTAAAGAAACCGCTGCTGC-3′

Supplementary figures

Figure S1. Sequence comparison of old yellow enzymes PETNR and NemR-PS. The secondary structural elements of PETNR (α-helices, β-strands, T-turns, and η-helices) were indicated above the aligned sequences. The numbering shown was from PETNR. A red background highlights conserved residues. ▲ for the active site. The figure was produced using ESPript 3.0.
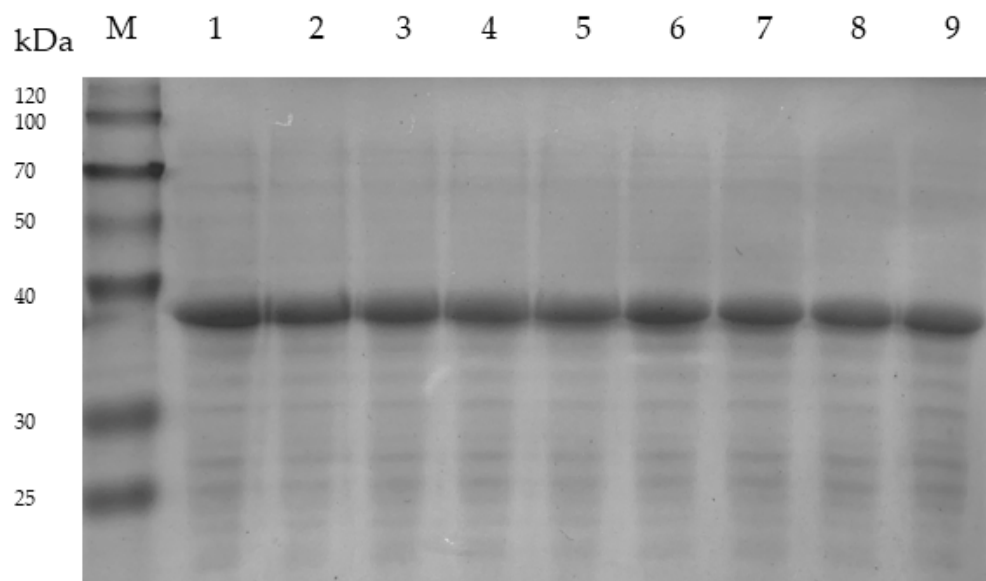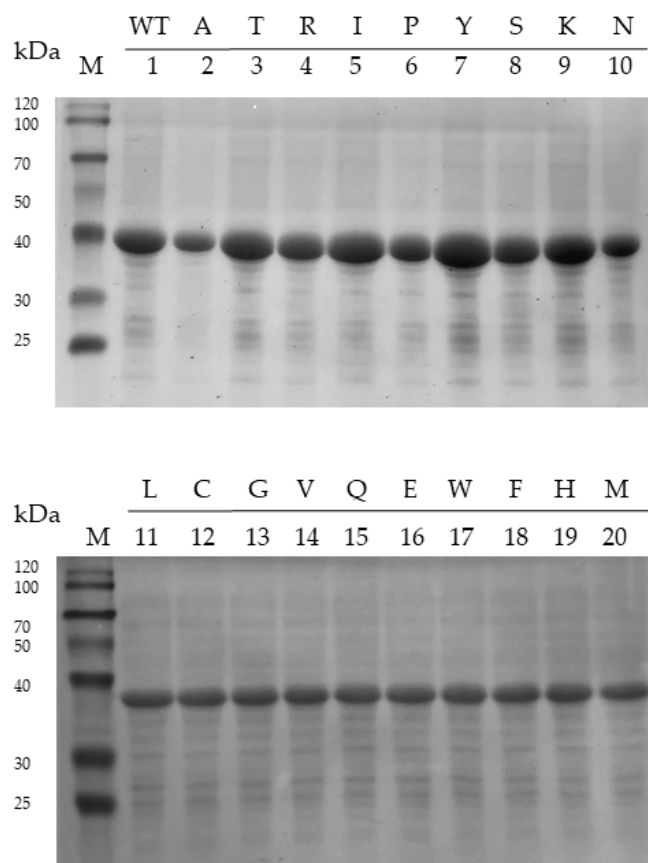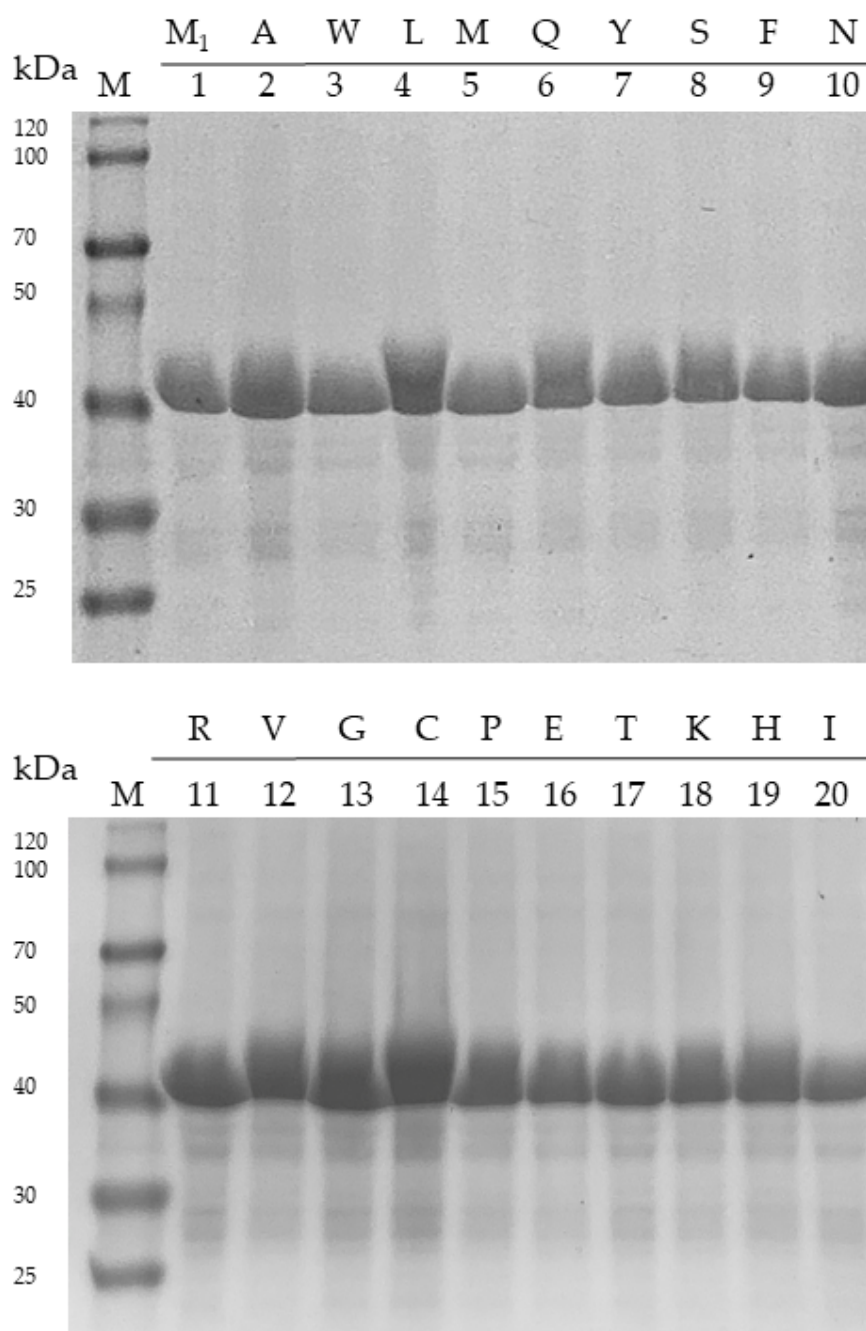

(a)

(b)

Figure S2. The model evaluation through the Verify-3D analysis (**a**) and the Ramachandran plot (**b**).

Figure S3. SDS-PAGE analysis of NemR-PS variants in alanine scanning. Lane M, **Blue Plus II Protein Marker.** Lane 1 to 9 (from left to right): W103A, R143A, F241A, Q242A, S272A, D275A, W276A, F351A, Y352A.
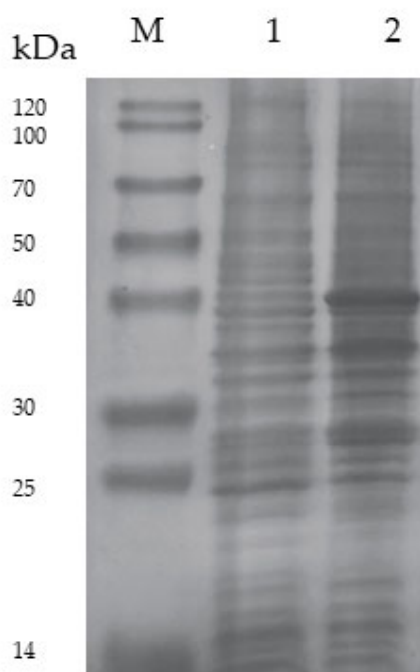

Figure S4. SDS-PAGE analysis of NemR-PS variants in site-saturation mutagenesis of the residue D275. Lane M, **Blue Plus II Protein Marker**. Lane 1, wild type. Lane 2-20 (from left to right), the specific substitution labeled right above the number.
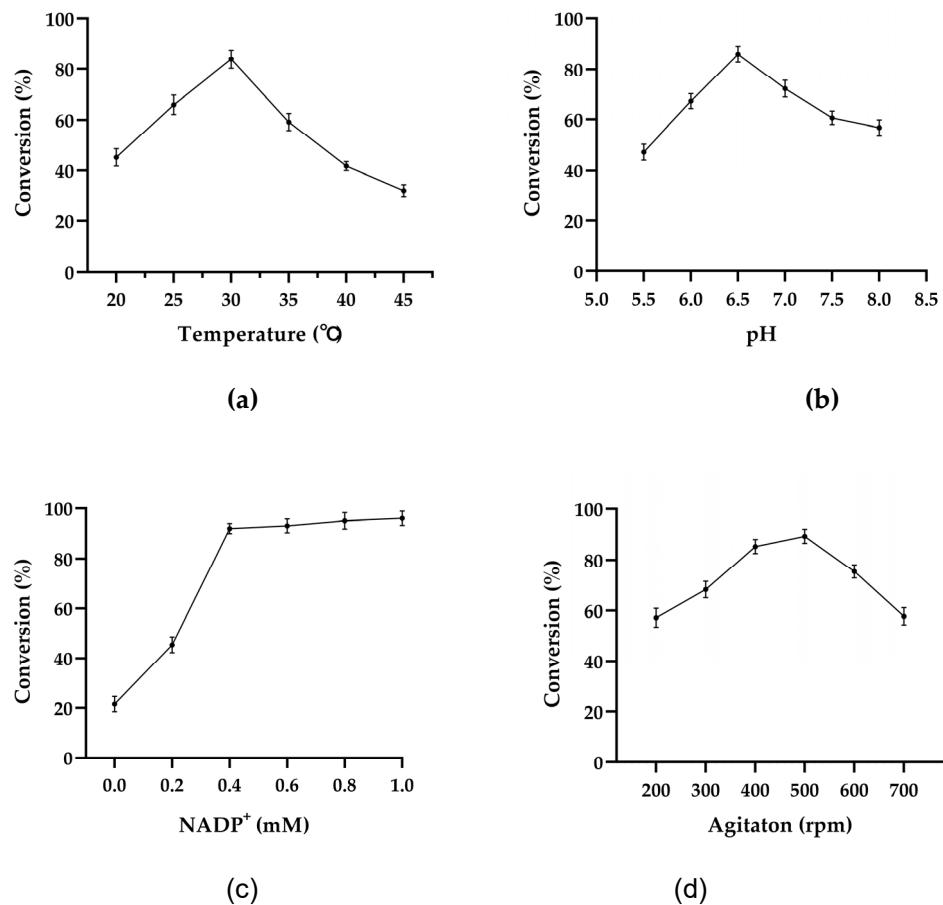
Figure S5. SDS-PAGE analysis of NemR-PS variants in iterative site-saturation of D275G and the residue F351. Lane M, **Blue Plus II Protein Marker.** Lane 1, NemR-PS variant D275G (M1). Lane 2-20 (from left to right), the specific substitution labeled right above the number.
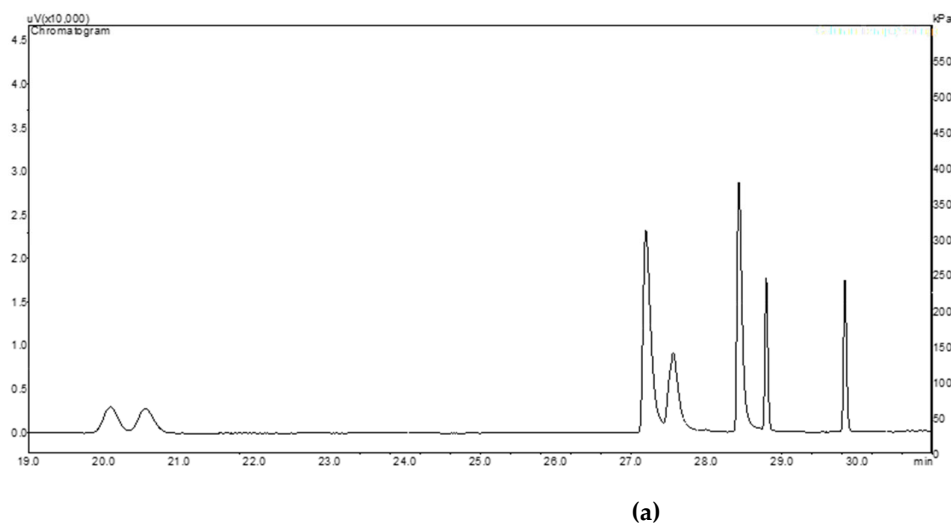
Figure S6. SDS-PAGE analysis of the purified NemR-PS and its variant D275G/F351A. Lane M, mareker; lane 1, NemR-PS; lane 2, NemR-PS variant.
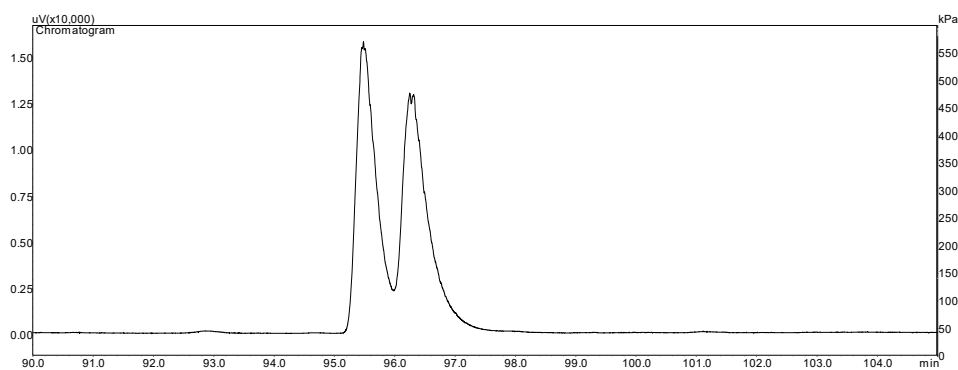

Figure S7. SDS-PAGE analysis of the strain co-expressing NemR-PS D275G/F351A, YsADH and BmGDH$_{M6}$**.** Lane M, Blue Plus II Protein Marke. Lane 1, the strain without the induction. Lane 2, the strain co-expressing NemR-PS D275G/F351A (39.9 kDa), YsADH (36.8 kDa) and BmGDH$_{M6}$ (28.1 kDa).
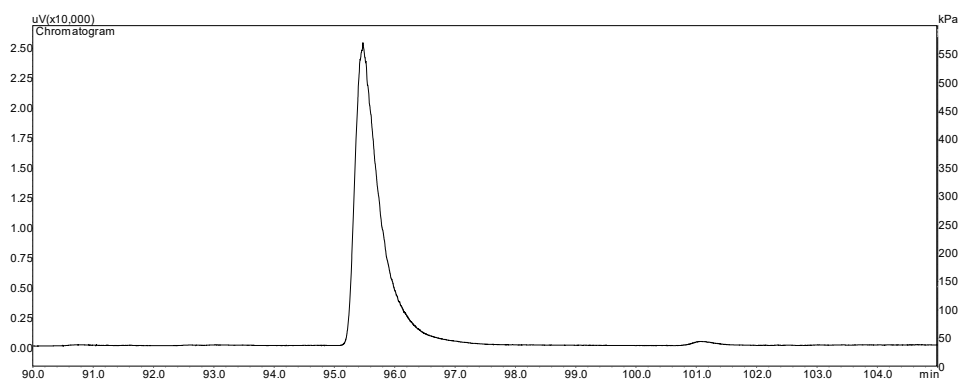
Figure S8. Factors affecting the reduction of (*E*/*Z*)-citral to (*S*)-citronellol. Standard deviations are indicated in the diagram (n=3). (**a**), temperature; (**b**), pH; (**c**), NADP$^+$; (**d**), agitation.
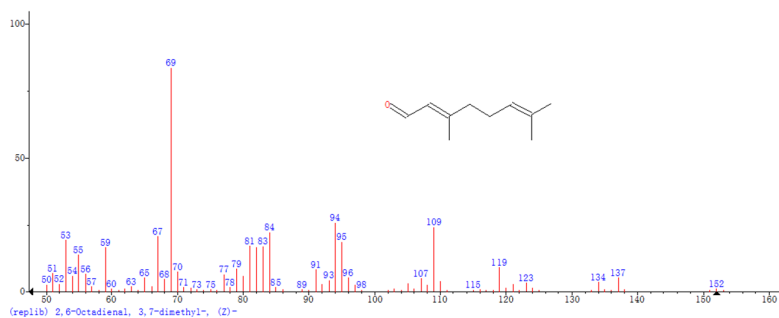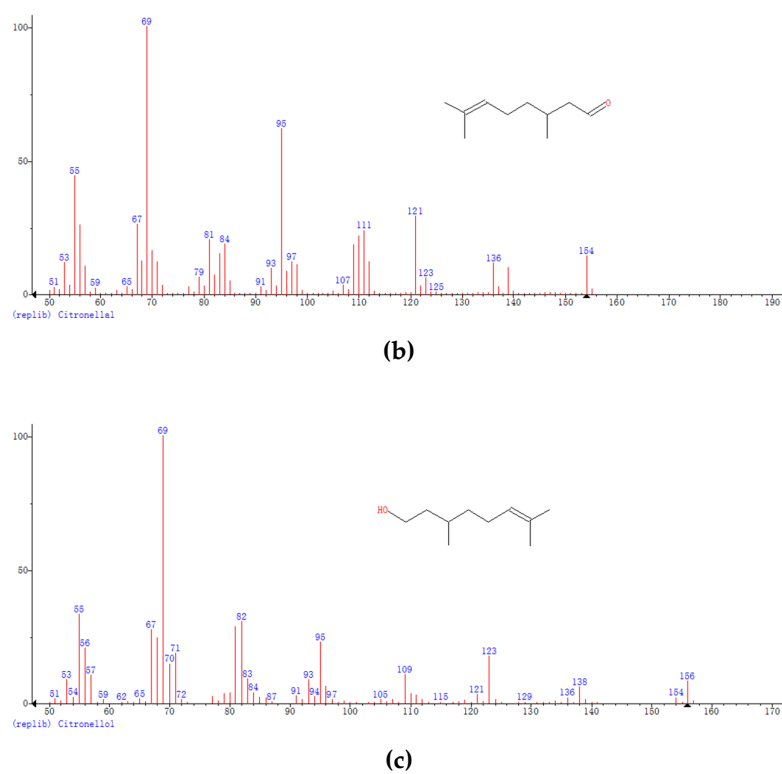

(a)

(b)

(c)

Figure S9. GC analyses of substrate, intermediate, product and by-product. (**a**), GC chromatogram for (*S*)-citronellal, 20.091 min; (*R*)-citronellal, 20.542 min; nerol, 27.196 min; citronellol, 27.556 min; geraniol, 28.433 min; (E)-citral, 28.796; (Z)-citral, 29.840 min. (**b**), GC chromatogram for (*S*)-citronellol, 95.501 min; (*R*)-citronellol, 96.249 min. (**c**) GC chromatogram for reaction solution (95.501 min).

(a)

(b)

(c)

Figure S10. GC-MS analyses of substrate, intermediate and product. (a), GC-MS chromatogram for citral (MW 152). (b), GC-MS chromatogram for citronellal (MW 154). (c), GC-MS chromatogram for citronellol (MW 156).