One-pot photoenzymatic synthesis of maleic acid and its derivatives from biobased furfural via catalytic cascades

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Materials

Furfural (99%), HFO (98%), D-MalA (99%), EY (80%), TiO_2 (P25, 20 nm), HRP (Rz > 3.0), and NaOH (96%) were purchased from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). FCA (98%), $NADP^+$ (97%), $HAuCl_4$ (Au 23.5 ~ 23.8% in dilute HCl), $NaBH_4$ (98%), ABTS (98%), and Na_2CO_3 (99.5%) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). TEMPO (98%), xanthine oxidase (lyophilized powder, ≥7 units/mg solid) and catalase (2000-5000 U/mg protein) from bovine liver were from Sigma-Aldrich (USA). MA (>98%), FA (>99%), isopropyl β-D-1-thiogalactopyranoside (IPTG), DNA marker, protein marker, kanamycin, and ampicillin were from Sangon Biotech Co., Ltd. (Shanghai, China). *DpnI* endonuclease was purchased from TaKaRa Biotech Co., Ltd. (Dalian, China). Phanta Max Master Mix (Dye Plus) DNA polymerase was obtained from Nanjing Vazyme Biotech Co., Ltd. (Nanjing, China). $CuSO_4$ (≥99%), NaH_2PO_4 (≥99.5%), and Na_2HPO_4 (≥99.5%) were from Guangzhou Chemical Reagent Co., Ltd. (Guangzhou, China). The resin NKA-III was obtained from Qinshi Technology Co., Ltd. (Zhengzhou, China). All other chemicals used in this study were of analytical grade. The recombinant strains *E. coli*_pRSFDuet-1_GO $M_{3\text{-}5}$, *E. coli*_pET-28a(+)_HbzIJ, *E. coli*_pET-24a(+)_MaiA, and *E. coli*_pET-28a(+)_*Pa*ALDH were recently constructed by us.[1-3] The gene of Ssl1 was optimized and synthesized by GenScript Biotechnology Co., Ltd. (Nanjing, China). The gene sequences of GO $M_{3\text{-}5}$ and Ssl1 were showed as below.

Preparation of AuNPs@TiO_2

AuNPs@TiO_2 was prepared according to a previous method.[4] Briefly, 0.5 g of TiO_2 powder was added to 100 mL water containing 0.063 g of $HAuCl_4$, followed by addition of 4.25 mL of L-lysine (0.5 M) in 10 min with vigorous stirring. After stirring for 1 h, 10 mL aqueous solution of $NaBH_4$ (0.0265 g) was slowly added into the dispersion in 10 min. Then, the mixture was stirred for 24 h. Finally, the solid was collected, washed with distilled water and ethanol, and then dried at 60 °C for 12 h under vacuum.

GO variants cloning

Whole plasmid PCR was used to introduce mutations to the GO M_{3-5} gene by site-directed mutagenesis. The primers (Table S1) were designed on the basis of the gene sequence of the plasmid pRSFDuet-1_GO M_{3-5} and synthesized by Sangon Biotech (Shanghai, China). PCR was performed by Phanta Max Master Mix (Dye Plus) DNA polymerase in the presence of both forward and reverse primers, with pRSFDuet-1_GO M_{3-5} as the template. The PCR products were subjected to *DpnI* digestion to remove the template DNA and transformed into *E. coli* DH5α. The resulting variants plasmids were verified by colony PCR and nucleotide sequencing.

Table S1. The primers used for the introduction of mutations to GO M_{3-5}

Variant	Mutations	Primer name	Primer sequence(5'-3')
M_4	M_{3-5} +Y329L/M330F	Y329L/M330F-F	GGTCTGctgtttAGCGATAATCATGCCTGGCTG
		Y329L/M330F-R	ATCGCTaaacagCAGACCCTGTTTATCTGCGGTC
M_{5-1}	M_4+V477D/A626S	V477D-F	TCTATgatCCGGAACAGGATACCTTTTATAAAC
		V477D-R	CTGTTCCGGatcATAGATTTCCGGGGTAAAAACCG
		A626S-F	GAATAGTagcGGCGTTCCGAGCGTTGCAAGCA
		A626S-R	GAACGCCgctACTATTCATCACAAACAGCATCCAA
M_{5-2}	M_4+N318D/C383T/Y436H/V477D	N318D-F	AAAAGTGgatCCGATGCTGACCGCAGATAAAC
		N318D-R	GCATCGGatcCACTTTTGCATTCGGCAGGCTG
		C383T-F	TGCCATGaccGGTAATGCCGTGATGTATGATGC
		C383T-R	CATTACCggtCATGGCATCCGGTGCAACACCA
		Y436H-F	TGGTCTGcatTTTGCACGCACCTTTCATACCA
		Y436H-R	GTGCAAAatgCAGACCATTGCTTGCAAAAACG
		V477D-F	TCTATgatCCGGAACAGGATACCTTTTATAAAC
		V477D-R	CTGTTCCGGatcATAGATTTCCGGGGTAAAAACCG

Enzyme expression

GO variant expression. The recombinant plasmid pRSFDuet-1_GO variant was transformed into *E. coli* BL21 (DE3) and pre-cultivated in 30 mL LB medium containing 50 mg/L kanamycin at 37 °C and 180 r/min overnight. Then, 100 mL LB medium containing 50 mg/L kanamycin was inoculated with 1 mL of an overnight culture. Cells were cultivated at 37 °C and 180 r/min. When OD600 reached 0.6-0.8, IPTG was added to induce enzyme expression at the final concentration of 0.5 mM, followed by incubation at 26 °C and 160 r/min for 48 h. The cells were harvested by centrifugation (8500 r/min, 5 min, 4 °C) and washed twice with 0.85% NaCl solution for the subsequent use.

Ssl1 expression. The recombinant plasmid pET-22b(+)_Ssl1 was transformed into *E. coli* BL21 (DE3) and pre-cultivated in 30 mL Terrific Broth (TB) medium containing 100 mg/L ampicillin at 37 °C and 180 r/min overnight. Then, 100 mL TB medium containing 100 mg/L ampicillin was inoculated with 1 mL of an overnight culture. Cells were cultivated at 37 °C and 180 r/min. When OD600 reached 0.6-0.8, IPTG and $CuSO_4$ were added to induce enzyme expression at the final concentrations of 40 μM and 2 mM, respectively, followed by incubation at 25 °C and 160 r/min for 20 h. The cells were harvested by centrifugation (8500 r/min, 5 min, 4 °C) and washed twice with 0.85% NaCl solution for the subsequent use.

Expression of HbzIJ, MaiA, and *Pa*ALDH was conducted according to previous methods.[1, 2]

Enzyme purification

The harvested cells were resuspended in binding buffer (pH 7.0, 0.1 M NaPi buffer, 0.5 M NaCl and 0.05 M imidazole) and disrupted by ultrasonication for 20 min (30% power, 3 s on and 5 s off at 4 °C). The cell lysate was centrifugated at 4 °C (12 000 r/min) for 20 min, and the supernatant was loaded onto a HisTrap™ FF crude column (GE, USA) equilibrated with binding buffer. Then, the samples were eluted with buffer (pH 7.0, 0.1 M NaPi buffer, 0.5 M NaCl and 0.1 M imidazole) at a flow rate of 1 mL/min to remove the impure proteins. Next, the elution of the target enzymes was conducted with an elution buffer (pH 7.0, 0.1 M NaPi buffer, 0.5 M NaCl and 0.4 M imidazole) with a flow rate of 3 mL/min. The fractions containing target enzymes were desalted by HiTrap™ desalting column (5 mL, GE, USA) with desalting buffer (pH 7.0, 0.1 M NaPi buffer). The purified protein fractions were subjected to SDS-PAGE analysis to determine its purity (Figures S5 and S6). Additionally, the protein concentrations were measured by the Bradford protocol.

Preparation of GO $M_{3\text{-}5}$ CFE

After expression, 1 g of GO $M_{3\text{-}5}$ wet cells were resuspended in 10 mL NaPi buffer (0.1 M, pH 7) and then disrupted by ultrasonication for 20 min. The cell lysate was centrifugated at 4 °C (12 000 r/min) for 20 min, and the supernatant was frozen at -80 °C and vacuum lyophilized to produce cell free extract (CFE). Approximately 0.28 g CFE was obtained from 10 mL of crude enzyme supernatant.

HPLC analysis

The reaction mixtures were analyzed on a Zorbax Eclipse Plus C18 column (4.6 mm × 250 mm, 5 μm, Agilent, USA) by using a reversed phase HPLC equipped with a Waters 996 photodiode array detector (Waters, USA). The quantitative analysis for furfural (maximum absorption wavelength: 278 nm, retention time: 21.1 min), FCA (246 nm, 10.3 min), HFO (210 nm, 6.4 min), and MA (210 nm, 5.9 min) were performed with the mixture of acetonitrile/0.4% $(NH4)_2SO_4$ solution with pH 3.5 (5:95, v/v) as the mobile phase. The mixture of acetonitrile/0.4% $(NH4)_2SO_4$ solution with pH 3.5 (10:90, v/v) was used as the mobile phase for quantifying FA (210 nm, 4.5 min). The column temperature and the flow rate of the mobile phase were 35 °C and 0.6 mL/min, respectively.

The quantitative analysis for D-MalA were analyzed on a Titank C18 column (4.6mm×250 mm, 5 μm, Phenomenex, USA) with Waters 2489 UV/Visible detector (Waters, USA). The retention times and detecting wavelength for D-MalA were 7.9 min and 220 nm with 20 mM phosphoric acid solution as mobile phase, respectively. The column temperature and the flow rate of the mobile phase were 35 °C and 0.6 mL/min, respectively.

Some representative HPLC chromatograms are attached as follows.

Enzyme assay

The GO variant activity was spectrophotometrically determined using a UV2550 UV-visible spectrophotometer (Shimadzu, Japan) at 25 °C by measuring the ABTS oxidation at 420 nm (ε of oxidized ABTS: 36000 L M^{-1} cm^{-1}) in 2 mL NaPi buffer (0.1 M, pH 7) containing 50 mM HFO, 1 mM ABTS, 5 μg/mL HRP, and appropriate amount of purified GO variant. One unit (U) is defined as the amount of enzyme which oxidizes 1 μmol ABTS per minute under the standard assay conditions. The specific activities of GO $M_{3\text{-}5}$, M_4, and $M_{5\text{-}1}$ were around 0.15, 0.57, and 0.92 U/mg, respectively.

Oxidation of furfural by XO and AuNPs@TiO_2

XO: 2 mL NaPi buffer (0.1 M, pH 7) containing 20 mM furfural, 0.11 mg/mL XO (1 U) and 1 mg/mL CAT was incubated at 250 r/min and 30 °C. Aliquots were withdrawn at specified time intervals from the reaction mixture. The samples were treated at 100 °C for 10 min to denature the enzymes, and then diluted with the corresponding mobile phase prior to HPLC analysis. All the experiments were conducted in duplicate, and all the data were the averages of experimental results. The conversion and yield were determined by reversed-phase HPLC, based on the corresponding calibration curves.

AuNPs@TiO_2: 2 mL NaPi buffer (0.1 M, pH 7) containing 20 mM furfural, 25 mg AuNPs@TiO_2 and 0.1 M Na_2CO_3 was irradiated under 30 W green LEDs (530-540 nm) at 250 r/min and 25 °C. Aliquots were withdrawn at specified time intervals from the reaction mixture. The samples were diluted with the corresponding mobile phase prior to HPLC analysis. All the experiments were conducted in duplicate, and all the data were the averages of experimental results. The conversion and yield were determined by reversed-phase HPLC, based on the corresponding calibration curves.

GO-catalyzed oxidation of HFO

Typically, 2 mL NaPi buffer (0.1 M, pH 7) containing 10-20 mM HFO, 0.1-1 mg/mL purified GO variant, 0.02 mg/mL HRP, 0.35 mg/mL CAT and 0.01-2 mM $CuSO_4$ was incubated at 250 r/min and 30 °C. Aliquots were withdrawn at specified time intervals from the reaction mixture. The samples were treated at 100 °C for 10 min to denature the enzymes, and then diluted with the corresponding mobile phase prior to HPLC analysis. All the experiments were conducted in duplicate, and all the data were the averages of experimental results. The conversion and yield were determined by reversed-phase HPLC, based on the corresponding calibration curves.

Photoenzymatic oxidation of furfural by *Pa*ALDH and EY@NKA

2 mL NaPi buffer (0.1 M, pH 7) containing 20 mM furfural, 1 mg/mL purified *Pa*ALDH, 0.5 mM $NADP^+$ and 5 mol% EY@NKA was irradiated under 30 W green LEDs (530-540 nm) at 250 r/min and 25 °C. Aliquots were withdrawn at specified time intervals from the reaction mixture. The samples were treated at 100 °C for 10 min to denature the enzyme, and then diluted with the corresponding mobile phase prior to HPLC analysis. All the experiments were conducted in duplicate, and all the data were the averages of experimental results. The conversion and yield were determined by reversed-phase HPLC, based on the corresponding calibration curves.

Concurrent photoenzymatic oxidation FCA to MA by EY@NKA and GO M_4/M_{5-1}

Typically, 2 mL NaPi buffer (0.1 M, pH 7) containing 20 mM FCA, 5 mol% EY@NKA, 0.02 mg/mL HRP, 0.35 mg/mL CAT, 0.1 mM $CuSO_4$, and 0.7 mg/mL purified GO variant was incubated at 25 °C and 250 r/min under light irradiation by green LEDs (30 W, 530-540 nm). Aliquots were withdrawn at specified time intervals from the reaction mixture. The samples were treated at 100 °C for 10 min to denature the enzymes, and then diluted with the corresponding mobile phase prior to HPLC analysis. All the experiments were conducted in duplicate, and all the data were the averages of experimental results. The conversion and yield were determined by reversed-phase HPLC, based on the corresponding calibration curves.

One-pot three-step synthesis of FA

1 mL NaPi buffer (0.1 M, pH 7) containing 40 mM furfural, 1 mg/mL purified *Pa*ALDH, 0.5 mM $NADP^+$ and 5 mol% EY@NKA was incubated at 25 °C and 250 r/min under light irradiation by green LEDs (30 W, 530-540 nm). After 6 h, 0.7 mg/mL purified GO M_{5-1}, 0.02 mg/mL HRP, 0.35 mg/mL CAT and 0.1 mM $CuSO_4$ were supplemented, followed by the reaction under green light irradiation at 25 °C and 250 r/min. After 36 h, pH of the reaction mixtures was to 8.5 by NaOH. Then 0.1 mg/mL purified MaiA were added and incubated at 450 r/min and 25 °C. Aliquots were withdrawn at specified time intervals from the reaction mixture. The samples were treated at 100 °C for 10 min to denature the enzymes, and then diluted with the corresponding mobile phase prior to HPLC analysis. All the experiments were conducted in duplicate, and all the data were the averages of experimental results. The conversion and yield were determined by reversed-phase HPLC, based on the corresponding calibration curves.

One-pot three-step synthesis of D-MalA

1 mL NaPi buffer (0.1 M, pH 7) containing 40 mM furfural, 1 mg/mL purified *Pa*ALDH, 0.5 mM $NADP^+$ and 5 mol% EY@NKA was incubated at 25 °C and 250 r/min under light irradiation by green LEDs (30 W, 530-540 nm). After 6 h, 0.7 mg/mL purified GO M_{5-1}, 0.02 mg/mL HRP, 0.35 mg/mL CAT and 0.1 mM $CuSO_4$ were supplemented, followed by the reaction under green light irradiation at 25 °C and 250 r/min. After 36 h, pH of the reaction mixtures was to 8.5 by NaOH. Then 50 mg/mL (wet cell weight) *E. coli*_HbzIJ cells were added and incubated at 450 r/min and 25 °C. Aliquots were withdrawn at specified time intervals from the reaction mixture. The samples were treated at 100 °C for 10 min to denature the enzymes, and then diluted with the corresponding mobile phase prior to HPLC analysis. All the experiments were conducted in duplicate, and all the data were the averages of experimental results. The conversion and yield were determined by reversed-phase HPLC, based on the corresponding calibration curves.

Table S2. Screening of biocatalysts for HFO oxidation[a]

Entry	Enzyme	Abbreviation	Specific activity (U/mg)
1	Galactose oxidase M_{3-5} from *Fusarium graminicola*	GO M_{3-5}	0.15[b]
2	Alcohol dehydrogenase from *Pichia finlandica*[5]	*Pf*ADH	n.a.[c]
3	Alcohol dehydrogenase from *Rhodococcus erythropolis*[6]	*Re*ADH	n.a.
4	Alcohol dehydrogenase from *Synechocystis* sp.[7]	*Syn*ADH	n.a.
5	Methanol dehydrogenase from *Cupriavidus necator* N-1[8]	*Cn*MDH	n.a.
6	Horse liver alcohol dehydrogenase[7]	HLADH	n.a.
7	Carbonyl reductase from *Streptomyces coelicolor*[9]	*Sc*CR	n.a.

[a] Assay conditions: 10 mM HFO, 0.2 mM NAD^+, 0.1-0.5 mg purified enzyme, 2 mL NaPi buffer (100 mM, pH 7), 25 °C.

[b] 50 mM HFO, appropriate amount of GO variant, 1 mM ABTS, 5 μg/mL HRP, 2 mL NaPi buffer (0.1 M, pH 7), 25 °C.

[c] no activity.

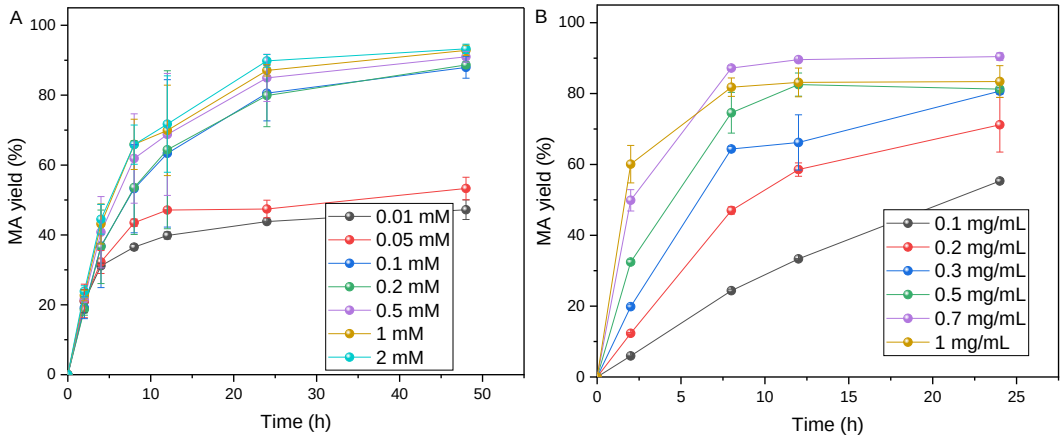

Figure S1. Effect of Cu^{2+} (A) and GO M_{3-5} (B) concentrations on enzymatic oxidation of HFO to MA. General reaction conditions unless otherwise stated: 10 mM HFO, 0.7 mg/mL purified GO M_{3-5}, 0.02 mg/mL HRP, 0.35 mg/mL CAT, 0.1 mM $CuSO_4$, 2 mL NaPi buffer (0.1 M, pH 7), 30 °C, 250 r/min; (A) 0.5 mg/mL purified GO M_{3-5}, 0.01-2 mM $CuSO_4$; (B) 0.1-1 mg/mL purified GO M_{3-5}.

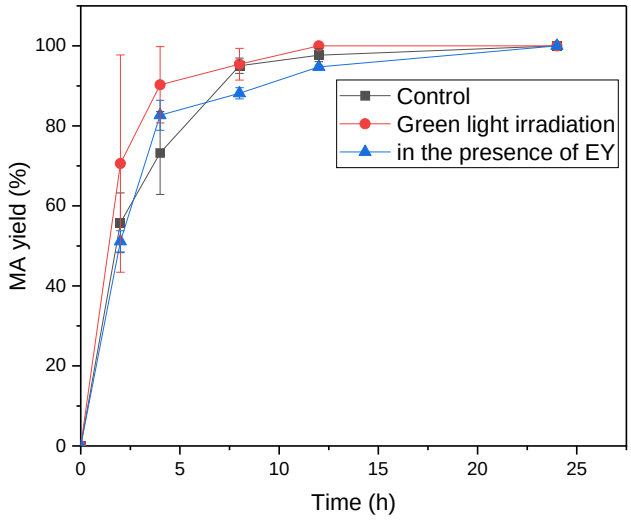

Figure S2. Effect of green light irradiation and the presence of EY on GO M_{3-5}-catalyzed oxidation of HFO. Reaction conditions: 10 mM HFO, 2 mg/mL GO M_{3-5} CFE, 0.02 mg/mL HRP, 0.35 mg/mL CAT, 0.1 mM $CuSO_4$, 2 mL NaPi buffer (0.1 M, pH 7), 25 °C, 250 r/min; green light or 2 mol% EY.

Table S3. Specific activities of GO variants toward HFO[a]

Entry	Enzyme	Specific activity (U/mg)
1	GO M_4	0.57
2	GO M_{5-1}	0.92
3	GO M_{5-2}	n.a.[b]

[a]Assay conditions: 50 mM HFO, appropriate amount of purified GO variant, 1 mM ABTS, 5 μg/mL HRP, 2 mL NaPi buffer (0.1 M, pH 7), 25 ℃.

[b] no activity.

Table S4. GO-catalyzed oxidation of HFO

Enty	Catalyst	Time (h)	MA yield (%)
1	GO M_4	12	96
2	GO M_{5-1}	8	>99

Reaction conditions: 20 mM HFO, 0.7 mg/mL purified GO variant, 0.02 mg/mL HRP, 0.35 mg/mL CAT, 0.1 mM $CuSO_4$, 2 mL NaPi buffer (0.1 M, pH 7), 25 °C, 250 r/min.

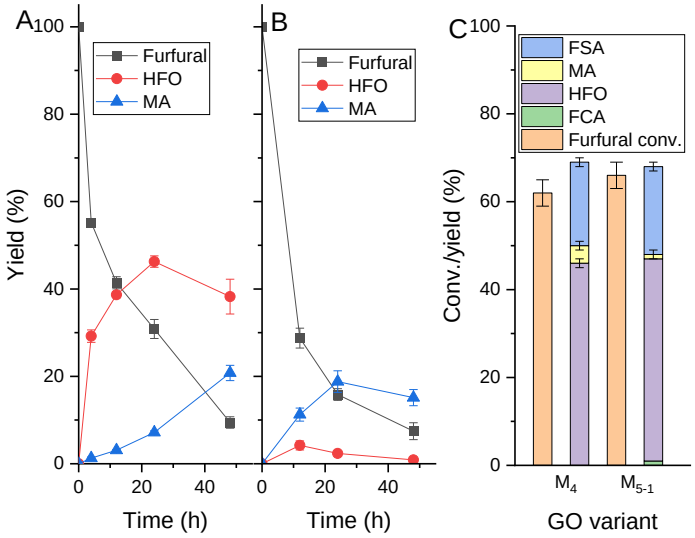

Figure S3. One-pot concurrent photoenzymatic conversion of furfural into MA by XO coupled with EY@NKA and GO $M_{3\text{-}5}$ (A), by AuNPs@TiO_2 coupled with EY@NKA and GO $M_{3\text{-}5}$ (B), and by *Pa*ALDH, EY@NKA and GO variant (C). Reaction conditions (A and B): 20 mM furfural, 0.33 mg/mL XO (3 U) (A)/25 mg AuNPs@TiO_2 and 0.1 M Na_2CO_3 (B), 5 mg/mL GO $M_{3\text{-}5}$ CFE, 0.02 mg/mL HRP, 0.35 mg/mL CAT, 0.1 mM $CuSO_4$, 5 mol% EY@NKA, green light, 2 mL NaPi buffer (0.1 M, pH 7), 25 °C, 250 r/min; reaction conditions (C): 20 mM furfural, 1 mg/mL purified *Pa*ALDH, 0.5 mM $NADP^+$, 0.7 mg/mL purified GO variant, 0.02 mg/mL HRP, 0.35 mg/mL CAT, 0.1 mM $CuSO_4$, 5 mol% EY@NKA, green light, 2 mL NaPi buffer (0.1 M, pH 7), 25 °C, 250 r/min, 12 h.

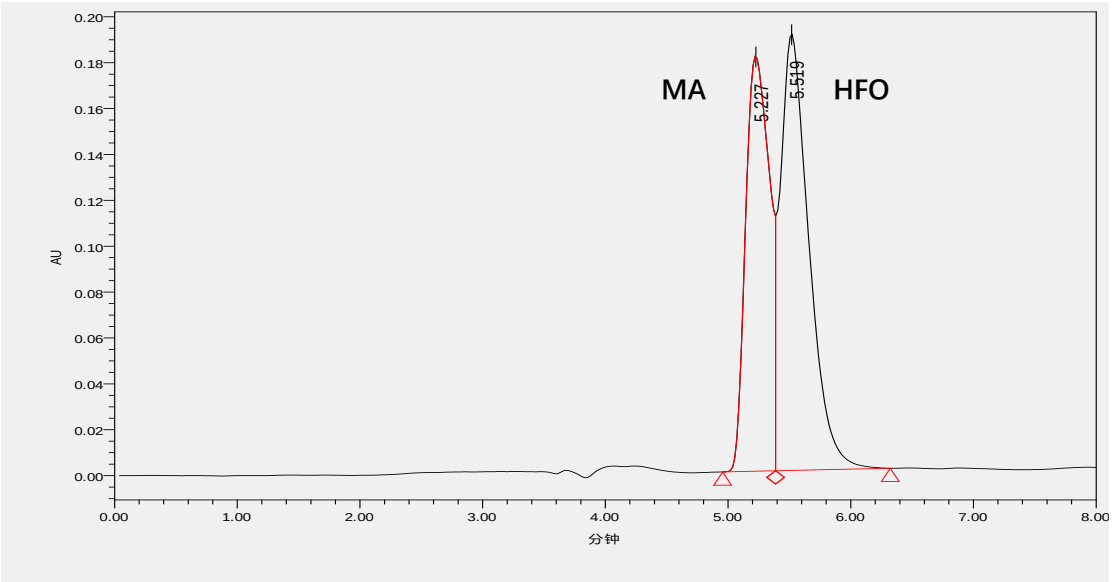

Figure S4. HPLC chromatogram with unsatisfactory chromatographic resolution of MA and HFO

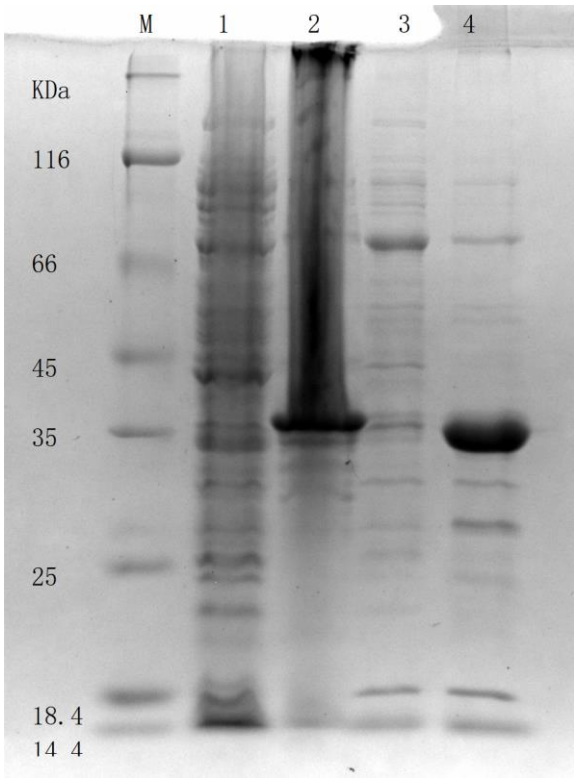

Figure S5. SDS-PAGE analysis of Ssl1. Lane M: protein marker; lane 1: the supernatant upon ultrasonication of the cells; lane 2: the precipitant upon ultrasonication of the cells; lane 4: purified enzyme

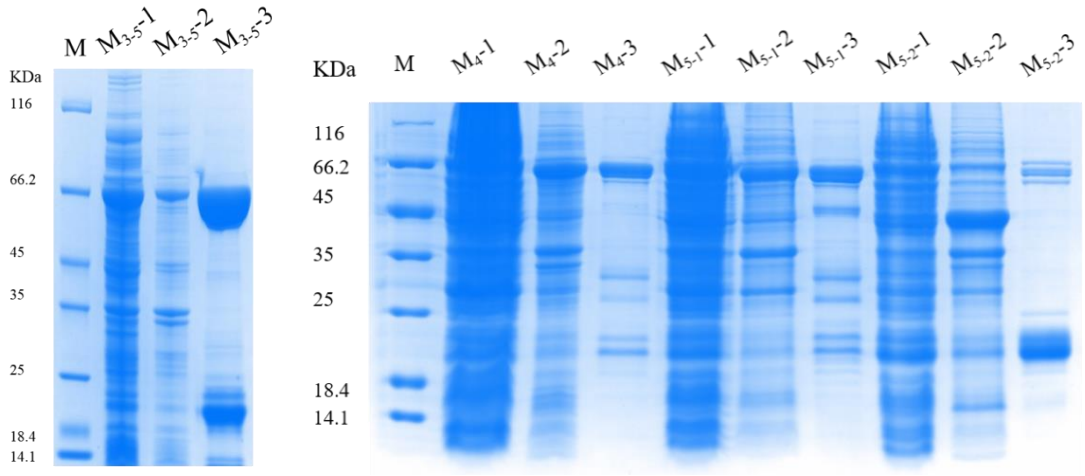

Figure S6. SDS-PAGE analysis of GO variants. Lane M: protein marker; lane 1: the supernatant upon ultrasonication of the cells; lane 2: the precipitant upon ultrasonication of the cells; lane 3: purified enzyme

HPLC chromatograms

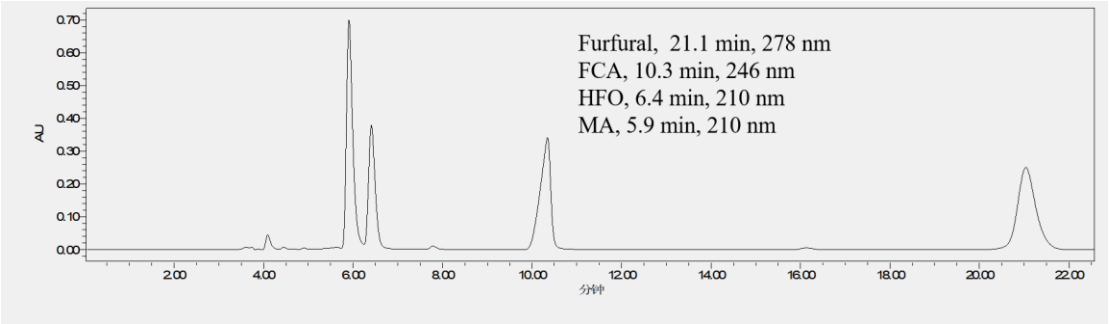

Figure S7. Representative HPLC chromatogram for quantitative analysis of MA

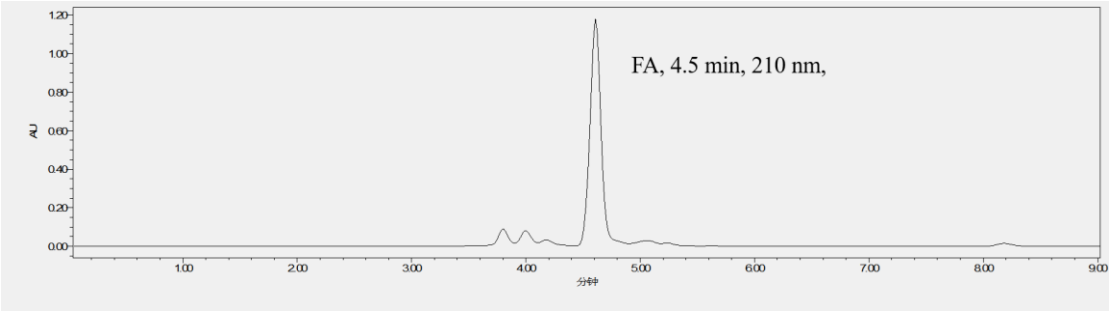

Figure S8. Representative HPLC chromatogram for quantitative analysis of FA

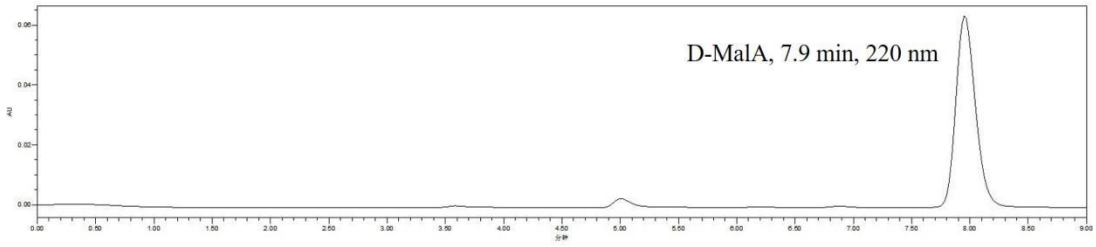

Figure S9. Representative HPLC chromatogram for quantitative analysis of D-MalA

GO $M_{3\text{-}5}$ gene sequence

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CGCACAGAGTGGCAATGAATGCAATAAGGCAATTGATGGTAATAAGGATACCTTTTGGC
ATACCTTTTATGGCGCCAATGGTGACCCGAAACCGCCGCATACCTATACCATTGATATGA
AAACCACCCAGAATGTTAATGGTCTGAGTGTGCTGCCGCGTCAGGATGGCAATCAGAA
TGGTTGGATTGGCCGTCATGAAGTTTATCTGAGTAGCGATGGCACCAATTGGGGTAGTC
CGGTGGCCAGTGGCAGTTGGTTTGCCGATAGCACCACCAAATATAGCAATTTTGAAAC
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CGATAGCGGTGTTGCACTGCCGGGTTATTGGATGCTGTTTGTGATGAATAGTGCAGGCG
TTCCGAGCGTTGCAAGCACCATTCGTGTGACCCAGTAA

Ssl1 gene sequence

ATGCATCACCACCACCACCATGCTCCCGGAGGCGAAGTTCGTCGCATCAAACTGTATG
CGGAACGTCTGGCGGACGGCCAGATGGGTTACGGCTTGGAGAAAGGTCGTGCCACCA
TCCCGGGACCGCTGATTGAGCTGAATGAAGGTGATACCCTCCACATTGAGTTCGAGAA
CACCATGGACGTGCGCGCGAGCCTGCACGTTCATGGCCTGGACTACGAGGTGAGCAG
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GCAAGGGTTTGTACGGTCCGGTTATCGTGCGTAGAAAGGGCGACGTTCTGCCGGATGC
AACCCACACCATTGTCTTTAATGATATGCTGATCAACAACCGTCCGGCGCATAGCGGTC
CAAATTTCGAGGCAACTGTAGGCGACCGTGTTGAGTTCGTGATGATTACGCACGGTGA
ATATTACCACACCTTCCACATGCATGGTCATCGTTGGGCAGACAACCGCACCGGCATGC
TGACCGGTCCAGATGATCCGAGCCAAGTGGTTGATAACAAGATCGTTGGTCCGGCGGA
CTCTTTTGGTTTTCAGGTGATCGCCGGCGAGGGCGTCGGTGCGGGTGCTTGGATGTATC
ACTGCCATGTCCAATCACATTCCGACATGGGTATGGTTGGCTTATTTCTGGTTAAAAAG
ACCGATGGGACGATCCCGGGCTACGAGCCGCATGAACACTCTGGTCAGCGTGCGGAG
CACCACCACTAA

HbzIJ and MaiA gene sequences can be obtained from ref. 2.

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