Electronic Supplementary Material (ESI) for Sustainable Energy & Fuels.

Supporting Information

Production of fatty alcohols from non-edible oils by enzymatic cascade reactions

Yunjian Ma,[a] Xizhen Zhang,[b] Yongru Li,[a] Peilin Li,[a] Frank Hollmann,*[c] and Yonghua Wang*[a]

[a] School of Food Science and Engineering; Overseas Expertise Introduction Center for Discipline Innovation of Food Nutrition and Human Health (111 Center), South China University of Technology, Guangzhou 510640, China.

[b] School of Biology and Biological Engineering, South China University of Technology, Guangzhou 510006, China.

[c] Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629HZ, Delft, The Netherlands.

F.Hollmann@tudelft.nl (Frank Hollmann); yonghw@scut.edu.cn (Yonghua Wang)

Experimental Procedures

Chemical reagents and materials

All chemicals were purchased from Aladdin, TCL etc. in the highest purity available and used without further purification (Table S1). Lipase ROL (*Rhizopus oryzae* lipase on resin) was friendly provided by Guangdong VTR Bio-Tech Co., Ltd (Zhuhai, China), (the immobilized enzyme of ROL contains 20 mg pure enzyme per gram, the hydrolytic activity of immobilized ROL enzyme is 200000 U g^{-1} immobilized enzyme). Castor oil were friendly provided by Guangzhou Zhizhiyuan Oil Industry Co., Ltd. (Guangzhou, China). Water was purified with Millipore (Bedford, MA) Milli-Q water system.

Table S1. Detail of all chemicals used in this experiment.

Chemical	**Purity**	**Manufacturer**
Octanol	99%	Aladdin, China
Ethyl acetate	99%	Aladdin, China
HCl	37%	Guangzhou Chemical Reagent Factory, China
Tris	AR	Sixin, China
DMSO	AR	Fuyu, China
Isopropanol	AR	Aladdin, China
Formic acid	AR	Kermel, China

N-hexane	AR	Richjoint, China
12-hydroxystearic acid	98%	Aladdin, China
16-hydroxystearic acid	98%	TCL, China
Ricinoleic acid	98%	Aladdin, China

Details of product separation and detection

The products after enzymatic hydrolysis of castor oil were analyzed using a HPLC (Waters, 1525) equipped with a phenomenex luna silica column (250 mm × 4.6 mm i.d., 5 μm particle size, Phenomenex Corporation, Torrance, CA, America) and a refractive index detector (Waters, 2414). Injection quantity: 10 μL. The mobile phase was a mixture of *n*-hexane, 2-propanol and formic acid (33: 1: 0.003, v: v: v) and performed with a flow rate of 1 mL min^{-1} at 30°C. The retention time for castor acid and castor oil were 6.51 min and 8.88 min, respectively (Figure S1A). Waters 2695 integration software was employed to calculate the peak-areas percentages.

An Agilent 7890B GC system (Agilent Technologies, Palo Alto, CA, USA) was used together with an KB-FFAP GC column (Kromat Corporation, 4 Providence Court, Delran, NJ08075, USA. 30 m length × 0.25 mm I.D. × 0.25 μm film thickness) for the detection of alkanes / alkenes and free fatty acids of the bienzymatic synthesis. The injector temperature: 250°C; split mode: 30:1; detector temperature: 280°C; GC oven temperature program: initial 110°C, hold for 3.4 min, then from 110°C to 190°C at a ramp rate of 25°C min^{-1}, hold for 2.1 min, from 190 to 230°C at a ramp rate of 25°C min^{-1}, then hold for 2 min, from 230 to 250°C at a ramp rate of 30°C min^{-1}, then hold for 20 min. The effect diagram of test and analysis is shown in Figure S1B. The retention time for ethyl acetate, n-octanol (internal standard) and (*R, Z*)-heptadec-9-en-7-ol were 1.66 min, 4.81 min and 10.01 min, respectively.

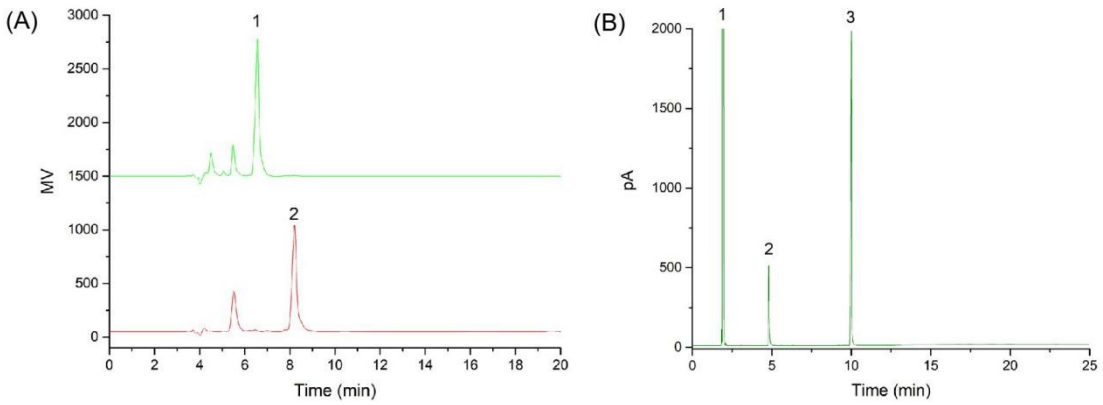

Figure S1. Chromatographic charts of various reaction substrates and products.

(A): HPLC chromatography of castor oil and castor acid: 1. castor acid, 2. castor oil; (B): GC chromatography of (*R, Z*)-heptadec-9-en-7-ol: 1. ethyl acetate, 2. n-octanol (internal standard), 3. (*R, Z*)-heptadec-9-en-7-ol)

Preparation of the cell free extract containing *Cv*FAP (CFE *Cv*FAP) and whole cell *Cv*FAP (*Cv*FAP@*E. coli*)

Production and isolation of recombinant *Cv*FAP enzyme conducted in *Escherichia coli* according to the method previously reported with minor modification [1-3].

Cultivation protocol:

(1) Pre-culture: pre-culture was inoculated with *E. coli* BL21 (DE3) cells harboring the designed pET28a-His-Trx-*Cv*FAP plasmid, and overnight culture (ONC) in terrific broth medium (TB medium: (yeast extract 2.4% (w/v), peptone 1.2% (w/v), glycerol 0.4% (v/v), 72 mM K_2HPO_4, 17 mM KH_2PO_4) containing 50 µg mL^{-1} kanamycin, incubation at 37°C under 200 rpm.

(2) Main culture: the main culture consisted of 500 mL TB medium and 50 µg mL^{-1} kanamycin in 2 L Erlenmeyer flask, inoculated with the corresponding volume of the ONC constituting a start OD_{600} of 0.1. The main culture was incubated at 37°C, 200 rpm for approx. 3 - 4 h until reach an OD_{600} value of 0.7 - 0.8.

(3) Induction: after reaching the desired OD_{600}, protein production was induced by the addition of 0.5 mM IPTG and the cells were left at 17°C and 180 rpm for about 20 h.

(4) Harvest: The cell pellet was collected by centrifugation (4000 × g, 30 min, 4°C) followed by supersonic treatment to get the cell free extract containing *Cv*FAP (CFE *Cv*FAP). Washed with Tris-HCl buffer (50 mM, pH 8, containing 100 mM NaCl), and centrifuged again (10000 × g, 20 min, 4°C), The cell pellet was resuspended in the same buffer, and 1 mM PMSF and 5% glycerol (w/v) was added to the soluble fraction, the cell extract was separated into aliquots, frozen in liquid nitrogen, and stored at -20°C. In order to collect the whole cell *Cv*FAP (*Cv*FAP@*E. coli*), the cell pellet was collected by centrifugation (4000 × g, 30 min, 4°C). Washed with Tris-HCl buffer (50 mM, pH 8, containing 100 mM NaCl), and centrifuged again (10000 × g, 20 min, 4°C). The cell pellet was resuspended in the same buffer, and 1 mM PMSF and 5% glycerol (w/v) was added to the soluble fraction, the *Cv*FAP@*E. coli* frozen in liquid nitrogen, and stored at -20°C. 1 g of wet *Cv*FAP@*E. coli* corresponds to 250 mg dry *Cv*FAP@*E. coli*. As a control, a whole cell of *E. coli* BL21 (DE3) cells harboring an empty pET28a vector was prepared according to the same protocol.

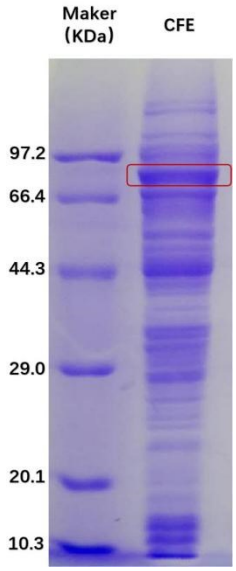

Figure S2. SDS-PAGE analysis of the CFE *Cv*FAP[3].

From left to right: (1) Molecular weight marker; (2) CFE *Cv*FAP (Molecular weight of the *Cv*FAP construct 6xHisTrxA-TEV-*Cv*FAP(short-length): 77.0 kDa).

Photoenzymatic decarboxylation of hydroxy fatty acids catalysed by CFE *Cv*FAP and *Cv*FAP@*E. coli*

The hydroxy fatty acids standard photoenzymatic decarboxylation reactions catalysed by CFE *Cv*FAP and *Cv*FAP@*E. coli* were performed at 30°C (unless indicated otherwise) in a total volume of 1.0 mL Tris-HCl buffer (pH 8.5, 100 mM) containing 30% DMSO as cosolvent.

For CFE *Cv*FAP photoenzymatic decarboxylation reaction, 700 μL of Tris-HCl buffer (pH 8.5, 100 mM) containing 8 mmol CFE *Cv*FAP and 300 μL of hydroxy fatty acids stock solution in DMSO were added to a transparent glass vial. The final conditions of this reaction were: DMSO 30% (v: v), [CFE *Cv*FAP] = 8 μM, [hydroxy fatty acid] = 50 mM. For *Cv*FAP@*E. coli* photoenzymatic decarboxylation reaction, 700 μL of Tris-HCl buffer (pH 8.5, 100 mM) containing 0.35 g wet *Cv*FAP@*E. coli* and 300 μL of hydroxy fatty acid stock solution in DMSO were added to a transparent glass vial (total reaction volume is 1 mL).The final conditions of this reaction were: DMSO 30% (v: v), [Wet *Cv*FAP@*E. coli*] = 0.35 g mL^{-1}, [hydroxy fatty acid] = 50 mM.

Reaction mixtures were thermostatted at the reaction temperatures indicated, stirred at 500 rpm and illuminated with blue LEDs (10 W, 220 V) for 24 h, the reaction temperature is 30°C. Afterwards, the entire reaction mixtures were extracted with ethyl acetate (containing 25 mM of 1-octanol as internal reference) in a 2: 1 ratio (v: v) and analyzed via GC chromatography. The homemade experimental setup is shown in Figure S3.

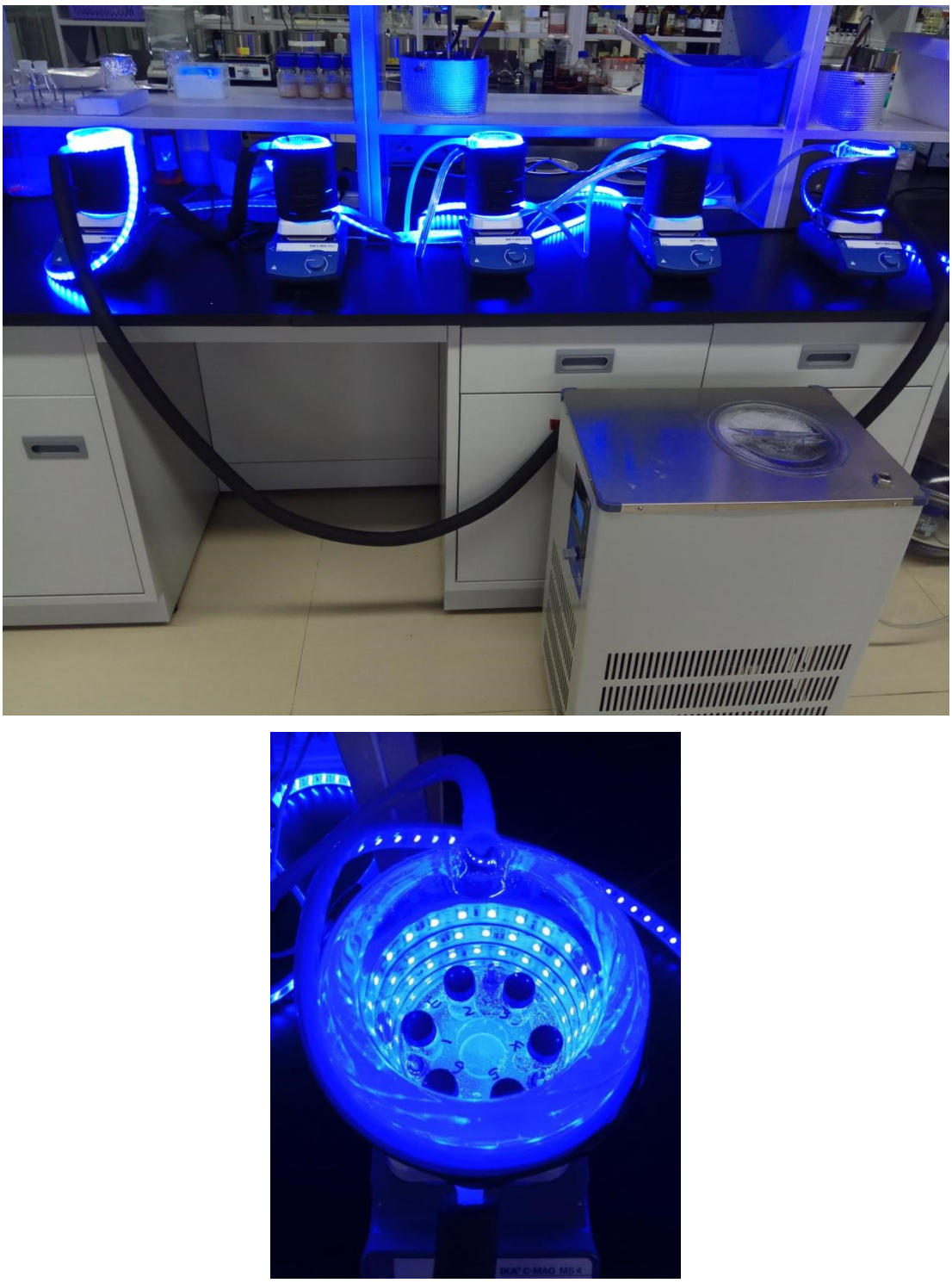

Figure S3. Image of homemade photoenzymatic decarboxylation reaction setup used in the experiment[3].

Enzymatic hydrolysis reactions of castor oil

The enzymatic hydrolysis reactions of castor oil were performed in a 5 mL glass bottle containing 500 μL of castor oil, 500 μL Tris-HCl buffer (pH 8.5, 100 mM) together with 100 mg of Lipase ROL (*Rhizopus oryzae* lipase on resin). The reaction flasks were placed into oil bath with 500 rpm of the rotate speed at 30°C for 24 h. After the reaction is over, the mixture was centrifuged at 10000 × g for 3 minutes. The upper layer (reservoir) will be used for high performance liquid chromatography (HPLC) analysis. Absorbing 30 μL upper reservoir into the 1.5 mL chromatographic bottle, then dissolve and mix well with 970 μL mobile phase (mixture of *n*-hexane, 2-propanol and formic acid (33: 1: 0.003, v: v: v)) for HPLC analysis.

One-pot-one-step hydrolysis/decarboxylation reaction cascade transforming castor oil into fatty alcohols

700 μL of Tris-HCl buffer (pH 8.5, 100 mM) containing 0.35 g wet *Cv*FAP@*E. coli*, 50 mg ROL and 50 μL of castor oil were added to a transparent glass vial (total reaction volume is 1 mL). The final conditions of this reaction were: [Wet *Cv*FAP@*E. coli*] = 0.35 g mL^{-1}, [ROL] = 0.05 g mL^{-1}, [Castor oil] =50 μL mL^{-1}. Reaction mixtures were thermostatted at the reaction temperatures indicated, stirred at 500 rpm and illuminated with blue LEDs (10 W, 220 V) for 24 h. Afterwards, the entire reaction mixtures were extracted with ethyl acetate (containing 25 mM of 1-octanol as internal reference) in a 2: 1 ratio (v: v) and analyzed via GC chromatography.

Table S2. The investigations of key factors to influence castor oil hydrolysis catalyzed by lipase ROL

Entry	ROL（mg mL^{-1}）.	Reaction time（h）	pH	Phase ratio （oil：buffer（vol:vol））	T（°C）	Degree of hydrolysis （%）[a]
1	10	24	8.5	500：500	30	43.5±8.0
2	20	24	8.5	500：500	30	83.6±7.7
3	30	24	8.5	500：500	30	91.5±5.8
4	50	24	8.5	500：500	30	95.5±1.6
5	75	24	8.5	500：500	30	97.1±1.1

6	100	24	8.5	500：500	30	97.4±0.7
7	50	0	8.5	500：500	30	1.1±0.2
8	50	2	8.5	500：500	30	83.1±0.8
9	50	4	8.5	500：500	30	85.4±1.0
10	50	6	8.5	500：500	30	85.5±4.5
11	50	8	8.5	500：500	30	85.3±2.2
12	50	12	8.5	500：500	30	88.4±1.5
13	50	24	8.5	500：500	30	94.5±2.3
14	50	36	8.5	500：500	30	97.9±0.3
15	50	48	8.5	500：500	30	97.1±0.9
16	50	24	7.0	500：500	30	91.4±5.1
17	50	24	8.0	500：500	30	95.3±0.8
18	50	24	8.5	500：500	30	96.2±0.4
19	50	24	9.0	500：500	30	89.5±8.3
20	50	24	8.5	50：950	30	95.0±1.1
21	50	24	8.5	100：900	30	90.2±1.3
22	50	24	8.5	300：700	30	97.6±0.3
23	50	24	8.5	500：500	30	97.2±1.5
24	50	24	8.5	900：100	30	85.2±5.1
25	50	24	8.5	500：500	20	96.1±1.7[b]
						93.0±0.2
26	50	24	8.5	500：500	30	98.1±0.2[b]
						97.7±0.4
27	50	24	8.5	500：500	37	95.7±4.3[b]
						95.8±0.4

[a] Degree of hydrolysis：$[substrate]^{-1}_{initial}\times$ ($[substrate]_{final}-[substrate]_{final}$). [b] performed under blue light（λ=450nm），all other non-special instructions indicate normal daylight. Mean ± SD (n=3).

Table S3. Activity of *Cv*FAP crude cell extracts or whole cells on several hydroxy fatty acids.

Substrate	16-Hydroxy- Hexadecenoic acid	12-HydroxyStearic acid	Ricinoleic acid
*Cv*FAP crude cell extracts	17.3±1.6	30.8±11.8	40.9±0.4
*Cv*FAP whole cells	13.9±0.5	30.5±0.3	37.9±6.6

Conditions: 700 μL of Tris-HCl buffer (pH 8.5, 100 mM) containing 0.35 g wet *Cv*FAP@*E. coli* (or 8 mmol *Cv*FAP crude cell extracts) and 300 μL of hydroxy fatty acids with hydroxyl groups stock solution in DMSO were added to a transparent glass vial (total reaction volume is 1 mL). The final conditions of this reaction were: DMSO 30% (v: v), [Wet *Cv*FAP@*E. coli*] = 0.35 g mL-1 or [*Cv*FAP crude cell extracts] = 8 μM, [hydroxy fatty acid] = 50 mM, 30°C, 10W blue LED, 24 h. Mean ± SD (n=3).

Table S4. Influence of the key factors on the bienzymatic synthesis of (R, Z)-octadec-9-en-7-ol[a].

Entry	Stirring speed（rpm）.	pH	Phase ratio（oil：buffer（vol:vol））	T（°C）	$Con_{(R, Z)\text{-octadec-9-en-7-ol}}$ [mM][b]
1	500	8.5	50：950	30	41.7±5.7
2	800	8.5	50：950	30	37.0±6.4
3	1000	8.5	50：950	30	22.7±5.1
4	500	7.0	50：950	30	8.6±2.7
5	500	8.0	50：950	30	6.9±2.0
6	500	8.5	50：950	30	35.1±10.6
7	500	9.0	50：950	30	44.8±11.5
8	500	10.0	50：950	30	56.7±1.1
9	500	8.5	50：950	30	36.0±16.6
10	500	8.5	100：900	30	42.6±10.2
11	500	8.5	200：800	30	22.9±4.9
12	500	8.5	500：500	30	24.6±5.5
13	500	8.5	50：950	20	11.0±6.5

14	500	8.5	50：950	30	31.4±2.3
15	500	8.5	50：950	37	17.9±7.6

[a] The final conditions of this reaction were: [Wet *Cv*FAP@*E. coli*] = 0.35 g mL^{-1}, [lipase ROL] = 50 mg mL^{-1}, 10 W blue LED, 24 h. [b]Mean ± SD (n = 3).

REFERENCES

[1] M. Huijbers, W. Zhang, F. Tonin, F. Hollmann, *Angew. Chem. Int. Edit.,* **2018**, *57*, 13648-13651.

[2] W. Zhang, M. Ma, M. Huijbers, G. Filonenko, E. Pidko, M. Schie, S. Boer, B. Burek, J. Bloh, W. Berkel, W. Smith, F. Hollmann, *J. Am. Chem. Soc.,* **2019**, *141*, 3116-3120.

[3] Y. Ma, X. Zhang, W. Zhang, P. Li, Y. Li, F. Hollmann, Y. Wang, *ChemPhotoChem*, **2020**, *4*, 39–44.