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# More efficient enzymatic cascade reactions by spatially confining enzymes via the SpyTag/SpyCatcher technology

Xuanru Zhong [a], Yunjian Ma [a,b,*], Xizhen Zhang [c], Jiahui Zhang [a], Bin Wu [c], Frank Hollmann [d], Yonghua Wang [a,e,*]

[a] School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, China
[b] Neher's Biophysics Laboratory for Innovative Drug Discovery, State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Taipa, Macau, China
[c] School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, China
[d] Department of Biotechnology, Delft University of Technology, van der Maasweg 9, Delft 2629HZ, the Netherland
[e] Guangdong Youmei Institute of Inteligent Bio-manufacturing Co., Ltd, Foshan, Guangdong 528200, China

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ABSTRACT

Hydrocarbon synthesis from (waste)oils enabled by a cascade of lipase-catalysed hydrolysis and decarboxylase-catalysed decarboxylation has become an active area of research en route to alternative, biobased fuels. However, Poor substrate transport efficiency is a major issue causing low reaction rates. This study focused on a protein self-assembly strategy based on SpyTag/SpyCatcher to overcome diffusion limitations. For this, two fusion proteins, TLL-Linker-SpyCatcher based on the lipase from *Thermomyces lanuginosus* and *Cv*FAP-Linker-SpyTag based on the fatty acid photodecarboxylase from *Chlorella variabilis* were designed. A covalent multi-enzyme complex (TLL-*Cv*FAP) was formed spontaneously by self-assembly of each enzyme. The effects of temperature, pH and molar ratio of self-assembled components on assembly efficiency were investigated. The results showed that the multi-enzyme complex TLL-*Cv*FAP reached about 60% after 12 h of assembly, and the enzyme activity of the multienzyme complex was increased by about 50% compared to that of the corresponding non-assembled enzymes. Under optimized conditions 10 mM soybean oil were converted into 25 mM of the corresponding hydrocarbons, suggesting a good potential of biofuel synthesis.

## 1. Introduction

Cascade reactions are gaining increasing attention in (bio)catalysis as alternatives to classical multi-step syntheses [1]. Performing several conversions simultaneously not only supersedes isolation and purification of intermediate products but also results in significant savings in the total reaction time. Cascade reactions therefore are an attractive approach for more economical and environmentally less demanding syntheses.

Especially in the field of biocatalysis cascade development is an ongoing research trend. It has been pointed out that the spatial confinement of different biocatalysts operating in parallel or in sequence can drastically reduce diffusion distances of the reagents and thereby accelerate the rate of biocatalytic cascade reactions [2]. Next to co-immobilization of different enzymes [3] or confinement in vesicles [4] also genetically fused enzymes [2,5,6] have been investigated. From these studies it can be concluded that generally, spatial proximity represents an advantage for cascade catalysis as diffusion distances of the individual reagents are reduced.

Therefore, we became interested in the recently developed SpyTag/SpyCatcher technology [7–10] to covalently link individual proteins through a newly formed covalent (isopeptide) bond. Hence, the SpyTag/SpyCatcher technology may also represent an interesting approach to accelerate enzymatic cascade reactions.

To test our hypothesis, we chose a cascade of lipase-catalysed hydrolysis of natural triglycerides [11,12] and photocatalytic decarboxylation of the liberated fatty acids using a recently reported fatty acid photodecarboxylase [13–15] (Scheme 1).

* Corresponding authors at: School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, China.
*E-mail addresses:* femayj@mail.scut.edu.cn (Y. Ma), yonghw@scut.edu.cn (Y. Wang).

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## 2. Experimental

### 2.1. Chemical reagents and materials

All chemicals were purchased from Sigma-Aldrich, TCI or Aladdin in the highest purity available and used without further purification. Waste cooking oil, soybean oil and other vegetable oils were a kind gift from provided by Guangzhou Zhizhiyuan Oil Industry Co., Ltd. (Guangzhou, China). Water was purified with a Millipore (Bedford, MA) Milli-Q water system.

### 2.2. Experimental set-up and operating conditions

#### 2.2.1. Experimental set-up

The homemade experimental setup is shown in Fig. S3. The blue LEDs (10 W) were purchased from Midea Co., Ltd. (Foshan, China), The distance between light and reaction bottle was 2.5 cm, light intensity was 5000 μWcm$^{-2}$. A representative hydrolysis/decarboxylation reaction cascade transforming oils into hydrocarbons: pH 7.0 (20 mM sodium phosphate) buffer, 10 μL oil (corresponding to 30 mM fatty acid) with 20 μM enzyme (20 μM TLL-*Cv*FAP, or 20 μM TLL and 20 μM *Cv*FAP) were mixed and were added to a transparent glass vial (total reaction volume was 1 mL). Reaction mixtures were thermostatted at the reaction temperatures indicated, stirred at 500 rpm and illuminated with blue LEDs. Afterwards, the entire reaction mixtures were extracted with ethyl acetate (containing 25 mM of 1-octanol as internal reference) in a 1: 1 ratio (v: v) and analyzed via GC chromatography.

#### 2.2.2. For the detection of products after enzymatic hydrolysis of oils

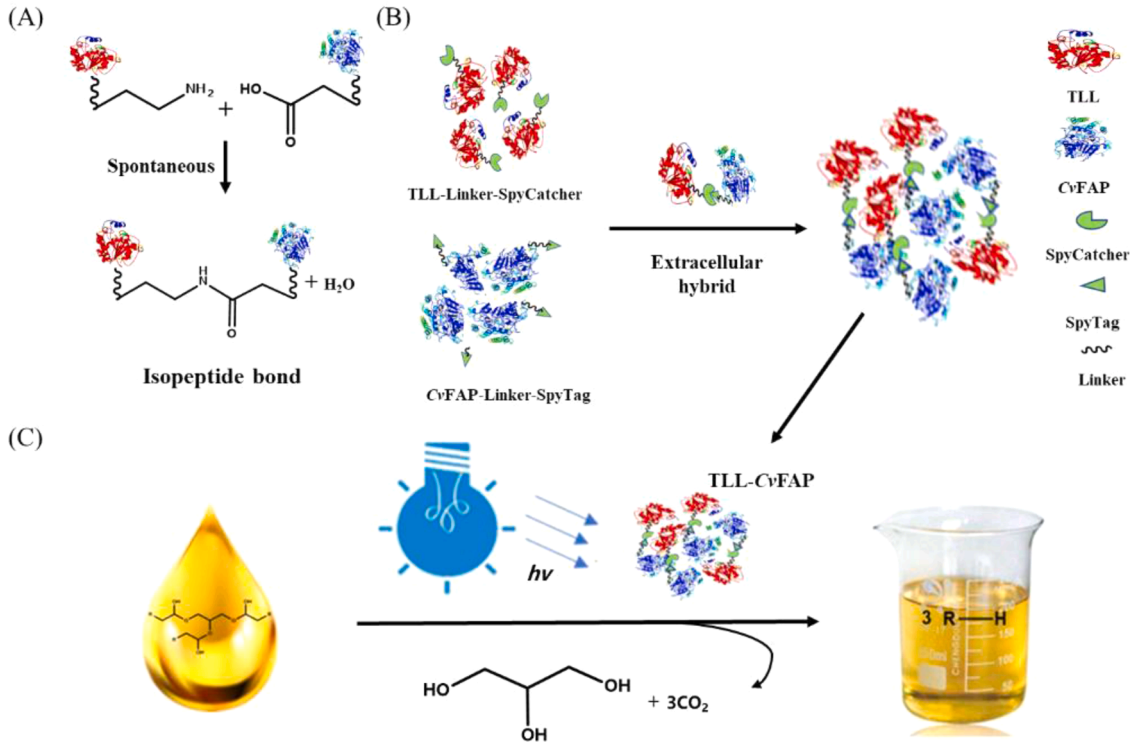
The enzymatic hydrolysis reactions of oils were performed in a 4 mL glass bottle containing 50 μL of oil, 500 μL Tris–HCl buffer (pH 8.5, 100 mM) together with 10 μM lipase TLL. The reaction flasks were placed into oil bath with 500 rpm of the rotate speed at 30 °C for 12 h. The mixture was centrifuged at 10,000 × g for 3 min. The upper layer (reservoir) was submitted to high performance liquid chromatography (HPLC) analysis by diluting 50 μL of the sample in 950 μL or the mobile phase. The products after enzymatic hydrolysis of oils were analysed 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 (18: 1: 0.003, v: v: v) and performed with a flow rate of 1 mL min$^{-1}$ at 30°C. The retention time for triglyceride; free fatty acid; 1,3-glycerol diester; 1,2-glycerol diester were 3.66 min, 4.04 min, 4.75 min and 6.05 min, respectively. Waters 2695 integration software was employed to calculate the peak-areas percentages.

#### 2.2.3. For the detection of alkanes/alkenes and free fatty acids

Refer to Section 2.2.1 for the operating conditions of the reaction. 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. Method: 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 12 min. Retention time as list in Table S2.

### 2.3. Genes and plasmids

Previously reported pET28a-TLL containing the *Thermomyces lanuginosus* lipase (TLL) gene and pET23a-SpyCatcher containing the SpyCatcher gene from *Streptococcus pyogenes* fibronectin-binding protein FbaB were used [16]. pET28a-*Cv*FAP plasmids containing *Chlorella variabilis* fatty acid photodecarboxylase (*Cv*FAP) gene were the same as




**Scheme 1.** Using the SpyTag/SpyCatcher technology to accelerate the bienzymatic cascade transforming natural triglycerides into alkanes. (A&B) Based on SpyTag/SpyCatcher technology to prepare the covalent multienzyme (TLL-CvFAP) catalyst; (C) the resulting hybrid enzyme catalyses the hydrolysis of trigycerides and light-driven decarboxylation of the liberated fatty acids yielding alkanes.

reported previously [17]. PrimeSTAR Max DNA polymerase, restriction endonucleases, and polymerase chain reaction (PCR) reagents were purchased from TaKaRa (Dalian, China).

### 2.4. Construction of fusion genes

In order to construct pET28a-*Cv*FAP-$(EAAAK)_2$-SpyTag, the plasmid of pET28a-*Cv*FAP was used as template and $(EAAAK)_2$-SpyTag was cloned into this vector by PCR amplification. Accurately sequenced plasmids were stored for later use. In order to construct the pET28a-TLL-(GGGGS)2-SpyCatcher the plasmid pET28a-TLL containing NotI and SalI was used as template. 5′-$(GGGGS)_2$-SpyCatcher-3′ was cloned into this vector between Not I/Sal I. All target expression vectors were transformed into *E.coli* Top 10 through heat shock transformation. The primers used in plasmid construction listed in Table S1. All of the plasmids were verified by sequencing and then transformed into *E. coli* BL21 (DE3) for recombinant protein expression.

### 2.5. Protein expression and purification

Recombinant *E. coli* BL21 (DE3) strains containing *Cv*FAP-$(EAAAK)_2$-SpyTag were cultured in Terrific Broth (TB) containing 50 μg $mL^{-1}$ kanamycin at 37 °C. When the $OD_{600}$ reached 0.8–1, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and cells were incubated at 18 °C for 20 h. Then harvested by centrifugation at 10,000 rpm for 20 min and resuspended in lysis buffer (50 mM Tris–HCl pH 8.0). Resuspended cells were lysed by a homogeniser at 4 °C. After centrifugation at 12,000 rpm for 30 min, the supernatant was loaded into Ni-NTA agarose gravity-flow column, and then were eluted by the buffer consisting of 50 mM Tris–HCl (pH 8.0), 0.5 M NaCl and 250 mM imidazole. Elution fractions were desalted and concentrated.

The protein TLL-$(GGGGS)_2$-SpyCatcher were obtained by the same steps as *Cv*FAP-$(EAAAK)_2$-SpyTag.

The proteins were analysed using SDS-PAGE, and protein concentrations were determined using the Bradford method. Proteins without Tag, *Cv*FAP and TLL, were expressed and purified as the *Cv*FAP-$(EAAAK)_2$-SpyTag and TLL-(GGGGS)2-SpyCatcher, respectively. A protein assay kit was purchased from Sangon Biotech (ShangHai, China). The chromatographic columns His Prep™ FF16/10, HiPrep™ 26/10 and AKTA purifier were from GE Healthcare (Uppsala, Sweden).

### 2.6. Bioconjugation in vitro and SDS-PAGE assay

After determination of the protein concentrations, equal molar of TLL-(GGGGS)2-SpyCatcher and *Cv*FAP-(EAAAK)2-SpyTag were mixed in the buffer containing 20 mM PBS (pH 8.0) for 12 h at 4 °C. The assemblies were subjected to SDS-PAGE for analysis.

### 2.7. Optimization of the bioconjugation conditions and biocatalytic properties of TLL-CvFAP

The effects of molar ratio ([TLL-$(GGGGS)_2$-*SpyCatcher*]: [*Cv*FAP-$(EAAAK)_2$-SpyTag] = 3:1, 2:1, 1:1, 1:2 and 1:3), temperature (4, 20, 30, 37 and 45 °C), pH(5, 6, 7, 7.4, 8 and 9) and time (0.5, 1, 2, 4, 6, 8, 12 and 24 h) of self-assembled components on assembly efficiency were investigated. And its catalytic effect on the conversion efficiency of hydrocarbon generated from oils was investigated. The reaction time (from 1 to 24 h), enzyme dosage(10, 20, 30, 40 and 50 μM) and substrate scope (soybean oil,waste cooking oil and other vegetable oils) was obtained by oil with the self-assembling multienzyme complex.

## 3. Results and discussion

### 3.1. Design and construction of the tagged enzymes

The SpyTag/SpyCatcher approach is based on the formation of a covalent linkage (isopeptide bond) between the SpyTag (being a short, 13 amino-acid peptide sequence) and the SpyCatcher (being a 12.4 kDa protein) [10]. To utilise this approach to covalently link TLL and *Cv*FAP, we have created C-terminal fusions of *Cv*FAP with the SpyTag and TLL with the SpyCatcher peptide, respectively. To ensure accessibility of both fragments, small, flexible amino acid linkers were inserted between the enzymes' C-termini and the SpyTag and SpyCatcher, respectively. Hence, TLL-(GGGGS)2-SpyCatcher and *Cv*FAP-(EAAAK)2-SpyTag sequences were cloned into pET28a plasmids and overexpressed individually in *E. coli* BL21(DE3) (see materials and methods section for details). For the convenience of discussion, in the following, we record TLL-(GGGGS)2-SpyCatcher as TLL-Linker-SpyCatcher and *Cv*FAP-(EAAAK)2-SpyTag as *Cv*FAP-Linker-SpyTag.

### 3.2. Characterization of the tagged enzymes

To investigate a possible effect of the newly added tags to the wild-type enzymes, we first compared the properties of the wt-enzymes with those of their tagged derivates. The recombinant expression of both enzymes was not significantly impaired by the added linkers and tag (Table 1, Fig. 1).

Also the catalytic activities of tagged- and non-tagged TLL and *Cv*FAP were very comparable (Table 1) indicating that the catalytic activity of the tagged enzymes was not impaired.

### 3.3. Establishing the bioconjugation reaction

To generate the envisioned bifunctional enzyme, we decided to perform the bioconjugation of *Cv*FAP-Linker-SpyTag with TLL-Linker-SpyCatcher directly from the crude cell extracts of the individual enzymes. On the one hand, avoiding purified enzymes significantly simplifies the biocatalyst preparation while on the other hand, especially *Cv*FAP has previously been demonstrated to be less stable in purified form [18]. Indeed, SDS gel analysis revealed that upon mixing both tagged enzymes a new band between 100 and 140 kDa appeared, corresponding to the calculated molecular weight of the covalent conjugate (125 kDa), this band was not observed upon mixing of the wt-enzymes (Fig. 2). For the convenience of discussion, we abbreviate the assembled multi enzyme complex TLL-Linker-SpyCatcher-SpyTag-Linker-*Cv*FAP as TLL-*Cv*FAP.

**Table 1**
Comparison of the catalytic activity of the wild type enzymes and their tagged derivates.

| Enzyme | Final cell density [g $L^{-1}$] | Amount of purified enzyme [mg] | Specific activity [U $mg^{-1}$][a] |
|---|---|---|---|
| wt-TLL | 10 | 43.2 | 141.5 ± 4.4 |
| TLL-Linker-SpyCatcher | 12.4 | 53.8 | 152.1 ± 4 |
| wt-*Cv*FAP | 42.5 | 8.7 | 0.375 ± 0.0002 |
| *Cv*FAP-Linker-SpyTag | 45.4 | 6.8 | 0.363 ± 0.0003 |

The total fermentation volume was 4 L, assay conditions: TLL or TLL-Linker-SpyCatcher activity: an emulsion of 4 g of olive oil with 5 mL potassium phosphate buffer (20 mM, pH 7.4) was supplemented with an appropriate amount of 10 μM TLL or TLL-Linker-SpyCatcher and mixed vigorously at 25 °C for 5 min. The reaction was terminated by the addition of 15 mL ethanol (95%). The concentration of free fatty acid was determined by titration with 50 mM NaOH; *Cv*FAP or *Cv*FAP-Linker-SpyCatcher activity: 50 mM of palmitic acid were dissolved in an aqueous Tris–HCl buffer (100 mM, pH 8) containing 30% (v/v) DMSO, add an appropriate amount of *Cv*FAP or *Cv*FAP-Linker-SpyCatcher enzyme solution (10 μM), the mixed solution was illuminated with blue light (220 V, 10 W) for 2 h at 30 °C, the reaction bottle was place in a homemade photoenzymatic decarboxylation reaction setup (Fig. S3). The reaction mixtures were extracted with ethyl acetate and analysed via gas chromatography. All experiments were performed in triplicate. [a] 1 unit (U) is defined as the rate at which 1 μmol of product are formed per minute.

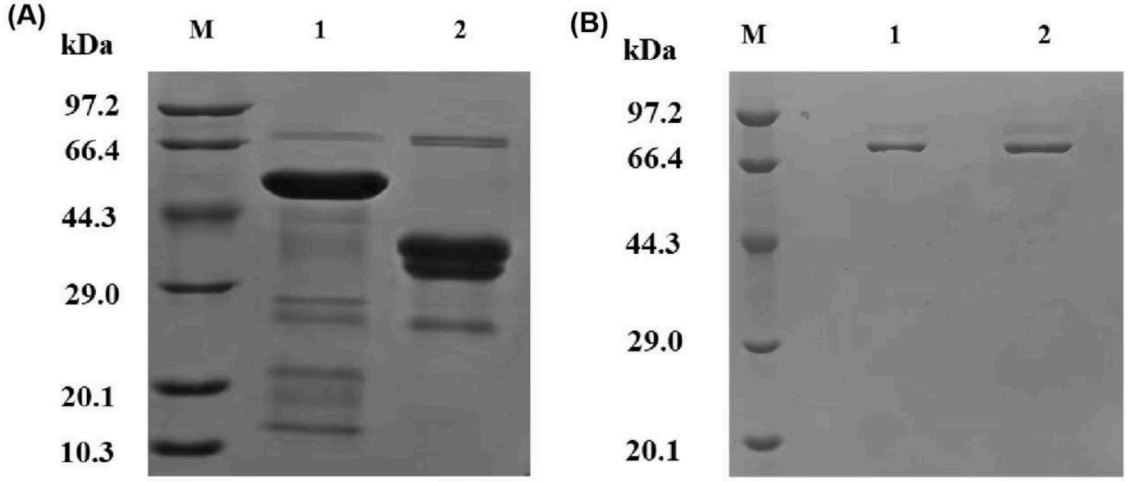



**Fig. 1.** SDS-PAGE analysis of the purified samples of the fused enzymes and the non-fused corresponding enzymes.
((A) SDS-PAGE analysis of purification of TLL and TLL-Linker-SpyCatcher (line M: protein marker; line 1: TLL-Linker-SpyCatcher after purification; line 2: TLL after purification); (B) SDS-PAGE analysis of purification of CvFAP and CvFAP-Linker-SpyTag (line M: protein marker; line 1: CvFAP-Linker-SpyTag after purification; line 2: CvFAP after purification)).

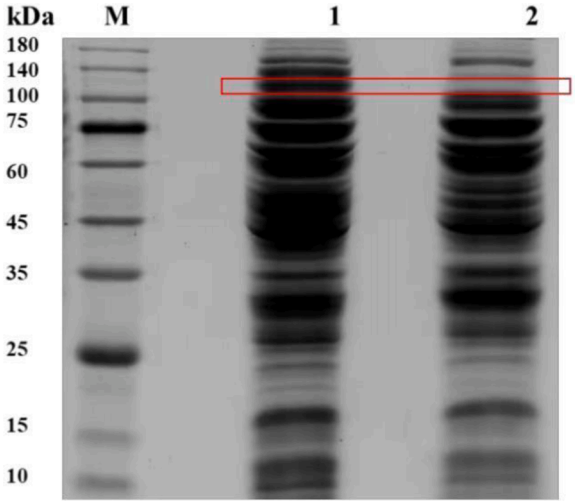



**Fig. 2.** SDS-PAGE analysis of multi-enzyme complex self-assembly.
(line M: protein marker; line 1: multienzyme complex formed by mixing crude cell extracts containing CvFAP-Linker-SpyTag with crude cell extracts containing TLL-Linker-SpyCatcher; line 2: mixing crude cell extracts of the wt-enzymes. (Assembled (TLL-Linker-SpyCatcher and CvFAP-Linker-SpyTag) and unassembled (TLL and CvFAP) samples were prepared by mixing two enzymes at 10 μM submit concentration at 4 °C and potassium phosphate buffer (20 mM, pH 7) for 12 h, and then subjected to SDS-PAGE analysis.)

Varying the molar ratio of both enzymes had no influence on the molecular weight distribution of the resulting bioconjugate (only the expected band around 125 kDa was observed) confirming that the stoichiometry of the coupling reaction was indeed 1:1, this is also confirmed by a densiometric quantification of the enzymes present after the bioconjugation at varying molar ratios of the proteins (Table 2 and Fig. S4).

### 3.4. Optimization of the bioconjugation conditions

Next, we systematically investigated the influence of reaction parameters on the efficiency of the bioconjugation. These experiments were followed semi-quantitatively by SDS gel analysis (Fig. 4, S5, 6 and Tables S3–5). For a more quantitative evaluation, we tested the resulting conjugated, bifunctional enzyme for its hydrocarbon synthesis activity

**Table 2**
Densiometric analysis of non-reacted TLL-Linker-SpyCatcher andCvFAP-Linker-SpyTag and the resulting bioconjugate.[a]

| Lane | Molar ratio | TLL-Linker-SpyCatcher | *Cv*FAP-Linker-SpyTag | TLL-*Cv*FAP |
|---|---|---|---|---|
| 1 | 1:3 | 12,411.58 | 23,398.38 | 7886.42 |
| 2 | 1:2 | 10,127.27 | 19,889.46 | 10,619.44 |
| 3 | 1:1 | 23,978.97 | 19,306.29 | 10,759.37 |
| 4 | 2:1 | 18,617.51 | 16,105.66 | 10,235.80 |
| 5 | 3:1 | 25,778.11 | 16,062.39 | 10,637.49 |

[a] Quantified using ImageJ software (1.8.0) values shown are in arbitrary intensity units.

from soybean oil and compared it to the activity of an equiconcentrated mixture of wild type enzymes treated under the same conditions as the bioconjugation experiment (Figs. 3, and 5–9).

Increasing the duration of the bionjugation reaction increased the yield of the desired bifunctional enzyme (Fig. S5). In parallel, the productivity of the bienzymatic cascade increased steadily with increasing bioconjugation duration (Fig. 3). After approx. 12 h of bioconjugation no further increase of the catalytic activity of the bifunctional enzyme was observed indicating that within this time frame the bioconjugation reaction was complete.

Noteworthy, the catalytic activity of the bioconjugate was approximately 2 times higher than of the isolated wild type enzymes under otherwise identical conditions. We interpret this as a consequence of the channelling effect caused by the spatial proximity of both biocatalysts.

The pH of the bioconjugation reaction mixture had no distinct influence on the efficiency of the bioconjugation as judged by SDS gel analysis (Fig. 4).This is in line with previous reports describing a broad pH range for the isopeptide formation in the SpyTag-SpyCatcher system [10].

In contrast, the catalytic performance of the bioconjugate showed a very pronounced pH optimum around 7 (Fig. 5). This optimum is the result of the differing pH optima of the lipase (around pH 7–9) [16] and the photodecarboxylase (around pH 8) [13,19,20].

The optimal temperature for the bioconjugation was found to be 30 °C (Fig. 6). Performing the bioconjugation reaction at 4 °C resulted in very little accumulation of the desired bifunctional coupling enzyme (Fig. S7) whereas the yield did not increased significantly at coupling temperatures above 20 °C. Increasing the bioconjugation temperature above 30 °C resulted in high coupling efficiency but decreased catalytic

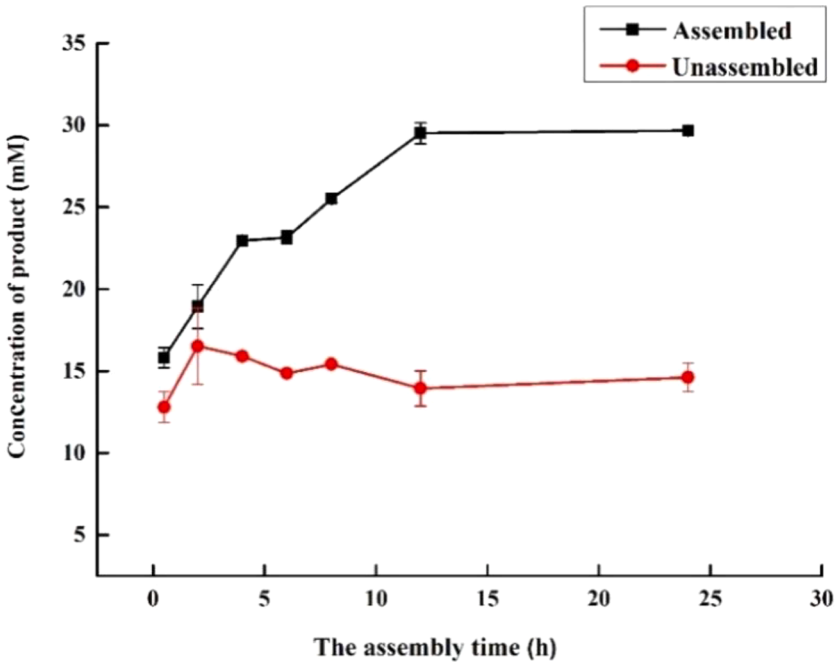



**Fig. 3.** Comparison of the product formation of the bifunctional TLL-*Cv*FAP conjugate (black) and the wt-enzymes (red) depending on the assembly time.
(Reaction conditions: TLL-Linker-SpyCatcher and *Cv*FAP-Linker-SpyTag were mixed and assembled at a molar ratio of 1:1, the enzyme concentrations were 20 μM, the assembly temperature was 30 °C, and the assembly time was 0.5, 1, 2, 4, 6, 8 and 12 h, respectively, pH 7 (20 mM sodium phosphate);10 μL soybean oil (30 mM fatty acid) with 20 μM enzyme were mixed and under gentle magnetic stirring (500 rpm) at 30 °C in a total volume of 1 mL under the homemade photoenzymatic decarboxylation reaction setup (illumination with blue light (10 W, Fig. S3).

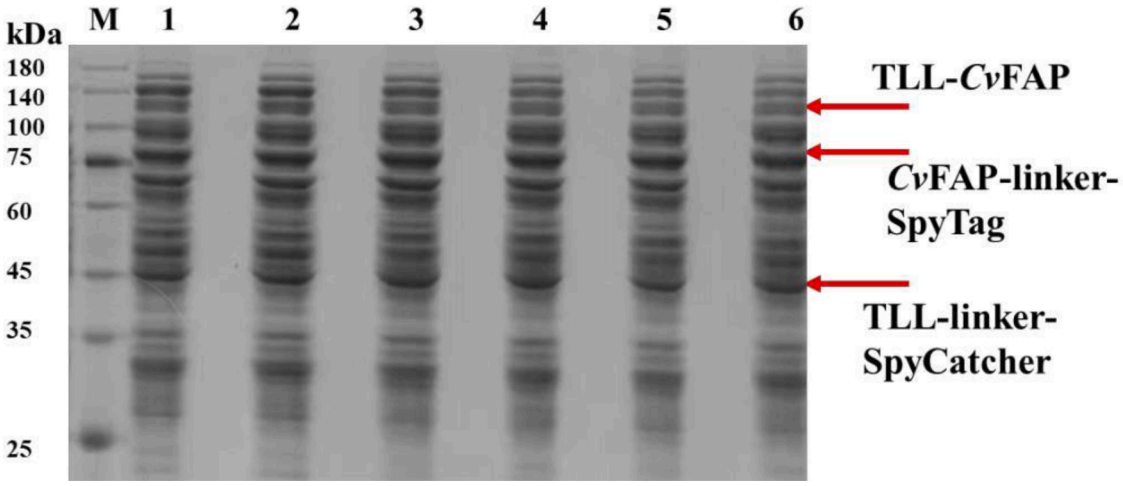



**Fig. 4.** SDS gel analysis of the bioconjugation reactions performed at different pH values.
(line M: protein marker; line1–6: the pH was 5.0, 6.0, 7.0, 7.4, 8.0 and 9.0 in turn; Buffers with different pH values: pH 5.0 (20 mM citrate); pH 6.0–7.5 (20 mM sodium phosphate); pH 8.0 (20 mM Tris–HCl) ;pH 9.0 (20 mM glycine sodium hydroxide), TLL-Linker-SpyCatcher and CvFAP-Linker-SpyTag were mixed and assembled at a molar ratio of 1:1,the enzyme concentrations were 20 μM, the assembly temperature was 30 °C, and the assembly time was 12 h).

activity of the bifunctional enzyme. Considering the modest thermal robustness of *Cv*FAP, this may be attributed to the thermal inactivation of the photodecarboxylase catalyst.

The comparison of the bioconjugated enzyme with the isolated enzymes of the previous experiments already indicated that the catalytic efficiency of the bioconjugate was higher. We therefore also conducted a kinetic experiment comparing both enzyme preparations (Fig. 7).

The bifunctional catalyst was well-behaved in terms of concentration-dependency of the overall product formation rate (Fig. 8) and an approximately linear relationship between catalyst concentration and product formation rate was observed.

Finally, we compared the substrate scope of the bifunctional enzyme with the one of the isolated individual enzymes (Fig. 9). Interestingly, the rate-acceleration observed in the previous experiments could not be validated with all starting materials evaluated. Especially for castor oil, waste cocking oil and sea buckthorn oil, the overall reaction rates of both catalyst preparations were comparable. At present time, we are lacking a plausible explanation for this observation.

## 4. Conclusion

In this study, the lipase photodecarboxylase multi enzyme complex (TLL-*Cv*FAP) was constructed by SpyTag/SpyCatcher self-assembly system for the first time. Compared with double free enzyme catalysis, the efficiency of TLL-*Cv*FAP in oil synthesis was increased by about 50%, and the storage stability of TLL-*Cv*FAP after self-assembly was also significantly improved. By changing the length and type of flexible linker, the mass transfer rate between enzymes in cascade reaction is adjusted to tune the catalytic efficiency. Firstly, TLL linker Spycatcher and *Cv*FAP linker Spytag fusion proteins were constructed. The soluble expression of the fusion protein was realised with *E. coli* as the expression strain, and the enzyme activity was not affected before and after

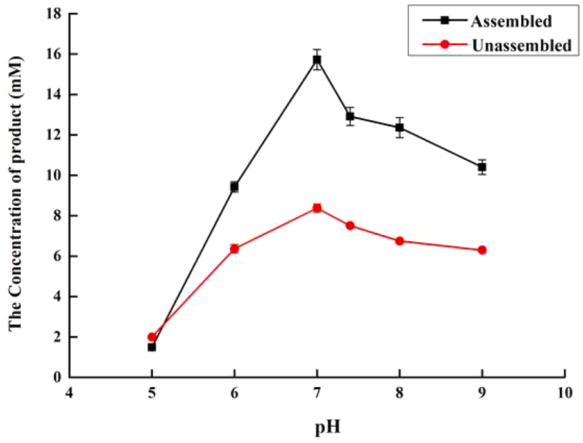



**Fig. 5.** pH profile of the bienzymatic hydrolysis/decarboxylation reaction catalysed by the bioconjugate, bifunctional enzyme (black) and the isolated wt-enzymes (red).
Reaction conditions: TLL-Linker-SpyCatcher and *Cv*FAP-Linker-SpyTag were mixed and assembled at a molar ratio of 1:1, the enzyme concentrations were 20 μM, the assembly temperature was 30 °C, and the assembly pH was 5, 6, 7, 7.4, 8 and 9, respectively, the assembly time was 12 h;10 μL soybean oil (30 mM fatty acid) with 20 μM enzyme were mixed and under gentle magnetic stirring (500 rpm) at 30 °C in a total volume of 1 mL under the homemade photoenzymatic decarboxylation reaction setup (illumination with blue light (10 W, Fig. S3).

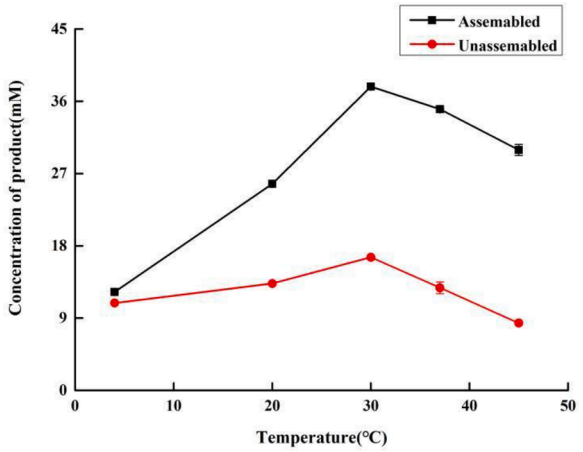



**Fig. 6.** Influence of the bioconjugation temperature on the catalytic efficiency of assembled enzyme preparation (TLL-CvFAP) and unassembled enzyme preparation.
Reaction conditions: TLL-Linker-SpyCatcher and CvFAP-Linker-SpyTag were mixed and assembled at a molar ratio of 1:1, the enzyme concentrations were 20 μM, the assembly time was 12 h, and the assembly temperature was 4, 20, 30, 37 and 45 °C, respectively, the assembly time was 12 h, pH 7 (20 mM sodium phosphate); 10 μL soybean oil (30 mM fatty acid) with 20 μM enzyme were mixed and under gentle magnetic stirring (500 rpm) at 30 °C in a total volume of 1 mL under the homemade photoenzymatic decarboxylation reaction setup (illumination with blue light (10 W, Fig. S3).

fusion. Secondly, the multi enzyme complex TLL-*Cv*FAP was successfully constructed by mixing self-assembly units *in vitro*. By optimising the assembly conditions, the assembly efficiency of TLL-*Cv*FAP was about 60% when assembled at 30 °C, pH 7 and the molar ratio of self-assembled elements was 1:1 for 12 h, and the activity of TLL-*Cv*FAP was increased by about 50% compared with double free enzyme. Moreover, the conversion efficiency of TLL-*Cv*FAP catalysed oil to hydrocarbons was evaluated. Under the optimal conditions, 25 mM of hydrocarbon corresponding to 83.3% conversion was achieved using 10

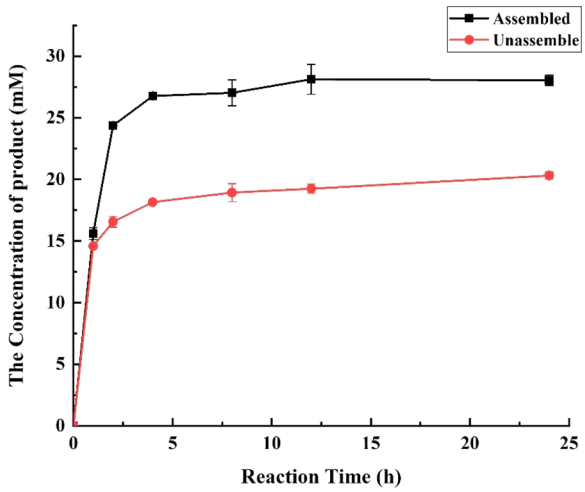



**Fig. 7.** Time courses of the combined hydrolysis/decarboxylation reaction using the bioconjugate bifunctional enzyme and the isolated individual wt-enzymes.
Reaction conditions: 20 μM TLL-CvFAP, 20 μM TLL and 20 μM CvFAP, pH 7.0 (20 mM sodium phosphate, pH 7); 10 μL soybean oil (that is, 30 mM fatty acid) with 20 μM enzyme were mixed and under gentle magnetic stirring (500 rpm) at 30 °C in a total volume of 1 mL under the homemade photoenzymatic decarboxylation reaction setup (illumination with blue light (10 W, Fig. S3).

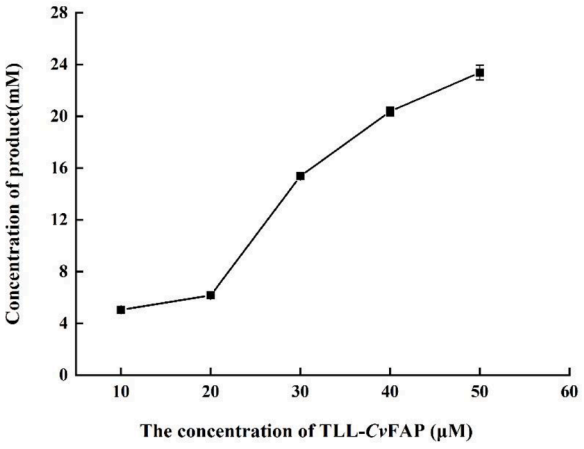



**Fig. 8.** Rate-dependency of the bienzymatic hydrolysis/decarboxylation reaction.
Reaction conditions: 10 μL soybean oil (30 mM fatty acid) with 20 μM TLL-CvFAP enzyme were mixed and under gentle magnetic stirring (500 rpm) at 30 °C, pH 7 (20 mM sodium phosphate); in a total volume of 1 mL under the homemade photoenzymatic decarboxylation reaction setup (illumination with blue light (10 W, Fig. S3).

mM soybean oil as substrate.

Admittedly, there are still many questions demanding further in-depth study, such as the efficient purification of photodecarboxylase and the separation, purification and characterization of multi enzyme complex, and the crystals of multi enzyme complex. Also the poor photostability of *Cv*FAP certainly represents an issue that needs to be addressed by e.g. enzyme engineering.

## CRediT authorship contribution statement

**Xuanru Zhong:** Conceptualization, Visualization, Validation. **Yunjian Ma:** Conceptualization, Visualization, Validation. **Xizhen Zhang:** Formal analysis. **Jiahui Zhang:** Formal analysis. **Bin Wu:** Formal analysis. **Frank Hollmann:** Writing – original draft. **Yonghua Wang:**

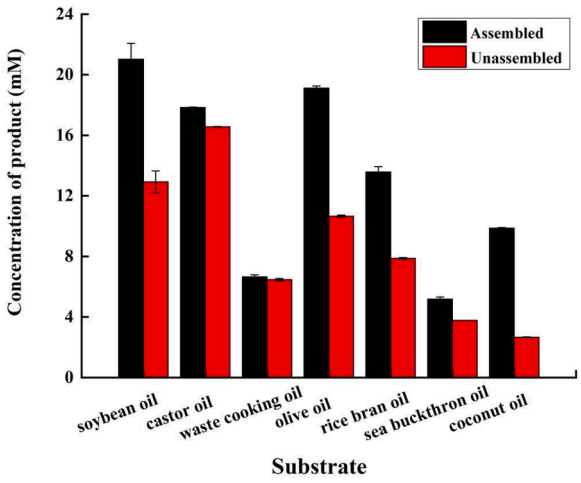



**Fig. 9.** The substrate scope of TLL-CvFAP (Assembled) or TLL and CvFAP (Unassembled).
Reaction conditions: 20 μM TLL-CvFAP, 20 μM TLL and 20 μM CvFAP, pH 7 (20 mM sodium phosphate);10 μL oil (that is, 30 mM fatty acid) with 20 μM enzyme were mixed and under gentle magnetic stirring (500 rpm) at 30 °C in a total volume of 1 mL under the homemade photoenzymatic decarboxylation reaction setup (illumination with blue light (10 W, Fig. S3)).

Visualization, Project administration.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mcat.2022.112188.

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