Light-Driven Enzymatic Decarboxylation of Fatty Acids

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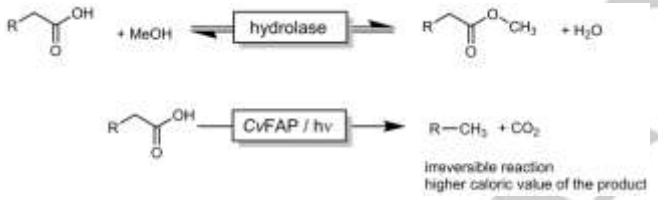
Light-driven enzymatic decarboxylation of fatty acids

Mieke M.E. Huijbers[+], Wuyuan Zhang[+], Fabio Tonin, and Frank Hollmann*

Abstract: Photoenzymatic decarboxylation of fatty acids into alkanes is proposed as alternative approach for biodiesel synthesis. Using a recently discovered photodecarboxylase from *Chlorella variabilis* NC64A (*Cv*FAP) we demonstrate the irreversible preparation of alkanes from fatty acids and triglycerides. Several fatty acids and their triglycerides are converted by *Cv*FAP upon illumination with blue light in near-quantitative yield and exclusive selectivity. In this proof-of-concept study very promising turnover numbers of up to 8000 were achieved.

The conversion of (waste) fatty acids and oils into biofuels has been in focus of research for decades.[1] The most widely used approach is to transform fatty acid (esters) into the corresponding methyl- and ethyl esters (FAMEs and FAEEs, respectively). Especially hydrolase-catalysed transesterification of oils and fats has been in focus due to its mild reaction conditions. Equilibrium issues, however, still challenge the practicability of this approach. Decarboxylation of fatty acids into the corresponding C1-shortened alkanes may be an interesting alternative to the (trans)esterification strategy (see Scheme 1).



Scheme 1. Enzymatic transesterification (upper) and decarboxylation (lower) reactions for synthesis of biofuels.

On the one hand, the specific heat of combustion of alkanes is somewhat (ca. 9%) higher than that of corresponding FAMEs.[1] On the other hand, the irreversible decarboxylation of fatty acids should lead to simpler reaction schemes as compared to the reversible (trans)esterification procedure where issues such as water content (leading to saponification and catalyst inactivation) and equilibrium (generally significant molar surpluses of methanol or ethanol are required to achieve near-full conversion) arise.

Established chemical methods for the decarboxylation of fatty acids require rather harsh reaction conditions and rare-metal catalysts, which will challenge the overall eco-efficiency of the proposed alkane synthesis.[2] Recent advancements in photochemical decarboxylation using Pd-doped TiO_2 operating under much milder reaction conditions.[3] However, one common issue of all classical chemical processes for fatty acid decarboxylation is their rather poor selectivity yielding complex product mixtures due to Kolbe- and Hofer-Moest-type side reactions.

Enzymatic counterparts so far are limited to the oxidative decarboxylation of fatty acids to terminal alkenes.[4] or to activated carboxylic acids such as malonic acids[5] or aromatic carboxylates.[6]

Very recently Beisson and coworkers reported on an algal fatty acid photodecarboxylase from *Chlorella variabilis* NC64A (*Cv*FAP).[7] This flavoenzyme catalyses the light-dependent decarboxylation of some long-chain fatty acids to the corresponding C1-shortened alkanes. *Cv*FAP requires photoactivation by blue light (450 nm) indicating that only the photoexcited FAD in the enzyme's active site is capable of the reaction. Examples of photoenzymes are quite rare: next to *Cv*FAP, only flavin-dependent DNA-repair enzymes[8] and protochlorophyllide oxidoreductases,[9] are known today.

We therefore set out to explore the preparative potential of *Cv*FAP for the production of alkanes from biobased fatty acids and triglycerides.

Two variants of *Cv*FAP were recombinantly produced in *Escherichia coli*: one variant comprises the complete sequence of *Cv*FAP (residues 1-654, full-length, Figure S1), while the second variant lacks a predicted chloroplast targeting sequence and comprises residues 62-654 of *Cv*FAP (short-length, Figure S1). After production in *Escherichia coli*, the crude extract as well as the purified enzyme were used for catalytic experiments. While short-length *Cv*FAP showed a good overproduction in *E. coli* and was purified in one step to a purity of about 40%, full-length *Cv*FAP showed almost no overproduction in *E. coli* and there was no clear band on SDS-PAGE after purification (Figure S2). Though both enzyme preparations showed significant decarboxylation activity (Figures S5 and S6) we continued our investigations using the short-length *Cv*FAP. Interestingly, crude enzyme preparations exhibited a higher activity and robustness of *Cv*FAP as compared to purified preparations. The activity of the purified enzyme was increased by a factor of 2 when reconstituted with filtered *E. coli* cell extract. Pre-incubating the enzyme in blue light causes a loss of activity, which is less pronounced when pre-incubating the enzyme in filtered E. coli cell extract (Table S1). At present time we no satisfying explanation for stabilization effect and further experiments will be necessary to shed more light on this. For all further experiments crude cell free extracts were used. It is worth mentioning that no background activity was found in the crude extracts prepared from *E. coli* cells containing an empty vector. Furthermore, performing experiments either in the absence of blue light or in the absence of *Cv*FAP (or using thermally inactivated cell extract) gave no determinable conversion of e.g.

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palmitic acid. Performing the photoenzymatic reaction under different atmospheric conditions (air, Ar, N_2 or H_2) gave essentially the same result (i.e. full conversion of palmitic acid within 3h, Table S2). To increase the solubility of the hydrophobic fatty acids such as palmitic acid, DMSO was applied as a cosolvent. *Cv*FAP tolerates up to 50% (v/v) DMSO (Figure S7). In further studies, the reaction mixture contained 30% (v/v) of DMSO.

Under these conditions, we were pleased to observe a smooth conversion of palmitic acid into pentadecane in the presence of *Cv*FAP and blue light (Figure 1). The reaction was strictly light-dependent.

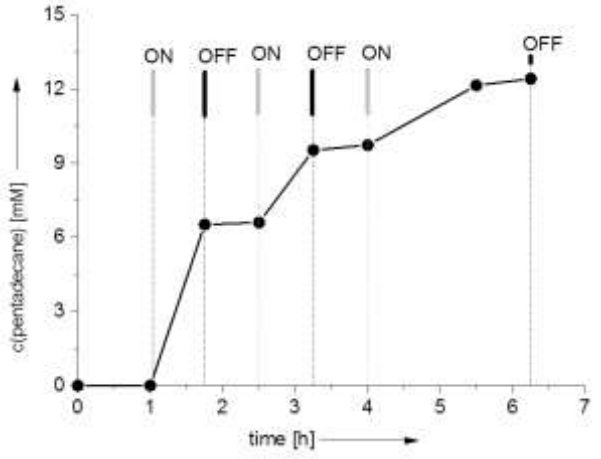


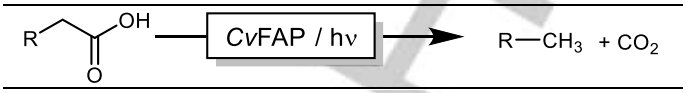
Figure 1. Photoenzymatic decarboxylation of palmitic acid into pentadecane. Conditions: [*Cv*FAP] = 6.0 μM, [palmitic acid] = 13 mM, Tris-HCl (pH 8.5, 100 mM), pH 8.5, 30 % DMSO, blue light illumination.

Further characterisation experiments showed that the rate of pentadecane production depended on the enzyme concentration, the intensity of the light source and, to some minor extent, the reaction temperature (Figures S5, S8 and S9).

Next, we explored the scope of the photoenzymatic decarboxylation reaction somewhat further (Table 1). Generally speaking, a broad range of different fatty acids was converted. For some of them full conversion was observed also resulting in quite favourable turnover numbers (TON, i.e. concentration of product divided by concentration of the biocatalyst) for the enzyme.

In accordance with previous observations[7a] *Cv*FAP showed the highest activity with long-chain fatty acids (C>14). Interestingly, the conversions of oleic acid and linoleic acid were significantly lower than of the fully saturated counterpart (stearic acid). We therefore investigated the relative activity of *Cv*FAP towards some isomers of oleic acid (*cis*/*trans*- and regioisomers, Table S3) and docked these isomers into the crystal structure of *Cv*FAP (PDB: 5NCC, Figures S14-17).[7a] A good correlation between the distance of the substrate carboxylate group to the flavin cofactor and the initial rate of the decarboxylation rate was found (Table S3). Hence, the differences in conversion and rate observed for the different substrates may be assigned to differences of the substrate binding in *Cv*FAP's access channel and positioning of the reactive group towards the cofactor.

Table 1. Substrate scope of the photoenzymatic decarboxylation reaction.



Substrate	[Product] [mM]	Conversion [%][a]	TON (*Cv*FAP)[b]
$C_{12}H_{24}O_2$ (Lauric acid)	3.0	11	500
$C_{14}H_{28}O_2$ (Myristic acid)	6.9	25	1150
$C_{16}H_{32}O_2$ (Palmitic acid)	27.7	96	4610
$C_{17}H_{34}O_2$ (Margaric acid)	28.7	96	4780
$C_{18}H_{36}O_2$ (Stearic acid)	26.1	92	4350
$C_{18}H_{34}O_2$ (Δ9) (Oleic acid)	17.7	65[c]	2950
$C_{18}H_{32}O_2$ (Δ9, 12) (Linoleic acid)	14.6[c]	49[c]	2600
$C_{20}H_{40}O_2$ (Arachidic acid)	25.7	90	4580

Reaction conditions: [substrate] = 30 mM, [*Cv*FAP] = 6.0 μM, Tris-HCl buffer (pH 8.5, 100 mM) , 30 % DMSO, blue light illumination (intensity = 13.7 μE L^{-1} s^{-1}) for 14 hours. [a] Conversion: $[product]_{final}\times([product]_{final}+[substrate]_{final})^{-1}$; [b] TON: $[product]_{final} \times [CvFAD]^{-1}$. [c] Due to the lack of standard reference, the conversion was calculated by using: conversion = $([substrate]_{initial} - [substrate]_{final}) \times [substrate]_{initial}^{-1}$.

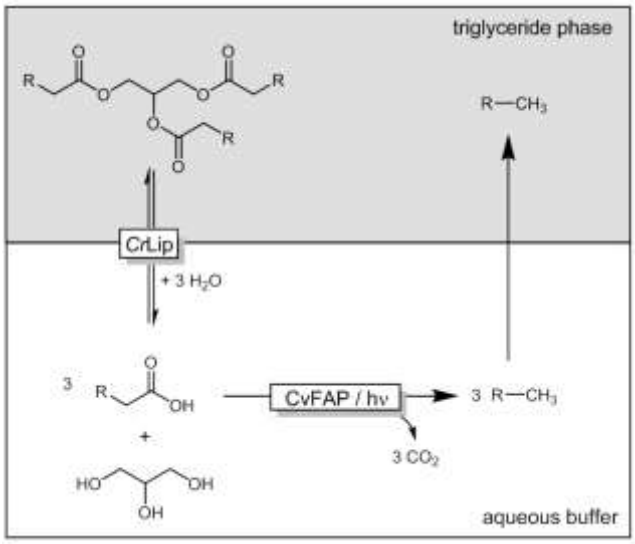
A preparative-scale synthesis was performed under the optimal conditions (see Supporting Information). 155 mg of pentadecane was obtained, giving 79% of conversion (TON of *Cv*FAP 7916) and 61% of isolated yield (Figures S11 and S12).

In view of the envisaged production of alkanes from (waste) oils and fats we also investigated a bi-enzymatic cascade comprising lipase-catalysed hydrolysis of triolein to the free oleic acid and glycerol followed by the *Cv*FAP-catalysed photodecarboxylation reaction (Table 2).

The two-step cascade using homogeneously dissolved triolein (20 mM) gave a satisfying overall yield of more than 80% (Table 2, entry 5) and respectable turnover number for the photodecarboxylase of 8280, which encouraged us to proceed to a cosolvent-free two liquid phase system. A first experiment using 890 U of lipase gave a relatively low product yield of 16 mM, which was attributed to a drop of pH in the aqueous phase to 5.2 leading to a decreased *Cv*FAP activity (Table 2, entry 1). Indeed, either lowering the lipase activity or adjusting the pH value after the hydrolysis reaction lead to significantly increased product formation (Table 2, entries 2&3). Performing both steps simultaneously (one-step) (Table 2, entry 4) was shown to be practical though the product yield and consequently the TON of

*Cv*FAP were rather modest. Again, a pH drop in the aqueous phase was observed. Optimisation of the relative activities of lipase and photodecarboxylase will circumvent this limitation and reveal the full potential of the proposed photobiocatalytic synthesis of alkanes from triglycerides.

Table 2. Photobiocatalytic cascade for the transformation of triolein into (*Z*)-heptadec-8-ene.



Entry	conditions	[*Cr*Lip] [U ml^{-1}]	[Product] [mM]	TON (*Cv*FAP)
1	two-step, pH 7.5	890	16.4	1366
2	two-step, pH 7.5	89	27.7	2308
3	two-step, adjustment of pH after step 1	890	34.5	2875
4	one-step, pH 7.5	890	19.8	1650
5	homogeneous, two-step, pH7.5	2500	49.7 (83% conversion)	8280

General conditions two-step procedure: triolein : Tris-HCl buffer (pH 8.5,100 mM) phase ratio = 1:1 (v/v); *Cr*Lip: Lipase from *Candida rugosa*; T= 37 °C; step 1: reaction time = 12h; followed by step 2: [*Cv*FAP] = 6 µM; irradiation with blue light (450 nm; intensity = 13.7 µE L^{-1} s^{-1}); reaction time = 20h. TON: $[product]_{final} \times [CvFAP]^{-1}$.

Overall, we have demonstrated the synthetic potential of the novel photodecarboxylase from *Chlorella variabilis* NC64A. We are convinced that further optimisations of the reaction setup and of the biocatalyst(s) will yield a practical approach to valorise non-edible triglycerides and acids into biofuels.

Experimental Section

Cloning of *Cv*FAP

For production of the *Cv*FAP in *E. coli*, two constructs were designed based on a previously reported construct.[7a] The constructs both consist of sequentially a 6x His-tag, thioredoxin (TrxA) tag, tobacco etch virus (TEV) protease cleavage site and the gene coding for *Cv*FAP (GenBank: KY511411.1). The first construct comprises the full-length sequence (residues 1-654) of *Cv*FAP, while the second construct lacks the residues encoding for a predicted chloroplast targeting sequence and thereby comprises residues 62-654 of *Cv*FAP (Fig. S1). The sequence coding for *Cv*FAP was codon optimised for expression in *E. coli*. The construct was synthesised by Baseclear (Leiden, the Netherlands) and cloned into a pET28a vector using *Nde*I and *Hind*III restriction sites. Competent *E. coli* BL21 (DE3) cells (NEB) were transformed with the plasmid for recombinant enzyme production.

Preparation of the cell free extract containing *Cv*FAP (full-length/short-length)

10 mL pre-cultures were inoculated with *E. coli* BL21 (DE3) cells harboring the designed pET28a-His-TrxA-*Cv*FAP plasmid. These cultures were grown overnight in terrific broth (TB) medium, containing 50 µg/mL kanamycin. The pre-cultures were used to inoculated large cultures (500 mL TB + 50 µg/mL kanamycin in 2 L shake flasks). Cells were grown at 37 °C, 180 rpm, until an OD_{600} between 0.7-0.8 was reached. Protein production was induced by the addition of 0.5 mM IPTG and the cells were left at 17 °C, 180 rpm, for about 20 hours. Cells were harvested by centrifugation (11000 g at 4 °C for 10 min), washed with Tris-HCl buffer (50 mM, pH 8, containing 100 mM NaCl) and centrifuged again. The cell pellet was resuspended in the same buffer, and 1 mM PMSF was added. Cells were lysed by passing them passed twice through a Multi Shot Cell Disruption System (Constant Systems Ltd, Daventry, UK) at 1.5 kbar, followed by centrifugation of the cell lysate (38000 g at 4 °C for 1 h). After centrifugation, 5% glycerol (w/v) was added to the soluble fraction, the cell extract was aliquoted, frozen in liquid nitrogen and stored at -80 °C.

The total protein content of the cell extract was determined by a BCA Assay (Interchim), using BSA as a standard. *Cv*FAP production was analysed by SDS-PAGE (Figure S2) using a Criterion™ Cell electrophoresis system (Bio-Rad). As a molecular weight marker, Precision Plus Protein Standard (Bio-Rad) was used. The gel was analysed using a gel imaging system (GBox, Syngene, Cambridge, UK) and the amount of *Cv*FAP in the cell extract was estimated from the relative intensity of the bands on gel.

As a control, a cell free extract of *E. coli* BL21 (DE3) cells harboring an empty pET28a vector was prepared according to the same protocol.

Photocatalytic setup

The photoenzymatic decarboxylation reactions using *Cv*FAP were performed at 37 °C in total volume of 1.0 mL Tris-HCl buffer (pH 8.5, 100 mM) containing 30% DMSO as cosolvent. Unless mentioned otherwise, 200 µL of DMSO containing 65.5 mM of palmitic acid, 100 µL of pure DMSO, 500 µL of Tris-HCl buffer (pH 8.5, 100 mM) and 200 µL of *Cv*FAP stock solution (30 µM cell extract in Tris-HCl buffer) were added to a transparent glass vial (total volume 5.0 mL).The vial was sealed and exposed to blue LED light under gentle magnetic stirring. The homemade setup is shown in Fig. S3. The final conditions of this reaction were:

[palmitic acid] = 13.1 mM and [*Cv*FAP] = 6 μM in Tris-HCl pH (8.5, 100 mM), 30 % DMSO. At intervals, aliquots were withdrawn and the substrates and products were extracted with ethyl acetate (containing 5 mM of 1-octanol as internal reference) in a 2:1 ratio (v/v). The remaining organic phase was analysed using Gas Chromatography.

Enzymatic cascade reactions transforming triglycerides into alkanes

Two-step approach: a certain amount of lipase from *Candida rugose* in 500 μL of Tris-HCl buffer (pH 8.5, 100 mM) and 500 μL of triolein as an organic phase were added to a transparent glass vial (total volume 5.0 mL). The hydrolysis of triolein was then performed at 37 °C a thermal shaker (700 rpm). After 12 hours, 200 μL of *Cv*FAP stock solution (30 μM cell extract in Tris-HCl buffer) was added and the mixture was exposed to blue LED light under gentle magnetic stirring at 37 °C. After 20 hours, 10 μL of organic phase was withdrawn and treated with 30 μL of NaOH (12M) at 70 °C for 1.5 h. Subsequently, the mixture was extracted with ethyl acetate (containing 5 mM of 1-octanol as internal reference) and analysed using Gas Chromatography.

One-step approach: using the same reaction condition as these described in two-step approach, except that after the addition of all reaction components into the reaction vial, the mixture was illuminated by blue LED light under gentle magnetic stirring at 37 °C.

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Keywords: biocatalysis • biofuels • decarboxylation • fatty acids • photocatalysis •

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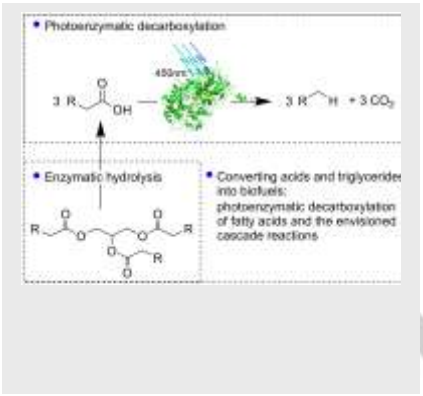

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