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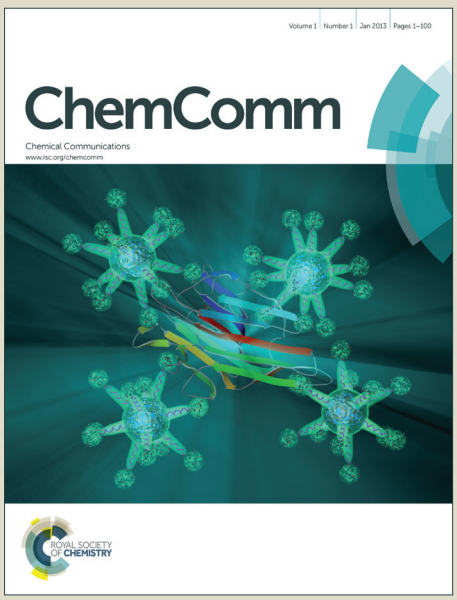

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COMMUNICATION

Photobiocatalytic decarboxylation for olefin synthesis

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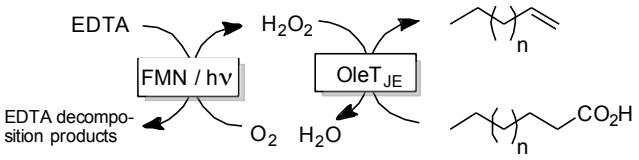
Here, we describe the combination of OleT$_{JE}$ with a light-driven in situ H_2O_2-generation system for the selective and quantitative conversion of fatty acids into terminal alkenes. The photobiocatalytic system shows clear advantages regarding enzyme activity and yield, resulting in a simple and efficient system for fatty acid decarboxylation.

The development of sustainable energy and material production processes has been widely acknowledged as a key challenge for the 21st century. The dwindling of fossil resources and the increasing emission of greenhouse gases urge mankind to find new possibilities for the production of fuel and platform chemicals. Amongst them fatty acids are the most important renewable raw materials available for the chemical industry.[1] The number of chemical transformations starting from fatty acids, however, still is rather limited. Well-known and established are (trans)esterification reactions leading to esters with applications ranging from fuel to personal care products.[2] Beyond this, only a few other transformations such as reduction of the carboxylate group[3] or oxidative cleavage of C=C double bonds of poly-unsaturated fatty acids have been reported.[4]

Apparently, there is a need for more synthetic transformations to turn fatty acids into building blocks for organic synthesis. For example, terminal alkenes, being valuable building blocks for polymers[5] and performance additives[6] have been obtained from carboxylic acids by means of transition metal catalyzed decarbonylative dehydration of fatty acids.[7] However, these methods mostly require precious transition metal catalysts, stoichiometric activation of the fatty acid as anhydride and relatively harsh reaction conditions. The formation of internal olefins can only partly be suppressed and often limits the yield. For olefin synthesis, enzyme catalysts may be a viable alternative to the aforementioned transition metal catalysts also circumventing their limitations.[8] Therefore, we were inspired by a recent contribution by Schirmer and co-workers who reported on a new P450 fatty aciddecarboxylase (OleT$_{JE}$) from *Jeotgalicoccus* sp. ATCC 8456 catalyzing the oxidative decarboxylation of a range of natural fatty acids into the corresponding, chain-shortened terminal alkenes.[9a] OleT$_{JE}$ is thus a promising catalyst for the preparation of terminal olefins with uneven chain length. The enzymatic decarboxylation is highly selective and does not yield internal alkenes.

OleT$_{JE}$ has high structural similarity to CYP450 hydroxylases from the CYP152A2 clan.[9b] A fusion variant of OleT$_{JE}$ with a reductase domain from *Rhodococcus* directly accepts NADPH as electron donor.[9c] This 'traditional' approach however bears some intrinsic disadvantages for the practical application of OleT$_{JE}$ (Scheme 1). First, the overall sequence depends on the costly and instable nicotinamide cofactor ($NADP^+$) that requires an enzymatic regeneration system for its use in catalytic amounts. Second, the electrons from NADPH (or the sacrificial electron donor such as glucose) are delivered to OleT$_{JE}$ via a rather complicated electron transport chain comprising yet another reductase and an electron mediator such as ferredoxin. Next to making the overall reaction scheme more complicated, such electron transport chains are also very vulnerable to the reaction with molecular oxygen in futile cycles. As a result, not only precious reducing equivalents are wasted but also harmful reactive oxygen species are formed.[10] Interestingly enough, OleT$_{JE}$ can also utilize the hydrogen peroxide shunt pathway to form the catalytically active hydroperoxo iron species directly from H_2O_2.[11]

Overall, we envisioned a reaction system comprising recombinantly expressed OleT$_{JE}$ with H_2O_2 to mediate the oxidative decarboxylation of natural fatty acids with high selectivity and under mild reaction conditions as a straightforward, sustainable reaction for the synthesis of terminal alkenes (Scheme 1).



Scheme 1. Photocatalytic *in situ* generation of H_2O_2 to promote OleT$_{JE}$ catalyzed oxidative decarboxylation of fatty acids (n=9-15). Flavin mononucleotide (FMN) serves as photocatalyst, which in its excited state (λ=450 nm) oxidizes the sacrificial electron donor (e.g. ethylenediaminetetraacetate (EDTA); the resulting reduced FMN is reoxidized by O_2 yielding H_2O_2.

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First, we constructed an *Escherichia coli* expression system for $OleT_{JE}$. The gene of the fatty acid decarboxylase $OleT_{JE}$ was ordered as synthetic gene (Life Technologies, Germany) and subcloned with an N-terminal His-tag into the vector pASK-IBA37plus.To avoid non-desired degradation of fatty acids by the expression strain, we used *E. coli* JW5020 from the Keio Collection with inactivated acyl-coenzyme A dehydrogenase FadE of the fatty acid *β*-oxidation complex. Simple shake-flask expression of the recombinant enzyme in the presence of 0.5 mM δ-aminolevulinic acid resulted in an $OleT_{JE}$ yield of 7.5 mg L^{-1} of the fermentation broth. For further studies we used either the crude cell extract or the His-tag purified $OleT_{JE}$ (His-tag purified, approx. 90 % pure). The cell-extract was filtered using centricons with a 15 kDa in order to remove small molecules that might interfere with hydrogen peroxide formation and electron transfer.

Assays for the light-driven biocatalysis were carried out in clear glass tubes. To obtain a constant temperature tubes were placed in a water bath. A clear glass light bulb (120 W) was used as continuous irradiation source with a 15 cm distance to the reaction tubes. Samples were acidified with hydrochloric acid (1 M), extracted twice with ethyl acetate and finally derivatized with an equal volume of N-Methyl-N-(trimethylsilyl) trifluoroacetamide. Formation of olefins and *β*-hydroxy fatty acids was monitored by gas chromatography with MS and FID detection.[9a] To rule out any olefin formation during the derivatization, *β*-hydroxystearic acid (Abcam, Cambridge, UK) was re-extracted from an aqueous solution (0.5 mM) and derivatized and injected to GC/MS. No olefin formation was observed.

Using crude cell extracts we observed the conversion of stearic acid (1 mM) in the presence of stoichiometric amounts of H_2O_2 into heptadec-1-ene after 2 h reaction time. However, the conversion (5%, Figure 1,■) was disappointingly low. Repeating the experiment with two- or twenty-fold molar excesses of H_2O_2 did not lead to significant improvements of the result. To rule out degradation of hydrogen peroxide by endogenous catalases, we performed the reaction using a solution of purified $OleT_{JE}$ and 1 mM hydrogen peroxide. However, total conversion remained below 4.6 % after 2 h. Possibly, the known limited stability of P450 enzymes towards high concentrations of hydrogen peroxide may account for this.[12] Indeed, stepwise addition of H_2O_2 resulted in slightly better conversion of 8 % after 2 h using crude extract (Figure 1,▲) and 8.6 % using purified enzyme. Continuous addition of hydrogen peroxide requires advanced technical equipment and leads to a dilution of the reaction mixture. Searching for an easy and robust method, we reasoned that continuous *in situ* generation of H_2O_2 may be the method of choice to obtain higher titers of terminal olefins. For this we chose the recently established photochemical system for flavin-mediated reduction of O_2 (Scheme 1).[13]

Indeed, this procedure resulted in more than 70% conversion of the stearic acid starting material after 2 h (Figure 1,◆). Using 10 mg stearic acid, a conversion of 95% was achieved. 1-Heptadecene was identified by ^{1}H and ^{13}C NMR. His-tag purified $OleT_{JE}$ (100 µg mL^{-1}) catalyzed the light-driven reaction with high conversion (80 %) within 2 h. As expected, we found a good correlation between the conversion and the amount of purified enzyme (compare Figure S1 in the supporting information), suggesting that the enzymatic decarboxylation / hydroxylation is overall rate-limiting.As an aside it is worth mentioning here that performing the experiments in the darkness, as well as in absence of either $OleT_{JE}$ or FMN, did not result in any significant product formation. $OleT_{JE}$ catalyzes the oxidative decarboxylation of fatty acids to the corresponding (chain-shortened) terminal alkenes. A known side-product of this reaction is the corresponding *β*-hydroxy acid, which had been observed recently by Schirmer *et al.*[9a] In case of stearic acid, the ratio between *β*-hydroxy acid and 1-alkene was approximately 1:3, which was also observed throughout the experiments reported here. Other side-products (such as internal alkenes etc.) were not observed, which is a clear advantage of the enzymatic reaction. Interestingly, the ratio of hydroxy acid to 1-alkene strongly depended on the starting material used (*vide infra*).The influence of varying FMN (photocatalyst) concentrations on the rate of the photoenzymatic decarboxylation of stearic acid is shown in Figure 2. A positive correlation between [FMN] and the overall reaction rate was observed, which we interpret with the increasing H_2O_2 formation rate with increasing photocatalyst con-

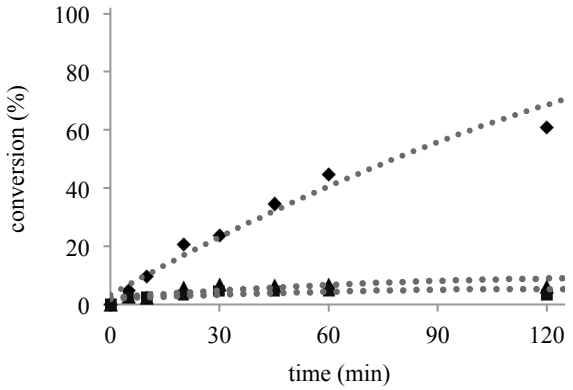

Figure 1 Comparison of photobiocatalytic and H_2O_2-driven $OleT_{JE}$ catalyzed conversion of stearic acid using crude extract. General conditions: buffer: Tris-HCl (50 mM Tris, 200 mM NaCl, pH 7.5), T=25 °C, $OleT_{JE}$ (200 µM) crude cell extracts, stearic acid (0.5 mM) H_2O_2 (■): 1 mM; (▲): 1 mM with dosage of 1 mM H_2O_2 every 10 minutes; (◆):FMN (50 µM), EDTA (50 mM), illumination with white light.

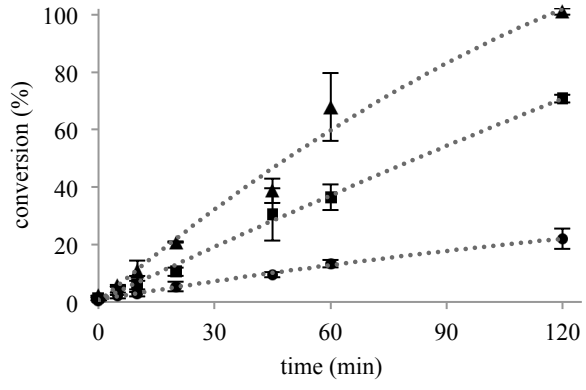

Figure 2 Dependence of the product formation rate of the photoenzymatic olefin production system on the concentration of photocatalyst (FMN) applied. General conditions: buffer: Tris-HCl (50 mM Tris, 200 mM NaCl, pH 7.5), T=25 °C, $OleT_{JE}$(200 µM) crude cell extracts, stearic acid (0.5 mM), EDTA (50 mM), illumination with white light. FMN (●): 2.5 µM; (■): 5 µM;(▲):10 µM.

centration. Above a FMN concentration of 10 µM, however, only marginal increases were observed, which we attribute to O_2 diffusion into the reaction medium becoming overall rate limiting.[12b] In previous studies total turnover numbers of at least 10,000 were determined.[13c] Therefore, at least at the present stage, photodegradation of the photocatalysts does not appear to be a major limitation. It is also worth mentioning here that the FMN degradation product (lumiflavin) also contains a chromophore.[14]

Finally, we also investigated the substrate spectrum of $OleT_{JE}$ in the photoenzymatic approach. As shown in Table 1, next to stearic acid, also a range of other long-chain carboxylic acids were converted. Interestingly, the ratio of decarboxylation and hydroxylation increased with growing chain lengths. The recent elucidation of the crystal structure of $OleT_{JE}$ and mutagenesis studies[9b] indicate that the exact accommodation of the carboxylic group towards the heme cofactor decides on the outcome of the reaction. Long-chain fatty acids interact with hydrophobic amino acid residues in the tunnel-shaped entrance of the active site. It is easy to imagine that shorter chain lengths modify this interaction, which in turn also alters the exact positioning of the carboxylic group close to the heme-cofactor.It would be very desirable to extend the substrate scope of the reported reaction to short-chain organic acids, where the volatile reaction products such as 1-propene can be easily removed from the reaction solution. Interestingly, the $OleT_{JE}$ reductase fusion reported by Liu *et al.* showed a significant preference towards medium chain-length fatty acids,[9b,9c] thus demonstrating the possibility to influence the substrate acceptance of $OleT_{JE}$ by protein engineering. Further works in this direction can be expected soon.

Table 1. Substrate scope of $OleT_{JE}$.

Chain length	conversion (%)	Ratio 1-alkene to β-hydroxy acid
12:0	> 10	no alkene
14:0	> 10	alkene traces
16:0	30	alkene traces
17:0	42	2.3
18:0	99	3.3
18:1Δ9*cis*	0	no product
18:1Δ9*cis* Δ12*cis*	0	no product
18:1Δ9*trans*	31	1.3
19:0	31	6.0
20:0	10	9.4

General conditions: buffer: Tris-HCl (50 mM Tris, 200 mM NaCl, pH 7.5), T=25°C, $OleT_{JE}$(100 µg mL^{-1}), fatty acid (0,5 mM), FMN (10 µM), EDTA (50 mM), 2 h illumination with white light.

Attempts to convert 'natural' fatty acids such as hydrolysed butter (Figure 3) were unsuccessful and the starting material was fully recovered. We suspected that oleic acid present in the complex starting material might inhibit $OleT_{JE}$. In fact, control experiments converting stearic acid in the presence or absence of oleic acid revealed that oleic acid has an inhibitory effect on the $OleT_{JE}$ catalysed oxidative decarboxylation of stearic acid (full and no conversion of stearic acid in the absence or presence of oleic acid, respectively under otherwise identical conditions).To avoid this, hydrolysed hardened palm oil was used yielding appreciable conversion (Figure 3). Hence, we are confident that also complex natural fatty acid mixtures may represent a viable feedstock for OleT-catalysed reactions. The inhibitory effect of oleic acid on $OleT_{JE,}$ may be ascribed to *cis*-configuration of the double bound as the corresponding trans isomer (elaidic acid) was converted. The access tunnel of $OleT_{JE}$ appears to favor zig-zag shaped alkyl chains (as in saturated- and *trans*-alkenes). Oleic acid may bind to $OleT_{JE}$ but may not be ideally positioned towards the heme moiety for conversion.

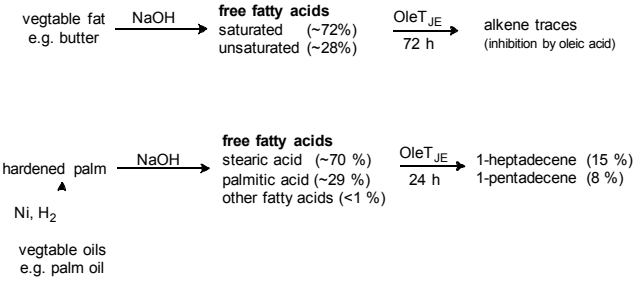

Figure 3. Conversion of natural derived fatty acids using the photoenzymatic olefin production system showed only barely conversion in the presence of unsaturated fatty acids. In contrast, using hardened palm oil exclusively consisting of saturated fatty acids yielded in the production of 1-heptadecene and 1-pentadecene. General conditions: buffer: Tris-HCl (50 mM Tris, 200 mM NaCl, pH 7.5), T=25 °C, $OleT_{JE}$ (100 µg mL^{-1}), fatty acid mixture (~ 1 mM), EDTA (50 mM), FMN (10 µM), illumination with white light.

In conclusion, we have demonstrated that $OleT_{JE}$ may well represent an interesting starting point for the conversion of natural fatty acids into terminal alkenes as building blocks for the chemical industry. Also the proposed *in situ* H_2O_2-generation method was demonstrated to be a facile solution to balance the opposing demands of H_2O_2 by heme-dependent enzymes. The results presented here are still rather preliminary and further optimization will be necessary to achieve a truly practical system for the clean and benign conversion of fatty acids into terminal alkenes. However, already at this early stage of development the proposed photoenzymatic method exhibits some promising features making it a viable alternative to the existing chemical methods (Table 2). Particularly, we believe that the Pd-free nature of our system will result in significant cost-advantages of an optimized photobiocatalytic system. Especially the cost-structure of the biocatalysts' production (particularly, if produced at scale) will make render the biocatalytic alternative more attractive than the ones depending on (scarce and putatively ever more expensive) precious metal catalysts. As 100 eq. of EDTA as used in the present study will neither be economically nor ecologically viable, further studies aiming for water as sacrificial electron donor[15] are currently underway in our laboratories.

Table 2. Comparison of the present system with established chemical decarbonylation systems.

Catalyst (mol-%)	Co-Catalyst (mol-%)	Reagent(s) (eq)	Reaction conditions	Product / selectivity	reference
$PdCl_2(PPh_3)_2$ (0.05)	Xantphos (0.06) $(t\text{-}Bu)_4$-biphenol (0.5)	Ac_2O (1.53)	Neat, 132°C, 5 mM Hg	40-80% yield up to 67% 1-alkene	7a
$PdCl_2$ (0.01)	$(PPh_3)_2$ (0.5)	Ac_2O (1-2)	Neat, 230°C	60-75% yield 99% 1-alkene	7c
$PdCl_2$ (3)	PDEPhos (9)	Ac_2O (1-2)	110°C	84% yield 70% 1-alkene	7d
$OleT_{JE}$ (0.4)	FMN (2)	EDTA (100 eq) O_2	Aqueous media, ambient conditions	Up to 99% conversion Up to 79% 1-alkene	This study

Acknowledgements
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Photobiocatalytic decarboxylation for olefin synthesis

The oxidative decarboxylation of fatty acids to terminal alkenes was accomplished with high selectivity by combining a fatty acid decarboxylase $OleT_{JE}$ with the light-catalyzed generation of the cosubstrate hydrogen peroxide, resulting in a simple and effieient system for the light-driven cleavage of C-C bonds.