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# Immobilization and Application of Fatty Acid Photodecarboxylase in Deep Eutectic Solvents

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Since its discovery in 2017, the fatty acid decarboxylase (FAP) photoenzyme has been the focus of extensive research, given its ability to convert fatty acids into alka(e)nes using merely visible blue light. Unfortunately, there are still some drawbacks that limit the applicability of this biocatalyst, such as poor solubility of the substrates in aqueous media, poor photostability, and the impossibility of reusing the catalyst for several cycles. In this work, we demonstrate the use of FAP in non-conventional media as a free enzyme and an immobilized preparation. Namely, its applicability in deep eutectic solvents

(DESs) and a *proof-of-concept* immobilization using a commercial His-tag selective carrier, a thorough study of reaction and immobilization conditions in each case, as well as reusability studies are shown. We observed an almost complete selectivity of the enzyme towards C18 decarboxylation over C16 when used in a DES, with a product analytical yield up to 81% when using whole cells. Furthermore, when applying the immobilized enzyme in DES, we obtained yields >10-fold higher than the ones obtained in aqueous media.

## Introduction

In the last years, photocatalysis has experienced an outstanding growth, emerging as a milder, environmentally friendly alternative to light-independent strategies.<sup>[1]</sup> Therefore, attempts to combine this discipline with another massively studied field, such as biocatalysis, is a logical step that aims to bring together the best of two worlds. These studies have particularly focused on light-driven cofactor regeneration, the use of phototrophic organisms and, to a lesser extent, the application of photoenzymes.<sup>[2]</sup>

Photoenzymes are a rare type of protein, which require a constant flux of photons to catalyze chemical reactions.<sup>[2,3]</sup>

Currently, four types of photoenzymes are known, which are the photosystem,<sup>[4]</sup> photolyases,<sup>[5]</sup> protochlorophyllide-reductases,<sup>[6]</sup> and photodecarboxylases,<sup>[7]</sup> of which only the latter has applications in biocatalysis.<sup>[8]</sup> This enzyme has been discovered in 2017,<sup>[7]</sup> and can be found in the microalgae *Chlorella variabilis* NC64A<sup>[9]</sup> and *Clamydomonas reinhardtii*,<sup>[10]</sup> participating in lipid metabolism.<sup>[11]</sup> It catalyzes the formation of C1-shortened alka(e)nes from fatty acids (FAs, Scheme 1) (hence its name, fatty acid photodecarboxylase (FAP)), through a flavin-dependent radical mechanism.<sup>[12]</sup>

After its discovery, CvFAP has been applied to different biotransformations, such as the resolution of racemic mixtures, or the synthesis of long chain secondary alcohols, amines and esters,<sup>[13]</sup> with significant efforts focused on the synthesis of drop-in fuels.

In comparison to other fatty acid decarboxylases widely studied to produce drop-in biofuels sustainably, probably the biggest advantage of CvFAP is that it is not oxygen dependent. Additionally, it is also redox-neutral, which eliminates the need of a continuous supply of reducing equivalents and electron transfer systems, which tend to be inefficient,<sup>[14]</sup> since the flavin adenine dinucleotide (FAD) cofactor does not need any external electrons for regeneration. To sum up, CvFAP is able to produce strategically relevant alka(e)nes from a renewable source, such as FAs, by generating radicals simply using light. Furthermore, there is potential for its practical application, with total turnover numbers (TONs) up to 9,000 recently reported in literature,

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Scheme 1. CvFAP catalyzed photodecarboxylation of fatty acids.

although TONs must still increase, and catalyst production cost must decrease for this strategy to be widely applied.<sup>[15]</sup>

On the other hand, biocatalysis in non-conventional media is a broadly used strategy to cope with the challenges inherent to the discipline, being the use of deep eutectic solvents (DESs) one of the most attractive possible alternatives, with several interesting examples of its application in recent literature.<sup>[16]</sup>

There is currently a controversy regarding the nature of DESs, with some authors claiming they are a subclass of ionic liquids, and others that they are a different type of solvent.<sup>[17]</sup> DESs are easily prepared by mixing hydrogen bond acceptors (HBAs), such as ammonium salts, and hydrogen bond donors (HBDs), such as polyols. Owing to the formation of intermolecular hydrogen bonds, which leads to a decreased melting point, DESs remain liquid and stable at room temperature. It is easy to tell that the number of possible starting materials is immense, which allows to conveniently tune the properties of the solvent to be useful for a specific application. Additionally, DESs can be made of natural (in which case they are sometimes denominated NADESs), non-toxic, cheap, abundant and sustainable compounds, which might tip the scale towards their use when planning a sustainable synthesis pathway.<sup>[18]</sup> It is important to mention that in a previous report,<sup>[19]</sup> the use of DES as a co-solvent has been applied to photodecarboxylation reactions, with some interesting results. But since the amount of water used was too high, the DES structure was likely disrupted,<sup>[20]</sup> and therefore, that system as a whole cannot be considered a "true" DES. Considering these results, we believed it would be interesting to analyze the behavior of FAP in a system with a lower proportion of water.

Another important aspect to consider when designing a biocatalytic process is the stability and reusability of the catalyst, especially when using an unstable enzyme such as FAP, which is easily inactivated when exposed to light in the absence of substrate. This photoinactivation is attributed to the formation of radicals within the protein.<sup>[21]</sup> Moreover, since the catalyst is in a heterogeneous form after immobilization, its separation from the reaction mixture gets significantly easier.<sup>[22]</sup> In this context, enzyme immobilization tends to be beneficial, although activity usually decreases, and is currently a widely studied topic for photobiocatalytic applications.<sup>[23]</sup> To the best of our knowledge, there are no reports of FAP immobilization on a solid carrier, although there is an interesting recent report of FAP immobilization on *Bacillus subtilis* spores.<sup>[24]</sup>

Enzyme immobilization can be performed using either crude extract preparations or using a purified form; but purification is a particularly tedious process for FAP.<sup>[21a]</sup> Therefore, the use of carriers that can bind the protein selectively from a crude extract (CE) is an attractive alternative. Recently, a hybrid controlled-porosity glass (CPG) carrier coated with a functionalized polymer was developed (EziG®).<sup>[25]</sup> The polymer has chelating groups that bind metals, in this case Fe<sup>3+</sup>, which is selective towards the His-tag present in the enzyme.

In this study, we evaluated for the first time CvFAP immobilization on EziG® and its application in the decarboxylation of fatty acids as a *proof-of-concept*. In parallel, we performed photodecarboxylation reactions in DESs, evaluating

different combinations of HBDs and HBAs, as well as water amounts. We performed a thorough study of immobilization and reaction conditions, evaluated three different carriers and three different DESs, each with different external amount of water added.

## Results and Discussion

### Enzyme immobilization

We observed that reactions using whole cells (WC) performed the best amongst all fractions (Figure S1, Supporting Information), therefore we immobilized them using calcium alginate. This immobilization method was chosen owing to its light-transparency, capacity of working in high solvent concentration, and simplicity of the overall process.<sup>[26]</sup>

The alginate beads were produced according to the protocol detailed in materials and methods section and had a diameter of approximately  $1.60 \pm 0.02$  mm (Figure S4, Supporting Information). After 20 h of reaction, full conversion of both C18 (stearic acid) and C16 (palmitic acid) substrates was observed, but no product could be retrieved. This led us to suspect that the substrate could be adsorbed in the alginate matrix, thus never reaching the cells to be converted. To evaluate this hypothesis, the beads were physically broken and extracted with ethyl acetate (containing 5 mM 1-octanol as an internal standard), but we could not see neither product nor substrate in this way. Considering these results, we decided to focus our efforts on a specific carrier-bound immobilization technique involving the use of His-tag specific carriers, such as EziG®.

The EziG® carriers consist of an inert controlled porosity glass (CPG) core with different organic polymer coatings, or lack thereof, which results in different hydrophobicity and functionality. On the surface of the carrier, Fe (III) cations can bind to the His-tag of heterologous proteins, which allows to perform both the purification and immobilization in a single step, directly from crude extract. The characteristic of the three carriers used in this work are detailed in Table S2 (Supporting Information).

The reaction protocol had to be slightly adapted to work with these carriers, since magnetic stirring was no longer a viable option as it would break the solid support. Therefore, we decided to use a sunflower shaker and accommodate the vials horizontally. An experiment was run to check if both set-ups were comparable, and we could see that the yields were similar.

Following the EziG® immobilization protocol, we firstly looked for the most suitable carrier amongst the three possible options, using a low protein-to-carrier ratio (10%wt. with respect to the target enzyme). Since we previously obtained better yields with C18 substrate, that was used to evaluate and optimize enzyme immobilization. Results of this experiment are summarized in Table 1.

From these results we could conclude that Opal carrier is the least adequate for the immobilization of this enzyme, since the immobilization yields obtained are the lowest for both

**Table 1.** Protein loading, immobilization yield, and reaction results from EziG® (Amber, Coral, and Opal), immobilizing CvFAP from CE and CFE aiming at 10% wt. enzyme-to-carrier ratio. Reaction conditions: 5 mM C18 (stearic acid) substrate in 1 mL reaction media, Tris-HCl buffer (100 mM, pH 8.5), 15% vol. EtOH, 30 mg of beads, 30 °C, 20 h and 40 rpm (sunflower shaker).

Carrier	Protein loading [mg/g <sub>carrier</sub> ]	Immobilization yield (%)	Conversion (%)	Product yield (C17) (%)
Amber (CE) <sup>[a]</sup>	117.8 ± 0.1	84 ± 6	92 ± 4	2.8 ± 0.1
Coral (CE)	119.3 ± 0.1	85 ± 6	80 ± 2	1.2 ± 0.1
Opal (CE)	46.3 ± 0.1	33 ± 6	ND	ND
Amber (CFE) <sup>[b]</sup>	110.7 ± 0.1	77 ± 6	86 ± 1	0.6 ± 0.1
Coral (CFE)	113.3 ± 0.1	85 ± 6	78.1 ± 0.9	0.2 ± 0.1
Opal (CFE)	59.4 ± 0.1	33 ± 6	ND	ND

[a] Crude extract (CE) prepared via sonication before centrifugation. [b] Cell free extract (CFE) prepared from CE after centrifugation to remove cell debris. ND: not determined.

evaluated fractions. For this reason, no further research was conducted using Opal carrier. On the other hand, both Amber and Coral yielded similar results, with Coral apparently binding slightly more protein than Amber. A result also supported by SDS-PAGE analysis.

Despite the slightly higher protein loading for Coral, the product yield is higher for Amber in both CE and CFE preparations. One possible explanation for this observation may be the unselective binding of proteins over the carrier, but SDS-PAGE analysis ruled out this possibility, since the CvFAP band relative intensity in the Coral immobilization supernatant is significantly smaller than that of Amber.

A difference in conversion and product yield was also observed between the CE and CFE preparations, the first one showing the highest conversion and yield. Even though the immobilization process was always performed under red dim light, it might be the case that the enzyme was photo-inactivated to some extent. Furthermore, we expect photo-inactivation to be a bigger challenge when using CFE in comparison to CE, since it is known that cell debris has a stabilizing effect on the enzyme.<sup>[27]</sup> For all the exposed reasons, we decided to move forward using the Amber carrier.

According to the manufacturer, with the evaluated carriers we can expect an active enzyme mass loading between 15–60% wt. To maximize the enzyme loading, immobilization was carried out at three different pH values (7.5, 8.0 and 8.5), using 80% wt. protein-to-carrier ratio to ensure an enzyme surplus. The obtained results are summarized in Table 2.

There is a clear difference between the protein loading obtained at pH 7.5 and the other two pH values; however, both conversion and yield seem to have no dependency on the pH during immobilization. This indicates that the binding at pH 7.5 is the most unspecific. Therefore, we decided to perform further immobilizations at pH 8.0, aiming at the most specific immobi-

lization taking place (based on the yield obtained per immobilized enzyme).

Surprisingly, the protein loading obtained when trying to maximize the protein-to-carrier ratio is significantly lower than that obtained during the carrier screening, which might be due to a more thorough washing of the CPG carriers. Nevertheless, this also indicates that the carriers are saturated at a much lower protein loading than what was expected. Despite this, the obtained product yields improved significantly when the protein loading was optimized.

SDS-PAGE and protein analysis (Figures S6 and S7) showed significant amounts of residual CvFAP in the immobilization supernatants resulting from both experiments (with 10% wt. and 80% wt. protein-to-carrier ratio). Therefore, for the following experiments, a protein-to-carrier ratio of 20% wt. was chosen as a compromise between maximizing the protein loading and the immobilization yields.

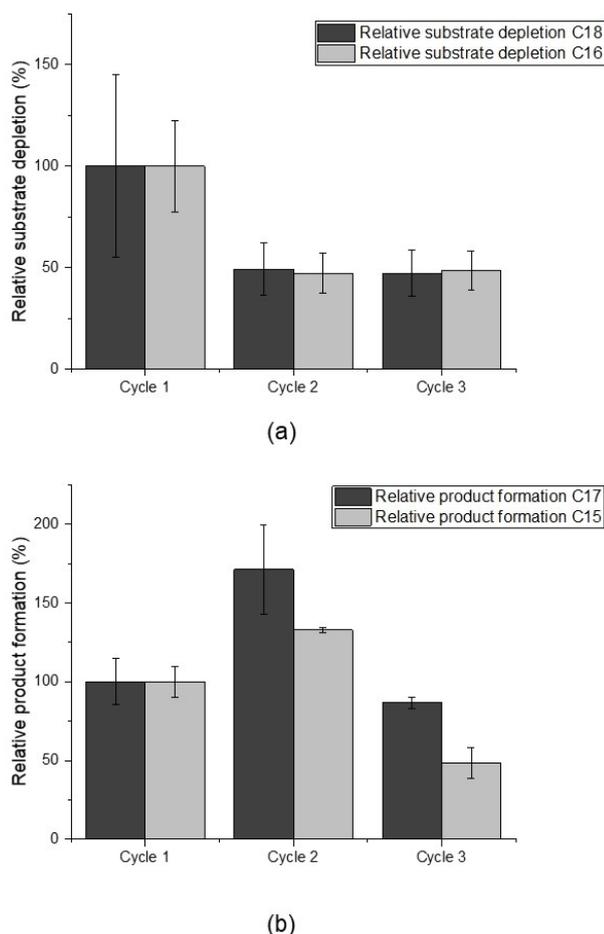
### Reusability studies

When working with CvFAP, its poor photostability is one of the major challenges that limit its application. On the other hand, even though we could see that either whole cells or lysate fractions perform better towards decarboxylation reactions in aqueous media when compared to the immobilized enzyme, unfortunately they cannot be reused. Considering this, we were curious about the stability and reusability of the immobilized enzyme, and therefore we conducted an experiment in which we used the same carriers in three consecutive cycles.

The data presented in Figure 1 shows a decrease in relative substrate depletion after the first cycle, which could be explained by the substrate adsorbed on the carriers' surface. This could also explain the observed differences between

**Table 2.** Protein loading, immobilization yield, and reaction results from EziG® Amber, immobilizing CvFAP from CE. Reaction conditions: 5 mM C18 (stearic acid) substrate in 1 mL reaction media, Tris-HCl (100 mM, pH 7.5 to 8.5), 15% vol. EtOH, 30 mg of carrier, 30 °C, 20 h, and 40 rpm (sunflower shaker).

Carrier	Protein loading [mg/g <sub>carrier</sub> ]	Immobilization yield (%)	Conversion (%)	Product yield (C17) (%)
Amber (pH 7.5)	170.80 ± 0.02	18 ± 2	89 ± 2	4.0 ± 1.0
Amber (pH 8.0)	84.11 ± 0.01	9 ± 1	88 ± 2	4.8 ± 0.6
Amber (pH 8.5)	96.72 ± 0.02	10 ± 2	88 ± 1	4.8 ± 0.5



**Figure 1.** Reuse of immobilized CvFAP for the photodecarboxylation of C16 and C18 fatty acid (FA) substrates over three consecutive reaction cycles in aqueous media. (a) Relative depletion of fatty acid and (b) Relative production of C-shortened alkane products. Each reaction cycle takes 20 hours, experiments are duplicates. Reaction conditions: 5 mM FA (C18 or C16) in 1 mL reaction media, Tris-HCl (100 mM, pH 8.5), 15% vol. EtOH, 30 mg of carriers, 30°C, and 40 rpm (sunflower shaker).

conversion and yield that we consistently obtained in aqueous media. Between cycles the CPG carriers were noticeably sticky and clumping together, even after washing several times, which could mean that the carriers were saturated with substrate after the first cycle and could explain why the depletion of the acid in cycle two and three are almost identical. The analysis of the washing fractions showed that after three consecutive washing steps, the amount of substrate eluted was negligible. Since the mass balance is not yet closed, this supports the hypothesis of substrate adsorption on the carrier.

An increase in relative product formation is observed between cycle one and cycle two, followed by a decrease from cycle two to cycle three. The reason for this rise in product formation is likely interconnected with the aforementioned phenomena, in which in the first cycle the carriers compete with the enzyme for the substrate, in the second, with the carriers already saturated, the enzyme has more substrate available, and finally in the third cycle inactivation of the enzyme makes the activity decrease.

Protein quantification by Bradford assay, and SDS-PAGE analysis of the reaction supernatants and the washing supernatants show no enzyme leakage between reaction cycles.

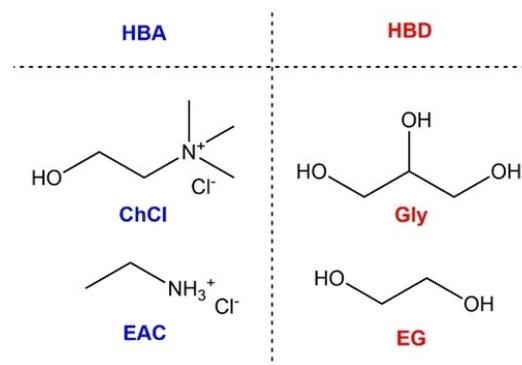
Even though immobilization of CvFAP is challenging, and the obtained yields are much lower than when using whole cells (4.8% in the first cycle), it is interesting to note that in previous literature, total enzyme inactivation is reported after as little as 4 h for reactions that used free catalyst.<sup>[15a]</sup> In our experiment, the catalyst was under continuous light exposure for 63 h, and maintained between 52% and 87% relative product formation for the photodecarboxylation of C18 and C16 respectively, as seen in Figure 1(b).

### CvFAP catalysis in deep eutectic solvents (DESs)

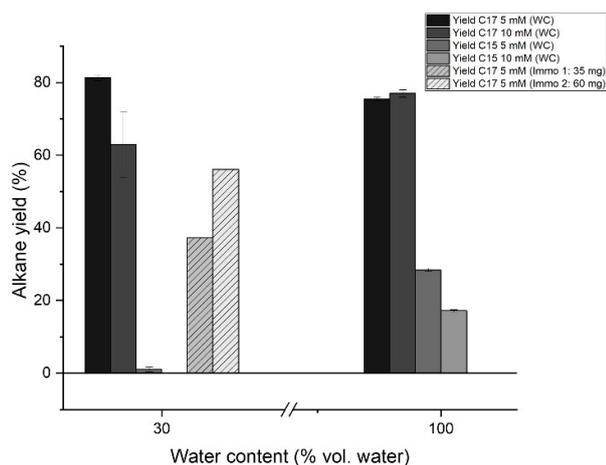
Different DESs were evaluated as reaction media to perform the photodecarboxylation of C18 and C16 catalyzed by CvFAP; three combinations of choline chloride (ChCl) and ethylammonium chloride (EAC) as hydrogen-bond-acceptors (HBAs) with different hydrogen-bond-donors (HBDs) such as glycerol (Gly) and ethylene glycol (EG) (Figure 2). Additionally, since water tends to significantly lower the inherent viscosity, while keeping the nature of the solvent as well as other important properties, such as the capacity to dissolve certain substrates,<sup>[16b,28]</sup> the effect of its addition in the reaction was studied.

As detailed in the introduction, there are several reasons to use DESs as reaction media in biocatalysis, but it seems particularly advantageous in our set-up considering how challenging it is to dissolve fatty acids in an aqueous media.

The DESs composed of ChCl:EG (1:2, mol:mol) and EAC:Gly (1:1.5, mol:mol) showed no product formation neither with 20% vol. nor with 30% vol. water content for C18 nor C16 using concentrations up to 10 mM. But on the other hand, we obtained very promising results using ChCl:Gly (1:2), as seen in Figure 3. There is a very clear dependency of the reaction regarding substrate type (C18 vs. C16), substrate concentration (5 mM vs. 10 mM), and water content (20% vol. vs. 30% vol.). The first mentioned aspect is particularly interesting, since it shows that the catalyst performs differently indeed in DES than



**Figure 2.** Different DES components used in this study as an hydrogen-bond-acceptor (HBA) or as an hydrogen-bond-donors (HBD). ChCl: Choline chloride, EAC: ethylammonium chloride, Gly: glycerol, EG: ethylene glycol.



**Figure 3.** Comparison between the product yields obtained with C18 and C16 substrates with the DES ChCl:Gly (1:2) 30% vol. water and pure water (100% vol. water). Whole cells (WC) or immobilized heterogeneous biocatalyst (Immo) were used for the experiments. Immo 1 corresponds to the results obtained using 35 mg of immobilized FAP on Amber carrier, whereas Immo 2 corresponds to that of 60 mg. Reaction conditions described in experimental section. Experiments done in duplicates unless otherwise stated.

in aqueous media, with a notable substrate specificity towards C18. This effect is also observed when both FAs are present at the same time in the reaction media. Furthermore, the amount of product obtained was even slightly higher in ChCl:Gly (1:2) 30% vol. water than in the reactions in aqueous media (81% yield vs. 75%), which could be due to the higher solubility of substrates in the DES (Table S3, Supporting Information).

When using ChCl:Gly (1:2) 20% vol. water, we could detect the formation of C17 only when using C18 5 mM as a substrate, but the obtained yield was not significantly different from zero. No product formation was detected at higher concentrations of C18, nor with C16 as substrate.

The best results were obtained when using 30% vol. of water, which has a better mass transfer than the media with 20% vol. water, likely due to the lowered viscosity at higher water amounts. The significant difference in reactivity across different DES compositions could also be explained by the beneficial effect that glycerol tends to have towards enzyme stability.<sup>[16e]</sup>

Following these promising results, we evaluated the behavior of the immobilized FAP (Amber carrier) in ChCl:Gly (1:2). We utilized the optimal immobilization conditions described previously to obtain the heterogeneous biocatalyst, and used them for the photodecarboxylation of C18 substrate in ChCl:Gly (1:2) with 30% vol. water. To our satisfaction, we observed an acceptable yield (37%) when performing the reaction, as seen in Figure 3 (Immo 1, 35 mg carrier). Encouraged by this, we decided to increase the number of carrier beads, and the yield increased up to 56% (Immo 2, 60 mg carriers).

Following these results, we decided to evaluate the photostability of the immobilized enzyme by pre-incubating the beads under blue light for 60 min. and 120 min. Unfortunately, the immobilized enzyme preparations were inactive in both

cases. Additionally, we performed reusability studies of the immobilized enzyme in ChCl:Gly (1:2) DES, but in this case we could not observe product formation after the first cycle.

## Conclusion

It has never been clearer that we need to start producing goods and wealth in the most efficient way possible.<sup>[29]</sup> In this context, photo(bio)catalytical processes might be a useful tool to help us achieve the sustainability goals that we so dearly need, and FAP in particular, is a promising catalyst to focus efforts on, given the potential it has for the production of drop-in biofuels.

Regarding fuels, it is sometimes desirable to have a specific type of hydrocarbons in its composition, since this determines their physicochemical properties, but it is not an easy task to decarboxylate similar chain length FAs by traditional means. Additionally, separation of both the fatty acids and alkanes, for example by distillation, tends to be highly energy consuming.

In this work, we have shown that just by changing the solvent, and without performing any mutations on the FAP, it is possible to tune the decarboxylation reaction selectivity towards C18 substrate. Furthermore, this is the first report on the use of FAP in a true DES, and using whole cells we obtained results for the decarboxylation of C18 that are comparable, and even slightly better, than those obtained in aqueous.

Additionally, we have immobilized FAP on a solid commercial carrier for the first time, and proved that this allows to reuse the catalyst, which is an interesting *proof-of-concept* for this methodology. Even though the amount of alkane obtained was significantly lower in aqueous media, we obtained very promising results by performing this reaction in a DES, although in this latter case the immobilized enzyme was unfortunately inactivated upon reuse. In this case, the reaction performed significantly better than in aqueous media using the heterogeneous catalyst.

We believe that further research on immobilization might open new possibilities for the application of this catalyst in different (larger-scale) reactors, in particular those which have proven to be efficient, such as flow systems.<sup>[23a,30]</sup> Also, we expect that improving the light provision to the system will yield good results for the synthesis, and therefore in future works we intend to evaluate different reactor configurations and light sources.<sup>[31]</sup>

## Experimental Section

### Materials

Chemicals, cultivation media components and reagents were purchased from VWR (Søborg, Denmark), Merck (Darmstadt, Germany) and Carl Roth (Karlsruhe, Germany) and used as received. SDS PAGE gels ExpressPlus™ PAGE Gel, were purchased from GenScript (Piscataway, United States). BCA protein quantification kit was obtained from Thermo Fisher Scientific (Schwerte, Germany).

## Protein expression

We used an *E. coli* codon-optimized pET-28a-CvFAP construct containing a truncated CvFAP gene (62–654) with N-terminal fused His-tag and tobacco etch virus (TEV) cleavage site.<sup>[27]</sup> Depending on the case thioredoxin was present or not (Table S1 and Figure S2). The protein expression protocol was adapted from previous literature.<sup>[32]</sup> BL21-Gold (DE3) *E. coli* cells transformed with the plasmid containing CvFAP were grown in TB media under continuous shaking (150 rpm), with a temperature of 37 °C until an OD<sub>600</sub> of 0.6 was reached. Then, they were induced with 0.5 mM IPTG and incubated for 20 h at 17 °C and 80 rpm. Cells were harvested by centrifugation (10 min, 4700 g, 4 °C), immediately resuspended to a theoretical OD<sub>600</sub> of 500 in 0.5 M Tris-HCl buffer (pH 8.5, 5% (v/v) glycerol), yielding a concentration of 50 mg/mL whole cells. Crude extract was prepared from the whole cells, resuspending as mentioned above but to an OD<sub>600</sub> of 200, followed by a subsequent sonication with an ultrasonic homogenizer SONOPLUS HD2200 (BANDELIN electronic GmbH & Co. KG, Berlin, Germany). To obtain the cell free extract (soluble fraction, CFE) and the pellet (insoluble fraction, SP), crude extract was centrifuged at 17,000 g, and 4 °C for 10 min. Protein expression was evaluated by SDS-PAGE analysis. Whole cells and crude extracts were stored in aliquots at –80 °C prior to analysis.

## Enzyme immobilization

Regarding whole cells, a suspension of the cells (OD<sub>600</sub> = 500) was mixed with a 4% w/w alginate solution. This suspension was slowly dripped on a magnetically stirred 10% w/w CaCl<sub>2</sub> solution using a syringe with a narrow needle. Once the beads were formed, they were vacuum filtered, washed and used for reactions. The immobilization procedure for EziG® carriers was adapted from the official EnginZyme AB (Solna, Sweden) protocol. It was performed under red dim light illumination to avoid inactivation (Figure S5). Different fractions were prepared (CE or CFE) and incubated with the respective carrier for 30 minutes using an end-over-end wheel at room temperature. Afterwards, the carriers were separated by centrifugation (1000 × G, 1 minute), and washed twice with immobilization buffer (KP, 20 mM, pH 8, 500 mM NaCl, 30 mM imidazole). Then, the carriers were recovered by vacuum filtration. In order to analyze protein content and loading, SDS-PAGE, digital band intensity analysis and Bradford analysis were performed to all the relevant fractions to calculate total protein concentration, and estimate FAP content.

## DES preparation

Process conditions for the preparation of deep eutectic solvents were implemented from recent literature.<sup>[16b]</sup> Adequate amounts of each component were mixed into a flask with a water bath. The mixture was magnetically stirred at 300 rpm, and 80 °C until a clear liquid appeared, which happened after between 30 minutes and 1 h. When finished, the DESs were stored in 250 mL capped flasks, at room temperature.

## Activity assays and product analysis

Prior to attempting immobilization and reactions in DESs, the system was optimized in aqueous media for the decarboxylation of C18 (stearic acid) and C16 (palmitic acid) using whole cells. While performing these experiments, we noticed that the yield obtained when using C18 as a substrate instead of C16 was 1.4-fold higher. Additionally, all the different fractions shown in Figure S1 were evaluated towards the photodecarboxylation reaction of both fatty

acids, observing that with the different fractions of cell lysates (CE, CFE and SP) a lower product yield was obtained when using C16 as substrate, whereas with C18 the values were similar in all fractions, except for SP, which was 1.6-fold lower. These results might be due to the presence of non-lysed cells, and aggregated CvFAP, since this was less noticeable when performing reactions with construct A, instead of construct B (see Supplementary Information). If our hypothesis was correct, then increasing the number of sonication cycles could decrease the activity of the pellet, and hopefully increase the activity of the rest of the fractions. Then, to investigate this, we increased the number of sonication cycles from six to eight. When comparing the results for the decarboxylation of C18 substrate when performing more cycles, we noticed that indeed the obtained yield when using SP decreased significantly (around twofold), while the one obtained when using CE or CFE increased slightly (1.1-fold). Regardless the number of sonication cycles, the activity of the fractions towards C16 substrate was always extremely low, although it has been reported as an adequate substrate for CvFAP.<sup>[7,21a]</sup> The lower yields obtained when using the lysed cells could be due to photoinactivation of the catalyst. It might happen that outside the cell the active site of the enzyme is 'empty', which leads to the formation of radicals that in turn damage the structure of the enzyme.<sup>[21a]</sup>

Initial assay conditions were adapted from previous literature.<sup>[7,27,32]</sup> In a 4 mL glass vial with lid, we combined Tris-HCl Buffer (500 mM, pH 8.5) with the co-solvent, which was absolute ethanol, the corresponding FA substrate diluted in ethanol and either the whole cells or the lysate to reach a total volume of 1 mL. If the reaction was performed using immobilized enzyme, 100 µL extra Tris-HCl (500 mM, pH 8.5) were added along with the carrier to reach the desired total reaction volume. When performing the reusability studies, the carriers were washed three times in between each cycle using Tris-HCl buffer (100 mM, pH 8.5). In the case of reactions in DES, the protocol was adapted to use the adequate water ratios. The reaction vials were incubated in an in-house built photobioreactor and irradiated with blue LED-lights (62 µmol<sub>photon</sub> · m<sup>-2</sup> · s<sup>-1</sup>) for 20 hours at 30 °C and constant magnetic stirring (300 rpm) (Figure S3). When using the heterogeneous biocatalyst, instead of using magnetic agitation a lab-made device consisting of a sunflower mini-shaker with the photobioreactor on top was used. The reaction mixture was extracted by adding a 1:1 proportion of ethyl acetate (5 mM 1-octanol as internal standard). Then, the samples were vortexed and centrifuged at 13,400 rpm for a minute. After doing this, the organic supernatant is taken with a pipette, dried with anhydrous MgSO<sub>4</sub>, centrifuged again, and the remaining solvent transferred to a GC vial with a low volume insert for its analysis. Which was performed by GC-FID in an Agilent GC 6850 with a Phenomenex GC column ZB – 1MS 30 m × 0.25 mm × 0.25 µm and an inlet temperature of 250 °C, 1:20 split ratio and 1 mL/min helium flow rate. The GC method is detailed in Supporting Information (Table S4).

## Light stability assay

100 µL of WC suspension (OD<sub>600</sub> = 500) and 35 mg of immobilized FAP on Amber carrier were put in 4 mL glass vials and irradiated with blue light for 60 and 120 minutes at 452 PPFD, 30 °C and continuous agitation (40 rpm, sunflower shaker). Afterwards, the rest of the reaction components were added in the same fashion as the other activity assays.

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## Conflict of Interest

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords:** deep eutectic solvents · drop-in biofuels · fatty acid · immobilization · photobiocatalysis

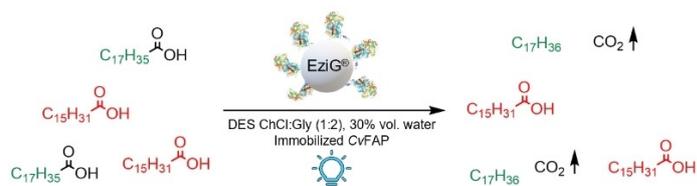
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The recently discovered fatty acid photodecarboxylase shows great promise for the synthesis of drop-in fuels, but several factors limit its applicability, such as poor photostability

and poor solubility of the substrates. In this work we show, for the first time, its immobilization in a solid carrier, and its applicability in deep eutectic solvents.

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**Immobilization and Application of Fatty Acid Photodecarboxylase in Deep Eutectic Solvents**

