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Continuous-flow *Cv*FAP photodecarboxylation of palmitic acid under environmentally friendly conditions

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ABSTRACT

The fatty acid photodecarboxylase from *Chlorella variabilis* NC64A (*Cv*FAP) promotes the elimination of CO_2 from fatty acids (C_n) producing the corresponding hydrocarbon ($_{Cn-1}$). Therefore, this enzyme is of great biotechnological interest since it can be used in alternative biofuel production routes matching the concept of green chemistry. However, due to its recent discovery, this reaction still requires optimizations, which was the focus of the present work together with the application of continuous flow system. The results in batch reactors showed the importance of using high power LED lamps (300 W) to reduce the reaction time for full conversion (30 min, >99%). In another approach, a continuous flow system demonstrated high potential, as it enabled full conversion with a half concentration of enzyme extract in a very short residence time of 15 min. Furthermore, the use of less expensive and sustainable light sources, not previously reported for reactions with *Cv*FAP, were evaluated with full conversion (>99%) after 1 h for continuous flow reactions using 300 W common white LED lamp and based preliminary batch reactions investigations using direct sunlight. Thus, important advances and new perspectives for *Cv*FAP photodecarboxylation reactions could be achieved with the present report.

Introduction

Light induced chemical reactions have been explored since the 18th century, but only recently with the achievements of visible-light photoredoxcatalysis and the growing interest in greener synthetic methodologies it came back to the main stage of synthetic organicchemistry [1,2]. Unfortunately, photo(bio)catalytic reactions are still hampered by the high energy demand of mostly artificial light sources and the poor penetration depth of external illumination into the bulk reaction mixture resulting in slow reactions and non-homogeneous irradiation of the reaction mixture [3].

In recent years much attention has been given to continuous-flow protocols and the advantages of flow chemistry are well documented by several reviews published in recent literature [4,5]. Performing photochemistry reactions in continuous flow environments has been demonstrated as a suitable method to perform photochemical process on larger scales, using the advantage of micro- or meso channels to overcome the penetration depth problem usually observed on batch reactors [6]. Together with the use of continuous-flow reactors, recent advances in LED technology providing very efficient pseudo-monochromatic light sources allowed photo induced reaction to reduce energy losses in non-absorbed light [7] and, more importantly, in heat-transforming photochemistry as a powerful tool for organic chemistry [8,9]. In agreement with the advances mentioned above, the quest for a more sustainable and environmentally friendly chemical processes is still a challenge. In this way the use of visible light photo catalysis is an interesting way to meet green chemistry concepts [10] since it enables the use of sunlight as a renewable light source which in combination with continuous flow reactors could unlock the full power of this technology.

Therefore, we became interested in applying the experience of our group in the development of continuous flow biocatalytic process to a photobiocatalytic fatty acid decarboxylation process catalyzed by *Cv*FAP. To meet (some of) the green chemistry requirements, we decided to use less energy demanding light sources (Blue and White LEDs) and a solar panel to capture solar light and use it as energy source for our

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photodecarboxylation reaction.

Methods

Strain, vector and materials

Escherichia coli DH5α was used for DNA manipulation [7]. Competent *E. coli* BL21 (DE3) cells were transformed with the plasmid containing the *Cv*FAP gene (short length; comprises residues 62-654) [7] for recombinant enzyme production. Kanamycin was purchased from Fluka. Palmitic acid was purchased from Sigma-Aldrich.

Preparation of the enzymatic extract

Enzyme expression was performed as described by Huijbers et al. [7]. After enzyme expression, cells were harvested by centrifugation (11000 g at 4 °C for 10 min), washed twice with washing buffer (50 mM Tris-HCl buffer, pH 8, containing 100 mM NaCl), resuspended in the same buffer with the addition of 1 mM PMSF and 20% glycerol, and stored at -80ºC. The cells were prepared for lysis by centrifugation and resuspension in lysis buffer (100 mM Tris-HCl buffer, pH 8.5, containing 1 mM PMSF and 5% glycerol). Cells lysis were performed by VIBRA-CELL VCX 500 Sonics (USA) instrument using the following settings: 30% amplitude; 15 min sonication; ON/OFF pulses of 10s on and 30 s off. The total extract obtained were frozen with liquid nitrogen and stored in -80 ºC to be used in the photocatalytic reactions. The total protein concentration of the extracts was determined and standardized by the Bradford assay.

Photocatalytic reactions in batch

Photoenzymatic decarboxylation reactions were performed at 37 °C in a total volume of 1.0 mL Tris-HCl buffer (pH 8.5, 100 mM) containing 30 vol% DMSO as cosolvent. To a transparent glass vial (total volume 1.5 mL) were added 0.3 mL DMSO, palmitic acid (13 mM), 0.7 mL Tris-HCl buffer (pH 8.5, 100 mM) containing the cell extract (5.6 mg.mL^{-1} of total protein). The vial was sealed and exposed at 6 cm from LED source (different powers were used: blue - 20, 36, 50, 100, 200 and 300 W white with solar panel – 300 W) under constant and gentle magnetic stirring. At intervals, aliquots were withdrawn and the substrates and products were extracted with twice the volume of ethyl acetate. The remaining organic phase was analyzed by gas chromatography.

Continuous flow photocatalytic reactions with a 300 W blue LED

A mixture with 3 mL of DMSO, palmitic acid (13 mM) and 7 mL of Tris-HCl buffer (pH 8.5, 100 mM) containing the cell extract (2.8 mg mL^{-1} of protein) was pumped (Asia Syringe Pumps) through a 10 mL FEP-coil (1/16) at different flow rates and 37°C. The coil was exposed at 6 cm from 300 W blue LED source to give the product (>99% yield). At intervals, 20 μL aliquots were withdrawn and the substrates and products were extracted with twice the volume of ethyl acetate. The remaining organic phase was analyzed by gas chromatography.

Photocatalytic reactions in flow reactor with a 300 W solar LED lamp

A mixture with 3 mL of DMSO, palmitic acid (13 mM) and 7 mL of Tris-HCl buffer (pH 8.5, 100 mM) containing the cell extract (5.6 mg mL^{-1} of protein) was pumped (Asia Syringe Pumps) through a 10 mL FEP-coil (1/16), at different flow rates and 37°C. The coil was exposed at 6 cm from 300 W Solar LED source to give the product (99% yield). At intervals, 20 μL aliquots were withdrawn and the substrates and products were extracted with twice the volume of ethyl acetate. The remaining organic phase was analyzed by gas chromatography.

Photocatalytic reactions in batch with solar light

The photoenzymatic decarboxylation reactions using solar light irradiation were carried out in an open glass jacketed reactor (total volume 25 mL) at 35 ºC. The reactions were performed in a total volume of 10.0 mL Tris-HCl buffer (pH 8.5, 100 mM) containing 30 vol% DMSO as cosolvent and 13 mM of palmitic acid as substrate. The enzymatic extract were used in a 5.6 mg mL^{-1} final concentration. The reactor was exposed to sunlight at summer season under constant and gentle magnetic stirring (the experiment was conducted on 23.03.2022 starting at 11 am (local time) in Rio de Janeiro (-22.8601423, -43.2297134) for 2 h. Aliquots (200 μL) were withdrawn, and the substrates and products were extracted with 1 mL of ethyl acetate. The remaining organic phase was analyzed by gas chromatography.

Gas chromatography analysis

Samples were prepared by diluting 20 μL of reaction crude in 980 μL of ethyl acetate. Conversion percentages were analyzed by chromatogram areas using the Shimadzu GC2014 GC-FID – Cpsil 5 CB column (50 m × 0.53 mm × 1.0 μm). Injection temperature 260 °C, injection split ratio 20.0, carrier gas was N_2, pressure 89.0 kPa, column flow 4.86 mL min^{-1}. The oven temperature setting was: 110 °C, heated at 25°C min^{-1} to 190 °C for 3 min, and remained heating at 25°C min^{-1} to 280 °C. Conversion percentages were analyzed by chromatogram area based on a calibration curve.

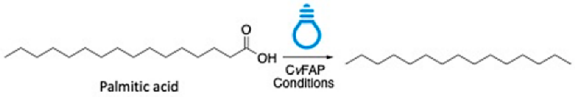
Results

We began our studies evaluating the batch decarboxylation reaction of palmitic acid catalyzed by *Cv*FAP under the irradiation of low power blue LED (20, 36 and 50 W). In this first set of experiments we tried to mimic the conditions already published by other groups [7,8] and evaluate how our reaction set-up would respond in terms of conversions towards the desired product. Reactions were carried out at 37ºC for 1 h (Table 1).

Initial results have shown a very interesting behavior of *Cv*FAP under the conditions used showing the importance of light intensity on the transformation rate in agreement with previously reported results [9]. But our goal was to intensify the decarboxylation reaction to its maximum and therefore decided to move forward to evaluate more powerful blue LED sources. With this objective in mind, we decided to use 100, 200 and 300 W blue LEDs and monitor product conversion during the first hour of reaction (samples taken every 10 min) since initial results with 50 W have shown full conversion after this time. Same reactions conditions were applied and results are presented on Table 2.

Results obtained with 100, 200 and 300 W shown that reductions on

Table 1
Initial evaluation of 20, 36 and 50 W blue LED on batch photodecarboxylation reaction of palmitic acid mediated by *Cv*FAP.



Entry	Blue LED (W)	Conversion (%)
1	20	60
2	36	80
3	50	>99

Reaction conditions: To a transparent glass vial (total volume 1.5 mL) were added 0.3 mL DMSO, palmitic acid (13 mM) and 0.7 mL Tris-HCl buffer (pH 8.5, 100 mM) containing the cell extract (5.6 mg mL^{-1} of total protein), reactions were carried out at 37 ºC for 1 h and analyzed by gas chromatographybased on product formation.

Table 2
Influence of LED power on the batch conversion of palmitic acid photodecarboxylation reaction mediated by C*v*FAP.

Entry	Reaction Time (min)	Conversion (%)		
		100 W LED	200 W LED	300 W LED
1	10	7	42	51
2	20	48	70	84
3	30	73	78	94
4	40	93	95	97
5	50	98	>99	>99
6	60	100	>99	>99

Reaction conditions: To a transparent glass vial (total volume 1.5 mL) were added 0.3 mL DMSO, palmitic acid (13 mM) and 0.7 mL Tris-HCl buffer (pH 8.5, 100 mM) containing the cell extract (5.6 mg mL^{-1} of total protein), reactions were carried out at 37ºC for 1 h and analyzed by gas chromatography.

reaction time are possible by increasing the power of light source, allowing to achieve excellent conversions in just 30 min of reaction using 300 W blue LEDs (Entry 3, Table 2). We have not observed difference between the different light sources as have delivered excellent conversions after 40 min of reaction (Entry 4, Table 2). Expectedly, the initial rate of the transformation correlated with the power of the light source used. With these results in hands we have also performed reaction with decreasing amounts of total protein limiting the reaction time at 1 h without having success in maintaining the excellent conversions previously observed (46% for 2.8 mg mL^{-1} and 1% for 0.56 mg mL^{-1}). The reagent concentration is also an important parameter for process optimization. In order to maximize production of the desired molecule and we have also evaluated different palmitic acid concentration and the effect of its increase on reaction conversion. Results are summarized in Table 3.

The effect of increasing concentrations of palmitic acid have shown that the reaction protocol used is suitable for concentrations of palmitic acid up to 26 mM, with a slightly decrease on reaction conversion (Entry 3, Table 3). Reaction performed with 19.5 mM of palmitic acid (Entry 2, Table 3) led to similar conversion of our standard condition (Entry 1, Table 3). Increasing the palmitic acid concentration to values higher than 26 mM have shown a dramatic decrease on reaction conversion which will have an important effect on reaction productivity. Possibly, the reaction pH was impaired by the relatively high concentration of fatty acids thereby not being optimal for C*v*FAP [10,11] or an increase in turbidity at higher concentrations of the substrate could have an effect on conversions.

As we mentioned during the introduction, eventually sunlight should serve as energy source for photo(bio)catalytic reactions to eliminate fossil-based power sources [12,13]. Therefore, we evaluated using a solar-powered white LED (300 W) light source for the transformation (Table 4).

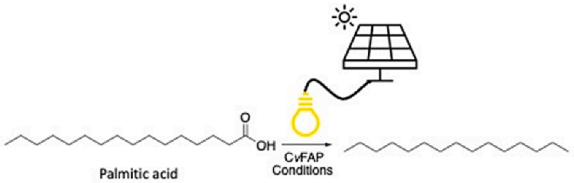
The use of a solar panel linked to a 300 W white LED was able to assist the photodecarboxylation of palmitic acid mediated by C*v*FAP in excellent conversions after a short reaction time (40 min, entry 4, Table 3) a result comparable with the one obtained by using 200 W blue

Table 3
Effect of palmitic acid concentration on batch product conversion at 100 W blue LED irradiation.

Entry	Palmitic acid (mM)	Conversion (%)
1	13	>99
2	19.5	96
3	26	92
4	34.5	32
5	39	16

Reaction conditions: To a transparent glass vial (total volume 1.5 mL) were added 0.3 mL DMSO, palmitic acid (mM) and 0.7 mL Tris-HCl buffer (pH 8.5, 100 mM) containing the cell extract (5.6 mg mL^{-1} of total protein), reactions were carried out at 37 ºC for 1 h and analyzed by gas chromatography.

Table 4
Evaluation of 300 W white LED powered by solar panel on batch conversion of palmitic acid photodecarboxylation reaction mediated by C*v*FAP.



Entry	Time (min)	Conversion (%)
1	10	7
2	20	48
3	30	73
4	40	93
5	50	98
6	60	>99

Reaction conditions: To a transparent glass vial (total volume 1.5 mL) were added 0.3 mL DMSO, palmitic acid (13 mM) and 0.7 mL Tris-HCl buffer (pH 8.5, 100 mM) containing the cell extract (5.6 mg.mL^{-1} of total protein), reactions were carried out at 37 ºC for 1 h and analyzed by gas chromatography.

LED (Entry 4, Table 2) connected to a standard power supply. It is important to note that no difference on conversions was observed between 300 W white LED connected to the solar panel and the one under standard power supply.

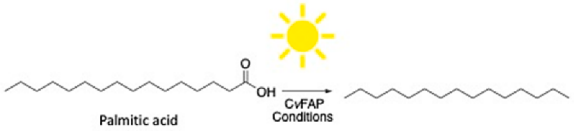
Next, sunlight was used directly to promote the photobiocatalytic decarboxylation reaction (Table 5).

The results obtained by the irradiation of sunlight in our experiment have shown very interesting results leading to almost full conversion towards the desired product in one hour of reaction (Entry 1, Table 5). It is important to note that reaction temperature was kept at 35 ºC using a cooling system in order to mimic the conditions already developed under the use of high-power LEDs. Further investigations are under development to completely disclose the full potential of sunlight irradiation on biocatalyzed photodecarboxylation reactions.

Besides the interesting results achieved under our optimization studies, scale-up remains a challenge using batch-type photodecarboxylation reactions. Therefore, we further investigated the possibility of performing the reaction in flow reactors [14]. The set-up used was simple, reagents were added to a light-protected flask and pumped at different flow rates through a tubular reactor (FEP tubing) in contact with the desired high-power LED (white or blue) and product was collected in the end of the tubing (Table 6).

Results presented on Table 6 show that continuous-flow approach

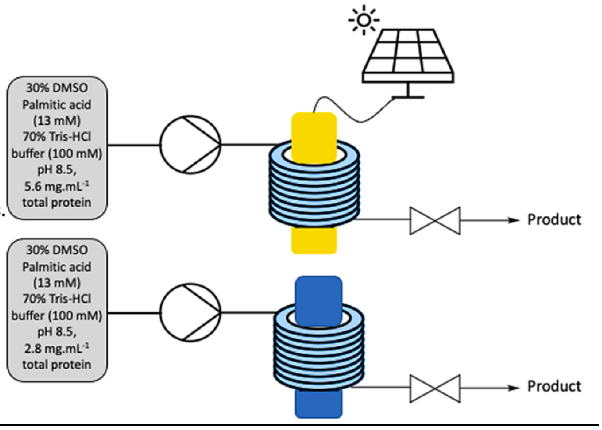
Table 5
Palmitic acid photodecarboxylation batch reaction mediated by C*v*FAP exposed to sunlight.



Entry	Conditions	Conversion (%)
1	Stand. Cond.[a]	96
2	Control exp.[b]	no conv.
3	Stand. Cond. without sunlight	no conv.

Reaction conditions:[a]To a transparent glass vial (total volume 1.5 mL) were added 0.3 mL DMSO, palmitic acid (13 mM) and 0.7 mL Tris-HCl buffer (pH 8.5, 100 mM) containing the cell extract (5.6 mg mL^{-1} of total protein), reactors were connected to a cooling system in order to keep reaction temperature at 35ºC for 1 h and analyzed by gas chromatography,
[c] Same conditions as entry 1 but protected from sunlight irradiation.
[b] same conditions as mentioned before without enzyme

Table 6
Continuous flow approach to photodecarboxylation of palmitic acid under C*v*FAP catalysis.



Entry	Power (W)	Residence Time (min)	Conversion (%)
1	300 (blue)	10	92
2		15	>99
3	300 (white)	15	32
4		60	>99

Reaction conditions: A mixture with 3 mL of DMSO, palmitic acid (13 mM) and 7 mL of Tris-HCl buffer (pH 8.5, 100 mM) containing the cell extract was pumped (Asia Syringe Pumps) through a 10 mL FEP-coil (1/16) at different flow rates and 37 °C. The coil was exposed at 6 cm from light source to give the product reactions were carried out at 37°C for 1 h and analyzed by gas chromatography.

can be an effective way of scaling up photodecarboxylation process catalyzed by C*v*FAP enzyme for both white LED connected to a solar panel and blue LED plugged in conventional power supply, with full conversion achieved under short to moderate residence times. Continuous flow reactor irradiated by 300W blue LED lead to further improvement on reaction time when compared to the batch process with a 50% reduction on reaction time using half the standard concentration of the enzymatic extract, 2.8 mg mL^{-1}. Unfortunately, the same behavior was not observed for the white LED connected to the solar panel where the residence time needed for full conversion was similar to the one obtained in batch mode using the standard concentration of the enzymatic extract. This result can be explained since blue LED promoted a much higher heating then white LED on reaction zone and under batch conditions this effect is not observed because heat transfer limitations. But when working in continuous flow reactors the higher surface-to-volume ratio can use this additional heating of the blue LED to enhance product conversion and allow a further decrease on reaction time.

Conclusions

In this work we were able to apply continuous flow conditions to the photodecarboxylation of C*v*FAP with full conversion in very short residence times (15 min) by using high power blue LED source (300 W). We have also shown that high power white LED (300 W) or even sunlight could be used to promote this reaction with full conversion achieved after 1 h. Our approach has also shown that solar panel connected to the light source can be an alternative for the use of renewable energy to help promoting biocatalysis under the green chemistry principles.

CRediT authorship contribution statement

Luiza A.D. Benincá: Methodology. **Alexandre S. França:** Methodology, Validation, Investigation. **Gabriela C. Brêda:** Methodology, Validation, Formal analysis. **Raquel A.C. Leão:** Methodology, Validation, Writing – review & editing. **Rodrigo V. Almeida:** Supervision, Writing – review & editing. **Frank Hollmann:** Supervision, Resources, Writing – review & editing. **Rodrigo O.M.A. de Souza:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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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.2022.112469.

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