AGRI-WASTE VALORIZATION FOR BIOFUELS THROUGH ENZYMATIC HIDROLYSIS AND PHOTODECARBOXYLATION

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ABSTRACT

Research on hydrocarbon synthesis through biochemical pathways is expanding, emphasizing the significance of the fatty acid photodecarboxylase (FAP) enzyme. This study explores the synthesis of diesel-type hydrocarbons from low-cost raw materials using *Chlorella variabilis* fatty acid photodecarboxylase (CvFAP), optimized through ultraviolet light irradiation and a continuous flow system. Palm oil deodorizer distillate (PODD), a byproduct of palm oil refining, is rich in free fatty acids (FFA), particularly palmitic acid, making it a promising but underexplored substrate for photodecarboxylation reactions. A full conversion (>99%) was achieved in 20 minutes of residence time during the photodecarboxylation step in continuous flow using PODD as the substrate. Two other oleaginous industrial wastes, distillery corn oil (DCO) and palm oil mill effluent (POME), were also evaluated for enzymatic hydrolysis in a continuous flow system, achieving full conversion with a 1:2 oil-to-water ratio and 10% immobilized catalyst after 2 hours of reaction. The hydrolyzed oils present high potential for photodecarboxylation cascade reactions considering the fatty acids composition obtained. These findings highlight the potential of using waste materials in drop-in fuel synthesis via CvFAP photodecarboxylation, paving the way for more sustainable second-generation biofuel production.

Keywords: *Chlorella variabilis* fatty acid photodecarboxylase (CvFAP). Palm fatty acid distillate (PFAD). Distillery corn oil (DCO). Palm oil mill effluent (POME). Hydrocarbons.

1 INTRODUCTION

The energy sector largely depends on fossil fuels, but growing environmental, political, and social concerns are prompting significant investments in the transition to renewable energy sources [1–3]. Among the alternatives to reduce reliance on fossil fuels, biofuels stand out as promising candidates. Notably, biodiesel (comprising methyl or ethyl alkyl esters) and green diesel are key substitutes for fossil diesel. Green diesel's composition is similar to petroleum diesel, which is driving advancements in its production technologies [4–6]. Traditionally, green diesel production requires harsh conditions with high pressure and temperature using metallic catalysts [7]. However, the use of enzymes capable of converting specific substrates into hydrocarbons offers a potential biological pathway that is environmentally safer [7,8]. The fatty acid photodecarboxylase (EC 4.1.1.106), a recently identified enzyme, shows significant potential for hydrocarbon production. This enzyme converts fatty acids (Cn) into their corresponding hydrocarbons (Cn-1) under light irradiation, with optimal performance in violet light (397 nm) [9]. Due to its effectiveness with lipid-based and residual feedstocks, it is gaining increasing attention. Industrial residues and by-products, such as palm oil deodorizer distillate (PODD), are often underutilized despite their valuable compounds. PODD is emerging as a viable low-cost feedstock for biofuel production to replace conventional diesel [10]. Additionally, distillery corn oil (DCO) and palm oil mill effluent (POME), which are by-products from the corn ethanol industry and palm oil refinery respectively, contain triglycerides suitable for processing. This study aims to utilize CvFAP photodecarboxylation to synthesize green diesel-type hydrocarbons from PODD, while also enhancing the enzymatic hydrolysis of DCO and POME to establish a sustainable cascade process in continuous flow systems.

2 MATERIAL & METHODS

CvFAP Expression in *E. coli* and biocatalyst preparation: Fatty acid photodecarboxylase from *Chlorella variabilis* (CvFAP) was expressed in *Escherichia coli* BL21(DE3) using the pET28a-His-TrxA-CvFAP vector. After CvFAP expression, the 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 -80 ºC. The whole-cell CvFAP biocatalyst was adjusted by spectrophotometric analysis (600 nm) to 11 mg.mL^{-1} of dry cell weight in reaction buffer (100 mM Tris-HCl buffer, pH 8,5, 1 mM PMSF and 5 % glycerol).

Photodecarboxylation for Hydrocarbon Production: Using whole-cell CvFAP biocatalyst, photodecarboxylation reactions were conducted under ultraviolet LED light, utilizing palmitic acid and PODD (84% acidity) as substrates. PODD was kindly provided by AgroPalma and presented a fatty acids composition of 43.65% palmitic; 42.82% oleic; 8.49% linoleic; 4.43% stearic; 0.6% myristic. The conversion of fatty acids to hydrocarbons was quantified by GC-MS, according to [11].

Hydrolysis for Fatty Acids Production: Canola oil, DCO and POME were enzymatically hydrolyzed using a commercial lipase (free lipase from *Candida rugosa*, obtained from Sigma-Aldrich). The reaction conditions were 1:2 oil-to-water ratio and 10% biocatalyst at 40°C with preliminary batch reactions using free lipase and the enzyme after immobilization in Amberlite™ XAD7HP and oxalate-based (OA) supports. The immobilization protocol consisted of an overnight incubation of 800 mg crude CRL in 10

mL buffer solution (20 mM pH 6.0 phosphate buffer solution) with 1 g Amberlite™ XAD7HP. In the case of OA, 24 mg of crude CRL was brought in contact with 100 mg of OA using the same buffer and incubation time.

Continuous Flow System Reactions: The photodecarboxylation and hydrolysis reactions were performed in tubular (FEP-coil; 1/16) and packed bed reactors respectively at different flow rates (Figure 1). The photodecarboxylation reactions were performed according to [11] using 20 mM DDOP. The packaged bed was prepared using a 10 mL iron column filled with alternate layers of OA (3.0 g of total weight) and CRL (0.2 g of total weight), resulting in a volume empty of 2.7 mL. In isolated flasks, water and canola oil were pumped (Asia Syringe Pumps) using PTFE-coil (1/16), mixed and passed through the iron column at 0.1mL flow rates and 40 °C to a residence time of 120 min. 100 µL aliquots were withdrawn and the substrates and products were extracted with twice the volume of heptane. The remaining organic phase was analyzed by gas chromatography.

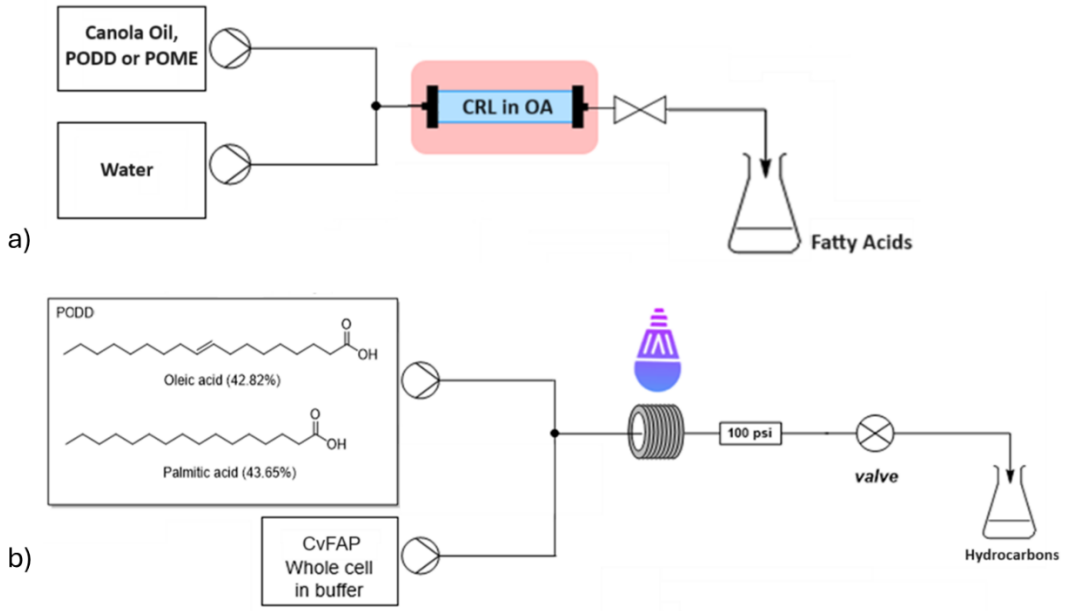

Figure 1: Continuous flow system reactions schemes. a) Hydrolysis of canola oil, PODD and POME to fatty acids using packed-bed reactors with *Candida rugosa* lipase (CRL) immobilized in oxalate-based (OA) support. b) Photodecarboxylation of PODD fatty acids using CvFAP whole cell biocatalyst pumped into tubular reactor under UV light irradiation.

Hydrocarbons and Fatty Acids Analysis: The determination of produced hydrocarbons and fatty acids was carried out by gas chromatography-with flame ionization detection (GC-FID), calculating the conversion rate of substrates to desired products**.** The conversion of substrate into the product was estimated by calculating the percentage area of hydrocarbons about the percentage area of fatty acids (reconversion %) (Equation 1). This approximation was carried out based on the work carried out by [12].

$$area_{\,Conversion\ \%} = \frac{\sum areas\ of\ hydrocarbons}{\sum areas\ of\ hydrocarbons + \sum areas\ of\ fatty\ acids}\ x\ 100\ \%\quad \text{(Equation. 1)}$$

3 RESULTS & DISCUSSION

The conversion of fatty acids to hydrocarbon through CvFAP photodecarboxylation has been reported particularly for palmitic acid as the standard substrate. However, the efficient use of different fatty acids has been also proven, highlighting CvFAP as a versatile enzyme with potential to be used in fatty acids mixtures, such as industrial waste. A hitherto unexplored Agri-waste for CvFAP enzyme, PODD, was tested in 30-minute photodecarboxylation batch reactions (Table1) in comparison to palmitic acid demonstrating to be a potential raw material. The impurities present in the residue did not inhibit the reaction.

Table 1. Conversion of fatty acids into hydrocarbons in photodecarboxylation reactions with *Cv*FAP using 20 mM PODD as substrate.

Substrate	Conversion (%)
Palmitic acid	>99
PODD	>99

Reactions conditions: Transparent glass vials were used with 30% DMSO, palmitic acid (13 mM) or PODD (20 mM) and Tris-HCl buffer (pH 8.5, 100 mM) containing CvFAP whole cell (11 mg.mL^{-1}) at 37ºC. 50W Violet LED was used as light source.

To intensify the reaction PODD photodecarboxylation was then explored using continuous flow system (Figure 1b) using different residence times (Table 2). The results demonstrated that short residence times such as 10 minutes, presented high conversion values of 86%.

Table 2. Conversion of fatty acids into hydrocarbons in photodecarboxylation reactions with CvFAP using 20 mM PODD as substrate.

Residence Time (min)	Conversion (%)
30	>99
20	>99
10	86 ± 1
5	56 ± 7.9

Reactions conditions: A 10 mL mixture of 30% DMSO, PODD (20 mM) and Tris-HCl buffer containing CvFAP whole cells (11 $mg.mL^{-1}$) was pumped (Asia Syringe Pumps) through a 10 mL FEP-coil (1/16) at different flow rates and 37 °C for up to 30 min. 50W Violet LED was used as light source.

Other raw materials containing triglycerides, such as DCO and POME, were explored for potential future use in photodecarboxylation reactions. The hydrolysis step of these residues is crucial for establishing a cascade process. Preliminary batch reactions were conducted using free CRL enzyme to determine the optimal reaction conditions, achieving conversions of 99% and 98% for canola oil and DCO, respectively, after 2 hours of reaction. Subsequently, CRL was immobilized on two different supports (Amberlite™ XAD7HP and OA), with OA showing satisfactory performance over canola oil (Table 3). The enzyme loading for OA was lower than that used for reactions with free lipase and the enzyme immobilized in Amberlite™ XAD7HP.

Table 3. Conversion of canola oil into fatty acids in hydrolysis reactions with immobilized CRL in different supports.

Support	Conversion (%)	Selectivity (%)
Amberlite™ XAD7HP	29 ± 0.6	55 ± 3.2
Oxalate-based	>99	98 ± 0.2

Reactions conditions: The reactions were performed in a glass reactor (total volume 4 mL) using a mixture of 1 mL water and 0.5 mL of residue oil as substrate. The mixture was stirred until the emulsification and then 10% biocatalyst was added and heated at 40 ºC for 2 h. The final mixture was extracted with 3 mL of heptane. The remaining organic phase was derivatized and analyzed by gas chromatography.

Hydrolysis reactions in a continuous flow system (Figure 1a) were performed under the optimal conditions determined from batch reactions for canola oil, DCO, and POME as substrates. The results showed complete conversion (>99%) and high selectivity (98%) for both residues used. This demonstrates the potential for utilizing these hydrolyzed raw materials in photodecarboxylation reactions to stablish an enzymatic cascade process.

4 CONCLUSION

In conclusion, this study demonstrates the feasibility of utilizing fatty acid photodecarboxylase (CvFAP) for converting palm oil deodorizer distillate (PODD) into valuable hydrocarbons, showcasing its potential as a viable and sustainable feedstock for green diesel production. The results from continuous flow systems reveal that short residence times can achieve high conversion rates, while the enzymatic hydrolysis of by-products like distillery corn oil (DCO) and palm oil mill effluent (POME) further supports the development of an efficient cascade process. This approach not only highlights CvFAP's versatility but also paves the way for integrating these important industrial wastes into novel renewable energy solutions.

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ACKNOWLEDGEMENTS

The authors thank to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), UFRJ), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Instituto SENAI de Inovação em Biosintéticos e Fibras (SENAI CETIQT).