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Extraction of recombinant fatty acid photodecarboxylase-*E. coli* and its use for biohydrocarbon synthesis

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Abstract

The use of fossil fuels still becomes a problem of unsustainable and environmental issues, so it is crucial to use renewable energy. Biohydrocarbons as renewable energy, could be generated from biomass that contains fatty acids and produce compounds such as alkanes and alkenes. *Chlorella variabilis* Fatty Acid Photodecarboxylase (*Cv*FAP) enzyme from *E. coli* recombinant is a remarkable recent technique for producing bio-hydrocarbons. According to extensive studies, this enzyme can change free fatty acids when induced by blue light and accompanied by the addition of substrates. This research aims to synthesize bio-hydrocarbons that focus on the enzyme activation process with variations in protein concentration, activation time, and the type of substrate CPO compared with palmitic acid regarding bio-hydrocarbon concentration produced. The research focuses on cloning the synthetic gene of *Cv*FAP in *E. coli* and the *Cv*FAP enzyme produced was used to convert palmitic acid to pentadecane, which was measured using Gas Chromatography. This study has confirmed the potency of producing bio-hydrocarbons in the form of pentadecane with a yield of 16.44%. The synthesis of pentadecane can run optimally using the light activation method with several substantial factors needed in the activation process, namely the optimum growth medium for TB, protein volume of 1777.5 μL, activation time of 3 hours, and a substrate preference of 50% CPO.

[Keywords: biocatalyst, cap, light-driven enzyme, recombinant protein, renewable energy]

Introduction

Fossil-based fuels make up the majority of the energy sources used by the population, particularly by the Indonesians. Unfortunately, the widespread use of these fuels leads to the depletion of fuel supplies and significant emissions of greenhouse gases that are harmful to the environment (Pugazhendhi et al., 2017). To solve those issues, shifting to renewable energy, a bio-hydrocarbon that uses renewable resources, should be a priority. Bio-hydrocarbons are an example of a potentially renewable energy resource that is less exposed and is believed to be an alternative energy for the present and the future. Bio-hydrocarbons illustrate a possibly renewable energy source that is less exposed and is thought to be alternative energy for the present and the future. Shifting towards renewable energy should be prioritized to tackle those problems.

Bio-hydrocarbons are hydrocarbons generated from biomass that contain fatty acids and produce compounds such as alkanes and alkenes. Fatty acids can be obtained from one of Indonesia's largest vegetable oil commodities, palm oil. Through the process of decarboxylation, these fatty acids are converted into alkanes and alkenes. Alkanes and alkenes with various carbon chains are important compounds for the development of biotechnology, especially in the energy sector. It has previously been observed that these compounds are the main components of commonly used fuels, such as gasoline (C_5-C_9), jet fuel (C_5-C_{16}), and diesel (C_{12}-C_{20}) (Bernard et al., 2012; Lee & Suh, 2013).

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Production of bio-hydrocarbons can be categorized into two methods: chemical and biological. Several chemical production approaches for bio-hydrocarbons have been proposed, such as hydro processing, catalytic cracking, and hydrodeoxygenation processes with reducing agents. However, a major challenge with this kind of application is the requirement of enormous energy and complex tools (Naqvi et al., 2017). By contrast, a biological synthesis of bio-hydrocarbons is preferred as it is easier to produce and more environmentally friendly. This biological method requires the help of natural catalysts, such as enzymes found in several organisms (Liu & Li, 2020). Previous research has established that one of the enzymes capable of manufacturing bio-hydrocarbons, especially alkanes in one step, is the fatty acid photodecarboxylation enzyme obtained from the microalgae *Chlorella variabilis* NC64A (Ng et al., 2023).

This enzyme, better known as *Cv*FAP (*Chlorella variabilis* Fatty Acid Photodecarboxylase), needs some desirable factors to achieve its function in decarboxylating fatty acids. Sorigué et al. (2017) demonstrated that decarboxylation of *Cv*FAP enzyme can occur when this enzyme is subjected to blue light (λ=400-520 nm). Another factor is the concentration of proteins, which are the main intermediates for converting substrates (Sorigué et al., 2017). Likewise, the activation time also needs to be observed because it determines the number of substrate conversion cycles that ensue until it reaches its saturation point. While some experiments have been carried out on bio-hydrocarbon production, the selectivity of products, specifically pentadecane, is still far from success. The prior study revealed a yield value of 61% with a conversion rate of 79% (Huijbers et al., 2018).

The unsatisfactory bio-hydrocarbon production results are linked to recent findings from Lakavath et al. (2020), which suggest that the *Cv*FAP enzyme may undergo light inactivation. This phenomenon might occur when light excitation of the *Cv*FAP enzyme occurs due to insufficient substrate for fatty acid conversion. Wu et al. (2021) also proposed that the solution to this obstacle is to keep enzymes occupied by ensuring an adequate substrate concentration.

Based on the problems and conditions that have been presented, it is necessary to conduct further studies regarding the better conditions of bio-hydrocarbon synthesis to obtain higher selectivity. Following up on the plan, an investigation was carried out on the activity of the *Cv*FAP enzyme derived from the extraction of recombinant products from *Escherichia coli*. The use of this recombinant protein aims to accelerate the cultivation of the protein source used. This research provides bio-hydrocarbons synthesis that focuses on the enzyme activation process with variations in protein concentration, activation time, and the type of CPO substrate compared with palmitic acid regarding bio-hydrocarbons concentration produced.

Materials and Methods

Materials

2-aminoethyl-benzene sulfonyl fluoride hydrochloride (AEBSF), crude palm oil (CPO) from Cikasungka, PT Perkebunan Nusantara 8 oil palm mill**,** $CuSO_4.5\ H_2O$, *Cv*FAP gene expression in *E. coli,* Folin Fiacalteu, IPTG, kanamycin, $KNaC_4H_4O_6$, *β-mercaptoethanol*, NaOH, Na_2CO_3, $5H_2O$, NdeI and HindIII restriction enzyme, palmitic acid terrric broth, Tris HCl Buffer.

Cloning recombinant DNA for Cv*FAP gene expression in* E. coli

For the production of *Cv*FAP in *E. coli,* a synthetic gene construct designed based on GenBank: KY5111411.1 was used. The construct consisted of a 6X His-tag sequence, thioredoxin (TrxA) tag, a tobacco etch virus (TEV) protease cleavage site, and a gene encoding *Cv*FAP. Codon-optimized *Cv*FAP sequence coding for expression in *E. coli*. The constructs were synthesized and cloned into the pET28a vector using the NdeI and HindIII restriction sites. Competent *E.coli* BL21 (DE3) cells were transformed using a recombinant plasmid for enzyme production (Huijbers et al., 2018).

Escherichia coli *colony culture*

E. coli containing the *Cv*FAP gene were cultured using 500 mL of liquid Terrific Broth (TB) media into a small Duran bottle containing 20 mL of media and 10 µL of kanamycin 50 ppm. The bottle is then shaken at a speed of 180 rpm with a temperature of 37°C overnight. After incubating overnight, the colony culture is re-mixed into the initial container and shaken at the same speed and temperature until the optical density (OD) is 0.7 to 0.8. Once the OD was fulfilled, the media was given 0.5 mM IPTG and shaken again for 20 hours (Huijbers et al., 2018).

Protein extraction

The media was harvested by centrifugation using a falcon tube at a speed of 11,000xg at 4°C for 10 minutes. The centrifuged pellet was then crushed with liquid nitrogen and added Tris HCl Buffer 50mM pH 8.0, AEBSF 0.5 mM, and β-mercaptoethanol 1.5 mM. The supernatant was separated using centrifugation at a speed of 16,000xg at 4°C for 30 minutes. After centrifugation, the resulting supernatants was stored in the refrigerator (Bruder et al., 2019).

Lowry testing

Prior to Lowry testing, necessary reagent such as reagent A (100 mL of aquadest; 0.44 gram of NaOH; and 2.04 gram of Na_2CO_3, reagent B (10 mL of aquadest; 0.05 gram of $CuSO_4.5\ H_2O$ and 0.1 gram of $KNaC_4H_4O_6.\ 5H_2O$, reagent C (mixture of reagent A and reagent B in a ratio of 50:1 (v/v), and reagent D (folin and aquadest with a ratio of 1:1 (v/v) need to be prepared. The next step is to set up Lowry's standard solution from 300 ppm BSA. After the Lowry standard had been provided, each standard series was pipetted as much as 200 µL (samples used 190 µL of aquadest and 10 µL of samples), then 1000 µL of reagents C were added and waited for 10 minutes. Following previous incubation, each standard series was added with 100 µL of reagent D to resuspend the solution and waited for another 30 minutes. The last step is each standard series was measured using a UV-Vis MultiskanGO spectrophotometer (ThermoFisher Scientific) at a wavelength of 750 nm (Martina et al., 2015).

SDS-PAGE

Samples were taken at 15 µL and mixed with 5 μL buffer loading. The sample was then denatured in a water bath at a temperature of 99°C for 5 minutes. After denaturation, the sample was placed into the acrylamide gel along with 15 µL SDS marker ladder. The type of gel used was acrylamide 15% using *Thermo Scientific™ Page ruler™ Protein ladder* with a reference ranging from 10 to 170 kDa. SDS-PAGE operation was carried out for two hours using 20 mA in the stacking gel step and another two hours using 50 mA in running gel step. On completion of the SDS-PAGE operation, the gel was stained with Coomassie blue dye for one night. The final step was decolorization by immersing the gel in water until the protein bands were clearly visible on the gel (Simpson, 2006).

Enzyme activity testing

A simple light reactor has been established, which adopts the incubator design used in the experiment by Sorigué et al. (2017). The reactor can be seen in Figure 1. The next step is to prepare a test sample containing 250 µL palmitic acid 10 mM, 40 µL DMSO, 711 µL protein, and 1500 µL Tris HCl with 8.5 pH in vials. Then, the vial is placed into the reactor. Following that action, the reactor circuit was turned on with agitation of 550 rpm. The reactor temperature is kept at 30-35°C with an activation time of 3 hours. After 3 hours, the vial was taken from the reactor and given an additional 20 µL of 10 M NaOH. The vial was then vortexed for 5 minutes and added with 1000 µL hexane. After mixing, the bubbles in the vial were removed using a sonicator bath. Then, the sample was left overnight until there was a phase separation. The upper phase or hexane phase was then taken and analyzed using GC-FID and GC-MS to determine the presence and concentration of the resulting hydrocarbons (Bruder et al., 2019). In this experiment was conducted the optimization of activation time (0, 1, 3, and 5 hours) variation of protein enzyme volume (0; 711; 1066.5; 1422; and 1775.5 µL) and use of CPO (50 and 100%) compared to palmitic acid with duplication in each experiment.

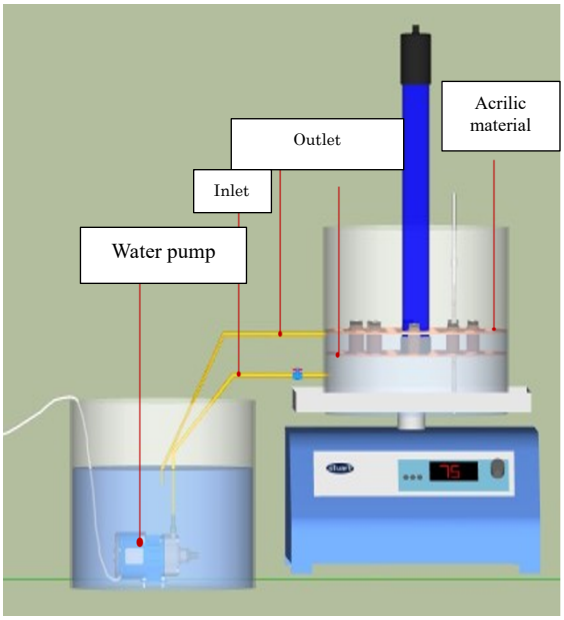


Figure 1. The reactor used for *Cv*FAD activity assay

Result and Discussion

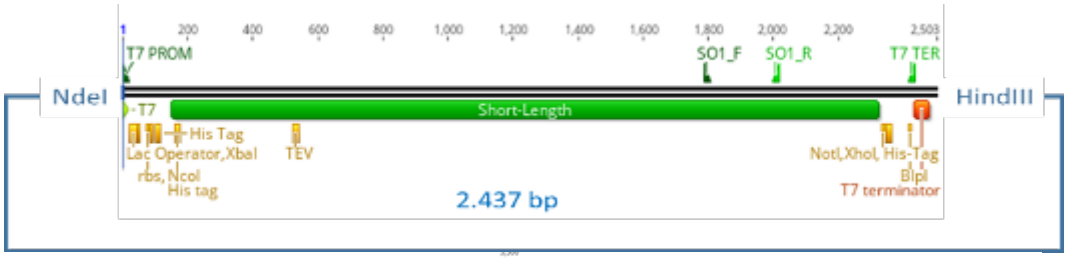
Cloning recombinant DNA for Cv*FAP gene expression in* E. coli

The *Cv*FAP construct consisted of a 6X His-tag sequence, a thioredoxin (TrxA) tag, a tobacco etch virus (TEV) protease cleavage site, and a gene encoding CvFAP with different codon and his-tag optimization. The total recombinant plasmid measured was 7,444 bp with a Kanamycin-resistant selection marker and a T7 promoter. Figure 2 shows the construct consists of a thioredoxin (TrxA) tag (blue background), tobacco etch virus (TEV), protease cleavage site (purple background), and the gene coding for *Cv*FAP (gray background).

Transformation and characterization of E. coli *carrying* Cv*FAP recombinant DNA*

The *Cv*FAP version 2 gene construct was transformed on *E. coli* BL21 (DE3) according to the protocol of Sigma-AldrichHT96™ BL21(DE3) Competent Cells. After one night, the growing colonies were carried out on agar media, and 10 colonies were produced (Figure 3). Colonies were subcultured on the same medium and tested by PCR using primers T7, F1/R1, F2/R2, and F3 to confirm successful transformation (Figure 3).

From PCR results, all 10 colonies showed positive with *Cv*FAP primer. Positive colonies were confirmed by constructing gene sequences by sequencing using primers F1R1, F2R2, and F3R1 (Figure 4). The sequencing results showed a *Cv*FAP nucleotide of 2204 bp and a coverage percentage of 93.9% (Figure 5).



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GCGAGCGCTGCGGCGCCGGCGACCGTTGCGGCGTAA

Figure 2. Construct *Cv*FAP gene (His-Tag, TrxA, dan TEV) in PET28a+ plasmid in NdeI and HindIII restriction site. (grey colour: the synthetic gene of *Cv*FAP, blue colour: gene of pET28a plasmid)

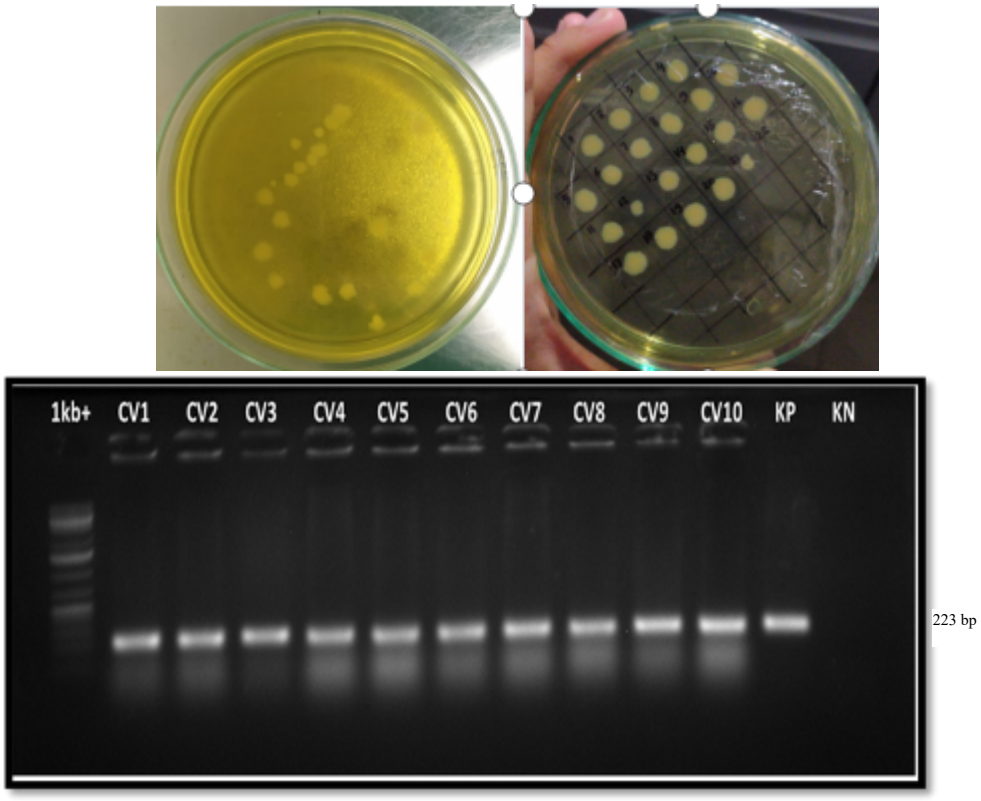

Figure 3. E. coli BL21 colony that transformed with pET-28a(+) plasmid: *Cv*FAP (top) and the PCR result of 10 kanamycin resistance positive colonies.

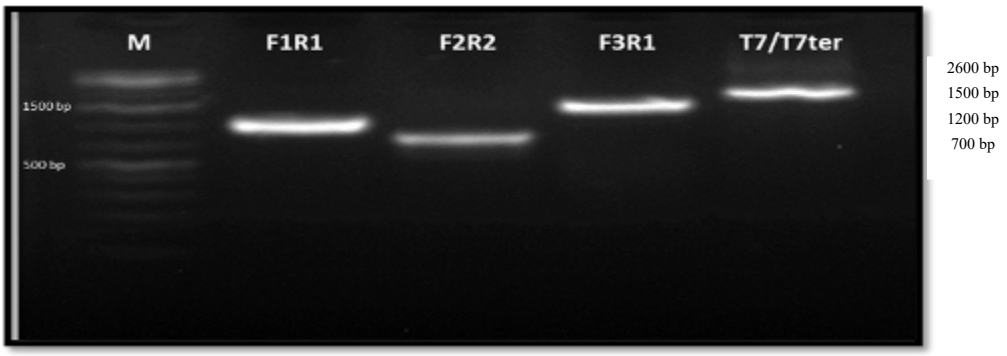

Figure 4. Validation of construct pET-28a(+):CvFAP in *E. coli* BL21 recombinant

Synthesis of CvFAP Protein

SDS-PAGE and the Lowry test are the most common protein identification procedures. The results of the SDS-PAGE visualization in Figure 6 display the qualitatively suspected type of protein in the sample. M is a protein ladder marker, E. cv TB is an extraction of *E. coli* protein that uses TB as a medium, whereas E. cv LB is *E. coli* which uses Lauria Bertani as a medium. With the help of Gel Analyzer 19.1, the Retardation factor (Rf) value is obtained, which will be used as a regression equation to determine the suspected type of protein-based on molecular weight (MW). Protein results based on their MW can be seen in Figure 6. It can be observed in the table the suspected protein of CvFAP that has been identified by Sorigué et al. (2017), having a molecular weight range of 60 to 65 kDa was found in the sample. In addition, that particular range also involved a photosystem complex or photosystem complex-PS, which presumed protein contains a protein-chlorophyll complex for capturing light (Sukenik et al., 1993; Bateman et al., 2021). This conjecture allows the great potential for the protein extracted from this study to provide target hydrocarbons in the form of alkanes using light activation.

Figure 5. Pairwise alignment between consensus sequence and *Cv*FAP gene (*Cv*FAP = 2204 bp vs Consensus = 2062 bp, Coverage = 93.6%)

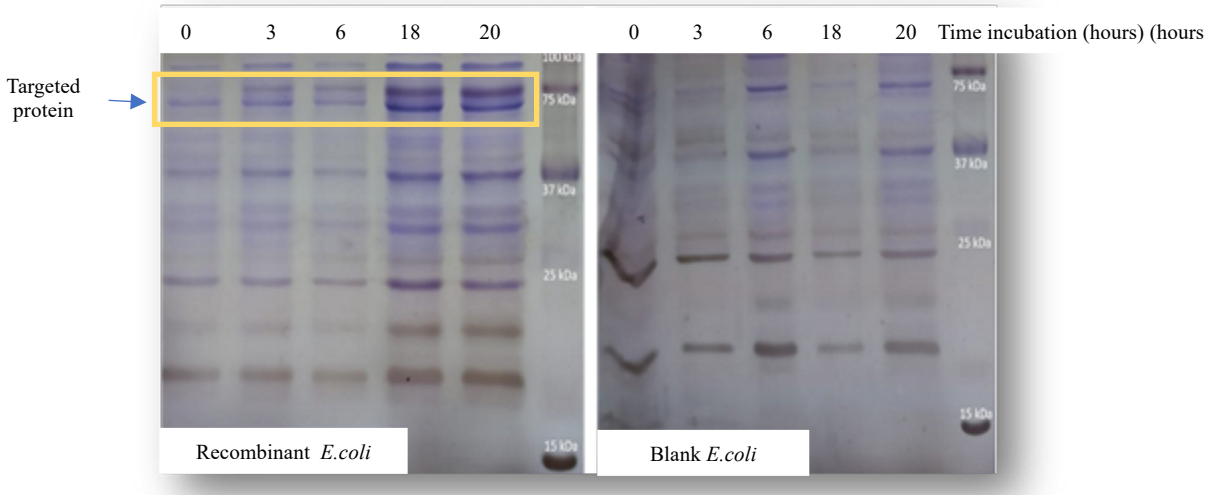

Figure 6. SDS PAGE result of *Cv*FAP recombinant with the variation of IPTG induction time (0,3,6, 18, and 20 hours)

Having defined by qualitative analysis using SDS-PAGE, turning now to the quantitative approach using Lowry testing. Data was collected from the processing concentration of each test sample based on the Lowry. E. cv TB has a much higher concentration value than E. cv LB. These results are similar to those reported by Santner et al. (2021) when he and his team compared LB and TB culture media. In that study, TB media produced significantly higher expression levels (Santner et al., 2021). The value is possibly influenced by changes in cell physiology owing to different metabolisms from different media components (Kram & Finkel, 2015). E. cv TB is used as a control sample because it has a higher protein content.

Once the SDS-PAGE and Lowry test was completed, the favorable protein was activated using a light reactor. The activation process was carried out with three variations, namely testing for variations in protein concentration, variations in time, and variations in substrate types. Following the activation, GC-FID and GC-MS instruments are used to locate the presence and concentration of the resulting hydrocarbons.

Effect of protein volume on pentadecane concentration

Figure 8 dictate that there is a clear trend of increasing value of pentadecane concentration with the highest value found in samples with the highest protein concentrations, that is 1777.5 μL. This result is associated with research conducted by Ma in 2020 which published that the photocatalyst concentration positively affects product formation (Ma et al., 2020). The reason behind the statement is due to the greater the number of enzymes contained in the activation sample, the more biocatalysts or intermediaries that can convert the existing substrate into the desired target hydrocarbon. Another significant advantage of high enzyme concentration is the ability to eliminate saturation resulting from blue light irradiation, which inhibits the rate of product formation (Duong et al., 2021).

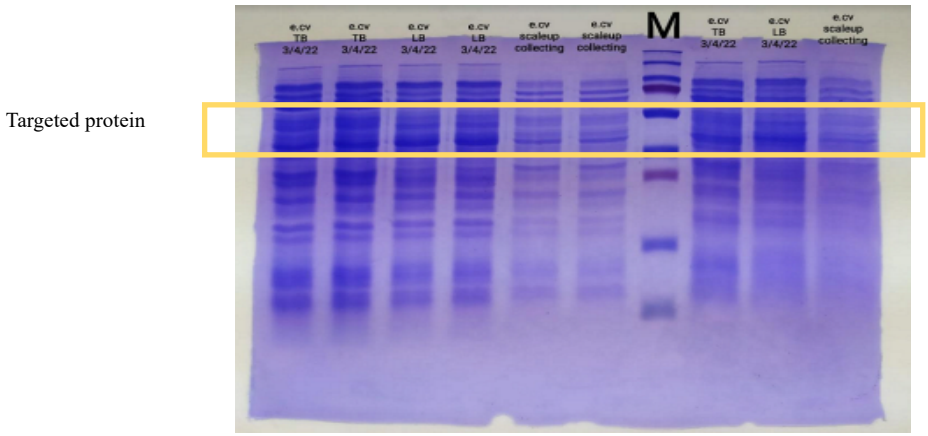

Figure 7. SDS PAGE result of *Cv*FAP recombinant with variation growth media Terrific Broth (TB) and Luria Bertani (LB).

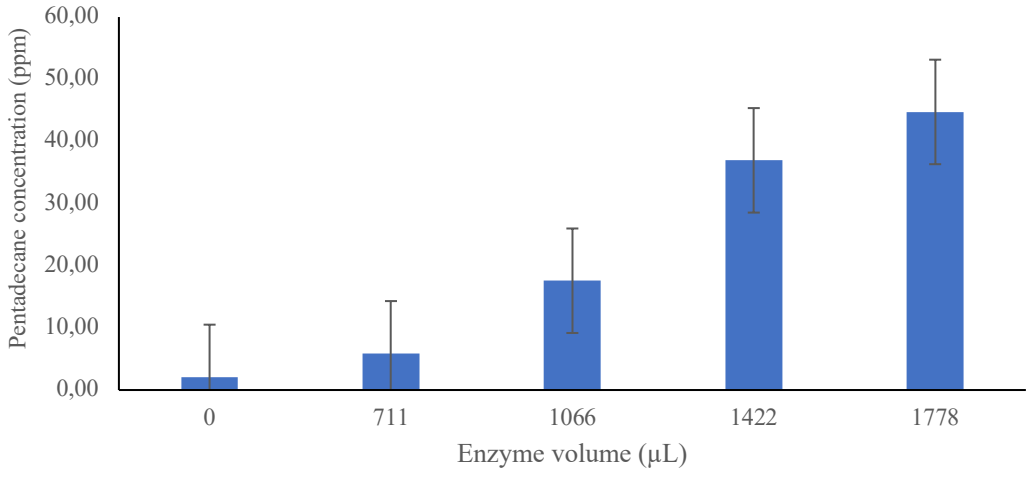

Figure 8. Pentadecane concentration with variation of protein concentration with activation time 3 hours, pH 8.5, 23000 lux, 28ºC and substrate palmitic acid.

Effect of activation time pentadecane concentration

Time variation testing indicates a continuing increase in pentadecane concentration until it reaches a peak during 3 hours of activation time (Figure 9). However, when the activation is applied for 5 hours, there is a significant decrease in the concentration of pentadecane compared to the results of the 3-hour activation. This phenomenon is suspected to ensue because continuous blue light induction might result in saturation of *Cv*FAP enzymes so that they undergo deactivation or even denaturation that caused the enzyme to be unable to convert substrate into hydrocarbons (Winkler et al., 2020). This experiment has results that are quite similar to research held by Guo and his team in 2022 which stated that the production of pentadecane using *Cv*FAP experienced a peak of activation at a time of 3 hours and at a longer time, there was a loss of enzyme activity ability due to prolonged lighting (Guo et al., 2022).

Effect of substrate pentadecane concentration

Studies on various types of substrates showed that palmitic acid had a much higher pentadecane concentration value than the other two single fatty acid compounds (Figure 10). This is likely due to the *Cv*FAP enzyme having an affinity for reacting with carbon compounds with chain lengths of 16 and 17 (Sorigué et al., 2017). Hollmann and his co-workers pointed out that the preference is influenced by the distance of the carboxylate group from the substrate to the flavin cofactor. Single fatty acids with longer chains will bind more easily to the *Cv*FAP access channel. In addition, the reactive group (*COOH) is more easily exposed to the cofactor (to produce the radical pair CH3COO*···FAD*) which results in higher conversion (Zeng et al., 2021)

Despite that, CPO as the substrate should be used because of the efficiency process and the grade that we used was the CPO with high free fatty acid that is not appropriate for frying oil. The experiment indicated that the CPO substrate which is a mixture of both 50% and 100% w/w in ethanol substances produces significantly higher pentadecane concentration than a single fatty acid. We also used oleic acid (C-18) as a control and to prove the ability to convert only fatty acid with chain lengths 16-17. Thus, it is not surprising that the CPO substrate will produce a high concentration value as well. The highest concentration of pentadecane is found in 50% CPO substrate. This is likely because 100% CPO is too concentrated, causing the enzymes to have difficulty converting the substrate into pentadecane due to the limited contact surface with the enzyme. The high pentadecane concentration of 50% CPO is also probably affected by the solvent used because fatty acids are difficult to dissolve in water, and solvents are needed to increase the availability of the substrate to the enzymes in the reaction mixture (Santner et al., 2021).

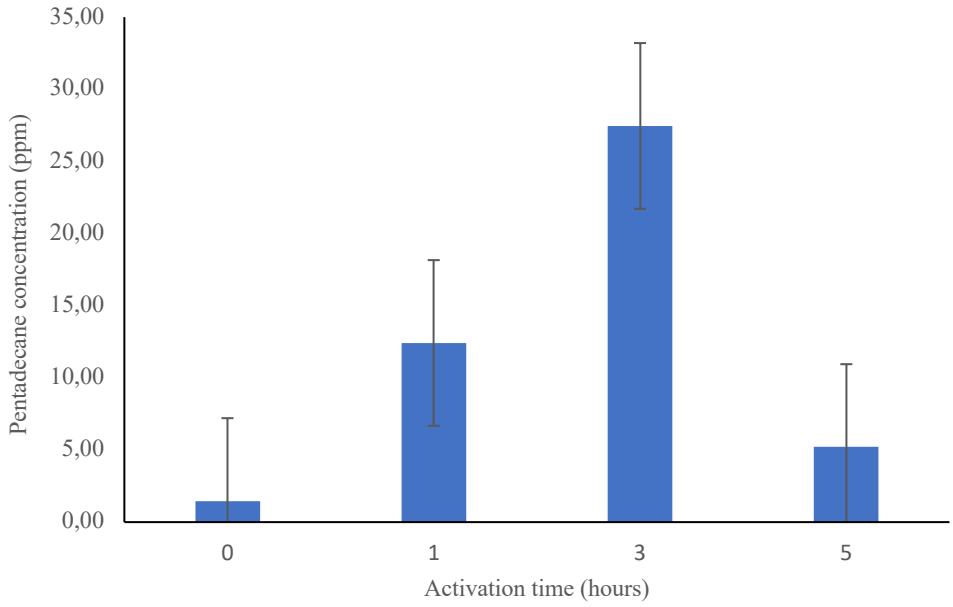

Figure 9. Pentadecane concentration with variation of time activation with palmitic acid as substrate, pH 8.5, 23000 lux, 28ºC and concentration of protein volume 17775 µL

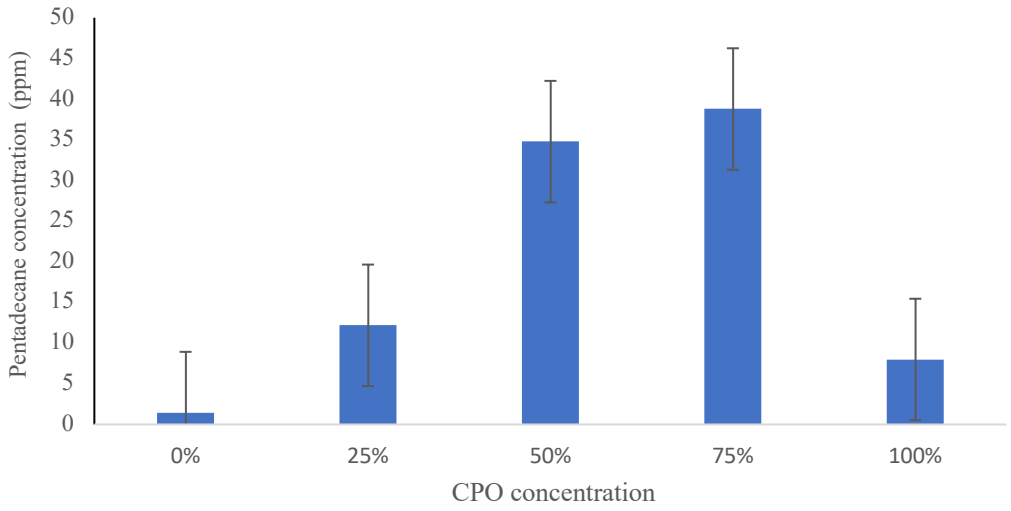

Figure 10. Pentadecane concentration with the variation of substrates (lauric acid, oleic acid, palmitic acid and CPO), pH 8.5, 23000 lux, 28°C, 3 hour's time activation in protein volume 1777.5 µL.

Conclusion

These experiments confirmed that the production of pentadecane was potentially carried out by the light activation method using the *Cv*FAP enzyme with a yield of 16.44%. These results came from the FAP protein which is the main component of the success of enzyme activation detected in samples with a molecular weight of 60-65 kDa. The results of this investigation show that the synthesis of pentadecane can run optimally using the light activation method with several substantial factors needed in the activation process, namely the optimum growth medium for TB, protein volume of 1777.5 *µ*L, activation time of 3 hours, and a substrate preference of 50% CPO. Further research should be undertaken to explore the upscale condition of this synthesis method of bio-hydrocarbons.

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