# Direct Transesterification of Microalgae Cells using an Acid Catalyst for the Production of 1-[Methoxy(methyl)phosphoryl]oxyheptane

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The depletion of petroleum-based fuels has led researchers to explore alternative biofuels. Microalgae are a potential alternative for biodiesel feedstock due to its cost-effective cultivation, low carbon emissions, and high biomass. As a third-generation feedstock, microalgae have become an attractive option to the energy field. This study investigated direct transesterification using microalgae and characterized the fatty acid methyl ester (FAME) produced by this method. An acid catalyst was chosen to prevent saponification which could promote the loss of products. The research methodology encompassed four main stages: microalgae cultivation, direct transesterification, biodiesel purification, and analysis of biodiesel components using gas chromatography and NMR spectroscopy. Sulfuric acid (5 % v/v) was used to acidify ethanol for direct transesterification of microalgae cells with a solids loading of 20 % (w/w). The collected FAME was characterised as heptyl methyl methylphosphonate (HMMP). The IUPAC name of this methyl ester was 1-[methoxy(methyl)phosphoryl]oxyheptane and its molecular formula was $C_9H_{21}O_3P$. HMMP is a component in biodiesel production.

**Keywords**: FAME; direct transesterification (in-situ); Spirulina Sp.; microalgae; acid catalyst, heptyl methyl methylphosphonate

*Received: June 2024; Accepted: August 2024*

The current global energy landscape relies primarily on non-renewable fossil fuels, which significantly contribute to $CO_2$ emissions and environmental issues. The combustion of fossil fuels accounts for approximately 52 % of human-induced $CO_2$ emissions, highlighting the urgent need for research into alternative energy sources to mitigate the consequences of greenhouse gases [1]. Biodiesel is increasingly seen as a viable alternative to conventional diesel, presenting significant advantages for both economic returns and environmental benefits. Its manufacturing process results in reduced carbon monoxide emissions, thereby contributing to its positive ecological profile. Biodiesel plays an essential part in mitigating the environmental consequences of fossil fuels and advocating for a more sustainable energy landscape [2]. Biodiesel production involves converting fatty acids or oils and fats through processes such as esterification or transesterification with short-chain alcohols.

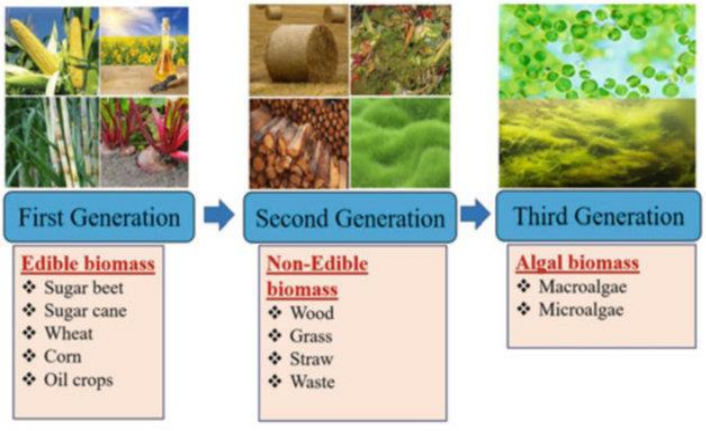


**Figure 1.** Type of Feedstock for Biodiesel Production [4].

Biodiesel is recognised based on its feedstock source [3]. The first generation of biodiesel was produced using edible sources such as rapeseed, palm, wheat, sunflower and soybean. The second generation, on the other hand, was derived from non-edible sources like castor, rubber seed, tobacco seed, jojoba oil and jatropha. In contrast, the third generation involved producing biodiesel from algae including microalgae, macroalgae, and fungi.

Microalgae, a third-generation feedstock, are considered the most promising option for biofuel production due to their unique characteristics. They have rapid growth rates with short generation times and can thrive in diverse environments, showing excellent cell production in controlled vessels to open ponds [5][6] without any significant growth drawbacks. One significant advantage is their ability to absorb carbon dioxide through very active photosynthesis, contributing to $CO_2$ reduction, which promotes environmental sustainability. Apart from their rapid growth, they can adapt to various water sources such as wastewater and seawater without disrupting the food chain [7] or any growth element readily occurring in the water system. Microalgae have high lipid productivity and are capable of giving about 10 times of product per acre of plantation compared to classical production using non-algae sources. This characteristic makes them a major contender for the future of biofuel production [7].

Since microalgae have the potential to become a biofuel source, there are a lot of studies that have been done to produce biodiesel from microalgae biomass. The most common method for extracting biodiesel is the two-step conventional process. Although widely used, this method has several drawbacks that provide valuable lessons. These include being time-consuming, yielding a low amount of biodiesel, involving a complex process, and posing environmental risks. According to Silas *et al.,* [8] the Soxhlet extraction of green fuels using hexane is performed at 60-70 °C for about 18 hours. Even though hexane is not a great choice of solvent as the US Environmental Protection Agency (EPA) has identified it as a hazardous compound, it is still recognized as the best solvent for some standard transesterification processes. Apart from this, the produced biodiesel needs purification, which extends the overall processing time. These factors contribute to the drawbacks of the two-step conventional transesterification process.

Various literature reviews, on the other hand, have indicated that the direct transesterification (*in situ*) technique is more cost-effective and energy-efficient compared to the conventional method [9]. Commonly, the direct transesterification method is utilized using a base catalyst as it provides greater conversion efficiency compared to acid catalysts [10]. However, base catalysts can lead to saponification due to the presence of free fatty acids and a high water content. In comparison, acid catalysts may cause corrosion problems and slower reaction rates compared to base catalysts [9].

To overcome these limitations, the direct transesterification technique was explored. This method is more practical for large-scale biodiesel production because it eliminates the need for a separate or standalone lipid extraction process prior to transesterification. As a result, lipid losses during extraction can be minimized, leading to higher biodiesel yields. Additionally, the direct transesterification technique reduces processing time since extraction of lipids and transesterification occur in a single step. The *in situ* method also reduces overall costs. Therefore, the objective of this study was to assess the biodiesel yield using sulfuric acid ($H_2SO_4$) as an acid catalyst in the direct transesterification (*in situ*) technique for microalgae biodiesel production. This research is essential for gaining further insight into the application of acid catalysts that could be commercially utilized to maximize biodiesel production yields and support the field of energy production. Methanol was selected as the extraction solvent and acylation agent in this study. This is because the general criteria for a direct transesterification solvent is that the chemical can disrupt or break microalgae cell walls (great penetration ability), directly convert lipid into biodiesel, react quickly and has a low cost [11]. Methanol was commonly used in biodiesel production even before the direct-transesterification method was introduced, during the first generation of biodiesel feedstock. It is also commonly used for extraction in Folch and Bligh and Dyer's method [12]. Hence, methanol is a good solvent for direct-transesterification due to its extraction and transesterification capabilities.

## EXPERIMENTAL

### Chemicals

Sulfuric acid (95 % purity, laboratory grade), methanol (95 % purity, laboratory grade), chloroform (99 %, HPLC grade), n-hexane (95 % purity, laboratory grade), all fatty acids for HPLC calibration, and anhydrous sodium sulphate, were obtained from Sigma-Aldrich.

### Culture Conditions, Microalgae Cultivation and Wet Microalgae Harvesting

Spirulina sp. was cultivated in 9 sets of 1 L beakers containing 700 mL growth solution. A growth solution was prepared using minimal ions and nutrients based on the method by Badar et al. (2017), with some modifications. The growth solution mixture contained 0.15 % potassium nitrate ($KNO_3$), 0.15 % calcium nitrate tetrahydrate ($Ca(NO_3)_24H_2O$), 0.15 % potassium dihydrogen phosphate (KH2PO4), 0.15 % boric acid ($H_3BO_3$), 0.15 % zinc sulphate heptahydrate

($ZnSO_47H_2O$), and 0.15 % copper sulfate pentahydrate ($CuSO_45H_2O$)[13]. The beaker, equipped with a fluorescent lamp (1800 lux) and air pump (0.5 vvm) for the circulation of carbon dioxide at a constant room temperature (25 °C), served as a mini functional photobioreactor for cultivation purposes. Growth was observed daily for nine days. The harvesting process was done using a Büchner funnel and flask, vacuum pumps, and filter paper. Residues of the microalgae collected on the filter paper were dried naturally under sunlight for 24 hours. The collected dried sample was ground into a fine powder using a mortar and pestle, and stored for lipid extraction.

**Lipid Extraction**

The method by Wahlen *et al.*, [14] was modified. The extraction of lipids from the microalgae cell membranes was performed using a mixture of solvents at equal concentrations. n-Hexane, tetrahydrofuran and chloroform were chosen based on the quality of extraction produced with lipids from many types of photosynthetic cells. Sonification of cells was performed in a 5.00 mL solvent mixture at 150 W for 1 minute, with 10 second intervals. The mixtures were centrifuged for supernatant collection. This step was repeated and re-suspension was done followed by sonification. The extracted solution was ready for GC analysis.

**Analysis of Lipids**

Analysis was performed using a Shimadzu GC-2010 with automatic flame ionization detector (FID) and 20i auto-injector (AOC-Shimadzu). The column size was 0.53 nm (RTX_Restek). The analysis was accomplished using a column flow of 2.55 mL/min with a detector temperature of 380 °C. 1.0 mL of the lipid solution was injected into the column at a temperature range of 60 °C to 380 °C. The rate was calibrated at 10 °C/min with a constant temperature held for 10 minutes. The injection concentrations were calibrated between 0.025–0.300 mg/mL with a 0.100 mg/mL standard solution of octacosane.

**Statistical Analysis**

All experiments were carried out in triplicate. The results were reported as the average value with a standard deviation representing its percentage error.

**Direct Transesterification (*in situ*) Process**

This process was carried out using 5 g of microalgae (Spirulina Sp.) powder as the lipid source, a 60 ml mixture of methanol (0.35 % v/v) in distilled water as the acylation agent and 2.2 mL sulphuric acid (0.04 M) as the catalyst for the transesterification process. These were mixed in a beaker and covered with aluminium foil to avoid any losses during the reaction. The beaker was heated at 60 °C and stirred at 500 rpm for 8 hours.

**Purification of Fatty Acid Methyl Esters (FAME)**

The reacted mixture was filtered using filter paper, a Büchner flask and vacuum pump to separate the microalgae from the solvent mixture. The residue was then washed twice using a resuspension technique in 30 ml of methanol for 10 minutes each. Next, the obtained filtrate solution was added to 50 ml of distilled water and transferred to a separating funnel. The water was used to dilute the polar and hydrophilic compounds before extracting the non-polar compounds. Then, 30 ml of hexane was added to the separating funnel and mixed with the filtrate. The separating funnel was shaken and the stopcock was opened to release any accumulated pressure during the mixing process. The extraction process was performed for 15 minutes and repeated thrice. Two layers were formed during the extraction process, a polar layer and a non-polar layer. The FAME product was extracted in the non-polar layer due to its non-polar characteristics. The non-polar layer (containing mostly hexane) usually emerges as the top layer due to its lower density compared to the polar layer (containing mostly water).

The two layers were separated to collect the desired product in the top (non-polar) layer, followed by washing with 50 ml distilled water in a separating funnel. Then, the mixture was shaken to mix them together. This procedure was performed to remove impurities in the product such as any unreacted methanol and acid catalyst. After the separation of the desired non-polar layer, 1 g of anhydrous sodium sulphate was added as a drying agent. The solid-liquid mixture was left for 10 minutes before the separation process using filter paper in a Büchner funnel and vacuum pump. The remaining non-polar solvent, which contained hexane and the FAME, was put through an evaporation process at 70 °C using a hot plate in a fume hood until only 10 ml of the sample was left. This process ensured that all the hexane was removed. Finally, the solution was transferred to a universal sample bottle.

**Characterization of Fatty Acid Methyl Esters**

The FAME produced was analyzed using gas chromatography-mass spectrometry (GCMS) and nuclear magnetic resonance (NMR) spectroscopy. The data obtained was interpreted using the Automated Mass Spectral Deconvolution & Identification System (AMDIS) software by the National Institute of Standards and Technology (NIST).

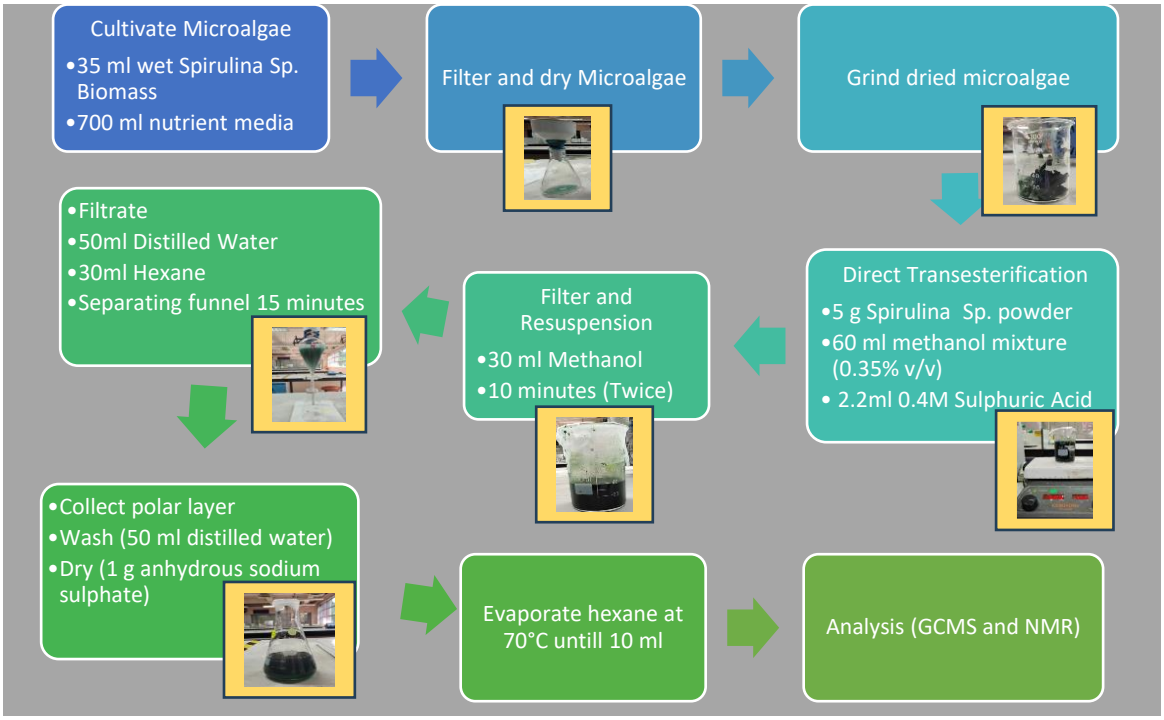


**Figure 2.** Summary of Direct Transesterification (*in situ*) Process

## RESULTS AND DISCUSSION

### Microalgae Cultivation

**Figure 3** displays the data for the dry weight of microalgae that was cultivated over nine days. Theoretically, the difference in microalgae dry weight data across the growth curve can provide significant information regarding its growth phases [15]. This is because as the number of cells increases, the microalgae dry weight should also increase. The growth phases include the lag, exponential, linear, stationary and death phases. The results revealed that the highest growth rate occurred in the first 24 hours, with a recorded dry algae weight of 0.014 g. The findings indicated that Spirulina sp. underwent three distinct phases within a short time frame of just 24 hours: a lag phase, an exponential phase, and a linear phase. The lag phase was typically attributed to either the presence of non-viable cells or the adaptation of microalgae to a new environment [16]. This suggests that microalgae can adapt to a new environment in less than 24 hours. Subsequently, the microalgae entered an exponential and then a linear growth phase where cell duplication occurred rapidly. This was due to the nutrients provided for the microalgae to undergo photosynthesis, which led to an increase in microalgae activity. In 24 hours, the microalgae dry weight increased 57.14 % from 0.006 g to 0.014 g, the highest recorded dry weight during that period (exponential and linear phase). Theoretically, after the exponential and linear phases, there is a stationary phase where the microalgae growth remains constant. However, in this case, growth declined between the time interval of 24 to 72 hours from 0.014 g to 0.011 g, or 21.42 % in 48 hours. This may be due to the diminishing nutrient source available for microalgae growth. Due to the population increase during the first 24 hours, there was greater competition for the limited nutrients in this pre-stationary phase. This argument is further strengthened by the fact that all the basic needs and the environment for microalgae growth such as light, temperature and carbon dioxide from air were constantly supplied. The only difference was the amount of nutrients available. This is because the nutrients were only provided at the beginning of the experimental startup. Thus, the microalgae need to survive with a limited nutrient supply from the beginning until their death phase in a constant environment. The stationary phase was then observed from 72 hours (0.011 g) to 120 hours (0.011 g). During this phase, microalgae growth was constant due to the exhaustion of nutrients in the medium culture. The microalgae would need to survive with the carbon sources that they accumulated as carbon storage including starch and neutral lipids. This carbon storage would be used as the energy for the microalgae to survive before undergoing the death phase. The death phase of the Spirulina sp. occurred from 120 hours (0.011 g) to 192 hours (0.003 g). In this phase, the population of microalgae decreased rapidly due to the lack of an energy source. Hence, the lowest dry weight of microalgae recorded in the data was 0.003 g at 192 hours.

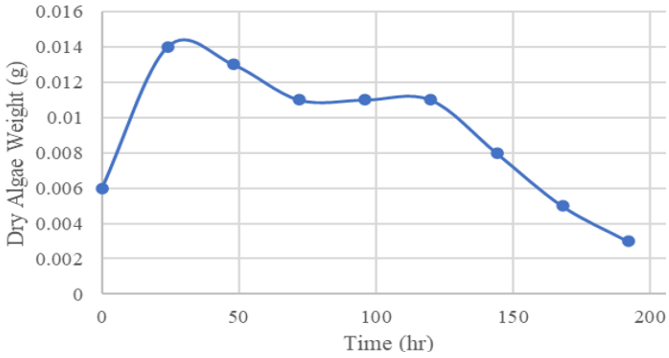


**Figure 3.** Microalgae Growth Curve

Each phase of microalgae growth is influenced by environmental conditions, including the nutrients received by the microalgae and the cell population. In a medium that lacks nutrients, the lifespan of the microalgae would be negatively affected and become shorter. As a result, the declining growth phase and the death phase would occur earlier than the expected. The cell population could also affect the growth rate; when the microalgae population increases, competition for the same nutrients and other life factors cause a slower growth rate. This scenario has been reported by Rinanti [17].

**Direct Transesterification**

The harvesting of microalgae was done during its highest mass. Lipid extraction and transesterification were performed at the same time using direct transesterification. This technique was used to avoid the loss of lipids during the extraction and transesterification process. **Figure 4** shows the steps involved, from the collection of algae biomass until the FAME was extracted, while **Figure 5** shows the reaction steps of ester production from triglyceride. To produce biodiesel, the lipid must be reacted with an acylation agent such as methanol or ethanol. Lipids contain mostly triglyceride which forms an ethyl ester biofuel such as Fatty Acid Methyl Ester (FAME) when it undergoes transesterification. Theoretically, the lipid would undergo three reversible transesterification steps to produce ester and glycerol. In the first step, the triglyceride would be reacted with alcohol to produce diglyceride and ester. In the second step, the diglyceride would be reacted with alcohol to produce monoglyceride and ester. In the third step, monoglyceride was used as the reactant. The main product is the ester produced in every reaction step, and the by-product is glycerol.

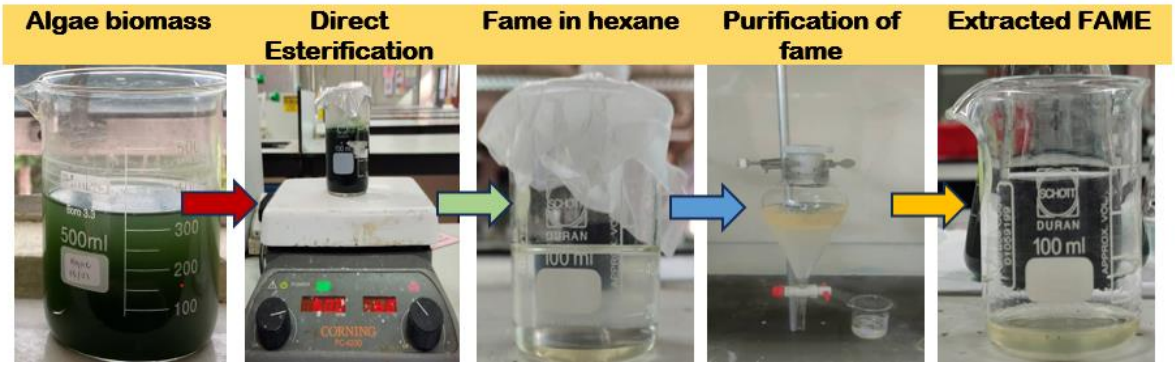


**Figure 4.** FAME Production from Algae Biomass.

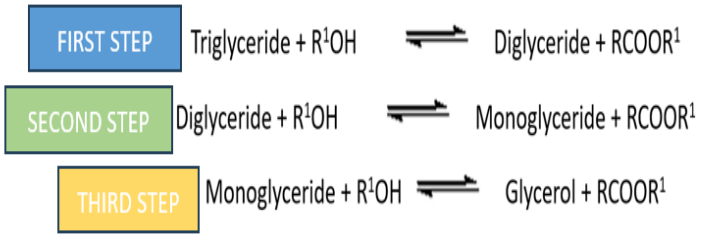


**Figure 5.** Transesterification Reaction Steps

Generally, the extraction solvent should have a high polarity to access regions of ion dipole interactions, and form hydrogen bonds to disrupt this interaction [18]. Methanol is one of the best candidates in this study as it is primarily used to disrupt the electrostatic forces or hydrogen bonding network between proteins and lipids in the extraction process [18-19]. Hence, it may be used for both lipid extraction and transesterification to produce Fatty Acid Methyl Ester (FAME). Saini *et al.* [18] has suggested that methanol could be substituted with ethanol or isopropanol as the extracting solvent. Ethanol has a similar polarity, and thus works like methanol [20]. Isopropanol however, may have weaker disrupting activity due to its hydrophobic moiety [18].

Sulphuric acid was used to increase the efficiency of the extraction process by disrupting the cell and acting as an acid catalyst to produce FAME. The fatty acid methyl ester would need to undergo several purification steps to obtain a FAME yield of high purity [21]. The purification step was performed after the extracted solvent was filtered. The extracted material from the microalgae was split into two parts: polar and non-polar compounds. Lipids from microalgae may appear in both polar and non-polar substances. Through the transesterification process, the lipids were converted directly into biodiesel as a non-polar product.

The medium polarity and excess methanol in the FAME extract may cause difficulty in separating the biodiesel layer. Hence, it is important to choose a solvent that has the ability to form an aqueous two-phase system in order to remove non-lipid (polar) compounds from the medium [18]. Usually, the extraction process is successful when a non-polar solvent is used. The effectiveness of non-polar solvents has been reported with extraction of lipids such as triglyceride and biodiesel [22]. The most non-polar solvent is hexane, with a polarity index of 0 (PI=0)[18], making it the most suitable solvent in extracting lipids (non-polar). **Figure 6** and **Figure 7** show the results of the FAME extraction process using hexane from the methanol mixture, before and after the evaporation of hexane. A very thin layer of yellow liquid can be observed settling at the bottom of the beaker in Figure 6. As mentioned by Guldhe *et al.* [23], the thin yellowish layer could be a sign of the existing lipids or fatty acids that are mostly present in a neutral lipid, triglyceride. Based on its appearance, the product may be fatty acid methyl ester **(Table 1).** Confirmation was done using gas chromatography (GC) and NMR spectroscopy.

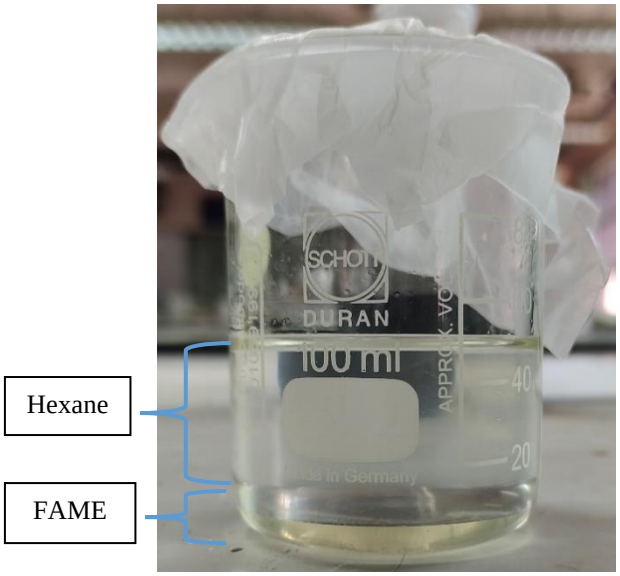


**Figure 6.** Extracted FAME in Hexane before Evaporation.

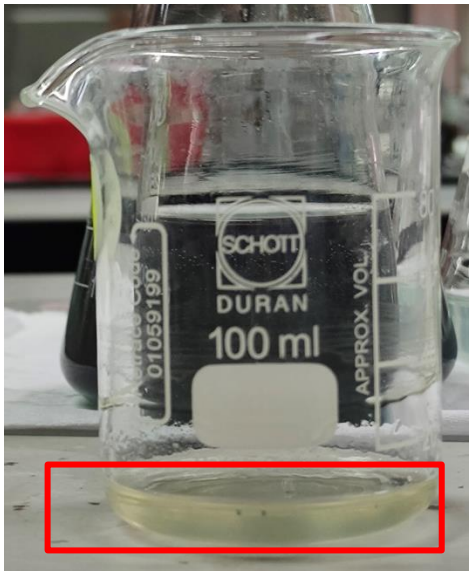


**Figure 7.** Extracted FAME after evaporation of Hexane.

**Table 1.** Physical Characteristics of FAME Produced.

| Physical Characteristics | Results |
|---|---|
| Volume | 10 mL |
| Product Phase | Liquid |
| Colour | Yellowish |

**Chemical Characterization of the Produced FAME**

The microalgae-produced lipid components were analysed by GCMS. **Figure 8** shows the components of the produced lipid layer after the purification process. Several compounds were present with different abundance percentages and retention times. The most abundant component was recorded at 14.88 min (retention time) with peak of model 111 m/z (Figure 8, highlighted in the red oval). This indicates that the sample contained a high concentration of Fatty Acid Methyl Ester (FAME). The interpreted data showed that the chemical compound at 14.88 min with 111 m/z of peak model was heptyl methyl methylphosphonate (HMMP). **Figure 9** shows the $^{1}$H NMR spectrum of heptyl methyl methylphosphonate.

The presence of methyl compounds in the sample indicated that the Fatty Acid Methyl Ester (FAME) was produced during the transesterification reaction.

Small changes in the electrons of the nucleus causes changes in the magnetic field of the nuclei. The chemical shifts in the $^{1}$H NMR spectra of the FAME sample recorded at 90 MHz in $CDCl_3$ are shown in **Table 2.** The $^{1}$H NMR spectrum of heptyl methyl methylphosphonate (with chemical shifts and assignments) is shown in **Figure 9.** By comparing Table 1 and Figure 9 with some physical details of HMMP, the FAME was assigned the molecular structure shown in **Figure 10.**

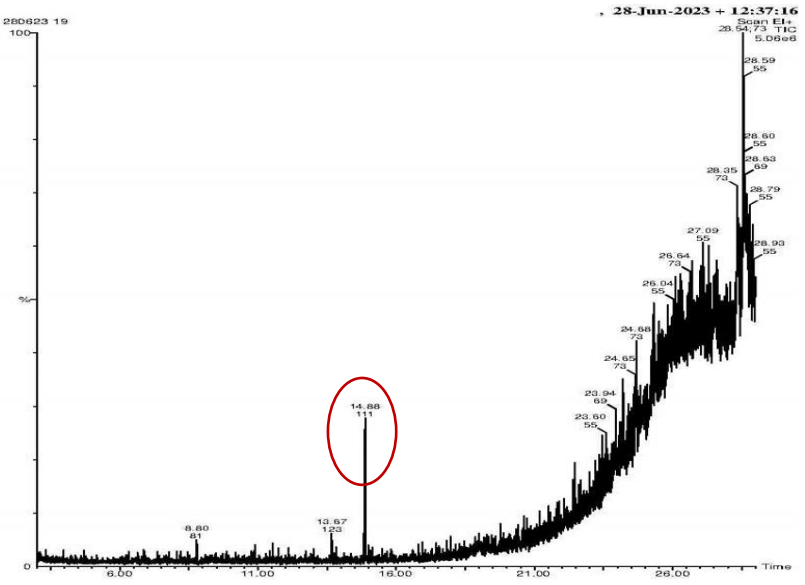


**Figure 8.** Gas Chromatography peaks analysis.

**Table 2.** $^{1}$H NMR chemical shift values.

| **$^{1}$H shift chemical (ppm)** | **Multiplicity** | **Assignment** |
|---|---|---|
| 1.376 | Singlet | -O-$CH_2$ (B) |
| 3.856 | Doublet | -P-O (D) |
| 1.481 | Singlet | -P-$CH_3$ (C) |
| 3.741 | Singlet | -O-$CH_3$ (A) |

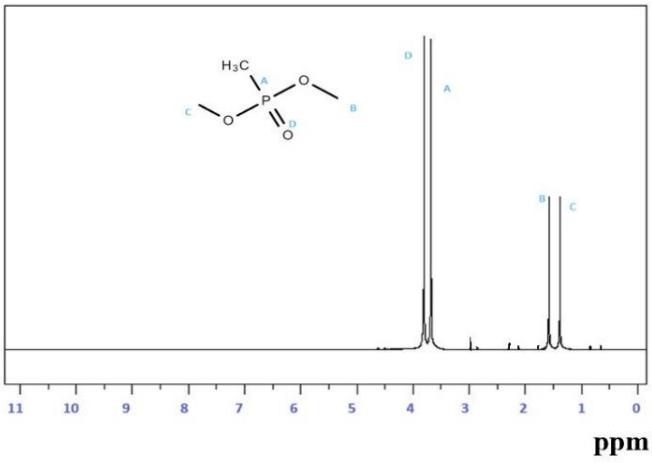


**Figure 9.** $^{1}$H NMR Spectrum of Heptyl Methyl Methylphosphonate (chemical shift and assignment).

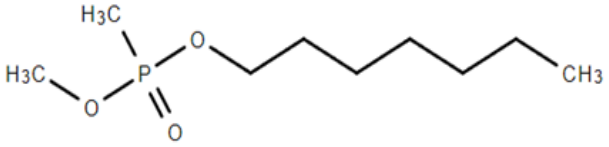


**Figure 10.** Molecular Structure of Heptyl Methyl Methylphosphonate Produced by Direct Transesterification of Spirulina sp.

**Table 3.** Properties of Heptyl Methyl Methylphosphonate in FAME Extracted from Spirulina sp.

| Properties | Value |
|---|---|
| Molecular Weight | 208.23 g/mol |
| Hydrogen Bond Acceptor Count | 3 |
| Rotatable Bond Count | 8 |
| Heavy Atom Count | 13 |

HMMP consists of a tetrahedral phosphorus centre bonded to oxygen, a methyl group, and two hydroxyl groups (-OH). It is classified as an ester due to its formation from three functional groups: carbonyl, alkoxy, and alkyl. The HMMP had a molecular weight of 208.23 g/mol and possessed notable hydrogen bonding properties, featuring 3 hydrogen bond acceptor sites and 2 hydrogen bond donor sites. A hydrogen bond acceptor is an electronegative atom that can form a hydrogen bond by accepting a hydrogen atom from a neighbouring molecule or ion, making it possess a lone pair of electrons. Hence the HMPP molecule possessed 3 lone pairs. These acceptor sites enhance HMMP's ability to interact with other molecules through hydrogen bonding, contributing to its overall chemical behaviour and reactivity. HMMP also consisted of 8 rotatable bonds which allow free rotation. The presence of these rotatable bonds allows for flexibility and freedom of movement within its molecular structure. In addition, HMMP contained 13 heavy atoms. A heavy atom is defined as a non-hydrogen atom which refers to the phosphorus, oxygen, and carbon atoms in the HMMP molecule. The properties of HMMP from the produced FAME are summarized in **Table 3**.

## CONCLUSION

The direct transesterification (*in situ*) process of Spirulina sp. microalgae self-cultivated with specific nutrients under a constant environment (1800 lux light intensity, 0.5 vvm air pump and 25 °C room temperature) produced fatty acid methyl esters. The cultivated microalgae were harvested during the highest peak of its growth, at 24 hours after cultivation.

Methanol, used as an acylation agent, was used for both extraction and transesterification purposes because it can disrupt the electrostatic forces between the proteins and lipids of the microalgae for the extraction, and directly interact with lipids for the transesterification process. Sulfuric acid also had two functions, to disrupt the microalgae cell walls and as a catalyst for transesterification. Water and hexane were used for the purification of the FAME. Water, acting as a polar solvent, removed polar impurities such as glycerol and excess methanol. Hexane was used to extract the FAME layer from the direct transesterification mixture, due to its non-polar characteristics, and then removed by evaporation. The FAME extract was analysed by GCMS, which showed the existence of a methyl compound identified as heptyl methyl methylphosphonate with a retention time of 14.88 min, peak model of 111 m/z and 53 % purity, which was confirmed by NMR. The $^{1}$H NMR results showed that the heptyl methyl methylphosphonate derived from Spirulina sp. had 3 hydrogen bond acceptors, 8 rotatable bonds and 13 heavy atoms.

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge the School of Chemical Engineering, College of Engineering, UiTM for providing all necessary chemicals and equipment for this research, and express great thanks to the supervisor and the team for their support in this endeavour.

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