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THE EFFECT OF IRON (II) CHLORIDE IN MICROALGAE CULTIVATION FOR BIO-OIL EXTRACTION

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Abstract: The world is facing a problem regarding the use of petroleum fuels that has led to a search for a suitable alternative fuel source. Researchers have come up with the idea of producing biofuel to overcome this problem. In this study, microalgae were explored as a high potential feedstock to produce biofuel. In order to produce a large quantity of biofuel with low cost at a short time, the manipulation of nutrients is a factor in microalgae cultivation. In this study, Iron (II) Chloride ($FeCl_2$) was added to the nutrients to initiate a stressful condition during growth which contributes to the produce of lipid. Isolated microalgae species were identified as Scenedesmus sp. During mass cultivation, the microalgae cultures were scaled up to 2 L of culture. Three flasks of microalgae culture were labelled with S1, S2, and S3. Flask S1 acts as a control without the addition of $FeCl_2$, while another two flasks acted as experimental flasks. Flask S2 was supplemented with 0.5 mg $FeCl_2$ while Flask S3 was supplemented with 1.0 mg of $FeCl_2$. With the addition of Iron (II) Chloride, microalgae entered a stationary phase at day 9 and day 10 as compared to the control flask which enters the stationary phase at day 7. This also affects the dry weight. Flask 3 produces 0.8658 g of microalgae powder compared to Flask 1 and 2 which produced 0.4649 g and 0.5357 g respectively. Lipid analysis was done by using GCMS and GC-FID. Flask 3 produced various types of fatty acids which can be used for biodiesel production compared to other cultivates. In Flask 1, docosanoic acid which is a saturated fatty acid was detected. While in Flask 2 (S2), with the addition of 0.5 mg of $FeCl_2$, docosapentaenoic acid was produced. In the last flask which involved the addition of 1.0 mg of $FeCl_2$, more fatty acid was detected. In GC-FID data, 6 types of fatty acids were detected. Linolein acid, linolenic acid, stearidonic acid, docosapentaenoic acid, docosahexaenoic acid and docosanoic acid were produced at different retention times. Most of the fatty acids produced are polyunsaturated fatty acid (PUFA). In transesterification, the fatty acid reacts with methanol and acid catalyst. The reaction produces fatty acid methyl ester. In Flask 1, the control flask, without the addition of $FeCl_2$, no fatty acid methyl esters (FAME) was produced. However, in Flask 2 and 3 which were added 0.5 mg $FeCl_2$ and 1.0 mg $FeCl_2$, n-hexadecanoic acid methyl ester which is also known as palmitic acid was produced. Palmitic fatty acid can be used for biodiesel production.

Key words: *Bio-oil, Microalgae, Scenedesmus, Lipid*

INTRODUCTION

Bio oils, which are also termed as biofuels are fuels that are primarily produced from biomass and can be used to replace fossil fuels [1]. Biofuels are generated from biological materials which are also defined as renewble sources of carbon. Depletion of oil stocks combined with the increase of worlwide demand has forcds several countries to divert their exploration towards biofuels [2]. As there has been an incline in worry over the greenhouse gas emission's impact on the environment, it has come to pass that biofuels are a greener choice as carbon balance is almost neutral as oppossed to that of fossil fuels.

Biofuels can be classified by three generations. For first generation, biofuels are produced directly from food crops such as wheat and sugar by extracting the oils for use in biodiesel [3]. However, first generation biofuels can give rise to a contentious issue. In the past

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two years, it can be seen that when the demand of biofuels increase, global food prices increases too. This is attributed due to biofuels being derived straight from food crops, thereby diverting from the global food market masses of crops Eventually, this can threaten food supply and biodiversity [4].

Due to the aforementioned limitations of the first generation biofuels, the second generation has been developed. Unlike the first generation, the second generation does not generate biofules that are of food crop origin. Instead the production of biofuels come from the likes of wood, organic waste, crop residue and crops of a specific biomass. This can help in overcoming the limitation of the previous generation. The second generation's aim is for the extension in sustainable biofuel production amount [5].

Derived from algae, third-generation biofuels are different from previous generations on the count of their growth yield. Biofuels from the later generation differ from the former as seen by the advances made to biomass manufacturing. These biofuels are derived from microalgae [6]. Another advantage of biofuels derived from algae, is the diverse types of fuels produced; which include petrol, diesel as well as jet fuel.

Microalgae, which are also known as microphytes, are microscopic algae that exist in freshwater and marine habitats. Microalgae exist unicellularly as part of a group, part of chains, or on their own. They also vary in size that can range up to hundreds of micrometers. They are different from other higher plants due to the absence of roots, stems and leaves. Besides, they are also able to perform photosynthesis which can help in producing atmospheric oxygen. As a means of photoautotrophical growth, the oxygen produced in used concurrently with carbon dioxide. They can also produce storage lipids in the form of triacylglycerols (TAGs). Due to the content of lipid of dry cell weight [4] and raised productivity of 48% and 7.4 g/L/d respectively, certain species, for example *Chlorella protothecoides* are specifically scouted for [1].

A cornocorpia of advantages can be obtained using microalgae for the production of biofuel which are of higher yield of lipid as well as high scale production. This means that more biofuels can be produced in the current generation as compared to previous generations. Microalgae also have a faster growth rate amongst other species where they can double from 1 to 3 hours in 24 hours. This species is able to grow in variable environment conditions with simple nutrient requirements [7]. Addition of Iron (II) Chloride during the cultivation of microalgae can affects the growth rate of the species. The presence of Iron (II) Chloride can stimulate the microalgae culture and increase the growth of microalgae. This can cause more lipid being produced for the production of biofuel. Under stressful conditions, microalgae produces maximum lipid by accumulating the lipid inside them. With the presence of Iron (II) Chloride, microalgae will grow under stressful conditions and more lipid will be accumulated.

METHODS

Isolation of microalgae

Microalgae were collected from Kuantan coastal water samples. After primary cultivation, pure cultures were isolated by performing serial dilutions. Pure Microalgae suspensions were then spread on a petri dish of BG 11 medium with pH 7.5. All microalgae samples were allowed to grow for 2 weeks. Then, a single colony of microalgae was picked and inoculated in test tubes that have 20 ml of BG 11 medium. The inocolum was incubated further to promote the growth of microalgae cells.

Morphology Analysis

The microalgae cells were observed under fluorescence microscope (Olympus, BX53) for their morphological features and other cellular details to identify the genus. The cells were further studied using field emission scanning electron microscope (FESEM) (JEOL, JSM-7800F, Japan). The basic steps for sample preparations were fixed with 3% glutaraldehyde in buffered phosphate. It was then dehydrated in various concentration of ethanol (30-100%), air dried before being mounted on a specimen stub coated with Carbon and then coated with Platinum before being examined under the microscope.

Mass culture of Microalgae

Pure microalgae strains were cultured in 100 mL of BG11 liquid medium in a 250 mL Erlenmeyer flask on a rotary shaker (100 rpm) until near stationary growth occurs. The culture was left to multiply and monitored frequently before scaling up to 1 L conical flask with 500 mL of fresh medium. Cultures were then grown in batch mode in a 2 L conical flask containing 1 L of sterilized algal medium. Three batch culture flasks were labeled with S1, S2, and S3. Flask S1 acted as control without the addition of $FeCl_2$ while the other two flasks acted as the experimental flasks. Flask S2 was supplemented with 0.5 mg $FeCl_2$ while Flask S3 was supplemented with 1.0 mg of $FeCl_2$. In mass cultivation, all growth conditions were optimized including pH at

7.5, temperature ranging 20 °C ± 5 °C, photoperiod 16 hours' light / 8 hours' dark and constant aeration.

Microalgae harvesting

Microalgae growth was measured at 2 days intervals. The growth curve was used to determine the suitable phase for harvesting. The samples were then collected by centrifugation (8,000 rpm, 8 min) in falcon tubes. The precipitated algal cells were collected and kept at −80 °C before freeze dried into powder form.

Lipid extraction

The microalgae lipid extraction procedure was described by protocol Bligh and Dryer in 1959. The microalgae tissue was homogenized first with chloroform/methanol (2/1) to a final volume 20 times the volume of the tissue sample (1 g in 20 ml of solvent mixture). Following the addition of chloroform and methanol, the whole mixture was agitated for 15-20 minutes in an orbital shaker at room temperature. The mixture was then filtrated to recover the liquid phase, and then washed. Following the vortex of the mixture for a few seconds, said mixture was separated into two phases through centrifugation with less than averages speeds at 2,000 rpm of 5 minute duration. The top phase was claved and the bottom phase contained lipid. The lipid was prepared for the next step which is transesterification and screening of lipid profile.

Screening of lipid profile

Flame Ionization Detector (FID) was used to study the lipid profile. One microliter of the sample was injected in a split less mode at a flow rate of 1 ml/ min with Nitrogen as the carrier gas onto a J&W 122-7062, DB-WAX with column (250 m x 250 micro m x 0.25 micro m, total run time 65 min). Individual temperatures were allocated with 250 °C for the Injector and 275 °C paired with the Detector. It was possible to identify lipids through the comparisons of the peak as well as retention time.

RESULTS AND DISCUSSION

Isolation of microalgae

Water samples from the coast of Kuantan were collected and microalgae species were isolated from the sample. BG11 medium with pH 7.5 was used to culture isolated microalgae on a petri dish (Figure 1). After 2 weeks, microalgae growth can be seen on the petri plate and they were isolated and inoculated into test tube with BG11 medium. They were left for 2 weeks for further cultivation.

Figure 1: Microalgae growth (green colonies) on petri plate

Strain Identification

Characterization of microalgae was performed using fluorescent microscope. By referring to Figure 2 and Figure 3, morphology was referred to microalgae library to identify their strain. The isolated green algae were identified as *Scenedesmus* sp.

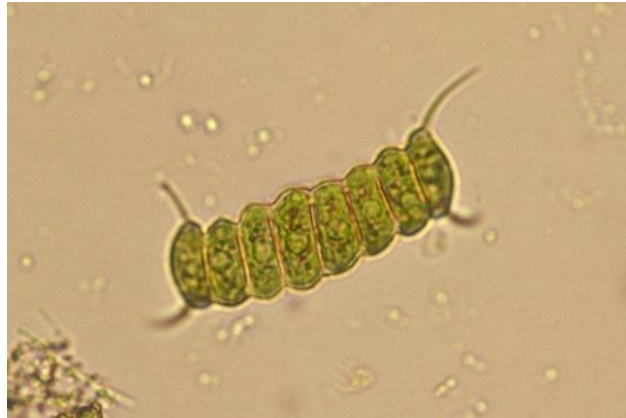

Figure 2: Image of *Scenedesmus* sp. from fluorescent microscope

This species was non-motile and colonial. It is looked to as a forerunner amongst the species to produce biodiesel [8,9], as it contains lipid that has a range from 18.8 to 29.3 % dwt for a medium rich in nutrients and up to 42 % dwt in mediums deficient in nutrients. The colonies of this species most often have two or four cells but may have 8, 16 or rarely 32 and are occasionally unicellular. The study by [10], suggested that among the tested strains, *Scendesmus* sp. was found to be the best candidate for biofuel production due to high lipid content and high lipid productivity. [11], concluded that the selected species, *Scenedesmus obliquus*, was done so as it is form of microalgae that shows great promise in the production of large scale lipids. This is due to the biomass production that results in high lipid as well as fatty acid productivity.

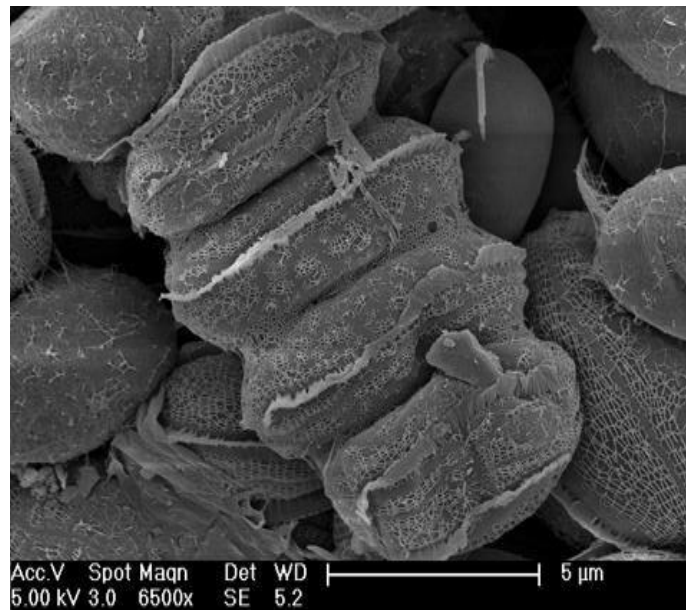

Figure 3: Image of *Scenedesmus* sp. by using SEM

Growth analysis

During mass cultivation, microalgae growth was measured using the GENESYS 10S UV-Vis spectrophotometer to obtain optical density (OD) of the culture at 665 nm. Figure 4 indicates the growth phase of microalgae. Flask 1 which was labelled as S1 acts as a control and shows increase of optical density from day 1 until day 7. From day 7, the microalga culture starts to enter the stationary phase as indicated by the absence of increasing culture concentration. Day 7 to day 9 show a slight decrease of the optical density measurement. For

Flask 2, S2, which was supplemented with 0.5 mg $FeCl_2$ show the increase of optical density measurements from day 1 until day 9 were observed. Beginning from the day 9, the concentration starts to decrease and show that the culture was in stationary phase and eventually reached death phase. Flask 3, S3, which was added with 1.0 mg of $FeCl_2$, shows almost the same increase in concentration with S1 and S2 but culture in the Flask S3 entered the stationary phase at day 10.

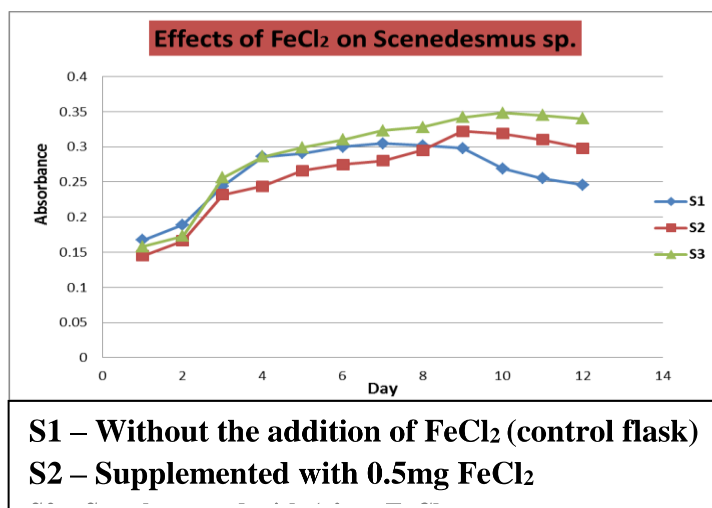

Figure 4: Growth curve of *Scenedesmus* sp with effects of $FeCl_2$ on the microalgae culture

By comparing all three flasks, the experimental flask, Flask S2 and S3, entered the stationary phase later as compared to control flask. By adding iron into the culture, the period of the log phase is lengthened and the final cell density increased [12]. Log phase recorded the most rapid growth of microalgae and at that state, accumulation of lipid was done by microalgae. Hence, the addition of iron can give rise to a stressful condition in order for microalgae to produce and accumulate more lipids compared to the control flask which was without addition of $FeCl_2$.

Microalgae stores lipid synthesized through the photosynthesis process in the cell membrane and later convert it into energy. Increase of the algal biomass and bio-oil content is the primary goal in bio-oil production. Therefore, alterations in nutrients components is a vital method to provide a stressful condition to microalgae and further produce lipids more than usual conditions. It should be noted that it is challenging to produce high bio-oil content during optimal growth conditions; although the increase of algal biomass was observed. Thus, while depletion of certain compounds, such as nitrogen and phosphorus increases, increase in lipid production and biomass usually happens. Alternately, the increase of iron in nutrients usually promotes the increase of lipid storage in microalgal cells [13]. Low iron concentration in algal nutrients further decreases its chlorophyll concentration. Such decrease will reduce further the biomass as well as lipid content in microalgal cells. It was reported that, at 30M of ferrous sulphate in 12 days old culture about 21.9 % CDW lipid could be produced from *Skeletonema costatum* [14]. When iron was added into culture medium during late exponential groth phase of *Chlorella vulgaris*, the cells were able to increase the total lipid content up to 56.6 % CDW [13].

Microalgae harvesting

Harvesting of microalgae cells was done by centrifugation of the culture for 15 minutes (Figure 5). Pellets containing microalgae cells were then collected and placed into the freezer with temperature -80 °C to remove the water content. Then, they were placed into freeze dryer machine to undergo the freeze-drying process. From this process, dry weight of the sample was obtained (Figure 6).

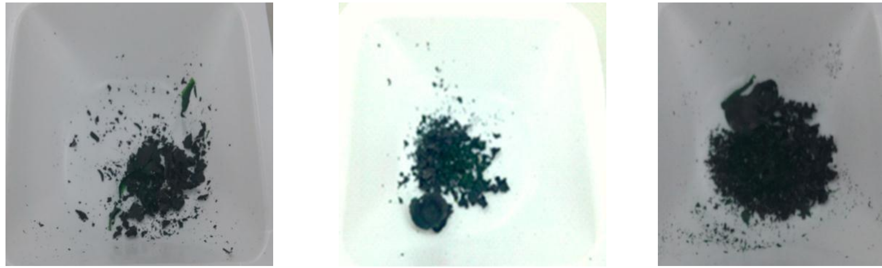

Figure 5: Pellet containing microalgae cells obtained after centrifugation.

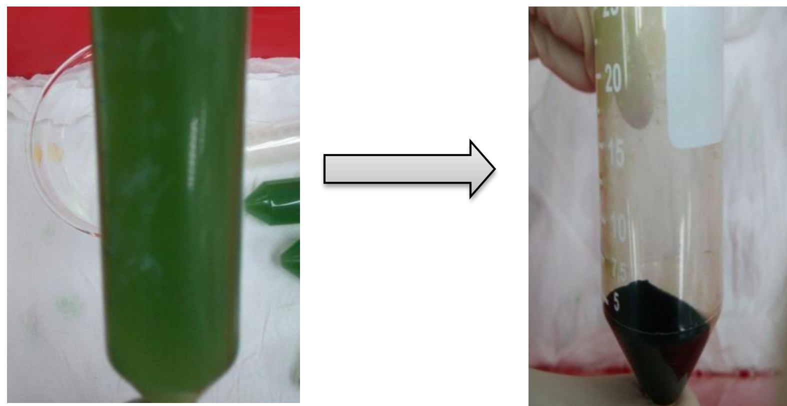

Figure 6: Microalgae cells in powder form (dry weight). Sample S1, S2 and S3 from left to right.

Dry weight of the microalgae cells in Flask S3 which was supplemented with 1.0 mg $FeCl_2$ is 8685 g. While Flask S1 and S2 produced 0.4649g and 0.5357g respectively (Figure 7). The dry weight of cells from each flask indicated the different growth range of microalgae. Microalgae accumulate lipids in log phase. Therefore, more lipids are produced when the log phase is lengthened. Hence, the final cell density is also affected by the addition of $FeCl_2$. A mathematical model derived by [15], done to study the effects that iron possess to grow *Chlorella vulgaris* as well as produce lipid optimally , concluded that the increase of dissolved iron in the culture medium is able to influence bio-oil productivity. It was confirmed that the lipid content rose from 9.6 % to 10.6 % in terms of dry weight and this occurs as iron concentration in said medium expended from 0 to 25 gm^{-3}.

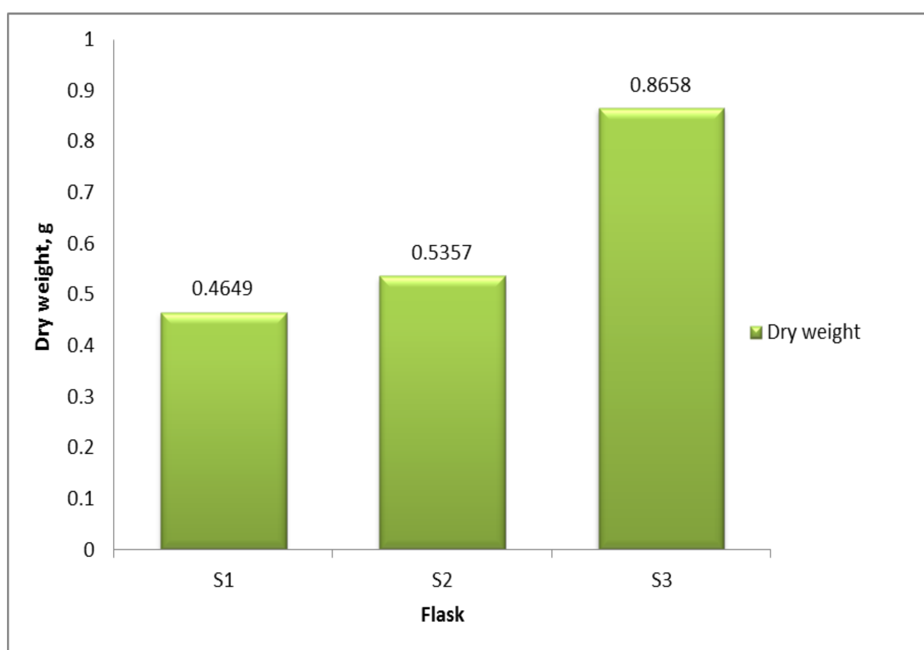

Figure 7: Bar chart shows dry weight of microalgae cells in different flask

Screening of lipid profile

Figure 8 shows simplified diagrams of the lipid extraction pathway. Lipids that were obtained from the bottom layer were analyzed by using Flame Ionization Detector (FID). Figure 9 shows the bio-oil extracted from *Scenedesmus* sp. The FID results indicated that all the samples were able to produce fatty acid. However, S3 which was supplemented with 1.0 mg $FeCl_2$ produced various types of fatty acids compared to S1 which was without the addition of $FeCl_2$ and S2 with addition of 0.5 mg $FeCl_2$.

Table 1 and Figure 10 indicate that the S1 sample produced 2 peaks at the retention time of 5.497 and 45.710 minutes respectively. While peak 5.497 belongs to solvent, peak at 45.710 indicates the presence of fatty acid known as docosanoic acid and is a saturated fatty acid. Docosanoic acid is a carboxylic acid with the formula $C_{21}H_{43}COOH$. It is a long chain fatty acid and can be used in biodiesel production.

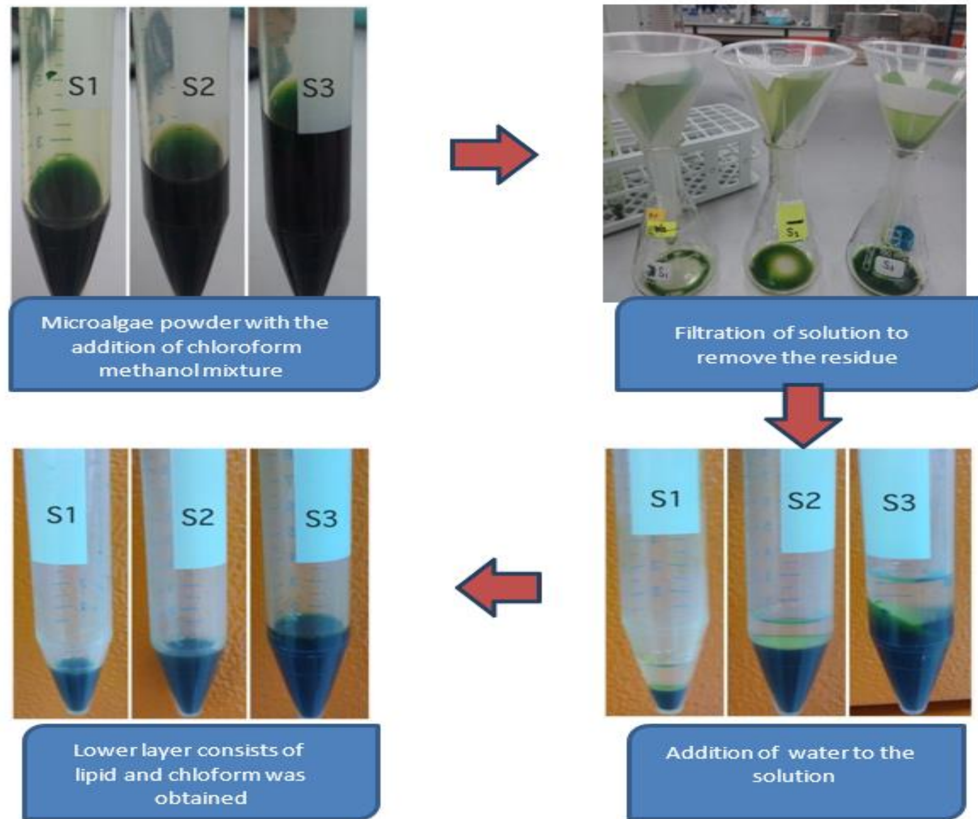

Figure 8: Pictogram simplified lipid extraction of microalgae by using chloroform, methanol and water.

Figure 9: Bio-oil collected from *Scenedesmus* sp.

Table 1: Fatty acid analysis of *Scenedesmus* sp. from flask 1 (S1) by using GC-FID

Peak	Retention Time	Fatty Acid Name	Type of Fatty Acid	Carbon
1	5.497	ND	ND	ND
2	45.710	Docosanoic acid	SFA	C21:0

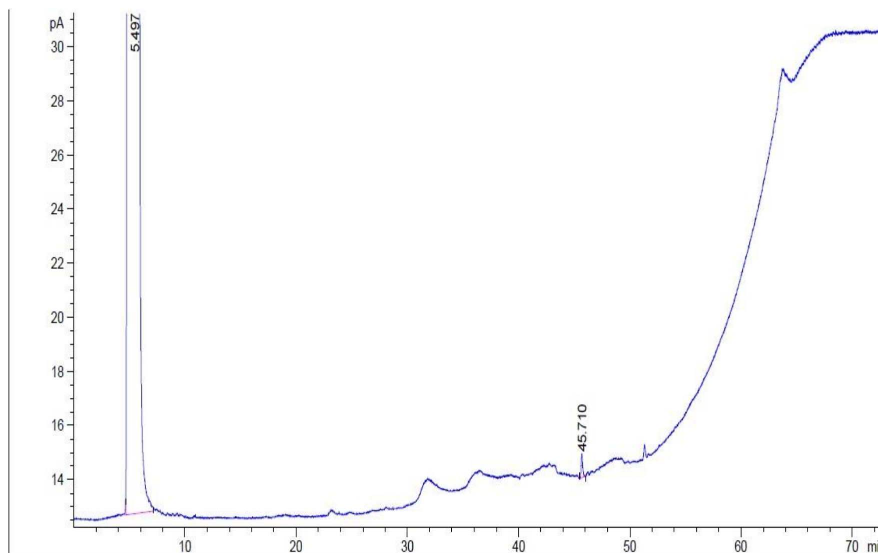

Figure 10: GC-FID results for screening lipid profile of *Scenedesmus* sp. in flask S1 (without addition of $FeCl_2$)

On the other hand, two peaks at retention time of 5.493 and 45.692 minutes were observed for S2 (Table 2 and Figure 11). The second peak at 45.692 minutes was identified as docosapentaenoic acid. Docosapentaenoic acid is a dietary omega-3 fatty acid mainly found in fish oil, seal oil and red meat. Johnson, (2009) revealed that, the major FAMEs contained biodiesel were esters of docosapentaenoic acid (C22:5), myristic acid (C14:0), palmitic acid (C16:0) and docosahexaenoic acid (C22:6).

Table 2: Fatty acid analysis of *Scenedesmus* sp. from flask 2 (S2) by using GC-FID

Peak	Retention Time	Fatty Acid Name	Type of Fatty Acid	Carbon
1	5.493	ND	ND	ND
2	45.692	Docosapentaenoic acid	PUFA	C22:5

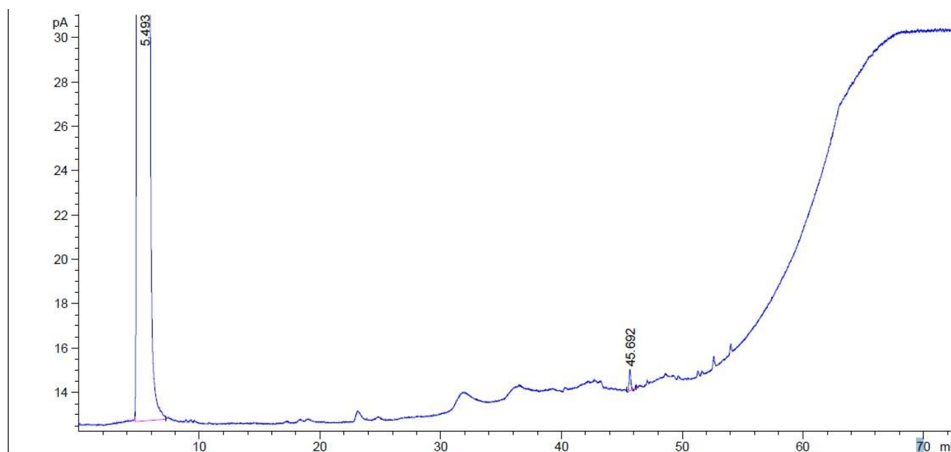

Figure 11 : GC-FID results for screening lipid profile of *Scenedesmus sp* in flask S2 (with addition of 0.5mg $FeCl_2$)

While for S3, due to the addition of $FeCl_2$, more fatty acids were produced from microalgae under stressful conditions (Table 3). Seven peaks were produced at different retention times (Figure 12). The peak obtained

at retention time 18.362 minutes identified to be linoleic acid, a compound similar to carboxylic acid apart from the fact that it has an 18-carbon chain as well as two cis double bonds present. The primary double bone is situated at the sixth carbon from the end containing the methyl-group. It is a polyunsaturated and essential fatty acid. Linoleic acid's molecular formula is $C_{18}H_{32}O_2$ with molar mass 280.45 g/mol, a melting point at -5 °C and boiling point at 230 °C. Linoleic acid (C18:2) has been acclaimed to be the most familiar fatty acids found amongst biodiesel thus securing a fair distribution of fuel characteristics.

At retention times 19.007 minutes, the presence of α-linolenic acid was detected. α-linolenic acid is a carboxylic acid containing a 18-carbon chain with a triplet of cis double bonds. The primary double bond is found situated where the third carbon is from the end where the methyl-group is amongst the fatty acid chain, referred to as the n end. Therefore, α-linolenic acid is a polyunsaturated n−3 (omega-3) fatty acid. It is an isomer of gamma-linolenic acid, a polyunsaturated n−6 (omega-6) fatty acid. For both linoleic and α-linolenic acid, it is proven by a study by [17], in 2009 that they were produced mostly in green algae including *Scenedesmus* sp.

At retention time 23.166 minutes, stearidonic acid was produced. Molecular formula of stearidonic acid is $C_{18}H_{28}O_2$ with a molar mass of 276.40 g/mol. This fatty acid can be found naturally from blackcurrant, corn and also microalgae species. In previous study by [18], stearidonic acid was proven to be present in *Scenedesmus* sp. At retention time 45.696 minutes docosapentaenoic acid was detected. Docosapentaenoic fatty acid was also detected in Flask 2 (S2) which the addition of 0.5mg $FeCl_2$ was present. The results showed that docosapentaenoic only exist in the experimental flask, which had the addition of Iron (II) Chloride and not exist in control flask. Therefore, the addition of $FeCl_2$ may enhance the production of docosapentaenoic acid in *Scenedesmus* sp.

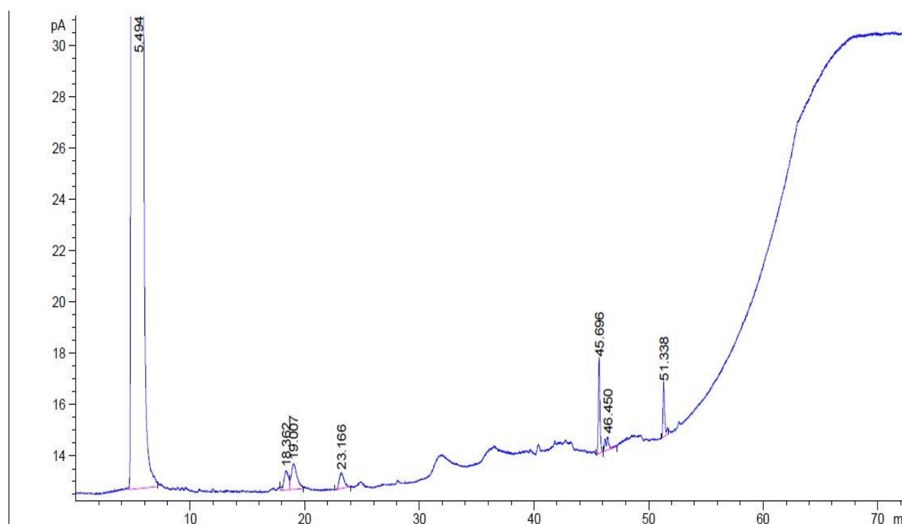

Figure 12: GC-FID results for screening lipid profile of *Scenedesmus sp* in flask S3 (with addition of 1.0mg $FeCl_2$)

Additionally, at retention times 46.450 minutes, there was the presence of docosahexaenoic acid. Docosahexaenoic acid is carboxylic acid containing 22 carbon chain present with 6 cis double bonds. The primary double bond is situated where the third carbon is at the end containing the methyl. Naturally, this fatty acid can be found in cold-water oceanic fish oils and most of docosahexaenoic acids originated from photosynthetic and heterotrophic microalgae. This kind of fatty acid can also be used for biodiesel production.

Table 3: Fatty acid analysis of *Scenedesmus* sp. from flask 3 (S3) by using GC-FID

Peak	Retention Time	Fatty Acid Name	Type of Fatty Acid	Carbon
1	5.494	ND	ND	ND
2	18.362	Linoleic acid	PUFA	C18:2
3	19.007	Linolenic acid	PUFA	C18:3
4	23.166	Stearidonic acid	PUFA	C18:4
5	45.696	Docosapentaenoic acid	PUFA	C22:5
6	46.450	Docosahexaenoic acid	PUFA	C22:6
7	51.338	Docosanoic acid	PUFA	C22:7

In general, unsaturated fats are oils that contain at least one double bond in between the carbons on the chain. In monounsaturated fats, it can be seen that they possess just one double bond between the carbon whilst polyunsaturated fats have multiple double bonds. However, monounsaturated fatty acids are the most suitable fatty acid to be used in biodiesel production due to low temperature fluidity and oxidative stability [19]. Furthermore, low gelling point of unsaturated fatty acid characteristics allows it to be used as an excellent product to produce biodiesel (Daniel *et al*., 2010). In order for fatty acids to be converted into biodiesel, they should be composed of triglycerides. Triglycerides possess three long fatty acid chains as well as a glycerol molecule to which said chains are attached to. As long as the fatty acid has that basic structure, they can be turned into biodiesel. Palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid are said to be the most commonly found fatty acid methyl esters available. In this study, lipids that can be converted into biodiesel are produced by *Scenedesmus* sp. under $FeCl_2$ stress condition. By adding $FeCl_2$ to the medium, *Scenedesmus* sp was able to produce linoleic acid, linolenic acid and hexadecaenoic acid specifically.

[8] indicated that *Scenedesmus obliquus* presents the most adequate fatty acid profile, namely linolenic and other polyunsaturated fatty acids. High content of saturated fatty acids is present in *Scenedesmus dimorphus* under dark conditions. However, PUFAs are highly present in *Scenedesmus dimorphus* under light condition [20]. This explains why most of fatty acids produced by *Scenedesmus* sp. in this study were polyunsaturated fatty acid (PUFA).

CONCLUSIONS

Biofuel from microalgae is believed to be able to help the world in becoming the alternative to fossil fuel. Rapid growth of microalgae gives them the advantage in producing biofuel. In this study, microalgae species were isolated from coastal regions of Kuantan and the species was identified as Scenedesmus sp. This species is believed to be the most suitable strain for biofuel production due to its high lipid content and productivity. In this study, it has been proven that the addition of iron to the Scenedesmus sp. culture can increase the growth of microalgae as well as produce biofuel compatible fatty acids.

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