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Research Article

Improvement in Oil Extraction from Microalgae/Algae for Biodiesel Production using Microwave Assisted Oil Extraction with Methyl Ester

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Abstract: The prospects of producing carbon-neutral biofuels from microalgae appear bright because of their unique features such as high CO_2-sequestering capability and ability to grow in wastewater/seawater/brackish water and high-lipid productivity. Extraction of lipids from microalgae/algae is still considered a challenging process due to the difficulties faced during extraction. The commercial production of microalgae biofuels including biodiesel is still not feasible due to the low biomass concentration and costly downstream processes. This study reports the solvent effectiveness of methyl ester (biodiesel) for microalgal lipid extraction together with microwave irradiation. Two co-solvent systems, BD20 (20% Methyl Ester and 80% Ethanol) and BD40 (40% Methyl Ester and 60% Ethanol) were experimented at time intervals of 5, 10 and 15 min, by Microwave-Assisted Extraction at 100 Watts. Microwave irradiation led to disruption of the algal cell walls which facilitated lipid extraction. Results were compared to another system, Chloroform 33% and Ethanol 67% by Microwave-Assisted Extraction as well as conventional Soxhlet extraction as the control. The results obtained from the experiment shows that BD40 has the highest lipid yield for all time intervals compared to BD20, Chloroform + Ethanol. When compared to the control, all of the samples that were extracted with the microwave had a higher lipid yield than Soxhlet Extraction.

Keywords: Co-solvent, lipid extraction, methyl ester, microalgae, microwave irradiation

INTRODUCTION

Today, different types of sources of energy are being used to meet the ever increasing demand of the 21st century. Most of the world's energy output is produced by fossil fuels. This type of energy is the most dominant as compared to the other types due to the availability of the technology and the dependability of the fuel in terms of energy output. According to "BP Statistical Review of World Energy" (2015), the Asia Pacific region accounted for the largest increment to global primary energy consumption and continues to account for the largest share (41.3% of the global total). The region accounted for over 71% of global coal consumption in 2014 and coal remains the region's dominant fuel. Renewable energy sources in power generation, accounted for 6.0% of global electricity in 2014. Global bioethanol production increased by 6.0%, while biodiesel production increased by 10.3% in 2014 (BP Statistical Review of World Energy, 2015).

Support for biomass-based fuels started to increase slowly as to reduce the reliance on petroleum imports by using resources available domestically (Raman and Mohr, 2014).

A concern with the production of first generation of biofuels (biofuels produced from food sources) was that it would require a lot of arable agricultural lands; which means lesser lands for food production, hence reducing food supply (Chisti, 2007; Alam *et al*., 2012). The second generation of biofuels consisted of agricultural waste residues. The issue with the second generation biofuel production was that it is not profitable as the expensive technologies including special use of pre-treatment processes are required to convert the biomass into fermentable sugars (Brennan and Owende, 2010). Algae and aquatic biomass has the potential to provide a new range of "third generation" biofuels, including jet fuels. This generation is an excellent alternative energy source because it is able to compensate for the flaws of both first and second generation of biofuels. Their high oil and biomass yields, widespread availability, absent (or very reduced) competition with agricultural land, their efficient use as a mean to capture CO_2 and their suitability for wastewater treatments make algae one of the most promising and attractive renewable sources for a fully sustainable and low-carbon economy portfolio

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(Demirbas, 2011; Abd Al Baky *et al*., 2014; Saifuddin *et al*., 2015). Microalgae also take considerably shorter harvesting cycle compared to crops that grow on land. This allows harvesting continuously throughout the year, which results in increased yields (Chisti, 2007; Muthu *et al*., 2011). There are, however, various technological and economic obstacles which have to be overcome before industrial-scale production of microalgae biodiesel can take place.

There are two types of lipids found in the Microalgae, which are the neutral and polar lipids. Phospholipids and glycolipids, which are both polar lipids, are found in the membrane structure of photosynthetic organisms. Mono, di and triglycerides are neutral lipids and are located inside lipid reserves organelles such as chloroplasts. The lipid content obtained using different lipid extraction techniques varies dramatically (Lohman *et al*., 2013) and this effects the yield of the products of transesterification. Algae-based biofuel can be commercialized on a larger scale with the development of a suitable cost-effective growth medium, low-energy-intensive harvesting method and effective lipid extraction method. Among the difficulties involved in commercial deployment of microalgal biofuel technology, cost effective and efficient extraction of lipids remains a major bottleneck. Extraction of oil from microalgal cells is an important and costly procedure, which often involves the use of toxic solvents. The development of an effective and energetically efficient lipid extraction process from the microalgal cells is critical for the successful upscaling of the downstream processes (Halim *et al*., 2011).

The conventional method for biodiesel production from algae begins with lipid extraction using organic solvents followed by transesterification of extract (Kasim *et al*., 2010). Nevertheless, a large amount of solvent is necessary for traditional oil extraction, which causes environmental pollution and increases costs. The use of solvent extraction requires extra energy input to recover the solvents and it has the potential to contaminate the algae solids, thereby restricting options for their end use (Lee *et al*., 2010). In recent years newer methods have been reported applying additional processes to enhance the extraction such as supercritical extraction, ultrasonic extraction, microwave extraction, high-pressure homogenizer extraction, hydrothermal liquefaction and solvent extraction (Iqbal and Theegala, 2013; Reddy *et al*., 2014; Bucy *et al*., 2012; Toor *et al*., 2013; Reddy *et al*., 2013). A brief review of the various extraction methods is given by Ranjith Kumar *et al*. (2015). Microwaves (MW) are non-ionizing radiation and a part of the electromagnetic spectrum with frequencies ranging from 300 MHz to 300 GHz corresponding to wavelength range of 1 mm to 1 m (Mishra *et al*., 2013). The basic mechanism of microwave heating involves agitation of polar molecules or ions that oscillate under the effect of an oscillating electric or magnetic field. In the presence of an oscillating field, particles try to orient themselves or be in phase with the field. Microwave processing has been suggested to provide a more uniform method of heating as the heating occurs due to the rotation of dipolar molecules and vibrations of ions in solution in an electromagnetic field. This mode of heating can reduce residence times, increase reaction rates and provide more accurate control of reaction conditions (Tsubaki *et al*., 2012). It was showed that the addition of halide salts within hydrothermal hydrolysis of cellobiose increases hydrolysis reaction of carbohydrates, results in a reduction of unwanted side reactions and energy consumption (Tsubaki *et al*., 2012).. It is therefore hypothesized that algae, which are naturally high in salts, could prove to be a promising feedstock for microwave processing. Microwave processing could either be used to facilitate extractions of valuable compounds such as lipids.

This study investigates the use of low toxicity solvent, which has the least potential of toxicity to human health and ecosystem. One of such solvents is biodiesel (methyl soyate: monoalkyl esters of fatty acids). Biodiesel is rapidly biodegradable and is a non-toxic solvent. Solvency power of biodiesel has been proved successfully in recent studies (Hu *et al*., 2004; Spear *et al*., 2007; Salehpour *et al*., 2009; Knothe and Steidley, 2011). The good solvency potential of biodiesel is due to the partial polar behavior imparted by carbonyl oxygen in alkyl-ester molecule (Knothe and Steidley, 2011). Very limited literature is available on the solvent potential of biodiesel for extraction purposes. The study will also investigate the co-solvent extraction system coupled with microwave assisted extraction using reflux system for lipid extraction from microalgae. This study will potentially contribute to the development of a rapid greener method of extraction of lipid which would reduce the cost, time, labor and very importantly avoiding thermal degradation of lipids.

MATERIALS AND METHODS

Materials: *Nannochloropsis* sp., microalgae (100 mL) was obtained from Algae tech Sdn Bhd in Bukit Jalil, Malaysia. The microalgae were cultivated in F/2 growth medium. Artificial seawater was prepared by mixing 1 L of reverse osmosis water with 33 g of Red Sea Salt. The water was stored in dark for 24 h at a room temperature. The water was autoclaved in 2-L Schott bottles. After cooling to room temperature the water was filtered with 0.22 μm membrane paper disc filters to remove any waste or plankton materials.

The growth medium used was F/2 medium. The chemicals used in the growth medium were of analytical grade. The F/2 medium is a slight

Table 1: F/2 concentrated nutrients media (reproduced from Saifuddin *et al*., 2015)

Number	Stock Solutions	Per L distilled water (dH_2O)	
S1	Nitrate stock solution $NaNO_3$	150.0 g	
S2	Phosphate stock solution $NaH_2PO_4.2H_2O$	11.3 g	
S3	Trace metals stock solution (mg/L)		Add each of the constituents to ~750mL dH_2O, mixing thoroughly between additions to dissolve. Finally made up to 1L
	$CuSO4.5H_2O$	19.6 mg	
	$ZnSO4.7H_2O$	44.0 mg	
	$CoCl2.6H_2O$	22.0 mg	
	$MnCl2.4H_2O$	360.0 mg	
	$Na_2MoO4.2H_2O$	12.6 mg	
S4	Fe citrate stock solution:	g / L	
	Ferric citrate ($FeCl_3.6H_2O$)	9.0 g	
	Citric acid	9.0 g	
S5	Vitamins stock solution		
	(i)Working Stock Solution	to 100 mL of distilled water, add the following	
	Biotin	1.0 mL primary stock	
	Vitamin B12	1.0 mL primary stock	
	Thiamine HCl	20.0 mg	
	(ii) Primary Stocks		
	Vitamin B12	10.0 mg /100 mL dH_2O	
	Biotin	10.0 mg /100 mL dH_2O	

modification of the original F medium, where it is prepared at half strength of the F medium (Guillard, 1975). The composition of F/2 concentrated nutrient medium is shown in Table 1. All stock solutions were stored in the refrigerator. To prepare Medium F/2 concentrated nutrients media; five milliliter of each stock solution (S1-S5) were mixed. The volume made up to 100 mL with distilled water. This was then filter sterilized using a 0.22 m filter into a sterile 250 mL Schott bottle. The F/2 growth medium was prepared by adding 1 mL of the F/2 nutrients media solution above to 100 mL of autoclaved and filtered (0.22 μm) artificial seawater.

Sodium bicarbonate solution (10%) was prepared by dissolving 10 g of $NaHCO_3$ in sufficient water to make the final volume of 100 mL. All organic solvents (n-hexane, chloroform, ethanol and methanol) were of analytical grade. The Biodiesel to be used as solvent was palm oil derived biodiesel purchased from Sime Darby (M) Sdn. Bhd.

Microalgae Strain and Culture Condition: The experimental cultures were grown using 2 L Schott flasks containing 1000 mL of F/2 growth medium. Fifty milliliters (mL) of *Nannochloropsis* sp. microalgae (obtained from Algaetech Sdn Bhd in Bukit Jalil, Malaysia) were cultivated in the F/2 growth medium as prepared previously. The cells were cultivated for 12 days. For all the studies, before inoculation the pH of each flask was adjusted to 7.6. Temperature throughout the culturing period was around at 25-28°C. Algae cultures were exposed to artificial fluorescent light (20 W at a distance of 25 cm). Light intensity was 60 μmol $m^{-2}sec^{-1}$ measured using a lux meter (YORCO Lux meter, YSI 606). The distance from the fluorescent light to the algal suspension was sufficient to minimize photo inhibition. Photo-period was divided into 12 h of light and 12 h of dark cycle. For soluble carbonate salts, 20 mL of the 10% bicarbonate solution was added. The cultures were hand shaken two to three times daily to avoid adherence of microalgae to the sides of the culture flasks and accelerate growth.

Growth monitoring: Growth was determined in terms of increase in optical density and biomass was estimated in terms of dry weight. Duplicate samples of 1 mL were collected at 24 h intervals and biomass concentration was determined by measuring optical density at 750 nm (Kwon *et al*., 2005), using a UV/VIS Spectrophotometer (Optizen Pop from Mecasys Co., Ltd., Daejeon, Korea). Known volumes of algae cell suspension after suitable dilutions (if the OD values were higher than 1.3, the sample was diluted with F/2 media) were used to determine the OD using UV/VIS Spectrophotometer at 750 nm (The OD obtained at this wavelength would not be interfered by chlorophyll absorbance). The actual OD was calculated by multiplying by the dilution factor. The dry weight was measured by filtering 20 mL of the culture sample using a pre-weighed 0.45 μm cellulose nitrate filter (Sartorius Stedim, Germany), rinsing with dH_2O and drying filter at 85°C in the oven and cooling in desiccators prior to weighing to determine the oven dry weight. All dry weight measurements were carried out in duplicate. To generate a standard curve of OD vs. dry weight, five serial dilutions were made from the stock and the OD at 750 nm measured. The dry weight was determined and the relationship between OD at 750 nm (y) and cell dry weight (x) of *Nannochloropsis* sp. microalgae was established. Estimations on dry weight were then calculated from the optical density measurements at other time points.

Preparation of extraction solvents: The first step in the extraction process involves the preparing the solvent systems to be used in the extraction of the lipid from the algae. Three types of solvent systems were prepared. Three extraction time intervals were chosen

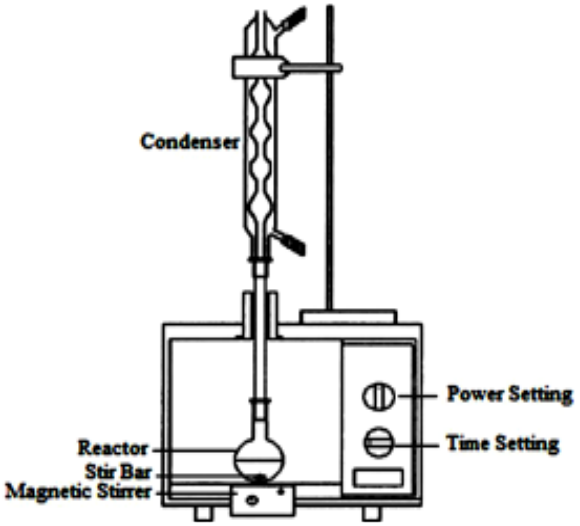

Fig. 1: The experiment setup for microwave assisted extraction process. Three solvent systems were used separately to extract the lipid

(5, 10 and 15 min). Two of the solvent systems contained biodiesel (palm oil biodiesel) as co-solvents, while the other one had no biodiesel. The 3 types of solvent systems were as follows:

BD20: This extraction solvent consists of 20% Biodiesel with 80% Ethanol. In a total volume of 50 mL, 10 mL of Methyl Ester was mixed with 40 mL Ethanol.

BD40: This extraction solvent consists of 40% Biodiesel with 60% Ethanol. In a total volume of 50, 20 mL of Methyl Ester was mixed with 30 mL Ethanol.

Chloroform+Ethanol: This extraction solvent consists of 33% Chloroform with 67% Ethanol. In a total volume of 50 mL, 17 mL of Chloroform was mixed with 33 mL Ethanol. This solvent system will be used to compare the difference in lipid yield between biodiesel based co-solvent with non-biodiesel co-solvent.

Microwave assisted extraction: The microwave as energy source was performed to produce biodiesel conducted in a batch reactor. The design of equipment used in this study is shown in Fig. 1. The reactor was a flat bottom flask made from Pyrex glass equipped with magnetic stirrer. The microwave irradiation was carried out using a domestic microwave oven (Samsung, CE2877N, S. Korea) with an operating frequency of 2450 MHz. The microwave can provide microwave radiation at variable power levels of 100, 180, 300, 450, 600 and 850 W and time setting from 0-30 mins. Approximately 8.0 g of wet paste of algae was loaded into a borosilicate bottle. In the first extraction 50 mL of BD20% solvent (20% Biodiesel with 80% Ethanol) was used. The paste was swirled slowly to allow it to mix completely with the solvent. The solution was reacted in a 500 mL reaction flask attached to a reflux set apparatus. The microwave oven was modified by making hole on the top of the casing to put the condenser. The power was set at 180 W and 5 min. The reaction mixture was stirred at 200 rpm. The process was repeated for different time setting (10 and 15 min) while keeping the power at 180W.

Two other extractions were performed using similar method but the solvent was BD40% (40% Biodiesel with 60% Ethanol) and Chloroform + Ethanol (33% Chloroform with 67% Ethanol). This extraction was also performed using the same exposure time to microwave irradiation of 5, 10 and 15 min at the power of 180W.

Post-extraction process: The extracts were transferred to 50 mL centrifuge tubes. To 10 mL of the extracts, 5 mL of DI-water and 5 mL of hexane were added to obtain a biphasic system. The extracts were then centrifuged with the Rotofix 32 (Hettick zentrifugen, Germany) at 3000 rpm for 5 min to fully separate the layers.

After the centrifugation process was complete, the bottom layer, containing water and ethanol, was removed and discarded. The separated top layer contained extracted lipids, with other solvents and chlorophyll contents was dissolved in hexane.

Soxhlet extraction: Conventional Soxhlet extraction of the algal oil was performed using the Soxhlet extractor (Konte, USA). The Soxhlet extraction was performed according to the method mentioned by Luque de Castro and García-Ayuso (1998). Briefly, 8.0 g of algal paste was placed in a cellulose extraction thimble (Whatman # 2800-338). The well-known co-solvent system which consisted of 50 mL of chloroform, 100 mL of ethanol and 40 mL of DI water was used. The extraction was performed for 7 h to achieve a complete extraction. The extracts were transferred to a stoppered graduated cylinder and 40 mL of DI water was added. The cylinder was inverted 30 times and allowed to settle for 1 h to recover the bottom layer containing lipids and chlorophyll dissolved in chloroform. The chloroform layer was transferred to a 250 mL flask to evaporate the excess chloroform using a rotary evaporator. The solvent was removed under vacuum at 400 mbar in a rotary evaporator (Eyela, N–N Series, Rikakikai Co. Ltd., Tokyo, Japan). The temperature was maintained at 45°C. The Soxhlet extraction was used to compare the yields of all the different solvent system in the microwave assisted extractions.

Chlorophyll *a* analysis: The last step in the experimental procedures is the analysis of the lipid.

Efficiency of extraction of all samples is evaluated in terms of lipid yield and by Chlorophyll *a* analysis.

Chlorophyll *a* was determined to evaluate the efficiency of the extraction method in terms of cell destruction. Chlorophyll *a* was determined using US EPA method 446.0, using Jeffrey and Humphrey's Trichromatic Equations (Arar, 1997). The UV–VIS Spectrophotometer (Optizen Pop from Mecasys Co., Ltd., Daejeon, Korea) was calibrated using a chlorophyll standard (MP Biomedicals, OH, USA; Catalog# 210221). Absorbance was measured at 750, 664, 647 and 630 nm. Chlorophyll *a* (mg/L of extract) was calculated according to the following equation:

$$Chl_a = 11{:}85\ (Abs_{664}–Abs_{750}) \\ –1.54\ (Abs_{647}–Abs_{750})\ –0.08\ (Abs_{630}–Abs_{750})$$

where, Chl_a is the concentration (mg/L) of chlorophyll *a* in the extracts, converted to mg/g dw.

Scanning Electron Microscope (SEM): The morphological changes of the cells during microwave irradiation were analyzed by SEM images of the biomass, taken using a scanning electron microscope (Joel, JSM-6610LV; Tokyo, Japan) as mentioned in Balasubramanian *et al*. (2011). Briefly, 5 mL of the cell suspension (before or after extraction) was fixed for 1 h with 5 ml of 4% glutaraldehyde and 2% formaldehyde solution in 0.2 M cacodylate buffer (pH 7.2). One mL of the mixture was diluted with 9 mL of 2% glutaraldehyde, 1% formaldehyde in 0.1 M cacodylate buffer solution. The solution was filtered through 5-lm pore polycarbonate filter and fixed for an additional 1 h. The filter membrane was rinsed with 0.1 M cacodylate buffer followed by DI water and then dehydrated in ethanol. The membrane was dried with liquid CO_2 in a Denton DCP-1 critical point dryer, mounted on aluminum SEM stubs, coated with gold: palladium (60:40) in an Edwards S150 (Crawley, England) sputter coater and imaged with JSM-6610 (JEOL Ltd., Japan) high vacuum mode SEM (Balasubramanian *et al*., 2011).

Fourier Transform Infrared Spectroscopy (FTIR) analysis: The Fourier Transform Infrared Spectroscopy (FTIR) IRPrestige-21 (Shimadzu Corporation, Japan) was used to investigate the functional groups of the extracted oil. The FTIR spectrometer is equipped with temperature controlled DLATGS (deuterated, L-alanine doped triglycerine sulfate) detector. The samples were then scanned with the settings as follows; resolution: 8/cm, accumulation: 20 scans, measurement mode: transmittance (T%), wave number 4000-650/cm. Each oil sample was dropped on top of an ATR (Attenuated Total Reflectance) crystal disc (Diamond Type II crystal) at ambient temperature (23±1°C). The ATR was cleaned with propanol after every sample analysis. All spectra were referenced against the background spectrum of air. IR resolution software (IR solution-window based version 1.4-Shimadzu) was used to analyze the spectra produced.

RESULTS AND DISCUSSION

Cell growth: Throughout the cultivation period, the strain supplied with 10% (v/v) bicarbonate (as dissolve CO_2 source) showed increasing growth, with a maximum dry biomass weight of 0.411 g/L at day 9 (Fig. 2). On the other hand, the control sample, which was allowed to grow at atmospheric CO_2 levels

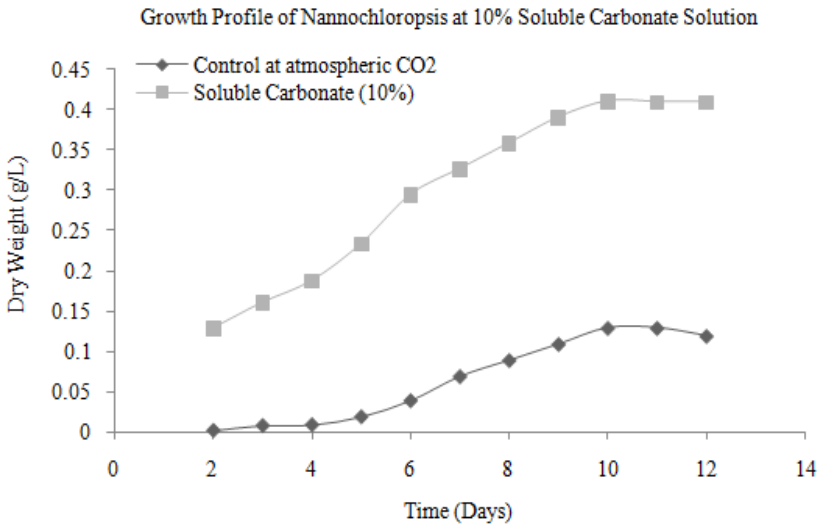

Fig. 2: *Nannochloropsis* sp. strain was cultivated with soluble carbonate concentrations of 10% (v/v). The OD at 750 nm was measured at different intervals and the biomass dry weights were estimated from standard graph of OD 750 nm vs dry biomass

Table 2: The amount of lipid extracted with BD20, BD40 and chloroform with ethanol using microwave assisted extraction and conventional Soxhlet extraction (n = 3)

	Lipid yield from microwave assisted extraction at different time (g/8 g wet algal paste)			Lipid yield from Soxhlet system
Solvent system	5 min	10 min	15 min	(7 h extraction)
BD 20 (20% biodiesel+80% Ethanol)	3.84±0.4 g	4.02±0.5 g	4.71±0.3 g	-
BD 40 (40% biodiesel+60% Ethanol)	4.22±0.5 g	4.51±0.7 g	5.23±1.5 g	-
Chloroform+Ethanol (33% Chloroform with 67% Ethanol)	3.84±0.7 g	3.92±0.8 g	4.02±0.2 g	3.71±1.1 g

(~0.04%) experienced poor growth, having maximum of 0.13 g/L biomass dry weight during the same period. It is inferred from the results that high CO_2 concentrations (10%) boosts photosynthetic efficiency, so that microalgae can reproduce within a shorter period with a greater quantity of biomass. It is generally accepted that increased atmospheric CO_2 concentrations can stimulate growth of many microalgal species (Tang *et al*., 2011). It has been known that the CO_2 level in ambient air (0.03%) is suboptimal for higher plants and algal growth. Although, most of the plants can tolerate up to 0.1 CO_2%, many microalgae species could tolerate to high CO_2 level up to 12.0% (Chiu *et al*., 2009). Previous result of *Nannochloropsis sp.* microalgae grown at 10 % level of carbon dioxide had shown higher levels of growth and lipid content (Saifuddin *et al*., 2015). Other reports also confirmed elevation of CO_2 level with increase in lipid biosynthesis in many microalgae species (Tang *et al*., 2011; Abd El Baky *et al*., 2014). It is thought that under high CO_2 concentration, biosynthesis of lipid compounds may be increased over other components, mainly proteins compounds, by fixing more carbon, as a reserve form of energy for the algal growth.

Microwave assisted extraction with biodiesel and ethanol co-solvent system: The recovery of lipid from *Nannochloropsis sp*. using 7 h Soxhlet extraction was approximately 46.3% of the total wet microalgal paste (w/w). Similar results were also obtained by Iqbal and Theegala (2013) which used the similar soxhlet extraction procedure. The Soxhlet extractions were used to normalize all microwave assisted extractions. Table 2 shows the amount of lipid extracted for all three solvent systems with microwave assisted extraction at time intervals 5, 10 and 15 minutes. For comparison, the control value is the amount extracted by the Soxhlet extraction procedure. The chloroform and ethanol solvent system in Microwave Assisted Extraction (MAE) with 15 min exposure yielded approximately 50% of lipid, which was slightly higher compared to that of the Soxhlet extraction. The solvent system containing 40% Biodiesel (palm methyl ester) and ethanol co-solvent (BD40), indicated better results than those of chloroform plus ethanol as well as Soxhlet extraction (Table 2). With the initial amount of 8 g of wet algal paste, the extraction with BD40 (for 15 min) produced 4.71±1.5 g of lipids compared to 3.71±1.1 g of the Soxhlet extraction (Table 2). The BD 40 solvent system with microwave assisted extraction yielded approximately 65.4% of lipid from 8 g of algal biomass. This is about 19.03 % higher relative to the Soxhlet method. Balasubramanian *et al*. (2011), recovered 77% of oil from microalgae *Scenedesmus obliquus* using continuous microwave system at 95°C with hexane. The data obtained suggest that increasing the biodiesel proportion in the solvent system and longer exposure of microwave irradiation lead to more efficient extraction. Previous studies have shown that chemical solvents method is by far the most commonly used, but less effective when microalgae are still wet (Samorì *et al*., 2010). It was reported that drying microalgae prior to lipid extraction could require 2.5 times more energy than a process without drying (Lardon *et al*., 2009). Using microwave irradiation seems to be the most promising techniques to increase the lipid yield of wet or dry microalgae biomass. As an example, Lee *et al*. (2010), increased the lipid extraction yield of *Botryococcus sp.* microalgae in water phase by 3 folds using a 5 min microwave pretreatment. Consequently, chemical solvent extraction can be stimulated in the presence of water in the microalgae biomass when treated with microwave irradiation.

The key aspect of microwave-assisted extraction is the good solvent contact during the whole extraction step and easy manipulation of the procedure. The procedure also overcomes the shortcomings of conventional Soxhlet by great reduction of the extraction time, minimizing environmental pollution due to the small amount of solvent released into the atmosphere, faster start-up and increased yield. Unlike conventional solvent extraction, heat and mass transfers occur in the same direction for microwave assisted extraction; from the inside of the material to the bulk solvent (Virot *et al*., 2008). In addition, completeness of analyte extractions not always achieved with conventional methods is assured by this approach due to reflux process in a closed vessel system. A dielectric or polar material introduced in a rapidly oscillating electric field, such as that produced by microwaves, will generate heat because of the frictional forces arising from inter- and intra-molecular movements (Amarni and Kadi, 2010). Intracellular heating results in the formation of water vapor, which disrupts the cells from within. This in turn leads to the electro oration effect, which further opens up the cell membrane. Thus, rapid generation of heat and pressure within the

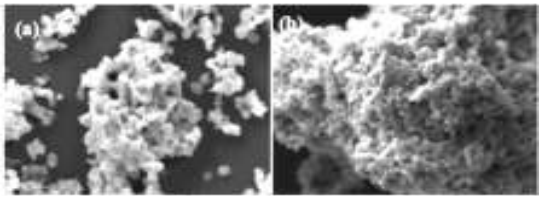

Fig. 3: SEM images of untreated *Nannochloropsis* (a) and microwave irradiated *Nannochloropsis* (b)

biological system forces out compounds from the cell matrix, resulting in the production of good-quality extracts with better target compound recovery. The chosen power (180 W) during MAE has to be set correctly to avoid excess temperatures, leading to possible solute degradation. The reflux system is able to increase the efficiency while using the low power microwave irradiation.

Scanning Electron Microscope (SEM) analysis of *Nannochloropsis* revealed that microwaving leads to more compact clustering of cells and individual cells became increasingly less recognizable (Fig. 3). The onset of cell disruption will have influence on the recoveries of different biochemical compounds such as lipids. Cell disruption by microwaves as seen by SEM analysis (Fig. 3) can lead to much higher recovery of lipids from microalgae than conventional solvent extraction alone. A study by Lee *et al*. (2010) identified microwave cell disruption as the most simple and efficient disruption method for the recovery of lipids form *Botryoccocus sp*., *Chlorella v*. and *Scenedesmus sp*.

The efficiency of microwaves can be very poor when either the target compounds or the solvents are non-polar. The dielectric constant of a solvent is a relative measure of its polarity. Water is a very polar molecule and has a dielectric constant of 80.1 at 20°C. On the other hand, n-hexane, being a very non-polar molecule, has a dielectric constant of 1.89 at 20°C. Diesel and biodiesel have dielectric constants of 2.2 and 3.35, respectively (Prieto *et al*., 2008; Sorichetti and Romano, 2005). The dielectric constant for biodiesel is close to conventional solvent chloroform (4.8). It has been reported that biodiesel has a potential to be an alternative solvent to n-hexane or chloroform in extraction of lipids (Iqbal and Theegala, 2013). However, solvent penetration power of biodiesel is low compared to n-hexane and chloroform (Spear *et al*., 2007; Hu *et al*., 2004). Therefore, use of biodiesel as co-solvent with another polar solvent like ethanol, improves the penetration power of biodiesel. Methyl esters (biodiesel), when used as a co-solvent with ethanol, readily absorb microwave energy to yield a hot solvent in contact with extracting materials, which improve the extraction efficiency. The amount of microwave absorbing inorganics, such as halide salts, present in the sample increases the efficiency of heating. The approach of using wet microalgae (with salt content) gives advantage of increase in heating by ionic conductance resulting in lower energy requirements to heat the reactants to the desired processing temperature. This provides saving in term of energy usage for the microwave process. The main disadvantages of conventional Soxhlet extraction include:

- The extraction time is long
- A large amount of solvent is used
- Agitation cannot be provided in the Soxhlet device to accelerate the process.
- The large amount of solvent used requires an evaporation/concentration procedure; and
- The possibility of thermal decomposition of the target compounds cannot be ignored as the extraction usually occurs at the boiling point of the solvent for a long time.

The FTIR analysis was performed as it is a reasonably fast method to determine the chemical bonding structure of the lipid sample extracted. FTIR scan was also performed on a control sample of sunflower oil (cooking oil). Figure 4 shows the scan of the lipid extracted using the microwave assisted extraction using the binary extraction solvent BD40 (40% biodiesel +60% Ethanol). The spectra (Fig. 4) have strong absorptions between 3000-2800/cm and also in the fingerprint region of 1500-700 cm^{-1}. The bands at around 710 and 3340/cm are *cis*-isomer, as expected from algal lipid. The absorptions observed in the region of 3000-2800 cm^{-1} are attributed to the asymmetric C-H stretching and symmetric C-H stretching of methyl and methylene groups. A characteristic broad band in the 3300-3000/cm region, assigned to the hydrogen bonded O-H of most carboxylic acids, overlaps the C-H stretching region. Common bands for all short-chain fatty acids in the finger print region were observed between 1400-930/cm indicating C-O-H in-plane bending, C-O stretching and O-H out-of-plane bending, respectively (Koca *et al*., 2007). A strong and broad absorption band in the region of 1860-1550/cm is characteristic of C = O stretching. The spectra for algal lipid extract shown here had similar characteristics to those found by Miglio *et al*., 2013.

As a comparison the FTIR scan for sunflower oil was also performed. A carbonyl stretch, C = O of a carboxylic acid appears with a higher intensity band appears at 1760-1690/cm as shown in Fig. 5. Bands present in the range 1400-1000/cm show characteristics of C-O stretching in esters. The characteristic broad band in the 3300-3000 cm^{-1} region, assigned to the hydrogen bonded O-H of most carboxylic acids is observed with much lower intensity compared to the algal lipid. This may also be indicative of the presence of triglycerides in these samples. All fatty acids chains in the finger print region were observed between 1400-930 cm^{-1} indicating C-O-H in-plane bending, C-O stretching and O-H out-of-plane bending, respectively (Koca *et al*., 2007). These bands had much lower intensities compared to the algal lipid.

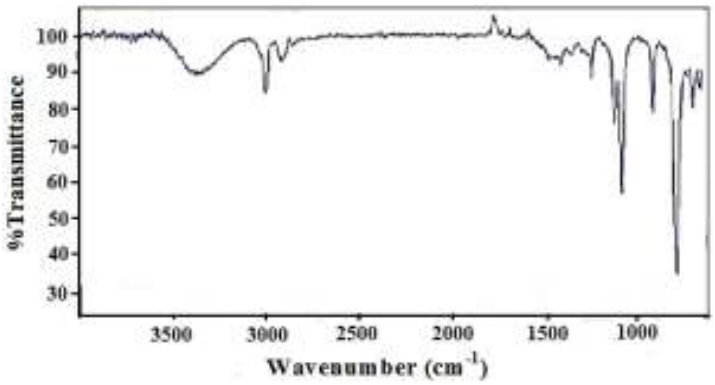

Fig. 4: FTIR scan of microwave extracted lipid by binary mixture of BD40 (40% biodiesel+60% Ethanol)

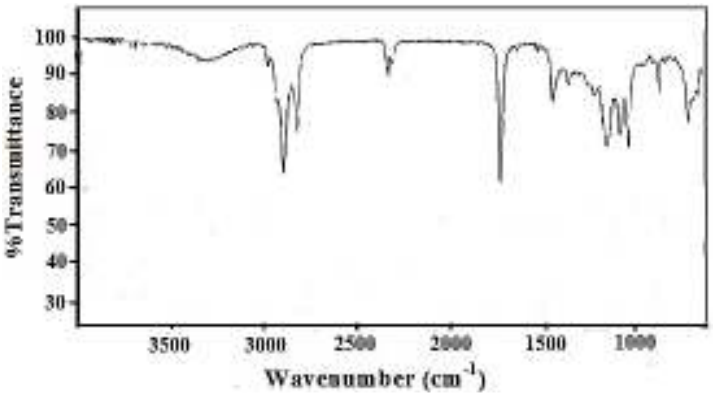

Fig. 5: FTIR scan of sunflower oil (cooking oil)

Table 3: The amount of Chlorophyll *a* content extracted with BD20, BD40 and chloroform with ethanol using microwave assisted extraction (n = 3)

	Chlorophyll *a* Yield from Microwave Assisted Extraction at Different Time (mg/8 g wet algal paste)		
Solvent System	5 min	10 min	15 min
BD 20 (20% biodiesel +80% Ethanol)	7.84±0.6 mg	8.13±0.5 mg	8.45±0.3 mg
BD 40 (40% biodiesel+60% Ethanol)	9.08±0.5 mg	9.21±0.7 mg	9.32±1.5 mg
Chloroform+Ethanol (33% Chloroform with 67% Ethanol)	7.11±0.7 mg	7.48±0.6 mg	7.72±0.4 mg

The amount of chlorophyll in algae ranges from 0.1 to 1.0% (Hosikian *et al*., 2010). Chlorophyll *a* was determined to evaluate the efficiency of the extraction method in terms of cell destruction. As shown in Table 3, the higher amount of chlorophyll *a* produced by BD40 solvent shows that the BD40 is a more efficient solvent than the BD20. Chlorophyll *a* concentrations in the extracts are adversely effected by high temperature. Exposure to heat may have caused degradation of thermally labile compounds. It has been found that cell disruption, achieved through grinding, homogenization, ultrasound or sonication, significantly improves the effectiveness of chlorophyll extraction (Macías-Sánchez *et al*., 2009; Cravotto *et al*., 2008; Balasubramanian *et al*., 2011). It has been reported that without cell disruption, only 25 - 40% of the potential chlorophyll *a* was able to be extracted by an optimal method (Cravotto *et al*., 2008; Balasubramanian *et al*., 2011).

Another major advantage of using the BD 40 (40% biodiesel +60% Ethanol) as extraction solvent for lipids from algae is when the extracted oils are converted to methyl ester (Biodiesel). As in usual lipid extraction, it is performed with solvents like n-hexane, a non-polar solvent; however, the use of biodiesel as the co-solvent reduces one important step of separating the extracted lipids for subsequent transesterification reaction where ethanol is used as one of the reactants to produce biodiesel. It has also been reported that the use of biodiesel as co-solvent for transesterification reaction can increase the reaction rate for solid acid catalyst system (Lam and Lee, 2010).

CONCLUSION

Reflux microwave-assisted Soxhlet extraction provides the following advantages:

- Substantial shortening of the extraction time (from 6 -8 h to 35 min).
- Use of samples as received, without the moisture adjustment usually required in conventional Soxhlet methods. Residual water in wet Algae becomes an excellent organic polar solvent which have excellent microwave absorption properties.
- Extraction efficiencies and precision comparable to or better than those provided by conventional Soxhlet extraction.
- Lower Energy Consumption (for Extraction) (no drying required) compared to conventional Heating.

Biodiesel, along with ethanol as a co-solvent system was found to yield comparable results to those of chloroform plus ethanol or conventional 8 h Soxhlet extraction. This study confirms that toxic solvents like hexane and methanol can successfully be substituted with relatively less toxic, environmentally acceptable and biodegradable solvents to extract oil from microalgae. Employing such a solvent system is anticipated to be comparatively economical; avoiding the need for separating the co-solvents from the extracted lipids, as all the solvents and lipids are involved in the transesterification reaction.

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