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## Sequestration of High Carbon Dioxide Concentration for Induction of Lipids in Microalgae for Biodiesel Production

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### ABSTRACT

Microalgae can provide solutions to the twin challenges of energy security and environmental pollution. They can capture carbon dioxide in the flue gas thereby reducing greenhouse gas and also producing algal biomass, which can be converted into biofuel. The present study evaluated the effects on growth and lipid content of *Nannochloropsis* sp., under reduced nitrogen content and enrichment with sodium carbonate/bicarbonate derived from flue gas. Studies have been conducted to identify and develop efficient lipid induction techniques in microalgae, such as nutrients stress (e.g., nitrogen and/or phosphorus starvation), osmotic stress, radiation, pH, temperature, heavy metals and other chemicals. Our results showed that the total lipid productivity of *Nannochloropsis* sp. cultures aerated with five different CO<sub>2</sub> levels (1, 10, 15, 20 and 25% CO<sub>2</sub>) had the highest lipid level of 18.93 mg L<sup>-1</sup> day<sup>-1</sup> for the 15% CO<sub>2</sub>. The growth using soluble carbonates was slightly better than when using only CO<sub>2</sub> gas as carbon source. The 20% flue gas solution gave maximum yield of dry biomass of 0.55 g, while the maximum yield of dry biomass for 15% dissolved CO<sub>2</sub> gas was around 0.44 g. In terms of nitrogen level; the concentration of nitrogen at 0.882 mM gave the best growth and biomass amount. Those with lower nitrogen had their growth and biomass reduced by almost 5 times. Lipid productivity increased when exposing the culture to nitrogen starvation condition. The 100% N cultures (0.882 mM) yielded the lowest lipid productivity, while the 25% N cultures (0.353 mM) showed the highest productivity of lipid. The cells that grew in the lowest nitrogen content showed slightly more than twice the productivity of oil production compared with the one with 100% N cultures. This work has described the favorable conditions for lipid production by *Nannochloropsis* sp. in respect to CO<sub>2</sub> gas, soluble carbonates and different nitrogen concentrations.

**Key words:** Lipid content, biodiesel, flue gas, CO<sub>2</sub> mitigation, *Nannochloropsis* sp.

### INTRODUCTION

Continuous and increasing consumption of petroleum based transportation fuels accounts for roughly one-third of CO<sub>2</sub> (a major greenhouse gas) emissions, which is thought to be a major contributor to global climate change (Owen *et al.*, 2010). Biodiesel produced from rapeseeds, corns, soybeans and palm oil has been rapidly developing in recent years as

one of the most promising biofuel. However, sustainable production of renewable energy is an issue that needs to be addressed as well. The first generation biofuels, primarily produced from food crops and mostly oil seeds, compete for arable land, freshwater or biodiverse natural landscapes and are limited in their ability to achieve targets for biofuel production. These concerns have generated the interest in developing second and third generation biofuels, such as

lignocellulosics and microalgae, respectively, which do not need to compete for arable land and precious freshwater (Schenk *et al.*, 2008; Chisti, 2007).

Algae for biofuels have been studied for many years for production of hydrogen, methane, vegetable oils (triglycerides, for biodiesel), hydrocarbons and ethanol. Algae has the advantages of rapid growth, high oil yield per unit area and can be cultivated on land that are unsuitable for cultivation. Microalgae are widely distributed and present high photosynthesis efficiency and favorable environment adaptability as opposed to oil crops and animal fat. In addition, some of the microalgae strains are able to accumulate significant amount of lipid within their cells, in which the lipid can be converted to biodiesel through transesterification reaction (Schenk *et al.*, 2008; Iqbal and Theegala, 2013). The microalgae have the ability to grow fast, produce large quantities of lipids, carbohydrates and proteins, thrive in poor quality waters, sequester and recycle carbon dioxide from industrial flue gases and remove pollutants from industrial, agricultural and municipal wastewaters (Chisti, 2007). The microalgae could be cultivated to help in the reduction of atmospheric CO<sub>2</sub> and as a promising source for biofuel production and other valuable added products. Depending on the type of microalgae species, the algae can produce different lipids, hydrocarbons and other complex oil content which is suitable for the production of biodiesel. The known total lipid content of microalgae varies from 5-77% and can yield 10-30 times higher the amount of biodiesel than any first generation feedstock crops (Gouveia and Oliveira, 2009; Yeh *et al.*, 2010). Therefore, microalgae could be used to develop strategies for CO<sub>2</sub> emissions mitigation as well as utilization for the replacement feedstock for the biofuel industry. Most recently, a strong initiative in bioenergy research has been taken up to investigate methods for enhancing productivity and metabolic processes for biomass production and biorefining of biomass for production of biofuels, energy and other added value products.

The growth of microalgae requires CO<sub>2</sub> as one of the main nutrients to carry out photosynthesis. Carbon dioxide sequestration refers to the removal or reduction of CO<sub>2</sub> from the atmosphere which is generated from fossil fuels being burned by industries. Flue gases generated from industrial power plants consist of nitrogen (N<sub>2</sub>), carbon dioxide (CO<sub>2</sub>), oxygen (O<sub>2</sub>), water vapor, minor amounts of carbon monoxide (CO), sulfur oxides (SO<sub>x</sub>) and nitrogen oxides (NO<sub>x</sub>). Among all these flue gases the most global environmental concern is the enormously increased amount of CO<sub>2</sub> concentration in the atmosphere. The CO<sub>2</sub> in flue gases from a coal-fired power plant can be fed directly to algae cultivated for biofuel production. Central to the concept of algal biofuels production is the use of power plant flue gas or a similar nearby available, enriched source of CO<sub>2</sub>. Different species have various CO<sub>2</sub> tolerances. High CO<sub>2</sub> concentration may result in growth inhibition while lower concentration could limit microalgae cell growth (Salih, 2011; Sobczuk *et al.*, 2000; Yang and Goa, 2003). Atmospheric CO<sub>2</sub> of 0.0387% v/v is too

low for microalgae growth, therefore it requires supplement with carbon sources. The carbon sources include CO<sub>2</sub>, H<sub>2</sub>CO<sub>3</sub>, HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> (Sobczuk *et al.*, 2000). Apart from carbon sources, light intensity is necessary for microalgae growth. Light is the limiting factor for both the microalgae growth and lipid composition. The growth of microalgae is directly proportional to the increased in light intensity, however, when the microalgae cells are exposed to a high light intensity for a long period it causes photoinhibition. For example, in a report by Liang *et al.* (2009), when *Chlorella vulgaris* was cultured using different light intensities ranging from 0-185 μmol m<sup>-2</sup> sec<sup>-1</sup>, the light intensity of 90 μmol m<sup>-2</sup> sec<sup>-1</sup> was the optimal and anything above that will cause photoinhibition. High lipid accumulation and biomass productivity are the two most desired features in algae for the feasibility of utilizing algal oil biodiesel production. However, various studies conducted under nutrient depleted (nitrogen deprivation) conditions have demonstrated that biomass productivity and lipid accumulation are negatively related (Li *et al.*, 2008). Optimizing oil production would therefore require a balance between supporting cell growth and stimulating lipid yields. Within this context, nitrogen depletion can be still considered as a good strategy for increasing lipid accumulation in algae (Spolaore *et al.*, 2006). There have been studies showing that electromagnetic fields can elicit *in vivo* and *in vitro* effects in many biological systems (Cellini *et al.*, 2008; Zrimec *et al.*, 2002). Increasing attention is being directed towards bio-electromagnetic stimulation of living cultures for biotechnology and bioenergy applications using the low frequency electromagnetic fields (EMF) (Breckenkamp *et al.*, 2003; Kappe and Stadler, 2005; Sheppard *et al.*, 2008).

The lipid content obtained using different lipid extraction techniques varies dramatically (Lohman *et al.*, 2013) and therefore, the products of transesterification of different extracts also differ. The conventional method for biodiesel production from alga begins with lipid extraction using organic solvents followed by transesterification of extract (Kasim *et al.*, 2010). Various methods are applied for extracting microalgae, including supercritical extraction, ultrasonic extraction, microwave extraction, high-pressure homogenizer extraction, hydrothermal liquefaction and solvent extraction (Iqbal and Theegala, 2013; Reddy *et al.*, 2013, 2014; Bucy *et al.*, 2012; Toor *et al.*, 2013). Nevertheless, a large amount of solvent is necessary for traditional oil extraction, which causes environmental pollution, increases costs and consumes much energy in the extraction process. To circumvent this problem, a method for direct transesterification of whole cells without extraction was developed (Wahlen *et al.*, 2011). Previous studies have demonstrated that transesterification of wet biomass in biodiesel processing saves the energy consumed in biomass drying and lipid extraction (Ehimen *et al.*, 2010). Griffiths *et al.* (2010) achieved the direct transesterification of wet biomass via the addition of a water scavenger to ensure anhydrous reaction conditions. Wet biomass transesterification

under supercritical conditions also has been explored (Reddy *et al.*, 2014). However, several milligrams or even larger amounts of lyophilized microalgae are usually demanded for such transesterification reactions. A transesterification approach that uses only micro-scale samples and can be applied to fresh cells would be valuable for daily fatty acid analysis in microalgal cultivation. Microwaves reveal characteristics of even and rapid heating, little consumption of solvents and short extraction time. Microwave irradiation have been used in the past and is a constructive method to extract oils from biomass, animal fats and vegetable feedstock (Pan *et al.*, 2002; Kiss *et al.*, 2000; Amaro *et al.*, 2011).

The present study will investigate, effect of cultivating *Nannochloropsis* sp., microalgae under different conditions to produce high-yield biomass with high lipid content, which would be converted into biodiesel fuel in tandem with the mitigation of high carbon dioxide concentration. The effects of using CO<sub>2</sub> gas at various percentages concentration and flue gas in the form of sodium bicarbonate/carbonate on the microalgae growth and lipid production were studied.

## 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 for 24 h prior to the experiment at a room temperature of 25°C, covered with a black PVC cover to exclude light and thereby growth of phytoplankton. The water was autoclaved in

2 L Schott bottles at 121°C and 1.03 bar (15 psi) for 15 min. 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 modification of the original F medium of Guillard and Ryther (1962), where it is prepared at half strength of the F medium (Guillard, 1975). The nitrogen concentration of 0.882 mM is 100% of the recommended recipe for preparing the standard culture F/2 medium and is used here for comparison and control.

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. This will give F/2 growth medium with nitrogen concentration (NaNO<sub>3</sub>) of 0.882 mM.

The effect of a lower nitrogen concentration of 0.705 mM (80% of the original recipe), 0.530 mM (60%), 0.353 mM (40%) was chosen for investigation in this study. To prepare variable nitrogen content F/2 concentrated nutrient media; five milliliter of each stock solutions (S2-S5) were mixed. Then 5.0, 4.0, 3.0 or 2.0 mL of S1 stock solution was added to create NaNO<sub>3</sub> concentrations of 0.882, 0.705, 0.530 or 0.353 mM. The F/2 growth media was prepared as mentioned above.

Table 1: F/2 concentrated nutrients media

Stock solutions	Per liter distilled water (dH <sub>2</sub> O)	
<b>S1</b>		
Nitrate stock solution NaNO <sub>3</sub>	150.0 g	
<b>S2</b>		
Phosphate stock solution NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	11.3 g	
<b>S3</b>		
Trace metals stock solution (mg L <sup>-1</sup> )		
CuSO <sub>4</sub> ·5H <sub>2</sub> O	19.6 mg	Add each of the constituents to ~750 mL dH <sub>2</sub> O, mixing thoroughly between additions to dissolve. Finally made up to 1 L
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	44.0 mg	
CoCl <sub>2</sub> ·6H <sub>2</sub> O	22.0 mg	
MnCl <sub>2</sub> ·4H <sub>2</sub> O	360.0 mg	
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	12.6 mg	
<b>S4</b>		
Fe citrate stock solution:	g L <sup>-1</sup>	
Ferric citrate (FeCl <sub>3</sub> ·6H <sub>2</sub> O)	9.0 g	
Citric acid	9.0 g	
Vitamins stock solution		
<b>S5</b>		
(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 <sub>2</sub> O	
Biotin	10.0 mg/100 mL dH <sub>2</sub> O	

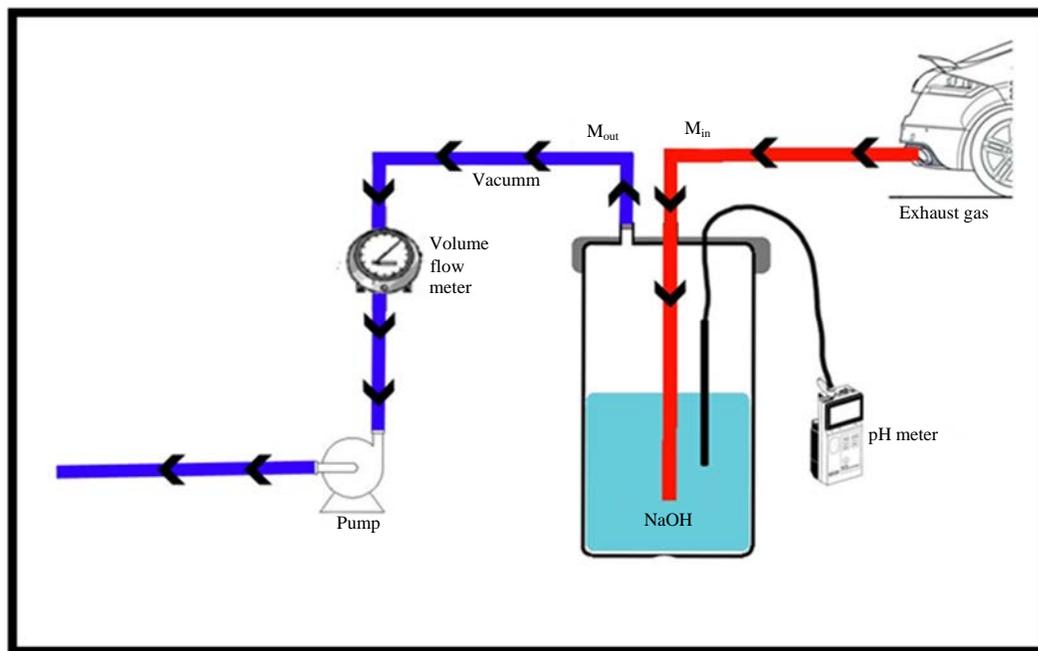


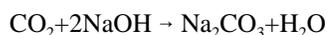
Fig. 1: Schematic diagram for flue gas collection from vehicle exhaust

Three factors studied on algae growth (biomass) and lipid production were CO<sub>2</sub> level, bicarbonate/carbonate concentration and nitrogen concentration. The response variables were quantified as Optical Density (OD), dry weight, specific growth rate and lipid production.

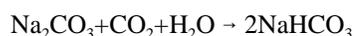
## Methods

### Production of sodium bicarbonate and sodium carbonate using carbon dioxide

**Captured from flue gas:** The flue gases emitted by motor vehicles constitute around 13-15% of CO<sub>2</sub> (Sakai *et al.*, 1995). Most of the microalgae species were found to tolerate SO<sub>x</sub> and NO<sub>x</sub> in the flue gas up to 150 ppm. The process of producing sodium bicarbonate can be adapted to convert CO<sub>2</sub> from flue gas of fossil fuel power systems (such as car) to bicarbonates. Sodium bicarbonate (NaHCO<sub>3</sub>) may be obtained by the reaction of carbon dioxide with an aqueous solution of sodium hydroxide. The initial reaction produces sodium carbonate:



Further addition of carbon dioxide produces sodium bicarbonate:



One liter Duran bottle containing 200 mL of 1 M sodium hydroxide (NaOH) solution was used to collect the flue gas from the exhaust of a 1.6 L Ford Fiesta Sport Vehicle. Prior to connecting the Duran bottle to the exhaust, the pH value of the NaOH solution was recorded. The setup for this process is

illustrated in the schematic diagram below (Fig. 1). The flue gas collection was stopped when the pH reached 6. In this work, no attempt was made to evaluate the efficiency of carbon dioxide capture or the amount of CO<sub>2</sub> captured, as it was not within the scope of this work. This flue gas solution with dissolved carbonate salts was used for further experiments.

### Cultivation of microalgae and effect of carbon dioxide concentration

**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 using the F/2 growth medium. For all the studies, before inoculation the pH of each flask was adjusted to 7.6.**

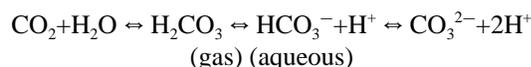
Temperature throughout the culturing period was around at 25-28°C. Algae cultures were exposed to artificial fluorescent light (20 W at a distance of 25 cm). Light intensity was 60 μmol m<sup>-2</sup> sec<sup>-1</sup> measured using a lux meter (YORCO lux meter, YSI 606). The distance from the fluorescent 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. 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.

Proper carbon source is very essential for growth of microalgae along with other nutrients. Carbon dioxide gas was supplied periodically to the cultures by maintaining a steady pH of the cultures. Cultures were aerated with gaseous CO<sub>2</sub>

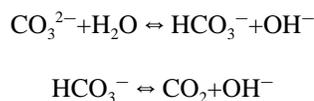
(99.9%) mixed with ambient air through an air diffuser (Aqua-Tech, Moorpark, CA) via bubbling from the bottom of the flask at 100 mL min<sup>-1</sup>. The flow rate of CO<sub>2</sub> was controlled using Omega FLD C3502ST flow meter. Several experiments were carried out to determine the effects of CO<sub>2</sub> concentration on biomass growth and lipid content. The strain was cultivated in five different flasks with varying amount of dissolved CO<sub>2</sub> concentrations of 1, 10, 15, 20 and 25% (v/v), which was measured using a dissolved CO<sub>2</sub> meter (Model 7001, Telaire-General Electric, California, USA). Higher than 25% of CO<sub>2</sub> concentration was not tested because the expected composition of CO<sub>2</sub> from the flue gas is about 15%. Parameters kept fixed for this experiment were growth medium concentration (per volume basis) and pH of the cultivation environment. At each carbon dioxide gas concentration a certain pH (measured using digital pH meter-Mettler Toledo, MP220) was reached. These certain pH values were maintained throughout the cultivation time by periodical addition of CO<sub>2</sub> gas daily for between 20-30 min. Biomass increase per day (mg L<sup>-1</sup> day<sup>-1</sup>), cell number increase per day and Optical Densities (OD) (Genesys 10 UV-Vis spectrophotometer-Thermo-Fisher) were taken daily for growth characteristics of the strain. The strain was checked for 12 days of growth period in varying amount of carbon dioxide gas.

**Cultivation using flue gas in the form of soluble carbonate salt solution:** The three principal forms of dissolved inorganic carbon are carbon dioxide (CO<sub>2</sub>), bicarbonate (HCO<sub>3</sub><sup>-</sup>) and carbonate (CO<sub>3</sub><sup>2-</sup>). Algae can utilize dissolved inorganic carbon such as CO<sub>2</sub> and bicarbonate (HCO<sub>3</sub><sup>-</sup>) and carbonate (CO<sub>3</sub><sup>2-</sup>) for photosynthesis.

The solution of atmospheric CO<sub>2</sub> into water and the corresponding equilibrium reactions are presented below:



The equation below shows what happens when carbonate and/or bicarbonate are added to water:



Further experiment was carried out using only inorganic carbon source from the flue gas solution containing soluble carbonate salt (sodium carbonate and sodium bicarbonate salt). For soluble carbonate salts growth study of the aforementioned strain, the flue gas solution (pH 6.0) was added in various quantities 20, 50, 100 and 150 mL (2, 5, 10, 15 and 20%) were added to five different flasks whose final volume was made up to 1 L by adding F/2 growth medium. The CO<sub>2</sub> gas was not added. All other conditions were as described earlier. The

flasks were corked with cotton wool to prevent contamination. To determine the effect of dissolved carbonate salts on the growth, 1 mL of algae was sampled by mixing the jar thoroughly and OD was determined daily using the spectrophotometer at a wavelength of 750 nm. The OD was determined in three replicate samples. Biomass dry-weight and lipid content were measured for 12 days of the growth period.

**Cultivation in growth media containing variable amount of nitrogen:** To study the effect of nitrogen deficiency stress on cell biomass and lipid accumulation, *Nannochloropsis* sp. cells were grown at different concentrations of sodium nitrate. The *Nannochloropsis* sp. were cultivated (10%, v/v) in the F/2 growth medium with the different concentration of sodium nitrate in media. The concentrations of sodium nitrate were 0.881, 0.661, 0.441 or 0.220 mM and prepared as described earlier. The cultures were supplied with flue gas solution containing soluble carbonate salt at 20% concentration as the carbon source. Cell growth, biomass weight and lipid content were measured for 12 days of the growth period. Each experiment was repeated three times.

**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 Perkin Elmer Lambda 35 UV/VIS Spectrophotometer (Perkin Elmer Inc. Massachusetts USA). Known volumes of concentrated cell suspension after suitable dilutions (if the OD values were higher than 1.3, the sample was diluted with F/2 media, so as to fall within the linear range of measurement) were used to determine the OD using UV/VIS Spectrophotometer at 750 nm. The actual OD was determined by multiplying by the dilution factor. The OD obtained at this wavelength would not be interfered by chlorophyll absorbance. 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<sub>2</sub>O and drying filter at 90°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. Standard curve of OD vs. dry weight was generated at specified times in the growth cycle. Five serial dilutions were made from the stock and the OD at 750 nm measured in duplicate. 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.

**Lipid assay:** Determination of lipid was carried out using the spectrophotometric method as described by

Table 2: Lipid assay reagents from Chen and Vaidyanathan (2012)

Reagents	Comments
25% methanol in 1 M NaOH (R1)	Saponification
Chloroform: methanol (2:1, v/v) (R2)	Solvent extraction
1 M Triethanolamine: 1 M acetic acid (9:1, v/v) (R3)	Complex formation
1 M Triethanolamine: 1 M acetic acid: 6.45% (w/v) Cu(NO <sub>3</sub> ) <sub>2</sub> ·3H <sub>2</sub> O (9:1:10, v/v) (R4)	Complex formation
Mix 0 = R1+R2+R3	Measurement
Mix 1 = R1+R2+R4	
Mix 2 = Samples+R2+R3	
Mix 3 = Samples+R2+R4	
$Abs_{\text{samples}} = Abs_{\text{mix3}} - Abs_{\text{mix2}}$	

Chen and Vaidyanathan (2012). The protocol was followed without any modification. Table 2 below summarizes the reagent preparation procedures.

Briefly, it consists of cell lysis, saponification of lipids, extraction of FA and the formation of Cu-FA complexes. For each experiment performed, 1.5-2 mL was collected on the first and last day of culturing. These algal suspensions were centrifuged at 12,000 rpm for 10 min in an Eppendorf 5424 centrifuge (Eppendorf, Hamburg, Germany) and cell pellets were stored at -20°C until lipid extraction. Next, the harvested cell pellets were dissolved in 20 µL phosphate buffer (0.05 M, pH 7.4). To this 0.48 mL of R1 (25% methanol in 1 M NaOH) and 0.25 mL of 400 µm glass beads (Sigma-Aldrich) were added and the cells were disrupted by bead-beating using a vortex mixer for 10 min. This was followed by addition of another 0.5 mL of R1 and the mixture was saponified at 100°C for 30 min (shaking, every 5 min) to release fatty acids. After saponification, the mixture was allowed to cool to room temperature and an aliquot (600 µL) of sample was introduced to an eppendorf tube containing 900 µL of a solvent mixture R2 (chloroform/methanol, 2:1 v/v) and vortexed for 2 min. The mixture was centrifuged at 12,000×g for 2 min. Then, 200 µL of the organic phase was transferred to two separate tubes, one containing 200 µL of R3 (TEA) and the other 200 µL of R4 (Cu-TEA), respectively. The mixture was vortexed for 2 min again and centrifuged at 12,000×g for 2 min. Finally, the organic phase was transferred to a quartz cuvette and directly read at 260 nm in a UV/visible spectro-photometer (GENESYS 10 UV-Vis spectrophotometer-Thermo-Fisher). Background absorbance correction was performed for the sample with the copper-less complexing reagent (Mix 2). This absorbance (TEA-FA blank) was subtracted from the absorbance of the sample (Mix 3) that contained the copper (Cu-TEA-FA), in order to obtain the absorbance due to the fatty acids in the microalgal sample.

The FA standards (Shikimic acid, sodium decanoate, sodium dodecanoate, sodium palmitate and linoleic acid from Sigma-Aldrich) were used as standards. These five fatty acids of carbon length C7-C18 were prepared as 5 mM stock solutions in a solvent mixture of chloroform and methanol (2:1). Serial dilutions were prepared and 300 µL of each dilution was subjected to quantification as described above.

**Production of biodiesel from wet microalgae through direct transesterification using microwave irradiation:** The microalgae were harvested using the centrifugation method, at a speed of 2000×g for 1 h. Four milliliter of chloroform, 4 mL of methanol and 0.2 mL of 1 M sulphuric acid were added into the harvested paste and mixed thoroughly (Cheng *et al.*, 2013). Subsequently, the contents were transferred into glass vials for microwave pre-treatment using Samsung Microwave Oven (2.45 GHz). The microalga cell paste was heated via microwave irradiation for 1 min at 180 Watts (W). The paste was then shaken and allowed to cool for 1 min. These two steps were then repeated again for another 9, 14 and 24 cycles (microwave treatment for 15, 30 and 45 min, respectively).

The organic layer containing the biodiesel was again transferred into centrifuge tubes to separate the layer containing biodiesel from other aqueous layers. 10 mL of distilled water was added to the organic layer before centrifuging at 6000 rpm for 1 h. The significance of this step is so that to ensure that the aqueous phase was completely removed. Finally, the top-most layer of the centrifuge was removed using a dropper for further analysis.

**Fourier transform infrared spectroscopy (FT-IR):** Fourier Transform Infra-Red Spectrometry (FT-IR) were used to determine the suitability of *Nannochloropsis* sp. lipid to be used as a biodiesel feedstock. For the FT-IR analysis, the instrument model used was Shimadzu IRPrestige-21 (Shimadzu Corporation Japan) equipped with temperature controlled DLATGS (deuterated, L-alanine doped triglycine sulfate) detector. The scan settings were set as follows; resolution: 4 cm<sup>-1</sup>, accumulation: 20 scans, measurement mode: Transmittance (T %), wave number 4000-650 cm<sup>-1</sup>. In a typical analysis, a drop of sample (using a Pasteur pipette) was deposited on the surface of the horizontal Attenuated Total Reflectance (ATR) crystal disc (Diamond Type II crystal) at controlled ambient temperature (23°C). A background measurement of air spectrum was performed. Spectra were processed using IR solution-window based software version 1.4 (Shimadzu). After every scan, a new reference air background spectrum was taken. After each analysis, the sample was removed with dry tissue and the surface of crystal disc was washed with acetone and finally, it was dried and cleaned with tissue.

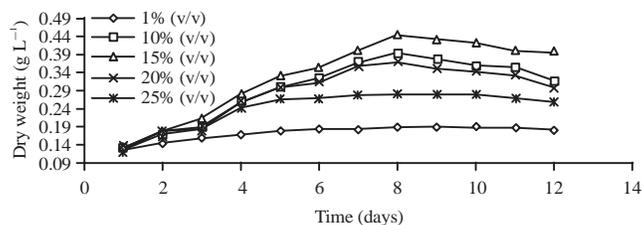


Fig. 2: *Nannochloropsis* sp. strain was cultivated in five different flasks with CO<sub>2</sub> concentrations of 1, 10, 15, 20 and 25% (v/v). The OD at 750 nm was measured at different intervals and the biomass dry weights were estimated from standard graph of OD 750 nm vs dry biomass

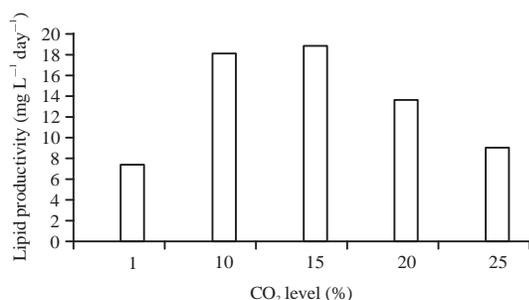


Fig. 3: *Nannochloropsis* sp. strain was cultivated in five different flasks with CO<sub>2</sub> concentrations of 1, 10, 15, 20 and 25% (v/v). The total lipid was determined after 8 day for each cultured using the spectrophotometric method

## RESULTS AND DISCUSSION

**Growth of *Nannochloropsis* sp. with different concentrations of CO<sub>2</sub>:** As seen in Fig. 2, the effect of CO<sub>2</sub> on growth was clearly observed. The ideal CO<sub>2</sub> concentration for *Nannochloropsis* sp. growth is 15% (v/v), which produced the highest dry biomass weight of 0.441 g L<sup>-1</sup> at day 8. Throughout the cultivation period, the strain supplied with 10% (v/v) also showed increasing growth, with a maximum dry biomass weight of 0.392 g L<sup>-1</sup> at day 8. On the other hand, the control sample, which contained 1% (v/v) CO<sub>2</sub>, experienced poor growth, with a relatively constant growth curve. However, cultures provided with 20 and 25% (v/v) CO<sub>2</sub> initially showed increasing growth, but the growth level slowed after 8 days. Their growth levels were much lower than that of (15% CO<sub>2</sub>), indicating that 15% CO<sub>2</sub> is somewhat saturation point and any higher amounts of CO<sub>2</sub> may not be beneficial. The maximum biomass growth at day 8 was 0.19 g L<sup>-1</sup> (1% CO<sub>2</sub>), while the maximum for 15% (v/v) CO<sub>2</sub> was 0.441 g L<sup>-1</sup>.

According to researchers, atmospheric CO<sub>2</sub> levels [~0.0387% (v/v)] are not adequate to support the high microalgal growth rates and productivities needed for full-scale biofuel production (Kumar *et al.*, 2010). Since, waste gases from combustion processes typically contain

>15% (v/v) CO<sub>2</sub> (which is the optimum concentration for microalgal growth shown from this study), industrial flue gas can be used as a source of CO<sub>2</sub> for large-scale microalgae production. Other investigations have discovered that the specific growth rate of *Nannochloropsis* sp. was increased from 0.33-0.52/day (increased by 58%) when supplied with atmospheric air with 15% CO<sub>2</sub> (Jiang *et al.*, 2011; Kaewkannetra *et al.*, 2012). Correspondingly, at CO<sub>2</sub> concentrations of 15%, maximum biomass of 2.3 g L<sup>-1</sup> was achieved. Thus, it is inferred from all the results that high CO<sub>2</sub> concentrations 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<sub>2</sub> concentrations can stimulate growth of many microalgal species. It is also important to note that certain algae strains have the ability to adapt to the high CO<sub>2</sub> concentrations (Tang *et al.*, 2011). The growth enhancement in these algae by elevated CO<sub>2</sub> can be attributed to accelerated photosynthetic carbon fixation during increased CO<sub>2</sub> availability (Jiang *et al.*, 2011; Xia and Gao, 2005). It has been known that the CO<sub>2</sub> level in ambient air (0.03%) is suboptimal for higher plants and algal growth. Although, most of plants can tolerate up to 0.1 CO<sub>2</sub> %, many microalgae species could tolerate to high CO<sub>2</sub> level up to 12.0% (Chiu *et al.*, 2009). However, the algae growth can be affected by very high CO<sub>2</sub> levels and the concentration of dissolved oxygen evolved by photosynthesis pathway.

Lipid productivity increased at 10% level of carbon dioxide and this effect is shown in Fig. 3. The total lipid productivity of *Nannochloropsis* sp. cultures aerated with five different CO<sub>2</sub> levels (1, 10, 15, 20 and 25% CO<sub>2</sub>) was determined after 8 days of cultivation (Fig. 3). The results revealed that high lipid level were 18.93 mg L<sup>-1</sup> day<sup>-1</sup> for the 15% CO<sub>2</sub>. This result can be correlated to the dry mass amount. There are no significant differences between 10% and 15% CO<sub>2</sub> on the lipid weight. The 20% CO<sub>2</sub> level shows slight lower lipid content and even much lower for 25% CO<sub>2</sub>. It is thought that under high CO<sub>2</sub> 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. This result is also

confirmed by other reports that have correlated elevation of metabolic of CO<sub>2</sub> with increase in lipid biosynthesis in many microalgae (Tang *et al.*, 2011; Abd El Baky *et al.*, 2014). Similarly, it is also possible that the high lipid content found in many species of algae is a result of an adaptive strategy for mitigation of CO<sub>2</sub> in its natural environment. The values of total lipid productivity obtained in this work were within the ranges of many other total lipid productivity values reported in the literature for microalgae species that grew under the same conditions (Yoo *et al.*, 2010; Mandal and Mallick, 2009). From the perspective of fuel applications, the lipid productivity seems to be more important performance index due to its usage for production of biodiesel from microalgal (Griffiths and Harrison, 2009).

**Effect of using flue gas in the form of soluble carbonates on the growth and lipid content of *Nannochloropsis* sp.:**

Microalgae can absorb carbon directly from the dissolved CO<sub>2</sub> or in the form of soluble carbonates in the culture medium. Dissolved inorganic carbon, such as free CO<sub>2</sub>, bicarbonates and carbonates, are the carbon sources for the growth of microalgae via photosynthesis. Microalgae have higher photosynthetic efficiency than terrestrial plants because of the active transport system of inorganic carbon through an enzyme carbonic anhydrase. This mechanism enables algae to utilize carbon dioxide in various forms (Xia and Gao, 2005). Under normal condition, the growth of algae is slow due to the limited availability of CO<sub>2</sub>, because of the relatively low atmospheric CO<sub>2</sub> concentration (less than 400 ppm) and coupled with relatively low amount of CO<sub>2</sub> gas able to dissolved in water (i.e., 1.25 g L<sup>-1</sup> at 30°C and 1 atm) (Putt *et al.*, 2011). The algae growth can be promoted by feeding with exhaust gases (flue gases) from industrial plants, which significantly increases the CO<sub>2</sub> bioavailability for algae. The flue gas containing CO<sub>2</sub>, has a drawback if it is directly fed to the algae culture system, because of the poor absorption efficiency of CO<sub>2</sub> in water. In this study, the ability of algae to grow with CO<sub>2</sub> and carbonate salt (sodium bicarbonate/carbonate from flue gas) was investigated. Soluble carbonates solution (from flue gas solution prepared) was added in varying amounts.

The results show that the addition of flue gas solution was able to support the growth of *Nannochloropsis* sp. This was indicated by the increase in chlorophyll (increase in green colour of the culture) produced from day 2-10. As seen in Fig. 4, there is a positive increase on the dry biomass weight as the concentration of the flue gas solution (HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup>) increased. The growth rate increased by almost 5 times as the HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> concentration increased from 2-20% of the flue gas solution concentration in the media. It is also interesting to note that the growth using soluble carbonates is slightly better than when using only CO<sub>2</sub> gas as carbon source. The 20% flue gas solution gave maximum yield of dry biomass of 0.55 g, while the maximum yield of dry biomass

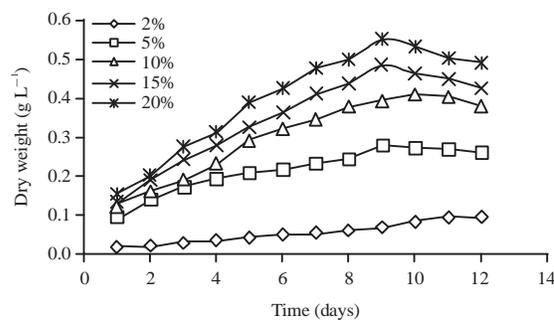


Fig. 4: Comparison of growth curves of microalgae *Nannochloropsis* sp. cultured in an enriched solution with variable amount of sodium bicarbonate/sodium carbonate from flue gases

for 15% dissolved CO<sub>2</sub> gas was around 0.44 g. These results shows that microalgae culture with carbon source as sodium bicarbonate/carbonate promote better algal growth of *Nannochloropsis* sp. compared in cultures with carbon source using only dissolved CO<sub>2</sub> gas. Similar results were also reported by other researches, where there was a 13-45% better biomass productivity of algae in media supplemented with NaHCO<sub>3</sub> as compared with those with just CO<sub>2</sub> gas as carbon source (Elvira-Antonio *et al.*, 2013; Sirakov *et al.*, 2013; Nayak *et al.*, 2013; Prabakaran and Ravindran, 2012).

The capture of CO<sub>2</sub> emitted from industries by alkali absorption and stored in liquids as HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> is an alternative way to support the growth of microalgae. It has been shown that carbonic anhydrase is essential to use organic carbon and this capacity is stimulated by low concentrations of CO<sub>2</sub> gas and alkaline pH. However, it has been reported that the levels of susceptibility are dependent on the algal-species (Xia and Gao, 2005). This enzyme is also produced in larger amounts by many microalgae when grown under limited CO<sub>2</sub> conditions. The CO<sub>2</sub> from the flue gas could be directly fed into microalgae culture for biofixation but sometimes it is difficult to obtain a stable and consistent supply of CO<sub>2</sub> unless the location of microalgae cultivation system is very close to a factory or power plant. So, one of the alternative ways to prepare a large amount of inorganic carbon source for microalgal growth is to capture the CO<sub>2</sub> emitted from the industries by alkali absorption and stored it in the liquid as HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> ions (Nayak *et al.*, 2013).

The lipid productivity and the effect of varying amounts of flue gas solution can be seen in Fig. 5. Higher amount of the flue gas solution was found to increase the lipid productivity of *Nannochloropsis* sp. with light of 60 μmol m<sup>-2</sup> sec<sup>-1</sup> and high soluble carbonate/bicarbonate systems, the microalgae performed well, producing higher lipid productivity. The 20% flue gas solution gave maximum yield of lipid productivity and it was 27% higher than that produced by the 2% flue gas solution. As reported from previous research studies, an

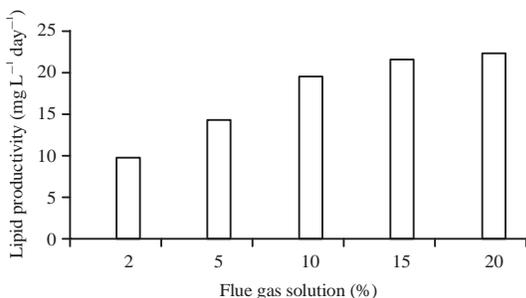


Fig. 5: Comparison of lipid productivity microalgae *Nannochloropsis* sp. cultured in an enriched solution with variable amount of sodium bicarbonate/sodium carbonate from flue gases

increase in carbon source helps accumulation of higher lipid contents in microalgae cells (Sharma *et al.*, 2012). The ability of microalgae to survive in diverse and extreme conditions is reflected in the tremendous diversity of cellular lipids obtained from these microalgae. Essentially, microalgae biomass and triglycerides compete for photosynthetic assimilate and a reprogramming of physiological pathways is required to stimulate lipid biosynthesis. Under stress conditions many microalgae alter their lipid biosynthetic pathways towards the formation and accumulation of neutral lipids (20-50% dry cell weight), mainly in the form of triglycerides (Schuhmann *et al.*, 2012; Sharma *et al.*, 2012).

**Effect of nitrogen concentration on growth and lipid accumulation of *Nannochloropsis* sp.:** There exists a great challenge, when it comes to maximizing oil production in microalgae. The production of fatty acids by photosynthetic microalgae cultures are sensitive to a number of environmental factors, including temperature, nitrogen concentrate and light intensity. A high lipids concentration is achieved when the algae are under environmental stresses, in particular nutrients limitation (Huntley and Redalje, 2007). Nitrogen is the single most critical nutrient affecting lipid metabolism in algae. A general trend towards accumulation of lipids, particularly triglycerides, in response to nitrogen deficiency has been observed in numerous species or strains of various microalgae (Yeh and Chang, 2012; Praveenkumar *et al.*, 2012). Moreover, all the microalgae species studied so far seem to increase triglycerides production under nitrogen stress (Sharma *et al.*, 2012). A total of four nitrogen concentrations using NaNO<sub>3</sub> were employed (0.882, 0.705, 0.530 or 0.353 mM). The Optical Density (OD) were taken at 750 nm at different intervals up to 8 days and was related to dry weight as described before. The highest biomass dry weight under lowest nitrogen regime was 0.121 g L<sup>-1</sup>, compared with 0.431 and 0.380 g L<sup>-1</sup> for medium with highest and second highest nitrogen regimes as shown in Fig. 6. As expected, the

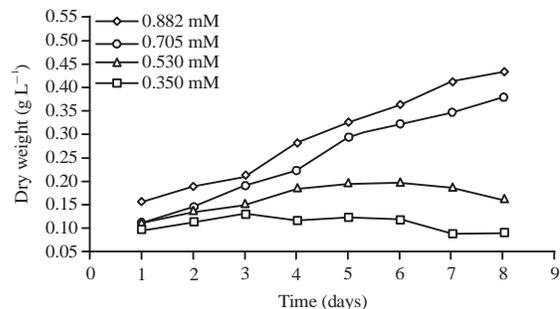


Fig. 6: *Nannochloropsis* sp. strain was cultivated in four different flasks with nitrogen content as NaNO<sub>3</sub> of concentrations of 0.355, 0.53, 0.705 and 0.882 mM. Carbon source was flue gas solution at 20% concentration. The OD at 750 nm was measured at different intervals and the biomass dry weights were estimated from standard graph of OD 750 nm vs dry biomass

concentration of nitrogen at 0.882 mM gave the best growth and biomass amount. Those with lower nitrogen had their growth and biomass reduced by almost 5 times. These results confer the importance of nitrogen composition in the medium to the growth and biomass production of the microalgae. When the nitrogen is exhausted from medium culture, the cell-nitrogen is used in enzymes and essential cellular structures and a portion of the carbon dioxide content is converted to lipid and carbohydrates (Liu *et al.*, 2008; Liang *et al.*, 2006).

Other studies suggest that because chlorophyll is a nitrogen-rich compound, it is possible that the nitrogen exhausted from medium causes that the cells to use chlorophyll-nitrogen for the synthesis of cell material for further division and/or growth (Zhila *et al.*, 2005). However, it should be noted that the chlorophyll is an essential component of the photosynthetic mechanism, which is responsible for the capture of CO<sub>2</sub> to generate energy and metabolic fluxes for both growth and accumulation of lipids, therefore, low critical levels of chlorophyll may affect the algal-growth. Nitrogen is the most growth-limiting factor for eukaryotic microalgae and would be one of the first nutrients to be depleted during algae cultivation. It has been observed that in low concentration of nitrogen source, all algae were able to grow, but growth was limited. It has been commonly noticed that when a nutrient element is insufficient for the protein synthesis required by growth, excess carbon from photosynthesis would be channeled into such storage molecules as triglycerides. Environmental stress condition, when nutrients are limited, invariably cause a steadily declining cell division rate. Surprisingly, active biosynthesis of fatty acids is maintained in some algae species under such conditions, provided there is enough light and CO<sub>2</sub> available for photosynthesis.

Figure 7 shows the lipid productivity obtained at the end of 15 days during normal nutrition and during nitrogen starvation incubation of *Nannochloropsis* sp. (CO<sub>2</sub> gas level was 10%). The 15 days incubation was employed since it had been previously reported that longer time of nitrogen starvation results in higher accumulation of lipid inside the cell (Widjaja *et al.*, 2009). As shown in Fig. 7, lipid productivity increased, when exposing the culture to nitrogen starvation condition. Since, accumulation of lipid occurs at nitrogen depletion condition under which the growth was much slower or even no growth was encountered, compromising between increasing lipid content and harvesting time was necessary to obtain higher values of both the lipid content and lipid productivity. The 100% N cultures (0.882 mM) yielded the lowest lipid productivity, while the 25% N cultures (0.353 mM) showed the highest productivity of lipid. The cells that grew in the lowest nitrogen content showed slightly more than twice the productivity of oil production compared with the one with 100% N cultures. Consistent with many other studies conducted under nutrient stress, the cell growth was arrested and the lipid content exhibited a rise compared with the control (Illman *et al.*, 2000). The results suggest that the nitrogen starvation strategy can result in the most favorable lipid productivity.

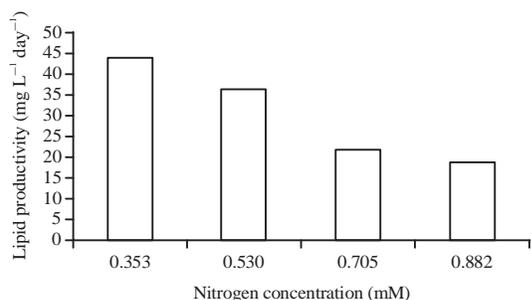


Fig. 7: *Nannochloropsis* sp. strain was cultivated in four different flasks with nitrogen content as NaNO<sub>3</sub> of concentrations of 0.355, 0.53, 0.705 and 0.882 mM. Carbon source was flue gas solution at 20% concentration. The lipid productivity was calculated as total lipid (mg) obtained per volume (L) of culture per total incubation time of 15 days

**One-step direct transesterification and the enhancement of transesterification reaction using microwave irradiation:**

The two-step method of traditional solvent extraction and transesterification usually consumes a tremendous amount of energy because of algae dewatering and grinding into powder. Algae drying process accounts for 84% of the total energy consumption for biodiesel production from microalgae. The one-step method directly extracts the algal lipids and converts them into biodiesel through the chemical process of transesterification. This approach processes dry algae into biodiesel, employing a supercritical methanol solvent with reaction time of 4-5 min (Patil *et al.*, 2011). The application of microwave in direct transesterification process is proved to be feasible. The in-situ transesterification simplifies the production cycle of biodiesel, since it eliminates the extraction process in the conventional two-steps method. The dewatering and grinding in the extraction process which are tedious and energy-intensive have been removed. The microwave treatment at 30 min showed a great improvement in the properties compared to that at 15 min. The curve of 45 min showed almost similar trend as that of 30 min. Therefore, the optimization of the microwave enhancement in the single transesterification process occurred at 30 min (Fig. 8).

Table 3 showed the FT-IR spectra in relation with specific groups and the wave number range that it occurs. Each peak in the FT-IR spectra was assigned to a functional group. Carbohydrates and lipid were characterized by vibration of the C-H bonds at 2839 and 2949 cm<sup>-1</sup>, C-O-C of polysaccharides at 1012 cm<sup>-1</sup>, respectively (Ponnuswamy *et al.*, 2013).

The samples are characterized by strong peaks at 1450 cm<sup>-1</sup> which shows the bending of methyl lipids. The protein spectra of *Nannochloropsis* sp. was characterized by the strong peaks at 3251 and 1643 cm<sup>-1</sup> (amide I), which were due to C = O combined with N-H and C-H stretching vibration. The presence of methyl peak indicates the presence of methyl ester (biodiesel). The other major peaks found were 2839 and 2949 cm<sup>-1</sup>. These peaks show the presence of methyl and methylene groups, which is an indication of methyl ester formation. From these results, it is concluded that the optimum time of microwave treatment for *Nannochloropsis* sp. for biodiesel production is 30 min.

Table 3: FT-IR spectra and its assigned functional groups

Bands	Main peak (cm <sup>-1</sup> )	Typical band	Wave number range (cm <sup>-1</sup> )
1	3251.98	Water (O-H) stretching	
		Protein (N-H) stretching	3029-3639
2	2839.22, 2949.16	C-H: Methyl and methylene groups	2800-2960
3		CO <sub>2</sub>	2000-2800, 700
4		C = O stretching	
		Carbonylic compounds (aldehydes, acids, etc.)	1540-1870
		Esters	1735-1750
5	1643.35	Protein amide I (C = O) stretching	1583-1709
6	1450.47	Bending of methyl lipids	1425-1477
7		H <sub>2</sub> O	1200-1800
8	1012.63	C-O-C ethers	900-1200

Adapted from: Ponnuswamy *et al.* (2013) and Elumalai and Sakthivel (2013)

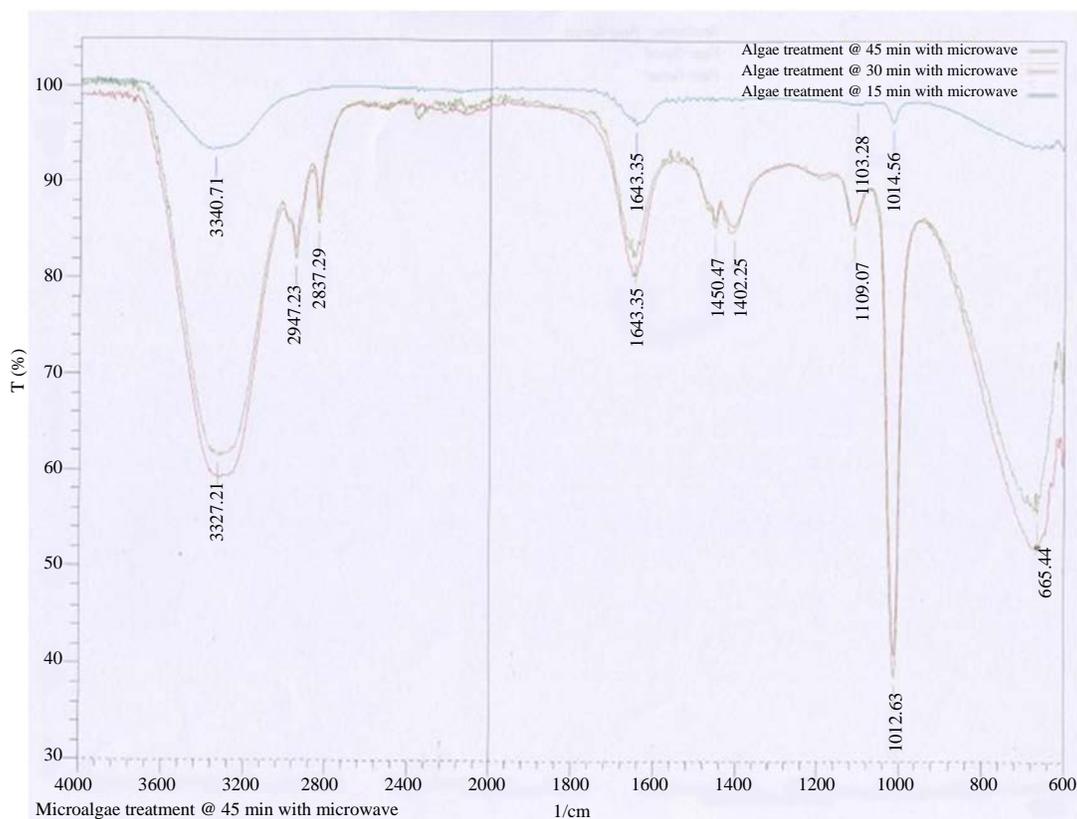


Fig. 8: FT-IR spectra at 3 different duration of microwave treatment

Microwave enables lipid to be released easier by speeding up the disruption of microalgae cells, besides being a faster and efficient heating method due to the molecular diffusion and mass transfer (Cheng *et al.*, 2013). Methanol acts as a strong microwave absorption solvent to extract lipid from biomass, in addition, it also functions as a reactant to convert lipids to Fatty Acid Methyl Esters (FAME) (Cheng *et al.*, 2013; Wahlen *et al.*, 2011). Sulphuric acid is an effective catalyst at converting both TAG and Free Fatty Acids (FFA) into FAME (Wahlen *et al.*, 2011).

### CONCLUSION

The robust characteristics of microalgae are shown in this work, illustrating the ability of microalgae to adapt and survive under different carbon source treatments and nitrogen levels. The primary goals was to increase the algae biomass and lipid accumulation for biodiesel production in tandem with sequestration of high CO<sub>2</sub> concentration. Based on the outcomes of this research, it is concluded that the optimum CO<sub>2</sub> gas concentration for *Nannochloropsis* sp. microalgae is 15% (v/v). At higher CO<sub>2</sub> concentration, growth under normal nutrition gave higher lipid productivity. It is also interesting to note that the growth using soluble carbonates is slightly better

than when using only CO<sub>2</sub> gas as carbon source. The 20% flue gas solution gave maximum yield of dry biomass of 0.55 g, while the maximum yield of dry biomass for 15% dissolved CO<sub>2</sub> gas was around 0.44 g. Higher amount of the flue gas solution was found to increase the lipid productivity of *Nannochloropsis* sp. The 20% flue gas solution gave maximum yield of lipid productivity and it was 27% higher than that produced by the 2% flue gas solution.

The better growth characteristics between flue gas solution and CO<sub>2</sub> gas dosing for the cultivation of *Nannochloropsis* sp., proves that this microalgae is a viable strain to be implemented for the abatement of CO<sub>2</sub> emissions.

The concentration of nitrogen at 0.882 mM gave the best growth and biomass amount. Those with lower nitrogen had their growth and biomass reduced by almost 5 times. The lipid productivity increased, when exposing the culture to nitrogen starvation condition. Since, accumulation of lipid occurs at nitrogen depletion condition, under which the growth was much slower. By reducing the nitrogen level in the nutrition, higher lipid productivity was achieved although the growth was decreased. Optimizing lipid production would therefore require a balance between supporting cell growth and stimulating total lipid yields. Direct transesterification simplifies the biodiesel production process by eliminating the

energy-intensive and time-consuming drying and extraction. The direct transesterification via microwave irradiation can save about 65% of the total energy consumption in the biodiesel production and shortens the production time drastically.

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