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Nile Red, an Alternative Fluorescence Method for Quantification of Neutral Lipids in Microalgae

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***Abstract*—**According to biodiesel from microalgae is an attractive fuel for several reasons such as renewable, biodegradable and environmental friendly. Thus, this study, green microalgae *Scenedesmus acutus* PPNK1 isolated from natural water, was selected based on high growth rates, easy cultivation and high lipid content. The Nile red fluorescence method has been successfully applied to the determination of lipids in *S. acutus* PPNK1. The combination of the method to the lipid composition in algal cells showed the yellow fluorescence under fluorescent microscope. Interestingly, maximum cell numbers and biomass concentration were obtained at 5.44×10^7 cells/mL and 1.60 g/L when it was cultivated in BG-11 medium while in case of BG-11 with nitrogen deprivation (N 0.25 g/L), accumulated lipid content in cells (44.67%) was achieved that was higher than that found in case of BG-11 medium at about 2 times (22.63%).

***Keywords*—**Biodiesel, Lipid, Microalgae, Nile red.

I. Introduction

THE dramatic situation in climate changes and fuel demands are bringing the worldwide attention to search for renewable and sustainable energy. Biodiesel, one of the most common biofuels, can replace diesel with little or none modifications of vehicle engines with several reasons; it is a renewable fuel, highly biodegradable, minimal toxicity, and environmentally friendly, resulting in very low sculpture release and no net increased release of carbon dioxide [1], [2]. One promising source of biomass for alternative fuels production is microalgae that have ability to grow rapidly and synthesize and accumulate large amount (20-50% of dry weight) of neutral lipid (mainly in form of triacylglycerol, TAG) stored in cytosolic lipids bodies [3]. Under suitable culture conditions, some microalgal species are able to accumulate up to 50–70% of oil/lipid per dry weight, the fatty acid profile of microalgal oil is suitable for the synthesis of biodiesel. The major attraction of using microalgal oil for biodiesel is the tremendous oil production capacity by microalgae, as they could produce up to 58,700 L oil per hectare, which are one or two magnitudes higher than that of any other energy crop [4].

The lipid content of microalgae could be increased under unfavorable environmental or stress conditions such as, phosphate limitation, high salinity, high iron concentration and nitrogen depletion [5], [6]. Normally a nitrogen-limiting or nutrient limiting condition was used to increase the lipid content in microalgae. In the absence of the nitrogen necessary for protein synthesis and cell growth, excess carbon from photosynthesis appears to be channel into storage molecules, such as starch and TAG [7]. The synthesis of neutral lipids as has been found to be a protective mechanism for cells against stressful conditions [8]. As a result, higher lipid content is usually achieved at the expense of lower biomass productivity. Thus, lipid content is not the sole factor determining the oil-producing ability of microalgae. Instead, both lipid content and biomass production need to be considered simultaneously. Hence, lipid productivity, representing the combined effects of oil content and biomass production, is a more suitable performance index to indicate the ability of a microalga with regard to oil production depletion [9].

The conventional methods for lipid determination involved solvent extraction and gravimetric determination. Further quantification of neutral lipid requires specialized equipment by Gas chromatography–mass spectrometry (GC–MS), or thin-layer chromatography (TLC) [10]. These techniques are time-consuming and do not allow high throughput therefore, detection of microalgal lipid is most often accomplished by staining techniques employing fluorescence microscopy.

A dye color of 9-diethylamino-5H-benzo[α] phenoxa-phenoxazine-5-one, normally called as Nile red is a lipid-soluble fluorescent dye which allows the *in situ* staining of lipids, has been frequently employed to evaluated the lipid content of animal cells and microorganisms, such as mammalian cells, bacteria, yeasts and microalgae [11]. The algal strains by the Nile red method, the color is penetrated cell walls, cytoplasmic membrane and ultimately dissolving in the intracellular neutral and polar lipids to give the desired fluorescence with the additive of dimethyl sulfoxide (DMSO) to microalgal samples as the strain carrier [12]. Fluorescent measurement by Nile red is simple, rapid and sensitive. However, it was recognized that various factors including algae species, the polarity of solvent to dissolve Nile red and different measure conditions might greatly affect the combination of Nile red to lipid component in microalgal cells and finally resulted in fluorescence intensity. The oil bodies in microalgal cells stained by fluorescent dye Nile red when observed using fluorescence microscope have characteristic yellow fluorescence and without staining cells have the red fluorescence [13].

The objectives of this study are to determine and characterize the quantification of intracellular neutral lipids in

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microalgae. The effect of nitrogen concentration, biodiesel production and fatty acid composition were also considered.

II. Materials and Methods

A. Microalgae Strain and Growth Medium

The freshwater unicellular green microalgae *Scenedesmus acutus* PPNK1 was isolated from a Mekong river at Nong Khai province, north eastern of Thailand. A medium used for photoautotrophic cultivation called as BG-11 was modified as follows (in g/L): $NaNO_3$ (1.5g), KH_2PO_4 (0.004g), $MgSO_4.7H_2O$ (0.075 g), $CaCl_2.2H_2O$ (0.027g), $EDTA.Na_2$ (0.001g), Citric acid (0.006g), Ammonium Ferric Citrate (0.006g), Na_2CO_3 (0.02g) and trace element solution 1mL. The pH of the medium was adjusted to 7.5 before autoclaving at 121°C for 15min.

B. Nitrogen Stress Conditions

Pure strain microalgae of *S. acutus* PPNK1 was cultivated in three different nitrogen concentration (nitrogen from $NaNO_3$) in BG-11 medium; BG-11 with $NaNO_3$ 0.75 g/L (BG-11 N 0.75 g/L), BG-11 with $NaNO_3$ 0.50 g/L (BG-11 N 0.50 g/L) and BG-11 with $NaNO_3$ 0.25g/L (BG-11 N 0.25g/L). The microalgae was cultured in a 500mL Erlenmeyer flask containing 250 working volume with initial cell 1×10^6 cells/mL (10%, v/v inoculums). The flasks were left at room temperature (28±3°C), aeration and mixing were achieved by air pump with a flow rate 5L/min. In addition, the light was supply by cool white fluorescent lamp with light intensity of 4,000 lux using interval periods of 16h: 8h in the dark, respectively.

C. Staining of Microalgae Cells with Nile Red Fluorescence Dye

A dye color of Nile red (9-diethylamino-5H-benzo [α] phenoxa-phenoxazine-5-one) were purchased from Sigma-Aldrich, acetone and DMSO were purchased from other commercial suppliers. All are analytical grade. Microalgae cells were cultivated until early stationary phase for accumulating neutral lipids. The cells then were harvested and centrifuged at 10,000rpm for 10min. Algal samples were washed three times with distilled water. Then, algal cells 20 µL were mixed with 400µL of 25% DMSO. The mixture was pretreated using a microwave oven for 1min. After the addition of 20µL of Nile red solution (200µg/mL in acetone), the mixture was placed in a microwave oven for 1min, and stained in the dark for 10min. Next viewed under a fluorescent microscope (Olympus, BH2-RFCA) with 100× objective lens were used to visualize the fluorescent yellow-gold lipid in microalgal cells. The microwave power was set at 1,350 W for both processes. For each treatment, three replicate determinations were performed.

D. Lipid Extraction

The strain *S. acutus* PPNK1 was cultured and reached to early stationary growth phase, and then cells were harvested. Total lipids were extracted from dry algal biomass by a solvent mixture of chloroform: methanol (1:2) using a modified Folch method. Dried algae samples (200mg) were mixed with 5mL of chloroform: methanol by magnetic stirring at 40°C, 1h. Then, the solvent mixture 5mL was added and left an extraction again for approximately 30min, extraction was repeated twice. The biomass was then collected at the bottom of the test tube by centrifugation and the solvent was removed to a weighed vial. The organic extractions were dried in the fume hood; the dried vials were weighed to establish the total lipid per dry cell weight.

E. Biodiesel Production and Fatty Acid Analysis

Biodiesel were produced via *in situ* transesterification reaction or one step extraction-transesterification. Dried algae samples (200mg) were weighed into 25mL screw-top glass bottles, 5mL of the mixture of methanol and concentrated sulfuric acid (4.4:0.6) and 5mL of hexane were added. The chemical reaction was carried out at 80°C with magnetic stirring for 120min. Then placed at room temperature for 15 min, warm distilled water 2mL and 10 % Na_2SO_4 1mL were added. Subsequently mixing and by centrifugation steps, the methanol and sulfuric acid partitioned with the water in the upper phase, while FAMEs, TAG, and other lipids partitioned with hexane in the lower organic phase. The residual biomass formed a layer at the boundary between these two phases. The hexane phase was removed with a gas tight syringe to a 10mL vial, then the solvent was evaporated and finally FAMEs were taken.

Fatty acids were analyzed by Gas Chromatography (GC), the samples were determined by EN 14103 method. Briefly, accurately weigh 250mg of FAME sample were mixed with 5 mL of 10mg/mL methyl heptadecanoate (C17:0) internal standard solution using n-heptane. Next one micro liter of the prepared solution was injected into a GC with a flame ionization detector and a Restek, FAMEWAX capillary column (30m, 0.32mm ID, 0.25µm). Chromatographic data were recorded and identified by comparison of the retention times of the eluting fatty acids to a standard FAMEs mix (C8-C24 Supelco).

F. Analytical Methods

1. Cell Concentration

The microalgae solution was determined regularly by measuring optical density at a wavelength of 680 nm using UV/ Vis spectrophotometer (PG Instruments) while cell numbers were count by haemacytometer under microscope (ZEISS).

2. Biomass Concentration and Biomass Productivity

The biomass concentration of microalgae was estimated by 20mL of algae solution were centrifuged and washed three times with distilled water. Then it was dried at 70°C in hot air oven until constant weight**.** The biomass productivity (*P*, mg /L/d) was calculated according to (1):

$$P = \frac{\Delta X}{\Delta t} \quad (1)$$

where ΔX is the variation of biomass concentration (mg /L) within a cultivation time of Δt (d) [5].

3. The Specific Growth Rate

The specific growth rate (μ) was calculated by plotting the concentrations of biomass on a logarithmic scale versus time in terms of the (2):

$$\mu = \frac{(1nX_1 - 1nX_0)}{(t_1 - t_0)} \quad (2)$$

where X_1 and X_0 were the biomass concentration at time t_1 and t_0 at the end and at the beginning of the cultivation [14], [15].

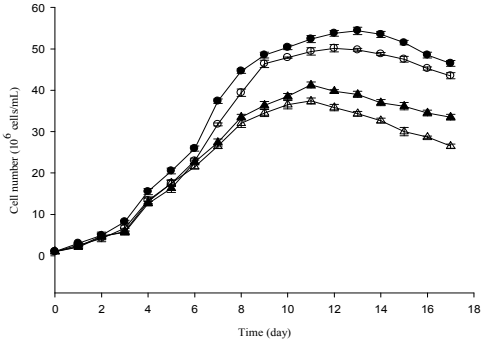
4. Lipid Yield (g/L)

The eventual lipid yield was determined in both biomass yield (g/L) and lipid content (%) [9].

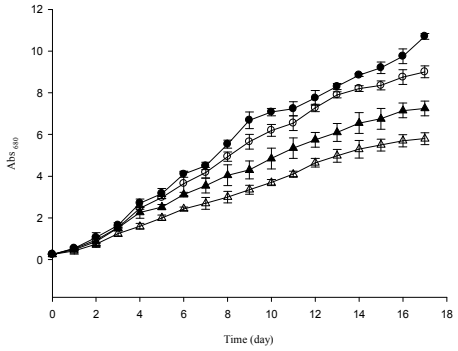
III. Results and Discussion

A. Microalgae Growth

S. acutus PPNK1 was cultivated under photoautotrophic cultivation with BG-11 medium, BG-11 N 0.75g/L, BG-11 N 0.50g/L and BG-11 N 0.25g/L. The maximum cell number reached at 5.44×10^7 cells/mL at 13 day cultivation of BG-11 medium (control or normal condition) and the lowest cell number found when microalgae was cultivated in BG-11 N 0.25g/L (3.74×10^7 cells/mL) see Fig. 1 (a), although absorbance continued to increase (Fig. 1 (b)) providing the cells swelling. The absorbance of algae in BG-11 medium, BG-11 with $NaNO_3$ 0.75g/L and BG-11 with $NaNO_3$ 0.50g/L increase two fold after four days but BG-11 with $NaNO_3$ 0.25g/L increase one fold. The result indicates that *S. acutus* PPNK1 required nitrogen for growth and for biomass accumulation. In addition, when compared with the maximum specific growth rate, biomass concentration, biomass productivity and cell number BG-11 medium without limitation of nitrogen showed the highest all of parameters (Table I and Fig. 2).



(a)



(b)

Fig. 1 Growth of *S. acutus* PPNK1 under four different medium (a) Cell density of all medium (b) Time course of the absorbance A $_{680}$ nm.; ● BG-11 medium, ○ BG-11 N 0.75 g/L, ▲ BG-11 N 0.50 g/L and Δ BG-11 N 0.25 g/L (average ± SD)

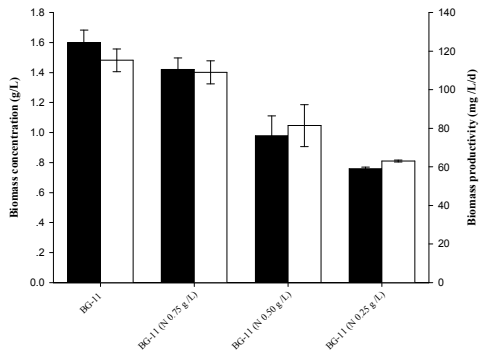


Fig. 2 Biomass concentration (g/L) and Biomass productivity (mg /L/d) of *S. acutus* PPNK1 under four different nitrogen concentration in BG-11 medium. The highest biomass concentration (Black columns) and the highest biomass productivity (White columns) reached 1.60 g/L and 115.23 mg/L/d in BG-11 medium.(average ± SD)

TABLE I
Maximum Specific Growth Rate, Biomass Concentration, Biomass Productivity and Cell Number of *S. acutus* PPNK

Condition	Maximum specific growth rate (μ_{max},day^{-1})	Biomass concentration (g/L)	Biomass productivity (mg /L/d)	Cell number (cells/mL)
BG-11 medium	0.681	1.60	115.23	5.44×10^7
BG-11 N 0.75 g/L	0.617	1.42	108.97	5.01×10^7
BG-11 N 0.50 g/L	0.597	0.98	81.40	4.13×10^7
BG-11 N 0.25 g/L	0.573	0.76	63.00	3.74×10^7

B. Fluorescence Characteristics of Nile red Stained Microalgae

To analyze the effect of nitrogen depletion on *S.acutus* PPNK1 grown under photoautotrophic condition with four medium, the accumulation of lipid bodies were examined when microalgae were stained with Nile red fluorescence dye**.** The characteristic of microalgae when observed under a fluorescent microscope showed a yellow fluorescent lipid in microalgal cells, while they were red without staining (Fig. 3).

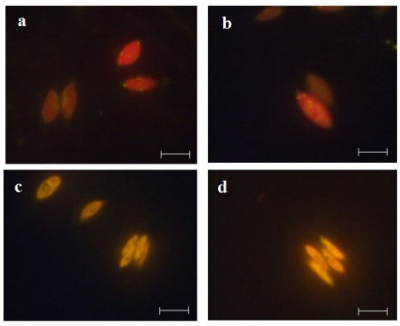


Fig. 3 *S. acutus* PPNK1 stained with Nile red fluorescence dye viewed under fluorescent microscope (100 ×), (a) cells in BG-11 medium, (b) cells in BG-11 N 0.75g/L, (c) cells in BG-11 N 0.50g/L and (d) cells in BG-11 N 0.25g/L show a yellow fluorescent lipid in microalgal cells. Scale bars = 20µm

C. Lipid Content and Lipid Yield

Lipid contents of *S. acutus* PPNK1 were analyzed, the neutral lipid content was increased as nitrogen concentration in medium decreased. The maximum lipid content reached at 44.77%, when the algae were cultivated with BG-11 reduced $NaNO_3$ to 0.25g/L (BG-11 N 0.25g/L) while the lowest lipid content was founded when BG-11 medium is used (22.63%). However, the eventual lipid yield was determined in both biomass yield and lipid content. Thus, the result indicates that BG-11 reduced $NaNO_3$ to 0.75g/L (BG-11 N 0.75g/L) was suitable to cultivate this microalgae strain because of the highest lipid yield (1.42g/L × 31.44 % = 0.45g/L) as seen in Fig. 4.

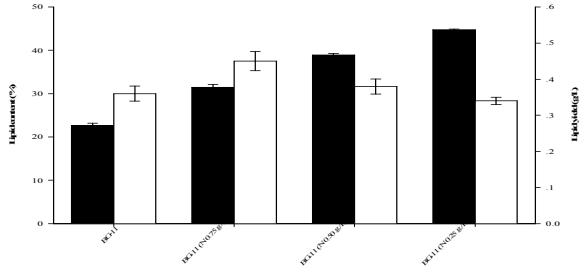


Fig. 4 Lipid content (%) and Lipid yield (g/L) of *S.acutus* PPNK1 in four medium. The highest lipid content reached 44.77% in BG-11 N 0.25 g/L (Black columns) and the highest lipid yield reached 0.45 g/L in BG-11 N 0.75 g/L (White columns)

D. Fatty Acid Compositions

Fatty acids of *S. acutus* PPNK1were primarily esterified, the compositions of biodiesel were determined using GC. The results showed that the most commonly synthesized fatty acids have chain lengths of C16 and C18 in all of medium (see Table II and Fig. 5).

TABLE II
COMPOSITION OF FATTY ACID METHYL ESTERS (FAMES) OBTAINED FROM *S. ACUTUS* PPNK1

Fatty acid methyl ester Contents (%)		BG-11	BG-11 (N 0.75 g/L)	BG-11 (N 0.50 g/L)	BG-11 (N 0.25 g/L)
C12:0	Lauric acid	0.86	2.25	3.81	2.84
C14:0	Myristic acid	2.25	1.40	4.32	1.56
C16:0	Palmitic acid	22.50	17.80	18.30	15.60
C18:0	Stearic acid	2.20	3.07	10.50	17.90
C18:1	Oleic acid	18.30	36.60	27.80	33.00
C18:2	Linoleic acid	10.30	12.00	12.30	2.37
C18:3	Linolenic acid	11.50	1.20	2.74	1.22
C20:0	Arachidic acid	11.07	14.65	1.02	3.48
	Others	21.02	11.03	19.21	22.03
	Total	100	100	100	100

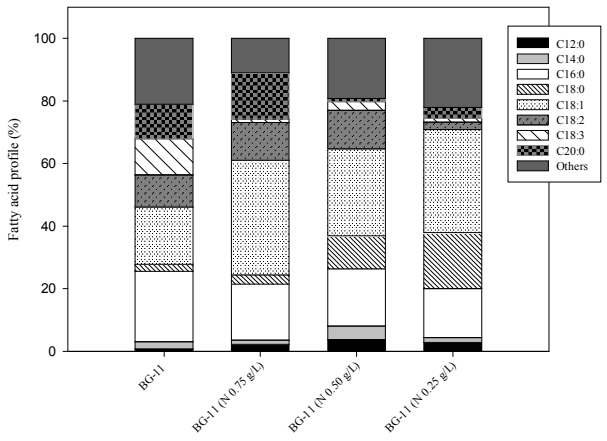


Fig. 5 The performance of fatty acid profile of *S. acutus* PPNK1 cultured on the different nitrogen concentration of BG-11 medium

IV. Conclusion

Successfully, isolated microalgae *Scenedesmus acutus* PPNK1, a valuable candidate of raw material for biodiesel production, was stained by a dye Nile red, a lipid-soluble fluorescence dye and showed brighter yellow fluorescence in the lipid body. The maximum cell numbers and maximum biomass concentration were obtained at 5.44×10^7 cells /mL and 1.60g/L when BG-11 medium was used while the maximum lipid content reached at 44.77% when microalgae was cultivated in BG-11+N 0.25g/L. Considerably, lipid yield (biomass yield × lipid content), the highest lipid yield reached 0.45g/L when BG-11+N 0.75g/L was used. Nitrogen deprivation is one of the stress condition is promoted via increased lipid accumulation in microalgal cells, in contrast biomass and cell growth are inhibited.

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