COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

Simultaneous Estimation of Chlorophyll *a* and Lipid Contents in Microalgae by Three-Color Analysis

Chia-Hung Su,[1] Chun-Chong Fu,[2] Yet-Chung Chang,[3] Giridhar R. Nair,[4] Jun-Liang Ye,[2] I.-Ming Chu,[1] Wen-Teng Wu[2]

[1]Department of Chemical Engineering, National Tsing-Hua University, Hsin-Chu, Taiwan

[2]Department of Chemical Engineering, National Cheng-Kung University, Tainan 701, Taiwan; telephone: 886-6-2376734; fax: 886-6-2344496; e-mail: wtwu@mail.ncku.edu.tw

[3]Department of Network Communication, Diwan College of Management, Madou, Taiwan

[4]Department of Engineering, The University of Waikato, Hamilton, New Zealand

Received 12 March 2007; revised 24 July 2007; accepted 1 August 2007

Published online 17 August 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21623

ABSTRACT: A method of rapid determination of chlorophyll *a* and lipid contents of microalgae based on colorimetric analysis of the digital images of the microalgae is proposed. The color variation of microalgae during cultivation is evaluated by the brightness of the three primary colors (red, green, and blue). The brightness values of the three primary colors are modeled as two linear correlation functions (RGB model) for microalgal chlorophyll *a* and lipid contents, respectively. The chlorophyll *a* and lipid contents predicted by the proposed model are compared with that determined by the standard methods. The good agreement of the model predictions with experimental results is demonstrated with a squared correlation coefficient (R^2) of 0.99 for chlorophyll *a* and lipid. The reliability of the RGB model was verified in real cultivations of the microalgae in a photobioreactor. Growth dynamics, contents of chlorophyll *a* and lipid corresponded very well with previously reported studies.

Biotechnol. Bioeng. 2008;99: 1034–1039.

KEYWORDS: microalgae; image analysis; chlorophyll; lipid; RGB model

Introduction

Photosynthetic microalgae are one of the organisms that efficiently capture light energy. The photosynthetic pigment, chlorophyll *a*, is the principal photochemically active compound, which functions as a receiver of light for driving photosynthesis. The content of this pigment in microalgae is therefore related to photosynthetic activity (MacIntyre et al., 2002). Photosynthetic efficiency and cell growth associated with quantification of chlorophyll *a* under various culture conditions has been studied (Flynn et al., 1993; Marin et al., 1998; Masojidek et al., 2000; Silva et al., 1998; Tremblin et al., 2000). The amount of light harvesting pigment influences the production of biomass and the accumulation of target products in the microalgae cultivated under different conditions.

Several species of microalgae are found to accumulate large amounts of lipids in the cells (oleaginicity), under nitrogen limited growth (Ratledge, 2002). Lipids are extremely attractive as a source of biodiesel, which is a renewable energy and in the production of nutritionally valuable biochemicals, such as polyunsaturated fatty acids (Chisti, 2007; Rocha et al., 2003). The lipid content of microalgae varies with environmental conditions, making it a potential indicator of the physiological state of these organisms.

The determination of chlorophyll *a* and lipid contents of microalgae would provide useful information concerning the growth status during cultivation. The conventional protocols for estimating microalgal pigment chlorophyll *a* and intracellular lipid contents are commonly carried out by using spectroscopic and chromatographic methods (Gilmore and Yamamoto, 1991; Lichtenthaler and Wellburn, 1983; Meireles et al., 2003). These methods are too laborious, time-consuming and a series of solvent extractions and chemical reactions are required for analysis. Hence, several improved methods such as image analysis for the estimation of microalgal pigments (Lopez et al., 2006), fluorometric method (Cooksey et al., 1987), and dielectric analysis

Correspondence to: W.-T. Wu

Contract grant sponsor: NSC

Contract grant number: 94-2214-E-006-015

(Higashiyama et al., 1999) for determination of lipid contents have been developed. However, these equipments are expensive and are not portable.

In this study, an image analysis method based on the intensity of the three primary colors, for the simultaneous prediction of chlorophyll *a* and lipid contents of microalgae has been developed. A photosynthetic microalga, *Nannochloropsis oculata*, has been chosen as the model organism, since it is known to accumulate high levels of intracellular lipids (Zou et al., 2000). To the best of our knowledge, this is the first report of a rapid and reliable method for simultaneous determination of the chlorophyll *a* and lipid contents of microalgae using image analysis based on RGB colorimetric indicators.

Materials and Methods

Microalgae and Growth

The marine nanoplanktic alga (Eustigmatophyta), *N. oculata,* was obtained from Fisheries Research Institute (Pingtung, Taiwan) and cultivated in artificial seawater enriched with 0.1% (v/v) Walne medium nutrients and 0.1% (v/v) trace metal solution. Walne medium contained (g/L): Urea, 100; $NaH_2PO_4{\cdot}2H_2O$, 20.0; Na_2EDTA, 4.0; H_3BO_3, 33.6; $MnCl_2{\cdot}4H_2O$, 0.36; $FeCl_3{\cdot}6H_2O$, 13.0; Vitamin B12, 0.001; Vitamin B1, 0.02; $NaSiO_3$, 6.6. Trace metal solution contained (g/L): $ZnSO_4{\cdot}7H_2O$, 4.4; $CoCl_2{\cdot}6H_2O$, 2.0; $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$, 0.9; $CuSO_4{\cdot}5\ H_2O$, 2.0. All solutions were prepared in sterilized water.

N. oculata was cultivated in 500 mL of culture medium in 1-L Duran bottles at room temperature for 6 days, under 300 μmol photon $m^{-2}\,s^{-1}$ illumination using cool white fluorescent lamps and at 1 vvm aeration. The photon flux was measured with a Li-Cor quantum sensor (model Li-190SA).

Biomass Concentration

The biomass concentration was determined spectrophotometrically (Beckman DU 530, Fullerton, CA) at 682 nm and using a linear correlation of optical density versus dry cell weight (DCW).

Chlorophyll *a* Content by Standard Analytic Method

Chlorophyll *a* content was determined spectrophotometrically, following extraction of the cells using 90% methanol. Chlorophyll *a* was determined in the methanol extracts using the modified equations of Lee and Shen (2004) as given below

$$\text{Chlorophyll } a\ (\text{mg L}^{-1}) = 13.43 \times \text{OD}_{665} \qquad (1)$$

where OD_{665} is the optical density measured at 665 nm.

Lipid Content by Standard Analytic Method

The microalgal lipid content was determined by the procedure previously reported (Su et al., 2006).

Chlorophyll *a* and Lipid Content by Colorimetric Method

The color variation in microalgae during cultivation has been observed by many researchers (Carvalho et al., 2004; Melis et al., 1998), mainly due to the change in the biochemical composition of the microalgae (Lopez et al., 2006). The major constituents, chlorophyll *a* and lipid, may dominate such color variance in oleaginic microalgae, and the color change will be proportional to the amount of these constituents. The proposed colorimetric method of determination of chlorophyll *a* and lipid contents is based on the trichromatic theory, which states that any specific color can be represented by combining the three primary colors red (R), green (G), and blue (B). Conversely, any color can be decomposed into the primary colors and the intensity of an individual color can be represented by the number of pixels of brightness, in a digital image. Thus it would be possible to develop a mathematical correlation between chlorophyll *a* and lipid contents of the microalgae and the brightness values of the primary colors. In this study, the images of the microalgal cells were captured under constant background conditions and selected regions of the digitized image were analyzed using a program written in Microsoft Visual Basic 6.0. The color intensities were then correlated to the chlorophyll *a* or lipid content using linear equations (RGB model). An environment consisting of identical biomass (0.5 g L^{-1}) in the samples analyzed and equivalent photon flux (25 ± 2 μmol photon $m^{-2}\,s^{-1}$) for image capture is considered as constant background conditions.

Microalgal cells from culture samples withdrawn at regular intervals were homogeneously suspended in distilled water to a final concentration of 0.5 g L^{-1}. The microalgal suspension was then carefully transferred to a quartz cuvette without air bubbles. The image of the cells in the cuvette was captured by a digital camera (DMC-FX8; Panasonic, Osaka, Japan). The digital image (5 mega-pixels) was analyzed using a computer program in a personal computer. A region of digital image of the microalgal suspension corresponding to 100×100 pixels was decomposed and the brightness of each of the red, green, and blue pixels was transformed to a 8-bit 256-level scale (0–255). The color components were processed separately and data were sorted based on the colors distribution shown as two-dimensional histograms (Fig. 1). The histogram represented the number of pixels occurring at each color image with a particular brightness value. The mean brightness values of the three-color components with standard deviations of a sample were obtained from the histogram and are presented in Table I. The small coefficients of variations for three primary colors (Table I), indicated that the mean brightness values were highly reproducible.

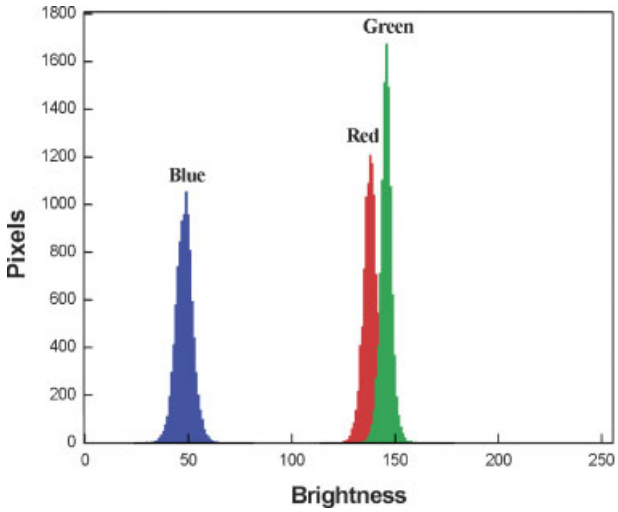

Figure 1. The two-dimensional histogram plot of the brightness value of the three primary colors (red, blue, and green) of *N. oculata* cultivated in 1 L Duran bottles under 300 μmol photon $m^{-2} s^{-1}$ illumination and at 1 vvm aeration. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

The mean brightness values of the three primary colors were linearly correlated to obtain the characteristic RGB model as shown below

$$Y = aR + bG + cB \tag{2}$$

where Y is the chlorophyll *a* or lipid contents of the microalgae; R, G, B are, respectively, the mean brightness value of each primary color; a, b and c are the model parameters.

Results and Discussion

Determination of the Parameters of RGB Models

The prediction of chlorophyll *a* and lipid contents of microalgae using the proposed RGB model (Eq. (2)) requires determination of the model parameters. Seven experimental runs were conducted for this purpose. The brightness values for the three primary colors and the chlorophyll *a* and lipid contents obtained from the seven experiments are shown in Table II. The first two experiments corresponded to the boundary limits of chlorophyll *a* and lipid contents. From experiments 3 to 5, the coefficient of variance of 0.05 and 0.07 respectively obtained for lipid and chlorophyll *a* contents indicated the reproducibility of the experiments. The model parameters were determined by the least square method by using the data from all seven experiments.

The parameters a, b, and c of Equation (2) were determined by using the matrix form of the RGB model as shown below

$$[a \quad b \quad c]^{\mathrm{T}} = \left[\underline{\underline{A}}_{\mathrm{RGB}}^{\mathrm{T}} \cdot \underline{\underline{A}}_{\mathrm{RGB}}\right]^{-1} \cdot \underline{\underline{A}}_{\mathrm{RGB}}^{\mathrm{T}} \cdot \underline{Y} \tag{3}$$

$\underline{\underline{A}}_{\mathrm{RGB}}$ represented the mean brightness values of the primary colors (Table II) and vector $\underline{Y}$ represented the actual chlorophyll *a* and lipid contents of the microalgae determined by the standard analytical methods (Table II). By using the least square regression of Equation (3), the parameters a, b, and c for the correlation between mean brightness values and lipid contents were −28.52, 24.65, and 9.09, respectively. Similarly, the parameters in the correlation for chlorophyll *a* were calculated as −1.32, 1.29, and −0.01, respectively.

Validity of Model Predictions

The chlorophyll *a* and lipid contents of 20 microalgal samples from batch culture were predicted by using the RGB model (Eq. (2)) with the model parameters determined (Table III) and the chlorophyll *a* and lipid contents of the same samples were measured by the standard analytical methods for comparison. An excellent agreement between model predictions and actual chlorophyll *a* (Fig. 2) and lipid (Fig. 3) contents was observed, which indicated the high predictability of the models for estimating the chlorophyll *a* and lipid contents in the microalgae.

Furthermore, the goodness-of-fit measure of the model was evaluated by the squared correlation coefficient (R^2) defined by the following equation

$$R^2 = 1 - \left(\frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{n} (y_i - \overline{y})^2}\right) \tag{4}$$

where n is the number of samples, y_i the actual experiment data of ith sample, $\hat{y}_i$ the model predicted data of ith sample and $\overline{y}$ is the average of all the experimental data. The R^2 was normalized between 0 and 1. In this evaluation, the R^2 of 0.99 demonstrated the reasonable agreements between

Table I. The mean brightness values with standard deviation and the coefficient of variation of the three primary colors of *N. oculata* cultivated in 1 L Duran bottles under 300 μmol photon $m^{-2} s^{-1}$ illumination and at 1 vvm aeration.

	Primary colors		
	Red	Green	Blue
Mean brightness value with standard deviation	138.02 ± 3.60	145.88 ± 2.80	48.14 ± 3.82
Coefficient of variation	0.03	0.02	0.08

DOI 10.1002/bit

Table II. The mean brightness value of the three primary colors, and the actual and predicted chlorophyll *a* and lipid contents determined by the standard methods and RGB models, respectively.

Exp. no.	Brightness value of primary colors			Actual lipid content (mg/g DCW)	Actual chlorophyll *a* content (mg/g DCW)	Predicted lipid content (Y_L)[a] (mg/g DCW)	Predicted chlorophyll *a* content (Y_C)[b] (mg/g DCW)
	Red	Green	Blue				
1	144.42	150.33	125.82	729.4	2.7	730.5	2.1
2	108.34	126.38	5.98	79.9	20.8	79.8	20.0
3	116.87	131.73	25.19	142.8	16.3	143.0	15.4
4	116.99	131.45	25.21	130.6	15.7	132.8	14.9
5	118.02	134.17	22.16	142.9	17.9	142.8	17.1
6	131.22	141.00	57.73	257.4	8.9	258.0	8.2
7	140.83	147.97	86.59	418.1	4.9	418.1	4.2

[a]RGB model for estimation of lipid: $Y_L = -28.52R + 24.65G + 9.09B$.
[b]RGB model for estimation of chlorophyll *a*: $Y_C = -1.32R + 1.29G - 0.01B$.

predicted and actual contents of chlorophyll *a* and microalgal lipid.

It is supposed that the differences in the cell size would not influence the chlorophyll *a* or lipid contents predicted by the proposed model, since the brightness values of the three primary colors of the microalgae were converted into chlorophyll *a* or lipid concentrations and not into the amount of chlorophyll *a* or lipid per individual cell (Miyanaga et al., 2000). During investigations on the cultivation of *Haematococcus pluvialis* (marine microalgae), Lopez et al. (2006) observed that the macroscopic characterization was not affected by size change from 18 to 30 μm. Miyanaga et al. (2000) has also observed that the characteristic features of RGB values depend on the type of pigment produced. The parameters of the RGB model proposed in this study have to be redetermined if the correlation is to be used for estimating the concentration of other biochemicals produced by the microalgae, for example astaxanthin.

Real Culture System Applications

The applicability of the RBG model for monitoring the time courses of chlorophyll *a* and lipid contents was tested by cultivating *N. oculata* in a real photobioreactor. The microalgae were cultivated in 2.7 L artificial seawater enriched with 0.1% (v/v) Walne medium and 0.1% (v/v) trace metal solution in a bubble column reactor under an illumination of 100 μmol photon $m^{-2} s^{-1}$. The temperature of cultivation was 28°C and aeration rate was 1 vvm. The color variation of *N. oculata* during cultivation was observed by capturing the digital images and chlorophyll *a* and lipid were quantified by the image analysis. The time courses of the mean brightness of three primary colures of the microalgae during cultivation were shown in Figure 4. The mean brightness of each of three primary colors was further used in the RGB models for estimating the chlorophyll *a* and lipid contents in the microalgae.

The time courses of microalgal biomass, chlorophyll *a* and lipid contents are presented in Figure 5. Lag, exponential and stationary phases of growth were obviously observed. The growth dynamics of microalgae was similar to the previously reported results during batch cultivation of *Nannochloropsis* sp. (Brown et al., 1993; Hu and Gao, 2003). After 4 days of cultivation, the growth rate decreased indicating the onset of stationary phase (Fig. 5). The chlorophyll *a* content of the microalgae in the photobioreactor started to decrease after 2 days of cultivation and

Table III. Parameters of RGB models for chlorophyll *a* and intracellular lipid content of *N. oculata*.

Component	Model parameters		
	a	*b*	*c*
Chlorophyll *a*	−1.32	1.29	−0.01
Lipid content	−28.52	24.65	9.09

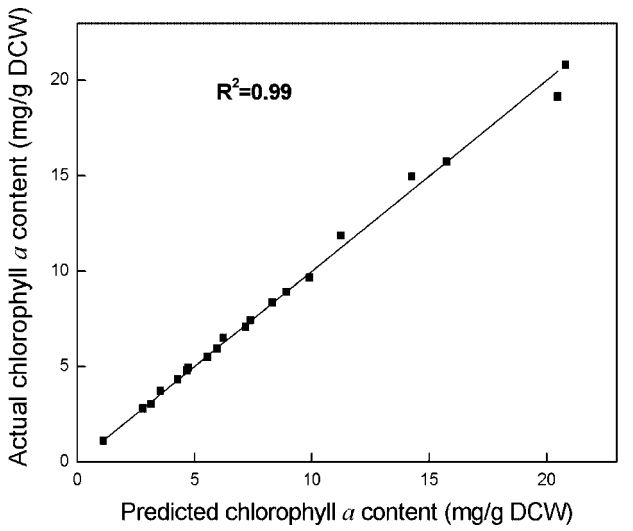

Figure 2. The agreement between model predicted and measured values of chlorophyll *a* content in *N. oculata* cultivated in 1 L Duran bottles under 300 μmol photon $m^{-2} s^{-1}$ illumination and at 1 vvm aeration.

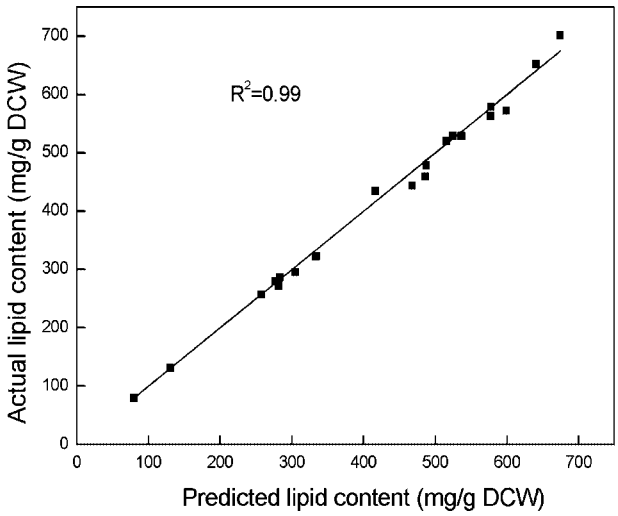

Figure 3. The agreement between model predicted and measured values of lipid content in *N. oculata* cultivated in 1 L Duran bottles under 300 μmol photon $m^{-2} s^{-1}$ illumination and at 1 vvm aeration.

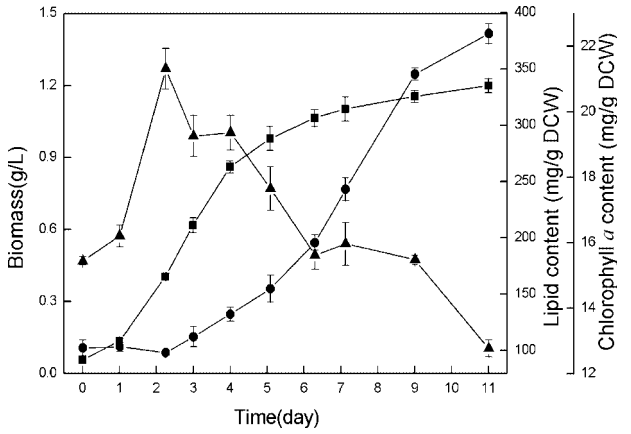

Figure 5. The time courses of biomass (■), lipid (●), and chlorophyll *a* contents (▲) of *N. oculata* cultivated at 28°C and 1 vvm aeration in bubble column photobioreactor under 100 μmol photon $m^{-2} s^{-1}$ illumination. The lipid and chlorophyll *a* contents were determined by using the RGB models. Error bars indicate the deviation in the readings between three experiments.

continued to decrease in the stationary phase. Most of the lipid accumulated in the stationary phase and it increased from 112.1 mg/g DCW after 3 days to 381.3 mg/g DCW after 11 days of cultivation. The decrease in chlorophyll *a* and increase in lipid might be due to nitrogen limitation (Flynn et al., 1993; Ratledge, 2002). Both of the above characteristics have been reported by Brown et al. (1993) and the results of the present study corresponded well with their findings. So the colorimetric method proposed in this study works reasonably well for the real time monitoring of chlorophyll *a* and lipid contents of microalgae and can be used in place of the standard analytical methods.

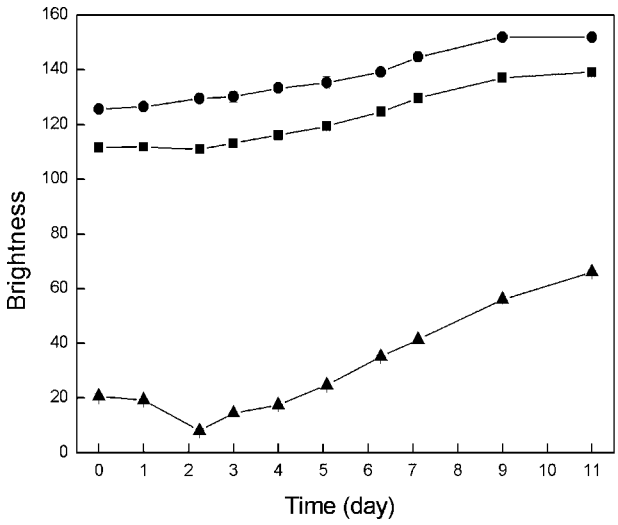

Figure 4. The time courses of variation of the mean brightness values of red (■), green (●), and blue (▲) colors. *N. oculata* was cultivated at 28°C and 1 vvm aeration in bubble column photobioreactor under 100 μmol photon $m^{-2} s^{-1}$ illumination. Error bars indicate the deviation in the readings between three experiments.

Conclusion

A colorimetric analysis was developed to rapidly determine the contents of microalgal lipid and chlorophyll *a*. *N. oculata* was the microalgae used as the model strain. A linear mathematical RGB model was proposed to correlate the mean brightness value of the primary colors and the contents of lipid and chlorophyll *a* in the microalgae. The good agreements between the predicted and experimental results are demonstrated with a squared correlation coefficient (R^2) of 0.99 for chlorophyll *a* and lipid. Finally, the proposed method and the model correlation were successfully applied in real microalgal cultivation for rapidly and effectively estimating the lipid and chlorophyll *a* contents of *N. oculata*, instead of using the standard analytic methods. The good agreement indicated that the proposed colorimetric analysis is reliable and is suitable for the simultaneous estimation of chlorophyll *a* and intracellular lipid contents of the microalgae. This method also exhibits the potential of on-line application to determining the chlorophyll *a* and intracellular lipid contents of the microalgae during cultivation.

This research was supported by grant (NSC 94-2214-E-006-015) from National Science Council of Taiwan.

References

Brown MR, Garland CD, Jeffrey SW, Jameson ID, Leroi JM. 1993. The gross and amino acid compositions of batch and semi-continuous cultures of

DOI 10.1002/bit

Isochrysis sp. (clone T.ISO), Pavlova lutheri and *Nannochloropsis oculata*. J Appl Phycol 5:285–296.

Carvalho JCM, Francisco FR, Almeida KA, Sato S, Converti A. 2004. Cultivation of *Arthrospira* (spirulina) *platensis* (Cyanophyceae) by fed-batch addition of ammonium chloride at exponentially increasing feeding rates. J Phycol 40:589–597.

Chisti Y. 2007. Biodiesel from microalgae. Biotechnol Adv 25:294–306.

Cooksey KE, Guckert JB, Willams SA, Callis PR. 1987. Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. J Microbiol Meth 6:333–345.

Flynn KJ, Davidson K, Leftley JW. 1993. Carbon-nitrogen relations during batch growth of *Nannochloropsis oculata* (Eustigmatophyceae) under alternating light and dark. J Appl Phycol 5:465–475.

Gilmore AM, Yamamoto HY. 1991. Resolution of lutein and zeaxanthin using a nonendcapped, lightly carbon-loaded C-18 high-performance liquid chromatographic column. J Chromatogr 543:137–145.

Higashiyama K, Sugimoto T, Yonezawa T, Fujikawa S, Asami K. 1999. Dielectric analysis for estimation of oil content in the mycelia of *Mortierella alpine*. Biotechnol Bioeng 65:537–541.

Hu HH, Gao KS. 2003. Optimization of growth and fatty acid composition of a unicellular marine picoplankton, *Nannochloropsis* sp., with enriched carbon sources. Biotechnol Lett 25:421–425.

Lee YK, Shen H. 2004. Basic culturing techniques. In: Richmond A, editor. Handbook of microalgal culture: Biotechnology and applied phycology. UK: Blackwell Science Ltd. p. 44.

Lichtenthaler HK, Wellburn AR. 1983. Determination of total carotenoids and chlorophyll a and b of leaf extract in different solvent. Biochem Soc Trans 603:591–592.

Lopez MCGM, Sanchez ED, Lopez JLC, Fernandez FGA, Sevilla JMF, Rivas J, Guerrero MG, Grima EM. 2006. Comparative analysis of the outdoor culture of *Haematococcus pluvialis* in tubular and bubble column photobioreactors. J Biotechnol 123:329–342.

MacIntyre HL, Kana TM, Anning T, Geider RJ. 2002. Photoacclimation of photosynthesis irradiance response curves and photosynthetic pigments in microalgae and cyanobacteria. J Phycol 38:17–38.

Marin N, Morales F, Lodeiros C, Tamigneaux E. 1998. Effect of nitrate concentration on growth and pigment synthesis of *Dunaliella salina* cultivated under low illumination and preadapted to different salinities. J Appl Phycol 10:405–411.

Masojidek J, Torzillo G, Kopecky J, Koblizek M, Nidiaci L, Komenda J, Lukavska A, Sacchi A. 2000. Changes in chlorophyll fluorescence quenching and pigment composition in the green alga *Chlorococcum* sp. grown under nitrogen deficiency and salinity stress. J Appl Phycol 12:417–426.

Meireles LA, Guedes AC, Malcata FX. 2003. Increase of the yields of eicosapentaenoic and docosahexaenoic acids by the microalga *Pavlova lutheri* following random mutagenesis. Biotechnol Bioeng 81:50–55.

Melis A, Neidhardt J, Benemann JR. 1998. *Dunaliella salina* (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells. J Appl Phycol 10:515–525.

Miyanaga K, Seki M, Furusaki S. 2000. Analysis of pigment accumulation heterogeneity in plant cell population by image-processing system. Biotechnol Bioeng 67:493–497.

Ratledge C. 2002. Regulation of lipid accumulation in oleaginous microorganisms. Biochem Soc Trans 30:1047–1050.

Rocha JMS, Garcia JEC, Henriques MHF. 2003. Growth aspects of the marine microalga *Nannochloropsis gaditana*. Biomol Eng 20:237–242.

Silva J, Santos R, Serodio J, Melo RA. 1998. Light response curves for *Gelidium sesquipedale* from different depths, determined by two methods: O_2 evolution and chlorophyll fluorescence. J Appl Phycol 10:295–301.

Su CH, Giridhar R, Chen CW, Wu WT. 2006. A novel approach for medium formulation for growth of a microalga using motile intensity. Bioresource Technol 98:3012–3016.

Tremblin G, Cannuel R, Mouget JL, Rech M, Robert JM. 2000. Change in light quality due to a blue-green pigment, marennine, released in oyster-ponds: Effect on growth and photosynthesis in two diatoms, *Haslea ostrearia* and *Skeletonema costatum*. J Appl Phycol 12:557–566.

Zou N, Zhang CW, Cohen Z, Richmond A. 2000. Production of cell mass and eicosapentaenoic acid (EPA) in ultrahigh cell density cultures of *Nannochloropsis* sp. (Eustigmatophyceae). Eur J Phycol 35:127–133.