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Direct spectrophotometric method to determine cell density of *Isochrysis galbana* in serial batch cultures from a larger scale fed-batch culture in exponential phase

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ABSTRACT

In this work, a very useful and accurate procedure, based on the spectrophotometric method published by the American Public Health Association in the Standards Methods for the Examination of Water and Wastewater, was developed to determine cell density of *Isochrysis galbana* performing a single direct absorbance measurement in exponential phase of growth, which is the desirable operating mode for any microalgae production plant. Thus, *Isochrysis galbana* was cultured in serial batch cultures from a larger scale fed-batch culture. The growth performance of this species of microalgae under laboratory conditions was analysed by spectrophotometry at different wavelengths and cell counting in a haemocytometer (Neubauer chamber) showing that doubling times and cell death increased with increasing initial cell density. Besides, it was demonstrated that the absorbance of these cultures followed a linear trend as a function of time and cell density during the exponential phase of growth, results in which the developed direct method is based on. .

KEYWORDS: Isochrysis galbana*, batch, fed-batch culture, spectrophotometry, haemocytometer, growth performance.*

RESUMEN

En este trabajo, se ha determinado un procedimiento muy útil y preciso basado en el método espectrofotométrico descrito por la Asociación Americana Pública de Salud en los Métodos Estándar para el Análisis de Aguas y Aguas Residuales, para calcular la densidad celular de cultivos de *Isochrysis galbana* realizando una única medida de absorbancia en fase exponencial de crecimiento que es el modo de trabajo deseable de cualquier planta de producción de microalgas. De este modo, se realizó una serie de cultivos de *Isochrysis galbana* en discontinuo a partir de otro cultivo a mayor escala con alimentación intermitente. Se ha analizado el nivel de crecimiento de esta especie de microalga por espectrofotometría a diferentes longitudes de onda y recuento de células en un hemocitómetro (cámara de Neubauer) mostrando que el tiemplo de duplicación y la muerte celular aumentó con el aumento de la densidad celular inicial. Además, se ha demostrado que la absorbancia de estos cultivos sigue una tendencia lineal en función del tiempo y densidad celular durante la fase de crecimiento exponencial, resultados en los cuales se basa el método directo desarrollado.

PALABRAS CLAVE: Isochrysis galbana*, cultivo discontinuo, alimentación intermitente, espectrofotometría, hemocitómetro, crecimiento.*

INTRODUCTION

The most prominent issue which has been dominating the applied microalgal world for the past few years is the great surge of interest in the mass cultivation of microalgae for promising products, first and foremost among which has been

microalgal fuel. Besides, the idea of microalgae as a food source for a growing world population to solve the future extreme protein deficiency predicted by the United Nations Advisory Committee has encouraged research activity in this field as a source of food and feed to avert hunger. Thus, a deficit of commodities such as fish meal is evolving rapidly. For example, for the expected total demand of 100 million tons of products from aquaculture in the coming decade, a deficiency of 10 million tons of polyunsaturated fatty acids (PUFA) is envisioned and an increase in food prices is thus to be expected without satisfying the growing demand [1].

The hatchery production of commercially valuable fish and shellfish is one application of microalgae because they constitute an almost exclusive live food for the larvae. Among many microalgae identified for aquaculture purposes, the genera of *Isochrysis* have been used extensively as food in most shrimp hatcheries in the last decades [2]. *Isochrysis* have been important for aquaculture hatcheries because they grow rapidly, provide rapid and healthy growth for shellfish, are easy to maintain in large volume cultures, and produce significant amounts of PUFAS [3].

Thus, *Isochrysis galbana* is widely cultured with applications in the bivalve aquaculture industry [4] and is gaining much attention in the last years for its high amount of Fucoxanthin (18.23 mg/g dried sample) [5]. A number of studies have examined the metabolism, safety, and bioactivities of fucoxanthin, including its anti-cancer, anti-obesity, antioxidant, anti-inflammatory, anti-diabetic, and anti-angiogenic activities [6, 7].

One of the most obvious characteristics of algae is their colour. Each phylum has its own particular pattern of pigments and an individual colour. With a few exceptions, the pigmentation of higher plants is thought to have evolved. However, in view of their phylogenetic age, it is obvious that they have developed additional pigments that are unique to them [1]. There is a possibility that the photosynthetic pigments and carotenoids that determine algal coloration are the constituents responsible for the antioxidant activity. Blaine and John stated that the concentration of microalgal pigments may vary depending on the species, thus illustrating that the activities of certain microalgae may also differ from one another [8]. Screening results also demonstrated that green algae and red algae are less active as antioxidants than brown algae [9, 10], and these facts are in good agreement with the results obtained by Natrah et al. in [11], where *I. galbana* (browncoloured algae) were found to be more active than the other microalgae in the ferric thiocyanate (FTC) and thiobarbituric acid (TBA) antioxidant chemical assays. High antioxidant activity from brown algae (*Sargassum polycystum*) was also found using the FTC method [12]. Besides, other than antioxidant activity, it was clearly showed that the species of microalgae studied in this work possess good proximate and biochemical content that would be beneficial to humans and animals. Thus, *I. galbana* had average percentage composition of protein, carbohydrate, and lipid as 47.9±2.5, 26.8±0.2 and 14.5±1.4% respectively. In addition, it contains high levels of omega-3 polyunsaturated fatty acids (PUFA) (28.0%±0.7), omega-6 PUFA (6.5%±1.8) and a high composition of essential amino acids [11].

Phytoplankton biomass can be estimated by determining the concentration of photosynthetic pigments in a water sample transferring an extract to a cuvette and measuring optical density at 750, 664, 647, and 630 nm, according to the American Public Health Association (APHA) in the Standards Methods for the Examination of Water and Wastewater [13]. The optical density readings at 664, 647, and 630 nm are used to determine chlorophyll a, b, and c, respectively and the absorption at 750 nm is a correction for turbidity. These spectrophotometric measurements are performed after following a chemical procedure to obtain an extract with the pigments.

The growth performance of Isochrysis galbana grown in batch culture under laboratory conditions was studied by Phatarpekar et al. in [14] showing that *I. galbana* had an initial rapid growth at 20±2°C. However, in this work, the growth of this microorganism was studied at higher temperature in serial batch cultures with inoculum taken from a larger scale fed-back culture in exponential phase, which is the desirable operating mode for any microalgae production plant.

MATERIALS AND METHODS

Isochrysis galbana was grown in 250 ml Erlenmeyer flasks in batch cultures using f/2 autoclaved medium [15] with a salinity of 35 g/l under continuous fluorescent illumination (6400k cool white type; two tubes of 18W) in a temperature controlled room at 27±3°C. The carbon source was provided via bubbling of 1% enriched CO_2 air into the culture

medium. Cultures used for the experiment were unialgal but non-axenic. Batch cultures were started with three equal inoculum of 50 ml taken from a 3 l fed-batch culture at different times. Once the first inoculum was taken, the second and third one were taken after 2 and 5 days respectively, adding 200 ml of f/2 autoclaved medium to start the 250 ml cultures from different points of the growth curve of the 3 l fed-batch culture. After taking inoculum of 50 ml, the same volume of f/2 autoclaved medium was added to the 3 l mother culture. These series of three 250 ml batch cultures will be called hereafter as C1.1, C1.2, C1.3 respectively and C3 for the 3 l culture. No more medium was added to the fed-batch culture after taking the inoculum of 50 ml for C1.3.

The cellular density of the three 250 ml batch cultures and that of C3 were determined by counting aliquots of cultures fixed with Lugol solution in a haemocytometer (Neubauer chamber) every two or three days. Duplicate samples were used for all estimations.

Spectrophotometric measurements were performed in a Perkin Elmer lambda bio + spectrophotometer. Thus, the direct spectrophotometric method followed in this work consisted of measuring optical density at 750, 664, 647, and 630 nm as the APHA method [13]. However, absorption measurements were performed directly in aliquots of cultures without carrying out any chemical extraction of pigments and another direct optical density reading was performed at 650 nm to have an additional absorption value inside the major absorption band of all chlorophylls.

Assuming that the cell growth of this microorganism can be considered as a first-order autocatalytic reaction, the growth rate of the cultures was calculated from the following expression.

$$k = (\ln N_t - \ln N_0)/t \quad (1)$$

where N_t = cell count at time 't', N_0 = initial cell count at time '0' and t = time (days).

Number of doublings (n) at a time interval t is determined by the relation t/t_d, where t_d is the doubling time or time required to achieve a doubling of the number of viable cells. Thus, the doubling time can be estimated with equation (3) in a growing culture after being incubated for some time t a number of cells (N_t).

$$N_t = N_0 2^n \quad (2)$$

$$t_d = (\ln 2)t / (\ln N_t / N_0) \quad (3)$$

RESULTS AND DISCUSSION

Cell density and growth rate

The cell density variations of the three 250 ml batch and the 3 l fed-batch cultures during the experimental period (23 days) are shown in Figure 1. C3 was only measured during 12 days to make sure that this fed-batch culture reached the continuous exponential phase of growth while *I. galbana* was cultured feeding 50 ml of f/2 autoclaved medium after two and five days (see Figure 1). However, since no more medium was fed in this 3 l fed-batch culture after five days, growth performance decreased more and more due to the depletion of nutrients in this mother culture (see Figure 2).

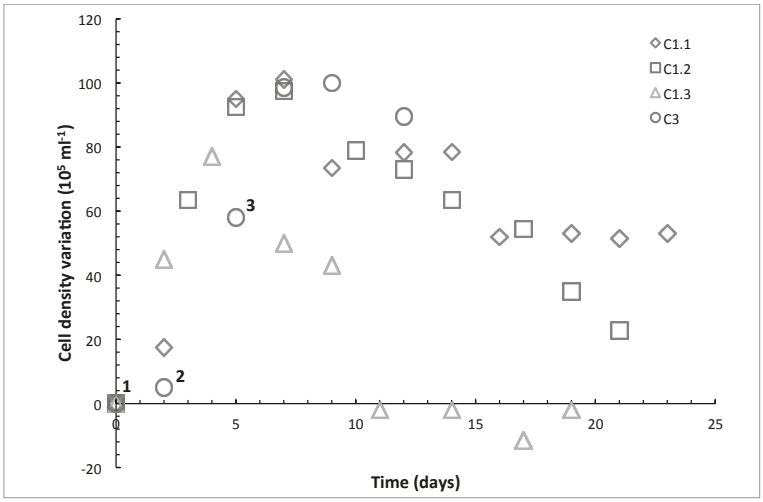

Figure 1. Cell density variation (cell density at time 't' - initial cell density) of *Isochrysis galbana* as a function of time for C1.1, C1.2 and C1.3 (beginning growth from point 1, 2 and 3 of the C3 growth curve respectively).

The growth rates of the four cultures were calculated with equation (1) and are depicted in Figure 2.

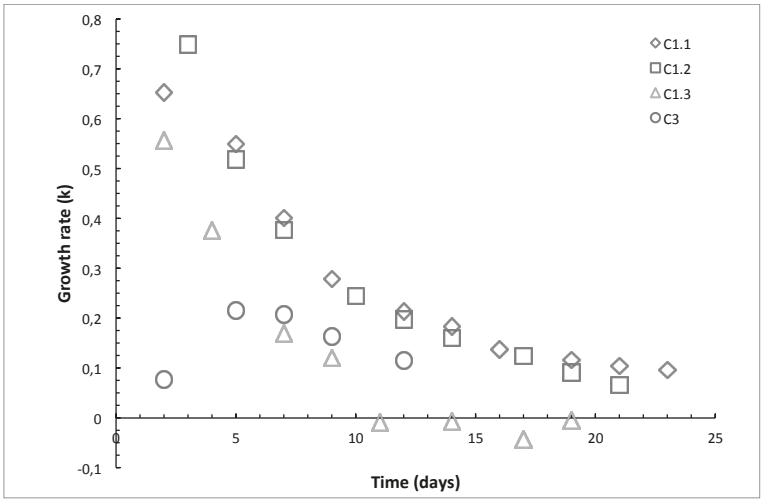

Figure 2. Growth rate of *Isochrysis galbana* as a function of time for C1.1, C1.2, C1.3 and C3.

Figure 2 shows that C1.1, which starts growing from point 1 at the lag phase of the C3 mother growth curve (see Figure 1), starts growing at a lower growth rate than the C1.2 culture. However, it reaches the maximum cell variation. The C1.2 culture starts growing at the highest growth rate due to point 2 belongs to the exponential phase of the C3 curve (see Figure 1). Finally, C.1.3 shows the lowest growth rate of the three 250 ml cultures and reaches a much lower cell density variation due to the initial cellular density of this culture being much higher than the other two (650000, 750000

and 2200000 cell/ml for C1.1, C1.2, C1.3 respectively), even though it also started growing from the C3 exponential phase. Thus, the higher initial cellular density, the sooner nutrient depletion occurs and more shadows are formed inside the culture decreasing light accessibility. For the same reason, the growth rate of the 3 l fed-batch culture (initial cellular density of 3000000 cell/ml) is the lowest until the fifth day of the experimental period and quite similar to C1.3 after that day. It can also be seen in these two Figures 1 and 2 that cell death also increases with increasing initial cell density being very significant in C1.3 with negative cell density variations and growth rates at the end of the experiment.

Doubling time

Equation (3) was applied to determine the doubling times after five days of culture. These results are shown in the following Figure 3.

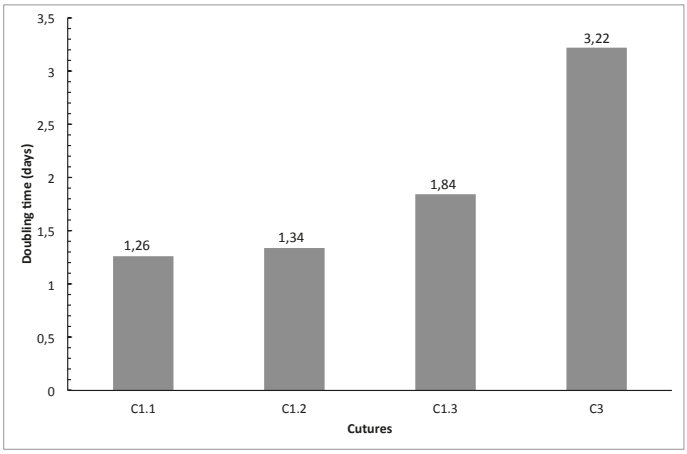

Figura 3. Doubling time of *Isochrysis galbana* determined from day 0 to 5 for C1.1, C1.2, C1.3 and C3.

These results show that doubling time increases with increasing initial cellular density. Besides, this trend is linear for the three batch cultures and the following equation (4) was determined from the fitting of these three doubling time-cell density points with a correlation coefficient of 0,9951.

$$t_d = 4 \cdot 10^{-7} \cdot CD_0 + 1{,}0462 \quad (4)$$

where CD_0 is initial cell density in cell/ml and t_d doubling time in days. Thus, this equation indicates that the minimum value of t_d could be approximately 1,05 by decreasing CD_0 to 1 cell/ml.

On the other hand, C3 shows the highest t_d because it started with the largest initial cell density. However, C3 did not follow the same linear trend as the other three batch cultures because of being a larger scale culture with the same light intensity and being fed intermittently. Therefore, these results are in very good agreement with those shown in Figure 1 and 2.

Spectrophotometry

Direct spectrophotometric measurements as a function of time were performed without any chemical procedure at 647, 664, 630, 650, and 750 nm in C1.1, C1.2, C1.3, and C3. Figure 4 shows theses results for the three batch cultures.

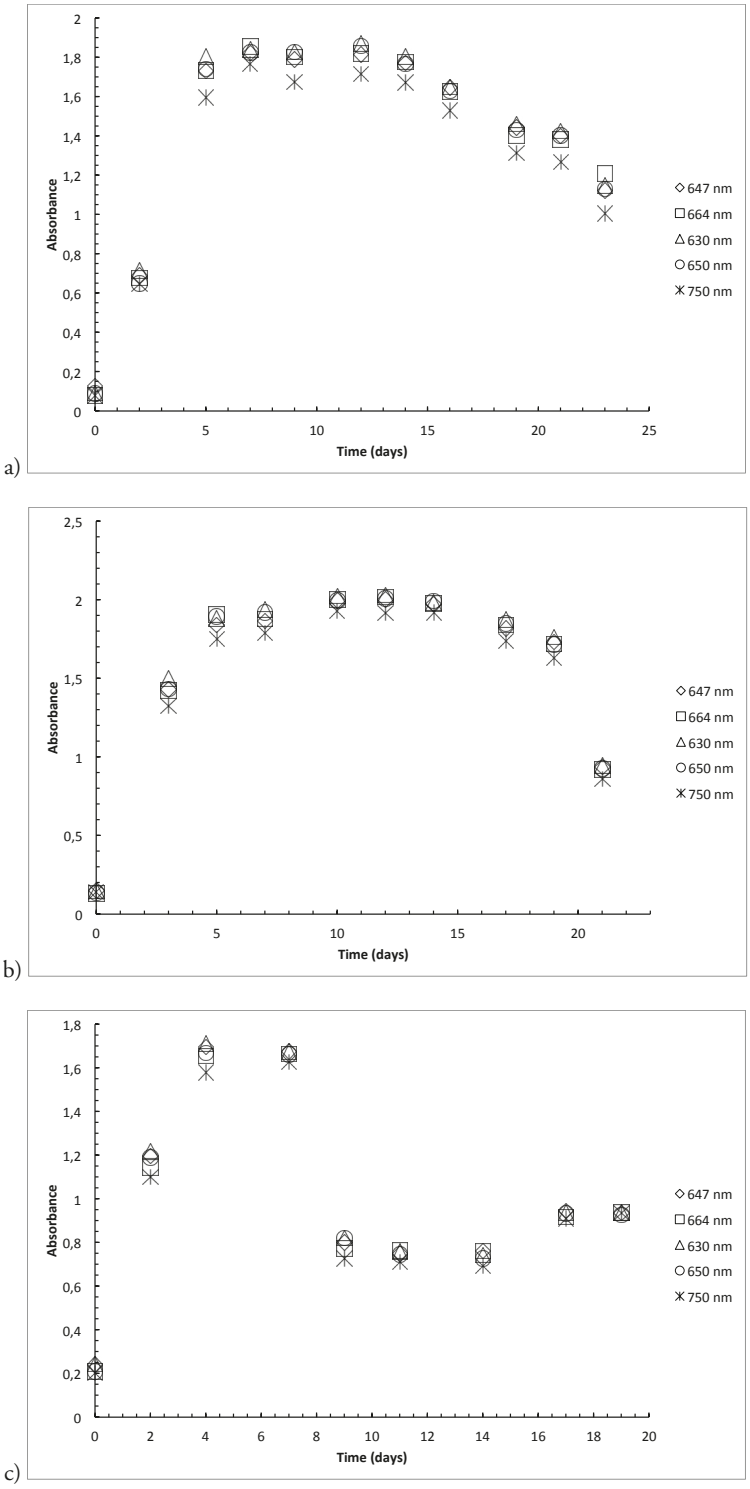

Figure 4. Absorbance as a function of time for C1.1 (*a*), C1.2 (*b*) and C1.3(*c*).

C1.1 and C1.2, which started growing with not very different initial cell densities, have similar absorbance-time growth curves. However, C1.3, which began growing from point 3 with an initial cell density more than three times larger, shows a sudden drastic loss of growth after the fifth day. Therefore, these results are in very good agreement with the cell counting results shown in Figure 1.

It can be seen in Figure 4 that the absorbance-time curves for C1.1, C1.2, and C.1.3 are linear during the first five days of culture. Besides, the C3 absorbance-time curve showed also a linear trend of growth until day 5 (curve not shown). Thus, if cell density is plotted against absorbance, a linear fit can be performed during this culture period, even though there is much dispersion after this growth period.

Table 1 shows the correlation coefficients (R^2) of these cell density-absorbance linear regressions for the four cultures.

Table 1. Correlation coefficients (R^2) of the cell density versus absorbance fittings for C1.1, C1.2, C1.3 and C3.

Wavelength (nm)	C1.1	C1.2	C1.3	C3
R^2 at **647**	0,9681	0,9942	0,9933	0,9981
R^2 at **664**	0,9642	0,9980	0,9949	0,9931
R^2 at **630**	0,9625	0,9900	0,9918	0,9947
R^2 at **650**	0,9724	0,9970	0,9901	0,9990
R^2 at **750**	0,9586	0,9973	0,9940	0,9844

It was shown in Figure 1 that C1.1 started growing with a lower cell density variation because their 50 ml aliquot was taken from the point 1 of the lag phase of the C3 mother growth curve. This fact could explain why the correlation coefficients of C1.1 are much worse than the other three batch cultures in Table 1. These results also show that the best linear fittings were performed at 650 and 664 nm (bold letters) probably because these wavelengths are the closest to the value of 682 nm, which was recently found to provide the maximum light absorbance for this species of microalgae [16].

Therefore, cell density can be determined directly during the exponential phase of growth measuring directly absorbance at 664 nm for the batch cultures and applying equation (5) with the corresponding m and n values shown in Table 2. This equation is valid for serial batch cultures from C1.2 on. The cell density of the fed-batch culture can also be calculated with this equation (5) but measuring absorbance at 650 nm to have a more accurate result.

$$CD = m \cdot A + n \tag{5}$$

where CD is cell density and A is absorbance.

Table 2. m and n values for C1.1, C1.2, C1.3 and C3 at the optimal wavelengths (650 and 664 nm).

Linear regression	**C1.2 at 664 nm**	**C1.3 at 664 nm**	**C3 at 650 nm**
m	$5 \cdot 10^6$	$5 \cdot 10^6$	$7 \cdot 10^6$
n	10773	995032	-604800

C1.2 and C1.3 show the best correlation coefficients of the three batch cultures and similar m value probably because both started growing from the C3 exponential phase of growth, even thought they had very different initial cell densities.

Therefore, it was determined a very useful and accurate method to determine cell density as a function of direct spectrophotometric measurement of a culture aliquot. This method is only valid when the batch cultures and the larger scale fed-batch culture are in exponential phase of growth, which is the optimal mode of production for the microalgae industry.

CONCLUSION

Three 250 ml batch cultures of this species of microalgae were carried out with different initial cell densities taken from a 3 l fed-batch culture showing that both doubling times and cell death increased with increasing initial cellular density. It was demonstrated by spectrophotometry and cell counting that cell death was very significant in C1.3 with negative cell density variations and growth rates at the end of the experiment due to the higher initial cell density of this culture. Besides, the doubling times of these three batch cultures followed a linear trend as a function of the initial cell densities, and an equation was obtained with a correlation coefficient of 0,9951. From this equation, a minimum doubling time of 1,05 days was deduced by decreasing the initial cell density to the minimum. However, C3 showed the highest t_d because of starting with the largest initial cell density, but did not follow the same linear trend as the other three batch cultures because it was a larger scale culture with the same light intensity and was fed intermittently. Finally the absorbance-time curves at different wavelengths showed linear trends during the first five days of culture for the four cultures. Thus, a direct method, based on the American Public Health Association method, was developed to directly measure cell density from the spectrophotometric absorbance of an aliquot when both batch and fed-batch cultures are in exponential phase of growth.

REFERENCES

[1] A. Richmond, Q. Hu (Eds.). *Handbook of Microalgal Culture: applied phycology and biotechnology, Second Edition.* Wiley Blackwell, 2013.

[2] L. L Smith, J. M. Fox, G. D. Treece. *Intensive algal culture techniques., Hand Book of Mariculture, Crustacean Aquaculture*, CRC Press, Boca Raton, 1993.

[3] V. Patil, T. Källqvist, E. Olsen, G. Vogt, H. R. Gislerød. Fatty acid composition of 12 microalgae for possible use in aquaculture feed. *Aquacult. Int.* **15** (2007) 1-9.

[4] M. M Helm, N. Bourne. Hatchery culture of bivalves: a practical manual. FAO Fisheries and Aquaculture Department. Food and Agriculture Organization of the United Nations, 2004.

[5] K. Sang Min, P. Cheol-Ho. Fucoxanthin as a major carotenoid in Isochrysis aff. galbana: Characterization of extraction for commercial application, *Journal of the Korean Society for Applied Biological Chemistry* **55** (2012) 477-483.

[6] K. Miyashita, S. Nishikawa, F. Beppu, T. Tsukui, M. Abe, M. Hosokawa. The allenic carotenoid fucoxanthin, a novel marine netraceutical from brown seaweeds, *J Sci Food Agric* **91** (2011) 1166-74.

[7] J. Peng, J. P. Yuan, C. F. Wu, J. H. Wang. Fucoxanthin, a marine carotenoid present in brown seaweeds and diatoms: metabolism andbioactivities relevant to human health, *Mar Drugs* **9** (2011) 1806-28.

[8] M. Blaine, W. P. John. Biologically active compounds from microalgae, *Enzyme Microb Technol* **8** (1986) 386-394.

[9] R. Matsukawa, Z. Dubinsky, E. Kishimoto, K. Masaki, Y. Masuda, T. Takeuchi, M. Chihara, Y. Yamamoto, E. Nike, I. Karube. A comparison of screening methods for antioxidant activity in seaweeds. *J Appl Phycol* **9** (1997) 29-35.

[10] X. Yan, Y. Chuda, M. Suzuki, T. Nagata. Fucoxanthin as the major antioxidant in Hijikia fusiformis, a common edible seaweed, *Biosci Biotechnol Biochem* **63** (1999) 605-607.

[11] F. M. I. Natrah, F. M. Yusoff, M. Shariff, F. Abans, N. S. Marina. Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value, *J Appl Phycol* **19** (2007) 711-718.

[12] Anggadiredja J, Andyani R, Hayati, Muawanah. Antioxidant activity of Sargassum polycystum (Phaeophyta) and Laurencia obtusa (Rhodophyta) from Seribu Islands, *J Appl Phycol* **9** (1997) 477-479.

[13] American Public Health Association. *Standards Methods for the Examination of Water and Wastewater 22nd edition*, 2012.

[14] P. V. Phatarpekar, R. A. Sreepada, C. Pednekar, C. T. Achuthankutty. A comparative study on growth performance and biochemical composition of mixed culture of Isochrysis galbana and Chaetoceros calcitrans with monocultures, *Aquaculture* **181** (2000) 141-155.

[15] R. R. L. Guillard, J. H. Ryther. Studies of marine planktonic diatoms. Cyclotella nana Hustedt and Detonula confervacea (Cleve) Gran. *Can. J. Microbiol* **8** (1962) 229-239.

[16] D. U. Santos-Ballardo, S. Rossi, V. Hernández, R. Vázquez Gómez, M. C. Rendón-Unceta, J. Caro-Corrales, A. Valdez-Ortiz. A simple spectrophotometric method for biomass measurement of important microalgae species in aquaculture, *Aquaculture* **448** (2015) 87-92.

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