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Original Article

Effects of culture medium on growth kinetics and fatty acid composition of *Chlorella* sp. T12

Kanokwan Chankhong[1], Wilaiwan Chotigeat[1, 2], and Jutarut Iewkittayakorn[1*]

[1] *Department of Molecular Biotechnology and Bioinformatics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand*

[2] *Center for Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand*

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Abstract

Many factors affect the growth performance of microalgae, especially culture medium impacts growth rate and fatty acid composition. Thus, the aims of this study were to investigate the effects of various culture media including NSIII, Chu No.10, BG11 and BBM on the growth kinetics, biomass productivity, lipid content, lipid productivity and fatty acid profiles of cultivated *Chlorella* sp.T12. The *Chlorella* sp.T12 was cultured at 25±2 ºC temperature under continuous 3,500 Lux light intensity for 14 days. The culture in BBM medium allowed the highest of the specific growth rate (0.83±0.69 d^{-1}), biomass productivity (0.20±0.05 g/l/d), lipid content of the dry weight (14.434%) and lipid productivity (0.029±0.01 g/l/d) and percentage of unsaturated fatty acid (67.56%). The unsaturated fatty acid meet requirement of fatty acid composition that suitable for biodiesel production. Although, the lipid content of cultivation in NSIII was the lowest, the percentage of saturated fatty acid (51.88%) was the highest that suitable for pharmaceutical and cosmetic production. Therefore, the BBM was the best medium choice for cultivating *Chlorella* sp.T12 to provide high productivity of biomass and lipids that can be used in biodiesel. NSIII was the best medium choice for cultivating *Chlorella* sp.T12 for pharmaceutical and cosmetic production.

Keywords: biodiesel, *Chlorella* sp.T12, fatty acids, lipid content, microalgae

1. Introduction

Microalgae are currently popular candidate feed-stocks for producing many products and biofuels (Abdeshahian *et al*., 2010). They have (1) higher photosynthetic efficiency than terrestrial plants, and some species can accumulate lipids in excess of 50% of their dry biomass (Sathya *et al*., 2012); (2) potentiality to grow in inappropriate conditions such as saline water, brackish, deserts arid and semi-arid land; (3) potentiality to use nitrogen and phosphorus in wastewater, for example agricultural drainage, Industrial wastewater, and urban wastewater; and potentiality to absorb carbon dioxide discharge on burning fossil fuels, in machine among others (Chisti, 2007; Knothe, 2005); and (5) they are considered to be non-food plants.

Chlorella is the most cultivated micro-alga, and popularly used in medicine and cosmetics industries due to its unique properties such as proteins, polysaccharides, carotenoids, vitamins, lipids, immune-stimulator compounds, anti-oxidants and minerals (Pienkos & Darzins, 2009). *Chlorella* can also be exploited in biodiesel production (Chisti, 2007). However, the development of *Chlorella* based products depends on many factors, the most important being the culture medium (Shay *et al*., 1987). Its chemical composition affects growth of the microalgae, biomass production, and fatty acid profile. NSIII medium, Chu No.10 medium, BG11 medium, and Bold's basal medium (BBM) were proposed to be good available media for microalgae cultivation (Borowitzka, 2005; Gong & Chen, 1997). These media reportedly greatly impact

*Corresponding author
Email address: jutarut.p@psu.ac.th

the growth of freshwater microalgae (Ilavasi *et al*., 2011). The extracted lipids from microalgae mainly contain fatty acid ranging from C8:0 to C24:1, while C16:0, C18:1 and C18:2 are reported as fatty acids suitable for biodiesel production (De la Pena, 2007).

Moreover, in large-scale production of *Chlorella*, growth kinetics, yields of biomass and lipid, are the main parameters required to evaluate the economic feasibility (Wijffels *et al*., 2010). Therefore, this present study focused on investigating alternative media for *Chlorella* sp. T12 cultivation. The culture media tested were NSIII, Chu No. 10, BBM and BG11, and their effects on growth kinetics, biomass, lipid content and fatty acid compositions were determined.

2. Materials and Methods

2.1 Microorganism and culture condition

The green microalgae, *Chlorella* sp. T12, obtained from Department of Biology, Faculty of Science, Prince of Songkhla University, Thailand, were cultivated in four alternative culture media: NSIII (Hosakul, 1972), Chu No. 10 (Chu, 1942), BG11 (Andersen, 2005), and Bold's Basal medium (BBM) (Bischoff & Bold, 1963). The individual compositions and concentrations (g/l) of each medium are listed in Table 1. 150 ml working volume of the medium were conducted in each 250 ml Erlenmeyer flasks for cultivation. The initial cell concentration was set at 0.2 of OD_{680}. The culture flasks were inoculated with 10% (v/v) inoculums and were incubated at 25±2 °C temperature under 3,500 Lux continuous light intensity (no dark period in cycle). Air was also fed continuously as the CO_2 source, and to prevent sedimentation of the algae.

2.2 Primary screening of microalgae cells

Nile red staining was used in the primary lipid screening to assess from stain intensity the lipid content of *Chlorella* sp. T12 grown in the four alternative culture media: Chu No 10, NSIII, BBM and BG11. Nile red (9-diethylamino-5H benzo [α] phenoxazin-5-one; Sigma-Aldrich, USA) was dissolved in acetone at 0.1 mg/ml concentration. Then, 10 µl of this Nile red solution was mixed with 100 µl of cell suspension in a microcentrifuge tube, before incubating for 10 minutes. The lipid staining was observed under a fluorescence microscope (Abdo *et al*., 2014; Neboh *et al*., 2014).

2.3 Lipid extraction of microalgae cells

Lipid extraction: The microalgae cells were collected by centrifugation at 4000 rpm for 10 min. The cells were washed twice with deionized water, freeze-dried and weighed. The cells were then extracted by methanol: chloroform blend (2:1, v/ v) and sonicated for 30 min. Methanol, chloroform and water blended in the ratio 2:2:1 (v/v/v) was added, and the mixture was centrifuged at 4,000 rpm for 15 min to separate phases. The chloroform phase was collected and evaporated at room temperature to remove the organic solvent. The obtained residual was weighed as the lipids in the sample (Bligh & Dyer, 1959).

Table 1. Nutrient compositions of the alternative media tested for *Chlorella* sp.T12 cultivation.

NSIII (Hosakul, 1972)	Chu No.10 (Chu, 1942)	BG11 (Andersen, 2005)	Bold's Basal (Bischoff and Bold, 1963)
pH 6.7	pH 7.1	pH 7.5	pH 7
101.1 g KNO_3	0.04 g $Ca(NO_3)_2$	15 g $NaNO_3$	2.50 g $NaNO_3$
6.2 g $MgSO_4.7H_2O$	0.025g$MgSO_4.7H_2O$	7.5 g $MgSO_4.7H_2O$	0.75 g $MgSO_4.7H_2O$
120 g KH_2PO_4	0.005 g KH_2PO_4	0.6 g $C_6H_8O_7$	1.75 g KH_2PO_4
142 g K_2HPO_4	0.02 g Na_2CO_3	4 g K_2HPO_4	0.75 g K_2HPO_4
7.4 g $CaCl_2.2H_2O$	0.025 g $2Na_2O$	3.6 g $CaCl_2.2H_2O$	0.25 g $CaCl_2.2H_2O$
0.6 g NaCl	0.008 g $FeCl_3$	0.6g $Fe(NH_4)_3(C_6H_5O_7)_2$	0.25 g NaCl
Trace element		0.1 g $EDTANa_2$	Trace element
0.750 g EDTA		2 g Na_2CO_3	5 g EDTA
0.415 g KCl		Trace element	3.1 g KOH
0.077 g H_3BO_3		2.86 g H_3BO_3	1.142 g H_3BO_3
0.05 g $MnCl_2.4H_2O$		1.81 g $MnCl_2.4H_2O$	0.144 g $MnCl_2.4H_2O$
0.144 g $ZnSO_4.7H_2O$		0.22 g $ZnSO_4.7H_2O$	0.882 g $ZnSO_4.7H_2O$
0.125 g $CuSO_4.5H_2O$		0.08 g $CuSO_4.5H_2O$	0.157 g $CuSO_4.5H_2O$
8.1 g $Fe(NO_3)_3$. $7H_2O$		0.39 g $Na_2MoO_4.2H_2O$	0.498 g $FeSO_4.7H_2O$
0.07g $CoSO_4.7H_2O$		0.05 g $Co(NO_3)_2.7H_2O$	0.09 g $Co(NO_3)_2.7H_2O$
0.658 g $NiSO_4.6H_2O$			0.071 g MoO_3
0.04g $(NH_4)_6Mo_7O_{24}.4H_2O$			
0.029 g of NH_4VO_3			
0.167g $Al_2(SO_4)_3$ $18H_2O$			
0.0212 g LiCl			
0.595 g KBr			

2.4 Determination of growth kinetics

The growth kinetics of *Chlorella* sp. T12 cultures were determined and specific growth rate, biomass productivity and lipid productivity are reported.

Specific growth rate: the *Chlorella* sp. T12 culture was observed every 2 days for 14 days by determining both the optical density at 680 nm, with a UV-vis spectrophotometer, and the cell number density, counted under a microscope using a hemocytometer (Sharma *et al*., 2015). The specific growth rate is the number of doublings per unit time, in the population number count of an exponentially growing culture. The following equation is commonly applied to calculate the specific growth rate:

$$\mu\ (d^{-1}) = \frac{(\ln O.D_t - \ln O.D_0)}{t - t_0} \quad (1)$$

where μ is the specific growth rate; $O.D_t$ is optical density at the last day of exponential phase; $O.D_0$ is optical density at the starting day of exponential phase; t is the final day of observation and t_0 is the starting day, during the exponential phase of population growth. The doubling time is obtained by a transformation of the specific growth rate:

$$\text{Doubling time} = \ln 2 / \mu \quad (2)$$

where μ is the specific growth rate (Sharma *et al*., 2015).

After cultivation for 14 days, the *Chlorella* sp. T12 cells were harvested by centrifugation at 4,000 rpm for 10 min before washing twice with deionized water, frozen overnight at -80°C and freeze-dried at -40°C, under 133×10^{-3} mbar pressure (i.e., vacuum) conditions, for 24 h. The pellet were then determined values of biomass productivity and lipid productivity.

Biomass productivity: the pellets were weighed and the results considered as the dry biomass. Biomass concentration (dry-weight of cell powder in the culture medium, g/l) was estimated from the following equation, which was periodically compared to the dry-weight determinations:

$$\text{DCW (g/l)} = 1.1649\ x - 0.0663 \quad (3)$$

where x is the optical density at 680 nm by an UV-vis spectrophotometer. Biomass productivity P_{Biomass} (g/l/day) was calculated as:

$$P_{\text{Biomass}} = \frac{X_t - X_0}{t_t - t_0} \quad (4)$$

where X_t and X_0 are the biomass weight concentrations (g/l) on days t_t (end point of exponential phase) and, t_0 (start point of exponential phase), respectively (Hempel *et al*., 2012).

Lipid productivity: the previous cells were extracted for lipids, according to Bligh & Dyer (1959). The lipid content was calculated using equation (5):

$$C_{\text{Lipid}}(\%) = \frac{W_L}{W_B} \times 100 \quad (5)$$

where C_{Lipid} is the lipid content (%), W_L is the extracted lipids' weight (g), and W_B is the dried cell weight of the algae (g) (Hempel *et al*., 2012). Then, the lipid productivity P_{Lipid} (g/l/day) was calculated by:

$$P_{\text{Lipids}} = C_{Lipid} \times \frac{P_{Biomass}}{100} \quad (6)$$

where P_{Lipid} is productivity of lipid C_{Lipid} is lipid content (%) and $P_{Biomass}$ is biomass productivity (Hempel *et al*., 2012).

2.5 Fatty acid analysis by gas chromatography

Crude lipid extracts were changed to fatty acid methyl esters following the protocol of Jham & Campos (Jham *et al*., 1982). The fatty acid compositions were determined using a gas chromatography (GC, Agilent 7890A Series) equipped with automatic liquid sampler, flame ionization detector, and select biodiesel for FAME (J&W Scientific) fused silica with bonded polyethylene glycol phase capillary column (30 m length × 0.32 mm inner diameter, 0.25 μm stationary phase film). The operational conditions programmed were: inlet temperature 290 °C, split ratio 20:1, helium flow rate 1.0 ml/min, initial oven temperature 210 °C, hold for 12 min; Ramp to 250 °C, Rate 20 °C/min, hold for 8 min, detector temperature 250 ° C, hydrogen flow rate 30 ml/min, air flow rate 300 mL/min, and makeup flow rate 25 mL/min. Each retention time of fatty acid peak was identified by comparison with of fatty acid methyl ester standards (Supelco, U.S.A).

2.6 Statistical analysis

Statistical analysis was conducted via software SPSS 11.5. One-way analysis of variance (ANOVA) and Tukey's method were used to consider significant differences between means ($p<0.05$).

3. Results and Discussion

3.1 Primary screening by lipid accumulation, extracted lipids

The primary screening was based on fluorescent staining assessed under a microscope, with representative images shown in Figure 1. It was noted that the Nile red dye interacted with the intracellular lipid globules in the *Chlorella* cells. The lipid globules emitted orange/yellow fluorescence when interacting with the Nile red dye, whereas the chlorophyll emitted red auto-fluorescence as reported in (Halim & Webley, 2015). Although the C*hlorella* cells cultured in Chu No10 medium had the most orange/yellow globules, followed by NSIII, BG11, and BBM, the results of lipid extraction showed that the *Chlorella* cells cultured in BBM had the highest oil content, followed by Chu No 10, BG11 and NSIII, in this order Table 2. This result indicated that the lipid staining with nile red dye is preliminary screening, the emitted fluorescent could not be qualified for the lipid content.

3.2 Effects of culture medium on growth curves

The growth curves of *Chlorella* sp. T12 cultured in NSIII, Chu No 10, BG11 and BBM media for 14 days can be seen in Figure 2. The growth curves showed mainly two stages of growth (lag and log phases), only Figure 2D showed three stages (lag, log and stationary phases). Figure 2A, 2B, and 2C showed the log phase after day 2 until day 14 of cultivation time. Figure 2D: lag phase (day 0-2), log phase (day 2-10) and stationary phase (day 10-14). It is observed that the *Chlorella* sp. T12 grew faster in BBM (Figure 2D) and BG11 (Figure 2C) than in the other media.

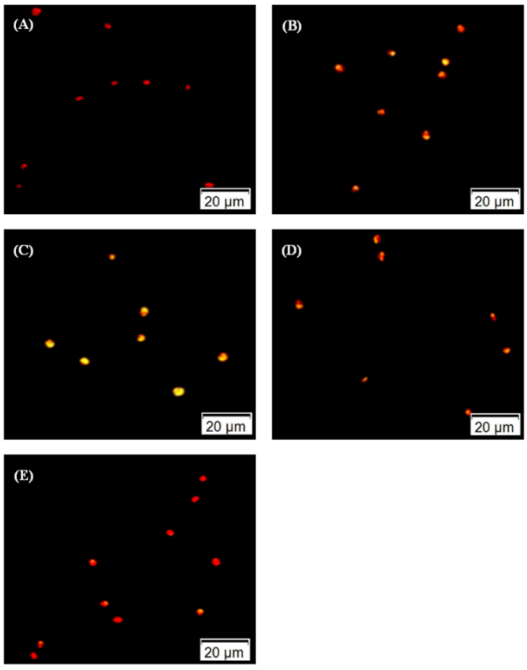

Figure 1. *Chlorella* sp. T12 stained with nile red fluorescence dye viewed under fluorescent microscope, (A) cell without nile red staining (control), (B) cells cultured in NSIII medium, (C) cells cultured in Chu No 10 medium, (D) cells cultured in BG11 medium, (E) cells cultured in BBM medium.

3.3 Effects of medium on growth kinetics and lipid production

Summarizing parameters describing the growth kinetics, namely specific growth rate, doubling time, biomass concentration, biomass productivity, lipid content, and lipid productivity are given in Table 2. It was found that *Chlorella* sp. T12 cultured in BBM medium gave the highest values of specific growth rate, biomass concentration, biomass productivity, lipid content and lipid productivity. Moreover, it is also found that the lipid content of *Chlorella* sp. T12 cultured in BBM (14.43 % w/w) were higher than that of *C. vulgaris* cultured in BBM (5.90 % w/w) (Converti *et al*., 2009). While the lipid content of *Chlorella* sp. T12 cultured in BG11 medium (11.35 % w/w) was about the same as *C. pyrenoidosa* cultured in BG11 medium (11.02%) (Shama *et al*., 2015), respectively.

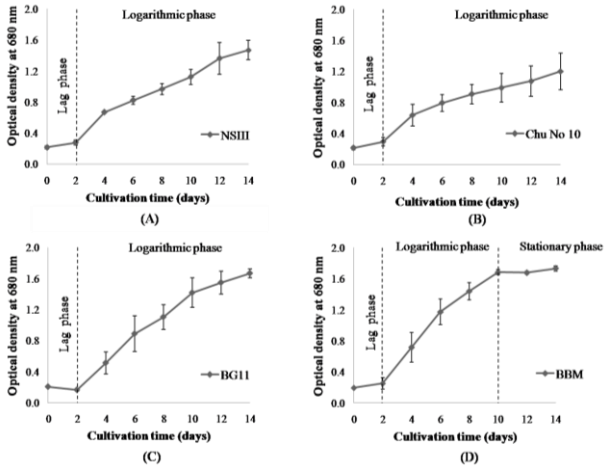

Figure 2. Growth curve of *Chlorella* sp.T12 in different media, (A) NSIII medium, (B) Chu No 10 medium, (C) BG11 medium and (D) BBM medium.

3.4 Effects of medium on fatty acid methyl esters on *Chlorella* sp. T12 lipids

The effects of culture medium on fatty acids, obtained from the extracted oils, were investigated for fatty acid profiles (C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C22:0, C22:1, C24:0 and C24:1) using gas chromategraphy. The fraction of fatty acid methyl esters in the total fatty acids is shown in Table 3.

The majority of the saturated fatty acids in *Chlorella* sp. T12, across all cases of culture medium, were caprylic acid (C8:0), nonanoic acid (C9:0), capric acid (C10:0), lauric acid (C12:0), palmitic acid (C16:0) and stearic acid (C18:0). In addition, the saturated fatty acids of *Chlorella* sp. T12 in culture medium NSIII, Chu No10 and BG11 were significant higher than the culture in BBM medium. The comparison between *Chlorella* sp. T12 and *Chlorella vulgaris* were culture in same BG11 (Bertoldi *et al*., 2006), the saturated fatty acids from *Chlorella* sp. T12 (47.82%) were found much higher than these from *Chlorella vulgaris* (38.35%). And the saturated fatty acids produced from *Chlorella* sp. T12 (43.76%) cultured in BBM were much more then *Chlorella sorokiniana* (25.82%) cultured in the same medium (Chader *et al*., 2011).

Table 2. Kinetic parameters of *Chlorella* sp. T12 cultivation in four media (n=3).

Culture media	Specific growth rate (μ/day^{-1})	Doubling time (day)	Biomass concentration (g/l)	Biomass productivity (g/l/d)	Lipid content % (w/w)	Lipid productivity (g/l/d)
NSIII	0.44±0.05^{a}	1.58±0.17^{a}	0.948^{a}	0.17±0.01^{a}	8.04±0.04^{a}	0.014±0.00^{a}
Chu no 10	0.38±0.09^{a}	1.88±0.46^{a}	1.088^{a}	0.15±0.05^{a}	11.56±0.04^{c}	0.017±0.01^{ab}
BG11	0.42±0.05^{a}	1.68±0.02^{a}	1.490^{b}	0.16±0.05^{a}	11.35±0.06^{b}	0.018±0.01^{ab}
BBM	0.83±0.69^{a}	1.23±0.76^{a}	1.869^{c}	0.20±0.05^{a}	14.43±0.08^{d}	0.029±0.01^{b}

Notes: Data are presented as mean ± standard deviation from 3 samples, ND is not detected, In each row, different lettering above paired values means that the values obtained from analysis of variance ANOVA are significantly different at $P<0.05$.

Table 3. Percentage of major fatty acid compositions of *Chlorella* sp.T12 cultivated under different media.

Fatty acid (Carbon atoms: double bonds)	Fatty acid composition (% of total fatty acids)					
	NSIII	Chu No.10	BG11	BBM	BG11*	BBM**
C8:0	1.74±0.85	1.42±0.08	2.82±1.31	1.10±0.11	ND	ND
C9:0	1.74±1.36	1.71±0.10	3.37±1.48	1.18±0.33	ND	ND
C10:0	1.35±0.57	1.13±0.09	2.06±0.93	0.88±0.07	ND	ND
C11:0	ND	0.41±0.03	ND	0.32±0.02	ND	ND
C12:0	0.84±0.36	1.06±0.31	1.33±0.61	0.92±0.14	ND	ND
C13:0	ND	ND	1.98±0.45	ND	ND	ND
C14:0	ND	0.61±0.01	0.81±0.08	0.73±0.05	ND	ND
C16:0	37.69±2.06	38.38±0.04	29.07±1.04	33.45±0.41	19.20	23.24
C17:0	ND	ND	ND	ND	4.19	ND
C18:0	8.29±0.32	5.55±0.04	7.32±0.30	5.28±0.13	14.60	2.54
C20:0	ND	ND	ND	ND	0.36	ND
Sum of saturated	51.88[b]	50.27[b]	47.82[ab]	43.76[a]	38.35	25.82
C16:1	1.19±0.08	0.52±0.01	1.62±0.06	1.14±0.03	0.87	ND
C18:1	8.43±0.24	17.43±0.16	3.01±0.13	10.00±0.26	12.66	30.99
C18:2	33.58±028	25.13±0.41	33.70±1.84	34.48±0.42	3.77	23.72
C18:3	7.61±0.60	6.66±002	14.39±0.93	10.61±0.12	21.09	12.85
C20:1	ND	ND	ND	ND	4.09	ND
C22:1	ND	ND	ND	ND	0.05	ND
Sum of unsaturated	48.12[a]	49.73[a]	52.18[ab]	56.24[b]	42.53	67.56
Total	100	100	100	100	80.88	93.38
SFA/UFA[b]	1.08	1.01	0.92	0.78	0.90	0.38

Note: Data are presented as mean ± standard deviation from 3 samples, ND is not detected, In each row, different lettering above paired values means that the values obtained from analysis of variance ANOVA are significantly different at $P<0.05$, SFA/ UFA is ratio of saturated fatty acids to unsaturated fatty acids. * denotes fatty acid compositions obtained from *Chlorella vulgaris* was reported by Cleber Bertoldi *et al*. (2006). ** denotes fatty acid compositions obtained from *Chlorella sorokiniana* was reported by Chader *et al*. (2011).

In contrast, the unsaturated fatty acids produced from *Chlorella* sp. T12 cultured in BBM were significantly higher than this microalgae was cultured in other medium (NSIII, Chu No 10). In addition, *Chlorella* sp. T12 (56.24%) produced the unsaturated fatty acids more than *Chlorella sorokiniana* (6.56%) when cultured in BBM. The ratio of SFA / UFA from *Chlorella* sp. T12 culture in BBM was less than that cultured in other medium, whereas the ratio was higher than that from *Chlorella sorokiniana*.

This could be concluded that the lipid products depend not only on the species of the microalgae but also the culture medium. The specific composition of the medium required for production saturated or unsaturated fatty acids should be further studied. The total percentages of identified saturated fatty acids were 51.88, 50.27, 47.82, and 43.76%, for the NSIII, Chu No10, BG11 and BBM media, respectively, whereas the rest was unsaturated fatty acids (56.24, 52.18, 49.73 and 48.12%, in the BBM, BG11, Chu No10 and NSIII, respectively). The approximate ratio of saturated to unsaturated fatty acids for BBM, BG11, Chu No10 and NSIII media cultures were 1.08, 1.01, 0.92, and 0.78, respectively. Knothe (2008) reported that palmitic, stearic, oleic and linoleic acid are the mainly common fatty acids in biodiesel. Therefore, the fatty acids observed in *Chlorella* sp. T12 cultured in BBM medium appear suitable for biodiesel production as well as often applications showed in Table 4.

Table 4. Fatty acids and their application (Guzman, 2013).

Fatty acid	Application
Caprylic acid (C8:0)	Lubricant ester, cosmetics, PVC stabilizers, perfumes and fungicides
Nonanoic acid (C9:0)	Cosmetics, pharmaceuticals and corrosion inhibitors
Capric acid (C10:0)	Lubricant ester, surfactants, cosmetics, PVC stabilizers, and fungicides
Lauric acid (C12:0)	Surfactant raw material, cosmetics, lauryl peroxides, and pharmaceuticals
Myristic acid (C14:0)	Detergent soap raw material and esters isopropyl myristate-cosmetics
Palmitic acid (C16:0)	Esters, shaving gels, pharmaceuticals and metallic pigments
Palmitoleic acid (C16:1)	Cleansing and cosmetic products
Stearic acid (C18:0)	Metal and specialty soaps, rubber industry pearling agent, candies and esters
Oleic acid (C18:1)	Cleansing soap raw material, metal soap for plastics and lubricant esters
Linoleic acid (C18:2)	Quick-drying oil for oil paints and varnishes
Linolenic acid (C18:3)	Quick-drying oil for oil paints and varnishes

4. Conclusions

The present work investigated the effects of alternative culture media, namely NSIII, Chu No.10, BG11 and BBM, on the growth kinetics and the fatty acid compositions of *Chlorella* sp. T12 cultures. It was observed that the BBM medium was practically suitable for culturing *Chlorella* sp. T12, giving the fastest growth with 0.54±0.57 d^{-1} specific growth rate, 0.20±0.05 (g/l/d) biomass productivity, 14.43± 0.08% lipid content, and 0.029±0.01 (g/l/d) lipid productivity. The major fatty acids extracted from *Chlorella* sp. T12 cultures that found in all media were palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid. This result showed that all media can provide major fatty acids that can be used in pharmaceutical and biofuel applications. The BBM was the best medium choice for cultivating *Chlorella* sp. T12 for biodiesel production

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