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Variations in lipid profile of *Isochrysis galbana*, a marine microalga, grown in different culture media for their use as live feed in mariculture

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Original Article

Abstract

The present work envisages close monitoring of variations in lipid profile and thereby analyzing the dietary status of the live feed *Isochrysis galbana*, a marine microalga by growing in 4 different standard culture media. Pure cultures of microalga were collected from the marine hatchery complex of the Central Marine Fisheries Research Institute, Kochi and cultured under four different media. Cultures were maintained in 4-litre haffkine flasks in triplicates under standard algal culture conditions. Fatty acid profiles were evaluated in microalgae cultured in all four algal culture media. Total saturated fatty acids ranged from 56.37% (Walne's medium), 62.32% (Miquel's medium), 75.02% (Chu#10 medium) to 79.35% (f/2 medium). Monounsaturated fatty acids were quantified as lowest of 12.21% in Miquel's medium as against the highest of 32.76% in Walne's medium. Polyunsaturated fatty acids (PUFAs) accounted for over 25.47% of the total fatty acids in Miquel's medium whereas it was only 6.05% for f/2, 7.21% for Chu#10 and 10.87% for Walne's media respectively. Statistical analysis of data brought to light that the composition of the fatty acid pattern of microalgae significantly ($p \leq 0.05$) varied with media of algal culture. By analyzing the results it can be confirmed that the nutritional value of *Isochrysis galbana* can be enhanced by culturing in Miquel's medium.

Keywords: *Microalgae, Isochrysis galbana, fatty acid profiling, monounsaturated fatty acids, polyunsaturated fatty acids, live feed*

Introduction

Screening and betterment of culture medium are preconditions for photoautotrophic cultivation of microalgae. Generally, the quality of the culture medium ascertains the growth performance of the microalgae (Prathima *et al*., 2011; Lam and Lee, 2012; Li *et al*., 2012). Vital variations in growth dynamics, biochemical composition and fatty acid layouts of microalgae have been noticed in response to growth media and time of harvest (Lincymol *et al*., 2012; Naseera *et al*., 2013; Lidiya *et al*., 2018; Aswathy *et al*., 2020). Manipulation of the dietary standing of the microalgae is possible by changing media composition and culture methods (Otero *et al*., 2006; Rivero-Rodriguez *et al*., 2007; Ilavarasi *et al*., 2011; Neethu and Dhandapani, 2016; Prabha *et al*., 2016). Changes within the carboxylic acid moiety within a given species in response to nutrient accessibility are also reported (Volkman *et al*., 1989; Fernandez *et al*., 1989; Dunstan *et al*., 1993). Since the composition and availability of macro and micro-nutrients within the media directly influence algal cultures, they should be supplied at an optimum level (Carvalho *et al*., 2006; Liu *et al*., 2008). As macronutrients such as nitrogen, potassium, magnesium, sulfur, and sodium are harmless to microalgal cells they can be used at larger concentrations. In distinction, essential trace elements such as Fe, Cu, Mn, Zn, Co and Mo are noxious at higher concentrations and growth-retarding at lower amounts (Spotte *et al*., 1979; Becker, 1994). The growth kinetics of microalgal cultures is affected by the availability of these trace elements as they play a major role in many metabolic channels (Sunda *et al*., 2005). The marine microalga, *I. galbana,* is a commonly used planktonic feed in the mariculture industry (Sukenik, 1991). Assimilation of microalgal cells by shellfish larvae is easier mainly due to their minuteness and lack of rigid cell walls. Because of their high proliferation rate, easiness in large-scale

cultivation, wide temperature and salinity endurance, and lack of toxins (Jeffrey *et al*., 1994) *Isochrysis* spp. have been used as a possible source of very long-chain polyunsaturated fatty acids (VLC PUFAs) such as Arachidonic acid, AA (C20:4n−6), Eicosapentaenoic acid, EPA(C20:5n−3), Docosahexaenoic acid, DHA (C22:6 n−3), etc. for nurturing many aquatic species such as finfishes and crustaceans, as well as their larvae (Guedes and Malcata, 2012).

It ought to be noted that the optimum concentration of nutrients in the culture media varies reckoning on the microalgal species likewise because of the processing and culture parameters. Therefore the medium should be optimized for every microalgal strain before use for mass cultivation. A detailed understanding of total lipid content and fatty acid profiles in terms of pattern in saturation and unsaturation in various microalgal culture media can help to estimate the changes in their nutritional value which can open prospects for the manipulation of culture conditions for particular microalgae. The present investigation aimed to assess four different standard culture media with different ingredients to enrich the nutritional status of microalga in terms of total fat and fatty acid profiles towards more unsaturation of marine microalga, *I. galbana*.

Material and methods

Pure culture of microalgae was obtained from ICAR-Central Marine Fisheries Research Institute (CMFRI), Kochi and was maintained in a standard f/2 medium (Table 1) to ensure maximum production of biomass. Since no single medium is suitable for higher growth and biochemical production in microalgal species, we attempted a few different formulations that are often used and found within the literature. About 600 ml (20% of the culture medium) of microalgal isolate at an initial inoculum density of 20-30x10^4 exponentially growing cells/ml (Pavlo *et al*., 2016) was transferred to previously autoclaved, properly capped and aerated borosilicate four-litre culture flasks (a set of 3 for each treatment) under aseptic conditions. For the illumination of the cultures, fluorescent tubes having an intensity of 1500 lux were utilized (Hoff and Snell, 1987). A light/ dark (L/D) cycle of twelve hours of light and twelve hours of darkness was used for maintaining the stock as well as major cultures that were controlled by an auto-timer (Barsanti and Gualtieri, 2006). Air-conditioned rooms having a fixed temperature of 25 °C were used for keeping the stock cultures. For cultures, seawater with a salinity of 33 to 34ppt was used (Barsanti and Gualtieri, 2006).

Table 1. Composition of f/2 medium (Guillard, 1975)

A	Sodium nitrate	7.5g/100ml
	Sodium orthophosphate	500mg/100ml
	Sodium silicate	3g/100ml
B	Ferric chloride	0.3gm/9.5ml
	Sodium EDTA	0.44gm/9.5ml
	Manganese chloride	18gm/100ml
	Zinc sulphate	2.2g/100ml
	Cobalt chloride	1g/100ml
	Copper sulphate	0.98g/100ml
	Sodium molybdate	0.63g/100ml
C	Thiamine	200mg/L
	Biotin	1mg/L
	Cyanocobalamin	1mg/L

The duration of the lag phase, log phase and stationary phases of the microalga could be monitored based on cell count, following the method of Andersen (2005). It was found that microalgae in f/2 medium attained exponential phase on the 42nd hour of inoculation; those in Walne's and Miquel's medium on the 48th hour and in Chu#10 medium, it was on the 96th hour after inoculation. After determining the cell concentration (Hoff and Snell, 1987), the fully grown culture was harvested during the late exponential phase (using High-Speed Refrigerated Centrifuge (Himac CR 22G). For this purpose, the algal suspension was centrifuged at 10000 rpm for one minute. The supernatant was discarded and the pellets were collected after multiple washing with seawater.

Total lipids were extracted as in Bligh and Dyer, 1959. About 500 mg to 1 gm of wet microalgal sample beside a pinch of Butylated Hydroxy Toluene (to prevent oxidation) was homogenized well in 5-10 ml distilled water using a pestle and mortar. The pulp was transferred to a 250 ml conical flask and mixed with a 20-30 ml chloroform-methanol (2:1 v/v) mixture and shaken well. The mixture was kept overnight at 4.0 °C preferably in the dark for complete extraction. At the end of this period, further addition of 20 ml chloroform and 20 ml distilled water was made. The resulting solution was subjected to centrifugation and three layers were obtained. Then the mixture was transferred to a separating funnel and the lower chloroform layer was carefully collected free of the interface by filtering through sodium sulphate using filter paper. It was concentrated in a pre-weighed round bottom flask at 40-45 °C using a rotary vacuum evaporator. Allowed to cool and the weight (w_1) was noted. Estimation of total lipid was done using the formula, Lipid = (w_1-w_2/w_3) x 100 (wherein w_1 = weight of flask + lipid, w_2 = weight of flask, w_3 = weight of sample taken). Fatty acids were analyzed as fatty acid methyl esters (FAMEs) following the methods of Metcalf *et al.* (1996). For this 5.0 ml of 0.5 N methanolic alkali was added to the extracted lipid and reflexed for five minutes in boiling water bath under a nitrogen atmosphere so that breakage of

ester bond (saponification of lipid) occurred. After cooling, 5ml BF_3 Methanol solution was pipetted out and slowly added into it, refluxed for another 5 min in a boiling water bath under a nitrogen atmosphere and the mixture was kept for cooling so that Fatty Acid Methyl Esters (FAMEs) were formed.

After cooling, 5-6 ml saturated NaCl was added to it and mixed well. FAMEs were mixed well with petroleum ether (double the volume of the solution) three times. Each time the lower layer was discarded. The upper petroleum ether layer containing FAMEs was washed thrice with distilled water, filtered through anhydrous Na_2SO_4, rotary evaporated and the concentrate was then reconstituted in a minimum amount of petroleum ether and used for injecting in GC. For injection, 1μl of the sample was used. The area of each component was obtained from computer-generated data. FAMEs were identified by comparison of retention times with the known standards (Supelco TM 37 Component FAME Mix, Catalog No. 47885-U), and the results were expressed as % Total Fatty Acid.

Statistical analysis

Statistical evaluation (to compare means) was carried out after applying arcsin transformation with the Statistical Program for Social Sciences (SPSS Inc, Chicago, USA, ver. 22.0). The differences between transformed mean values were compared according to Post-Hoc test (Tukey) taking p≤0.05 as significant.

Results and discussion

The amount of lipid produced in percentage from the harvested algal biomass of all experimental treatments is shown in Fig. 1. The highest lipid content (27.40%) was obtained from *I. galbana* culture in Chu#10 medium followed by f/2 medium (19.51%), Miquel's medium (18.30%) and the lowest was for Walne's medium (15.90%). Microalgae can utilize carbonate for the building up of cellular biomolecules (Wang *et al.,* 2008). The highest production of lipids in cultures of Chu#10 medium is due to the presence of sodium carbonate in the medium, which is lacking in the other three. This result corresponded with those obtained by Naseera *et al.* (2013). They cultured *I. galbana* in the same media and for maximum lipid production, the medium Chu#10 deserved mention. Analysis of data revealed that there is notable variance (p≤0.05) in the total lipid content of *I. galbana* culture in Chu#10 medium among the four media tested. The results in the other three media showed no significant difference (P<0.05). Since f/2 medium contains the optimum level of trace elements, growth and lipid production balance each other for the investigated algal species.

The gas chromatogram of the lipid profile of *I. galbana* grown in four different standard culture media is shown in Figs. 2

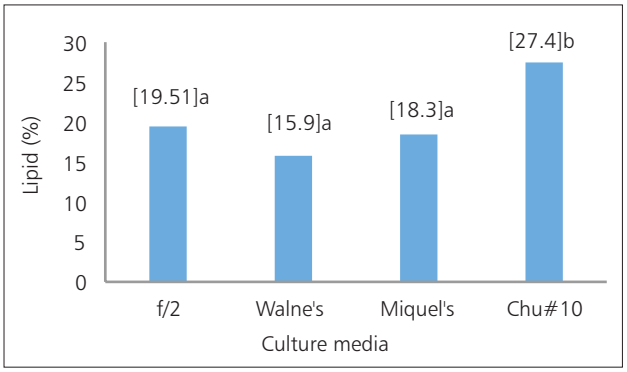

Fig. 1. Amount of Lipid produced by *Isochrysis galbana* when grown in 4 different standard culture media. Bar having different superscript in the mean value indicates significant difference (p≤0.05)

to 5. The fatty acids belonging to the classes of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) were analyzed in all experimental cultures and are graphically expressed as a percentage of total fatty acids in Fig. 6. The percentage of prominent fatty acids obtained and their transformed mean values are given in Tables 5 and 6 respectively.

Total saturated fatty acids ranged from 56.37% (Walne's

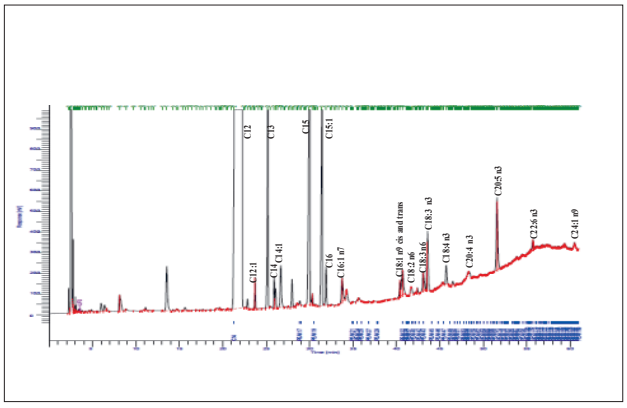

Fig. 2. Gas chromatogram of the lipid profile of *I. galbana* in f/2 medium

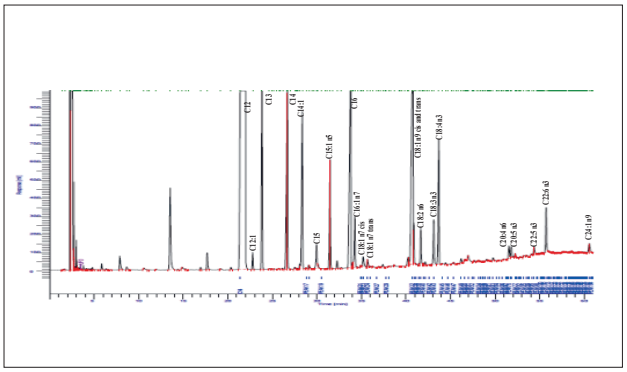

Fig. 3. Gas chromatogram of the lipid profile of *I. galbana* in Walne's medium

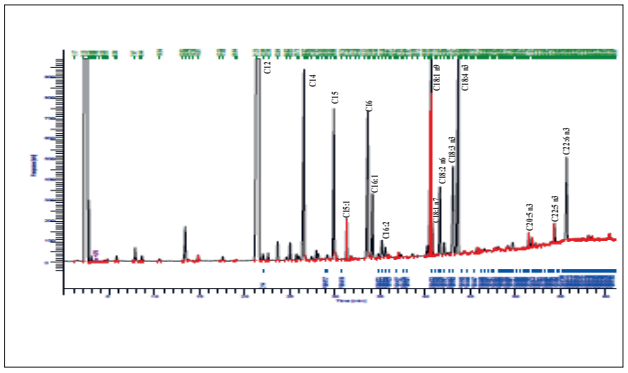

Fig. 4. Gas chromatogram of the lipid profile of *Isochrysis galbana* in Miquel's medium

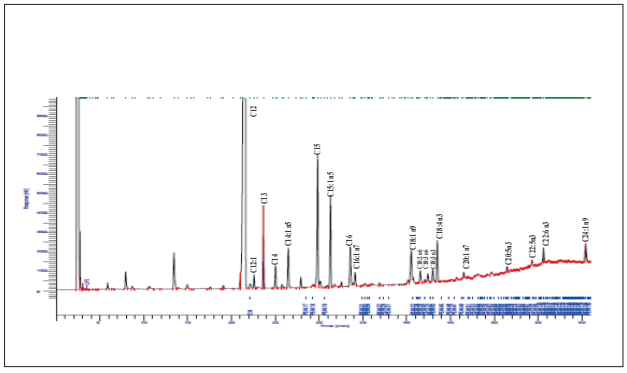

Fig. 5. Gas chromatogram of the lipid profile of *Isochrysis galbana* in Chu#10 medium

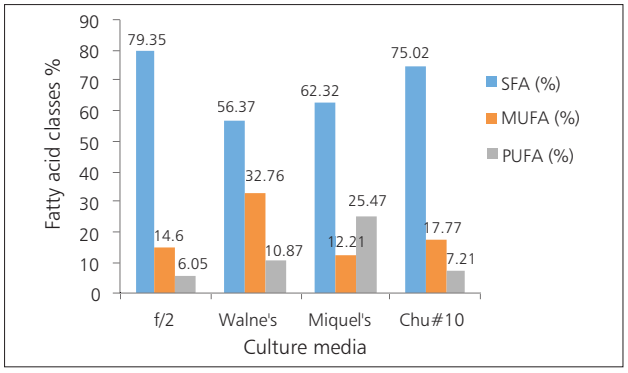

Fig. 6. Variations in fatty acid classes of *I.galbana* when grown in four different standard culture media

medium), 62.32% (Miquel's medium), 75.02% (Chu#10 medium) to 79.35% (f/2 medium). The findings of the current investigation showed that among saturated fatty acids, lauric acid (C12:0) is the major SFA in cultures of almost all media starting a value from 36.26% (Miquel's), 38.78% (Walne's), 55.54% (Chu#10) to 62.15% (f/2). Data analysis showed that there are remarkable variations ($p \leq 0.05$) regarding SFA content among the four tested media. In the current evaluation, the highest proportion of MUFA content corresponded to Walne's medium (32.76%) and the next highest to Chu#10 (17.77%) followed by f/2 (14.60%) and Miquel's (12.21%) media.

Iron (Fe) is taken as one of the foremost vital trace elements in microalgal growth media. Fe source of 2g/100 ml stock solution can account for the high lipid content of *I. galbana* cultures in Miquel's medium than those in the f/2 and Walne's media. This finding is supported by Liu *et al.* (2008), who recorded the lipid contents in *Botryococcus sudeticus*, *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Ettlia oleoabundans* showing 10, 60, 18, and 36% increases at high concentrations of Fe. It is expected that Fe will increase the overall production of lipids owing to the down-regulation of iron utilizing fatty acid desaturase enzymes. *Chlorella vulgaris* under laboratory conditions showed a considerable elevation in lipid radicals of the membranes when Fe was added, up to 500 μM (Estevez *et al.*, 2001). The addition of Fe in the form of inorganic salts will tend to precipitate and become unavailable to algae. As Miquel's medium contains Fe in the form of ferric chloride, it may shorten N assimilation thereby leading to lipid induction.

It is found that among MUFAs, Oleic acid (18:1 *n*-9) is the major one present in *I. galbana*, which attained maximum value in Walne's medium (14.53%), and minimum in f/2 (1.60%). In Miquel's medium, it is 6.89 and in Chu#10, the value is 3.35% of the total fatty acids obtained. Several previous studies have demonstrated that oleic acid is the major MUFA present in marine organisms (Walkowiak, 1979, Chen *et al.*, 2007, Barrento *et al.*, 2010). Ackman *et al.* (1968) reported the abundance of C18:1n-9 over C18:1n-7 in some prymnesiophytes. Univariate analysis showed that there is notable deviance($p \leq 0.05$) in the amount of MUFA among the four different treatments.

We observed nine categories of polyunsaturated fatty acids (PUFA) which are presented in Tables 5 and 6. It is seen that of all

Table 2. Composition of Walne's medium (Walne, 1974)

A	Potassium nitrate	10g
	Sodium orthophosphate	2g
	Sodium EDTA	4.5g
	Manganese chloride	0.036g
	Sodium EDTA	4.5g
	Ferric chloride	0.13g
	DW	100ml
B	Zinc chloride	0.42g
	Cobalt chloride	0.4g
	Copper sulphate	0.4g
	Ammonium molybdate	0.18g
	DW	100ml
C	Thiamine	0.1gm in 50ml DW
	Cyanocobalamin	0.005gm in 50 ml DW
	Both are mixed together	

Table 3. Composition of Miquel's medium (Miquel, 1892)

A.	Potassium Nitrate	20.2 g
B.	Sodium Ortho Phosphate	4 g
C	Calcium Chloride	2 g
	Ferric Chloride	2 g
	Hydrochloric Acid	2 ml
	Distilled Water	100 ml

Table 4. Composition of Chu # 10 medium (Chu *et al.*,1942)

A	Calcium nitrate	5.76g
B	Pottassium ortho phosphate	0.5g
C	Magnesium sulphate	2.5g
D	Sodium carbonate	2g
E	Sodium silicate	2.5g
F	Ferric chloride	0.08g

Table 5. Percentage of prominent fatty acids in *I. galbana* rown in four different standard culture media

Fatty acids	f/2 medium	Walne's medium	Miquel's medium	Chu#10 medium
C12:0	62.15±1.65	38.78±1.09	36.26±1.20	55.54±3.00
C13:0	1.06±0.1	nd	nd	2.81±1.00
C14:0	0.64±0.45	5.66±1.00	10.68±1.00	1.73±0.50
C15:0	13.78±1.00	1.06±0.50	6.87±0.09	11.17±1.78
C16:0	1.72±0.70	10.87±1.40	8.51±1.20	3.77±0.75
C12:1	0.56±0.01	6.53±2.10	nd	0.88±0.10
C14:1 n -5	2.31±0.80	6.28±2.00	0.40±0.01	3.49±1.00
C15:1 n- 5	9.22±2.10	2.65±1.00	0.89±0.21	7.30±2.00
C16:1 n- 7	0.88±0.01	1.89±0.10	3.08±1.00	1.12±0.01
C18:1 n -7*cis*	nd	0.47±0.18	0.94±0.09	nd
C18:1 n -7*trans*	nd	0.14±0.01	nd	nd
C18:1 n- 9*cis*	0.76±0.01	13.60±2.60	6.89±2.10	3.35±1.78
C18:1 n-9*trans*	0.84±0.10	0.93±0.27	nd	nd
C20:1 n-7	nd	nd	nd	0.40±0.01
C24:1 n -9	nd	nd	0.02±0.00	1.24±0.09
C18:2 n-6	0.03±0.003	1.37±0.230	3.37±1.000	1.07±0.025
C18:3 n-6	0.88±0.210	nd	4.76±0.980	0.61±0.090
C18:3 n-3	2.05±0.090	1.83±0.107	nd	1.06±0.100
C18:4 n-3	0.84±0.178	4.87±1.400	11.86±1.800	3.43±1.090
C20:4 n-3	0.03±0.002	nd	nd	0.26±0.070
C20:4 n-6	nd	0.48±0.230	nd	nd
C20:5 n-3	2.03±0.800	0.41±0.156	0.37±0.090	0.07±0.007
C22:5 n-3	nd	0.16±0.001	0.94±0.000	0.07±0.001
C22:6 n-3	0.19±0.004	1.77±0.580	4.16±0.890	0.64±0.100
n-3/n-6 Index	5.64	4.87	2.13	3.29

The data represent the mean values ± standard deviation of 3 replications. nd = not detected

the four media assayed, Miquel's medium produced the highest percentage of PUFA (25.47%) followed by Walne's medium (10.87%) and Chu#10 medium (7.21%). A marked variance ($p \leq 0.05$) is evident in the polyunsaturated fatty acid radicals among cultures in different media. Even though the level of SFAs and MUFAs were minimal for *I. galbana* cultures in Miquel's medium, it produced the highest level of polyunsaturated fatty acids (25.47%). A close examination of Table 3 reveals that it has sufficient quantity of macronutrients like N, P, K, Ca, etc. When N is adequate in the medium, microalgae synthesise membrane glycerolipids which occupy the cellular membrane systems (Piorreck and Pohl, 1984). These glycerol-based membrane lipids

Table 6. Transformed mean values of prominent fatty acids of *I.galbana* in four different culture media based on Post-Hoc Test (Tukey).

Fatty acids	f/2 medium	Walnes medium	Miquel's medium	Chu#10 medium
C12:0	0.9082^{c}	0.6722^{a}	0.6461^{a}	0.8409^{b}
C13:0	0.1029^{b}	0.0000^{a}	0.0000^{a}	0.1666^{c}
C14:0	0.0763^{a}	0.2397^{b}	0.3326^{c}	0.1311^{a}
C15:0	0.3802^{c}	0.1010^{a}	0.2652^{b}	0.3401^{c}
C16:0	0.1296^{a}	0.3356^{c}	0.2956^{c}	0.1948^{b}
C12:1	0.0746^{b}	0.2562^{c}	0.0000^{a}	0.0941^{b}
C14:1 n-5	0.1509^{b}	0.2513^{c}	0.0634^{a}	0.1868^{bc}
C15:1 n-5	0.3073^{b}	0.1614^{a}	0.0939^{a}	0.2719^{b}
C16:1 n-7	0.0940^{a}	0.1380^{bc}	0.1748^{c}	0.1060^{ab}
C18:1 n-7 *cis*	0.0000^{a}	0.0675^{b}	0.0970^{c}	0.0000^{a}
C18:1 n-7 *trans*	0.0000^{a}	0.0369^{b}	0.0000^{a}	0.0000^{a}
C18:1 n-9 *cis*	0.0872^{a}	0.3778^{c}	0.2635^{b}	0.1793^{ab}
C18:1 n-9 *trans*	0.0917^{b}	0.1027^{b}	0.0000^{a}	0.0000^{a}
C20: 1 n-7	0.0000^{a}	0.0000^{a}	0.0000^{a}	0.0629^{b}
C24:1 n-9	0.0192^{b}	0.0441^{c}	0.0134^{a}	0.1113^{d}
C18:2 n-6	0.0185^{a}	0.1170^{b}	0.1833^{c}	0.1038^{b}
C18:3 n-6	0.0935^{b}	0.0000^{a}	0.2193^{c}	0.0783^{b}
C18:3 n-3	0.1436^{c}	0.1355^{b}	0.0000^{a}	0.1032^{b}
C18:4 n-3	0.0915^{a}	0.2208^{b}	0.3510^{c}	0.1846^{b}
C20:4 n-3	0.0161^{b}	0.0000^{a}	0.0000^{a}	0.0504^{c}
C20:4 n-6	0.0000^{a}	0.0683^{b}	0.0000^{a}	0.0000^{a}
C20:5 n-3	0.1410^{b}	0.0632^{a}	0.0608^{a}	0.0258^{a}
C22:5 n-3	0.0000^{a}	0.0398^{c}	0.0971^{d}	0.0261^{b}
C22:6 n-3	0.0435^{a}	0.1320^{b}	0.2046^{c}	0.0798^{a}

Transformed mean values having the same superscript in the same row belong to the same homogeneous subset. Different superscripts in the same row indicate significant differences ($p \leq 0.05$)

are constituted particularly of long-chain unsaturated fatty acids containing various kinds of PUFAs and perform a structural role in the cell (Hu *et al*., 2008). Thus, the accumulation of PUFAs occurs in the exponential growth phase (Hu *et al*., 2008). The presence of a large amount of PUFA in Miquel's medium right here similarly testifies to the above-mentioned findings.

Eicosapentaenoic acid (EPA) (C20:5 n-3) became maximum in f/2 medium (2.03%). Camacho-Rodriguez *et al.* (2014) noticed that a growth medium with a low amount of Zn would be having a lesser value of EPA content in *Nannochloropsis gaditana*. They also stated that biotin (Vitamin B7) was important for EPA production whereas thiamine (Vitamin B1) and cyanocobalamin (Vitamin B12) were not. Another reason for the production of EPA in f/2medium cultures is none other than the presence of biotin in it. Since Miquel's and Chu#10 media are lacking certain microelements, the current investigation concurs with that of Richmond (1986) who unveiled that the absence of some trace elements such as Zn, B, Mo and Cu in the medium promoted increased production of some PUFA, like the EPA. It is reported that if the n-3/n-6 quotient comes within the range of 2-5, the biomass can have acceptable nutritional quality (Webb and Chu,1983). In the current study, *I. galbana* biomass from f/2 medium provided a maximum value of n-3/n-6 index (5.64) followed by Walne's medium (4.87), Chu#10 medium (3.29) and the least for that of Miquel's, which are shown in Table 5. The causes for the exceptional results are not known. But the findings ought not to be taken as typical of this species. Alterations in all probability arise from the culture conditions, analytical strategies or in the developmental phase sampled, making it difficult to compare the results presented by various authors and also to compare our's with those in the literature.

Conclusion

Based on n-3/n-6 parameter, f/2 and Walne's media are suitable for enriching the nutritional characteristics of the algal biomass. In our results regarding Miquel's medium, a clear parallel can not be established between the high value of PUFA content and the dietary potential of the biomass. The high percentage of DHA is achieved with Miquel's medium while

that for EPA with f/2 medium. The biomass of *I. galbana* will be more suitable for optimised larval rearing in the aquaculture industry when grown in f/2 as well as Miquel's media if a compromise between dietary characteristics and total PUFA content could be achieved. As *I. galbana* cultures raised in f/2 and Chu#10 media are rich in neutral lipids (TAGs) they can be used as a potential source of biofuel which is going to be a promising industry in future.

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