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Variations in lipid profile of the marine microalga *Nannochloropsis salina* in four different culture media

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ABSTRACT

The present work envisages close monitoring of variations in lipid content and fatty acid profile of the marine microalga *Nannochloropsis salina*, by growing it in 4 different culture media. Pure cultures of microalga were collected from the marine hatchery complex of the ICAR-Central Marine Fisheries Research Institute, Kochi and cultured in four different media *viz*, f/2 medium (Guillard,1975), Miquel's medium (Miquel,1892), Walne's medium (Walne, 1974) and Chu#10 medium (Chu, *et al*., 1942). Standard algal culture conditions *viz*, salinity 33 ppt, pH 8.0, culture room temperature of 25ºC, light intensity of 1500 lux with a photoperiod of 12 h and Light:Dark cycle of 12 h were maintained. Lipids extracted were subjected to gas chromatography to ascertain their fatty acid profile. Both Miquel's and f/2 media produced the maximum amount of lipid content (40.2 and 40% respectively). Total saturated fatty acids ranged from 39.17% (Miquel's medium) to 84.45% (Chu #10). For monounsaturated fatty acids, the highest value was obtained for Miquel's medium (42.79%) and minimum for Chu#10 (10.55%). Polyunsaturated fatty acids accounted for over 23.5% of the total fatty acids in Walne's medium followed by Miquel's (18.04%), f/2 (12.76%) and Chu#10 (5%) media. One way analysis of variance of the data brought to light that the medium composition can influence the fatty acid pattern of microalgae significantly.

Keywords: Fish culture, Live feed, Micronutrients, Omega-3, Omega-6

Introduction

The fast proliferation of some microalgae used as live feed in hatcheries of finfishes and shellfishes offers opportunity for consistent production of biomass rich in lipid, starch or protein (Atalah *et al*., 2007; Ganuza *et al*., 2008; Hemaiswarya *et al*., 2011; Velasquez *et al*., 2016), which indirectly helps to impart nourishment to human diet (Bold, 1942; Canizares *et al.*, 1994). Nearly all microalgal biomass are rich sources of omega-3/ omega-6 fatty acids and essential amino acids (leucine, isoleucine and valine).

Screening and optimisation of culture medium is among the foremost preconditions for photoautotrophic cultivation of microalgae. The performance of microalgae in culture is mostly determined by the quality of the medium used (Lam and Lee, 2012; Li *et al*., 2012; Prathima *et al*., 2012). Manipulation of the nutritional content of microalgae can be achieved by altering the media composition and culture strategies (Otero *et al.*, 2006; Ilavarasi *et al*., 2011; Lincymol *et al*., 2012; Naseera *et al*., 2013; Neethuand Dhandapani, 2016; Praba *et al*., 2016). The essential fatty acid profile of the algae could even be modified using these techniques (Kaladharan *et al*., 1999; Martinez-Fernandez *et al.,* 2006; Rivero-Rodriguez *et al.*, 2007; Lidiya and Joseph, 2018; Aswathy *et al*., 2020). The composition and availability of macro and microelements in the media directly affect algal cultures and hence they should be provided at optimal levels (Liu *et al*., 2008). Macronutrients *viz,* N, K, Mg, S and Na are non-toxic to algal cells and can be supplemented at higher concentrations. Whereas, essential trace elements like Fe, Cu, Mn, Zn, Co and Mo are growth-limiting at lower levels and toxic at higher doses. These trace elements play important roles in metabolic pathways that influence the performance of micralgae in culture (Sunda and Huntsman, 1998; Sunda *et al*., 2005).

The genus *Nannochloropsis* was first described by Hibberd (Hibberd, 1981) which is widely accepted in aquaculture (Roncarati *et al*., 2004; Bentley *et al*., 2008) owing to its rapid growth and nutritional value, especially high lipid content (Rodolfi *et al*., 2003; Olofsson *et al*., 2012). The high content of the omega-3 fatty acid, eicosapentaenoic acid (EPA, C20:5 n-3) in *Nannochloropsis* contributes to its significance in the aquaculture industry (Watanabe, 1979; Watanabe *et al*., 1983; Koven *et al*., 1990; Seto *et al*., 1992; Sukenik *et al*., 1993). Different species of *Nannochloropsis* have been found to facilitate relatively high rates of rotifer

multiplication which are important live feed for fish larvae (Hirayama *et al*., 1979; Yamasaki *et al*., 1989; Ahmad, 1991).

As the optimal nutrient concentration varies depending on the microalgal strain as well as the processing and cultivation parameters, the medium should be optimised for each microalgal strain prior to mass cultivation. The present investigation attempted to analyse the fatty acid profile of *Nannochloropsis salina* when grown in four different culture media to identify the optimal medium that increases unsaturation and thereby the nutritional status of the microalga.

Materials and methods

Pure culture of *N. salina* was obtained from ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI), Kochi, India and was maintained in standard f/2 medium in order to ensure maximum biomass production. Four different media *viz,* f/2 medium (Guillard, 1975); Miquel's Medium (Miquel, 1892); Walne's medium (Walne, 1974) and Chu#10 medium (Chu, 1942) were prepared as per standard published methodologies.

The experiment was performed following a completely randomised design (CRD), with three replications per treatment. Six hundred millilitres (20% of the culture medium) of *N. salina* having exponentially growing cells with an initial inoculum density of 20-30x10^4 cells ml^{-1} (Pavlo *et al*., 2016) was transferred to previously autoclaved, properly capped and aerated borosilicate culture flasks of 4 l capacity (a set of three per treatment) under aseptic conditions. For the illumination, fluorescent tubes of 1500 lux were employed (Hoff and Snell, 1987). A light/dark (L/D) cycle of 12 h of light and 12 h of darkness was used for maintaining the stock as well as major cultures which was controlled using a timer (Barsanti and Gualtieri, 2006). The indoor culture room for maintaining the stock cultures was air-conditioned to maintain the temperature at 25ºC. Seawater of salinity 33-34 ‰ was used for microalgal culture (Barsanti and Gualtieri, 2006).

The duration of lag phase, log phase and stationary phases of the microalga were monitored by counting cells, as per Andersen *et al*. (2005). After ascertaining cell density (Hoff and Snell, 1987), the culture was harvested during the late exponential phase by centrifugation at 10000 rpm for 1 min in a refrigerated centrifuge (HIMAC CR 22G). The supernatant was discarded and the pellets were collected after multiple washing with seawater.

For analysis of lipid profile of the microalgae harvested form differenrt treatments, total lipids were extracted following the protocol of Bligh and Dyer (1959). About 500 mg to 1 g of wet microalgal sample along with a pinch of butylated hydroxy toluene (added to prevent oxidation) was homogenised in 5-10 ml of distilled water in a pestle and mortar. The homogenate was transferred to a 250 ml conical flask and mixed with 20-30 ml chloroform-methanol (2:1 v/v) mixture and shaken well. The mixture was kept overnight at 4ºC in the dark and then 20 ml chloroform followed by 20 ml distilled water was added. The resulting solution was centrifuged and the three layers obtained were separated using a separating funnel. The lower chloroform layer was carefully collected free of the interface by filtering through sodium sulfate using a filter paper and then concentrated in a pre-weighed round bottom flask at 40-45°C using a rotary vacuum evaporator. The concentrate obtained was allowed to cool and the weight (w_1) was noted. Total lipid content was calculated using the formula:

$$\text{Lipid} = (w_1\text{-}w_2/w_3) \times 100$$

where w_1 = weight of flask + lipid, w_2 = weight of flask and w_3 = weight of sample taken.

Fatty acids were analysed as fatty acid methyl esters (FAMEs). For this 5 ml of 0.5N methanolic alkali was added to about 150-250 mg of the extracted lipid and reflexed for 5 min in a boiling water bath under nitrogen atmosphere, in order to break the ester bonds leading to saponification of lipids. After cooling, 5 ml BF_3 methanol solution was added slowly, refluxed for another 5 min in boiling water bath under nitrogen atmosphere and then allowed to cool so that FAMEs were formed (Metcalf *et al*., 1966). Subsequently, about 5-6 ml of saturated NaCl was added and mixed well to separate the FAMEs. FAMEs were then mixed well with petroleum ether (double the volume of the solution) three times. Each time lower layer was discarded and the top layer of petroleum ether having FAMEs was collected. The process of extraction was repeated three more times with distilled water. Finally, the lower layer was filtered through anhydrous Na_2SO_4, followed by rotary evaporation. The concentrate was then reconstituted in minimum volume of petroleum ether and 1 µl of the same was used for injecting in gas chromatograph (GC). The area of each component was obtained from computer-generated data. FAMEs were identified by comparison of retention times with that of the known standards (SupelcoTM 37 Component FAME Mix, Catalog No. 47885-U) and the results were expressed as percentage total fatty acid.

Statistical analysis

Statistical analyses were carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 22.0). The differences between treatments were analysed by one-way ANOVA, taking p=0.05 as significant, followed by Tukey's test.

Results and discussion

The lipid content (in percentage) of the harvested algal biomass of all the experimental treatments is given in Table 1. The highest concentration of lipid was obtained from *N. salina* cultures in Miquel's as well as in f/2 media (41.0%, 40.2% respectively) followed by Walne's (32.54%) and Chu#10 medium (26.13%). Analysis using one-way ANOVA revealed that there was significant difference ($p \leq 0.05$) in the total lipid content among cultures in different media.

Chiu *et al*. (2009) reported that under optimal growth conditions *N. oculata* had a lipid content above 50%. Bondioli *et al*. (2012) reported a lipid content of 39.1% in *Nannochloropsis* sp. (F&M-M24 strain) whereas Xu *et al*. (2004) observed 22-31%. Meng *et al*. (2015) stated a lipid content of 28-59% in *N. oceanica* IMET1, while Mitra *et al*. (2015) observed a lipid content of 22.3 to 38.6% in *Nannochloropsis gaditana*. The above reports regarding lipid content in *Nannochloropsis* spp. were in harmony with the results of the current study. However, the present results contradict the results obtained by Kaladharan *et al*. (1999) where they got only 11.28% of lipid content in *N. salina* cultures grown in Walne's medium. This may be because of the difference in the analytical procedures adopted and variations in culture environments such as temperature or photoperiod selected.

One of the most important microelements in microalgal culture media is iron (Fe). It is expected that Fe induces lipid accumulation due to down-regulation of iron requiring fatty acid desaturase enzymes. A substantial hike in lipid radicals in the membranes of *Chlorella vulgaris* has been reported by Estevez *et al.* (2001), when iron was added up to 500 µM. Hence, the highest level of Fe (2 g 100 ml^{-1} stock solution) in the Miquel's medium possibly resulted *N. salina* cultures having high lipid content. Being a constituent of cytochromes, Fe plays a key role in nitrogen assimilation and influences the synthesis of phycocyanin and chlorophyll. Fe added in the form of inorganic salts will tend to precipitate and become inaccessible to algae (Becker, 1994). Since Miquel's medium contains Fe in the form of ferric chloride, it may shorten nitrogen assimilation thereby leading to lipid induction.

Table 1. Total lipid content (%) in *N. salina* recorded in the four different culture media

Culture media	Lipid	Mean±SD
f/2	40	40±2.08
Miquel's	40.2	40.2±3.40
Waine's	32.54	32.54±2.35
Chu#10	26.13	26.13±3.56

p=0.001

The use of Fe as a chelated complex (Fe EDTA) is more useful in algal culture (Becker, 1994). Both f/2 medium and Walne's medium have Fe source in the form of chelated complex which helps in proper uptake of macronutrients especially nitrogen, leading to the high performance of photosynthetic pathway instead of lipid synthetic pathway. The higher lipid content (41.00%) in f/2 medium than that in Walne's (32.54%) could be attributed to the fact that f/2 contains lesser amount of Fe source than Walne's leading to reduced nitrogen assimilation and a substantial hike in lipid production. Omar (2002) observed that *Botryococcus sudeticus* accumulated more lipids (30%) when concentration of Zn was low. A close examination of the chemical composition of Miquel's medium reveals that it lacks the above trace element thereby increasing the lipid content.

Results of GC analysis of *N. salina* in four different culture media are depicted in Fig. 1a to d. The levels of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids recorded in the experimental treatments are presented as percentage of total fatty acids (% TFA) in Table 2, 3 and 4 respectively. Total SFAs ranged from 39.17% (Miquel's medium) to 84.45% (Chu#10). Lauric acid (C12) was found to be the major SFA in cultures maintained in all the four media followed by palmitic acid (C16:0) which ranged from 5.56% (f/2), 6.81% (Miquel's), 7.77% (Walne's) to 13.12% (Chu#10). Myristic acid (C14) levels recorded were almost similar in all the four media (2.29 to 3.37%). The presence of 0.8% stearic acid noted in Miquel's medium in the present study is in agreement to the results of Alicia *et al.* (2015) in *N. gaditana* where the fatty acid profile evidenced the presence of 4.4% myristic acid and 0.8% stearic acid. Statistical analysis of the data indicated significnat variations in SFA content among the algal cultures grown in four different media.

Highest level of MUFAs was obtained for Miquel's medium (42.79%) followed by f/2 medium (20.11%), Chu#10 (10.55%) and lowest for Walne's (9.39%) medium. One-way ANOVA revealed significant difference ($p \leq 0.05$) in MUFA content among the four different treatments. The observations of Carrero *et al*. (2011) and Alicia *et al*. (2015) in *N. gaditana* regarding MUFA content (above 40 and 46.5% respectively) are in tune with the findings of the current study.

Among the nine classes of MUFAs, oleic acid (18:1 n-9) is the major one present in *N. salina,* which attained maximum level in Miquel's medium (19.90%) and minimum in f/2 (1.27%). In Chu#10, both *cis* and *trans* forms were obtained (0.61 and 2.26% respectively) and in Walne's medium oleic acid content recorded was 2.06%. The fatty acid profile revealed the presence of

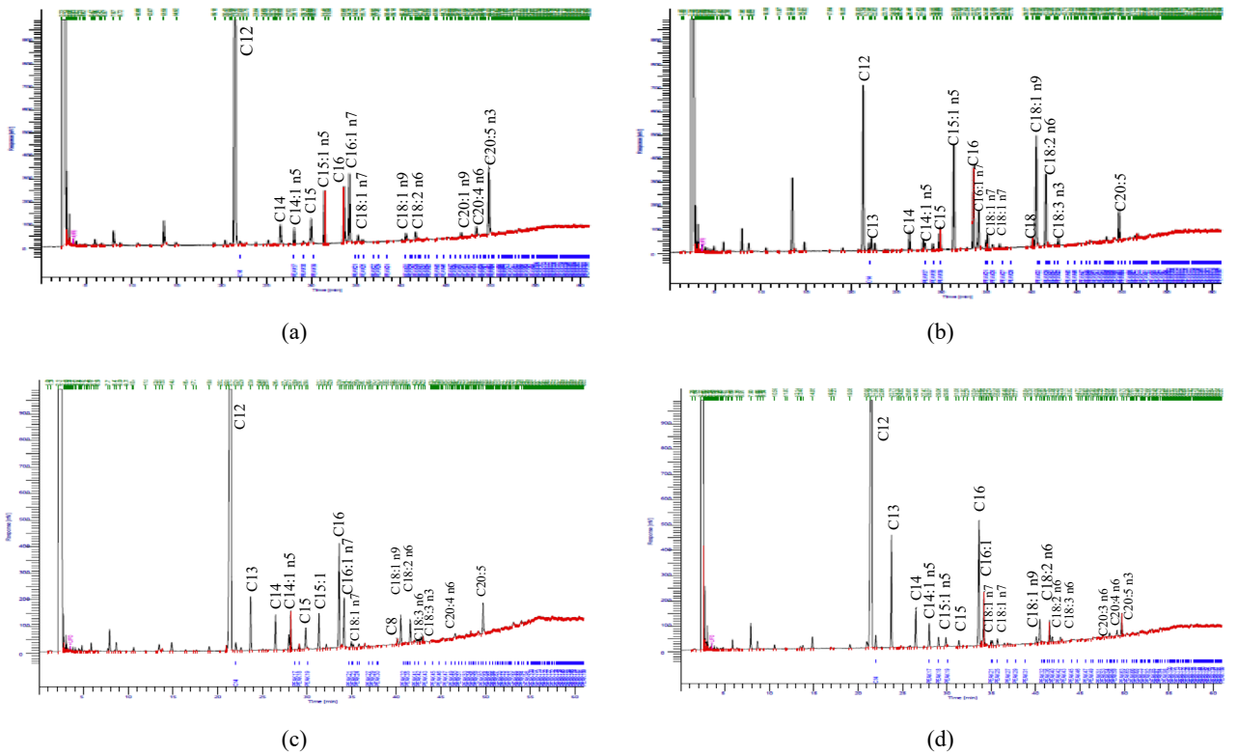

Fig. 1. Chromatogram showing fatty acid profile of *N. salina* in (a) f/2 medium, (b) Miquel's medium, (c) Walne's medium and (d) Chu#10 medium

Table 2. Saturated fatty acids content (%) of *N. salina* from four different culture media

Sl. No.	Fatty acids		f/2 medium	Miquel's medium	Walne's medium	Chu#10 medium
1	Lauric acid	C12	54.29 ±0.01	25.21±0.00	52.09 ±0.01	58.3 ±0.01
2	Tridecanoic acid	C13	nd	1.05 ±0.01	3.38 ±0.03	8.95 ±0.10
3	Myristic acid	C14	3.36 ±0.08	2.90 ±0.01	2.29 ±0.02	3.37 ±0.01
4	Pentadecanoic acid	C15	3.95 ±0.01	2.42 ±0.00	1.57 ±0.02	0.70 ±0.20
5	Palmitic acid	C16	5.56 ±0.32	6.81±0.71	7.77 ±0.09	13.12 ±0.21
6	Stearic acid	C18	nd	0.78 ±0.02	nd	nd
	Total SFA (%)		67.13 ±0.40	39.17 ±0.75	67.10 ±0.15	84.45 ±0.53

p=0.000; nd: Not detected

Table 3. Monounsaturated fatty acids content (%) of *N. salina* from four different culture media

Sl. No.	Fattyacids		f/2 medium	Miquel's medium	Walne's medium	Chu#10 medium
1	Oleolauric acid	C12:1 n-5	nd	nd	nd	1.07±0.01
2	Oleomyristic acid	C14:1 n-5	1.99±0.03	1.75±0.03	0.96±0.01	2.05±0.01
3	Penta decenoic acid	C15:1 n-5	4.281±0.09	17.50±0.01	2.40±0.10	0.56±0.40
4	Palmitoleic acid	C16:1 n-7	10.80±0.02	2.37±0.41	3.26±0.20	3.08±0.21
5	Vaccenic acid	C18:1 n-7 *cis*	1.23±0.01	1.26±0.01	0.40±0.02	0.48±0.01
6	Vaccenic acid	C18:1 n-7 *trans*	nd	nd	nd	0.45±0.03
7	Oleic acid	C18:1 n-9 *cis*	1.27±0.70	19.90 ±0.80	2.06±0.05	0.61±0.01
8	Oleic acid	C18:1 n-9 *trans*	nd	nd	nd	2.26±1.50
9	Eicosaenoic acid	C20:1 n-9	0.54±0.03	nd	0.33±0.04	nd
	Total MUFA (%)		20.11±0.87	42.79 ±1.25	9.39±0.42	10.55±2.26

p=0.000; nd: Not detected

Table 4. Polyunsaturated fatty acids content (%) of *N. salina* from four different culture media

Sl. No.	Fatty acids		f/2 medium	Miquel's medium	Walne's medium	Chu#10 medium
1	Linoleic acid	C18:2 n-6 cis	1.13 ±0.01	11.99 ±0.03	1.54 ±0.01	0.78 ±0.01
2	Linoleic acid	C18:2 n-6 trans	nd	nd	nd	1.15 ±0.00
3	α linolenic acid	C18:3 n-3	nd	1.05 ±0.00	0.36 ±0.02	nd
4	Gamma linolenic acid	C18:3 n-6	nd	nd	0.24 ±0.01	0.43 ±0.03
5	Dihomogamma linolenic acid	C20:3 n-6	nd	nd	nd	0.46 ±0.00
6	Arachidonic acid	C20:4 n-6	1.20 ±0.02	nd	0.14 ±0.01	1.14 ±0.01
7	Eicosapentaenoic acid	C20:5 n-3	10.43 ±0.80	5.00 ±0.60	21.22 ±0.09	1.04 ±0.02
	Total PUFA (%)		12.76±0.83	18.04±0.63	23.50±0.11	5.00±0.06

p= 0.000; nd: Not detected

oleomyristic acid at 0.96% in Walne's medium, followed by 1.75% in Miquel's, 2% in f/2 and 2.05% in Chu#10. This finding is in agreement with that of Alicia *et al.* (2015) in *N. gaditana.*

Polyunsaturated fatty acids (PUFAs) accounted for over 23.50% of the total fatty acids in Walne's medium followed by Miquel's (18.04%), f/2 (12.76%) and Chu#10 (5%). The total PUFA content of *N. salina* from Walne's medium is in agreement with that reported by Carrero *et al*. (2011) (above 20%) and Alicia *et al*. (2015) (23.8%) in *N. gaditana.* In the present study, significant difference ($p \leq 0.05$) in the PUFA content among *N. salina* cultures in different media was evident.

A close examination of the chemical combination of Walne's and Miquel's media revealed that they have sufficient amount of macronutrients like N, P, K and Ca. Usually, under conditions of nitrogen sufficiency, microalgae synthesise membrane glycerolipids which reside in the plasma membrane and endoplasmic membrane systems (Piorreck and Pohl, 1984). These membrane lipids are constituted mainly of long-chain unsaturated fatty acids, which play a structural role in the cell (Hu *et al.,* 2008). Thus, PUFA is accumulated more in the growth phase during which cell division actively progressed (Hu *et al.,* 2008). The presence of a large amount of PUFA in *N. salina* cultures grown in Walne's as well as in Miquel's media further affirms the above mentioned findings.

The nitrogen source in f/2 medium is sodium nitrate, in Chu#10 medium it is calcium nitrate while in Walne's and Miquel's it is potassium nitrate. *N. salina* cultures in Chu#10 medium had least content of total lipid, MUFA, PUFA and EPA. This is because the calcium nitrate in this medium is actually responsible for protein biosynthesis and thereby it accounts for algal growth and not for lipid synthesis (Ilavarasi *et al*., 2011). Another fact is that the PO_4 source (K_2HPO_4) enhances algal growth and not lipid synthesis (Turpin, 1986).

The present experiment revealed 6 classes of PUFAs in *N. salina,* among which C18:2 n-6 was highest in Miquel's medium (11.99%) compared to the other three media. The arachidonic acid content was more or less equal in f/2 (1.2%) and in Chu#10 (1.14%) media. DGLA (C20:3 n-6) was recorded only in Chu#10 medium. Hoffmann *et al.* (2010) observed that *N. salina* contains 2.5 to 4.5% PUFA content as well as 1.1-3.5% EPA content.

The content of palmitoleic acid (C16:1 n-7) of *N. salina* cultures grown in Walne's and Chu#10 media in the present study is similar to that reported in *N. limnetica* cultures (Krienitz and Wirth, 2006). Similarly, Volkman *et al.* (1993) reported 2.18% palmitoleic acid (C16:1 n-7) and 0.63% oleic acid (C18:1 n-9) in *N. oculata* CS 216 which is almost equivalent to 2.37 and 0.61% observed from *N. salina* cultures grown in Miquel's and Chu#10 media respectively, during the present study. Cultures in Walne's medium produced the maximum amount of EPA (21.22%) and minimum was recorded with Chu#10 (1.04%) medium. *N. salina* grown in f/2 medium had 10.43% EPA content while it was 5% for Miquel's medium.

Camacho-Rodriguez *et al.* (2014) found that culture media with low levels of zinc would be having lesser EPA content in *N. gaditana.* This finding is in tune with the present result because both Miquel's and Chu#10 media are lacking the said trace element and recorded low values of EPA (5 and 1.04% respectively). A higher quantity of Zn accounts for increased EPA content in Walne's medium cultures (21.22%) than those in f/2 medium (10.43%). They also reported that biotin was important for EPA production while thiamine and cyanocobalamin were not. Another reason for the higher content of EPA in f/2 medium cultures could be attributed to presence of biotin. Chen *et al.* (2013) also showed that trace elements are important for the growth and EPA production in *N. oceanica* CY2. A close perusal of the chemical composition of f/2 medium and Walne's medium indicates that both have a good balance of micronutrients. Many of these micronutrients are lacking in Miquel's and Chu#10 media. Therefore, the optimum balance of micronutrients in f/2 and Walne's media could be one of the reasons for the higher EPA content in *N. salina* grown in these media.

Different authors have reported the value of EPA content in different experimental setups and in different species of *Nannochloropsis* which are in tune with the results of the current study. Zou *et al*. (2000) reported 2.3-5.7% EPA content in *Nannochloropsis* spp. Chaturvedi and Fujita (2006) observed 2.4% EPA content in *Nannochloropsis oculata* ST-6 (wild type) having a PUFA content of 3%. Meng *et al*. (2015) obtained an EPA value as 2.7-5.2% for *Nannochloropsis oceanica* IMET1 where total lipid content was 28-59%. Krienitz and Wirth (2006) estimated an EPA value of 0.22-5.6 for *Nannochloropsis limnetica* SAG18.99 where total PUFA was 0.84-12.25%. Molino *et al*. (2019) and Mitra *et al*. (2015) reported an EPA value of 4.4 to 11% in *N. gaditana* cultures. In their experiment, *N. gaditana* cultures displayed 1.6-3.5% linoleic acid (18:2 n-6), 0.3-1.1% ALA (18:3 n-3) and 0.4 -3.4% AA (20:4 n-6). The findings of the present study are in agreement with these reports (Table 4).

There are reports regarding negative impacts of high concentrations of Zn on mocroalgal cell division, total chlorophyll content and ATPase activity (Omar, 2002). In addition, a high concentration of Zn was found to increase lipid peroxides in microalgae like *Pavlova viridis* (Li and Zhu, 2006), resulting in lipid depravity. The effects of molybdenum (Mo) on microalgal lipid production are still unclear and require further investigation. However, it is known that Mo is essential for the assimilation of nitrate (Raven, 1988) and for the conservation of homeostasis, while certain enzymes carry Mo cofactors conserved in eukaryotes. It was documented that Manganese (Mn) limitation suppresses photosynthetic activities in algae (Constantopoulos, 1970) and thereby enhances lipid synthesis. The present findings regarding lipid production is in agreement with the above results since Miquel's medium is lacking in the above trace elements.

The main goal of this work was to describe the lipid classes of *N. salina* and their composition in terms of esterified fatty acids when grown in different standard microalgal culture media. Since Walne's medium enriches polyunsaturated fatty acid content, cultures from this medium are suitable for optimised larval rearing in aquaculture. As *N. salina* is rich in neutral lipids, it can be used as a potential source of biofuel, which can become a promising industry in the future for which Chu#10 medium can be used. A detailed understanding of biochemical parameters and fatty acid expression patterns in different algal species can help to estimate their dietary potential and can open up possibilities for the manipulation of culture conditions.

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