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Production of Lipids Rich in Omega 3 Fatty Acids from the Halotolerant Alga *Dunaliella salina*

[1]Hanaa H. Abd El-Baky, [1]Farouk K. El Baz and [2]Gamal S. El-Baroty
[1]Agricultural Division, National Research Center, Dokki, Cairo, Egypt
[2]Department of Biochemistry, Faculty of Agriculture, Cairo University, Egypt

Abstract: The effect of nitrogen limitation and salt stress on total lipid and unsaponifiable contents as well as fatty acid composition of *Dunaliella salina* were studied. The contents of total lipids, unsaponifiables and fatty acid composition were basically depend on NaCl and nitrogen concentration in the culture. The highest yield of total lipids (37.69%) and unsaponifiables (29.02%) was obtained in cells grown at 16% NaCl combined with 2.5 mM nitrogen. While, minimum yield occurred in cells grown in a culture containing 8% NaCl and 5 mM N. Cells grown at 16% NaCl combined with 2.5 mM N produced relatively higher proportion of polyunsaturated fatty acids (PUFAs), in particular C18:3ω3 and C16:4ω3. Increasing NaCl combined with decreasing N levels in the growth medium increased the total unsaturated fatty acids (TU) at the expense of total saturated fatty acids. At higher salinity, the total amounts of carotenoids and α-tocopherol in unsaponifiable fraction were significantly increased to reaching up to 12.03 and 4.10%. The results obtained suggest that *D. salina* cells containing high amount of total lipid, rich in ω_3 polyunsaturated fatty acids and antioxidant compounds in unsaponifiable lipid fraction may used as a supplemental ingredient or as a complete food to enhance the performance and state of the human body or improve a specific bodily function.

Key words: *Dunaliella salina,* nitrogen, salt stress, fatty acids, ω3 polyunsaturated fatty acids

INTRODUCTION

Several of unicellular algae, e.g., *Chlorella, Dunaliella* and *Spirullina* accumulate appreciable amount of fat (up to 60% of dry weight basis) when grown under certain environmental conditions, such as high in both temperature and light intensity and rise in salinity[1-4]. The algal lipids are known to contain a relatively high amount of long chain polyunsaturated fatty acids (PUFAs), especially omega (ω) 3 and 6 of fatty acid series such eicosapentaenoic (EPA, 20:5 ω3), docosahexaenoic (DHA, 22:6 ω3), archidonic (AA, 20:4 ω 6), γ- linolenic (GLA, 18:3 ω 6) and α-linolenic (ALA, 18:3ω3) acid. These long chains PUFAs in algae have profound benefits and functions in dietetics and therapeutic uses[5-8]. They are believed to have a positive effects for the treatment of hypertension, premenstrual tension, various atopic disorders diabetes, coronary heart disease, skin disease, hypertonia, cancer, hyperlipidemia and number of the other cases[7-10]. In addition, GLA is the precursors of prostaglandins (E2 and F2) which possess potent vasodilators, antiflammatory and antiaggrgatory properties as well as may be useful to correct defects occur in metabolism of essential fatty acid and imbalance of eicosanoids formation[11]. Eicosapentaenoic acid (EPA) plays an important role in mammals as an agent to prevent blood platelet aggregation[12-14]. Docosahexaenoic acid [DHA, $C_{22:6}$ ω3] is important for the reception and transmission of impulses between brain cells[15]. Furthermore, dietary omega-3 (ω3) PUFAs especially EPA and DHA are play a major role in modulating the biosynthesis of eicosanoids and in controlling the levels of blood lipids and lipoproteins[16]. Thus, the principal clinical value of this fatty acid may be for the amelioration of atherogenesis and thrombogesis[17].

Unicellular algae can be recognized as an important source of ω3 PUFAs[18]. At present, the used algae as a nutritional source of ω3 PUFAs are expensive, however, it is free of cholesterol and fish odour and taste[18]. Microalgae become increasing attractive for use by commercial food companies and health-car practitioners[1], where microalgae can be grown in environments normally not used for agricultural purposes and are rapidly renewable source of food and fine chemicals[19].

The basic aim of the present study was to develop an efficient system to produce high amounts of oil rich in ω3 PUFAs. The effects of different concentrations of NaCl combined with nitrogen on the contents of total lipids,

Corresponding Author: Dr. Hanaa H. Abdel-Baky, Botany Department, National Research Center, Cairo-Dokki, Egypt
E-mail: abdelbakyh@hotmail.com

unsaponifiable matter and fatty acid composition of *Dunaliella salina* were also studied.

MATERIALS AND METHODS

Algal source: Marine microalgae *Dunaliella salina* was obtained from the Culture Collection of Botany Department, Texas University, Austin, Texas, U.S.A.

Growth conditions: *Dunaliella salina* was cultured in a 4 liter flask with 2.5 L of culture medium containing 8% NaCl and 5 mM nitrogen, pH 8.5 during spring season in National Research Center (NRC). All glass and plastic ware were washed with 1% HNO_3 and rinsed several times with distilled water. The cultures were gassed with 1.5% volume CO_2 in air. The masses of *D. salina* were obtained under the following conditions. Potassium nitrate served as nitrogen source at two different concentrations, i.e., 5 and 2.5 mM N in media containing 8, 12 and 16% NaCl, respectively. The cultures were continuously illuminated with cool white fluorescent lamps (Philips 40 W) and light intensity level was approximately 200 W m^{-2}. The algal masses were obtained from triplicate experiments at 22±3°C for 15 days[19,20].

Growth measurements: The dry weight method and optical density as described by Payer[21] were used to measure the growth rate of *Dunaliella salina*.

Harvesting: Under the above mentioned experimental conditions, the algal cell were harvested by centrifugation (6000 x g) at 4°C for 15 min and stored at -20°C.

Extraction of lipids: Lipids were extracted by a modified methods described by Xu and Beardall[3]. The cells (ca. 5 g) were extracted twice with a mixture of distilled H_2O, chloroform and methanol (8:10:20, v/v/v) and sonicated for 10min using a microtop of Microson Ultrasonic Cell Disruplor. Then, sonicated cells filtered on to 47 μm diameter GF/C Whatman glass microfibre filters. Chloroform (10 ml) and distilled water (10 ml) were added sequentially to the filtrate and sonicated again for 10 min. The resultant solution was filtered under vacuum through a 25 mm diameter Whatman glass filter microfiber. The filtrate was washed by 30 ml of 5% NaCl solution, then the lower layer of $CHCl_3$ was separated and dried over anhydrous sodium sulfate. The solvent was removed through evaporation at 40°C under reduced pressure. Then, the total lipids were weighed and stored at -20°C until analysis.

Separation of fatty acids and unsaponifiable matter: A known amount of algal lipids (0.2 g) was saponified with methanolic KOH (30 ml, 1N) containing BHT (1 mg) at 60°C for 1 h. under reflux. The unsaponifiables matter was extracted with petroleum ether (b.p 40-60°C), washed several times with distilled water and dried over anhydrous sodium sulfate. The solvent was evaporated and the unsaponifibale matter was weighed.

Separation of fatty acids: The soap solution was acidified with sulfuric acid (5 N), the liberated fatty acids were extracted with ether, washed several times with distilled water and methylated with diazomethane ethereal solution[22].

Determination of lipophilic antioxidant in unsaponifiable fraction: A known weight of the unsaponified matter was shacked with n-hexane and the total carotenoid and α-tocopherol contents were spectrophotometrically determined as reported by Abd El-Baky *et al.*[19].

Determination of algal total carotenoids: The total carotenoids was spectrophotometrically determined at 450 nm, β-carotene served as a standard compound was used for the proportion of calibration curve[23].

Determination of algal α-tocopherol: Tocopherols were determined by HPLC apparatus equipped with UV2000 detector at 290 nm and separated on a 250x4.6 mm (i.d) column packed with Vydac and eluted with acetonitrile : methanol mixture (9:1, v/v) at a flow rate of 1 ml min^{-1}. Standard of α- tocopherol (Sigma Co.) separated under the same conditions[24].

Identification of fatty acids: Fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) according to Farag *et al.*[22]. The chromatographic conditions were: supelcowax 10 fused silica capillary column (30 m x 0.32 mm, film thickness 0.25 μm), flame ionization detector, nitrogen as a carrier gas at flow rate of 30 ml min^{-1}, initial column temperature was 80°C increased to 180 °C at rate of 4°C $min^{-1}$and hold at 180°C for 10 min, temperature of injector and detector temperatures were 230 and 240°C, receptively. The fatty acids were identified by comparing their retention times with those of standard fatty acid methyl esters (purity 99% by GLC, Sigma Co.). Also, co-chromatography method and GC/MS were used to verify the peak identity and position of double bonds in a fatty acid molecule.

Statistical analysis: Data represent the means values. Results were analyzed by one- way ANOVA and Scheffe' *F*-test to identify significant differences between treatments. All analyses performed using Co Stat software version 4 (Abacus Concepts, Berkeley, CA).

RESULTS AND DISCUSSION

Influence of NaCl stress combined with nitrogen starvation on total lipid content: Data in Table 1 indicate the effect of NaCl and nitrogen concentration on total lipid content of *D. salina*. Generally speaking, the level of NaCl and nitrogen in growth medium markedly affect in the percentage of total lipids. The largest increase in total lipids occurred in cells grown in a medium containing 16% NaCl (highest NaCl concentration) and 2.5 mM nitrogen (nitrogen limitation), which being 37.69% (based on dry weight). The lowest amount of total lipids (5.23%) was obtained in cells grown at 8 % NaCl combined with 5 mM nitrogen. In generally, the amounts of total lipids were gradually decreased as a result of decreasing NaCl concentration combined with an increase of nitrogen levels in nutrient medium. For instance, the total lipid content in cells grown at 8% NaCl combined with 5, 2.5 and zero nitrogen levels were 5.23, 11.48 and 23.11%, respectively. While, the algal cells grown in medium containing 12% NaCl combined with 5 and 2.5 nitrogen levels accumulated 14.94 and 19.69% total lipids, respectively.

Effect NaCl stress combined with nitrogen starvation on unsaponifiable matter content

As shown in Table 1 *D. salina* contained small amounts of unsaponifiable matter when grown under optimum conditions. With increasing NaCl combined with decreasing nitrogen level, the amount of unsaponifiable significantly increased and the largest increase in unsaponifiable matters (29.02%) was obtained in cells grown at 16% NaCl and 2.5 mM N. Whereas the lowest amount (2.88 %) was found at 8 % NaCl combined with 5 mM N.

Influence of salinity and nitrogen limitation on lipophilic antioxidant: The levels of total carotenoids and α-tocopherol in *D. salina* were gradually increased by increasing and decreasing the concentrations of NaCl and nitrogen in media, respectively For instance, the values of these compounds were 120.2 and 41.2 mg g^{-1}, respectively in *D. salina* grown at 16% NaCl and 2.5 mM N, compared with values10.2 and 6.0 mg g^{-1} in cells grown at 8% NaCl and 5 mM N level. It is worth noting that, the increase of antioxidant quantity in unsaponifiable matter in *D. salina* was associated with the increase of total lipid content (Table 1).

Influence of salinity and nitrogen levels on fatty acids composition: Data in Table 2 revealed the influence of salinity and nitrogen levels on fatty acid composition of *D. salina*. The fatty acid composition was greatly influenced by the composition of the nutrient media. *D. salina* had variety of fatty acids ranging from short chain fatty acids (SCFA, C_8-C_{12}), medium chain fatty acids (MCFA, C_{14}-C_{16}) and long chain fatty acids (LCFA, C_{18}-C_{24}). The lipids of *D. salina* contained high proportions of C_{16} and C_{18} polyunsaturated fatty acids (PUFAs) and in particular, omega 3 acids when grown in a medium containing high salinity combined with low nitrogen concentration. In addition, the algal lipids contained a noticeable amount of long chain polyunsaturated fatty acids (LCPUFAs) C_{20}, C_{22} and C_{24}. Under optimum conditions, the main fatty acids (>10% of the total FA) in *D. salina* were $C_{16:4}\omega_5$ (24.26%), $C_{16:0}$ (14.12%) and $C_{18:3}\omega_3$ (14.79%). Whereas, $C_{14:1}\omega_5$, $C_{22:6}\omega_3$, $C_{20:4}\omega_6$, $C_{18:2}\omega_6$ and $C_{18:2}\omega 9$ were found as minor compounds (<10%). In contrast, the cells grown under high NaCl concentration (16%) combined with low nitrogen level (2.5mM), the most abundant fatty acids were $C_{18:3}$ ω_3 (63.24%) and $C_{16:4}\omega_3$ (28.77%) which amounted to 92.01% of the total fatty acids. It seems that the increase of NaCl concentration and decrease nitrogen level in the nutrient medium produce particular fatty acid patterns. For instance, at 8, 12 and 16% NaCl in the presence of 2.5 mM nitrogen (N. limitation) the percentage of $C_{18:3}\omega_3$ in *D. salina* cells were 23.65, 47.82 and 63.24%, respectively. The levels of these acid were about 1.6, 3.2 and 4.3 times as high as that in cells grown under optimum conditions. Thus, $C_{18:3}\omega_3$ quantity was positively associated with NaCl concentration. Furthermore, all ω3 and ω6 polyunsaturated fatty acids showed positive correlation with the increase the NaCl concentration and nitrogen starvation in the culture. The total ω3 PUFA levels were 44.8, 72.13 and 92.01% in *D. salina* cells grown at 8, 12 and 16% NaCl combined with 2.5 mM nitrogen, respectively. Other ω3 PUFAs, e.g., eicosapentaenoic (EPA) $C_{20:5\ \omega 3}$ and docosahexaenoic (DHA) $C_{22:6\ \omega 3}$ were present only in small or / and trace amounts (<1%).

Table 3 summarizes the effect of nutrient medium components and in particular NaCl and nitrogen concentrations on the fatty acid composition of *D. salina*. It has been reported that the fatty acid composition vary greatly as a results of stress conditions[4,25]. There is a clear correlation between the percentage of total monounsaturated (MUFAs) and total saturated fatty acids (SFAs) and concentrations of NaCl and nitrogen in nutrient media. The increasing of NaCl concentration combined with nitrogen limitation (2.5 mM) in culture medium led to significant decrease in the percentage of both total MUFAs and SFAs in *D. salina*. The most of SFAs were $C_{16:0}$, $C_{10:0}$ and $C_{12:0}$ while, oleic acid ($C_{18:1\omega 9}$) was the most dominate MUFA. The same conclusion can be laid with total PUFAs and ω3 total

Table 1: Influence of nitrogen and salt stress on total lipids of *Dunaliella salina*

			Lipophylic antioxidant			
		Total unsaponifiable	Total carotenoids		α- tocopherol	
Treatments	Total lipids %	matter %	%	mgg^{-1}	%	mgg^{-1}
Optimum nutrients 5mMN+8%NaCl	5.23	2.88	1.0	10.2	0.60	6.0
2.5 mM Nitrogen +8 % NaCl	11.48	7.61	3.30	33.4	0.92	9.2
Zero Nitrogen + 8 % NaCl	23.11	18.8	4.90	49.1	1.10	11.0
5 mM Nitrogen + 12 % NaCl	14.94	14.58	5.90	59.6	2.20	22.0
2.5 mM Nitrogen+12 % NaCl	19.69	24.84	11.00	110.4	2.80	28.0
5 mM Nitrogen +16% NaCl	27.33	19.01	7.63	76.3	2.93	29.3
2.5 mM Nitrogen + 16% NaCl	37.69	29.02	12.02	120.2	4.10	41.2

All values are significant at $P< 0.5$

Table 2: Fatty acid compostion of *Dunaliella salina* as affected by nitrogen and NaCl concentration in nutrient medium

Fatty acids Relative%	S1	S2	S3	S4	S5	S6	S7
$C_{8:0}$	0.41	0.19	0.17	tr	ND	ND	tr
$C_{10:0}$	0.13	0.18	tr	tr	ND	ND	tr
$C_{12:0}$	0.21	ND	tr	0.75	ND	ND	ND
$C_{14:0}$	tr	ND	tr	tr	ND	1.92	ND
$C_{14:1\omega_5}$	11.12	1.24	2.18	ND	ND	ND	ND
C_{14}	11.12	1.24	2.18	ND	ND	1.92	ND
$C_{16:0}$	14.12	25.22	10.57	8.29	3.48	2.79	1.43
$C_{16:1\omega 7}$	2.57	ND	ND	ND	1.48	ND	ND
$C_{16:4\omega 3}$	26.26	18.60	22.51	26.54	23.65	34.17	28.77
C_{16}	42.95	43.82	33.08	34.83	28.61	36.96	30.20
$C_{18:1_\omega 3}$	1.11	ND	ND	4.63	ND	ND	ND
$C_{18:1\omega 9}$	1.12	5.70	3.26	ND	ND	ND	0.31
$C_{18:2_\omega 9}$	1.35	ND	5.35	ND	ND	0.10	5.92
$C_{18:2\omega 6}$	2.11	15.22	19.47	15.05	16.14	6.15	
$C_{18:3w3}$	14.79	23.65	28.20	38.61	47.82	52.69	63.24
$C_{18:4w6}$	1.45	1.84	1.17	ND	1.43	ND	ND
$C_{18:4w3}$	0.89	1.13	0.17	1.38	0.66	0.46	0.14
C_{18}	22.82	47.20	57.65	59.67	66.05	59.40	69.61
$C_{20:0}$	1.23	ND	0.52	ND	ND	ND	ND
$C_{20:4\omega 6}$	4.17	3.48	3.12	2.80	4.13	ND	ND
C_{20}	5.40	3.48	3.64	2.80	5.13		
$C_{22:0}$	2.41	ND	ND	ND	ND	ND	ND
$C_{22:4\omega 6}$	2.11	ND	ND	ND	1.19	ND	ND
$C_{22:6\omega 3}$	6.92	1.42	tr	1.90	tr	tr	tr
C_{22}	11.44	1.42		1.90	1.19		
$C_{24:1w4}$	2.81	2.12	ND	tr	ND	ND	ND
$C_{24:1w11}$	2.12	ND	ND	ND	ND	ND	ND
C_{24}	4.93	2.12					

S1 Optimum nutrients 5mM N+ 8% NaCl
S2 2.5 mM Nitrogen +8 % NaCl S3 Zero Nitrogen + 8 % NaCl
S4 5 mM Nitrogen + 12 % NaCl S5 2.5 mM Nitrogen+12 % NaCl
S6 5 mM Nitrogen +16% NaCl S7 2.5 mM Nitrogen + 16% NaCl

PUFAs levels where their levels were gradually increased in increasing NaCl level in media. The presence of NaCl and nitrogen in culture media led to produce low levels of fatty acids with chain length greater than C20. It interesting to note that, the levels ω-3; $C_{18:3}$ (63.24 %, essential fatty acids) were progressively increased by increasing the salt concentration under nitrogen limitation.

In the other words, under the influence of salinity combined with nitrogen limitation, the amounts of total unsaturated acids or the degree of unsaturation (DU) in *D. salina* lipid was gradually increased (Fig. 1). This finding was also, evidenced by the gradual increase in total unsaturated FA /total saturated FA ratio (TU/TS).

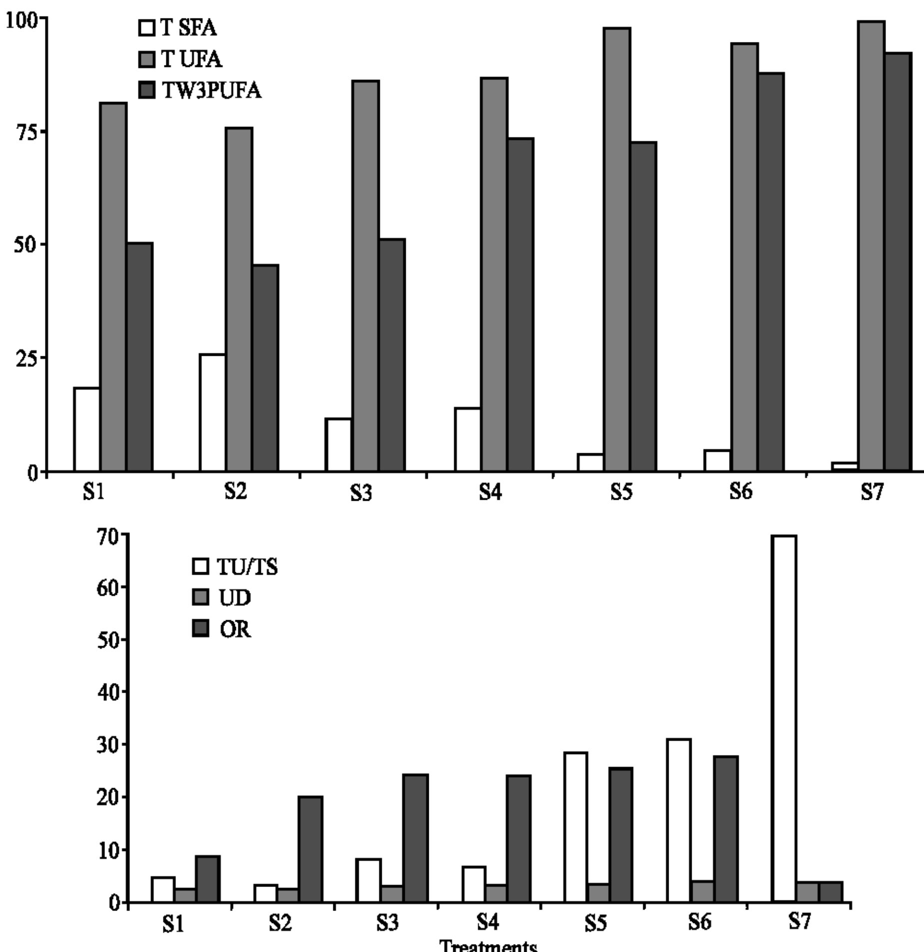
Table 3: Evaluation criteria of *Dunaliella salina* lipids

Parameter	S1	S2	S3	S4	S5	S6	S7
Total SFA	18.30	25.59	11.26	13.67	3.48	4.71	1.43
Total MUFA	20.85	9.06	5.44		1.48		0.31
Total PUFA	60.05	65.34	79.99	86.28	95.02	93.50	98.07
Total UFA	80.90	74.40	85.43	86.28	96.50	93.50	98.38
Total ω3PUFA	49.97	44.80	50.88	73.06	72.13	87.32	92.15
TU/TS	4.42	2.91	7.59	6.31	27.73	29.85	68.80
DU	2.20	2.20	2.50	2.84	2.83	3.10	3.20
RO	8.30	19.66	23.69	23.48	24.53	26.92	32.00

Total SFA: Total saturated fatty acids %,
Total MUFA: Total monounsaturated fatty acids %,
Total PUFA: Total polyunsaturated fatty acids %,
Total UFA: Total unsaturated fatty acids %,
Total ωPUFA: Total omega poly unsaturated fatty acids %,
TU/TS:Total unsaturated / Total saturated,
DU: Degree of Unsaturated, RO: Relative Oxidation,
Rat of oxidation = [%UFA 1 = x1 /100] + [%UFA 2 = x 12 /100]+ [%UFA 3 = x 25 /100]+[%UFA 4 = x 50 / 100]+ [%UFA 5 = x 75 / 100] (=) : Number of double bonds

Due to extremely susceptible of polyunsaturated fatty acids to autooxidation, the equation reported by Schultz *et al.*[26] was modified (as shows in Table 3) to calculate the relative oxidation (RO) values of *D. salina* lipids. The values of relative oxidation were 2.9, 3 and 3.3 in total lipids extracted from *D. salina* grown at 8, 12 and 16% NaCl combined with 2.5 mM nitrogen, respectively. This means that the proportion of different unsaturated fatty acids was depended upon the culture conditions.

As already mentioned both carotenoids and α-tocopherol were detected in *D. salina* cells grown at all experimental conditions (Table 2). The increase of these natural antioxidants compound was correlated with increase of NaCl and decrease of nitrogen concentrations in media, unsaponifiable fraction and total lipid content (Table 1). The antioxidant activity of carotenoids and α-tocopherol was noticed in various lipid model systems[27]. Several authors have shown that unsaponifiable compounds can act as antioxidant[27,28]. However, Mendoza *et al.*[29] found that the unsaturated fatty acids have positive correlation with carotenoids content in *D. salina* grown under nitrogen starvation. Also, in this study, the values of total unsaponifiable matter exhibited positive correlation with increasing amount of PUSFA in *D. salina* cells. The RO values for lipids extracted from *D. salina* grown at 8,12 and 16% NaCl combined with 2.5 mM



TSFA: Total saturated fatty acids %
TPUFA: Total polyunsaturated fatty acids%
Tw3PUFA: Total omega 3 poly unsaturated fatty acids
UD: Unsaturated degree
TMUFA: Total monousaturated fatty acids %
TUFA: Total unsaturated fatty acids %
TU/TS: Total unsaturated/Total saturated
OR: Oxidation rate

Fig. 1: Evaluation criteria of *Dunaliella salina* lipids

nitrogen were 19.66, 24.53 and 32.0, respectively. While, the total unsaponifiable in these lipids was 7.61, 24.58 and 29.02%, respectively. Accordingly increase these compounds might increase the stability of algal lipid toward oxidative damage.

The main roles of fatty acids in algae are related to cell membrane function and to other metabolic processes[3]. The degree of fatty acid unsaturation is important in the process of plant or algae adaptation to the growth environment. Our results revealed that *D. salina* cells can be manipulated the lipid content reached ca-38% when the algae grown at high salinity and low N contents and the algal lipid is characterized by high proportion of C_{18} as long chains polyunsaturated fatty acids (LCPUFAs). Therefore, under stress conditions, the major effect was an increasing the degree of desaturation mainly at the level of $C_{18:3_\omega3}$ that reached ca. 68% of the total fatty acids. Such an increase was at the expense of $C_{16:0}$ and to a lesser degree on $C_{18:1}$ and $C_{18:2}$. This phenomenon is related to the increase of enzyme activity that catalyze the desaturation and elongation of fatty acids in *D. salina* cells, which may be extremely sensitive to increased NaCl level. Also, similar results were obtained by Romano *et al.*[30] who found, that $C_{16:0}$ acids first elongated to $C_{18:0}$,which subsequently undergoes various degrees of desaturation. Seto *et al.*[31] suggested that the enzyme that carry out the elongation and desaturation of fatty acids may require NaCl. Al-Hasan *et al.*[32] and Peeler *et al.*[33] reported that the desaturated fatty acids was increased in *D. salina* cells grown in high salinity medium. According to Azachi *et al.*[34], *D. salina* cells grown in high salinity contained higher proportion

of C_{18} to C_{16} fatty acids and consequently higher content of desaturated fatty acids were found compared with low-salt-grown cells. Hence, salt-induce modifications not only in fatty acids chain elongation but also increased the desaturation process. Al-Hasan *et al.*[32] indicated a salt-related increase in the relative proportion of linolenic acid ($C_{18:3}$) in the total lipid of *D. salina*. Also, environmental conditions changed the qualitatively and quantitatively the lipid unsaponifiable matter including lipophilic antioxidant and sterols contents[3,19,20].

Generally, *D. salina* cells can be manipulated the lipid content which yield a maximum values of about 38% in cells grown at high salinity and low N contents. This lipid is characterized by high proportions of C_{16} and C_{18} polyunsaturated fatty acids (PUFAs) and in particular, by a high content of ω 3 PUFAs (92.15%). Furthermore, the increase in PUFAs was associated with the increase of lipophilic antioxidant in unsaponifiable fraction. These unsaponifiable compounds can act as antioxidant to prevent autooxidation of PUFAs. The ω 3 fatty acids are known to possess some biological activities, including anti-inflammatory, anticancer, antiplatelet and hypolidemic activities[7,35]. These beneficial effects obtained from ω 3 PUFAs have been recommenced to increase serum PUFA level that decrease the risk of many diseases[8,17]. Consequently, it is possible to produce useful materials, such as antioxidants and lipids rich in ω 3 PUFAs and LCPUFAs by *D. salina.*

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