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Biochemical profiling of three indigenous *Dunaliella* isolates with main focus on fatty acid composition towards potential biotechnological application

Nahid Hosseinzadeh Gharajeh[a], Mostafa Valizadeh[a,*], Ebrahim Dorani[a], Mohammad Amin Hejazi[b,*]

[a] Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
[b] Department of Food Biotechnology, Branch for Northwest & West region, Agricultural Biotehnology Research Institute of Iran, Agricultural Research, Education and Extension Organization (AREEO), Tabriz, Iran

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ABSTRACT

This study describes the biochemical composition of three isolates, *Dunaliella* sp. ABRIINW-B1, -G2/1 and -I1 towards the biotechnological potential. *Dunaliella* sp. ABRIINW- G2/1 and -I1 had a remarkable protein content (~40% dry weight). *Dunaliella* sp. ABRIINW-I1 contained a pigment fraction of 3.2% largely composed of chlorophyll a (1.9%) and carotenoid (1.1%). *Dunaliella* sp. ABRIINW-B1, -G2/1 and -I1 produced respectively 42, 36 and 47% lipid content. The occurrence of high lipid and low carbohydrate (4–7%) in the isolates demonstrated their cell tendency to store energy and carbon mainly in lipid form. The lipid profile of the isolates expressed adequate n3:n6 ratio and health indices. The biochemical analysis revealed that *Dunaliella* sp. ABRIINW-B1 and -G2/1 have potential applications in the food and freshwater aquafeed sector. While *Dunaliella* sp. ABRIINW-I1 owing to appropriate pigment, protein, and lipid level containing very-long-chain polyunsaturated fatty acids showed a great promise in nutritional, pharmaceutical and marine aquafeed industries.

1. Introduction

By increasing population with the outgrowing nutritional as well as energy demands and limitation of the conventional sources, alternative resources are pursued in support of environmentally responsible and protective practices. Moreover, the general preference toward naturally derived food and health supplements over the chemically synthesized counterparts, results in excessive pressure on the environment if not maintained in viable manner [1]. Microalgae, owing to occurrence of the various and unique metabolites with remarkable biological, and pharmacological qualities in addition to environmental friendly cultivation process lacking competition with agriculture industry, came the main alternative and sustainable source forwards [2,3]. Microalgae are great untapped resources with promising potential to revolutionize biotechnology benefitting human nutrition and health, as well as global marketing in the near future. The valuable bioproducts with microalgal origins are getting widespread use as their community and the constitutive components are progressively being discovered.

The members of the microalga *Dunaliella* are unicellular photosynthetic microorganisms with exceptional physiological properties [4]. In addition to the massive accumulation of carotenoids [5], this alga is a rich source of bioactive substances such as lipids, proteins and carbohydrates [6,7]. The compositional ration of each component within a cell, due to various cellular behavior, differs in species- and even strain level [8]. The microalgal pigments have pivotal role in human health as natural antioxidant, anticarcinogenic, antidiabetic, antiangiogenic, antihypertensive, anti-inflammatory, anti-obesity and cardioprotective agents [9]. The proteins from microalgae have complete profile of the essential amino acids (EAAs) and thus can be integrated into processed foods or be provided as health functional food and aquaculture feed [10,11]. They have additional biotechnological applications in analytical, diagnostic and pharmaceutical purposes such as enzymes, antibodies and biosensors, antibiotics and biocatalysts in food processing [12]. Further, algal saccharides are shown to have various antimicrobial, antitumor, antiviral, fibrinolytic and anticoagulant properties [13].

* Corresponding authors.
E-mail addresses: nahid.hosseinzadeh@tabrizu.ac.ir (N. Hosseinzadeh Gharajeh), mvalizadeh@tabrizu.ac.ir (M. Valizadeh), dorani@tabrizu.ac.ir (E. Dorani), aminhejazi@abrii.ac.ir (M.A. Hejazi).

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The accumulation of large quantities of lipids with miscellaneous fatty acid (FA) composition in microalgal producer organisms, makes them a unique and viable oil platform [14]. The lipid accumulation in addition to the physical, chemical and functional properties of the fatty acids is affected by the strain of microalgae [15,16]. Microalgae are original producers of long chain and very-long-chain polyunsaturated fatty acids (LC- PUFAs> 18 and VLC- PUFAs >20 carbon chain) which include n6 and n3 fatty acids [16]. They then enter the food chain through their consumption by fish which in fact is the subsequent source [17]. The prominent fraction of the human diet, land plants and animals are rich in n6 PUFAs and poor in n3 PUFAs which generally lack VLC-PUFAs [18,19]. The human body is not able to efficiently convert α-linolenic acid (ALA; C18:3) to n3 VLC-PUFAs and thus they are very essential part of the food diet [18,19]. The fatty acid composition in oily food is an indicative of its potential in retarding atherosclerosis and prevention of coronary diseases, cardiovascular disorders and plaque aggregation [20,21]. Similar to the pigments, proteins and polysaccharides derived from microalgae, the VLC-PUFAs, have applications as functional food additives and health products [22]. Microalgae are being used in aquaculture feed formulation which ultimately improves the food nutritional value and offers human health benefits [23].

The studies related to *Dunaliella* genus, as other microalgae, are commonly based on the popular species, remaining the native isolates unexplored [24]. In this research, the biochemical composition of three indigenous *Dunaliella* isolates were described with regard to their biotechnological potential. The studies covering exploration of the native isolates and their biochemical compounds would provide a profound contribution to outreach a collection of isolates with wide spectra of distinctive applications.

2. Material and methods

2.1. Microalgal source

Three native *Dunaliella* isolates, namely *Dunaliella* sp. ABRIINW-B1, -G2/1 and -I1 were originated from the south (Persian gulf), central region (Gavkhooni marsh) and northwest (Urmia lake) of Iran (Fig. 1). Urmia or Orumiye lake (37°42′N 45°19′E) is the world's second large salt-water lake located between the two provinces of East and West Azerbaijan. Gavkhooni salt marsh (32°08′N 52°54′E) is located in Iranian Plateau, east of the city of Isfahan [25]. The coastal Tiab area of Persian gulf (27°38′N 57°05′E) is in Kuh Shah Rural District, Hajiabad County and Hormozgan Province [26]. These isolates were previously characterized as the members belonging to *Dunaliella* genus based on internal transcribed spacer (ITS) region. The ITS sequences of *Dunaliella* sp. ABRIINW-B1, -G2/1 and -I1, are available with accession numbers of KX870020, HQ823665 and MH880103, respectively at the Genbank of National Center for Biotechnology Information (NCBI).

2.2. Culture condition

The isolates were uniformly cultured in the media described by Hejazi et al. [27] containing 1 M NaCl, 0.048 M $MgCl_2.6H_20$, 0.00036 M $CaCl_2.2H_2O$, 0.0225 M Na_2SO_4, 0.0049 M K_2SO_4 and 0.1 M Tris-base. To the 985 ml autoclaved media, 5 ml of each of the sterilized stock solutions of 1) 1 M KNO_3, 2) 0.1 M KH_2PO_4 and 3) trace elements including 0.0168 mM $CoCl_2.6H_2O$, 0.0036 mM $MnCl_2.4H_2O$, 0.0011 Mm $NaMoO_4.2H_2O$, 0.0136 M Na_2EDTA, 0.0047 M $FeCl_3.6H_2O$, 0.0364 mM $CuSO_4.7H_2O$ and 0.059 mM $CuSO_4.7H_2O$ was added to reach the final 1 L volume. The cultures were maintained in the experimental condition of 25 °C, 100 μmolphoton m^{-1} s^{-1}, and under light to dark period of 16-8 h. Sampling was performed from 24-day old culture for biomass and biochemical measurements.

2.3. Biomass production

The biomass parameter of the isolates was determined spectrophotometrically [28]. The 50 ml volume of the culture suspension was filtered through 1.2 μm filter (glass microfiber, Whatman, 1822-047) and incubated at 105 °C for 24 h. The biomass dry weight (DW) was recorded and an equation (Eq.1) was developed based on its relationship with OD_{730} to express the biochemical composition of the isolates as portion of DW.

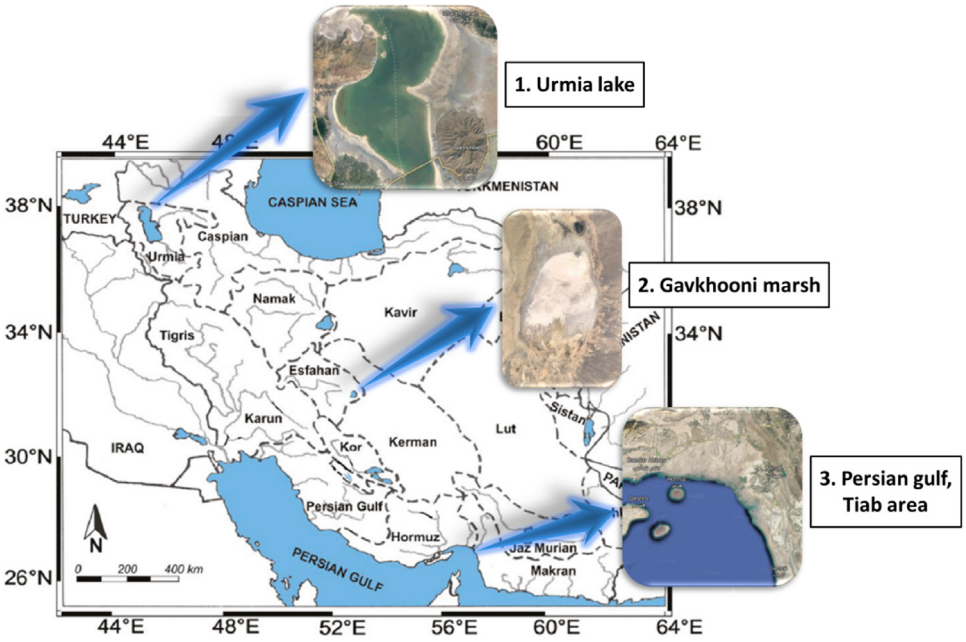

Fig. 1. The river map showing geographical origins of *Dunaliella* sp. ABRIINW-I1, -B1 and -G2/1, respectively taken from different regions of Iran: 1) hypersaline Urmia lake (Urmia), 2) Gavkhuni salt marsh (Isfahan) and 3) Persian gulf (coastal Tiab area).

$$DW\ (g\ L^{-1}) = OD_{730}{}^{*}0.8,\ R^2 = 0.95 \quad (1)$$

The dry biomass was further ashed in a furnace at 550 °C for 30 min. The relationship between OD_{730} and ash-free dry weight (AFDW) of algal biomass (mg L^{-1}) was established. The obtained regression equation (Eq.2) was used to calculate biomass concentration:

$$AFDW\ (g\ L^{-1}) = OD_{730}{}^{*}0.41,\ R^2 = 0.95 \quad (2)$$

The specific growth rate of the isolates was also calculated using Eq.3:

$$\mu = \ln(X_2/X_1)/(t_2 - t_1) \quad (3)$$

Where μ (day^{-1}) is the specific growth rate, and X_1 and X_2 are the biomass concentrations at respectively 1st (t_1) and 21st (t_2) day of cultivation, during the exponential growth phase.

2.4. Pigment content and profile

The pigment content of the isolates was extracted according to the method by Sedjati et al. [9]. Three ml of algal biomass was taken, then centrifuged at 800 g for 15 min. Three ml of 100% pure aceton was added to the wet pellet and was homogenazed by a vortex mixer. The mixture was recentrifugated at 800 g for 10 min to get a clear supernatant. The upper phase was used to measure chlorophyll a and b as well as β-carotene contents spectrophotometrically using the equations by Lichtenthaler and Buschmann [29].

$$Chla\ (\mu g\ ml^{-1}) = 11.24{}^{*}A_{662}\text{-}2.04{}^{*}A_{645} \quad (4)$$

$$Chlb\ (\mu g\ ml^{-1}) = 20.13{}^{*}A_{645}\text{-}4.19{}^{*}A_{662} \quad (5)$$

$$Cart\ (\mu g\ ml^{-1}) = (1000{}^{*}A_{470}\text{-}1.9{}^{*}Chla\text{-}63.14{}^{*}Chlb)/214 \quad (6)$$

$$T\ Chl\ (\mu g\ ml^{-1}) = Chla + Chlb \quad (7)$$

Where Chla: chlorophyll a; Chlb: chlorophyll b; T Chl: total chlorophyll; Cart: carotenoid

The obtained pigment concentrations as μg ml^{-1} were converted to the percent of DW biomass. The ratios of Chla/Chlb, Cart/Chla and Cart/Chlb were also calculated.

2.5. Total protein

Bradford [30] method was used to measure the total protein content. To the microalgal cell pellet of 100 mL cultures, 0.05 g polyvinylpyrrolidone (PVP) and 5 mL extraction buffer containing 50 μM potassium-phosphate buffer (pH 7) was added. The homogenate was centrifuged at 9,000 g for 30 min at 0 °C, and the resulting supernatant was used for protein assay. All the extraction procedures were performed at the temperature of 0–4 °C range. To the 20 μl protein extract, 2 ml of Coomassie Blue dye reagent was added and mixed well. Total protein determination was performed spectrophotometrically at 595 nm using a standard curve based on bovine serum albumin.

2.6. Total carbohydrate

To quantify the total catbohydrates (TCs), the modified phenol-sulfuric acid method [31] was applied. The TC measurements were performed spectrophotometrically (Perkin Elmer, lambda 35) at 485 nm using glucose standard. To the cell pellet of 50 ml culture, dried at 60 °C for 48 h, 15 ml ethanol 80% was added. The tubes were vortexed and centrifuged at 1100 g for 10 min. The supernatant was transferred to the new tubes and maintained at 50 °C to the complete alcohol evaporation. Then, to separate the pigments or other components from the residual algal material, in addition to10 ml distilled water, 470 μl Barium hydroxide (0.3 N) and 500 μl Zinc sulfate $ZnSO_4$ (5%) was added. The reaction mixtures were centrifuged for 10 min and the upper phase was removed to a new tube. Following the addition of 500 μl phenol 5% (w/w) and 2.5 ml H_2SO_4 96%, the mixture was allowed to stand for 45 min at room temperature; when the yellow-brownish color was established. The optical density was recorded at 485 nm and total carbohydrate was calculated based on calibration curve from a glucose standard.

2.7. Lipid extraction and GC analysis

Lipid content was extracted by the modified Bligh and Dyer method [32]. To assess fatty acid composition (as % of total fatty acids: TFA), the method by Duong et al. [33] was applied using the C4-C24 FAME standard (Sigma, 18919-1AMP Supelco). To extract total TFA, the biomass of 50 ml culture was transferred to a 2 ml container tube, to which 500 μl of sulfuric acid in methanol (1–50) was added. The vial lids were stringently covered with parafilm to avoid methanol evaporation. Vials were vortexed for 20 s and were then incubated for 2 h in 80 °C inside thermomixer and were cooled down for 5 min in the room temperature. 300 μl of 0.9% NaCl solution and 150 μl of Hexane were added to the vials, the mixture was vortexed for 20 s and centrifuged in 3000 rpm, 25 °C for 5 min to get two phases. From the upper phase of Hexane, an injection volume of 1 μl was injected to GC system (Varian company, Model 3800) equipped with a flame ionization detector (FID) detector, CP17973 column and 1079 injector. The flow rate of the carrier gas was set as 1 ml min^{-1} under application of the following ramp: 130 °C, hold for 4 m in. 180 °C, 8 min with the rate of 5 °C 4 min^{-1}; 220 °C, 10 min, with the rate of 4 °C min^{-1}; 220 °C, 20 min, 4 °C min^{-1}.

2.8. Nutritional value and health lipid indices

Based on the fatty acid composition, the lipid nutritional quality of n3:n6 ratio was determined for the isolates. Additionally, Atherogenic index (AI), thrombogenic index (TI) along with hypocholesterolemic index (HI) and hypocholesterolemic to Hypercholesterolemic (h/H) indices, were calculated using the equations described by Attia et al. [34]Lopes et al. [35] and Šimat et al. [36]:

$$AI = (C12{:}0 + 4 \times C14{:}0 + C16{:}0) / (\sum MUFAs + \sum PUFAs) \quad (8)$$

$$TI = (C14{:}0 + C16{:}0 + C18{:}0) / (0.5 \times \sum MUFAs + 0.5 \times \sum n6\text{-}PUFAs + 3 \times \sum n3\text{-}PUFAs + (\sum n3/\sum n6) \quad (9)$$

$$h/H = (C18{:}1n9 + C18{:}2n6 + C18{:}3n3 + C20{:}4n6 + C20{:}5n3 + C22{:}5n3 + C22{:}6n3)/ (C14{:}0 + C16{:}0) \quad (10)$$

$$HI = (C18{:}1n9 + C18{:}2n6 + C18{:}3n3 + C20{:}3 + C20{:}4n6 + C20{:}5n3 + C22{:}4 + C22{:}6n3)/ (C14{:}0 + C16{:}0) \quad (11)$$

2.9. Biodiesel properties

The biodiesel properties as degree of unsaturation (DU), long chain saturated factor (LCSF), cold filter plugging point (CFPP),

saponification value (SV), iodine value (IV) and cetane number (CN) were estimated for fatty acid profile of the isolates using mathematical models by Ramos et al. [37] and Francisco et al. [38]:

$$DU: MUFA + (2 \times PUFA) \quad (12)$$

$$LCSF: (0.1 \times C16:0) + (0.5 \times C18:0) + (1 \times C20:0) + (1.5 \times C22:0) + (2 \times C24:0) \quad (13)$$

$$CFPP\ (^{A}C): (3.1417 \times LCSF) - 16.477 \quad (14)$$

$$SV: \sum 560\ F / Mw \quad (15)$$

$$IV: \sum 245\ FD / Mw \quad (16)$$

$$CN: 46.3 + (5458 / SV) - (0.255 \times IV) \quad (17)$$

Where F is the amount of each fatty acid component (% TFA), D is the number of double bonds and Mw is the molecular weight of the corresponding FAME. The molecular weight of the FAMEs can be found in Møller [39].

2.10. Statistical analysis

Statistical analysis was carried out using SPSS v23.0 (IBM, Armonk, NY). One-way ANOVA was used in this study, with a confidence interval of 95%. The data were represented as mean values ± standard deviations.

3. Results and discussion

3.1. Biomass yield

The isolates, *Dunaliella* sp. ABRIINW-B1 and -G2/1 produced the high AFDW (and DW) as much as 1.2 g L^{-1} (2.34 g L^{-1} DW) and 1.18 g L^{-1} (2.3 g L^{-1} DW). This feature was significantly low as 0.55 g L^{-1} AFDW (equal to 1.1 g L^{-1} DW) in *Dunaliella* sp. ABRIINW-I1. The specific growth rate for *Dunaliella* sp. ABRIINW-B1 and -G2/1 was similar as 0.11, while for the isolate *Dunaliella* sp. ABRIINW-I1 it was obtained as 0.08. It should be pointed out that the estimated growth rates are measured in Erlenmeyer, rather than photo-bioreactor cultivation system and in the non-aerated autotrophic, rather than aerated mixo- or hetero-trophic condition. Since the growth rate is affected by numerous culture parameters [40], an improved growth rate would be expected for the isolates by promoting the culture conditions. In a study by Saha et al. [41] the growth performance of *D. salina* CCAP 19/20 at Erlenmeyer level was increased as 50% by exposing the culture to the appropriate light regime. While non-aerated Erlenmeyer cultivation of the oleaginous microalga *Monoraphidium sp.* Dek 19, presented a specific growth rate of 0.069 [42], it varies ranging from 0.1 to 0.4 day^{-1} in the commercial microalgae for scale-up production such as *D. salina*, *Nannochloropsis* sp., *Isochrysis* sp., and *Tetraselmis* sp. [14,43].

3.2. Biochemical composition of the isolates

The lipid, protein, carbohydrate, and pigment content of the isolates were measured as depicted in Fig. 2. Each cellular component is discussed in detail through the following sections.

3.2.1. Pigment content

Among the studied isolate, the pigment content was significantly high in *Dunaliella* sp. ABRIINW-I1 (3.21%). The other two isolates of *Dunaliella* sp. ABRIINW-G2/1 and -B1 contained the pigment of 1.85% and 1.55% DW, respectively. The pigments are reported to comprise 1–5 % of the algal dry weight [44]. The quantitative distribution of pigments revealed that chlorophyll was the major pigment observed in all three isolates (Table 1). As expected, chlorophyll a was the most abundant form of the chlorophyll. In the photosynthetic organisms, chlorophyll a is the primary light-harvesting complex as opposed to chlorophyll b which is the accessory light-harvesting pigment [45].

As seen in Table 1, the concentration of total chlorophyll (Chl) and carotenoid (Cart) varied as 1.17–2.15% and 0.38–1.06% DW in the isolates. The chlorophyll (including Chla and Chlb) and carotenoid content were within the ranges reported for *D. salina* [46]. The chlorophyll content in *Dunaliella sp.* ABRIINW-I1 (2.15%)

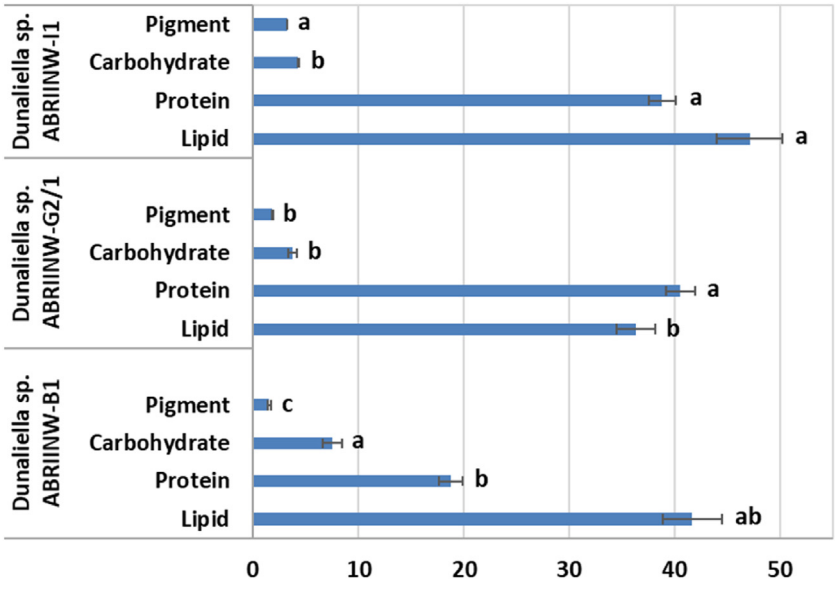

Fig. 2. Biochemical composition of the indigenous isolates, *Dunaliella* sp. ABRIINW-B1, -G2/1 and -I1 as lipid, carbohydrate, protein and pigment content (percent of dry weight) with the corresponding standard errors. Non-identical letters in a certain feature show significant statistical differences.

Table 1
Pigment content (% DW) in *Dunaliella* sp. ABRIINW-B1, -G2/1 and -I1. Chl: chlorophyll, T Chl: total chlorophyll and Cart: carotenoid.

Isolates	Pigment profile							Reference
	Chla	Chlb	T Chl	Cart	Chla/Chlb	Cart/Chla	Cart/Chlb	
Dunaliella sp. ABRIINW-B1	0.84	0.33	1.17	0.38	2.50	0.43	1.08	This study
Dunaliella sp. ABRIINW-G2/1	1.04	0.47	1.51	0.35	2.28	0.34	0.77	This study
Dunaliella sp. ABRIINW-I1	1.91	0.24	2.15	1.06	7.15	0.57	4.07	This study

was significantly higher than -B1 and -G2/1 and close to that of *Dunaliella sp.* (2.37%) [47]. The carotenoid content of *Dunaliella sp.* ABRIINW-I1 was higher than those of *Spirulina sp.*, *Chlorella sp.*, *Nannochloropsis sp.* and *Scenedesmus sp* [47]. Nevertheless, it is still way lower than that the improved 10% carotenoid production in *D. salina* (10%) under stimulating condition [10]. Not to mention that the carotenoid measurement in this study was performed under normal rather than stress condition. The carotenoid content of *Dunaliella sp.* ABRIINW-B1 (0.38%) and -G2/1 (0.35%) were similar to the data for *Scenedesmus sp.* (0.42%) [47] and higher than that in carrot (0.2%) [10].

Although the total chlorophyll, chlorophyll a and b contents varied between the two isolates of *Dunaliella* sp. ABRIINW-B1 and -G2/1, the chlorophyll a/b ratio was similar and close to those of the macroalga *Enteromorpha clathrata* and dark green leafy plants. As for *Dunaliella* sp. ABRIINW-I1 Chla/Chlb ratio (7.15) was three folds of the vegetables with dark green leaves [48].

The carotenoid to chlorophyll ratio was calculated to assess the active route of pigmentation. The Cart/Chla and Cart/Chlb ratios of three isolates were within the ranges recorded for *D. salina* under different conditions [46]. The Cart/Chlb ratio in *Dunaliella* sp. ABRIINW-I1 was ~4 folds of the corresponding values in -B1 and -G2/1,showing its active carotenogenesis pathway.

Due to their principal antioxidation bioactivity, the lipophilic pigment of chlorophylls and carotenoids are considered as natural food additives/colorants and the raw materials for nutraceutical, pharmaceutical, cosmetics, and aquaculture industries [9]. The chlorophyll consumption induces bile secretion, contributes to liver recovery and improves reproduction [49]. It is readily absorbed by the human body and enhances the metabolism of proteins, carbohydrates and lipids [50]. The dietary carotenoids have preservation and antioxidant function preventing cancer, arteriosclerosis, multiple sclerosis, arthritis, cataract, and aging macular degeneration [51].

3.2.2. Protein content

The protein content in *Dunaliella* sp. ABRIINW- G2/1 (40.5) and -I1 (38.8%) was significantly higher than -B1 (18.7%) ($p < 0.05$). As seen in Table 2, there is remarkable variability of protein contents within *Dunaliella* genus depending on the origin strain [10]. The reported protein content for *Dunaliella* species varies as 11.4% in *D. tertiolecta* [52], 12.6% in *D. primolecta* [53], 31–37% in *Dunaliella sp.* [54], 30–43% in *D.salina* [46] and 57% in *D. salina* [55]. The isolates *Dunaliella* sp. ABRIINW-B1 and -G2/1 represented an appropriate protein content higher than the conventional animal or plant sources of beef (17–22%), peanuts (26%), chicken and fish (19–24%) [10,56]. The isolates' protein production was less than *Chlamydomonas* (48%) [57], *Chlorella* (50–60%) and *Spirulina* (60–70%), though [10].

Table 2
Protein, carbohydrate and lipid content (as % DW) in *Dunaliella* sp. ABRIINW-B1, -G2/1 and -I1 and other green microalgae.

Isolates	Protein	Carbohydrate	Lipid	Reference
Dunaliella sp. ABRIINW-B1	19	8	42	This study
Dunaliella sp. ABRIINW-G2/1	41	4	36	This study
Dunaliella sp. ABRIINW-I1	39	4	47	This study
Dunaliella salina	40-57	32	6	[58]
Dunaliella bioculata	49	4	8	[59]
Dunaliella sp.	34	15	14	[47]
Chlamydomonas rheinharrdii	48	17	21	[57]
Chlorella pyrenoidosa	57	26	2	[58]
Chlorella vulgaris	51-58	12-17	14-22	[60]
Arthrospira maxima	60-71	13-16	6-7	[58]
Spirulina platensis	46-63	8-14	4-9	[58]
Tetraselmis maculate	52	15	3	[59]
Haematococcus pluvialis	48	27	15	[61]
Isochrysis galbana	50-56	10-17	12-14	[58]

Proteins, as the building blocks of human body, are essential macro-nutrients responsible for growth [10]. Microalgal derived proteins have considerable prospective for health food, aquaculture feed, medical and pharmaceutical applications [11].

3.2.3. Carbohydrate content

The carbohydrate content corresponded to 7.6% of the biomass in *Dunaliella* sp. ABRIINW-B1 which was higher than those in -I1 and -G2/1 (both 4%). The carbohydrate content of the isolates was low comparing to other microalgae and comparable with that of *Dunaliella bioculata* [59].

Microalgal carbohydrates are preferred as alternatives to the conventional sugars or treated lignocellulosic biomass in fermentation processes in food and medical industries. These less-value components have the potential to be converted into ethanol fuel production [62,63]. The low carbohydrate of the studied isolates does not primarily make them proper candidates for bioethanol production.

3.2.4. Lipid content

The lipid production of 47%, 42% and 36% was attained in the isolates of *Dunaliella* sp. ABRIINW-B1, -G2/1 and -I1, repectively. A range of 6–71% DW lipid production has been reported for different species of *Dunaliella* [3,64]. The lipid content in our isolates was above the middle data range for *Dunaliella* genus, similar to that of *D. salina* reported by Adarme-Vega et al. [24]. It also exceeded the lipid production of the model microalga *Chlamydomonas reinhardtii* (21%) (Table 2). The lipid content in *Dunaliella* sp. ABRIINW-I1 (47%) and *D.* sp. ABRIINW-B1 (42%) was close to that of *Chlorella vulgaris* (46%) and *Nannochloropsis* (50%) [65].

3.3. Lipid profile of the isolates

Comparison of the biochemical composition of the three studied isolates revealed that the cells tended to store the energy and carbon in lipid rather than carbohydrate form. Moreover, due to the large lipids quantities with unique and miscellaneous fatty acid composition in microalgae, the evaluation of the isolates' biotechnological application was mainly based on the lipid profile.

The preliminary FA analysis of all the three isolates revealed that palmitic acid (C16:0), along with the n3-PUFA constituent, α-linolenic acid (ALA: 18:3n3), respectively, dominate the FA spectrum, accounting for more than 50% of the TFA (54.3–63.9%). ALA is considered as the main representative of n3 PUFA [66]. The fatty acid profile in our isolates along with other *Dunaliella* species and phylogenetically close relatives is demonstrated in Table 3.

Table 3
Fatty acid profile (as % TFA) in *Dunaliella* sp. ABRIINW-B1, -G2/1, -I1 and other microalgae.

Reference	fatty acid pattern									Species
	EPA	DHA	UFA:SFA	n3: n6	∑n6 PUFA	∑n3 PUFA	∑PUFA	∑MUFA	∑SFA	
This study	–	–	2.1	4.2	8.6	36.2	45.1	22.8	32	*Dunaliella sp.* ABRIINW-B1
This study	–	–	2.32	5.81	6.8	39.5	46.3	23.4	30	*Dunaliella sp.* ABRIINW-G2/1
This study	1.4	0.6	1.2	3.6	9	31.9	45.4	7.8	46.3	*Dunaliella sp.* ABRIINW-I1
[67]	–	15.4	1.9	–	–	–	33.1	32.2	34.8	*D. salina* (1)
[68]	–	6.9	4.4	5.1	9.8	50	60.1	20.9	18.5	*D. salina* (2)
[69]	–	–	1	2.2	9.9	21.2	31.1	19.5	49.4	*D. salina* (3)
[70]	–	–	3.4	–	–	–	58.9	5.5	18.7	*D. salina* (4)
[2]	21.4	–	0.67	8.23	2.6	21.4	24	15.8	59.7	*D. salina* (5)
[71]	–	–	–	–	–	–	–	–	84.6	*D. salina* (6)
[72]	–	–	–	–	–	–	–	–	72.58	*D. salina* (7)
[73]	–	–	3.1	10.5	3.3	35	56.9	18.4	24	*D. salina* (8)
[74]	–	–	3.6	–	–	–	67.1	11.1	21.5	*D. salina* (9)
[75]	–	–	3.3	–	–	–	68.6	2.6	21.8	*D. tertiolecta* (1)
[33]	0.12	0.01	1.6	–	–	–	60.9	1.2	37.8	*D. tertiolecta* (2)
[76]	–	–	3.56	–	–	–	70.7	9.4	19.9	*D. tertiolecta* (3)
[77]	–	–	1.8	–	–	–	8	54.7	34.1	*Volvox carteri*
[78]	–	–	2.7	1	29	29	58	15	27	*Ch. reinhardtii*
[79]	1.9	3.5	1.9	0.5	17.2	7.9	25.1	39.3	34.3	*Spirulina platensis*
[79]	3.2	18.8	1.3	7.9	2.8	21.7	24.4	30	43.3	*Isochrysis galbana*
[79]	2.9	20.9	3.4	3	9.7	29.2	38.9	35.4	22.2	*Chlorella vulgaris*

A high content of PUFA (45.1–46.3%) and n3 PUFA (31.9–39.5%) was observed in the studied isolates. More importantly, among the isolates of our study, only *Dunaliella* sp. ABRIINW-I1 contained VLC-PUFAs of 9.8% of TFA as n6 FA of arachidonic acid (ARA; 7.8%) and n3 FAs of eicosapentaenoic acid (EPA; 1.4%) and docosahexaenoic acid (DHA; 0.6%). VLC-PUFAs have inhibitory and preventive effect on autoimmune disorders, atherosclerosis, thrombosis, Alzheimer, cancer, coronary and skin diseases [24,80]. DHA and EPA are influential factors on fetal, heart, brain and vision development [81]. Since the human body is not able to efficiently convert ALA to EPA and DHA, they should be externally provided [82].

Based on the literature, very few studies documented existence of n3 VLC-PUFA either as DHA or as EPA in *Dunaliella* species (Table 3). Among those, only *D.tertiolecta* (2) synthesizes DHA (0.01) as well as EPA (0.12) [33], both of which are less than those in the isolate -I1. No VLC-PUFA has been detected in the model microalga *Chlamydomonas reinhardtii* and *Volvox carteri*. However, in the microalgae of *Spirulina platensis*, *Isochrisis galbana* and *Chlorella vulgaris*, DHA and EPA of higher-level have been reported. The n6 PUFA of the three native isolates were in the same range of *D. salina* (2 and 3), and *Chlorella vulgaris* [67,68,79].

3.3.1. Lipid nutritional and health value

3.3.1.1. n3: n6 ratio. The n3 per unit of n6 ranged from 3.55 in *Dunaliella* sp. ABRIINW-I1 to the highest ratio of 5.81 in -G2/1. According to Table 3, the comparable n3:n6 ratio of 5.08 has previously been reported for *D. salina* (2) [68]. Comparing to the species listed in the table, the n3:n6 ratios were of satisfactory level in the isolates of our interest. The related ratio in most routinely consumed farmed/wild fishes is reported to be below 2 [83].

The consumption of oleaginous foods promotes a healthy life in a certain range of fatty acid ratio and composition. The n3:n6 ratio is documented to be an applicable index for oleaginous food sources with respect to their nutritional value [84]. Serious attention must be made to gauge equipoise between n3 and n6 fatty acids to keep healthy life because inappropriate consumption of n3 and n6 fatty acids is the main cause of cancer, diabetes neurochronic, cardiovascular, and inflammatory diseases [85]. The human body has evolved on a 1: 1 ratio diet and the n3: n6 ratio of more than 1:5 has been recommended by the World Health Organization (WHO) [86,87]. Our dominant food diet and the increased fried-/fast- food intake are insufficient in n3 fatty acids. Hence, the high n3:n6 ratios in our isolates show promises to benefit human health and compensate for the dire gap in n3 deficient nutritional habits [66].

3.3.1.2. Atherogenic, thrombogenic and hypocholesterolemic indices. The hypocholesterolemic index (HI), atherogenic index (AI), and thrombogenic index (TI) were calculated for the studied isolates. Based on GC results there was no detection of C20:3 and C22:4 included in HI and C22:5 in h/H calculation formulas, therefore these two mentioned indices in this research are identical. The isolates attained AI and TI of below 1, not surpassing 0.63, and the HI (h/H) of 1.71–1.86 range. The AI, TI and HI indicate respectively the anti-atherogenic, anti-thrombogenic and hypercholesterolemic potential of fatty acids in foods [20,21]. The lower AI and TI and the higher HI, the healthier the food source [35]. The nutritional sources with low AI and TI are useful in preventing the risk of cardiovascular disorders. Moreover, according to Grela et al. [88] cholesterol content in the human body is reversely correlated with total PUFA, HI as well as h/H ratio. The AI, TI and HI levels in our isolates were compared with food candidate microorganisms of *Dixioniella grisea, Isochrysis galbana*, *Leptocylindrus danicus, Nannochloropsis oculata, Nannochloropsis salina*, *Rhodomonas salina*, *Rhodella violacea* and

Table 4
Hypocholesterolemic (HI), atherogenic (AI), and thrombogenic (TI) health indices (Ind.) of fatty acid profile in *Dunaliella* sp. ABRIINW-B1, -G2/1, -I1 and nutritional candidate microalgae.

Health Ind.	AI	TI	HI
Dunaliella sp. ABRIINW-B1	0.45	0.24	1.71
Dunaliella sp. ABRIINW-G2/1	0.39	0.2	1.86
Dunaliella sp. ABRIINW-I1	0.63	0.42	1.85
Nannochloropsis oculata	0.66	0.46	0.95
Nannochloropsis salina	0.86	0.84	0.52
Rhodella violacea	0.57	0.24	1.89
Dixioniella grisea	0.69	0.39	1.6
Rhodella maculate	0.49	0.27	2.01
Rhodomonas salina	0.91	0.2	2.01
Isochrysis galbana	1.42	0.37	1.61
Leptocylindrus danicus	0.65	0.37	0.85

Table 5
Comparison of intrinsic biodiesel properties of the three native isolates of *Dunaliella* sp. ABRIINW-B1, *Dunaliella* sp. ABRIINW-G2/1 and *Dunaliella* sp. ABRIINW-I1 with those of standards (EN 14,214 and ASTM D6751) and other candidates.

Isolates	DU	LCSF	CFPP	SV	IV	CN	Reference
Dunaliella sp. ABRRNW-B1	113	4.7	−1.8	194.1	223.9	17.3	This study
Dunaliella sp. ABRRNW-G2/1	116	5.2	−0.2	194.8	237.3	13.8	This study
Dunaliella sp. ABRRNW-I1	98.5	12.3	22	194.2	226.7	16.6	This study
Chlamydomonas reinhardtii	73	–	–	165.9	64.8	64.6	[96]
Chlorella vulgaris	71	3.8	−4.6	225	65	57	[38]
Thraustochytrium sp.	70	5.8	1.9	165	157	44	[97]
Trichormus sp.	70	5	–	213	68	–	[98]
Sunflower	–	–	−3	193	132	50	[37]
Palm	–	–	10	205	57	61	[37]
EN 14,214	–	–	–	–	<120	>51	[99]
ASTM D6751	–	–	–	–	–	>47	[100]

Rhodella maculata [89] (Table 4). The AIs of *Dunaliella* sp. ABRIINW-G2/1 (0.39) and *Dunaliella* sp. ABRIINW-B1 (0.45) were less than the those reported for the eight nutritional microalgae. The AI of *Dunaliella* sp. ABRIINW-I1 (0.63) was typically lower than those of *N. salina*, *R. salina* and *I. galbana*. The HI of the three isolates (1.71–1.86) were comparable with *D. grisea*, *I. galbana* and *R. violacea*, and higher than *N. oculata*, *N. salina* and *L. danicus*.

Based on the fatty acid profile, all of the three isolates showed appropriate n3 PUFA, UFA: SFA, n3: n6 and nutritional lipid indices. However, *Dunaliella* sp. ABRIINW-I1 was the only isolate containing the VLC-PUFA and n3 VLC-PUFA of DHA and EPA, both of which are a very essential part of human diet. Oily products containing DHA and EPA are targeted as health food supplements and for medical biotechnology purposes [62].

3.3.2. Lipid aquafeed properties

The existence of PUFA and VLC-fatty acids in the lipid profile of the studied isolates rises their potential application in aquaculture for improved feed quality. Marine bio-destruction resulted from overfishing coupled with the rising population [90], have directed efforts toward improving aquaculture production. In parallel to the development of the aquaculture systems, attention must be paid to aquafeed ingredients and optimization of the food pellet formulation. That is because the fatty acid composition of aquafeeds has a large impact on the FA signature of the farmed fish [91], or the wild fish communities around coastal aquaculture [92]. An established correlation of PUFAs, n3 PUFAs and the VLC-PUFA, DHA, exists with the food digestion, growth, survival rate, sperm motility and viability of the fish [93,94]. Based on what mentioned, the feed sources with appropriate fatty acid content are of paramount importance in aquafeed purposes [16].

At present, most of the formulated diets in aquaculture are based on vegetable oil deficient in VLC-FA and VLC-PUFAs [95]. The utilization of common (vegetable/animal) oils in the aquafeed production is documented to lead to an increased level of terrestrial fatty acids in the farmed fish [92]. The fatty acid profile of the studied microalgae indicates that the isolate *Dunaliella* sp. ABRIINW-I1, due to high VLC-PUFA of nearly 10%, might be used in aquafeed production.

The feeding habit is largely affected by the species and bio-ecology of the fish being reared in aquaculture [16]. As for marine fish, the n3 VLC-PUFAs are of high essentiality whereas, linoleic acid and linolenic acid respond the feeding requirements of freshwater fish that can be subsequently converted to n3 VLC-PUFAs by the fish [16]. The isolate -I1, due to the presence of DHA and EPA, has the potential to meet the requirements in marine fish aquaculture. Whereas, the isolates *Dunaliella* sp. ABRIINW-B1 and -G2/1 due to higher linoleic in addition to α-linolenic acid as ∼43% of their TFA, are appropriate for the farming of freshwater fish.

3.3.3. Lipid biodiesel characteristic

The qualitative biodiesel properties of the isolates based on their fatty acid profiles, were estimated and compared with other sources (Table 5). The degree of unsaturation (DU), proportional indicative of unsaturated to saturated fatty acids, was higher in -B1 and -G2/1 in comparison to -I1. LCSF was relatively low in -B1 and -G2/1 isolates (∼5), however -I1 expressed higher value (12.3). The LCSF is an appropriate index to measure the LC-SFA content of the fatty acid profile that due to high melting points are undesirable. Since the biodiesel must maintain its liquid form at low temperatures, the low LCSF value and consequently the low CFPP is an ideal feature for biodiesel application. In the present investigation, the two isolates of -B1 and -G2/1 showed the fine CFPP values of -1.8 and -0.2, respectively. Whilst, the -I1 isolate demonstrated the highest CFPP of 22 which makes it inappropriate for biodiesel application.

The saponification values in the three isolates were uniformly obtained as about 194–195, very close to the SV of sunflower source (193). The high iodine values ranging from 224 in -B1 to 237 in -G2/1, suggests the isolates' low oxidative stability. According to the European Standard EN 14,214, the fatty acid pattern of the isolates did not meet the biodiesel standard requirement of IV, 120. The fatty acid profiles of the isolates resulting in high iodine and saponification values, lead to a high cetane number (CN). The CN criterion is an indicator of ignition quality in biodiesel fuel, which specifies the fuel readiness of auto-ignition and smoother run of the engine [67]. The isolates of -B1, -G2/1 and -I1 exhibited the CNs of as low as 17.3, 13.8 and 16.6, respectively. By this, they could not meet the reference standard criteria for biodiesel application by European Standard EN 14,214 and ASTM International standard D6751 (with CN > 51 and CN > 47, respectively). This is, in addition to low carbohydrate, an excessive confirmation on the non-proficiency of the studied isolates for biofuel purposes.

4. Conclusion

Determination of the biochemical composition of three isolates in this study supported their promising application in nutraceutical, pharmaceutical and aquaculture industries. *Dunaliella* sp. ABRIINW-B1 and -G2/1 due to medium to high protein and satisfactory lipid content with high n3 LC-PUFAs and appropriate health indices showed potential application in food and freshwater aquafeed sector. The production of high biopigment, protein and lipid consisting the VLC-PUFA and n3 VLC-PUFA befits the isolate *Dunaliella* sp. ABRIINW-I1 in nutritional, pharmaceutical, cosmeceutical and marine aquafeed industries. As *Dunaliella* sp. ABRIINW-I1 is a distinctive *Dunaliella* isolate capable of producing DHA as well as EPA, it can be the subject of complementary optimization studies. Photobioreactor cultivation and modification of the environmental condition may benefit biomass production

along with volumetric productivity of the intended biomolecules in the isolate of interest.

Conflict of interest

None.

CRediT authorship contribution statement

Nahid Hosseinzadeh Gharajeh: Methodology, Data curation, Writing - original draft. **Mostafa Valizadeh:** Conceptualization, Supervision, Funding acquisition. **Ebrahim Dorani:** Visualization, Investigation. **Mohammad Amin Hejazi:** Conceptualization, Project administration, Writing - review & editing.

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Appendix A. Supplementary data

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