ردیابی و شناسایی برخی از گونه های جنس سودوموناس عامل پوسیدگی نرم با استفاده از ژن tuf

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چکیده

مقدمه: بیماری پوسیدگی نرم در گیاهان که توسط عوامل باکتریایی بیماری‌زا ایجاد می‌شوند، هرساله سبب کاهش محصولات تولیدی و همچنین خسارت به محصولات انباری می‌گردند. تحقیق حاضر به‌منظور ردیابی و شناسایی عوامل باکتریایی عامل پوسیدگی نرم متعلق به جنس سودوموناس در برخی از محصولات صیفی و گیاهان تزیینی انجام شد.

مواد و روش‌ها: طی فصول زراعی در سال‌های ۱۳۹۴ تا ۱۳۹۶، نمونه‌های مشکوک و دارای علائم بیماری از میزبان‌های بادمجان، ذرت، برگ قاشقی، ذرت شیرین و گوجه‌فرنگی جمع‌آوری گردید. ویژگی‌های بیوشیمیایی و موفولوژیکی جدایه‌ها بر اساس روش‌های استاندارد باکتری‌شناسی بررسی شدند. همچنین ژن tuf در جدایه‌های نماینده با استفاده از آغازگرهای Bac-tuf-F و Bac-tuf-R به روش واکنش زنجیره‌ای پلی مراز تکثیر و پس از توالی یابی با بانک داده‌های موجود در سایت NCBI با نرم‌افزار بلاست تجزیه‌وتحلیل و مقایسه شدند.

نتایج: درمجموع ۱۲۰ جدایه باکتریایی از نمونه‌های موردنظر جداسازی گردید. بر اساس تولید آنزیم تجزیه‌کننده پکتین، پنج جدایه از جنس سودوموناس انتخاب شد. بر اساس نتایج آزمون‌های بیوشیمیایی و همچنین فیلوژنی بر پایه توالی یابی ژن tuf، این جدایه‌ها به‌عنوان گونه‌های Pseudomonas aeruginosa، P. entomophila، P. mosselii و P. putida شناسایی شدند.

بحث و نتیجه‌گیری: بر اساس دانش ما، این اولین گزارش از بیماری‌زایی P. aeruginosa در گوجه‌فرنگی، P. entomophila در ذرت شیرین و P. putida در بادمجان است. علاوه بر این، برای اولین بار P. aeruginosa و P. mosselii به‌عنوان عامل همراه بیماری به ترتیب در ذرت و بادمجان گزارش می‌شود.

واژه‌های کلیدی: بادمجان، ذرت،برگ قاشقی، ذرت شیرین، گوجه فرنگی

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The Effects of using Chemical Fertilizers along with Guillard (f/2) on Growth, Biochemical Compositions, Fatty Acid Profiles, Chlorophylls and Total Carotenoids of *Haematococcus pluvialis* in Brackish Water

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Abstract

Introduction: Using the standard methods for the productions of unicellular algae have high harvest costs and in many cases is not affordable. Therefore, chemical fertilizers could be considered as an appropriate alternative for the production of unicellular algae. The green microalgae *Haematococcus pluvialis* is capable of producing high levels of astaxanthin and lipid in resistant cells called spore.

Materials and methods: *H. pluvialis* with the initial density of 4.25×10^4 cell mL^{-1} transferred to the 3-liter containers. Fatty acid profiles, growth factors, chemical compositions and pigments of algae were investigated.

Results: The results showed that the specific growth rate, the cell division per day, carbon dioxide fixation efficiency, biomass, and total biomass efficiency were significantly higher and lower in treatment 5 (G: I= 50:50) and 9 (Gf= 100), respectively. The highest protein (40.68%), lipid (34.88%) and carbohydrate (42.04%) contents were observed in treatments of 9, 2 (G: R= 50:50) and 8 (I= 100), respectively. The highest chlorophyll a (71.29 mg/g FW) in T9 and chlorophyll b (85.60 mg/g FW) in T3 (G: R= 75:25) and total carotenoid (206.11 mg/g FW) in T6 (G: R= 75:25). Saturated fatty acid was significantly highest in T6, but its poly unsaturated fatty acid was significantly highest in T2.

Discussion and conclusion: The results indicated that the f/2 medium promotes biomass and lipid in T9 and T5, T6 made increase total carotene and SFA and T2 and T4 raised Mono saturated fatty acid and PUFA in *H. pluvialis* when it is cultured in brackish water.

Key words: *Haematococcus pluvialis*, Biochemical Composition, Fatty Acids, Chlorophyll, Total Carotenoid

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Introduction

Generally, microalgae are the microorganisms with high growth rates, which photosynthesis about 50% of the organic carbon in each year (1). The i algae are used in food industry as animal feed, food additives, health products and pigments (2). Among different groups of microalgae, the green algae, having a large number of marine and freshwater species, are the unique groups of algae typically used in industrial sectors, especially for the production of valuable nutrients and oils (3).

H. pluvialis is a single-celled green alga with two flagella that is able to produce cells resistant to stress conditions called cyst or aplanospore (4, 5), which can accumulate astaxanthin and lipid in cells (6-8). This alga and several species of microalgae are able to grow and survive in a wide range of environments that can alter lipid metabolism in response to diverse stress conditions (9-11) These microalgae are the ideal organisms for the production of non-polar triacylglycerols (TAGs). The lipid content is increased when algal cells are exposed to different conditions such as high salinity (12), depletion of nitrogen (13-15) and high light intensity (16). In addition, lipid compounds in microalgae depend on age and different life cycle stages (17). The green cells of *H.pluvilis* grow optically in terms of low light intensity at less than 100 µmol photons m^{-2} s^{-1} (18, 19) and saturated environment of nutrients (20). In addition, the accumulation of lipids takes place during cyst formation in microalgae under stressful conditions such as nutrient limitation, high light intensity and high salinity (15) and in this situation the Astaxanthin production increases (4, 5, 20, 8).

The green microalgae absorbs light for the purpose of photosynthesis through chlorophyll, which are at 630-675 nm and 450-475 nm, as well as carotenoids, are the pigments minor that absorbed light at 400-550 nm (21). Red light (600-700 nm) and blue (400-500 nm) are stimulating the growth of microalgae and growth rate. The lipid content varies with the intensity of light (22, 23). Changes in the composition of algae medium can change biochemical composition such as lipid, protein, carbohydrates, and pigments. The nutrient in the medium can significantly change the value of cell biomass produced by the cultivated algae (24). The salt concentration in the water influences the system through osmotic stress, salinity stress, and ionic cells ratio (25), but salinity in marine algae is expected to affect a series of activities such as lipid metabolism, especially in the cell membranes (26) and fatty acid composition (27). The profile of fatty acid in algae contains a mixture of saturated fatty acids, monounsaturated and polyunsaturated. The polyunsaturated fatty acids in algae mainly have 18 carbon atoms in their molecular chains that are essential for humans and animals. Polyunsaturated fatty acids, particularly the omega groups have a high biological activity, such as anti-inflammatory, rheumatoid arthritis and also have a high antioxidant activity in human and animal health (28, 29). On the other hand, the lipid is used to produce biofuels such as biodiesel, and the most common fatty acid compositions required for biodiesel production are C16-C18 (30).

The aim of this study was 1) to use chemical fertilizers with the N: P ratios equal of Gillard medium (f/2) to produce *H. pluvialis*, 2) to explore the possibility of cultivating *H. pluvialis* algae in brackish

water and 3) to evaluate biochemical and growth characteristics of *H. pluvialis* cultivated in brackish water using Gillard medium (f/2)[1] and fertilizers. Although microalgae cultivation does not require arable land, but due to the higher demand of water, fertilizer, and solar energy, they have the possibility to grow in limited places (31). Exploring the possibility of growing *H. pluvialis* in brackish water and using chemical fertilizers are likely to help eliminate the costs of water, fertilizer and energy in some areas of prone for culturing *H. pluvialis*.

Materials and methods

H. pluvialis (TAGACC006) was prepared from TAG BIOTEK, in Tehran, Iran. Three-liter flasks were used for growing algae. The cells with density of 4.25×10^4 cell ml^{-1} were reared at 7.5 ppt salinity, under 3500 lux light intensity with a primary pH of 8.00, 21ºC temperature and Guillard's medium (32) for rearing. Each flask was continuously and severely aerated with pipette filtered and the aeration rate was equal to 758 mL min^{-1}. For each of the treatments three replicates was considered as T1 100%Guillard (7.5 ppt), T2 50%G + 50%Rosier (7.5 ppt), T3 75%G + 25%Rosier (7.5 ppt), T4 25%G + 75%Rosier (7.5 ppt), T5 50%G + 50% (Urine + Superphosphate) (7.5 ppt), T6 75%G + 25% (Urine + Superphosphate) (7.5 ppt), T7 25%G + 75% (Urine + Superphosphate) (7.5 ppt), T8 100%Urine + Superphosphate (7.5 ppt) and T9 100%Guillard (in freshwater). The ratio N: P in treatments was considered as Guillard's medium equal to 10 to 1.

Analyzing nitrogen, phosphorus, and iron in chemical fertilizers: For analyzing the nitrogen, phosphorus and iron in the chemical fertilizers used in this study were used palintest photometer (Model 7100, made in England). The following values in Table 1 were obtained:

Measurement of cell growth: 1 ml of living algae was read from growing flasks at 750 nm daily (33). Then, by using the following equation to calculate biomass ($g.l^{-1}$):

Biomass ($g.L^{-1}$) *H. pluvialis* = 0.796 * OD750 – 0.1086 R2 = 0.977

Biomass productivity ($g.l^{-1}.d^{-1}$) was achieved from different biomass concentration ($g.l^{-1}$) divided by the specified time range (days) based on the following formula (34):

$Ps\ (g.L^{-1}.d^{-1}) = (X_1–X_0) / (T_1–T_0)$

Specific growth rate (d^{-1}) was calculated using the following equation:

$\mu=(\ln X_1–\ln X_0)/(T_1–T_0)$

Division time (day^{-1}) and Generation time (h^{-1}) was obtained using the following equation:

Divisions per day (day^{-1})=$\mu/0.9631$

Generation time (h^{-1})=$0.9631/\mu$

Where X_1 and X_0 were biomass concentration ($g.l^{-1}$) on T_1 and T_0 respectively.

CO_2 fixation efficiency was calculated by measuring the amount of carbon dioxide in microalgae (35):

$P\ CO_2\ (g\ l^{-1}d^{-1}) = 1.88 \times Ps$

Ps is biomass productivity ($g.l^{-1}.d^{-1}$).

Table 1- Chemical fertilizers analyzed

chemical fertilizer	NO_2^- ($mg.L^{-1}$)	NO_3^- ($mg.L^{-1}$)	NH_4^+ ($mg.L^{-1}$)	P_2O_5 ($mg.L^{-1}$)	Fe ($mg.L^{-1}$)
Urine	10.00	2.188	5.8	1.4	0.10
Superphosphate	0	0.705	4.6	11	0.09
Rosafert® (Granulated fertilizers)	3.2	5.246	8.2	14	1.6

Measuring of chlorophyll a, b and total carotene: 50 mL of algae culture was filtered with S&X filter (1micron) then weighed. The pigment is extracted with methanol 96% (50 ml for each gram algae). The samples were homogenized for one minute at 1000 rpm. The homogeneous mixture was passed through the S&X filter (1micron) and then was centrifuged for 10 min at 2,500 rpm. Supernatants were separated for readings at a wavelength of 666, 653 and 470 nm using the spectrophotometer. The following formula was used to calculate:

$C_a = 15.65A_{666} – 7.340A_{653}$

$C_b = 27.05A_{653} – 11.21A_{666}$

$C_c = 1000A_{470} – 2.860C_a – 129.2C_b /245$

Ca= chlorophyll a, Cb= chlorophyll b and Cc= total carotene in terms of micrograms per gram of fresh weight (36).

Lipid and ash content analysis: Laboratory methods were used to measure lipid content by ether extraction (37). For this purpose, a certain amount of samples was weighted and held for 7 hours on soxhlet within diethyl ether (98%). To calculate the ash content, samples were weighed and placed in an electric furnace for 6 hours at 550 °C (37).

Fatty acid profiles: 100-200 mg of algae was weighted into a glass tube with a Teflon lined screw cap. 1 ml of H2SO4 2.5% and methanol 98% (1:40, v/v) mixed solution was added to each cup and was incubated for one hour at 80 ° C. After cooling at room temperature (RT), 500 µl of hexane with 1.5 mL of NaCl 0.9% (w/v) were mixed and added to the samples to be extracted fatty acid methyl ester (FAME). Then samples were centrifuged for 10 min at 4000 rpm and were taken supernatant (this step was repeated three times to maximum fat extracted). The samples were injected into gas chromatography (GC) to determine the fatty acid profiles (38).

Protein content: To measure the protein content used by Slocombe et al. (2013), 5 mg of dried algae were mixed with 0.2 ml trichloroacetic acid (TSA) 24% (w/v), then the mixture was incubated for 15 min at 95 ° C and cooled at RT. The homogeneous sample was centrifuged for 20 min at 4 ° C with 15000g and the supernatant was discarded. The pellets were re-suspended in 0.5 mL Lowry reagent and were incubated for 3 h at 55 °C (supernatant was kept). After cooling samples at RT and centrifuging for 20 min at RT and 15000g, the supernatant was placed for 30 min in Lowry reagent and absorption was read 600 nm (39). The standard curve is drawn on the basis of 1 $mg.ml^{-1}$ BSA.

Total Carbohydrate content: First, 100 mg of algae was weighed into a boiling tube and 5 ml of 2.5 N HCl was added. Then, the samples were hydrolyzed to simple sugars by keeping it in a boiling water bath for 3 hours and then were cooled RT. After that, it was neutralize with solid Na2CO3 until the effervescence ceases. Next, the volume made up to 100 mL was centrifuged (5000 rpm for 5 min). 0.5 mL of supernatant was collected and made up to 1 mL with distilled water. Then, 4 mL of 0.2% anthrone reagent was added heated for 8 min in boiling water bath. The sample cools rapidly and reads the green to dark green color at 630 nm. The standard solution was prepared at the concentration of 100 µ.mL-1 glucose (40).

Statistical analysis: Normality of all data was checked by Kolmogorov-Smirnov and Bartlett tests. Mean (±SD) and ANOVA were also used. Tukey analysis was used at 5% ($P<0.05$). The calculated data and draw charts were held by SPSS (version 22) and Excel (2013 version), respectively.

Results

When G (100%) was used for the cultivation of *H. pluvialias* in fresh water, PH showed a large increase in range of 4 to 5 days (about 1.2 unit) that it can be associated with the number of cells in this timeframe, because at this time, an increase of 150,000 cells mL^{-1} in a day took place.

With using G: I (50:50) in brackish water and G (100) in freshwater, the average of SGR, 0.29±0.00 and 0.29±0.00 d^{-1}, CO2 fixation efficiency, 34.10±0.2 and 37.24±1.8 $mg.L^{-1}.d^{-1}$, biomass productivity, 0.0181 and 0.0198 $g.L^{-1}.d^{-1}$ and biomass, 0.1995 and 0.2179, were observed that were higher than other conditional culturing ($P<0.05$). When G: R (25:75) in brackish water was used, D (d^{-1}) and G (h^{-1}) had the most proliferation rate.

Figure 2 shows the number of *H. pluvialis* cell mL^{-1} that produced higher algal cells in the condition of using I (100%). In general, the minimum number of cell mL^{-1} was obtained in the presence of different ratios of R (25, 50 and75%).

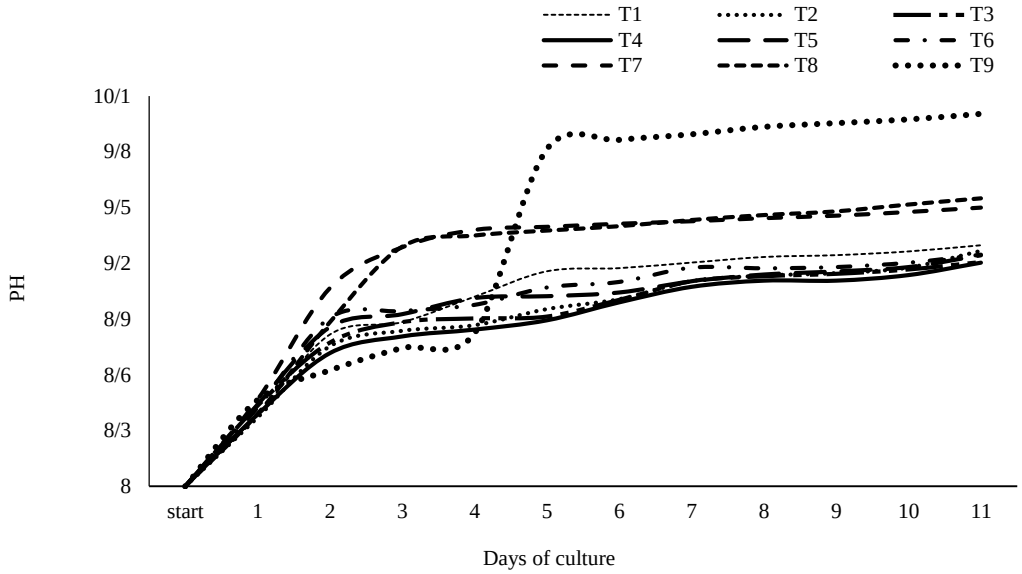

Fig. 1- Changes pH in *H. pluvialis* during cultivation time

Table 2- Effects of Gillard Medium (f/2) and fertilizers on SGR, D, G, CO2 fixation efficiency, Biomass productivity and Biomass in *H. pluvialis*

Treatments	SGR (d^{-1})	D (d^{-1})	G (h^{-1})	CO2 fixation efficiency ($mg.L^{-1}.d^{-1}$)	Biomass productivity ($\times10^{-2}$) ($g.L^{-1}.d^{-1}$)	Biomass ($\times10^{-2}$) ($g.L^{-1}$)
1 (G_b =100%)	0.26 ± 0.00^{c}	0.37 ± 0.00^{c}	2.72 ± 0.01^{a}	23.59 ± 0.28^{c}	1.26 ± 0.01^{c}	13.81 ± 0.16^{c}
2 (G:R = 50%:50%)	0.19 ± 0.00^{b}	0.28 ± 0.00^{b}	3.58 ± 0.01^{b}	11.25 ± 0.04^{b}	0.60 ± 0.00^{b}	6.58 ± 0.02^{b}
3 (G:R = 75%:25%)	0.15 ± 0.01^{a}	0.22 ± 0.02^{a}	4.56 ± 0.41^{c}	6.68 ± 1.23^{a}	0.36 ± 0.06^{a}	3.91 ± 0.72^{a}
4 (G:R = 25%:75%)	0.20 ± 0.00^{b}	0.28 ± 0.01^{b}	3.54 ± 0.08^{b}	11.56 ± 0.64^{b}	0.61 ± 0.03^{b}	6.76 ± 0.37^{b}
5 (G:I = 50%:50%)	0.29 ± 0.00^{d}	0.41 ± 0.00^{d}	2.41 ± 0.01^{a}	34.10 ± 0.26^{d}	1.81 ± 0.01^{d}	19.95 ± 0.16^{d}
6 (G:I = 75%:25%)	0.27 ± 0.00^{cd}	0.40 ± 0.00^{cd}	2.52 ± 0.02^{a}	29.55 ± 1.08^{cd}	1.57 ± 0.05^{cd}	$17.29\pm0.63c^{d}$
7 (G:I = 25%:75%)	0.20 ± 0.00^{b}	0.29 ± 0.00^{b}	3.50 ± 0.02^{b}	11.86 ± 0.13^{b}	0.63 ± 0.00^{b}	6.94 ± 0.08^{b}
8 (I = 100%)	0.27 ± 0.01^{cd}	0.39 ± 0.01^{cd}	2.56 ± 0.09^{a}	28.30 ± 3.20^{cd}	1.51 ± 0.17^{cd}	16.56 ± 1.87^{cd}
9 (G_f = 100%)	0.29 ± 0.02^{d}	0.42 ± 0.02^{d}	2.37 ± 0.15^{a}	37.24 ± 1.80^{d}	1.98 ± 0.47^{d}	21.79 ± 5.15^{d}

The same letters in each column represent no significant different (0.05). Guillard in brackish water: G_b, Guillard in fresh water: G_f, Rosier: R, Urine + Superphosphate (Inorganic fertilizer): I.

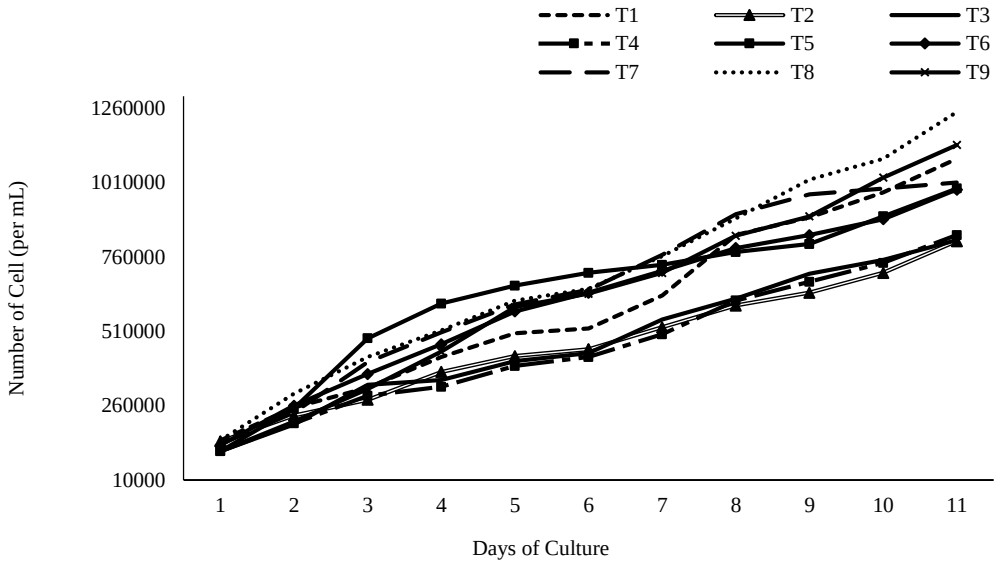

Fig. 2- Changes cell density in *H. pluvialis* during cultivation time

Adding G (100%) to the cultivation of *H. pluvialis* in freshwater increased the protein content up to 40.68 ± 2.23% ($P<0.05$). With using of I (100%) carbohydrate, content increased up to 42.04±0.21% and in T2 (G:R = 50:50) lipid content raised up to 34.88±0.21% in brackish water, which showed significant differences compared to other conditions ($P<0.05$). Rearing of *H. pluvialis* with I: G (50:50) showed a significant increase in ash content (16.53 ± 2.35 %) ($P <0.05$).

H. pluvialis cultivation with G in freshwater significantly increased the chlorophyll a (71.29 ± 0.37 $\mu g.g^{-1}fw$), but decreased chlorophyll b (33.36 ± 0.20 $\mu g.g^{-1}fw$) in this condition ($P <0.05$). However, the ratio of G: R or/and I (75:25) caused increasing of chlorophyll b, that were 56.53±2.62 and 58.97±1.69 $\mu g.g^{-1}fw$, respectively ($P <0.05$). Reduction of I (till 25%), increased total carotene up to 206.11±5.13 $\mu g.g^{-1}fw$ ($P <0.05$).

Table 3- Effect of Gillard medium and chemical fertilizers on protein, lipid and ash contents in *H. pluvialis*

Treatments	Protein (%)	Lipid (%)	Total Carbohydrate (%)	Ash (%)
1 (G_b =100%)	35.07±0.55^{b}	24.01±0.36^{a}	26.79±1.29^{ab}	11.14±0.58^{b}
2 (G:R = 50%:50%)	28.97±0.41^{a}	34.88±0.21^{e}	27.02±0.16^{b}	6.86±0.34^{a}
3 (G:R = 75%:25%)	27.45±0.72^{a}	29.68±0.08^{c}	31.86±0.14^{c}	7.11±0.31^{a}
4 (G:R = 25%:75%)	35.38±0.51^{b}	23.04±1.46^{a}	31.48±0.13^{c}	6.47±0.04^{a}
5 (G:I = 50%:50%)	31.08±0.73^{ab}	23.42±1.08^{a}	24.89±0.24^{a}	16.53±2.35^{c}
6 (G:I = 75%:25%)	34.56±0.32^{b}	26.03±1.36^{b}	24.58±0.18^{a}	12.99±0.38^{b}
7 (G:I = 25%:75%)	28.92±0.28^{a}	32.08±0.04^{d}	25.09±0.15^{ab}	8.30±2.47^{a}
8 (I = 100%)	27.53±0.21^{a}	23.09±1.6^{ab}	42.04±0.21^{d}	6.37±0.15^{a}
9 (G_f = 100%)	40.68±2.23^{c}	27.38±0.35^{b}	24.67±0.37^{a}	6.24±0.27^{a}

The same letters in each column represent no significant different (0.05). Guillard in brackish water: G_b, Guillard in fresh water: G_f, Rosier: R, Urine + Superphosphate (Inorganic fertilizer): I.

Table 4- Effect of Gillard medium and chemical fertilizers on chlorophyll a, chlorophyll b and total carotene content in *H. pluvialis*

Treatments	Chl.a (µg.g^{-1}fw)	Chl.b (µg.g^{-1}fw)	Total Carotene (µg.g^{-1}fw)
1 (G_b =100%)	23.71±1.21^{a}	48.10±1.29^{c}	114.62±3.05^{c}
2 (G:R = 50%:50%)	40.33±2.81^{c}	56.53±2.62^{d}	132.21±5.74^{d}
3 (G:R = 75%:25%)	56.47±6.40^{d}	82.88±5.95^{e}	184.26±12.40^{f}
4 (G:R = 25%:75%)	24.84±2.61^{ab}	33.49±1.89^{a}	74.28±4.21^{a}
5 (G:I = 50%:50%)	38.83±2.71^{c}	58.97±1.69^{d}	132.77±3.37^{d}
6 (G:I = 75%:25%)	40.06±1.38^{c}	85.60±2.13^{e}	206.11±5.13^{g}
7 (G:I = 25%:75%)	30.16±0.66^{b}	39.30±1.78^{b}	88.66±4.04^{b}
8 (I = 100%)	44.60±0.82^{c}	54.57±1.05^{d}	126.28±2.05^{cd}
9 (G_f = 100%)	71.29±0.37^{e}	33.36±0.20^{a}	147.00±1.58^{e}

The same letters in each column represent no significant different (0.05). Guillard in brackish water: G_b, Guillard in fresh water: G_f, Rosier: R, Urine + Superphosphate (Inorganic fertilizer): I.

Fatty acid profiles of *H. pluvialis* are shown in Table 5. DHA (C22: 6n3) and ARA (C20: 4n6) were 11.41±0.37% and 2.11±0.07%, of FAME when *H. pluvialis* grown in fresh water with G medium. EPA (C20: 5n3) was shown to be high (2.97±0.16%) with using of 50% ratio of R. Vaccine acid (C18: 1n7) only when G was used in freshwater (2.98 ± 0.09%). Saturated fatty acids (SFA) and total C16-C18 fatty acids increased in *H. pluvialis* when the ratio was G: I to 75:25 and were obtained 37.20 ± 1.07% and 69.20 ± 1.99%, respectively (P <0.05). Unsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) under conditions of using G: I = 50: 50 and G: R = 50: 50 were 1.40 ± 0.08% (lowest) and 40.46±2.20% (highest) (P <0.05).

Table 5- Effect of Gillard medium and chemical fertilizers on fatty acid profiles and SFAs, HUFAs and PUFAs in *H. pluvialis*

Treatments	1 (G_b =100%)	2 (G:R = 50%:50%)	3 (G:R = 75%:25%)	4 (G:R = 25%:75%)	5 (G:I = 50%:50%)	6 (G:I = 75%:25%)	7 (G:I = 25%:75%)	8 (I = 100%)	9 (G_f = 100%)
C14:0	0.47±0.03	1.06±0.05	1.41±0.08	0.53±0.03	0.85±0.05	1.08±0.03	0.88±0.05	0.87±0.05	1.15±0.03
C14:1n5	0.88±0.07	2.82±0.15	2.27±0.13	2.07±0.12	0.42±0.02	1.53±0.04	0.67±0.04	0.27±0.01	0.21±0.00
C16:0	2.52±0.21	2.15±0.11	0.97±0.05	4.04±0.24	2.56±0.15	21.35±0.61	2.78±0.17	2.81±0.18	0.26±0.00
C16:1n7	Nd	Nd	Nd	Nd	Nd	Nd	0.66±0.04	5.50±0.37	2.98±0.09
C18:0	28.09±2.36	27.19±1.47	25.15±1.44	23.58±1.40	13.30±0.82	14.21±0.41	12.22±0.77	8.83±0.59	20.40±0.67
C18:1n7	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	2.98±0.09
C18:1n9	5.74±0.48	Nd	2.24±0.12	5.57±0.33	0.98±0.06	0.78±0.02	1.16±0.07	0.97±0.06	1.92±0.06
C18:n26Cis	10.59±0.89	13.71±0.74	14.99±0.86	14.11±0.84	24.01±1.49	21.13±0.60	22.08±1.40	26.50±1.78	21.65±0.72
C18:3n3	3.78±0.31	17.20±0.93	14.47±0.83	20.16±1.20	13.31±0.82	11.72±0.33	9.57±0.60	10.99±0.73	0.48±0.01
C20:0	0.88±0.07	1.14±0.07	1.28±0.07	1.03±0.06	0.30±0.01	0.56±0.01	0.17±0.01	Nd	0.58±0.01
C20:2n6	0.92±0.07	1.36±0.07	1.02±0.05	0.93±0.05	0.17±0.01	0.71±0.02	0.11±0.00	Nd	1.34±0.04
C20:4n6	0.83±0.06	0.43±0.02	Nd	Nd	Nd	Nd	Nd	Nd	2.11±0.07
C20:3n3	0.59±0.04	0.72±0.03	0.68±0.03	4.12±0.24	0.16±0.00	0.36±0.01	0.19±0.01	0.11±0.00	2.36±0.07
C20:5n3	1.59±0.13	2.97±0.16	2.11±0.12	Nd	0.77±0.04	0.88±0.02	0.32±0.02	0.24±0.01	Nd
C22:0	0.48±0.04	0.82±0.04	0.77±0.04	Nd	Nd	Nd	Nd	Nd	Nd
C22:6n3	0.75±0.06	4.07±0.22	3.00±0.17	0.97±0.05	0.42±0.02	1.00±0.02	0.08±0.00	0.18±0.01	11.41±0.37
SFA	32.44±2.72^{cd}	32.36±1.76^{cd}	29.58±1.70^{d}	29.18±1.73^{d}	17.00±1.05^{b}	37.20±1.07^{e}	16.05±1.01^{b}	12.51±0.84^{a}	22.38±0.74^{c}
MUFA	6.62±0.55^{d}	2.82±0.15^{b}	4.51±0.26^{c}	7.63±0.45^{d}	1.40±0.08^{a}	2.31±0.06^{ab}	2.49±0.15^{ab}	6.75±0.45^{d}	5.12±0.17^{c}
PUFA	19.05±1.60^{a}	40.46±2.20^{c}	36.27±2.09^{bc}	40.29±2.40^{bc}	38.85±2.41^{b}	35.80±1.03^{b}	32.35±2.05^{b}	38.01±2.56^{b}	39.33±1.30^{b}
C16-C18	50.71±4.26^{a}	60.26±3.27^{bc}	57.82±3.33^{ab}	67.46±4.02^{bc}	54.17±3.37^{a}	69.20±1.99^{c}	48.46±3.07^{a}	55.60±3.74^{b}	47.68±1.58^{a}

The same letters in each column represent no significant different (0.05). Guillard in brackish water: G_b, Guillard in fresh water: G_f, Rosier: R, Urine + Superphosphate (Inorganic fertilizer): I.

Discussion and conclusion

The composition of culture medium have a significant effect on the growth, bloom and final concentration of microalgae (41). Hence, the production of microalgae on a commercial scale requires the provision of nutrients in sufficient amounts into the culture medium. There are many different methods used to increase mass production or growth rates of microalgae species (such as: NaHCO3, the fixation and size of carbon dioxide bubbles, salinity, organic carbon, and nutrient-absorbing compounds of algae) (42-44). On the other hand, water, nutrients, and carbon dioxide contain about 10-30% of production costs on algae growing commercial farms (45). Although algae farmers try to recycle the culture medium and use part of the recycled elements for new culture, but this method has its own problems, including contamination raised by algae pathogens or the aggregation of substances by secondary metabolisms, which ultimately lead to reduce algal yields. (46).

Our results showed that 50% of the standard culture medium (G) could be replaced by chemical fertilizers for use in massive culture of the *H. pluvialis*. So, no significant difference was observed in mass production of *H. pluvialis* cultured in I: G (50:50) and pure G medium

Under continuous exposure of lighting and temperature of 25 ° C after 14 days, the highest levels of chlorophyll a, beta-carotene and cell number were obtained for *Dunaliella* sp. 7.5 $mg.L^{-1}$, 5.2 $mg.L^{-1}$ and 6.5 × 106 $cells.mL^{-1}$ respectively (47). The cell division rate of *D.salina* was observed 0.43 in the modified Dewalne's medium, while in the Walnen's culture medium it was recorded about 0.40 (48). The highest cell density was observed in the use of I (100%) medium (1.2×10^6 cells. mL^{-1}) at the end of the eleventh day. Although, the growth and number of the cells increased during the culture period, but the biomass production was less than the G in freshwater and G: I (50:50). This is probably due to the reduced size of the algae cells produced in the culture medium I (100%). In the 24-hour light exposure of *Nannochloropsis gaditana*, cell density, SGR, biomass concentration and biomass productivity were obtained 4×10^6 $cells.mL^{-1}$, 0.50 d^{-1}, 0.30 $g.L^{-1}$ and 28 $mg.L^{-1}.d^{-1}$ in order (49). The growth of *H.pluvialis* cells was reported at constant concentrations of swine lactation, to be constant until nitrogen was depleted (50). Under the continuous exposure to lighting at 21 ° C, the highest SGR, division rate, CO2 fixation efficiency, biomass productivity and total biomass for *H.pluvialis* in cultivation of freshwater (Gf = 100%) and brackish water (G: I = 50: 50) were recorded to be 0.29 and 0.29 d^{-1}, 0.42 and 0.41 d^{-1}, 37 and 34 $mg.L^{-1}.d^{-1}$, 1.98 and 1.81 $mg.L^{-1}$ and 21.79 and 19.95 $mg.L^{-1}.d^{-1}$, respectively.

Growth and biochemical factors in different algal species can be strongly influenced by different growth culture conditions (51). For chlorophyll, the content was highest on the 18th day *D. tertiolecta* and on the 16th day *D. salina* culturing, while for chlorophyll b, the highest content occurred on the16th day for both species, so the total carotene on 26th day for *D. Tertiolecta* and on the 24th day for *D. salina* were obtained (52). Chlorophyll in algal cells is a place where light and energy storage in the form of ATP and NADPH occur, and it will increase with biomass increasing (53). Maity et al.

(2014) stated the chlorophyll content and biomass increase with increasing cultivation period and their change with light and temperature. Based on our results, the highest chlorophyll a content was observed on the eleventh day in Gf (100%) (71.29 µg.g-1.fw-1) culture medium while the highest chlorophyll b and total carotene content were recorded in the use of G: I (75:25). The chlorophyll content in algae is considered as an appropriate indicator to estimate the amount of their biomass production (54, 55). Based on our results, by synchronous increasing in chlorophyll a, content and biomass was occurred in Gf culture medium, but in contrast to the previous state, chlorophyll b decreased. It could be probably due to the increase of green non-photosynthetic pigments in algae, that is Non-photosensitive pigments are directly related to chlorophyll a and b content in algal cells (56). On the other hand, when algae are exposed to lower levels of nitrogen sources, nitrogenous compounds such as nitrates, ammonia, amino acids, proteins, and pigments are consumed in large quantities or completely (57,58) and these could also be due to using algae from nitrogenous compounds in standard culture medium rapidly. Therefore, the rapid growth of algae causes maximum reduction of the nitrogen combinations (NaNO3) in the Guillard medium, which results in the use of nitrogen compounds from the internal algae deposits, such as chlorophyll (which contain nitrogen-containing pufferins). This is probably the reason why chlorophyll is reduced, despite biomass being stable in particular of time.

With increasing the algae growth period time, the lipid content was increased, but the highest protein and carbohydrates contents were observed in the middle of growing time for *D. salina* and *D. tertiolecta* (52). The presence of algae exposed to high light intensity and higher photoperiod made increasing of growth rate and protein content (59). Seyfabadi et al (60) reported that when *Chlorella volgaris* were exposed to high light intensity and long-term photoperiod, the alga protein content would raise. The protein and carbohydrate contents produced in *H. pluvialis* cultured by G medium in freshwater and brackish water showed a higher amount, which may be probably due to the spending low energy for osmotic balance in freshwater, and also brackish water has led to higher carbohydrate production in it. Also, the lipid content was highest in the *H. pluvialis* cultivated with the combination of G: R (in ratios of 50:50) in brackish water, which is likely that salinity stress induces more fat production. On the other hand, the highest ash content with using G: I (50:50) was obtained, that the difference in the biochemical composition of algae could be attributed to the utilization of various chemical fertilizers (as nitrogen and phosphorus sources).

Previous research has shown *that Botryococcus braunii, D. tertiolecta, Nannochloropsis* sp., *N. oleoabundans* and *Porphyridium cruentum* are suitable species for the production of biomass (77% dry cell weight) and lipid production by the photosynthesis process (53,61-63). During the cultivation of *H. pluvialis* in brackish water, the lipid content production did not exceed more than 35% and this rearing condition could not be a suitable alternative for lipid production, but in order to produce biomass up to 0.2 $g.L^{-1}$ (DW), it could be considered as an appropriate condition in

brackish water. Differences in lipid content in algal are mainly due to the difference in algal species, the type and amount of nutrients and environmental conditions (water, light, temperature, carbon dioxide) (53). Rodolfi et al (64) demonstrated that there is an inverse relationship between biomass and lipid production under unfavorable conditions during the growth of microalgae.

Cheirsilp and Torpee (65) observed that the growth and lipid content in *chlorella* (marine species) was steadily rising to the highest level with an increase in light intensity of 112 $\mu mo.m^{-2}.s^{-1}$. Research has revealed that in *Chlorococcum* sp., *Microcystis* sp. and *Phormidium* sp. the total lipid content was 30.55%, 88.8%, and 18.66%, respectively, (lipid percentage in relation to biomass) (66). So far, there has been no report on the survey of *H. pluvialis* in brackish water (7.5 $g.L^{-1}$), and salinity just has been used for the induction of astaxanthin and lipid production. The results of this study indicated the inverse relationship between the lipid content and biomass concentration (in brackish water), which is in line with the results of Mahapatra and Ramachandra (2013) (66).

Various environmental factors (such as light, nutrients, and temperature) effect fatty acid profiles of microalgae (67). Omega fatty acids play an important role in the health of humans and animals. These fatty acids are essential for the structure and development of the activity of plasma membranes, heart tissue, brain cells, and neurons (68, 69). *H. pluvialis* fatty acid profiles showed that algae raised in brackish water produced higher ALA than the algae cultured in fresh water (less than 0.5% of total FAME). ARA was also highest in freshwater, and it was not seen in most of the other rearing conditions. EPA was observed highest when it was 50% R in medium, and DHA was significantly higher in freshwater than brackish water. On the other hand, due to the different growing conditions in this study, depending on the requirement, various fatty acids obtained in this study can be produced at the highest amounts. The global aquatic decreasing and environmental pollution are among serious factors that have unwittingly influenced the use of fish oil in the nutraceutical industry (70) and is considered in using algal oil. Various environmental factors can influence the saturated fatty acids (SFA), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) in various algal species. Mandotra et al. (71) reported that increasing the light intensity up to 6000 lux increases the SFAs in *S. abundans*, while increasing the phosphorus up to 60 $mg.L^{-1}$, increased MUFAs and the highest PUFAs was observed in PH 9. SFAs content that showed the highest value in *H. pluvialis* cultured by I (25%) and the highest level of MUFAs was registered in *H. pluvialis* grown in brackish water with G (100%), I (100%) and R (75%).

Conclusion

Our findings in this study suggested that 50% of the Guillard medium can be replaced with Inorganic fertilizers (Urine + Superphosphate) for the mass cultivation of various algal species. Replacing 25% of Guillard medium with Inorganic fertilizers leads to a maximum increase in carotenoids production. The Guillard culture medium (100%) increases the production of DHA (up to 11%) in *H. pluvialis* species grown in freshwater.

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[1]- (1) NaNO3= 75g/l; (2) NaH2PO4= 5 g/l; (3) Na2EDTA= 4.36 g/l + FeCl3.6H2O= 3.15 g/l; (3.1) CuSO4.5H2O= 1 g/100ml; ZnSO4.7H2O= 2.2 g/100ml; CoCl2.6H2o= 1 g/100ml; MnCl2.4H2o= 1.8 g/100ml; NaMoO4.2H2O= 0. 63 g/100ml; (4) Biotin= 10.0 mL of 0.1 mg mL^{-1} solution (1mg in 10ml); Vitamin B12= 1.0 mL of 1.0 mg mL^{-1} solution (1mg in 1ml); Thiamine HCL= 0.2 g/l. Add 1 ml of each solution from (3.1) into solution (3). Add 1 ml of each solution (1); (2); (3) and (4) into a liter algae medium.