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Full Length Research Paper

Comparative biodiversity and effect of different media on growth and astaxanthin content of nine geographical strains of *Haematococcus pluvialis*

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Haematococcus pluvialis **is a unicellular green volvocale alga living in temporary shallow freshwater ponds. It has many applications for humans, poultry and fishes due to its ability to produce astaxanthin. The main objective of this study was to evaluate the biodiversity and growth of nine strains of *H. pluvialis* originating from three countries: Iran, USA and Finland in distinct growth media (Bold, NIES, OHM, Mixotroph and COMBO) and their molecular marker investigation. The biomass, astaxanthin and chlorophyll concentration were determined in all strains. The results of this study showed that Mixotroph growth medium produced the highest biomass of 0.577 g dry weight/l followed by COMBO and OHM media, respectively. However, the highest astaxanthin concentration was obtained from COMBO and OHM growth media and Iran4 and USA3 strains. Hence, the Mixotroph is suitable for biomass production and OHM or COMBO is appropriate for astaxanthin production; so two steps cultivation is feasible to produce astaxanthin. The results depicted geographical differentiations of the strains with Iran4 and USA2 strains producing the highest biomass and Finland2 strain showing the slowest one. The inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) molecular markers were used as suitable tools for the purpose of finding out genetic variations and genetic discrimination of *H. pluvialis* strains. This technique differentiated the strains based on their geographical status.**

Key words: *Haematococcus*, growth medium, strain, astaxanthin, biomass, geographical, molecular marker.

INTRODUCTION

Algae have received much attention in recent decades because of its most useful natural resources which produce different bioactive compounds like antioxidants, vitamins, proteins, unsaturated fatty acids, carotenoid and astaxanthin. Astaxanthin production is limited to a few organisms such as *Haematococcus pluvialis*, *Phaffia rhodozima* (Domínguez-Bocanegra et al., 2007), *Paracoccus haeundaensis* (Young, 2011; Young et al., 2006), transformed *Escherichia coli* (Young et al., 2007), some other bacteria and *H. pluvialis* being the most important producer. Recently, astaxanthin biosynthesis ability was induced in *Sacharomycess cervisiae* yeast through gene transformation (Ukibe et al., 2009). Astaxanthin conjugates to peroxisome proliferator-activated receptors (PPARα) and modulates gene expression in the target cells which results in decreased amounts of lipids in the plasma (Yaoyao et al., 2011), and consequently reducing atherosclerosis risk (Ji-Young et al., 2011). Astaxanthin as the most powerful natural

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antioxidant can be used as a preventive medicine to slow down degenerative diseases, cardiovascular problems, cancers, immunological diseases, cholesterol and triglyceride levels and improve blood rheology, protect against cerebral ischemia, and neutralize both reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) (Garrett et al., 2004; Hughes, 1999; Lockwood et al., 2006; Miyawaki et al., 2008; Ji-Young et al., 2011; Hui et al., 2009). Probably, the zooplankton as intermediate of the food chain will accumulate astaxanthin in their body in the night which will serve as photo-protective pigment during the day (Andersson et al*.,* 2003). Animals and humans are unable to synthesize astaxanthin and they obtain it only through supplementation in their food (Sommer et al., 1991). The pink color of flamingo, salmonid fish and lobster is related to the amount of astaxanthin deposit in their muscle and feathers which is available in their rich diet (Ah, 1990; Dissing et al., 2011; Green et al., 2010).

With respect to cheap availability of synthetic astaxanthin in one hand and healthy concerns and demands for natural food pigments on the other hand, more researches are essential on alternative natural sources of astaxanthin (Higuera-Ciapara et al., 2006). *H. pluvialis* is a unicellular green alga from the order volvocales which shows great potential in astaxanthin production. However, the quest to provide astaxanthin from this alga has many obstacles due to its slow growth, complex life cycle and unpredictable yield under cultivation.

The first step in obtaining astaxanthin is mass production of *H. pluvialis* biomass. The vegetative phase of *H. pluvialis* is limited to the availability of nutrients and optimal light intensities. Lack of these conditions will cause a cyst stage transformation. The addition of certain organic substances such as acetate can boost their palmella growth stage (Hata et al., 2001), and indirectly increase astaxanthin production. The addition of 0.25% (W/V) acetate to the medium can enhance the growth of *H. pluvialis* but a 0.5% concentration can hinder its growth (Orosa et al., 2005; You-Chul et al., 2006).

Nutritional and environmental stresses would influence the kinetics of astaxanthin production. For example, nitrogen starvation can cause severe damage to the metabolism of the cell and thus it is more effective than phosphorous starvation in causing astaxanthin accumulation (Boussiba et al., 1999). The vegetative form of *H. pluvialis* changes to the cyst form under stressful conditions such as exposure to a diluted growth medium, decreasing nitrate and phosphate concentration, increasing light intensity, adding large amounts of acetate and using non-aerated culture which all consequently increase astaxanthin production (Fan et al., 1994; Domínguez-Bocanegra et al., 2007; Ranjbar et al., 2008). As mentioned by Kamonpan et al. (2007), the cell's growth is closely related to the types of used media and related constituents, including vitamin B_{12} levels which enhances the growth of *H. pluvialis.* The addition of this vitamin up to 12 µg L^{-1} was reported to boost the growth rate up to 55% in comparison to a medium without it (Kaewpintong et al., 2007).

There are various studies on the growth of *H. pluvialis* in different growth media and various cultivate milieus (Cerón et al., 2007; Imamoglu et al., 2009; González et al., 2009; Ping et al., 2007; Zhang et al., 1999). In this survey, the COMBO medium was used for the first time to cultivate *H. pluvialis.* COMBO is commonly employed in algal and zooplankton production resulting in excellent growth for both organisms. Therefore, there is no need to use separate media, and it can be used to study the interactions between animal and primary producers (Kilham et al., 1998).

The *Haematococcus* as airborne organism can be distributed to other water bodies by wind (Genitsaris et al., 2011). However, the variability of *H. pluvialis* growth is probably due to the different populations or genetic diversity of this alga in different parts of the world. It exhibits different biological properties such as growth rate, biomass production, cell wall thickness and astaxanthin production.

There have been some studies on algae using the inter simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) molecular markers (Bornet et al., 2005; Dayananda et al., 2007; House et al., 2008). ISSR markers are reliable, highly polymorphic, low cost and less laborious, needs only a small amount of DNA and are very fast when compared to most other molecular markers (Zietkiewicz et al., 1994). ISSR and RAPD do not require DNA sequence data and in terms of reproducibility, ISSR is comparable to SSR (Bornet and Branchard, 2001).

DNA molecular marker recently helped to distinguish a variety of *H. pluvialis* strains and made it feasible to find other strains with better biological attributes of astaxanthin production (Noroozi et al., 2011).

In this study, for the first time, nine strains of *H. pluvialis* from three countries (Finland, Iran and USA) of different continents, were cultured in five growth media namely Bold, OHM NIES, Mixotroph and COMBO (Kilham et al., 1998, Fabregas et al., 2000) to determine the responses of the different *H. pluvialis* strains when grown on them and the abilities of the different media to induce better growth of this alga. The main objectives of this study were to determine biodiversity and optimal medium and its effects on the biological characteristics of *H. pluvialis* such as biomass and astaxanthin production as well as to select the fastest growing strain of this alga and molecular marker investigation.

MATERIALS AND METHODS

Algal strain, growth conditions and inoculum preparation

The Iranian strains of *H. pluvialis* (Iran1, Iran2, Iran3 and Iran4) were isolated from freshwater ponds and temporary freshwater

Table 1. The origin and isolation place of ten strains of *H. pluvialis*.

Original name	Isolation place	Applied name
CCAP 34/6	Ostpicken,Island, Tvärminne, rain pool	Finland1
CCAP 34/7	Ostpicken Island, Tvärminne,	Finland1
CCAP 34/1D	Botanic garden, Univ. Basel	Switzerland (only molecular study)
CCAP 34/12	Ottowa, Kansas	USA1
CCAP 34/13	Ferrum, Virginia, bird bath,	USA2
CCAP 34/14	Cattonsville, Maryland, cement urn,	USA3
Iran1	Karaj, Golshahr Park, rain pool	Iran1
Iran2	Karaj, Kuye Karmandaneshomal, city park	Iran2
Iran3	Tehran, Shemiranat, swimming pool	Iran3
Iran4	Karaj, KuyeModares, stream	Iran4

bodies in Tehran and Karaj cities and the USA, Finland and Switzerland strains were purchased from CCAP (The Switzerland strain was not used in growth study due to subsequent contamination). After several subcultures using different isolation techniques, the Iranian strains were freed from contaminants like other algae, fungi and bacteria. The *Haematococcus* cells are sensitive to stresses such as strong light and nutrition deficiency and they were transformed to cyst form in unfavorable environmental conditions. Therefore, the *Haematococcus* cells in the water samples were exposed to strong light to transform into big and red color cyst cells (which were visible with naked eyes). The identification of *H. pluvialis* was based on the identification of key references (Guiry et al., 2007; John et al., 2002). Table 1 shows the original name and the isolation place of ten strains. The Switzerland strain was contaminated after achieving molecular marker experiments; therefore, this strain was not included in growth study.

Isolated *H. pluvialis* was cultured photoautotrophically at 23°C in 40-µmol photons $m^{-2}s^{-1}$ in a light dark cycle: 12-12 h in liquid Bold medium and aerated gently to get enough biomass for inoculation into the other media. After two weeks, 10 ml of the well mixed and homogenized cell suspension in Bold medium in the exponentially growing phase was transferred to the four media mentioned earlier (Table 2). All the cultures were in triplicates and were shaken daily by hand to mix the culture. It should be noted that aeration was not used for the culture media except for the Bold medium starter culture at the onset of the experiment.

Measurements of dry weight biomass, chlorophyll and astaxanthin levels

The growth of *H. pluvialis* in terms of biomass was determined by the dry weight method in which 10 ml of homogenized cell suspension after two weeks in the stationary were transferred to a membrane filter (Millipore) of 0.8 µm and vacuum filtered, rinsed with 10 ml distilled water and dried at 70°C overnight (Esperanza et al., 2005).

To determine the chlorophyll content, 10 ml of homogenized cell suspension in the stationary phase of growth were centrifuged for 5 min at 5000 rpm and the pigment concentration was measured by using a spectrophotometer based on the dimethyl sulfoxide (DMSO) method (Emile et al., 2003). The green biomass of *Haematococcus* was exposed to high intensity of light (345-µmol photon m^{-2} s^{-1}) for one week to transform the vegetative forms to the cyst form and to induce astaxanthin production. 10 ml of homogenized cyst suspension of each medium and strains were centrifuged at 3000 rpm for 5 min. The pellet was vortexed with 10 ml of DMSO and the mixture was retained in 70°C for one hour to extract astaxanthin (Sedmak et al., 1990). Astaxanthin was determined by using high performance liquid chromatography (HPLC) and spectrophotometer at 480 nm. An Agilent HPLC machine was used with Hypersil Silica column 250 mm length and the mixture of methanol, dichloromethane, acetone and H_2O (85, 5, 5 and 5%) as the mobile phase (Anarjan et al*.,* 2010). Astaxanthin with 98% purity from Sigma Company was used as the reference to detect astaxanthin concentrations in the different algal strains. Statistical Package for the Social Sciences (SPSS) version 17 was used to calculate statistical parameters such as analysis of variance (ANOVA), deterministic finite automaton (DFA), Duncan and correlation. The Duncan's test or multiple comparison test in an analysis of variance can be used to define the significant differences between group means.

Molecular experiments

Genomic DNA was extracted from *H. pluvialis* using the cetyl trimethylammonium bromide (CTAB) method. Polymerase chain reaction (PCR) was performed using 20 µl of reaction mixture solution containing 11.5 µl of autoclaved distilled water, 2 µl of 10X PCR buffer, 1.4 µl of $MgCl_2$ (50 mM), 0.6 µl of primer (5 mM), 0.4 µl of dNTP (25 mM), 1.6 µl of genomic DNA, 0.5 U of Taq DNA polymerase (Invitrogen) and 2 µl of Q-solution. PCR program used was: 97°C for 6 min followed by 35 cycles of 94°C for 45 s, 48 to 69°C for 45 s, 72°C for 60 s and 72°C for 5 min (Noroozi et al., 2011)**.** The genetic similarity was obtained by importing the data matrix from Excel to NTSYS pc version 2.10e. Jacard's similarity index is suitable for dominant markers to detect strain similarities. The dendrogram and bootstrap value was constructed using Paup software to discriminate different strains in clusters.

RESULTS

The obtained results reflected the effects of the growth media since all the cultures received uniform physical conditions such as light intensity and flask shape. Mixotroph medium with acetate as a carbon source induced the best growth for *H. pluvialis* followed by COMBO and OHM, while Bold and NIES media resulted in the lowest biomass (Figure 1). Duncan test categorized the media into three distinct groups: A, B and C. The highest biomass was obtained in Mixotroph (group C) for the strains Iran4, Finland1, USA2 and USA3 (0.43, 0.57, 0.54

Table 2. Composition of the different growth media used (g/L).

Content	Media				
	Bold	**NIES**	**Mixotroph**	**COMBO**	**OHM**
$NaNO_3$	0.2500000			0.0850000	
KNO_3		0.100000	0.5000000		0.410000
$Ca(NO_3)_2.4H_2O$		0.150000			
$Co(NO_3)_2.6H_2O$	0.0004900				
$K2HPO_4$	0.0750000			0.0087000	
KH_2PO_4	0.1750000				
Na_2HPO_4					0.030000
NaH_2Po_4			0.1950000		
H_3Po_4			0.1200000		
$CaCl_2.2H_2O$	0.0250000	0.004000	0.1830000	0.0360000	0.110000
NaCl	0.0250000				
$MgSO_4.7H2O$	0.0750000	0.040000	0.0616000	0.0370000	0.246000
$FeCl_3.6H_2O$		0.000600		0.0010000	
$Fe(III)citrateH_2O$					0.002620
$MnCl_2.4H_2O$	0.0014000	0.000110		0.0001800	0.000980
$CoCl_2.6H_2O$		0.000012	0.0000500	0.0000120	0.000011
H_3BO_3	0.0114000		0.0001200	0.0240000	
$FeSO_4·7H_2O$,	0.0049800		0.0209000		
$CuSO_4.5H_2O$	0.0015700		0.0006200		0.000012
$ZnSO_4.7H_2O$	0.0088000	0.000060	0.0007200	0.000022	
$MnSO_4H_2O$			0.0008500	0.000001	
$Na_2MoO_4.2H_2O$		0.000007	0.0000700	0.000022	0.000120
Cr_2O_3					0.000075
SeO_2				0.0000016	0.000005
KOH	0.0310000				
Acetate			1.2600000		
B-Na_2glycerophosphate		0.050000			
Trisaminomethane		0.500000			
EDTA-Na_2	0.0500000	0.003000	0.0186000	0.00430000	
Vitamin B_{12}	0.0000013		0.0000135	0.00000055	0.0000150
Thiamine-HCl	0.0000168	0.000010	0.0000168	0.00010000	0.0000175
Biotin	0.0000024		0.0000240	0.00000050	0.0000250
$NaHCO_3$				0.01200000	
KCl				0.00740000	
Na_3VO_4				0.00000180	
Na_2Moo_4	0.0000390				

and 0.44 g/L respectively); however, the lowest biomasses were from the NIES (group A) medium for strain Finland2 and from the Bold (group A) medium for USA3 strain (0.103 and 0.100 g/L, respectively). The OHM was grouped as AB and COMBO in group B.

Figure 1 shows the superiority of the Mixotroph medium and the dry biomasses of most strains grown in this medium were higher than those grown in the other media. The biomass for Iran4 strain in Mixotroph was 3.4 times more than its biomass in NIES and Bold. The discriminative function analyses (DFA) was used to predict group membership from the set of variables in which biomass, chlorophyll and astaxanthin contents were used as variables. The results showed the separation of the Mixotroph medium from the other media at the top of the graph, indicating their difference and confirmed the previous results and demonstrated the potency of the Mixotroph medium to produce biomass (Figure 2).

The COMBO medium has three ingredients which are absent in the other media: $NaHCO_3$, KCl and Na_3VO_4 (Table 2). The present sodium bicarbonate in COMBO supplied an extra carbon source which probably promoted algal growth. The stimulatory activity of

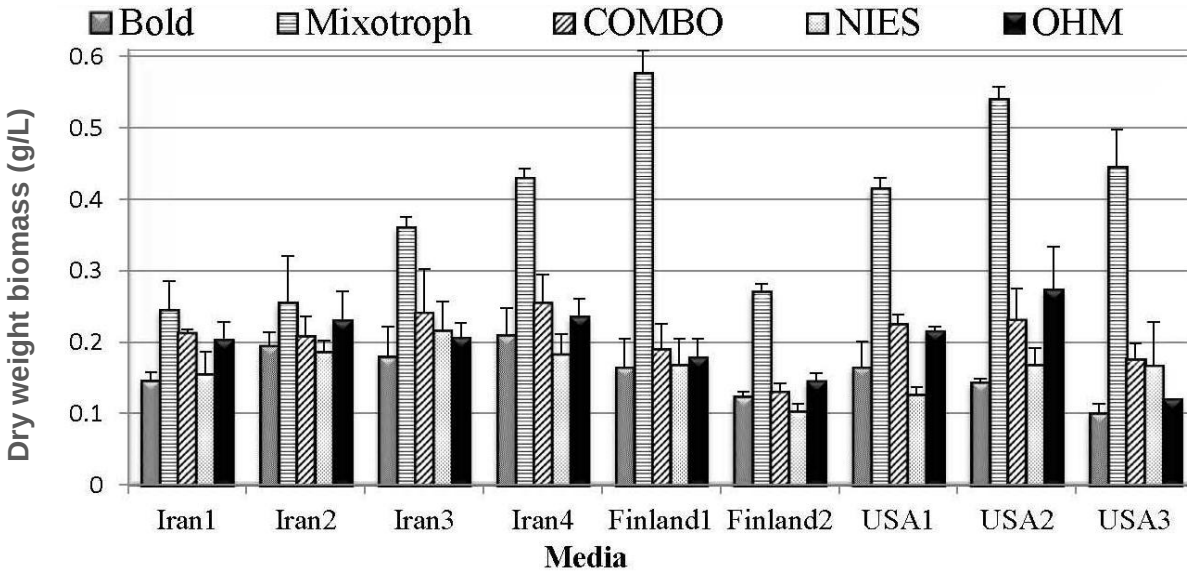

Figure 1. Dry weight biomass production by nine algal strains in five growth media.

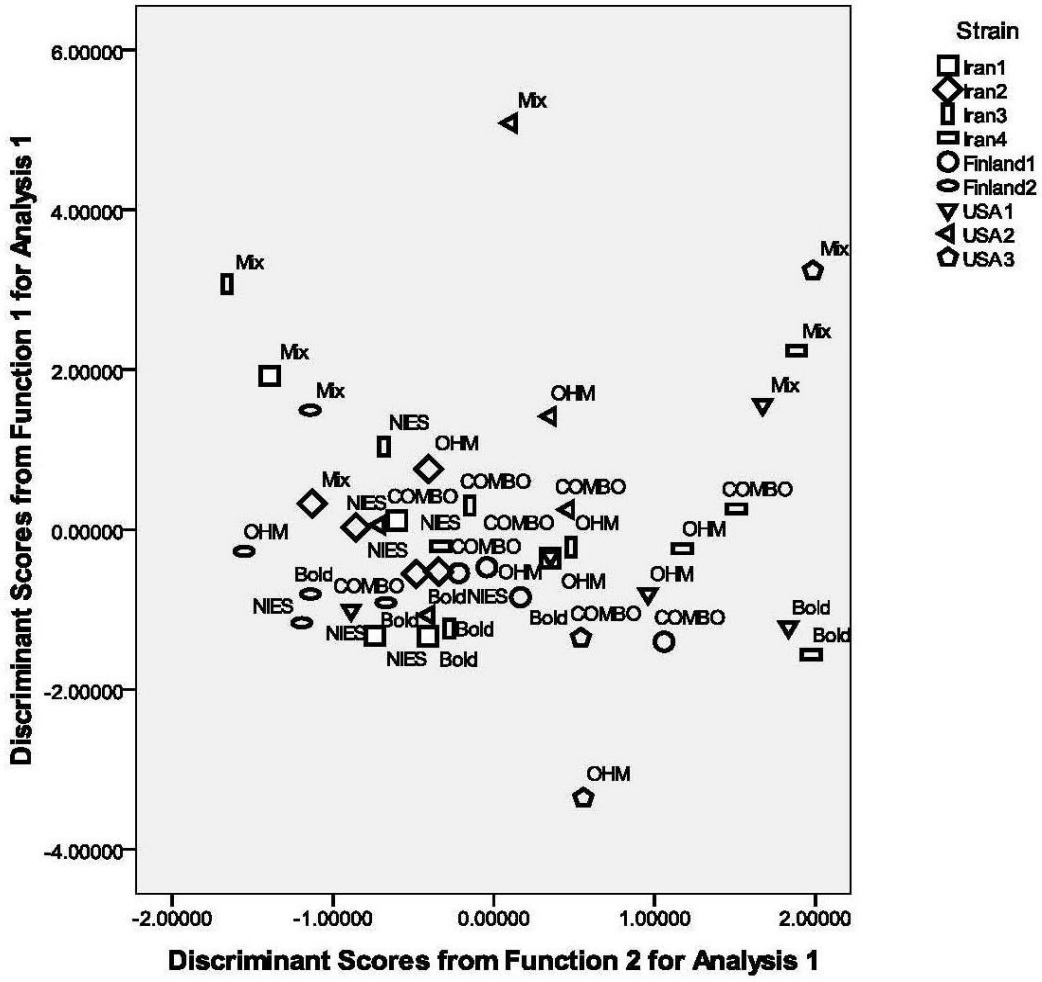

Figure 2. Discriminate score function in five different growth media for nine algal strains.

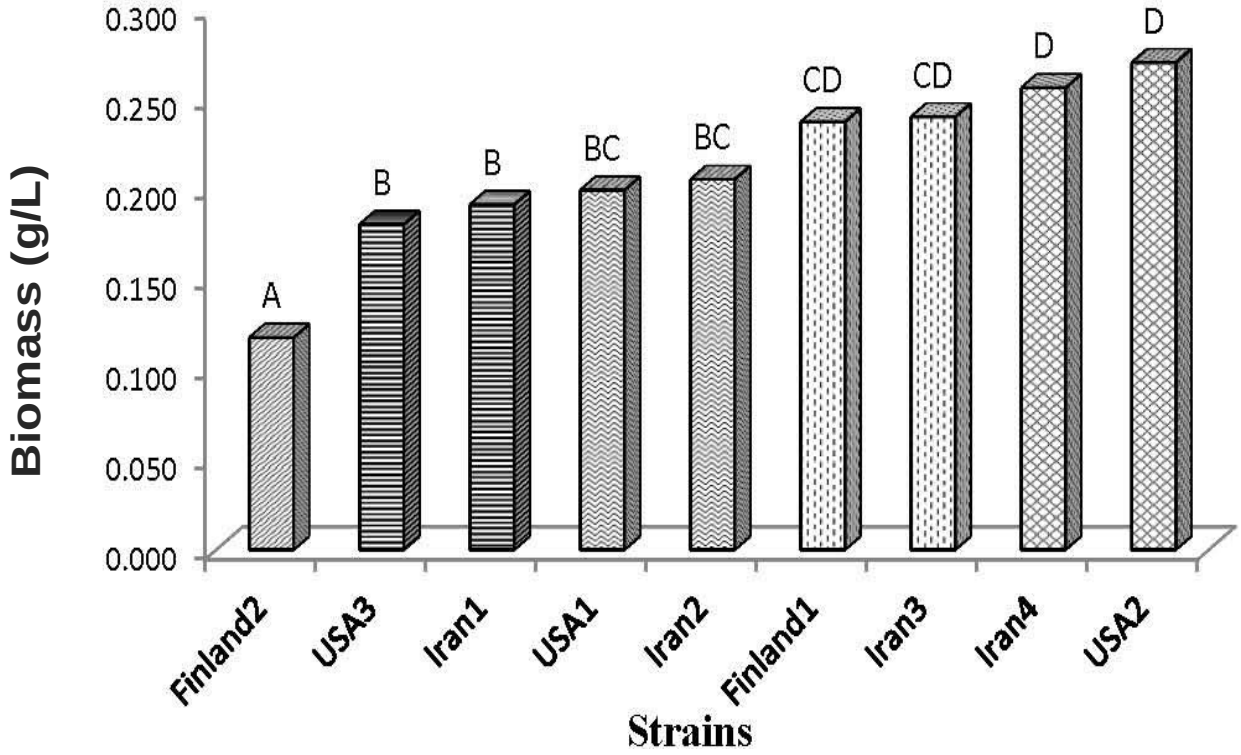

Figure 3. Categorization of strains based on Duncan coefficient.

COMBO on algal growth is the same as that of the OHM medium which is a special medium for *H. pluvialis*.

In terms of the strain investigation, the comparison of mean among strains based on biomass using Duncan multiple test categorized them into four groups in which the Finland2 strain produced minimum biomass in one group (A) and Iran4 and USA2 in separate group (D) with highest biomass production ($p<0.05$) (Figure 3).

The chlorophyll concentration varied among different strains and media. This difference could be seen even in the macroscopic appearances of the cultures; in the case of this study, the Mixotroph medium was pale green, while the other media, COMBO, Bold and OHM had higher chlorophyll concentrations (Table 3). The minimum ratio of chlorophyll to dry weight biomass belonged to the Mixotroph medium, while the maximum ratio belonged to the Bold medium. Growth quantity in terms of chlorophylls a and b and total chlorophyll was highest for Iran4 and USA1. The Pierson correlation coefficient between biomass and chlorophyll was 0.312, and between astaxanthin and chlorophyll was 0.616 ($p<0.01$). It means their correlation were significant; however, astaxanthin and chlorophyll content were more correlated to each other.

The astaxanthin concentrations ranged from 0.01 to 0.104 g/l in which the highest amount was obtained from the OHM and COMBO media (Table 4). According to this table, the COMBO, OHM and Bold were the best media for astaxanthin production. In contrast the NIES and Mixotroph media produced the lowest amount of astaxanthin in comparison with the other media; however, Mixotroph had the highest biomass production. The prominent astaxanthin producer strains were detected to be strains Iran4 and USA3 with 0.104 and 0.083 g/l, respectively. During HPLC experiment, the retention time for standard astaxanthin and unknown astaxanthin was 3.080 and 2.95, respectively and astaxanthin peak was sharp and pure (Figure 4).

Molecular diversity of strains was investigated using ISSR and RAPD methods. The pooled data of ISSR and RAPD molecular marker by UPGMA method for ten strains of *H. pluvialis* is depicted in Figure 5. In this dendrogram, the 0.74 coefficient refer line, separated the strains according to their geographical origin. The reliability of dendrogram and branches was calculated using 1000 replicates in Paup software. The Iran3, Iran4 and Finland1, Finland2 were separated into distinct group with high bootstrap support (100%). As a whole, the bootstrap values are not lower than 87% (USA2, 3), meaning the dendrogram is robust and reliable. The maximum Jacard's similarity index for pooled ISSR and RAPD markers is 0.68 and 0.64 between Finland 1 and Finland2 and between Iran3 and Iran4, respectively. The lowest similarity was 0.10 between Iran2 and USA1. The dendrogram based on pooled data of the ISSR and RAPD molecular marker separated the strains according

Table 3. Chlorophyll concentrations in different algal strains and growth media mg/l

Strain	Chlorophyll	Mean chlorophyll concentration in different growth media (mg/L)				
		Bold	NIES	Mixotroph	COMBO	OHM
Iran1	a	9.412	5.569	2.299	8.518	9.950
	b	3.563	1.955	0.866	2.981	3.512
	a+b	12.975	7.495	3.165	11.500	13.463
Iran2	a	10.346	5.405	4.996	8.755	8.942
	b	3.487	2.215	1.731	3.001	2.615
	a+b	13.834	7.621	6.727	11.756	11.557
Iran3	a	13.078	6.891	2.735	10.829	9.089
	b	3.585	1.998	1.540	3.592	2.713
	a+b	16.63	8.889	4.275	14.422	11.803
Iran4	a	19.754	9.030	14.629	10.769	12.528
	b	8.437	5.632	6.826	3.582	4.577
	a+b	28.191	14.663	21.455	14.351	17.106
Finland1	a	7.324	5.595	L S	11.141	5.680
	b	2.438	2.101	L S	4.040	2.352
	a+b	9.762	7.697	L S	15.181	8.033
Finland2	a	5.564	6.084	5.040	8.854	4.642
	b	1.928	2.027	1.360	2.995	1.576
	a+b	7.493	8.112	6.400	11.849	6.218
USA1	a	16.076	3.418	22.904	11.505	15.972
	b	6.9311	1.494	9.356	4.059	7.007
	a+b	23.008	4.913	32.260	15.565	22.979
USA2	a	7.8645	4.910	9.669	11.128	12.725
	b	2.8928	2.124	3.168	4.131	4.811
	a+b	10.757	7.034	12.837	15.259	17.536
USA3	a	L S	L S	16.802	11.427	8.592
	b	-	-	6.59	3.82	3.01
	a+b	-	-	23.39	15.25	11.60

LS: Losing sample and not detected.

to their geographical status (Figure 5).

DISCUSSION

The chlorophyll concentration in some of the media was low despite high biomass production; it is probably due to the presence of other pigment complementing chlorophyll. The akinet cells with red colour are full of astaxanthin and contain undetectable chlorophyll amounts (Dubinsky and Stambler, 2009). As a recommendation, chlorophyll concentration could be used as a biomass indicator in the vegetative phase of the cell cycle (not in the cyst form), because complementary pigments in the cyst form would replace chlorophyll and the concentration of chlorophyll will reduce as Borowitzka et al. (1991) suggested, high phosphate and low nitrogen sources would cause cells to transform into red palmella; albeit, nitrate was the best nitrogen source. In the addition of acetate to the medium, the pH could not rise rapidly due to CO_2 release. The nitrogen and phosphate contents in the Mixotroph medium were highest in comparison to the other four media and the presence of acetate probably resulted in higher growth in Mixotroph. Although, acetate

Table 4. Astaxanthin production in nine algal strains and different growth media.

Strain	Bold	NIES	Mixotroph	COMBO	OHM	Mean
Iran1	0.051	0.022	0.011	0.028	0.062	0.034
Iran 2	0.025	0.031	0.021	0.044	0.032	0.031
Iran 3	0.042	0.027	0.005	0.035	0.061	0.033
Iran 4	0.083	0.036	0.088	0.104	0.075	0.077
Finland1	0.069	0.058	0.072	0.091	0.063	0.071
Finland2	0.025	0.023	0.016	0.037	0.011	0.021
USA1	0.093	0.041	0.052	0.055	0.056	0.059
USA2	0.044	0.036	0.031	0.055	0.044	0.041
USA3	0.081	0.038	0.082	0.072	0.083	0.071
Mean	0.056	0.034	0.041	0.057	0.053	

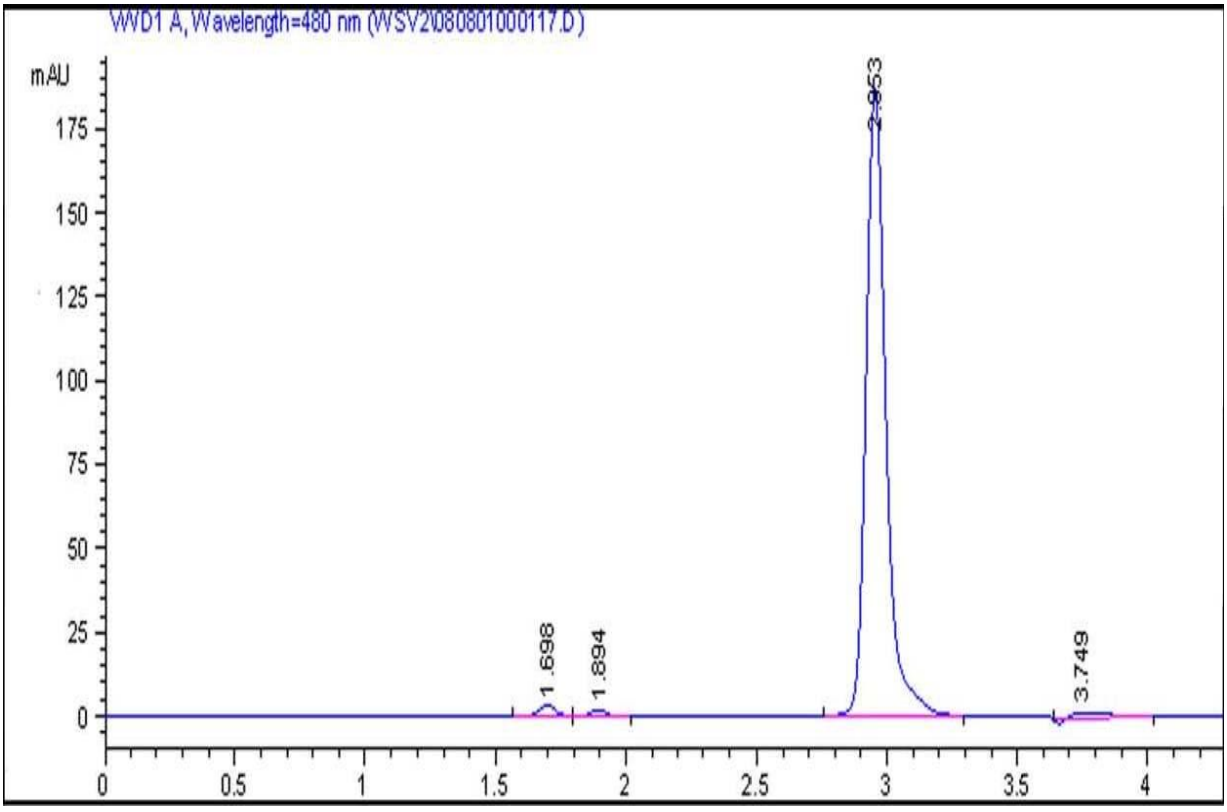

Figure 4. The sharp peak of total astaxanthin in HPLC.

as a carbon source can be used by other organisms such as bacteria and fungi, simultaneously it can also stimulate the growth of *H. pluvialis*. Some researchers claimed that mixtrophic organisms utilize acetate under dark conditions. The light had an inhibitory effect on the acetate uptake in *Chlorella* (Eduard et al., 1993) which meant acetate was utilized only during the dark period.

Hence, probably the mixotrophic biomass production was the sum of the autotrophic and heterotrophic cultures during the light and dark periods of growth (Kobayashi et al., 1993). The results of this study indicated that Mixotroph medium was the best for obtaining a high quantity of biomass. The dry biomass obtained in this study was relatively lower than those obtained elsewhere (Domínguez-Bocanegra et al., 2007; Esperanza et al., 2005; Zhang et al., 1999) since no aeration and additional CO_2 was given and the flasks were only shaken manually twice daily. However, the harvested biomass was higher than the biomass in the study of Cifuentes et al. (2003).

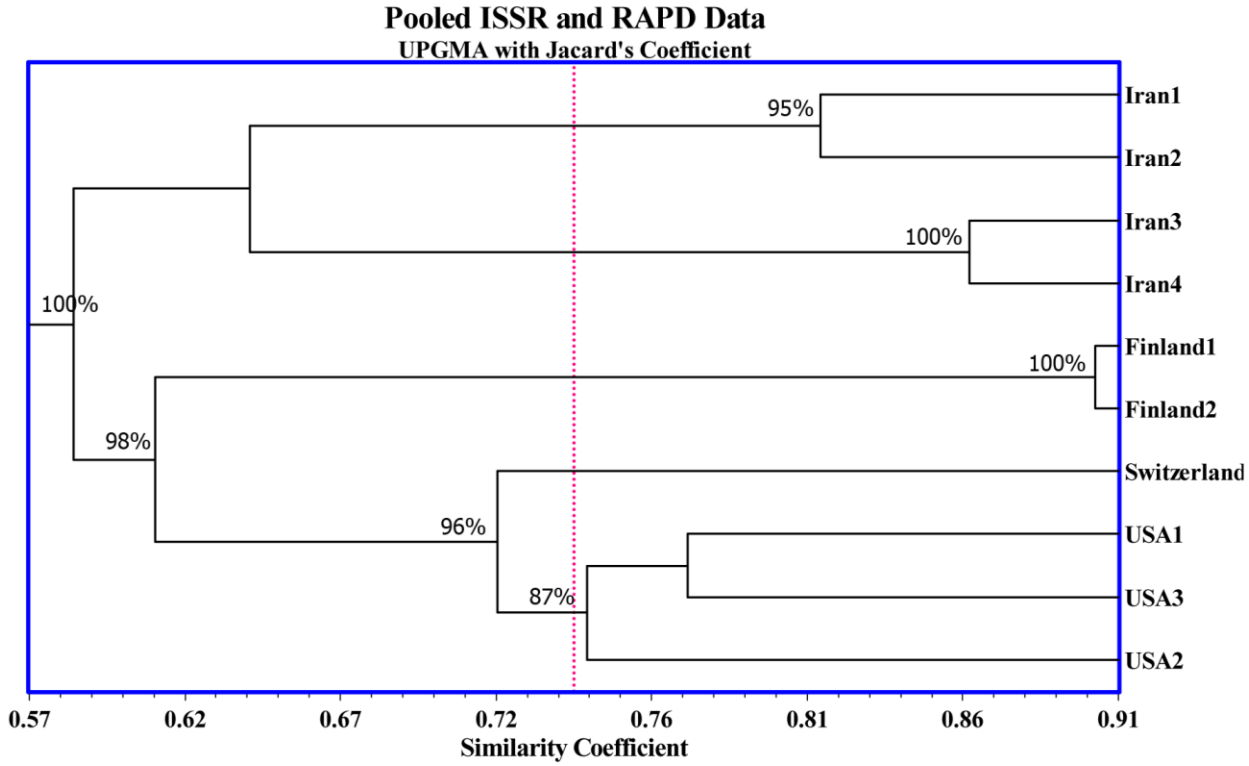

Figure 5. UPGMA dendrogram of 10 strains of *H. pluvialis* and bootstrap value based on pooled data of ISSR and RAPD markers.

This study evaluated the biomass production of *H. pluvialis* strains from different geographic locations which possessed different characteristics and various production abilities. As a part of our inquiry, the variations in the growth performances and the biochemical compositions of the numerous strains of the species were due to biodiversity and this necessitated the screening of new strains whenever they are isolated from the wild. *H. pluvialis* isolation and biodiversity characterization is a promising approach to discover new strains with high growth abilities and astaxanthin.

Astaxanthin production was weakly correlated with biomass of *Haematococcus* with 0.11 Pierson coefficient, and in some other cases, they acted reversely. Iran4 strain had the maximum biomass and the amount of astaxanthin in this strain was the highest in concentration. However, USA3 with a relatively lower biomass produced a large amount of astaxanthin.

The COMBO and OHM medium were intermediate in biomass production but they were prominent in astaxanthin production. As a choice for biomass production, Mixotroph medium could be used initially but the used growth medium at the next step should be changed to OHM to produce astaxanthin. Hence, it is concluded that Mixotroph is suitable for biomass production but it is not recommended to be used in pigment production. For future studies, we suggest other types of double stages cultivation. In the first stage, *H. pluvialis* should be grown in Mixotroph medium to obtain a high biomass but in the second stage, the culture should be transferred to OHM or COMBO media to produce astaxanthin. Furthermore, we recommend the simultaneous culturing of *H. pluvialis* and zooplanktons in COMBO medium to observe the transfer of astaxanthin to protozoa.

The ISSR and RAPD molecular markers were able to distinguish the strains geographically which were reinforced with high value of the bootstrap. Based on Figure 5, the Iran strains made up one clade, Finland one clade and USA strains, another separate clade which were supported by Jacard's similarity index.

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