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Full Length Research Paper

Comparative effects of autotrophic and heterotrophic growth on some vitamins, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, amino acids and protein profile of *Chlorella vulgaris* Beijerinck

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Chemical composition of *Chlorella vulgaris* Beijerinck including content of some vitamins (A, E and C), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, amino acids and protein profile was assessed under autotrophic and heterotrophic growth conditions as an indication of its nutritional value. Vitamin content (A, E and C) of heterotrophic cells increased by about 29, 52 and 20, respectively, as compared to its value for autotrophic cells. The free radical scavenging activity (RSA) was significantly higher for autotrophic cells than heterotrophic only at low concentrations (25 and 50 µl) of algal extract, but no significant difference was recorded at high concentration (100 µl). This result indicates non parallel relationship between the tested vitamins and RSA which suggest that other non-enzymatic antioxidants and/or antioxidant enzymes are involved. Concentration of essential and non essential amino acids in heterotrophic cells was double and 1.5 times, respectively its concentration in autotrophic cells. Histidine, lysine and phenylalanine constituted 77 and 44% of the total content of essential amino acids in heterotrophic and autotrophic cells, respectively. Methionine concentration was low in both types of cells. Proline content and non essential amino acid in heterotrophic cells was about 2.5 times its corresponding value in autotrophic cells. SDS-PAGE of protein extracts of autotrophic and heterotrophic *C. vulgaris* revealed two protein bands of molecular weight of ~75 and ~39 KDa. Higher intensity of the two bands was observed for autotrophic cells which may be associated with the effect of growth condition on the expression of regulatory genes. For valuable production of natural food supplement and/or natural pharmaceutical products, using heterotrophic cells rather than autotrophic cells for its richness in vitamins and essential amino acids is recommended.

Key words: Autotrophic, heterotrophic, *Chlorella vulgaris*, vitamins, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, amino acid, protein profile.

INTRODUCTION

Algae can be a very good natural source of new compounds with biological activity that could be used as functional ingredients. In this way, algae can be considered as genuine natural reactors (Plaza et al., 2008). In addition, the biochemical composition of algae (amino acids, protein and other pharmaceutical products) (Cardozo et al., 2007) appears to be the primary determinant in establishing food quality transferred through the food web, as algae are at the bottom of the aquatic food chain.

Many members of *Chlorella* are highly adaptable to life under marginal conditions. One of the most striking

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features of *Chlorella vulgaris* is its high biomass production; its doubling time has been reported to be less than 20 h (Griffiths and Harrison, 2008). *C. vulgaris* grown heterotrophically has been shown to have higher productivity than photo-autotrophic cells (Hosotani and Kitaoka, 1984). The cultivation conditions not only affect the cell productivity, but also affect cell composition and yield of specific products (Imamoglu et al., 2007). Successive studies revealed that *Chlorella regularis* S-50 produced many intracellular phytochemicals (carotenoids, chlorophyll, tocopherols, protein and others typical of green plants) under dark heterotrophic conditions (Endo et al., 1974; Fukuchi and Endo, 1975). Shi et al. (2002) reported that *C. protothecoides* is a good producer of luetin. *Chlorella zofingiensis* was reported to be a promising producer of the high-value carotenoids (Ip and Chen, 2005). *C. vulgaris* has been shown to produce many intracellular phytochemical (carotenoids, chlorophyll, fatty acids and protein) under autotrophic and heterotrophic conditions (El-Sheekh and Fathy, 2009).

Algae are exposed to production of reactive oxygen species (ROS) as a result of physiological processes (photosynthesis and respiration) as well as different environmental stresses. Under non-stressed conditions, production and scavenging of ROS in algae is in equilibrium (Apel and Hirt, 2004). However, different stress factors like pollution, drought, temperature, excessive light intensities and nutritional limitation are able to increase the production of ROS (Arorea et al., 2002; Rijstenbil, 2002) which may cause cellular and subcellular damage by reacting with macromolecules (Malanga et al., 1997). Oxidative stress is closely associated with these unstable but very reactive radicals (Fang et al., 2002). Algae have developed defiance system against photo oxidative damage by anti oxidative mechanisms to detoxify and eliminate these reactive oxygen species. This antioxidant defiance system includes hydrophobic substances (carotenoids and α-tocopherol) and antioxidant enzymes like superoxide dismutase, catalase, glutathione reductase, ascorbic peroxidase and peroxidase (Rao et al., 1996; Malanga et al., 1997; Rijstenbil, 2002). The DPPH method is described as a simple, rapid and convenient method for radical scavenging activity (Koleva et al., 2001). These advantages make the DPPH method interesting for testing microalgae as a natural source to scavenge radicals and to find out promising candidates for commercial sense.

Chlorella sp. can be applied in food industries because of its high nutritional value, natural pigment and anti-oxidant activity. Using the natural ingredients, exhibiting functional properties and providing specific health benefits beyond traditional nutrients, is a very attractive way to design the new food products. Therefore, the objective of this research was to assess the effect of growth conditions (autotrophic and heterotrophic) of *C. vulgaris*, in modified basal medium on the content of some vitamins (A, E and C), DPPH free radical scavenging activity, amino acids and protein profile.

MATERIALS AND METHODS

The stock culture of *C. vulgaris* Beijerinck was gotten from the algal culture collection of the Phycology Laboratory, Botany Department, Faculty of Science, Tanta University.

Cultural conditions

The modified basal medium (Chen et al., 1996; Wu and Shi, 2007) containing 1250 mg l^{-1} KH_2PO_4, 1000 mg l^{-1} Mg SO_4, 500 mg l^{-1} EDTA, 114.2 mg l^{-1} H_3BO_4, 111 mg l^{-1} $CaCl_2$, 49.8 mg l^{-1} $FeSO_4$, 88.2 mg l^{-1} $ZnSO_4$, 14.2 mg l^{-1} $MnCl_2$, 7.1 mg l^{-1} MoO_3, 15.7 mg l^{-1} $CuSO_4$ and 4.9 mg l^{-1} Co $(NO_3)_2$ and supplemented with 10 g L^{-1} glucose (as the carbon source) was used for heterotrophic cultivation of *C. vulgaris*. Cultivation of axenic *C. vulgaris* was carried out in 250 ml flasks (each containing 100 ml of medium). All media in the flasks were sterilized and autoclave at 121°C for 20 min, the cultures were incubated at 30°C with orbital shaking at 130 rpm under darkness. Autotrophic cultures were continuously illuminated with 40 W fluorescent tubes (70 µmol / m^2 / s). Each experiment was carried out in triplicates. Each culture was tested occasionally for contamination by bacteria or fungi by incubating an aliquot with peptonic agar at 30°C for at least 2 days in the dark. Cultures used for chemical analysis were harvested after 9 days during the exponential phase.

Effect of growth conditions on the production of vitamins and DPPH free radical scavenging activity

The intracellular concentrations of the vitamins and DPPH free radical scavenging activity were determined spectrophotometrically by using UV/Visible, Thermo Scientific "Heλiosσ" spectro-photometer.

Estimation of vitamin A

Vitamin A was extracted by the method of Beadle and Zscheile (1942), and determined in diethyl ether at 440 nm as mentioned in the method of Neeld and Pearson (1963), and expressed as IU vitamin A (the biological equivalent of 0.3 µg retinol or of 0.6 µg β-carotene or 1.2 µg of other provitamin-A carotenoids).

Estimation of vitamin E

Vitamin E was estimated by the method of Quaife and Harris (1948), and expressed as IU vitamin E (the biological equivalent of 2/3 mg d-α-tocopherol or 1 mg of dl-α-tocopherol acetate).

Estimation of vitamin C

Ascorbic acid (vitamin C) was extracted from the cells with 2% metaphosphoric acid, and determined spectrophotometrically using 2,6-di-chlorophenol indophenol dye (Augustin et al., 1985).

DPPH free radical scavenging activity (decolorization assay)

Free radical scavenging activity of algal extract (25, 50 and 100 µl)

was evaluated spectrophotometrically at 517 nm against the absorbance of the indicator, 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (20 mg L^{-1}). All reactions were carried out in triplicates and the degree of decolorization indicated the free radical scavenging activities of the algal extracts (Viturro et al., 1999). Silymarin was used as reference free radical scavenger and percentage of DPPH–decolorization was calculated using the following equation:

Free radical scavenging percentage = 1 - (Ac – As)/Ac × 100

Where Ac = Absorbance of control and As = absorbance of algal sample.

Estimation of amino acids

Algal sample of 3 g was prepared for hydrolysis according to Blackburn (1978) and Walker (1996) before determination of amino acids. Amino acid analyses were carried out using amino acid analyzer LC 3000 eppendorf / Biotronik using column type H 125 x.

The amino acid analysis was carried out in the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Protein profiles

Protein profile in algal sample were qualitatively analyzed by SDS-PAGE according to Laemmli (1970).

Statistical analysis

All data are expressed as means of triplicate experiments ± standard deviation, and the data were subjected to standard one-way analysis of variance (ANOVA) using SPSS Inc. software program version 15 (2006) at $p \leq 0.05$.

RESULTS AND DISCUSSION

Vitamins content (A as β-carotene, E and C) of heterotrophic cells of *C. vulgaris* was significantly increased by 29, 52 and 20%, respectively ($p \leq 0.05$) when compared with its corresponding value for autotrophic cells (Table 1). *Chlorella* was suggested to be a source of potential commercial application (example food production) (Anderson, 2005). The chemical composition of *Chlorella* was found to vary greatly as a result of variations in growth conditions (Spoehr and Milner, 1949; El-Sheekh and Fathy, 2009). Our results are in accordance with Potter (1997); who showed that *C. regularis* can produce carotenoids, under dark heterotrophic conditions. In the present study, the high production of intracellular vitamins (A, E and C) in heterotrophic cells may be related to the presence of glucose in the culture medium providing carbon skeleton for organic compounds. In this respect, Lee (2001) showed that some algal species can utilize organic carbon source such as glucose to produce organic molecules. Another possible explanation for the high content of vitamins in *C. vulgaris* may be the alterations in the ultrastructure of the photosynthetic apparatus reported for heterotrophically grown cells such as *Chlorella* sp. (Vladimirova, 1976) and *Chlamydomonas reinhardtii* (Dowidar, 1990) which were found to be associated with changes in cellular components (Ochiai and Hase, 1970; Dowidar, 1990).

The percentage of radical scavenging activity (RSA%) of *C. vulgaris* grown autotrophically was significantly higher than its corresponding value for heterotrophically grown cells only at low concentrations of algal extract (25 and 50 µl) but no significant difference was recorded at high concentration (100 µl) at $p \leq 0.05$ (Table 2). The results also show that there was no parallel relationship between the content of vitamins and the free radical scavenging activity. This result suggests that other non-enzymatic substances such as carotenoids and fatty acids or/and some enzymes like superoxidase dismutase, peroxidase and catalase are involved in scavenging ROS (Blokhina et al., 2003). El-Sheekh and Fathy (2009) reported that fatty acid content of heterotrophically grown *C. vulgaris* was 30% higher than that in autotrophically grown cells. Carotenoids and fatty acids are two examples for non-enzymatic classes of antioxidants which are able to protect the organism from oxidative damage (Sies and Stahl, 1995). The reactive oxygen species (ROS) example, superoxidase anion (O_2), hydroxyl radicals (OH^+), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) are formed as a result of normal metabolic activity and exogenous sources such as glucose used for growing algae heterotrophically (Halliwell and Gutteridge, 1986).

Results in Table 3 showed that there were 9 essential and 7 non essential amino acids forming the protein of *C vulgaris.* The concentration of the essential amino acids in heterotrophic cells was twice that in the autotrophic cells. Histidine, lysine and phenylalanine constituted about 77% of the total content of essential amino acids in heterotrophic cells, whereas their corresponding values formed 44% of the total content in autotrophic cells. On the other hand, isolucine, thereonine and valine (essential amino acids) had higher concentrations in autotrophic cells as compared to those in heterotrophic cells. Both types of cells had low content of methionine. This result is in agreement with Scheiler et al. (1953) for *Chlorella pyrenoidosa* and *C. vulgaris.* Total content of. non essential amino acids in heterotrophic cells was 1.5 times and proline was four times their corresponding values in autotrophic cells (Table 3). This result was in accordance with El-Sheekh and Fathy (2009) who found a pronounced increase in total free amino acids and proline in *C. vulgaris* grown heterotrophically. A possible explanation for the increase in essential and non essential amino acids under heterotrophic growth may include (i) stimulated synthesis and/or inhibited degradation of amino acids; (ii) the impaired protein synthesis and/or enhanced protein degradation (Rabe, 1990). In addition, accumulation of amino acids including proline is an expected behavior in algae subjected to

Table 1. Vitamins content of *Chlorella vulgaris* grown under autotrophic and heterotrophic conditions for 9 days.

Vitamin content	Autotrophic	Heterotrophic	F	P
Vitamin A (IU)	5422.58±6.2**	6995.20±11.5**	43290.5	0.000
Vitamin E (IU)	1250.41±7.2**	1896.43±4.7**	16879.2	0.000
Vitamin C (mg/100g)	514.92±5.2**	616.74±6.5**	452.5	0.000

** Results highly significant at $p \leq 0.05$.

Table 2. DPPH free radical scavenging activity (%) of *Chlorella vulgaris* grown under autotrophic and heterotrophic conditions for 9 days.

DPPH Decolouration	Standard	Autotrophic	Heterotrophic	F	P
At 25 µl	89.7±3.2	17.67±1.4*	13.37±1.8*	56.74	0.002
At 50 µl	90.8±4.1	19.36±2.3*	17.71±2.1*	21.32	0.010
At 100 µl	92.17±4.7	21.07±3.2	20.64±3.1	0.53	0.508

*Results significant at $p \leq 0.05$.

Table 3. Amino acids concentrations (µg/g) of *Chlorella vulgaris* grown under autotrophic and heterotrophic conditions for 9 days.

	Autotrophic		Heterotrophic	
Amino acid	**Rt**	**Concentration (µg/g)**	**Rt**	**Concentration (µg/g)**
Essential				
Arginine	59.46	191.72	58.82	146.82
Histidine	46.65	419.2	46.62	1605.12
Isoleucine	35.26	90.15	35.24	59
Leucine	36.73	174.95	36.94	131.85
Lysine	51.76	182.13	51.57	368.08
Methionine	32.73	33.82	32.69	26.68
Phenylalanine	42.53	17.94	42.55	84.74
Thereonine	16.9	151.14	16.72	122.28
Valine	29.69	135.37	29.81	117.4
Total		**1396.42**		**2661.97**
Non essential				
Alanine	26.6	175.17	26.22	190.15
Aspartic acid	13.29	193.48	13.02	165.82
Glutamic acid	19.33	156.71	19.2	185.71
Glycine	24.47	184.28	42.18	193.46
Proline	21.21	232.12	20.99	900.31
Serine	17.99	135.32	17.8	130.04
Tyrosine	31.01	102.74	41	40.69
Total		**1179.82**		**1806.18**

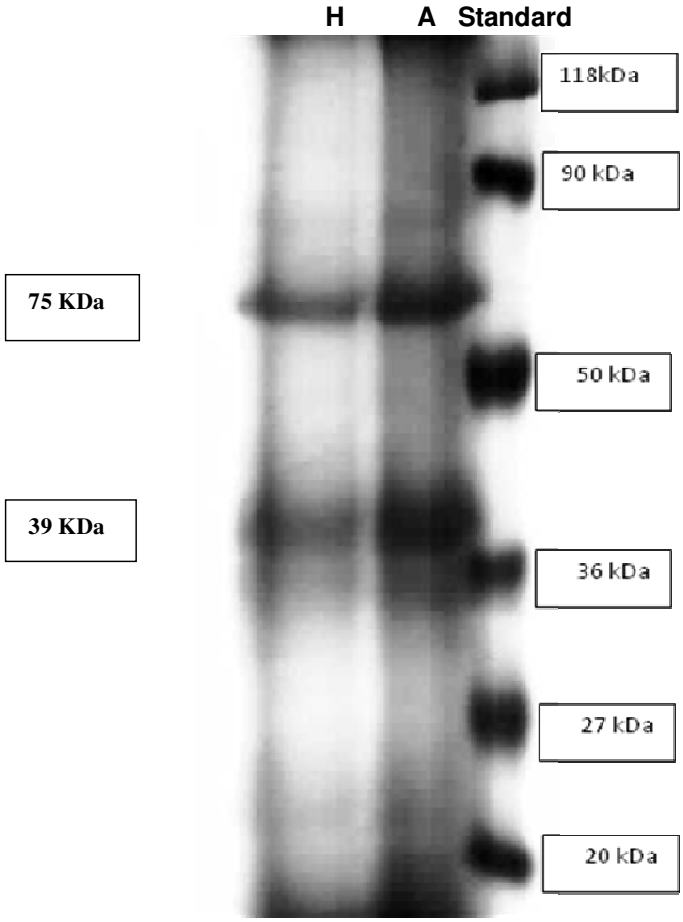

Figure 1. The SDS-PAGE profile of total protein of *Chlorella vulgaris* grown autotrophically (A) and heterotrophically (H).

different environmental stresses (Borowitzka, 1988; Sansawa and Endo, 2004). In response to environmental stresses, proline contributes to stabilization of sub-cellular structures, scavenging ROS and buffering cellular redox potential beside its action as a compatible solute (Ashraf and Foolad, 2007; Wahid and Close, 2007).

SDS-PAGE of protein extracts of *C. vulgaris* grown autotrophically and heterotrophically revealed two protein bands of molecular weights of ~75 and ~39 KDa in both types of cells (Figure 1). However, higher intensity of the two bands was observed for autotrophic cells. The variability in band intensity may be associated with an effect of the applied treatment on the expression of regulatory genes (El-Shazly, 1998; Badr et al., 1996). This result indicated that heterotrophic cells had lower production of protein and it is in accordance with results of El-Sheekh and Fathy (2009) who reported that heterotrophic growth decreased total protein content. Moreover, results of SDS-PAGE in the present study, correlated well with accumulation of total essential and non essential amino acids in this type of cells. Proteins are the primary effectors molecules that can be affected by environmental stresses such as herbicides (Dowidar et al., 2010), salinity (Apte and Bahgwat, 1989) and also physiological conditions (Sinhaand Häder, 1996; Sinha et al., 1996).

Conclusion

This work shows the differential ability of *C. vulgaris* to respond to different culture conditions. Heterotrophic growth induced higher production of some vitamins (A, E and C) and led to accumulation of essential amino acids when compared with autotrophic one. Moreover, the DPPH free radical scavenging activity of both types of cells was comparable at high concentration of algal extract.

Therefore, we recommend using heterotrophic growth, which is a cheap way, rather than autotrophic growth, for production of natural food supplements or/and natural pharmaceutical products.

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