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Variation of Some Nutritional Constituents and Fatty Acid Profiles of *Chlorella vulgaris* Beijerinck Grown under Auto and Heterotrophic Conditions

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Abstract: This study is an attempt to evaluate the nutritional value of *Chlorella vulgaris* Beijerinck grown under autotrophic and heterotrophic conditions concerning their content of carotenoids, protein, proline, total free amino acids and fatty acids. Chlorophyll a (Chl.a) content of autotrophic cells of *C. vulgaris* was double that estimated in heterotrophic cells, while chlorophyll b (Chl.b) content of autotrophic cells was nearly half the value recorded for heterotrophic cells. Carotenoids (Car.) content of heterotrophic cultures decreased by 30.82% compared to that value of autotrophic cells. There was a slight decrease in the protein content of *C. vulgaris* under heterotrophic conditions. When the composition of total free amino acids and proline of *C. vulgaris* grown under autotrophic conditions is compared to that grown heterotrophically, it was observed that a significant increase in total free amino acids and proline in heterotrophic cultures. The percentage of most fatty acids of heterotrophic cells was relatively higher than autotrophic ones. There was no qualitative difference between autotrophic and heterotrophic cultures, except for the fatty acid 16:02 which was absent under autotrophic conditions. Present results showed that *C. vulgaris* has quite a simple qualitative fatty acids composition compared to other chlorophyceae species, considering production of natural food supplements and/or natural pharmaceutical products, it is strongly recommended using autotrophic cells of *Chlorella* rather than using those of heterotrophic cells for such purpose.

Key words: *Chlorella vulgaris*, nutritional value of algae, green algae, light

INTRODUCTION

Natural products play an important role in the drug production (Cragg *et al.*, 1997). Thus, the investigation of new algal chemical compounds, as natural source of natural products, has proved to be a promising area of pharmaceutical study (Blunt *et al.*, 2005; Singh *et al.*, 2005; Cardozo *et al.*, 2007).

Carotenoids are natural pigments exercising important biological functions in algae, plants and animals (Polivka and Sundström, 2004; Cardozo *et al.*, 2007). For human nutritional purposes, some carotenoids offer provitamin A activity (Mayne, 1996). They are directly providing photoprotection against UV light photooxidation in the skin beside their key role factor in reducing the incidence of many diseases (Cantrell *et al.*, 2003; Astley *et al.*, 2004; Sies and Stahl, 2004; Aust *et al.*, 2005).

Polyunsaturated fatty acids (PUFAs), especially the essential omega-6 and omega-3 fatty acids, play key roles in nearly all cellular metabolic process in human body because there is no synthetic mechanism for their production inside the body (Funk, 2001; Sayanova

and Napier, 2004). Thus an external source is needed to provide the human body with its need of such essential fatty acids. Most of fatty acids production processes investigated to date have been based on photoautotrophic growth (Sánchez *et al.*, 2002; Molina *et al.*, 2003).

It is well known that algae are at the bottom of the aquatic food chain. The primary determinant in establishing the food quality transferred through successive levels of the food web appears to be the biochemical composition of the algae (fatty acids, amino acids, protein and other pharmaceutical products) (Droop, 1974; Brown and Miller, 1992; Morimoto *et al.*, 1995; Shibata *et al.*, 2003; Cardozo *et al.*, 2007).

Algae are considered as the main natural source for both carotenoids and essential fatty acids beside other valuable nutritional value. The green alga *Chlorella* has attracted considerable interest for commercial production of functional food such as polyunsaturated fatty acids by *C. sorokiniana* (Chen and John, 1991) and lutein by *C. protothecoides* (Shi *et al.*, 2002). *Chlorella zofingiensis* has been proposed as a promising producer

of the high-value carotenoids (Orosa *et al.*, 2000; Ip and Chen, 2005). The correlations between cultivation conditions and percentage of carotenoids, protein and fatty acids contents were subjects of many studies (Piorreck *et al.*, 1984; Vladimirova *et al.*, 2000; Petkov and Garcia, 2007).

Fatty acids yield and productivity in photoautotrophic systems are low, because the insufficiency of light caused by mutual shading of cells (Barclay *et al.*, 1994; Chen *et al.*, 1996). For enhancement of fatty acid production by microalgae, the development of a heterotrophic growth is desirable (Wen, 2001). The concept that autotrophic microorganisms can utilize only inorganic carbon dioxide as a carbon source has been modified over years (Shugarman and Appleman, 1966; Bennett and Hobbie, 1972; Cho *et al.*, 1981; Feng *et al.*, 2005).

In this study, an attempt was made to evaluate the nutritional value of *Chlorella vulgaris* grown under autotrophic and heterotrophic conditions concerning their content of carotenoids, protein, proline, total free amino acids and fatty acids. It was an attempt too, to answer the question: Does heterotrophy enhance fatty acids production? Also, validity of fatty acids composition as stable taxonomic feature for the genus *Chlorella* was briefly discussed as aside issue.

MATERIALS AND METHODS

Culture conditions: The modified basal medium (Chen *et al.*, 1996; Wu and Shi, 2007) containing 1250 mg L⁻¹ KH₂PO₄, 1000 mg L⁻¹ MgSO₄, 500 mg L⁻¹ EDTA, 114.2 mg L⁻¹ H₃BO₃, 111 mg L⁻¹ CaCl₂, 49.8 mg L⁻¹ FeSO₄, 88.2 mg L⁻¹ ZnSO₄, 14.2 mg L⁻¹ MnCl₂, 7.1 mg L⁻¹ MoO₃, 15.7 mg L⁻¹ CuSO₄ and 4.9 mg L⁻¹ Co(NO₃)₂ and supplemented with 10 g L⁻¹ glucose (as the carbon source) was used for heterotrophic cultivations of *Chlorella vulgaris*. Cultivation of axenic *C. vulgaris* was carried out in 250 mL flasks (each containing 100 mL of medium) (all experiments conducted from 2006 to 2008). All media in the flasks were sterilized in 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 lamps (70 µmol/m²/sec). Experiment for each trial 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.

Estimation of pigments: Algal cells were homogenized at 1,000 rpm for 1 min, using 100% acetone (50 mL for each g sample). The homogenate was filtered through two layer cheese cloths and was centrifuged at 2,500 rpm for 10 min. The supernatant was separated and the absorbance was read at 662, 645 and 470 nm for Chl.a, Chl.b and Carotenoids (Car.), according to the method described by Dere *et al.* (1998). The amount of these pigments was calculated according to the formulas of Lichtenthaler and Wellburn (1985):

$$\text{Chl.a} = 11.75 A_{662} - 2.350 A_{645}$$

$$\text{Chl.b} = 18.61 A_{645} - 3.960 A_{662}$$

$$\text{Car.} = [1000 A_{470} - 2.270 \text{ Chl.a} - 81.4 \text{ Chl.b}] / 227$$

Estimation of total free amino acids: Total free amino acids were estimated according to the method of Yemm and Cocking (1955). One milliliter aliquot of the acidified extract was mixed with 2 mL sodium acetate buffer (pH 6.5) and 1 mL freshly prepared ninhydrin reagent. The resulting color was then read at 570 nm with Perkin Elmer spectrophotometer. Leucine was used as a standard.

Estimation of free proline: Free proline estimation was carried out using the acid ninhydrin method of Bates *et al.* (1973). The absorbance was read at 520 nm using Perkin Elmer spectrophotometer and the proline concentration was calculated from a proline standard curve.

Estimation of protein: The protein was estimated according to Folin-Ciocalteu method described by Hartree (1972) and the resulting color was measured at 650 nm using Perkin Elmer spectrophotometer, related to a standard curve of bovine serum albumin.

Determinations of lipids and fatty acid composition: Fatty acids were analyzed by Gas Liquid Chromatography (GLC) (Schimadzu GC4-CM). The relative peak areas on the chromatogram were estimated after tracing them on sectional paper and thus the content (%) of different fatty acids was estimated. The conditions for GLC analysis were: column size: 3×3 mm id; carrier gas: nitrogen; fuel: hydrogen-air mixture; hydrogen flow rate: 1 mL min⁻¹; air flow rate: 0.5 mL min⁻¹; detector: FID; detector temperature: 270°C; column temperature: 180°C. Extraction and methanolysis were performed according to the method described by Chalvardjian (1964) and Moneam and Ghoneim (1986).

Experimental design and statistical analysis: All experiments were prepared in triplicate and each experiment was repeated 2 times. Pigment, protein, total

free amino acids, proline and fatty acid contents were analyzed in triplicate. The mean of all repetitions was calculated and analyzed by one-way analysis of variance (ANOVA) and correlation coefficients analysis, using the COSTAT 2.00 statistical analysis software manufactured by Cohort Software Company.

RESULTS AND DISCUSSION

Chlorophyll a (Chl.a) content of autotrophic cells was double that estimated in heterotrophic cells (4.481 and 2.672 mg g⁻¹ DW, respectively). On contrary, chlorophyll b (Chl.b) content of autotrophic grown cells was nearly half the value recorded for heterotrophic cells (0.563 and 1.071 mg g⁻¹ DW, respectively). The heterotrophic ratio of Chl.a to Chl.b lost about 68.65% of its value in autotrophic grown cells. Carotenoids (Car.) content of heterotrophic cultures decreased by 30.82% compared to its value for autotrophic cultures. The results in Table 1 showed that the significantly enhancement effect of light on Chl.a and Car., biosynthesis seemed remarkably under photoautotrophic conditions. However, under heterotrophic conditions, Chl.b content was significantly increased on the expense of the production of other pigments. These results suggest that there is an organic carbon source like glucose in the culture medium and in absence of light, *C. vulgaris* favor the formation of Chl.b. This agree with the study of Misonou and Pachlavuni (1986), who suggested that under heterotrophic conditions the synthesis pathways of Chl.a and carotenoids are blocked or at least slow down, while the synthesis pathway of Chl.b was greatly promoted. Another possible explanation was driven by Vladimirova (1976), who found ultra structural alterations in the photosynthetic apparatus of *Chlorella* sp. grown heterotrophically. It is possible that present result of the variation in pigment contents under different culture conditions, in this study, is related to such suggestion.

On comparing protein content of *C. vulgaris* cells grown under autotrophic and heterotrophic conditions, there was a slight decrease (6.05%) in the protein content (Table 2). When the composition of total free amino acids and proline of *Chlorella* grown autotrophically is compared to that grown heterotrophically, it was found that a significant increase in total free amino acids and proline (65.86 and 42.86%, respectively) (Table 2). It seemed that heterotrophy negatively affect the protein synthesis, while positively affect and promoted synthesis of total free amino acids and proline. That is may be due to the heterotrophic cells did not need to use the available amino acids to build up protein molecules or due to degradation of protein molecules. At the same time, the

Table 1: Contents (mg g⁻¹ DW) and correlation coefficients of chlorophyll a, chlorophyll b and carotenoids of *Chlorella vulgaris* grown under autotrophic and heterotrophic conditions for 9 days

Pigment	Autotrophic	Heterotrophic	Degree of significance
Chl.a	4.481±0.7	2.672±0.3	-
Chl.b	0.563±0.2	1.071±0.2	-
Chl.a/chl.b ratio	7.959±0.7	2.495±0.5	-
Carotenoids	6.064±0.8	4.195±0.4	-
Variables			
Chl.a			
Chl.b			**
Chl.a /chl.b			***
Carto.			***
Chl.b			
Chl.a/chl.b			**
Carto.			*
Chl.a/chl.b			
Carto.			***

Data expressed as Mean±SD (n = 3). *Mean values significant at p≤0.05; **Mean values significant at p≤0.01, ***Mean values highly significant at p≤0.001

Table 2: Contents and correlation coefficients of protein, proline and Total Free Amino Acids (TFAA) of *Chlorella vulgaris* grown under autotrophic and heterotrophic conditions for 9 days

Growth condition	Protein (mg g ⁻¹ DW)	Proline (mg g ⁻¹ DW)	Total free amino acids (mg g ⁻¹ DW)	Degree of significance
Autotrophic	388.95±21.3	301.97±16.1	6.438±0.9	
Heterotrophic	365.40±11.5	7040.59±23.4	9.776±2.2	
Variables				
Protein				
Proline				***
TFAA				***
Proline				
TFAA				***

Data expressed as Mean±SD (n = 3). ***Mean values highly significant at p≤0.01

increase of amino acids and proline was expected, due its known accumulation behavior in plants subjected to different environmental stresses as light availability (Hashimoto *et al.*, 1982; Borowitzka, 1988; Sansawa and Endo, 2004).

The composition of fatty acids of *C. vulgaris* is shown in Fig. 1 and 2. The percentage of most fatty acids of heterotrophy cells was relatively higher than autotrophic cells (Fig. 2). Only, three fatty acids (12:0, 14:0 and 20:0) showed different trend, where they increased under autotrophic conditions rather than heterotrophic conditions. However, except for the fatty acid 16:02 which was absent under autotrophic conditions, there was no qualitative difference between autotrophic and heterotrophic cultures, (Fig. 1, 2). Such quantitative differences could be accepted as temporary physiological response to variation in culture conditions (Shinichi *et al.*, 1983; Zhukova and Aizdaicher, 1995; Rosa *et al.*, 2005). The biological importance of the utilization mechanism of glucose by *Chlorella* is not completely understood, but whatever, the natural circumstances which make autotroph grown *Chlorella* switch to heterotrophy, a

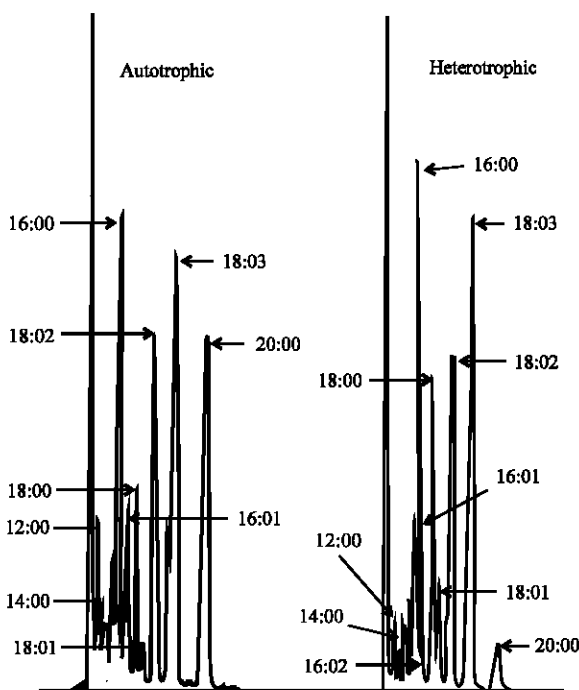


Fig. 1: GLC chromatogram of fatty acids extracted from *Chlorella vulgaris* grown under autotrophic and heterotrophic conditions for 9 days

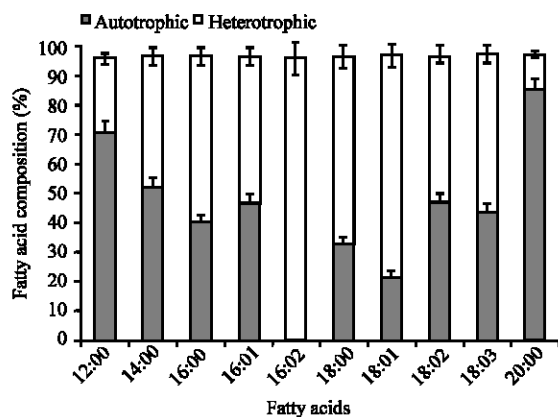


Fig. 2: Difference in percentage composition of individual fatty acids extracted from *Chlorella vulgaris* grown under autotrophic and heterotrophic conditions for 9 days

supply of exogenous carbon source might be provided (White, 1974; Cho *et al.*, 1981; Hashimoto *et al.*, 1982; Ip and Chen, 2005). In the presence of exogenous carbon like glucose, the growth and fatty acids production were enhanced (Cho *et al.*, 1981; Feng *et al.*, 2005).

However, the most important significant question is how one explains the qualitative variations in fatty acids

Table 3: Correlations coefficients for fatty acids extracted from *Chlorella vulgaris* grown under autotrophic and heterotrophic conditions for 9 days

Fatty acids	Variables								
	20:00	18:03	18:02	18:01	18:00	16:02	16:01	16:00	14:00
12:00	***	***	NS	***	***	**	*	***	*
14:00	**	**	NS	**	**	**	*	**	
16:00	***	***	NS	***	***	***	*		
16:01	*	*	NS	*	*	*			
16:02	***	***	NS	***	***				
18:00	***	***	NS	***					
18:01	***	***	NS						
18:02	NS	NS							
18:03	***								

NS: Not significant; *Mean values significant at $p \leq 0.05$; **Mean values significant at $p \leq 0.01$; ***Mean values highly significant at $p \leq 0.001$

of different species/phenotypes of the genus *Chlorella*? Present results showed that *C. vulgaris* has quite a simple qualitative fatty acids composition compared to other chlorophycean species. The qualitative composition of fatty acids varied not only at species level, but also varied in different phenotypes of the same species of *Chlorella* (Nichols, 1965; De Mort *et al.*, 1972; Antonyan *et al.*, 1986; Vladimirova *et al.*, 2000; Petkov and Garcia, 2007). Petkov and Garcia (2007) stated that there is no difference in qualitative composition of fatty acids of the genus *Chlorella*. Although, they believed in the stability of qualitative fatty acids composition as a characteristic feature of the genus *Chlorella*, they drove a lot of evidences that showed a great variability in qualitative fatty acid composition in different species of *Chlorella*. Meanwhile, they accept the idea of using qualitative fatty acids composition as a taxonomic feature of particular species of *Chlorella*; they presented many explanations of the variability in qualitative fatty acids composition (Table 3). For example, they revealed the presence of the fatty acids 15:0, 17:0 and 17:1 as results of culture contamination with bacteria, in agreement with others (De Mort *et al.*, 1972; Wacker *et al.*, 2002). Also, they explained the presence of the fatty acids 20:0, 20:1 and 20:2 on the basis of the presence of impurities with similar retention time in GLC which agreed with the results obtained by Antonyan *et al.* (1986) and Homova *et al.* (1986).

Due to the obvious variability in both qualitative and quantitative fatty acid compositions of *Chlorella* in this study compared to other recent reports, one should pay a lot of attention to come into acceptable conclusion for such issue. Thus, it is better to perform much detailed investigations on qualitative fatty acids composition for different *Chlorella* species using more sophisticated and precise techniques to come into acceptable conclusion.

Finally, considering production of natural food supplements or/and natural pharmaceutical products, it's strongly recommended using autotrophic cells of *Chlorella* rather than using those of heterotrophic cells for such purpose. This is because the richness in carotenoids, protein and fatty acids contents of autotrophic *Chlorella*.

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