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Photosynthetic pigments of *Chlorella* sp. K cultured under photoauto-, mixo- and chemohetero-trophic growth conditions

Taku MISONOU* and I. K. PACHLAVUNI

Department of Plant Physiology, Faculty of Biology, Moscow State University, Moscow, USSR, 119899

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The adaptive reaction of pigment synthesis to types of nutrition and spectral composition of light under photoautotrophic growth conditions was investigated in *Chlorella* sp. K. A comparative study was made for the pigment composition of the cells from various growth stages.

The types of nutrition and the spectral compositions of light had no effect on the pigment composition of this alga. The cells contained β-carotene, lutein, violaxanthin, loroxanthin, neoxanthin and 2 unidentified carotenoids and chlorophylls *a* and *b*. In all variants the content ratios of each carotenoid to the total carotenoid were stable. Although productivity of the photoautotrophic culture was at the lowest level, the pigment content of these cells was significantly higher than in the cultures grown with organic substrate. The cells of mixotrophic and heterotrophic conditions contained all pigments at practically the same level. The "green light" variant maximally accumulated all pigments.

These results suggest that 1) the pigment synthetic system of *Chlorella* sp. K is affected by the environmental conditions, and 2) these factors control the carotenoid synthesis at the pre-carotene stages.

Key Index Words: *Adaptation*; *carotenoids*; Chlorella *sp. K*; *chlorophylls*; *light condition*; *types of nutrition*.

The unicellular green algae are well known as suitable material for the study of photosynthesis and are used widely for investigations of the photosynthetic pigment systems. In these cases, however, most interests are directed to the chlorophyll pigments, and the "Second pigment" carotenoids are much less investigated.

The carotenoid pigments are widely distributed in the living world. In the last 30 years, the growing interest of chemists in this pigment has allowed the classification and identification of more than 500 carotenoid pigments from most natural sources. But there is very little information about the biological functions of these pigments. The role as the photosynthetic light-harvesting antenna was shown for β-carotene and some xanthophylls (PRÉZELIN and HAXO 1976, KAGEYAMA *et al.* 1977, KAGEYAMA and YOKOYAMA 1978, ÖQUIST *et al.* 1980, MATHIS and SCHENCK 1982) and the role of protecting chlorophylls against photodynamic action was shown for β-carotene and some photosynthetic bacterial xanthophylls (KRINSKY 1976, COGDELL 1978, ÖQUIST *et al.* 1980, MATHIS and SCHENCK 1982). But the functions of the remainder are still unclear.

The thermophilic strain *Chlorella* sp. K has been investigated for many years in Institute of Plant Physiology, USSR Academy of Sciences, and other laboratories. It

* Address for reprint requests.
Present address: Department of Biology, Faculty of Education, Tokyo Gakugei University, Koganei, Tokyo, 184 Japan.

has been shown that this *Chlorella* strain has very high metabolic flexibility. When the environmental conditions were varied, they easily changed the structure of their photosynthetic apparatus, contents of cell components or rates of respiration and photosynthesis etc. (SEMENENKO *et al.* 1966, VLADIMIROVA *et al.* 1968, VLADIMIROVA 1976, SEMENENKO 1982). The photosynthetic pigments of this strain, however, have not been studied in detail.

In this study, the adaptive reactions of the pigment system (especially about carotenoid pigments) of *Chlorella* sp. K under various growth conditions (types of nutrition and spectral compositions of light) were investigated.

Materials and Methods

A sterile culture of green alga *Chlorella* sp. K from the culture collection of Institute of Plant Physiology, USSR Academy of Sciences, was used for the investigation. This strain of Chlorella was isolated by KOSSIKOV through a selection method which increases the temperature and salinity of the medium (KOSSIKOV 1972). It is proposed, that this strain should be named *Chlorella vulgaris* BEIJERINCK var. *vulgaris* (SETRIC *et al.* 1975). This alga is thermophilic, grows at temperatures between 25 to 41°C, favours high intensity of light (light saturation—over 400 W/m²) and has a high productivity up to 30 g/*l* (VLADIMIROVA 1976).

The following composition of the culture medium was used: KNO_3, 5.0 g/*l*; $MgSO_4 \cdot 7H_2O$, 2.5 g/*l*; KH_2PO_4, 1.25 g/*l*; $FeSO_4 \cdot 7H_2O$, 3 mg/*l*; EDTA, 37 mg/*l* and microelement solution, 1 m*l*/*l* (Microelement solution: H_3BO_4, 2.86 g/*l*; $MnCl_2 \cdot 4H_2O$, 1.81 g/*l*; $ZnSO_4 \cdot 7H_2O$, 222 mg/*l*; MoO_3, 176.4 mg/10 *l*; and NH_4VO_3, 229.6 mg/10 *l*). The medium was autoclaved. In the cases of mixo- and chemoheterotrophic cultivation, 1% glucose was added to the culture medium.

The culture was grown in 500 m*l* flasks with 200 m*l* of medium in the shaker-incubator (200 rpm) maintained at 35°C. Autotrophic and mixotrophic cultures were continuously illuminated with fluorescent light (3500 lux). For the investigation of the effect of spectral compositions of light, the cells were cultured in a 250 m*l* incubation vessel with 100 m*l* of culture medium. The algal suspension was aerated with air (250 m*l*/min), and continuously illuminated with luminescent light through green, red or without acryl filters. The light intensity was fixed at 17 W/m². 2% of 10-day autotrophic culture (at the end of the log-phase) was used as the starting material.

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.

The cells were ground using a glass homogenizer with 0.3 mm glass beads, the modified method of that previously described (SEMENENKO and KASATKINA 1972), or disrupted by a sonication with quartz sand (400 W, 5 min) on Labsonic 1510 (Braun, USA).

The photosynthetic pigments were extracted as usual with acetone and then removed to diethyl ether. The content of chlorophylls *a* and *b* was determined using the following formulae,

$$\text{Chl } a \text{ (mg/}l\text{)} = 9.93 \times D_{660} - 0.78 \times D_{642.5};$$

$$\text{Chl } b \text{ (mg/}l\text{)} = 17.6 \times D_{642.5} - 2.8 \times D_{660},$$

where D_{660}=optical density at 660 nm and $D_{642.5}$=optical density at 642.5 nm (COMAR and ZSCHEILE 1942). The suspension was then saponified with 20% KOH in methanol in the dark at room temperature over night. Unsaponified carotenoid pigments were dissolved in diethyl ether and washed with distilled water, dried with Na_2SO_4, concentrated and used for further experiments. The total content of carotenoids was estimated spectroscopically, using $E^{1\%}_{1\,cm}=2500$ in diethyl ether at λ_{max} (HERTZBERG and LIAAEN-JENSEN 1966).

Individual carotenoids were isolated with silicagel thinlayer chromatography (TLC) on "Silufol UV-254" (Kavalier, Czechoslovakia)

and "Kieselgel-60" (Merk, West Germany). The isolated pigments were then immediately eluated from the TCL plate with ethanol.

Each pigment was identified using the following methods. 1. Comparison of the visible absorption spectra in some organic solvents with the data of previously described studies (FOPPEN 1972 DAVIES 1976,). 2. HCl-test for determination of epoxy groups (STRAIN *et al.* 1967). 3. %III/II for identification of the endogroups (HAGER and MEYER-BERTENRATH 1967). 4. Co-chromatography with β-carotene, violaxanthin, neoxanthin extracted from pea leaves and whole carotenoid fraction from *Scenedesmus obliquus*.

The content of individual carotenoid pigment wsa calculated using the following formula.

$$\text{Carotenoid (mg)} = \frac{E_{max} \times V_e \times V_a}{E^{1\%}_{1\,cm} \times V_s} \times 10,$$

where E_{max}=optical density at λ_{max}, V_e= volume of the pigment solution, eluated from TLC plate, V_a=total volume of aliquot, $E^{1\%}_{1\,cm}$=coefficient of extinction for each carotenoid and V_s=volume of the sample spotted on TLC plate (DAVIES 1976).

All experiments were performed under dim light using freshly distilled organic solvents. Absorption spectra were measured on Beckman 35 (Beckman, USA) and SF-14 (USSR) spectrophotometers.

Results

Fig. 1 shows the growth curves of *Chlorella* sp. K under various types of nutrition (A) and various spectral compositions of light (B). The photoautotrophic culture had a long exponential phase and reached the steady state at the 9th day, while the chemohetero- and the mixo-trophic cultures grew significantly faster and reached the steady state at the 2-3rd day. At the 8-9th day, the heterotrophic culture began to degrade.

The biomass at the steady state of the culture also depended on the types of nutrition. The maximal biomass of the culture of the auto-, the hetero- and the mixotrophic growth conditions was 0.4 g/*l*, 1.8 g/*l* and 5.3 g/*l*, respectively.

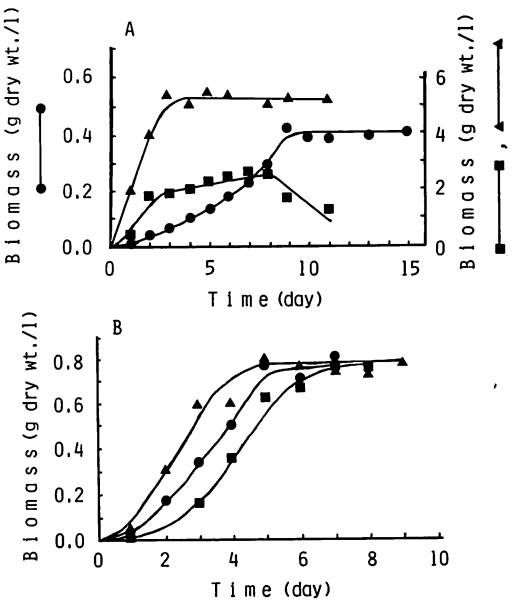

Fig. 1. Growth curves of *Chlorella* sp. K under various culture conditions. A) Under various types of nutrition. ●—●: Photoautotrophic, ▲—▲: chemoheterotrophic and ■—■: mixotrophic growth conditions. B) Photoautotrophic culture under various spectral compositions of light. ●—●: white, ▲—▲: green and ■—■: red light.

Cultures grown under white, green and red light accumulated about the same level of biomass (0.75 g/*l*) at the stationary phase. However, the "red" culture grew most rapidly, and the "green" cells grew slightly slower than the others.

The pigment contents of the cells under various types of nutrition are shown in Table 1. The carotenoid content of the photoautotrophic cells increased according to the growth curve of the culture and reached the maximum at the stationary phase. The content of chlorophyll *a* of this variant also coincided with the increase of biomass. The chlorophyll *b* content reached the maximum at the end of the exponential phase. The chlorophyll *a*/*b* ratios remained low level through the growth.

Table 1. The content of the photosynthetic pigments and the chlorophyll *a/b* ratios of *Chlorella* sp. K cultured under various types of nutrition.

Type of nutrition	Growth stage*	Chl *a*	Chl *b*	Total carotenoid	Chl *a/b*
		(mg/g dry wt.)			
Autotrophic	A	14.4	4.8	4.0	3.0
	B	23.3	11.7	7.1	2.0
	C	28.3	9.7	7.8	2.1
Heterotrophic	A	2.7	0.8	1.4	4.3
	B	9.4	2.2	1.6	4.3
	C	3.5	0.8	1.0	4.4
Mixotrophic	A	6.9	2.3	1.6	3.0
	B	6.4	2.2	1.8	2.9
	C	6.6	2.1	1.4	3.1

* Growth stage of the culture. A: The middle of the exponential phase. B: The end of the exponential phase. C: The beginning of the stationary phase.

Under heterotrophic conditions, the chlorophylls *a* and *b* content varied considerably although the content of carotenoid pigments was rather stable. The content of chlorophylls showed the maximum value at the end of the exponential phase, and decreased at the stationary phase of growth. Thus the decrease in chlorophylls precedes the degradation of the culture itself. The chlorophyll *a/b* ratios under this condition were higher than in the other culture variants through the growth stages.

The contents of all pigments in the mixotrophic cells were stable through the growing process and the values were closer to those of the heterotrophic culture. The chlorophyll *a/b* ratios were also stable and were between those of the hetero- and the autotrophic conditions.

Table 2 shows the contents of pigments in the cultures grown under various spectral compositions of light. The content of all the pigments in the "green" cells increased according to the growth stages of the culture, while the "white" cells and the "red" cells contained virtually steady amounts of the pigments through the growth phases. At the stationary phase, the "green" cells accumulated all the pigments respectively to a higher level than in any other variants

Table 2. The content of the photosynthetic pigments and the chlorophyll *a/b* ratios of *Chlorella* sp. K cultured under various spectral compositions of light.

Variation of light	Growth stage*	Chl *a*	Chl *b*	Total carotenoid	Chl *a/b*
		(mg/g dry wt.)			
White	A	21.6	9.6	3.7	2.3
	B	23.2	9.9	5.5	2.3
	C	20.9	9.8	5.6	2.1
Green	A	16.5	6.5	4.3	2.5
	B	24.1	9.9	6.6	2.4
	C	43.7	18.1	10.6	2.4
Red	A	19.5	6.8	3.7	2.9
	B	17.6	7.6	3.5	2.3
	C	17.2	6.7	2.9	2.6

* Growth stage of the culture. A: The middle of the exponential phase. B: The end of the exponential phase. C: The beginning of the stationary phase.

of the culture. The "red" cells seemed to contain less pigments than the others. The chlorophyll *a*/*b* ratios of all these cultures were similar to that of the photoautotrophic culture of Table 1 and varied within a range of 2.0-3.0.

The carotenoid composition did not vary with variation in culture condition. By the TLC with a solvent system of hexane/acetone (6 : 4), each of samples from all variants was separated into 7 spots as shown in Fig. 2. With more or less polar solvent systems, no other pigment spot appeared. Each pigment fraction was eluated from the zonal TLC and identified as follows.

Fraction 1: β-carotene

This pigment fraction was identified using HCl test (—), comparing the visible absorption spectrum with the data of previously described studies, co-TLC with pea leaves β-carotene.

Fraction 3: lutein

On the TLC plate, this fraction slowly changed color under HCl vapor from yellow to brown, with color-reversal when this vapor was removed (DAVIES 1976). Also this pigment was identified as lutein from the visible spectrum pattern and the medium Rf value.

Fraction 5: violaxanthin

This fraction showed rapid change of color from light-yellow to blue-violet with the HCl test. The visible spectrum pattern with a high %III/II (HAGER and MEYER-BERTENRATH 1967) and co-TLC with pea leaf violaxanthin showed that this pigment was violaxanthin.

Fraction 6: loroxanthin

From the β-carotene-like visible absorption spectrum, negative reaction to HCl test, and co-TLC with carotenoid fraction of *Scenedesmus obliquus*, known to contain loroxanthin (AITZETMÜLLER *et al.* 1969), this fraction was identified as the xanthophyll, specific to Chlorophyta—loroxanthin.

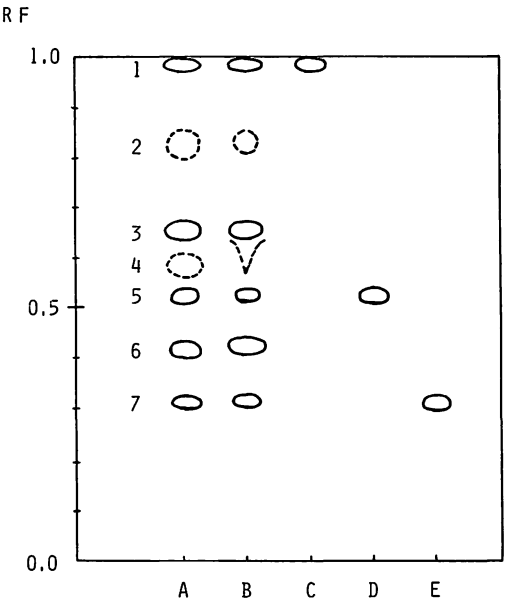

Fig. 2. The chromatogram of carotenoids on silicagel TLC plate. Solvent system—n-hexane /acetone (6 : 4). A: Carotenoid fraction of *Chlorella* sp. K, B: carotenoid fraction of *Scenedesmus obliquus*, C: pea leaves β-carotene, D: pea leaves violaxanthin and E: pea leaves neoxanthin.

Fraction 7: neoxanthin

Under HCl vapor, this fraction changed color from light-yellow to blue. And this fraction was identified as neoxanthin by the visible spectrum pattern, high %III/II value and co-TLC with pea neoxanthin.

Fractions 2 and 4

These trace fractions also showed a positive reaction to the HCl test. Their visible spectra had a typical pattern of degradative epoxide xanthophyll. We did not continue further investigations on these fractions with low content in the samples.

The relative contents of carotenoids in all the variants are shown in w/w percentages to the total carotenoid content in Table 3. Not only the pigment composition, but also the proportion of pigment content was practically identical among the cultures under various growth conditions and growth stages.

Table 3. The % ratios of individual carotenoid to the total carotenoid content.

Pigment	%(w/w) to total carotenoid content
β-carotene	12.9±2.3*
No. 2	0.9±1.0
lutein	53.0±4.9
No. 4	1.8±1.0
violaxanthin	4.9±2.0
loroxanthin	4.0±2.8
neoxanthin	17.4±3.6

* Standard deviation

Lutein was the dominant carotenoid of this strain and occupied up to 60% of the total carotenoid content. The epoxide xanthophylls (violaxanthin and neoxanthin) were comprised 23% and carotenes (only β-carotene), 13%.

Discussion

The conservatism of carotenogenesis in *Chlorella* sp. K was demonstrated in this study on the effect of environmental conditions on the pigment content of cells (Table 3). The composition and ratio of carotenoid content in the cells were fairly stable under various growth conditions. This suggests that in *Chlorella* sp. K, the content of the individual carotenoid varies in the same way. The results in Table 1 also show that light enhancement effect on pigment synthesis is seen remarkably under photoautotrophic condition and barely seen under mixotrophic condition. However the biomass of the mixotrophic culture was conspicuously high. These results suggest that when the environmental medium contains organic substrate, *Chlorella* sp. K synthesize the pigments only on the genetically provided basic level, i. e. as in the dark condition.

Furthermore, stability in the proportion of the individual carotenoid content suggests that carotenogenesis is regulated by culture conditions in the early stage at least, before the appearance of carotenes.

It is widely known that the quality of light has a notable effect on the chloroplast structure, the content of its component pigments, proteins and lipids and on other metabolic systems. In our study, the cells cultured under green light which is less effective for photosynthesis in green plants, accumulated considerably more pigments than other variants. This might be considered as one of the adaptive reactions to the environmental conditions of this alga.

For *Chlorella* sp. K, the ultrastructural alterations in the organization of photosynthetic apparatus was shown, when the culture was transferred to the chemoheterotrophic condition (VLADIMIROVA 1976). It is possible that our results of the variation in pigment content under various types of nutrition is related to this structural change.

The authors also studied the photosynthetic activity of each culture. The results show the different participation level of the pigments in photosynthesis (unpublished). EVANS and BRITTON (1983) suggested that in *Scenedesmus obliquus*, the carotenoids synthesized in the dark were not incorporated into the thylakoid membranes and did not function as the photosynthetic light harvesting pigment, unless exposed to light. Our results also supported this hypothesis. However, further investigations are required to clarify this problem.

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御園生　拓*・パフラヴニ，I. K.：光合成独立栄養，混合栄養および化学従属栄養条件下で培養した *Chlorella* sp. K の光合成色素

栄養条件および培養光のスペクトルに対する *Chlorella* sp. K の光合成色素システム（特にカロテノイド色素）の適応反応を調べるために，藻体を各条件で培養し，種々の生育段階における色素組成の比較を行なった。

栄養条件や光のスペクトル組成および生育段階にかかわらず，藻体には β-カロテン，ルテイン，ヴィオラキサンチン，ロロキサンチン，ネオキサンチンと2種の未同定カロテノイド，およびクロロフィル *a*, *b* が含まれていた。全カロテノイド量に対する各カロテノイドの含有比は常に一定であった。独立栄養条件下では，他に比べて生産量は低かったが色素含有量は高い価を示し，特に緑色光下では色素量は最高であった。

以上より，1) *Chlorella* sp. K の色素合成系は環境条件の影響を受けており，2) カロテノイド合成系はカロテン以前の段階で制御されていることが示唆された。(119899 ソビエト連邦モスクワ市モスクワ国立大学生物学部植物生理学科　*現住所：184 東京都小金井市貫井北町 4-1-1　東京学芸大学教育学部生物学教室 岡崎研究室気付)