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Studies of Growth according to the Concentration of Mineral Elements of Medium in Cyanophyte SG63

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배양액의 염도에 따른 남조식물 (SG 63)의 생장 연구

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ABSTRACT

The characteristic of Cyanophyte genus SG63 is similar to that of *Aphanot hece* sp. The optimal growth was found with the concentration of NaCl and $MgSO_4 \cdot 7H_2O$ on the culture medium. The most optimal condition is 56‰ of NaCl (S4 medium) and 20‰ of $MgSO_4 \cdot 7H_2O$ (M2 medium). The synthesis of chlorophyll *a*, phycocyanin and soluble proteins is affected by the concentration of the two mineral elements in culture. Especially, the content of chlorophyll *a* and phycocyanin decreases on the most highly saline medium. The identified principal carotenoids are β-carotene, echinenone, zeaxanthin and myxoxanthophyll. The rates of concentration of protein/chlorophyll *a* and phycocyanin/chlorophyll *a* are low on the S4 medium. Inversely, these rates are the highest on the M2 medium. Accordingly, the high concentration of $MgSO_4 \cdot 7H_2O$ provoke the synthesis of phycocyanin and total proteins.

INTRODUCTION

The vegetal organisms of several types grow on higher salinity medium than those of sea water: Cyanophytes (blue green algae, cyanobacteria), bacteria-halobacteria, photosynthetic bacteria and Chlorophyceae (*Dunaliella* sp.). Several Cyanophytes had been isolated from hypersalted water; *Aphanothece* (Eardey, 1938; Hof and Frémy, 1933), *Coccochloris* (Kao *et al.*, 1973), *Aphanocapsa* (Volcani, 1944). The halophytes had been also studied for their physiological and biochemical characters according to the variable levels of salinity. The relation between growth and metabolism of these plants was affected by physiochemical factors: for example, Na^+, K^+, Ca^{+2} and Mg^{+2} in the culture medium (Jay, 1976).

A halotolerant Cyanophyte, *Agmenellum* cultivated on the medium of ASP-2+90 g/*l* of NaCl decreased the content of lipids, hydrocarbonated substances and pigments: the activity of enzymes in Krebs' Cycle is inhibited by salinity (Maryk, 1968). Under the various conditions of the culture, it is necessary to study the acitivity of pigments and to specify photosynthesis and growth of the organisms.

In these studies, the goal of research is to observe the growth of a Cyanophyte type SG63 and to determine the optimal concentration of NaCl and $MgSO_4 \cdot 7H_2O$ in the mediums with analysis of parameters of chlorophyll, proteins and phycobiliproteins.

MATERIALS AND METHODS

Strains. The Cyanophytes SG63 originated from Salin de Giraud where they inhabit salt marsh. They form a considerable mass, ovoid unicells dispersed in a mucilaginous substance without clearly defined colonies. According to the criterion of classical taxonomy,

Table 1. Composition of medium S

Elements	Mediums (g/l)			
	S_1	S_2	S_3	S_4
NaCl	330	250	150	85
	*1(178)	(138)	(88)	(56)
X Cl.	13	10	6	3.3
Ca Cl_2	2.7	2	1.2	0.7
Mg SO_4 $7H_2O$	66	50	30	16
	*2(38)	(30)	(20)	(13)
Ca $(NO_3)_2 4H_2O$	1.5	1.5	1.5	1.5
X H_2PO_4	0.04	0.04	0.04	0.04
Fe EDTA	10 ml.	10 ml.	10 ml.	10 ml

Each medium mixed to the one volume of Erd-Schreiber. *1( ), Quantity of NaCl on the final medium (‰); *2( ), Quantity of $MgSO_4 \cdot 7H_2O$ on the final medium (‰); Quantity of NaCl estimated in seawater: 26 g/l. Quantity of $MgSO_4 \cdot 7H_2O$ estimated in seawater: 5 g/l.

Table 2. Composition of medium M

Elements	Mediums (g/l)			
	M_1	M_2	M_3	M_4
NaCl	150	150	150	150
	*1(88)	(138)	(88)	(56)
X Cl.	6	6	6	6
Ca Cl_2	1.2	1.2	1.2	1.2
Mg SO_4 $7H_2O$	40	30	20	10
	*2(25)	(20)	(15)	(10)
Ca $(NO_3)_2 4H_2O$	1.5	1.5	1.5	1.5
X H_2PO_4	0.04	0.04	0.04	0.04
Fe EDTA	10 ml.	10 ml.	10 ml.	10 ml

*1( ), Quantity of NaCl on the final medium (‰); *2( ), Quantity of $MgSO_4 \cdot 7H_2O$ on the final medium (‰).

these Cyanophytes are placed in genus *Aphanothece'* by reason of the atypical aspect of the colonies. These Cyanophytes SG63 had been isolated at the laboratory of Cellular & Vegetal Biomembranes and Surfaces in the Ecole Normale Supérieure of Paris (Grants of Mr. J.C. Thomas).

Culture medium. The salted mediums (S1 to S4)(M 1 to M4) were prepared by successive dissolution of salts indicated on the Tables 1 and 2. They were then mixed to Erd-Schreiber (volume to volume)(Foyn, 1934) according to the medium of Schreiber (1929). And then, the extract of soil was added in seawater (Provasoli *et al.*, 1957). The sodium appears in the form of sodium chloride and magnesium is under that of hydrated sulfate ($MgSO_4 \cdot 7H_2O$). In the first culture, the medium had been tried by the vartiation of the concentration of NaCl made between 330 g/l (S1) and 85 g/l (S4), which were modified by Erd-Schreiber, and constituted the medium so that the concentration of NaCl resulted in between 175 g/l and 56 g/l (Table 1). The ratios of the components of differant salts were constantly maintained with the formula nonmodified in the inonic balance.

In the second experiment, we studied the effect of the concentration of $MgSO_4 \cdot 7H_2O$. The NaCl of 88‰ was fixed, which gave satisfactory growth in the preceding experiment. The form $MgSO_4 \cdot 7H_2O$ was used under the various concentrations between 40 g and 10 g (Table 2). The erlenmeyer containing the medium was sterilized in an autoclave at 120℃ for 20 min.

Condition of the culture. The type SG63 was cultivated in an air-conditioned room and erlenmeyers of 500 ml containing 300 ml of each medium (medium S in 150 ml+Erd-Schreiber medium in 150 ml) or (medium M in 150 ml+Erd-Schreiber in 150 ml) at 21℃ (± 4℃) with the light in 23 $\mu m^{-2} s^{-1}$ (measured with a LiCOR photometer) from a fluorescent lamp (Mazdafluor) and the alternation of light (16 h) and dark (8 h). The homogeneity of the culture was maintained by magnetic agitation.

Measure of cellular density. Observation of the culture was taken every 3 days for 3 weeks. In spite of the vigorous agitation of the culture, their cells were not homogenized. The automatic counting with the apparatus of the Coulter type was then impossible. The number of cells had been carefully counted with the help of hemocytometer on an homogeneous condition. One identified volume of culture in 5 ml is each time homogenated.

Measure of dosage of pigments and proteins. The liposoluble pigments was extracted with 90% methanol after the breaking of cells with ultrasound and centrifugation in low speed (5000 rpm, 10 min.) in order to eliminate the cellular debris. The pigmental extracts were read on the spectrophotometer VARIAN DMS 90 and with the optimal density of 665 nm. The content of chlorophyll *a* was calculated with the help of the formula of Mackinney (1941) (quantity of chlorophyll *a* (mg/ml) =O.D./76 for an optical distance of 1 cm).

The hydrosoluble pigments were extracted by the breakage of cells with ultrasound in a phosphate buffer solution in 0.01 M and 0.15 M of NaCl. The extracts were clarified after the addition of streptomycin sulfate of 1% (Bryant, 1982) by the process of centrifugation (5000 rpm, 10 min.). The phycobiliproteins showed on the surface and these soluble proteins were read at 652 nm and 615

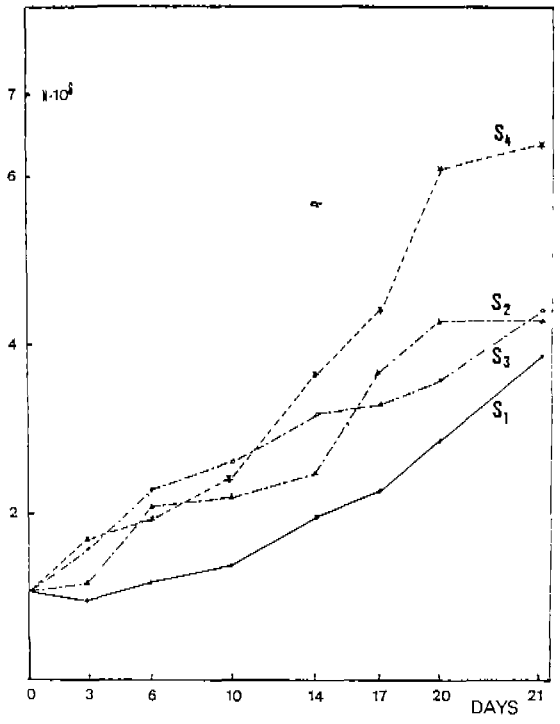

Fig. 1. Growth of Cyanophytes SG63 according to the concentration of NaCl (S1, Medium of NaCl in 178‰; S2, 138‰; S3, 88‰; S4, 56‰).
N×10⁶: N=Number of Cells.

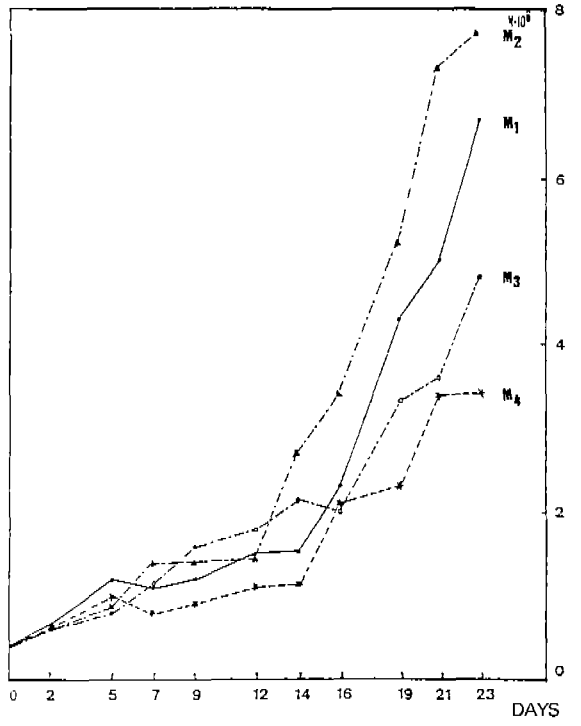

Fig. 2. Growth of Cyanophytes SG63 according to the concentration of $MgSO_4 \cdot 7H_2O$ (M1, Medium of $MgSO_4 \cdot 7H_2O$ in 25‰; M2, 20‰; M3, 15‰; M4, 10‰).

nm in DMS 90. The contents of phycocyanin and allophycocyanin were calculated with the formula of Benett and Bogorad (1973).

The aliquots were retaken in pure water and disintergrated in the apparatus of ultrasound. The proteins were measured by the colormetric method using the Bio-Rad protein assay reaction with ox albumin serum as standard protein.

After saponification according to the protocol, the insaponification was separated from the saponificated elements in a decantation ampule with sulfuric ether and 10% NaCl. The carotenoids were recovered from epiphase (sulfuric ether); after neutralization, they were dried, conserved under nitrogen, and frozen. The separation of carotenoids took place on the silica gel plate with chromatography (DC Fertigplatten, Kieselgel) with the mixed solvents (10 volumes of petrolic ether 40-60°, 2 volumes containers of benzene, 1 volume of ethanol) (Eichenberger and Grob, 1962) after migration. The bands of pigments separated were scratched and retaken in sulfuric ether (Rf>0.5) or ethanol (Rf<0.5). The absorption spectra had been measured between 600 nm and 350 nm.

RESULTS

Morphological modification of cells. The type SG63 was a colonical and unicellular Cyanophyte of the family of Chroococaceae, ovoid cells. At the end of the period of growth, the form and the size of the cells were lightly varied in accordance with the concentration of mineral elements in the culture. Particularly, the cultivated cells in the medium of 178‰ (S1) and 138‰(S2) of NaCl were more cylindric than those of 88‰ (S3) and 56‰ (S4) : S1, 8.7 μm (L), 3.5 μm (l); S2, 8.0 μm (L), 2.6 μm (l); S3, 6.99 μm (L), 3.7 μm (l); S4, 6.8 μm (L), 3.3 μm (l). That phenomenon was probably associated with the rhythm of cellular divisions. This modification of the cellular size was comparable to precedent observation of halotolerant Cyanophytes : *Anacystis, Agmenellum, Aphanothece, Coccochloris* (Batterton *et al.*, 1970; Yopp *et al.*, 1978). But the various concentrations of $MgSO_4 \cdot 7H_2O$ in culture hardly affected the celluar size.

Observation of cellular growth. The maximal rate

of growth was obtained on the medium S4 (56‰ of NaCl) and M2 (20‰ of $MgSO_4 \cdot 7H_2O$). On the S4, the exponential phase was from the 3th day to the 20th day. After 20 days of culture, the stationary phase began. On the other hand, the cellular density was the lowest on the S1 (NaCl in 178‰).

The latent phase was clearly observed on the most salted medium (S1 & S2) (Fig. 1). After 25 days of culture, it showed the exponential phase of growth on the S1 and the S3 medium, while the S2 and the S4 reached to the stationary phase. The time of cellular double division into two was the most rapid during the exponential phase of S4 (8 days).

The rate of growth was the highest on the medium of $MgSO_4 \cdot 7H_2O$ in 20‰ (M2) (Fig. 2). The stationary phase began at 21th day of culture with medium M4. The time of cellular duplication into two (8 days) was the most favorable on the mediums M1 and M2.

Evolution of the content of chlorophyll *a*. The photosynthetic apparatus of Cyanophyte includes only one kind of chlorophyll; chlorophyll *a*. The content was varied on each medium according to the concentration of $MgSO_4 \cdot 7H_2O$. As to the S mediums, the initial content of chlorophyll *a* (6.1×10^{-4} mg/m*l*) modified hardly on the mediums S1 and S2 but increased on the S3 and S4 (S3, 15×10^{-4} mg/m*l*; S4, 20×10^{-4} mg/m*l*) on the 21th day of culture. As to the medium M, the initial quantity of chlorophyll *a* was 2.2×10^{-4} mg/m*l*, which increased clearly after 5 days of culture (7.5×10^{-4} mg/m*l*). The maximum synthesis of chlorophyll *a* showed on the medium M3 (20.6×10^{-4} mg/m*l*) after culture of 20 days.

Evolution of phycobiliproteins. Their quantities were measured by the content of phycocyanin. These phycobiliproteins are very important accessory pigments collecting the luminescent energy for photosynthesis. About the medium S, the initial quantity (4.44×10^{-3} mg/m*l*) of phycocyanin decreased on the highest saline medium (S1, 2.1×10^{-3} mg/m*l*) and increased on the weakest concentration (S4, 7.8×10^{-3} mg/m*l*) at the 14th day in culture. The initial content of phycocyanin per cell (41×10^{-8} mg/cell) decreased abruptly on the first day of culture for every medium S (20×10^{-8} mg/cell). On the other hand, the mediums S1 and S2 provoke the stop of synthesis of phycocyanin. In all the medium M, the global phycocyanin continued to synthesize (initial quantity : 1.6×10^{-3} mg/m*l* to maximal quantity : 8.6×10^{-3} mg/m*l* of M2 after 20 days of culture).

Evolution of total proteins. The total proteins include phycobiliproteins, other soluble proteins, membranal

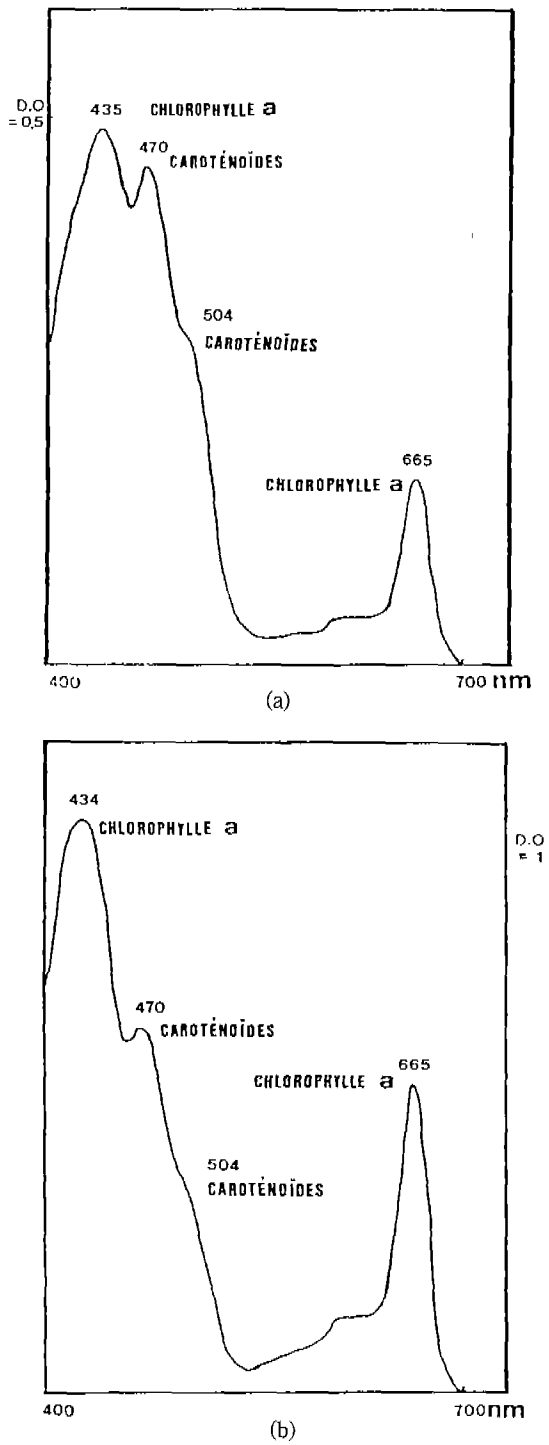

Fig. 3. Spectra of total absorption, liposoluble pigments of SG63.
a, Normal culture; b, Culture in strong light; O.D., Optical Density.

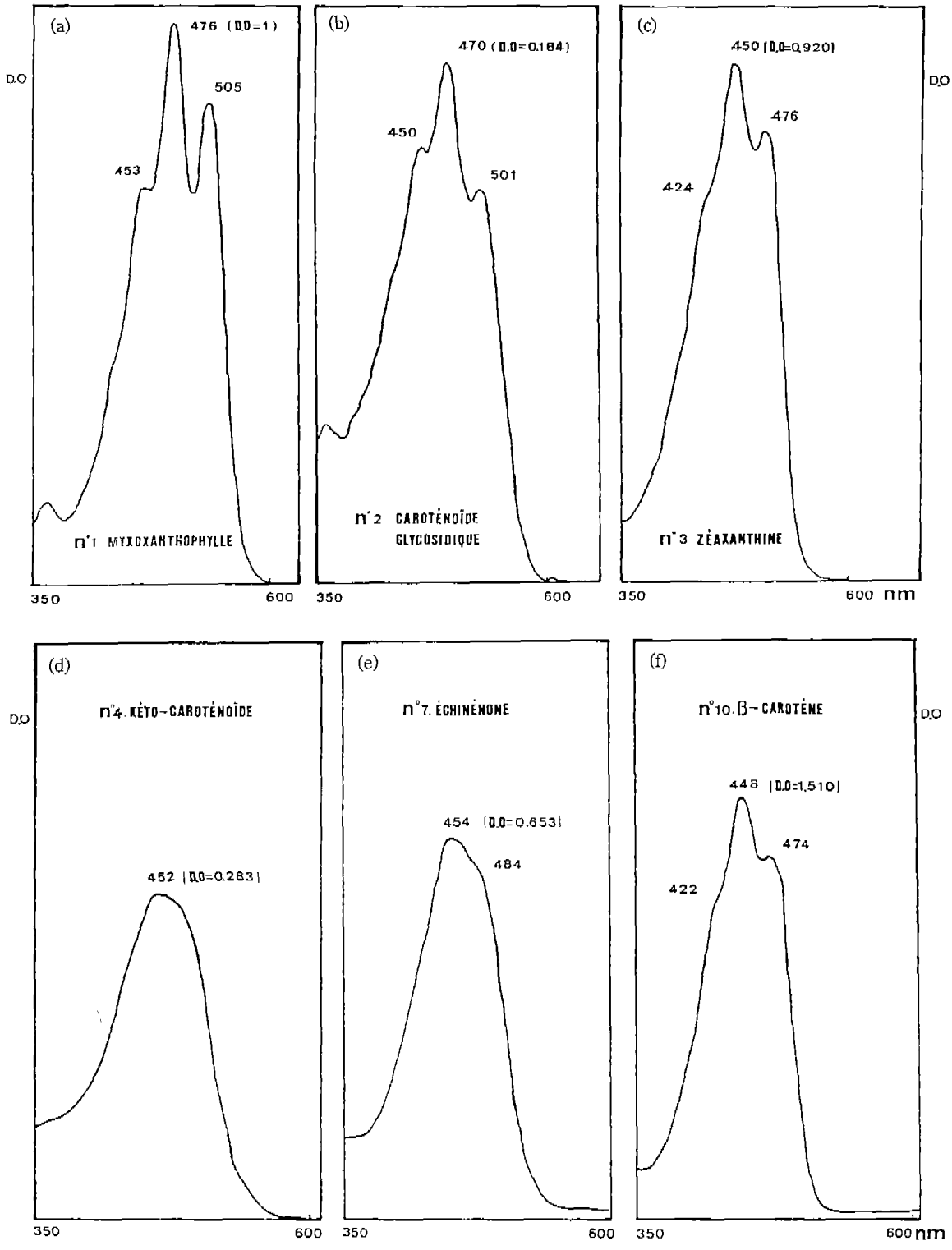

Fig. 4. Spectra of absorption of carotenoids.
a, Myxoxanthophyll; b, Glycosidic carotenoids; c, Zeaxanthin; d, Keto-carotenoids; e, Echinenone; f, β-carotene.

proteins, reserve proteins in Cyanophytes. The global synthesis of proteins was prominent on the S3 and S4 after 21th day of culture. At this time (initial quantity : 7.0×10^{-3} mg/ml to maximal quantity : 35.0×10^{-3} mg/ml of S 3), the weak quantity was in the S1 and S2 (S1, 11.8×10^{-3} mg/ml; S2, 8.3×10^{-3} mg/ml). In the medium M, the global content of protein was relatively active after culture of 20 days (initial quantity : 2.6×10^{-3} mg/ml to maximal quantity : 16.1×10^{-3} mg/ml of M2).

Characteristics of total carotenoids. Before saponification, the spectrophotometers of total absorption of methanolic extracts are represented in Fig. 3. They were extracted from the cells cultivated on the S3 medium at the end of exponential phase. Fig. 3a represents the total pigmental extract obtained from the culture realized in strong light. The quantity of carotenoids was more abandant in this culture than in the normal conditions (Fig. 3b). The identified principal carotenoids (β-carotene, echinenone, zeaxanthin and myxoxanthophyll) have spectral characteristics and comparable polarity (Fig. 4a-f) to former studies (Healey, 1968; Heztzberg, 1971; Palla, 1969).

DISCUSSION

Consideration of cellular morphology with regard to its salinity. The morphological variation had been observed in halophile Cyanophytes dependent on salinity (Brock, 1976). The form of cells in *Aphanothece halophytica* which is similar to SG63 was more cylindric on the medium of NaCl in 4 M than 3 M (Yopp *et al.*, 1978). We can also observe the most cylindric cells on the highest salinity mediums (S1 and S2).

Here, we must pay attention to this fact that the cellular modification provokes the taxonomical confusion of coccoidal and unicellular Cyanophytes : *Synechococcus, Gloeothece, Aphanothece* and *Aphanocapsa.*

Establishment of an optimum and definition of halophile organisms. On the whole, the time of cellular duplication with two of SG63 was slower than that of *Aphanothece* (2.5 days) (Yopp *et al.*, 1978). The optimal growth is in 56‰ of NaCl (S4) and 20‰ of $MgSO_4\cdot7H_2O$ (M4). The type SG63 can be developed on the more salted medium than *Anancystis nidulans* (less than 0.5 M of NaCl) and *Agmenellum quadruplicatum* (1-1.5 M of NaCl). The optimum of this algae SG63 is similar to that of *Aphanothece halophytica* isolated by Yopp (1978) (NaCl of 2 M) but not by Brock (1976) (NaCl of 3 M). The tolerance is then variable from one to another and does not have specific characteristics.

In general, the halophile Cyanophytes living in salt marche and intertidal regions can be submitted to variable salinity by the spray of waves and climatic factors. In order to survive in modified environments, these halophile Cyanophytes must be able to adapt. And then, we can not precisely define their distribution between halophile and halotolerant organisms.

Accordingly, we can merely suppose two categories of ecological factors related to this definition: first, those organisms which influence the growth dependent on ionic concentration of minerals on the culture medium and second, those which are related to the growth condition in order to survive on a hypersalted medium (Golubic 1980). Brown and Gibbons (1955) had indicated that halophile organisms were supported in the most salted medium and it was necessary that the concentration of Mg^{+2} (0.1-0.5 M : 2.4-12‰) in culture was moderately high. But we observed that the optimum of concentration in $MgSO_2\cdot7H_2O$ needs that of the medium S4 which has the best growth (13‰ of $MgSO_4\cdot7H_2O$).

Study of the relationship between cellular growth and pigmental content. The concentration of chlorophyll *a*, phycocyanin and soluble proteins is affected by the concentration of NaCl and $MgSO_4\cdot7H_2O$, namely, the global synthesis of those substances decreases on the highest salinity in culture. Conversely, the pigment and protein content is variable. It must be emphasized that the global synthesis on medium M2 ($MgSO_4\cdot7H_2O$ of 20 ‰) is more accented than that of M3 (15‰). The β-carotene representing 70-80% of the total quantity of carotenoids is strongly affected by the highest salinity. It is ascertained that the concentration of phycocyanin is dependent on the salinity.

As a result of these studies, the quantity of phycocyanin according to the concentration of NaCl decreases very suddenly at the beginning of culture. The ratios of the content of protein/chlorophyll *a* and that of phycocyanin/chlorophyll *a* are lower on an optimal medium (S4) than the S2 having the worst growth of cells. It means that the synthesis of proteins and phycocyanin is less important relatively than that of chlorophyll *a* on an optimal medium of S4. Accordingly, the content of chlorophyll *a* increases on optimal medium.

On the other hand, the ratio of the content of phycocyanin/chlorophyll *a* is higher on the optimal medium M2 (20‰ of $MgSO_4\cdot7H_2O$) than that of 10‰ (M1). The synthesis of phycocyanin seems to necessitate the highest concentration of $MgSO_4\cdot7H_2O$. It can be related to the

balance between divalent and monovalent ions.

CONCLUSION

We tried to determine the optimum of growth according to the concentration in NaCl and $MgSO_4 \cdot 7H_2O$. The medium S4 is the most favorable for Cyanphytes SG63. In this medium, they have good growth. In the most salted medium, the rate of growth is low (S1 and S2). In the medium M, the growth rate is highest on the M2. But in this case, the synthesis of pigment is low; conversely, the soluble protein is not affected. The increase of concentration in Mg^{+2} ion is necessary when the salinity increase in order to assure the best growth. The studies of growth in SG63 will be realized in the precision of the culture technique for the application of Cyanophytes. The ecological interest of this research in halophile Cyanophytes specifies the limits of proper development of organisms.

적 요

*Aphanothece*속과 분류학적 특징이 유사한 남조식물 SG 63을 대상으로 배양액의 NaCl과 $MgSO_4 \cdot 7H_2O$ 농도에 따른 최적 생리적 활성을 연구했다. SG63의 최고 성장율은 56% 의 NaCl과 20% 의 $MgSO_4 \cdot 7H_2O$농도의 배양액에서 각각 이루어졌다. 엽록소 *a*, phycocyanin과 수용성 단백질의 합성율은 두 무기염도에 따라 영향을 받았다. 특히, 엽록소 *a*와 phycocyanin의 농도는 NaCl 함량이 높은 배양액일수록 감소되었다. 남조식물 SG63의 carotenoid의 구성물질은 β-carotene, echinenone, zeaxanthin과 myxoxanthophyll이였다. 총단백질과 엽록소 *a*의 농도비율과 phycocyanin과 엽록소 *a*의 농도비율은 NaCl 함량에 비례하고 최고 성장율을 나타내는 20% 의 $MgSO_4 \cdot 7H_2O$ 배양액에서 가장 높았다. 따라서 배양액내에 $MgSO_4 \cdot 7H_2O$의 함량이 높을수록 phycocyanin과 단백질합성을 촉진한다. 위의 결과는 앞으로 남조식물의 대체식품개발과 의약품의 개발을 위한 새로운 합성물질의 추출에 기여하기 위한 mass culture에 응용될 수 있다고 본다.

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