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Factors Affecting the Mixotrophic Maximum Growth of Chlorella pyrenoidosa

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To obtain the economic benefits of biomass production, the maximum growth rate of *Chlorella pyrenoidosa* NT-11 under a high cell concentration (PCV >1 ml/l) was induced by use of the mixotrophic culture method in a 2-liter jar-fermentor system. The mixotrophic culture in the Roux-bottle (flat flask) system reached a maximum growth rate of PCV *4ml/l/day* while the autotrophic growth without supplemental acetic acid had a lower growth rate of PCV 2.5 *ml/l/day.* In the jar-fermentor system, a mixotrophic maximum growth rate of up to PCV 20*ml/l/day* and a final cell density higher than PCV*80ml/l* were obtained under the conditions of *25°C,* 10 Klux light intensity, and *pH 6.5 for* the medium. The carbon sources included 10% acetic acid in the feeding medium, and 5% C02 in the air used for aeration. A period of autotrophic growth prior to the mixotrophic maximum growth was necessary since neither the slant culture nor the maximally grown culture could be used directly or again for the purpose of maximum growth. *Other Chlorella* strains (C-28 and C-212) showed no such effects. The chemical compositions of the maximum growth cells were similar to those of normal ones. There were no significant ultrastructural differences between autotrophic growth cells and mixotrophic growth cells.

Key words: *Chlorella pyrenoidosa,* Maximum growth rate, Packed cell volume (PCV), Mixotrophic and autotrophic culture.

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INTRODUCTION

Chlorella has been cultivated for economic purposes for many years[1,2], 21 The first basic work on the growth rate of mass cultured *Chlorella* was reported by Tamiya *et* al.sup>(3) It is important, though difficult, to optimize mass-culture conditions for a high output rate and a high product yield when cultivating *Chlorella* outdoors. Although the maximum specific growth rate (U^{max}) *of Chlorella* cells at the logarithmic stage is quite high, whether in autotrophic, heterotrophic, or mixotrophic incubation(4,5), this means little for mass production of microalgae, because high growth rate is only possible under extremely low cell density (e.g., PCV< 1 ml/l)(6). As long as density is higher than PCV> 1 ml/l, growth will be very slow because cells do not reproduce in an exponential rate. It is still necessary, however, to maintain high cell density in a mass culture of *Chlorella* for the purpose of high biomass output rate as well as to reduce the cost of harvesting.

Our mixotrophic conditions were set as growth of *Chlorella* cells in light in the present C02 but with the additional use of acetic acids which were photometabolized during growth. This research program has studied the factors which control the growth of different *Chlorella* strains under different cell concentrations. We have succeeded in inducing a significantly higher cell growth rate in one strain (NT-11) by mixotrophic culture methods in a newly designed jar-fermentor system.

MATERIALS AND METHODS

Algal strains and maintenance conditions

Chlorella Pyrenoidosa NT-11 is a locally (Taiwan) isolated strain[7], and C-28 and C-212 were obtained from The Research Institute of Applied Microbiology, University of Tokyo, Japan. Algal cells were inoculated on agar slants and placed at room temperature under 2.5 Klux light intensity for 7-10 days. Thereafter, the well-grown slant cultures were maintained at 4°C in the dark before being inoculated into the liquid medium. The chemical composition of the maintenance medium is shown in Table 1. The pH value was adjusted to a suitable value with 40% NaOH.

Culture condition

Three types of culture systems were used in this research program: (1) an autotrophic culture in a Roux bottle; the detailed structure of this flat flask was described

TABLE 1
The chemical compositions of the maintenance medium, autotrophic medium, mixotrophic culture basal medium and feeding medium

Chemicals	Concentrations			
	Maintenance medium	autotrophic medium	Mixotrophic culture	
			Basal medium	Feeding medium
KNO_3 (g/*l*)	1.5	5		
$MgSO_4.7H_2O$ (g/*l*)	1.5	2.5	1.25	2.7
KH_2PO_4 (g/*l*)	1.5	1.25	0.34	3.5
$FeSO_4.7H_20$ (mg/*l*)	1	3	5	0.3
A_5 Sol'n (ml/l)*	2	1	1	
Agar (g/*l*)	18			
$CO(NH_2)_2$ (g/*l*)			1.5	10
K_2HP0_4 (g/*l*)			0.44	3.5
EDTA (mg/*l*)			7	
CH_3COOH (ml/l)				100
pH	6.8	6.5	6.5	6.5

As Sol'n: H_3BO_3, 2.18gli; $MnC1_2$-$4H_20$, 1.81g/*l*; $ZnSO_4$, 0.22g/*l*; $CUS0_4$, O.O8g/*l*; Na_2MoO_4, 0.021g/*l* conc. H_2SO_4, 1 drop/*l*.

in Tamiya's report[3];(2) a mixotrophic pure culture in a 2-liter jar-fermentor (Mituwa KMJ-2). The equipment and culture procedures of each system are illustrated in Figs. I and 2. In the first system, air containing 5%, CO_2'', was used for aeration without any additional carbon source. In the other two systems, a feeding medium containing 10% acetic acid as the organic carbon source for mixotrophic growth was added to the medium during incubation. In order to get a suitable feeding rate, the pH changes in the medium were controlled by a pH controller. In the third system, before incubation began, the slant culture was first inoculated into Roux bottles for a series of autotrophic growth and then transferred into the jar-fermentor. The compositions of the media for autotrophic or mixotrophic culture are shown in Table 1.

Determination of algal growth

A fixed volume of cell medium was collected in a Hematocrit tube and centrifugated at 4,000rbm for 30min to determine the packed cell volume *(ml/l)*[8] which was used as the index base of algal growth in this study.

Analysis of cell composition

The crude protein content was determined by the semimicro-Kjeldahl method[9], the total chlorophyll content by Richard and Thompson's method[10], the crude fat content by the Soxhlet method[9], and the lead and arsenic content by the atomic absorption spectroscopic method. The cellular powder was combusted at 500°C for 30min., and then the ash content was determined[11].

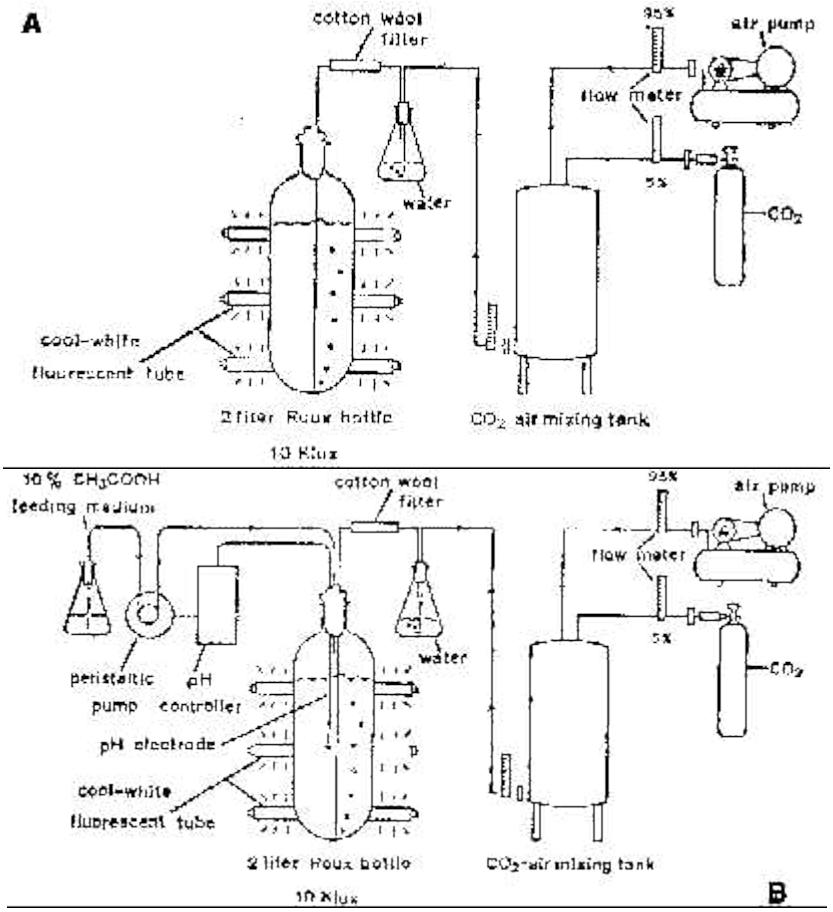


Fig. 1. Diagrams of the autotrophic Roux-bottle system (A) and mixotrophic Roux-bottle system (B) for culturing *Chlorella* **spp.**

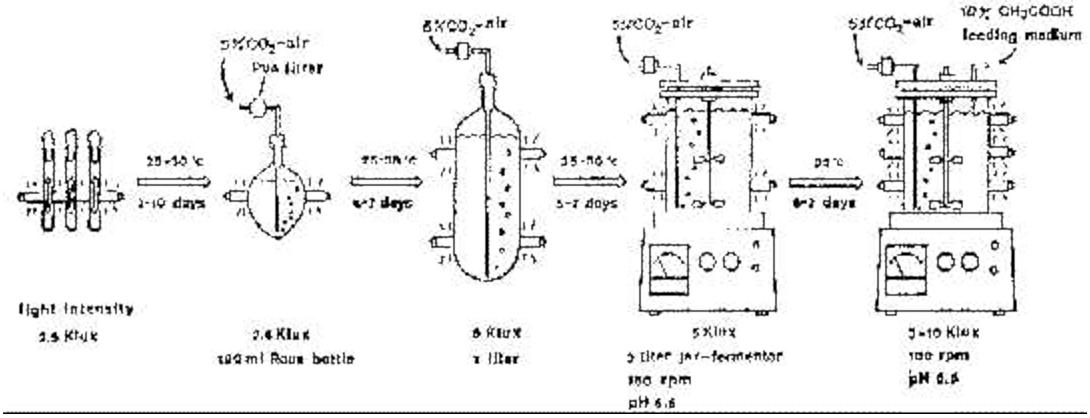


Fig. 2. The devices and procedures for the induction of the mixotrophic maximum growth of ***Chlorella*** in a jar-fermentor system.

Electron microscopic observations

As previous reported[12], algal cells were harvested by centrifugation (4,000rpm; 30min), and cell pellets were fixed in 2% glutaraldehyde. After post fixation in l% osmium tetroxide, the cells were dehydrated in a series of alcohol and embedded in Spurr's resin. The sections were post-stained with uranylacetate solution and examined using a Hitachi HU-12 transmission electron microscope at an acceleration voltage of 75 KV. Photographs were taken at an initial magnification of 10,000-12,000.

RESULTS

Figure 3 shows three growth curves of *C. pyrenoidosa* NT-11, which was all incubated in the Roux-bottle system but supplied with different carbon sources. The carbon source containing both acetic acid and enriched CO_2 (5% CO_2 in air, flow rate *1.0-2.nl/min, 20°C)* was best for growth, and *chlorella* growing on it had the highest final cell density (ca. PCV 27ml/l). The other two combinations of carbon sources (CH_3COOH plus air or only by 5% CO_2, in the air) had lower values of growth rate and final density.

A 2-liter jar-fermentor system (shown in Fig. 2) was devised for the study of *Chlorella* mixotrophic growth. It had many advantages in handling, harvesting, pH control, and prevention of contamination. In our developed procedures, *Chlorella* was transferred from slants, first into a 100ml Roux bottle, and then to a 1-liter bottle, each for a period of 5-7 days of autotrophic growth. The scaled-up culture

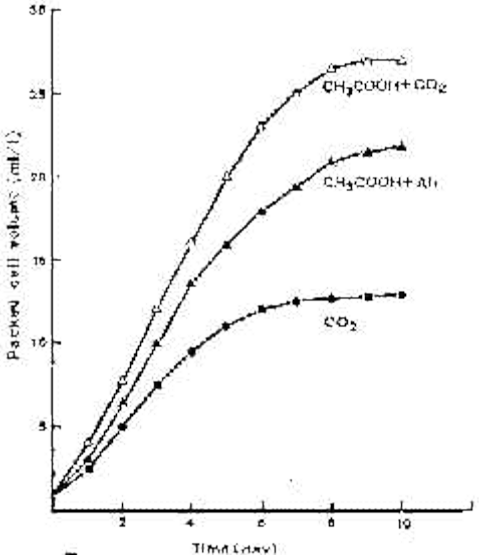


Fig. **3.** Growth curves **of** one autotrophic and two mixotrophic incubations (supplemented with or without 5% C02-air) of *C. pyrenoidosa* NT-11 in the Roux-bottle system.

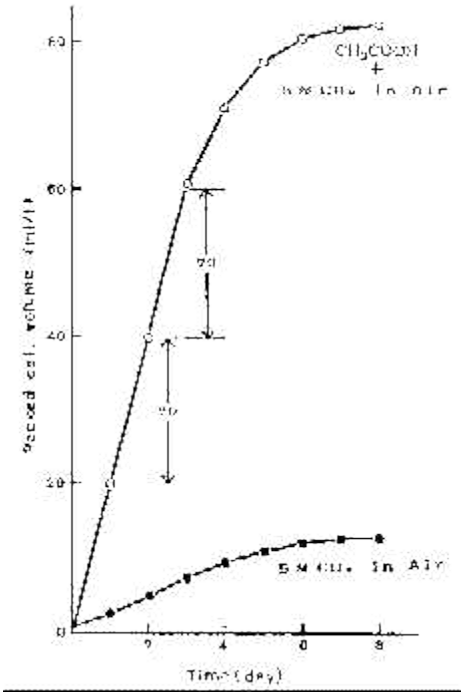


Fig. 4. The growth curve of the mixotrophic culture of *C. pyrenoidosa NT-1* I in the jar-fermentor system compared with that of the autotrophic culture in the Roux-bottle system.

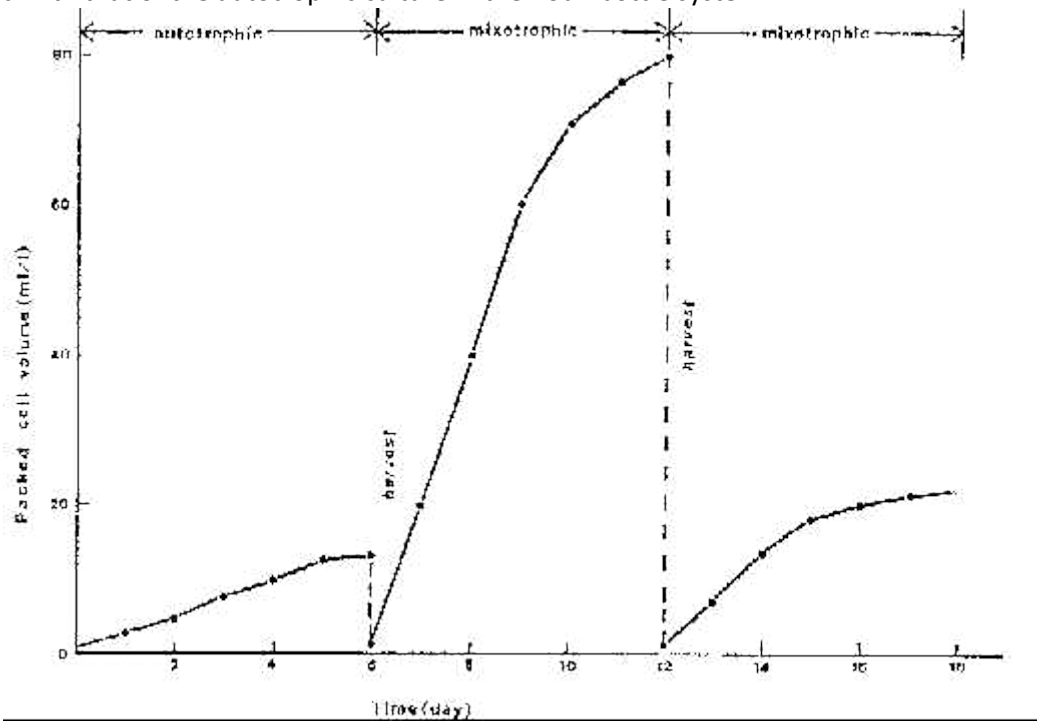


Fig. 5. The semi-continuous incubation of *C. pyrenoidosa* NT-11 in the jar-fermentor system.

was then inoculated into the 2 l jar-fermentor for another 5-7 days' of autotrophic growth we considered to be a necessary step for maximum growth. After the autotrophic growth period, part of the cell solution in the jar was harvested, and the rest was diluted with the basal medium to a density of about PCV 1(ml/l). Then, the feeding medium containing 10% acetic acid in basal distilled. H_2O was added according to the measured decrease in pH. This was caused by cell growth and detected by a pH meter attached to a pH controller. The pH of the cell solution was set and held at 6.5, the temperature at 25°C, and the light intensity was controlled initially at about 5 Klux (24 *hr)* and, finally, at 10 Klux when the cell density became higher. Under these conditions, cells grew very fast, at a rate of about PCV 20 ml/l */day.* After 6 days' inoculation, the cell density was above PCV *80ml/l* (Fig. 4). However, the high-density solution could not be diluted again to induce the same maximum growth. As shown in Fig. 5, the second mixotrophic maximum growth curve of NT-11 reached to one-fourth of the first cell density. Light intensity and temperature were found to be important factors which affected algal growth. The mixotrophic growth of NT-11 cells under different light intensities and temperatures, in the jar-fermentor system, was compared (Fig. 6). As the figure shows, the optimal light intensity and temperature for mixotrophic growth were above 10 Klux up to 50 Klux and 25-30°C respectively. Figure 7 shows two other mixotrophic growth curves of other algal strains C-28 and C-212, under the same set of aforementioned procedures and conditions in the jar-fermentor system. It is evident that neither of them could grow as fast and reach as high a cell density as NT-11 could. Only the *Chlorella*strain NT-11 was influenced **by** such autotrophic-mixotrophic growth induction treatment.

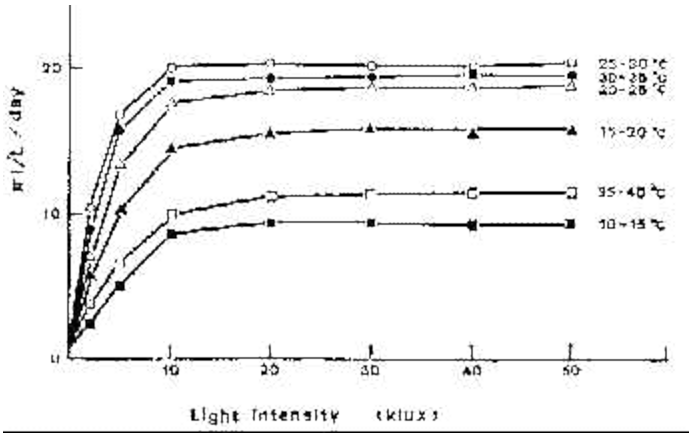


Fig. 6. The effect of light intensity and temperature on the mixotrophic growth rate of *C. pyrenoidosa* NT-11 in the jar-fermentor system.

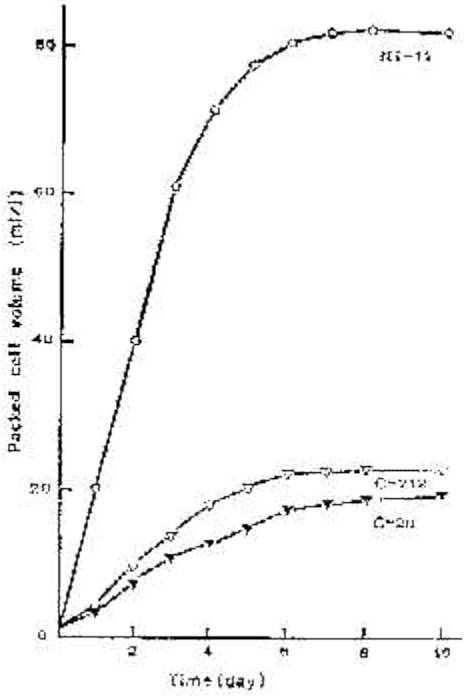


Fig. 7. The mixotrophic growth curves of three different *C. pyrenoidosa* strains in the jar-fermentor system.

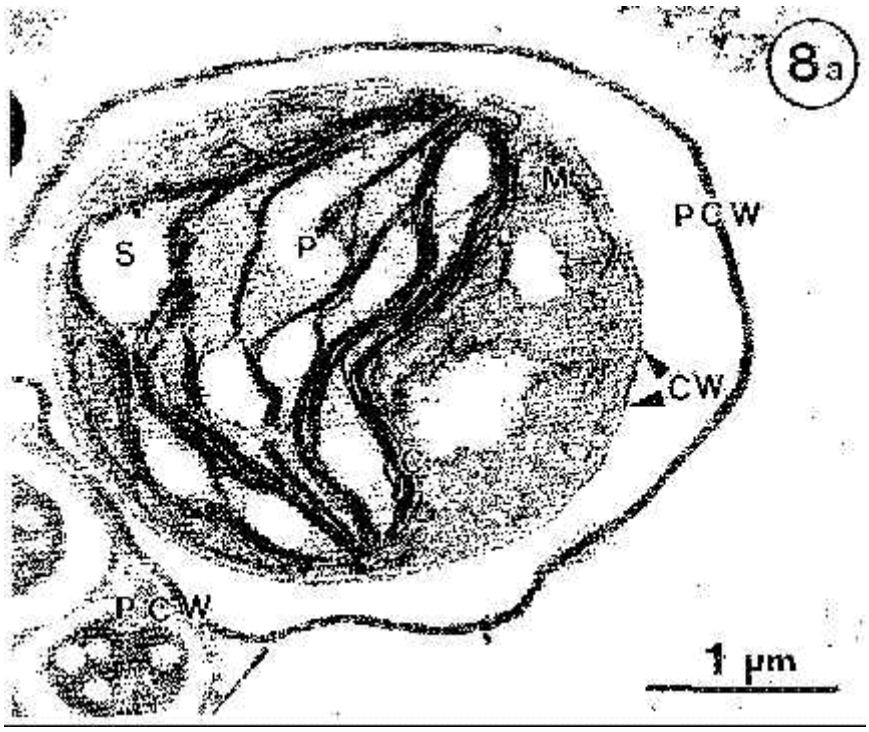
As indicated in Table 2, the compositions of the mixotrophic cells of strain NT11 incubated in the jar-fermentor system were similar to those in the Roux-bottle system, suggesting that the cells possessed equivalent nutritional value. There was, however, no significant structural difference between the two culture-type cells, as shown in Fig. 8.

The ultrastructure consisted of well-developed chloroplasts, nuclei, pyrenoids and mitochondria (Fig. 8a). Comparing it with the mixotrophically grown cells (Fig. 8b), there were no significant differences in the structures of organelles. Mixotrophically

TABLE 2
Cell compositions of C. pyrenoidosa NT-11 mixotrophically incubated in the Roux-bottle system and in the jar-fermentor system*

Component	Roux bottle	Jar-fermentor
Crude protein	58.2%	58.0%
Chlorophyll	3.8%	4.O%
Lipid	13.O%	13.2%
Ash	6.0%	6.5%
Heavy metal** (as Pb)	<1 ppm	<1 ppm
Arsenic	<0.1 ppm	<0. 1 ppm

Algal cells were harvested and freeze-dried for chemical analysis.
Total heavy metal contents are represented as the results of lead determinations.



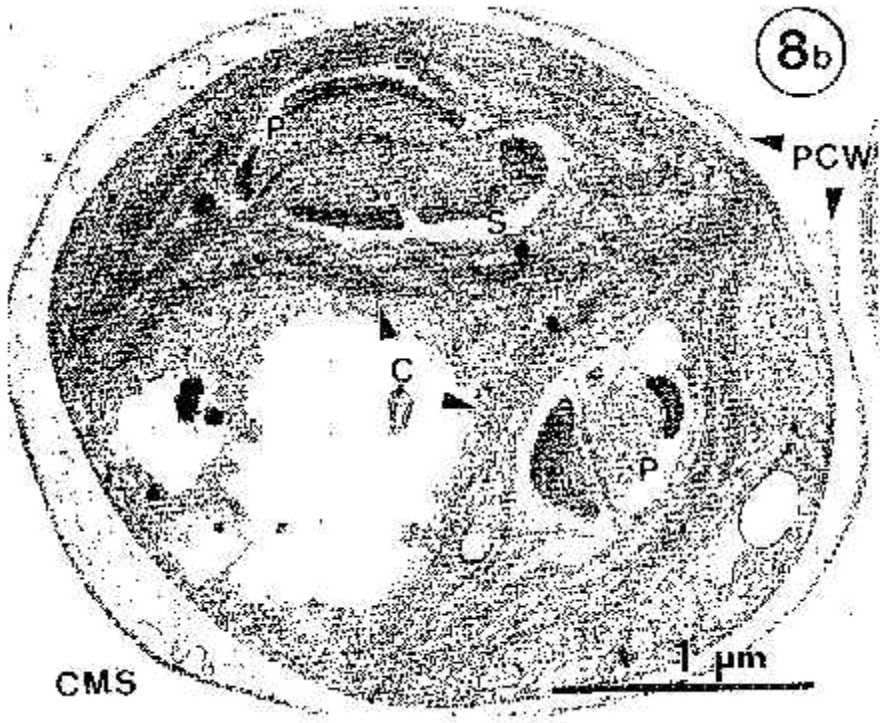


Fig. 8. Thin-sectioned electron micrographs of *C. pyrenoidosa* NT-11 cells: (a) autotrophic cells. Note a rather thick cell wall (CW) structure and a well-developed pyrenoid structure. (b) mixotrophic cells. Note scattered cellular materials (CMS) around the cells and a broken parental cell wall (PCW). Abbreviations, N: nucleus, S: starch materials, M: mitochondria, C: chloroplast.

grown cells in outdoor culture were described by Lin *et al.*[12];usually organic carbon sources induced faster cell division and secretion of mucoid materials around cells.

DISCUSSION

The rate of *Chlorella* growth is dependent on four main factors: (1) high light intensity, (2) temperature, (3) carbon sources, and (4) inorganic materials. The first and second factors are subject to both location and season in outdoor mass culturing; however, the other two factors can be controlled efficiently by culturing techniques.

In this experiment, we have succeeded in promoting mixotrophic growth of *C.* ***pyrenoidosa*** NT-11 by using acetic acid. In the initial growth period, the autotrophic growth rate in the Roux-bottle was very slow, even when supplemented with 5% CO_2 in air as the carbon source; then, even though acetic acid (as a carbon source) was supplied to proceed and enhance mixotrophic growth, the growth rate only increased from PCV 2.5 to 4*ml/l/day,* with a final density ranging from approximately PCV 12.5 to 27ml/l (Fig. 3). In our developed jar-fermentor system and procedures, the mixotrophic growth rate was significantly increased to PCV *20ml/l/day,* and the cell density reached PCV 80mlli, from an initial density of only PCV 1 ml/l (Fig. 4).

Firm evidence that *Chlorella* cells metabolize acetate was reported by Fujita(13), and the heterotrophic growth of *Chlorella* with acetate in a mass culture was possible("'. In our previous report[15], we examined the effects of cultural factors on the conversion of *C. pyrenoidosa* from an autotrophic to a mixotrophic culture.

Several points should be noted for the induction of the maximum growth of *Chlorella.* First, the incubation time of each preliminary stage should not be too long; e. g., 7-10 days is suggested for the slant culture stage, and 2-5 days for every autotrophic stage, especially for the autotrophic stage in the

jar-fermentor, which was considered to be indispensable for the maximum growth. Secondly, maximum growth can only be obtained shortly after the autotrophic stage in the jar-fermentor, as the slant culture could not be directly used to induce mixotrophic maximum growth, nor could the maximally grown cells have the same effect again. Finally, many other conditions such as light intensity, temperature, and the pH of the medium, are important though not the critical factors for the maximum growth. For example, light intensity must be high enough to complement the shielding effect under the very high cell density, but it should be maintained at a lower level at the beginning of the mixotrophic incubation when the cell density is still low. A similar result was also reported for other mass cultivated microalgae[16]. Cytologically, no obvious differences existed between autotrophically grown cells and mixotrophically grown cells. However, a thick cell wall was observed in autotrophic cells as compared to mixotrophic cells. As described in a previous paper[12], organelles existing in the mixotrophically grown cells of outdoor culturing ponds also possessed similar structures. Scattered cell surface materials were located around the mixotrophic growing cells. Whether these materials could have been produced because of a supplement of acetic acid is unknown. The later stage of wall formation was observed clearly as an inner layer of the autospore wall being formed with a ruptured layer of the parental walls. A similar result was reported by Atkinson *et al*[17]*.* As described in our previous report[18], a thorough understanding of*Chlorella* cells from chemical and structural analysis would be of great value for improving chemical processes for microalgal health foods to obtain better quality. The analytical results from present studies have indicated that mixotrophically grown cells possess suitable qualities for use as health foods. In conclusion, the applications of microalgal products in many fields, such as in the production of fine chemicals, biochemicals, medicals, etc., have made research into the mass-culture of microalgae promising, including our present study on the maximum output rate of *Chlorella.* As reported by Benemann *et al.*[19]*,* the future of microalgal culture in the jar-fermentor system could be a combination of industrial fermentation and agricultural cropping (photosynthesis), which would make these applications more feasible.

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REFERENCES

1. A. Richmond: Microalgae of economic potential. In *"CRC Handbook of Microalgal Mass Culture",* Ed. A. Richmond, CRCPress, Inc. Boca Raton, Florida. pp. 199-243 (1983).
2. **T.** Oh-Hama and S. Miyachi: *Chlorella. In "Microalgal biotechnology",* Ed. M.A. Borowitzka and L.J. Borowitzka, Cambridge University Press, Cambridge. pp. 1-26 (1988).
3. H. Tamiya, E. Hase, K. Shibata, A. Mituya, **J.** lwamura, T. Nihei and T. Sasaf: Kinetics of growth of *Chlorella,* with special reference to its dependence on quality of available light and on temperature. In *"Algal Culture-from Laboratory to Pilot Plant",* Ed. J.S. Burlen, Carnegie Inst. Wash. Pub., Wash. D.C. pp. 204-232 (1953).
4. H. Endo, H. Sanawa and K. Nakajima: Studies on *Chlorella regulars,* heterotrophic fast-growing strain II. Mixotrophic growth in relation to light intensity and acetic acid concentration. Plant *& Cell Physiol.* 18: 199-205 (1977).
5. H. Endo, K. Nakajima, R. Chino and M. Shirota: Growth characteristics and cellular components *for Chlorella regularis,* heterotrophic fast-growing strain. *Agr. Biol. Chem.* **38:** 9-18 (1974).
6. **C.** Sorokin and R.W. Krauss: The effect of light intensity on the growth rates of green algae. *Plant Physiol.* 33: 109-113 (1958).
7. S.W. Huang and L.P. Lin: Nutrient requirements on the growth of cultivated edible microalgae for mass production. ***J.*** Chinese Agri. Chem. Soc. 19: 208-217 (1981).
8. **C.** Sorokin: Dry weight, packed cell volume and optical density. In *"Handbook of Phycological Methods",* Ed. J. R. Stein, Cambridge University Press, Cambridge. pp. 321-343 (1973).
9. APHA, AWWA and WPCF: Standards for examination of water and wastewater. 16th ed. (WPCF, Washington, DC). pp. 411-412 (1985).

10. F. A. Richard and T. G. Thompson: The estimation and characterization of plankton population by pigment analysis. 11. A spectrographic method for the estimation of plankton pigments. J. *Mar. Res. 11:* 156-172 (1953).

11. J. Barber: Measurement of ionic content and fluxes in microalgae. In *"Handbook of Phycological Methods",* Ed. J. A. Hellebust and J. S. Craigie, Cambridge University Press, Cambridge. pp. 449462 (1978).

12. L. P. Lin, V. C. Liao and H. C. Chen: Ultrastructural cytology of the cultivated green algae *Chlorella pyrenoidosa. Memoir Coll. Agri., Natl. Taiwan Univ.* **20:** 86-100 (1980).

13. K. Fujita: The metabolism of acetate in *Chlorella cells. J. Biochem.* 46: 253-268 (1959).

14. H. Hosoya and T. Koibuchi: Growth yield of *Chlorella regularis* in dark-heterotrophic continuous culture using acetate. ***J.*** Ferm. Techniol. 55: 369-379 (1977).

15. S. W. Huang and L. P. Lin: Effects of the cultural conditions on conversion of *Chlorella pyrenoidas* from autotrophic to mixotrophic culture.***J.*** Chinese Agr. Chem. Soc. **21:** 71-81 (1982).

16. A. Vonshak, A. Abeliovich, S. Boussiba and A. Richmond: Production of *Spirulina* biomass: Effects of environmental factors and population density. *Biomass* 2: 175-181 (1982).

17. A. W. Atkinson Jr., B. E. S. Gunning and P. C. L. John: Sporopollenin in the cell wall of *Chlorella* and other algae: Ultrastructure, chemistry and incorporation of "C-acetate, studied in synchronous cultures. *Planta* 107: 1-32 (1972).

18. L. P. Lin: Microstructure of spray-dried and freeze-dried microalgal powders. *Food Microstructure* 4: 341-348 (1985).

19. J. R. Benemann, D. M. Tillett and J. C. Weissmann: Microalgae biotechnology. *Trends Biotechnol.* **5:** 47-53 (1987).