Fat Synthesis in Unicellular Algae.

Part I. Culture Conditions for Fat Accumulation in *Chlorella* Cells.

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The cultural conditions of *Chlorella ellipsoidea* were studied with special reference to lipide accumulation. It was ascertained that there is a close relation between the lipide content and the nitrogen content of cells. Cultivation with the nitrogen-free medium was applied to produce nitrogen deficient cells, and in this culture were studied the effects of light intensity, algal density and depth of algal suspension upon lipide accumulation. The annual yield of fat by mass-culture was roughly estimated.

The first investigation on the production of fat by the cultivation of unicellular algae was made in Germany during the last War, using diatom as the test organism. After the War, Spoehr and Milner¹⁾ found that *Chlorella pyrenoidosa* accumulates considerable amounts of fat in its body grown in the nitrogen deficient medium. At that time *Chlorella* was attracting world-wide attention as a new source of protein, and consequently it was also noticed as a fat producer and some discussions were made^{2, 3)}.

Fogg and Collyer⁴⁾ examined the characteristics of several species of green algae, and blue-green algae, including *Ch. vulgaris*, with reference to the accumulation of fat, and demonstrated that *Ch. pyrenoidosa* has overwhelming ability to accumulate fat, compared with all the other algae examined. The authors also examined *Ch. ellipsoidea* and obtained almost the same results as *Ch. pyrenoidosa*. And then some quantitative

investigations on the fat accumulation of *Ch. ellipsoidea* were pursued, while no quantitative investigation concerning the relation between the degree of fat accumulation and the light intensity or other environmental conditions had ever been made.

Experimental methods

The culture apparatus is shown in Fig. 1. The culture flask was a flat bottle of 3 cm thickness and of 700 ml. capacity, which was originally used by Tamiya et al⁵⁾. The culture bottle was immersed in a glass water bath to be maintained at 25°C, and was illuminated with a 300 W or 500 W flood lamp on one side from the outside of the water bath. The light intensity was regulated by changing the voltage or the distance between the lamp and the bottle. The illuminated area per 500 ml. medium in the bottle was 170 cm². In the initial stage of the experiments, four fluorescent lumps (white) per one side of the bottle were used instead of a flood lamp.

Five to four percent CO₂ rich air was used in aeration and the aeration rate was 500 ml. per 500 ml. medium per minute.

The composition of the basal medium was as follows:

KNO ₃	0.025 mol/l
MgSO ₄ ·7H ₂ O, KH ₂ PO ₄	0.01 mol/l

5) H. Tamiya, et al, *ibid*, Chap. 16, p. 204.

1) H. A. Spoehr and H. W. Milner, *Plant Physiol.*, **24**, 120 (1949).

2) F. Gummert, *Fette u. Seifen*, **52**, 453 (1950).

3) H. W. Milner, *J. Am. Oil Chem. Soc.*, **28**, 363 (1951).

4) G. E. Fogg and D. M. Collyer, "Algal Culture", Carnegie Inst. Washington Pub. 1953, Chap. 12, p. 177.

Fig. 1. Culture Apparatus.

Micro-elements*

Fe 10 ppm; B, Zn 4 ppm; Mn,
 Mo 0.8 ppm; Cu, Co 0.2 ppm
 Ethylenediamine tetraacetic acid 0.1g/l
 pH 5.5

When the nitrogen-free medium was used, the nitrogen source was just omitted from the basal medium.

Algal concentration was measured by the dry weight of algae in 10 ml. of algal suspension, drying for 24 hrs. at 100°C after centrifuging and washing twice by distilled water.

Semi-micro Kieldahl method was used for nitrogen determination, and Haskin's method⁶⁾ was applied to chlorophyll determination. Crude fat was determined by ether extraction. Using algal cells kept in a vacuum desiccator after dried on a tonplate, ether-extraction was carried out several times, inserting the process of grinding the algal cells in a mortar with emery-powder. This procedure is necessary for complete extraction.

Results and Discussions

Milner⁷⁾ once obtained *Chlorella* cells

* The following compounds were used: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, H_3BO_3 , $\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$.

⁶⁾ H. H. Haskin, *J. Biol. Chem.*, **144**, 149 (1942).

⁷⁾ H. W. Milner, *J. Biol. Chem.*, **176**, 813 (1948).

which contained lipides in 20–85% of dry matter. In this case, in order to obtain the *Chlorella* cells containing lipides in 75.5% of dry matter, he used a culture medium of low nitrogen concentration (N: 0.0025 mol/l, equivalent to the algal density 0.5 g/l, containing the total nitrogen in 7% of dry matter), and cultivated in Fernbach flasks, illuminating them from the bottom with 200 W tungsten lumps, for 75 days. The final algal concentration was reported to be 2.5 g/l and the total nitrogen of the cells was 1.23%. The normal *Chlorella* cells which can be obtained by culturing in a medium containing sufficient nitrogen source, contain total nitrogen in 7–8% of dry matter. So it is clear that the cells of high lipid content are of low nitrogen content compared with the normal cells.

The authors also tried to obtain nitrogen deficient cells by culturing in the media of diverse degrees of nitrogen deficiency, starting cultivation with a very small inoculum size. The result is shown in Table I. From this result, it is quite conceivable that *Chlorella* can grown in a completely-nitrogen-free medium and accumulates lipid during the growth. And it is also quite possible that the initial algal concentration, the depth of the medium and the light intensity will have influence on the final degree of nitrogen deficiency of the cells. Milner³⁾ reported that he had never obtained *Chlorella* cells of lipid content higher than 30% when residual nitrogen in the medium was more than 0.001 mol/l. the authors pursued the following experiments, having cultivated in a nitrogen-free medium with the inoculum of washed normal cells which had been cultivated in advance in the basal medium. As the result, it is quite clear that *Chlorella* can

Table I
Effect of the Nitrogen Source Concentration on Lipide Accumulation.

No.	1	2	3
A) KNO_3 (mol/l)	0.01	0.0025	0.00025
B) Maximum density to be reached by normal cells* (corresponding to A)	2.0 g/l	0.5	0.05
C) Inoculum size ($\times$ B)	1/50	"	"
D) Final density	6.98 g/l	3.35	0.39
E) Culture length (Days)	15	10	6
F) D/B	3.49	6.70	7.90
G) Lipide %	58.5	79.0	—

* Contain 7% total nitrogen.

Light intensity: 4000 lux on both sides of the flask.

grow and multiply remarkably, even in a completely nitrogen-free medium.

The difference of the final algal concentration, consequently that of the degree of nitrogen deficiency reached, corresponding to the different initial algal concentrations, is shown in Table II. The light intensity and the depth of culture are fixed in this experiment.

In culture No. 1, starting with the initial algal concentration of 4.58 g/l, the final algal concentration reached in ten days was only twice as high as the initial one, while in culture No. 7, starting with the initial concentration of one sixtyfourth of that of No. 1, namely 0.07 g/l, the final concentration reached after only four days was 11.4 times as high as the initial one. In the latter case, the lipide content was 84.5%. When culture No. 1, which had already reached maximum growth, was diluted with fresh nitrogen-free medium to one eighth and cultivation was continued, the growth curve after dilution showed quite a similar curve as culture No. 4 whose inoculum size was one eighth of No. 1, and the final concentration was 2.92 g/l, almost the same value as that of No. 4, 2.68 g/l.

This phenomenon is to be explained

by the fact that the amount of light energy absorbed by each cell in unit time is larger after dilution than that before dilution. It was ascertained that^{5, 8, 9)}, in the case of normal cells, the total increase of mass by photosynthesis per unit area is constant irrespective of the depth of culture and the algal concentration, if the incident light intensity is fixed and the light is completely absorbed by the suspension. Whereas if the light intensity is too weak, or if the algal density is too thick, it may happen that the net increase of mass does not correspond to the incident light energy, as the decrease of mass due to respiration becomes competitive to the increase due to photosynthesis⁹⁾.

The same phenomenon appears to occur in a greater extent in the case of nitrogen deficient cells, even when the light intensity is not so weak and the total algal mass per unit area is not so large. Considering the fact that further growth can be brought about by dilution even when the culture has reached maximum growth and does not show any more growth owing to the equilibrium described above, it seems that light energy

8) Y. Fujimoto, H. Iwamoto, A. Kato and K. Yamada This Bulletin, in print.

9) J. Myers, "Algal Culture", Carnegie Inst. Washington Pub. 1953 Chap. 4, p. 37.

Table II
Effect of the Initial Concentration of Algae on Nitrogen-free Growth.

No.	A Initial conc. g/l	B Final conc. g/l	C Culture length (Day)	D B/A	E Total N. %	F Lipide %
1	4.58	9.21	10	2.0	4.08	34.2
2	2.29	6.40	9	2.8	2.91	50.6
3	1.14	4.19	8	3.7	2.20	56.4
4	0.57	2.68	7	4.7	1.74	—
5	0.28	1.58	6	5.6	1.46	74.0
6	0.14	1.21	5	7.1	0.95	—
7	0.07	0.80	4	11.4	0.72	84.5

Light intensity: 4000 lux on both sides of the flask.

efficiency is very low in a nitrogen deficient cell compared with a normal cell. It was also mentioned by Milner³⁾ that a stronger light is required to obtained nitrogen deficient cells, while normal cells are able to grow even in a weak light.

In the case of culture No. 7, no further growth after dilution was observed. And so, it is believed that No. 7 had already reached the highest nitrogen deficiency which allowed no further growth. In this case the total nitrogen of the cells was 0.72%, in other words, the crude protein content was as small as 4.5%.

It is also conceivable that, if the depth of culture is varied, the final algal concentration will differ, even though both the initial concentration and the light intensity are not varied. This is true as indicated in Table III, the deeper culture showing the lower final concentration.

Further, it is quite reasonable to think that, if both the final concentration and the depth of culture are the same, the stronger the light intensity, the greater the growth rate is and the higher the final concentration would be reached. But the experiments showed, on the contrary,

Table III
Effect of the Depth of Culture on Nitrogen-free Growth.

No.	A Initial conc. g/l	B Final conc. g/l	C Depth. cm	D Culture length. day	E B/A
1	0.52	1.77	3.0	11	3.5
2	0.57	2.68	1.5	7	4.7

Light intensity: 4000 lux on one side of the flask.

Table IV
Effect of the Light Intensity on Nitrogen-free Growth. (I)

No.	Incident light. kilolux	Initial conc. g/l	Final conc. g/l	Culture length. day	Total N. %	Lipide. %
1	5	1.0	4.90*	18	—	—
2	10	1.0	5.05	18	1.56	63.7
3	30	1.0	4.27	18	1.81	55.0
4	50	1.0	3.97	18	2.02	50.6

* not maximum concentration.

Table V
Effect of the Light Intensity on Nitrogen-free Growth. (II)

No.	Incident light. kilolux	Initial conc. g/l	Final conc. g/l	Culture length. day	Total N. %	Lipide. %
1	10	1.75	6.00*	10	2.33	53.2
2	50	1.75	5.63*	10	2.49	51.0

* not maximum concentrations.

that not the stronger light but the weaker light results in the higher final concentration. It is seen, however, that the culture in the stronger light grows faster in the very initial stage than that in the weaker light. Tables IV and V, and Fig. 2 and 3 show these results.

As is seen in Fig. 3, the curves representing the potentiality in growth of the whole culture at every moment during cultivation, corresponding to the light intensities, 10 and 50 kilolux, intersect at

Fig. 2. Growth Curves in the Nitrogen Deficient Medium at Various Light Intensities.

Fig. 3. Degradation of Photosynthetic Capacity of Nitrogen-free Culture.

the point of about 60% nitrogen deficiency. And the final concentration reached at 10 kilolux is higher than that at 50 kilolux as shown in Fig. 2 (upper curves). This is indicated more clearly by the lower curves which give a comparison of the light intensities 5, 10, 30 and 50 kilolux.

These results can be explained by the difference in the degree of chlorosis which occurs along with the progress of nitrogen deficiency of cells corresponding to the difference in the light intensity. The chlorophyll content of *Chlorella* cells grown in a nitrogen rich medium and in an algal suspension of very low algal density, is much smaller in the case of the light intensity higher than 5–10 kilolux than in the case of the lower light

intensity¹⁰⁾. This phenomenon, however, is not observed in a very dense culture. But in the case of nitrogen deficient cells, it is seen, even in a very dense culture. In Fig. 4, is shown the correlation between the degree of chlorosis and the degree of nitrogen deficiency during the growth at two light intensities, 10 and 50 kilolux, in the nitrogen free culture, having started with the algal density of 2 g/l. B represents 10 kilolux and C 50 kilolux. Supposed that chlorophyll is neither newly synthesized nor decomposed during the nitrogen-free growth, the curve is to be A. From Fig. 4 it is quite clear that the degree of chlorosis is much larger at 50 kilolux than at 10 kilolux and this difference grows larger with the progress of nitrogen deficiency. This is considered to be the reason why photosynthetic capacity becomes smaller at stronger light intensity that at a weaker one in the later stage of cultivation.

Fig. 4. Change of the Degree of Chlorosis during Nitrogen-free Cultivation.

Finally, the relation between the nitro-

10) Y. Fujimoto, H. Iwamoto and T. Ishii, *This Bulletin*, in print.

gen content and the lipide content of *Chlorella* cells is shown in Fig. 5, which indicates a very close relation between them.

Fig. 5. Correlation between Nitrogen Content and Lipide Content of *Chlorella* cells.

Now, let us estimate the yield of fat on the basis of mass-production, calculating from Figs. 2 and 5. The yields of fat per 24 hours per square meter at various light intensities, and for various initial algal concentrations and date of harvest, are shown in Table VI. The time taken in producing the normal cells which is used in starting the nitrogen-free culture was taken into consideration in preparing this table. The percentage on the lower lines indicates the presumptive fat content on the harvest day.

It is interesting that there are no remarkable differences in the yields between the different conditions. The highest yield among them is 12 g/m²-day, which is obtained in the case of 50 kilolux, five days

Table VI
Yield of Lipide, g/m², 24 hrs.

Incident light (kilolux)		5		10		50	
Culture length (day)		5	10	5	10	15	10
Initial conc. (g/l)	1.0	5.2 (44%)	5.6 (59%)	8.4 (54%)	7.6 (64%)	6.1 (67%)	5.9 (56%)
	1.75			8.8 (45%)	7.8 (53%)	12.0 (47%)	7.8 (51%)

Depth of culture: 3 cm.

Temperature: 25°C.

of culture length and 1.75 g/l of initial concentration.

The annual yield per acre is estimated as 4.9 ton on the basis of the daily yield, $(8 \times 10^4) \text{ g/m}^2\text{-day.}$, which is roughly considered as the average daily yield, under the conditions that each day has ten hours of illumination period of 10–50 kilolux of incident light intensity and the initial algal density is 1.0–2.0 g/l and the depth of culture layer is about 3 cm and the culture length is 5–10 days. This value might be much greater than the actual yield expected in the operation of outdoor culture, but it is believed to give a general standard. Moreover we should say that *Chlorella* gives an overwhelming high yield of fat compared with soybean oil or peanut oil, the former being about 100 kg/acre-year and the latter about 150 kg/acre-year.

Summary

In order to examine the possibility of mass-cultivation of *Chlorella* for the purpose of fat production, the cultural conditions for fat accumulation were studied,

Chlorella ellipsoidea being used. It was seen that *Chlorella* grew in the nitrogen-free medium, accumulating lipide in its cells, and that the accumulation degree was greatly affected by the initial algal concentration, the depth of the suspension and the incident light intensity. In order to obtain cells of a high lipide content, it was required that the initial density is low and the layer of the suspension thin. As for the light intensity, it was found that a stronger light has a stronger retarding effect on the nitrogen-free growth in its later stage than a weaker light over 5–10 kilolux. This is explained to be due to more severe chlorosis caused by the stronger light. The presumptive yield of fat by mass-culture was roughly estimated as 4.9 ton per acre per year under favourable conditions.

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