CULTURAL AND PHYSIOLOGICAL STUDIES OF THE NITROGEN FIXING BLUE-GREEN ALGA, NOS TOC SPONGIAEFORME AG. I. THE MODIFICATION OF THE CULTURAL SOLUTION

CULTURAL AND PHYSIOLOGICAL STUDIES OF THE NITROGEN FIXING BLUE-GREEN ALGA, *NOSTOC SPONGIAEFORME* AG. I. THE MODIFICATION OF THE CULTURAL SOLUTION

By

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Blue-green algae are curious organisms which have the ability to carry out photosynthesis and fixation of elementary nitrogen at the same time. Therefore, it is important to pay attention to their physiology to clarify the mechanism of photosynthesis and nitrogen fixation. In the studies of photosynthesis, the green algae *e.g. Chlorella* and *Scenedesmus* were used. The photosynthetic behavior of the blue-green algae which contains phycocyanin in addition to Chlorophyll are hardly known.

This special organism may differ widely from *Azotobacter* and *Rhyzobium* in the behavior of elementary nitrogen fixation.

It is worthy to give attention to their physiology for the maintenance of soil fertilities in the paddy field as the fixed nitrogen by the algae is supposed to be the most important source of fertility and from the standpoint of utilization of algae for the food or feed.

Therefore, it is very important to treat these curious organism from many standpoints, and thus, both physiological and biochemical studies were made. Since it is most important to find a method for mass culture during the study, the experimental studies on the mass culture were also done in relation to cultural conditions.

Materials and Methods

This experiment was carried out to find the conditions of the mass culture and to investigate their physiology.

At first, the cultural solutions, which were proposed up to the present time were examined in detail. With regard to the composition of the cultural solution, Bortels (1) supposed that molybdenum is important for the fixation of elementary nitrogen or as growth promoting factor, and propped

to add molybdenum besides macro-elements. Chu (2, 3) devised new cultural solutions after a detail investigation on the mineral contents of cultural solution, and his No. 10 solution is famous for obtaining good results. Later, microelements were added to the basic solution. Jacobson (5), Myers (6) and Rodhe (4) reported that chelating substances were effective. Moreover, De (7), Allison *et. al.* (9, 8) and Watanabe (10) made their own cultural solution.

No. 101 solution (Table 1) devised by Katagiri in our laboratory was used as a standard solution and after many experiments it was modified. As the algal strain for the inoculation to the cultural solution, 12-D-A, isolated by Ishizawa, was used, and 2–3 times of the platiniumneedles of the alga were inoculated. This alga was identified as *Nostoc spongiaeforme* AG. by Negoro.

Table 1. No. 101 solution.

$Ca(NO_3)_2 \cdot 4H_2O$	0.040 g/*l*
K_2HPO_4	0.010 ,,
$MgSO_4 \cdot 7H_2O$	0.025 ,,
Na_2CO_3	0.020 ,,
Ferric citrate	0.003 ,,
Citric acid	0.003 ,,
H_3BO_3	1.43 mg/*l*
$MnSO_4 \cdot H_2O$	1.05 ,,
$ZnCl_2$	0.05 ,,
$CuSO_4 \cdot 5H_2O$	0.04 ,,
$H_2MoO_4 \cdot H_2O$	0.01 ,,

100 *ml* of No. 101 solution was applied in a 20 *ml* Erlenmeyer flask and autoclaved at 15 lbs for 10 min.

Considering the change of hydrogen ion concentration by the autoclaving, PH was adjusted to 9.0–9.5 before sterilization by the appropriate addition of N/2 NaOH.

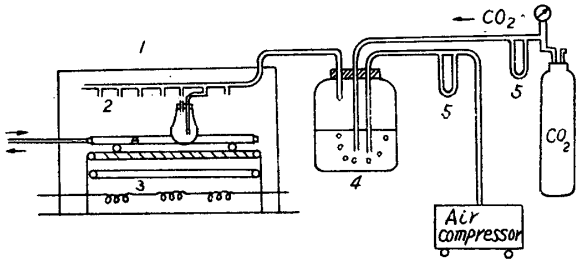

Fig. 1. Cultural equipment.
1. Thermostat
2. Branch tube
3. Fluorescent lamp
4. Gas mixing bottle in which gasses are cleaned and humidity added
5. Gas flow metre

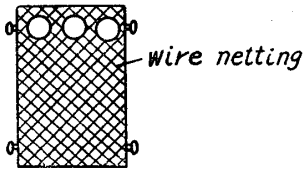

Fig. 2. A.

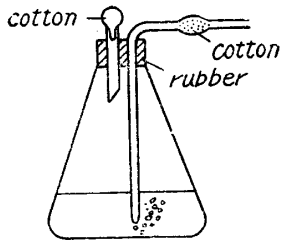

Fig. 3. Cultural flask.

An illuminating, reciprocating, and aerating shaker in a chamber of 32°C was adopted (Fig. 1, 2, 3 and 4). Illumination; *ca.* 4000 lux, 24 hrs/day by the fluorescent lamps. Reciprocation (shaking); 8 cm amplitude, 90 times oscillation/min. Aeration; 0.25 *l* air containing 3 per cent CO_2/*l* media/min.

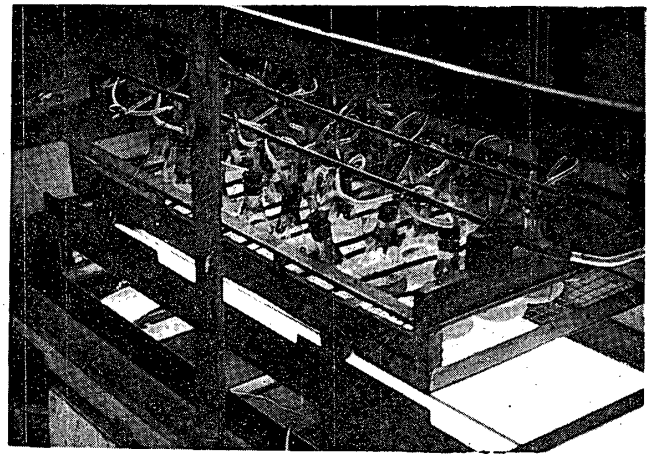

Fig. 4. Cultural equipment.

The dry weight of the algal cells were used as a measure of the growth rate. The dry weight was measured after centrifuging at 4000 rpm and separating of the supernatant solution. Nitrogen was estimated both in the algal cells and the supernatant.

PH and the residual P and K was examined using the supernatant, but especially in the case of Cu and Zn experiments, pH was measured before centrifuging on account of some experimental processes.

Results

(I) *The deficient cultures in relation to each macroelement.*

No. 101 solution was used as the standard complete group and $-N$, $-P_2O_5$, $-K_2O$, $-MgO$, $-CaO$, $-Fe$ and $-Na_2CO_3$ cultures were made from it.

Their cultural results are shown in Table 2.

Table 2. The deficient cultures in relation to each macroelement (result).

Treatments	Dry wt. mg	Fixed N mg	N in sol. mg	N in algae		Final pH	Absorbed quantity %			Fe	N.B.
				mg	%		Total quantity				
							P_2O_5	K_2O	etc.		
1. Complete	179.1	16.00		16.61	9.27						Uncentrifuged
2. -N	156.3	13.41	1.43	11.98	7.67	7.45	97.5	90.5	CaO 95 MgO 83	—	
3. $-P_2O_5$	Died										
4. $-K_2O$	Almost died										
5. -MgO	Little growth										
6. -CaO											
7. $-Na_2CO_3$	81.5	5.69	0.89	5.41	6.64						
8. -Fe-citrate	58.2	4.83	1.16	4.28	7.35						

Ca, Fe and $SO_4^=$ showed negative in the qualitative analysis as to the supernatant of normal growth, therefore it is supposed that these elements might be the limiting factors for the growth in the No. 101 solution. In the −N culture, growth was almost equal to the complete group and in −P, −K, −Mg and −Ca cultures, the algal growth was strongly affected by the deficiency of each element. Thus P, K, Mg and Ca are highly essential elements of the algal growth. Fe and Na_2CO_3 are considerably important factors for the algal growth as their deficiency brought about insufficient growth.

(2) *Experiment in relation to the increasing amounts of the essential macroelements.*

Considering from the results of Ex. 1, growth experiments were performed in the increasing amounts of each essential macroelement of No. 101 solution, and their results are shown in Table 3.

Table 3. Experiment in relation to the increasing amount of the essential macroelements.

Treatment	Dry wt. mg	Fixed N mg	N in sol. mg	N in algae mg	N in algae %	Final pH	Absorbed quantity % Total quantity P_2O_5	Absorbed quantity % Total quantity K_2O	Absorbed quantity % Total quantity etc.	Fe	N.B.
1. N.P.K. Ca.Mg×10	78.2	0	0.89	5.23	6.69	7.40	46.0	40.0		+	
2. ,, -N	115.6	7.83	0.74	7.07	6.13	6.60	17.5	56.0		−	
3. ,, ×5	169.6	8.35	1.12	10.29	6.09	7.45	60.5	68.0	CaO 95.0 MgO 78.0	−	
4. ,, -N	117.1	6.67	1.36	5.31	4.53	7.45	36.0	78.5	CaO 95.0 MgO 52.0	−	
5. ,, ×2.5	230.3	13.66		15.19	6.60						Uncentrifuged
6. ,, -N	187.6	13.12		13.12	6.99						Uncentrifuged
7. No. 101	165.5	9.98	1.43	9.16	5.53	7.60	89.5	87.5		−	
8. ,, -N	142.3	13.31	2.25	11.06	7.78	7.55	73.5	81.5		−	
9. P×2.5	104.4	4.79	0.76	4.64	4.44	6.95	42.5	60.5		−	
10. ,, -N	99.4	5.68	0.94	4.74	4.77	7.00	39.5	62.0		−	
11. K×2.5	137.3	9.24	1.63	8.22	5.99	7.30	58.5	75.5	CaO 95.0	−	
12. ,, -N	98.4	6.83	0.53	6.30	6.40	6.85	57.0	53.0		+	
13. Ca×2.5	162.8	12.95	1.79	11.77	7.23	7.40	72.5	90.5	CaO 95.0	−	
14. ,, -N	148.8	12.47	2.38	10.09	6.78	7.35	68.5	80.5		−	
15. Mg×2.5	210.1	15.01		14.48	6.89						Uncentrifuged
16. ,, -N	134.2	13.19	1.58	11.61	8.66	7.45	73.5	78.5		−	Uncentrifuged

The heaviest growth was obtained in No. 5, 6 and 15, hence, 2.5 fold concentration of N, P, K, Ca and Mg compared with No. 101 solution were adopted in the following experiments.

(3) *Exprimentals in relation to the increasing amounts of microelements (The deciding experiment of the concentration).*

The cultural experiments were carried out in the increasing amounts (0, 1, 2, 5, 10, 50 and 100 times) of the each microelement (Mn, B, Mo, Zn and Cu.) contained in the No. 101 solution. The results of the experiments

on each element are described in turn.

1. Manganese

Table 4. Mn experiment result.

Group		Treatment	Dry wt. mg	Fixed N mg	N in sol. mg	N in algae mg	N in algae %	Final pH	Absorbed quantity % Total quantity P_2O_5	Absorbed quantity % Total quantity K_2O	Fe	N.B.
1.	Complete	×100	103.8	8.79	1.81	8.51	8.19	7.15	69.5	73.5	—	
2.		× 50	110.4	8.57	1.40	8.44	7.64	7.00	75.5	66.0	—	
3.		× 20	166.9	13.66	1.54	13.67	8.18	7.50	73.5	78.0	—	
4.		× 10	220.1	15.79		17.32	7.86					Uncentrifuged
5.		× 5	186.9	13.44	1.53	14.97	8.00	7.60				
6.		× 2	157.9	10.32	0.91	10.32	6.53	7.70	67.5	70.5	—	
7.		× 1	143.3	8.57	1.13	9.19	6.41	7.10	83.0	67.0	—	
8.		× 0	138.4	7.12		7.52	5.43		63.5	75.5	—	
9.	-N	×100	111.2	8.04	0.88	7.16	6.43	6.75	77.5	81.0	—	
10.		× 50	139.6	10.14	1.00	9.14	6.54	7.40	72.5	79.5	—	
11.		× 20	176.5	15.78	1.85	13.93	7.89	7.10	83.5	76.5	—	
12.		× 10	195.3	16.39		16.39	8.39					Uncentrifuged
13.		× 5	183.2	10.43		10.43	5.69					
14		× 2	204.2	17.14		17.14	8.39					
15.		× 1	151.5	13.43	1.92	11.51	7.59	7.40	65.0	81.0	—	
16.		× 0	140.2	11.87	1.63	10.24	7.30	7.05	89.0	69.5	—	

As illustrated in Table 4, preeminent growth in the complete nutrient group were obtained in 5–10 fold concentration of No. 101 solution content and for the −N group in 2–20 fold concentration of No. 101 solution. Then, 10 fold of No. 101 solution which showed steady growth in both groups were adopted for the standard and the following B. experiment was performed.

2. Boron (Table 5)

Table 5. B experiment result.

Group		Treatment	Dry wt. mg	Fixed N mg	N in sol. mg	N in algae mg	N in algae %	Final pH	Absorbed quantity % Total quantity P_2O_5	Absorbed quantity % Total quantity K_2O	Fe
1.	Complete	×100	87.5	5.09	0.94	5.68	6.49	7.50	61.5	62.5	—
2.		× 50	89.1	5.98	0.47	7.04	7.90	8.20	60.5	78.0	—
3.		× 20	168.6	9.04	1.25	10.32	6.12	7.50	65.5	89.0	—
4.		× 10	143.6	6.60	0.83	7.30	5.08	7.15	87.5	67.0	—
5.		× 5	175.0	14.10	1.23	14.48	8.27	7.10	79.5	66.0	—
6.		× 2	158.8	7.04	0.98	7.59	4.77	7.25	83.0	63.0	—
7.		× 1	122.2	6.94	0.42	8.05	6.58	7.55	77.5	73.5	—
8.		× 0	119.5	6.35	0.74	7.14	5.97	7.35	65.5	73.5	—
9.	-N	×100	96.5	5.55	0.41	5.13	5.36	7.40	47.0	63.5	—
10.		× 50	112.5	6.86	0.53	6.33	5.62	7.45	63.5	59.0	—
11.		× 20	123.4	8.74	1.44	7.30	5.91	7.50	71.5	61.0	—
12.		× 10	122.6	7.77	1.11	6.66	5.44	7.60	79.0	78.5	—
13.		× 5	140.5	9.36	0.84	8.52	6.06	7.40	77.0	67.0	—
14.		× 2	126.6	8.64	1.49	7.15	5.64	7.25	74.0	70.0	—
15.		× 1	123.6	8.94	1.22	7.72	6.24	7.45	76.0	73.0	—
16.		× 0	102.8	7.19	0.41	6.78	6.59	6.90	52.0	71.5	—

The alga grew vigorously at 2–20 fold concentration in the complete nutrient group and 5 fold concentration in −N group. Therefore, 5 fold concentration of No. 101 solution content was adopted in the following experiments. But, in this culture, growth was generally insufficient but the reason was not clear.

It seemed that Fe was a somewhat limiting factor of such case.

3. Molybdenum (Table 6)

Excellent growth was atained at 5–20 fold concentration (of No. 101 solution content) in the complete nutrient group and 5 fold concentration in −N group. So the 5 fold concentration was adopted.

Table 6. Mo experiment result.

	Group	Treatment	Dry wt. mg	Fixed N mg	N in sol. mg	N in algae mg	N in algae %	Final pH	Absorbed quantity % Total quantity P_2O_5	Absorbed quantity % Total quantity K_2O	Fe	N.B.
1.	Complete	×100	77.1	5.21	1.03	5.71	7.41	6.90	81.5	49.0	—	
2.		× 50	108.2	9.82	1.18	8.17	7.55	7.45	73.5	55.5	—	
3.		× 20	137.3	11.38	1.65	11.26	8.20	7.10	78.5	75.5	—	
4.		× 10	156.7	12.04		13.57	8.65					Uncentrifuged
5.		× 5	142.9	11.08	1.13	11.49	8.03	7.45	80.0	76.5	—	
6.		× 2	130.2	9.48	0.89	10.11	7.76	7.45	77.5	73.0	—	
7.		× 1	125.2	9.81	1.37	9.87	7.88	7.60	73.5	64.5	—	
8.		× 0	107.3	5.53	0.57	6.49	6.05	7.45	55.0	61.0	—	
9.	−N	×100	89.6	6.91	0.41	6.50	7.25	7.40	92.5	55.5	—	
10.		× 50	103.0	8.31	0.99	7.32	7.11	7.35	87.0	59.0	—	
11.		× 20	104.1	8.67	0.79	7.89	7.57	7.65	80.0	52.0	—	
12.		× 10	123.3	10.53	1.19	9.34	7.57	7.55	80.0	65.5	—	
13.		× 5	163.5	10.64		10.64	6.50	7.45	67.5			Uncentrifuged
14.		× 2	139.9	11.94	1.24	10.70	7.44	7.10	68.0	56.0	—	
15.		× 1	136.7	10.51	1.16	9.35	6.83	7.50	49.0	62.5	—	
16.		× 0	118.6	8.43	0.68	6.75	5.69	71.5	55.5	62.0	—	

Through the Ex. 1, 2 and 3 yellowish color was observed compared with normal growth and Fe showed qualitatively negative in the supernatant. Therefore, this was considered to be the deficiency of Fe.

4. Iron

To ensure the supply of Fe, EDTA-Fe (11) which surpasses citrate as a chelating substance and resists the contamination of other microorganisms, was newly adopted and its optimum concentration was decided. The culture was performed in the concentrations illustrated in Table 7.

Since the alga grew most heavily at 2.25 ppm, the following experiments were carried out in this concentration.

5. Cobalt

Co is reported as essential for blue-greens (12), so it was added in the cultural solution and the optimum concentration was decided by the cultural

Table 7. Fe experiment result.

Group		Treat-ment	Dry wt. mg	Fixed N mg	N in sol. mg	N in algae		Final pH	Absorbed quantity %		Fe	N.B
						mg	%		Total quantity P_2O_5	Total quantity K_2O		
		ppm										
1.	Complete	45.00	little growth									
2.		22.50,,	115.0	6.72	0.87	7.37	6.40	7.30	57.0	78.0	++	
3.		9.00,,	184.0	14.95	0.94	15.54	8.44	7.10	78.0	91.5	+	
4.		4.50,,	198.5	12.45	0.93	13.05	6.57	7.20	89.0	97.0	+	
5.		2.25,,	252.3	18.24		19.77	7.89					Uncen-trifuged
6.		1.35,,	251.1	18.35		19.88	7.91					
7.		0.90,,	212.6	17.59		19.12	8.99					
8.		0.45,,	206.6	16.59		18.12	8.77					
9.		0.23,,	174.5	14.73	1.78	14.48	8.29	7.30	88.5	88.0	−	
10.		0 ,,	96.3	6.94	2.54	5.93	6.16	7.20	30.5	57.0	−	
		ppm										
11.	-N	45.00	little growht									
12.		22.50,,	100.3	8.16	0.71	7.45	7.44	7.20	36.0	61.0	++	
13.		9.00,,	248.3	19.88		19.88	8.00					Uncentrifuged
14.		4.50,,	174.1	14.14	0.93	13.21	7.58	6.80	72.0	75.5	++	
15.		2.25,,	226.7	16.74		16.74	7.87					Uncentrifuged
16.		1.35,,	183.7	16.54	1.81	14.73	8.02	6.85	90.0	97.0	+	
17.		0.90,,	184.7	18.11	1.74	16.37	8.86	7.15	91.5	96.5	+	
18.		0.45,,	178.1	16.86	1.66	15.20	8.54	7.00	95.0	75.5	−	
19.		0.23,,	186.6	15.84	1.85	13.99	7.49	6.90	90.0	94.5	−	
20.		0 ,,	123.8	9.70	2.53	7.17	5.75	7.20	62.5	62.5	−	

experiment shown Table 8.

4μg of Co added to 1l cultural solution, showed energetic growth, therefore the following experiments were performed in this concentration.

Table 8. Co expriment result.

Group		Treat-ment	Dry wt. mg	Fixed N mg	N in sol. mg	N in algae		Final pH	Absorbed quantity %		Fe	N.B
						mg	%		Total quantity P_2O_5	Total quantity K_2O		
1.	Complete	20 μg/l	123.7	9.99	1.13	8.59	6.94	7.50	88.0	58.5	+	
2.		10 ,,	191.4	13.98		15.45	8.02					Uncen-trifuged
3.		4 ,,	247.5	20.27		21.80	8.80					
4.		2 ,,	219.1	17.71		19.24	8.78					
5.		1 ,,	143.3	11.57	1.96	12.00	8.37	7.10	78.0	78.0	+	
6.		0.4 ,,	171.5	14.76	1.47	14.70	8.57	7.45	90.5	74.0	+	
7.		0.2 ,,	159.4	12.99	1.73	13.19	8.27	7.30	60.5	67.5	+	
8.		0 ,,	146.3	11.80	0.93	11.20	7.66	7.40	70.5	72.0	+	
9.	-N	20 μg/l	144.0	10.89	1.28	9.61	6.67	7.30	75.0	72.0	+	
10.		10 ,,	122.2	11.38	1.63	9.75	7.98	7.40	63.5	70.5	+	
11.		4 ,,	258.4	22.08		22.08	8.54					Uncentrifuged
12.		2 ,,	185.0	17.13	2.04	15.09	8.16	7.35	79.5	86.0	+	
13.		1 ,,	195.7	16.75		16.75	8.56					Uncen-trifuged
14.		0.4 ,,	201.2	17.16		17.16	8.53					
15.		0.2 ,,	141.6	13.73	1.15	12.58	8.88	7.40	90.0	80.0	+	
16.		0 ,,	163.0	14.33	0.91	13.42	8.23	7.10	83.5	66.0	+	

6. Copper (Table 9)

The best growth was obtained at 5 fold concentration of No. 101 solution content in both groups. This concentration was adopted in the following

Table 9. Cu experiment result

Group		Treatment	Dry wt. mg	Fixed N mg	N in sol. mg	N in algae mg	N in algae %	Final pH	Absorbed quantity % Total quantity P_2O_5	Absorbed quantity % Total quantity K_2O	Fe	N.B
1.	Complete	×100	149.0	11.38	1.41	11.50	7.72	7.55	64.0	84.5	+	
2.		× 50	218.0	13.01		14.54	6.66	7.30				Uncentrifuged
3.		× 20	286.7	17.80		19.33	6.74	7.15				
4.		× 10	281.8	18.58		20.11	7.13	7.40				
5.		× 5	309.5	17.74		19.27	6.22	7.20				
6.		× 2	272.7	17.13		18.66	6.84	7.15				
7.		× 1	225.8	11.29		12.83	5.67	7.25				
8.		× 0	201.8	13.35	1.08	13.80	6.83	7.55	74.0	90.0	+	
9.	-N	×100	146.5	13.09	1.13	11.96	8.15	6.55	90.0	83.0	+	
10.		× 50	175.9	14.39	1.07	13.32	7.57	7.45	71.0	86.0	+	
11.		× 20	234.9	15.52		15.52	6.60	7.25				Uncentrifuged
12.		× 10	283.1	17.98		17.98	6.35	7.15				
13.		× 5	299.4	17.18		17.18	5.75	7.10				
14.		× 2	224.6	16.08		16.08	7.15	7.50				
15.		× 1	196.6	13.81		13.81	7.02	7.35				
16.		× 0	165.1	13.45	0.98	12.47	7.54	6.85	80.5	80.0	+	

experiments.

7. Zinc (Table 10)

Table 10. Zn experiment result

Group		Treatment	Dry. wt. mg	Fixed N mg	N in sol.	N in algae mg	N in algae %	Final pH	Absorbed quantity % Total quantity P_2O_5	Absorbed quantity % Total quantity K_2O	Fe	N.B
1.	Complete	×100	173.3	12.10	1.28	12.35	7.56	7.95	63.5	82.0	+	
2.		× 50	257.3	16.26		17.79	6.91	7.15				Uncentrifuged
3.		× 20	301.2	20.92		22.45	7.45	7.50				,,
4.		× 10	255.2	15.05		16.58	6.49	7.35				,,
5.		× 5	249.2	16.33		17.86	7.16	7.45				,,
6.		× 2	232.5	14.98		16.51	7.10	7.55				,,
7.		× 1	247.7	13.14		14.67	5.92	7.65				,,
8.		× 0	240.3	14.98		16.51	6.87	7.70				,,
9.	-N	×100	196.5	14.66		14.66	6.46	7.75				Uncentrifuged
10.		× 50	228.8	18.22		18.22	7.96	7.20				,,
11.		× 20	302.9	22.31		22.31	7.36	7.15				,,
12.		× 10	271.2	18.29		18.29	6.74	7.10				,,
13.		× 5	286.6	17.98		17.98	5.27	7.25				,,
14.		× 2	260.5	17.79		17.79	6.82	7.00				,,
15.		× 1	262.5	17.18		17.18	6.54	7.15				,,
16.		× 0	254.6	15.93		15.93	6.25	7.15				,,

The heaviest growth was obtained at 20 fold concentration of No. 101 solution content in both groups. In the following experiments, this concentration will be used.

Considering the combination of the above results, the concentrations of nutrients in the cultural solution were modified and the new cultural solution was devised. It is given Table 11 and compared with No. 101 solution contents.

Table 11. The new cultural solution compared with No. 101 solution contents.

Salt	No. 101 Sol.	New Sol.
$Ca(NO_3)_2 \cdot 4H_2O$	40.00ppm	100.00ppm
K_2HPO_4	10.00 ,,	25.00 ,,
$MgSO_4 \cdot 7H_2O$	25.00 ,,	62.50 ,,
Na_2CO_3	20.00 ,,	20.00 ,,
Citric acid	3.00 ,,	
Fe-citrate	3.00 ,,	
Fe(Fe-EDTA)		2.25 ,,
H_3BO_4	1.43 ,,	7.15 ,,
$MnSO_4 \cdot H_2O$	1.05 ,,	10.50 ,,
$ZnCl_2$	0.05 ,,	1.00 ,,
$CuSO_4 \cdot 5H_2O$	0.04 ,,	0.20 ,,
$H_2MoO_4 \cdot H_2O$	0.01 ,,	0.10 ,,
$CoSO_4 \cdot 7H_2O$		0.02 ,,

Discussion

From the above experiments, excellent results were obtained for the nutrient solution of the *Nostoc spongiaeforme AG*, to perform the mass culture or to resolve the physiologically important problems such as the N fixation and the other biochemical questions.

Algal nutritional conditions differed widely from the normal ones of the plants.

As to common nutrients, the high concentration of environmental nutrients was required for the algal growth and also the growth was highly influenced by the contents of heavy metals, B, Mn, Fe, Cu and Zn respectively. Perhaps they would be important in the enzymatic catalytic metabolism of growing algae.

The environmental adaptation problem that algae may resist such high heavy metals concentration is expected to be important. Applying these properties, it is proposed that blue-greens may be used in the future as the source of food or feed containing much minerals.

This seems to be the best results when comparing with ones of the other N fixing organisms as N fixed per 100 ml solution in two weeks amounts to about 20 mg. N fixed seemed to be accumulated in the algal cells as the protein and the algal N content was observed in the range of 4.44–9.27 per cent, and the mean was within 6–8 per cent. Nitrogen content of the algal cells was increased by the addition of such elements as Fe, Co and Mo to the cultural solution, so it was considered that these elements would relate to the mechanism of N fixation directly or indirectly. It was already recognized that Mo has close connection with the enzymes concerning the mechanism of the N fixation (1). In the case of Fe, it may be interpreted that indirect action was combined with the synthesis of pigments such as chlorophyll. It was reported that the amount of N fixed was increased by the addition of

Co (12), but it was also considered to be the indirect affection. Concerning the mechanism of N fixation, it has been known that Mo is essential (1) and this power of N fixation is independent on light, for blue-greens can fix N readily in the dark if supplied with suitable carbohydrates (9, 13), but this ability is lost if the algae are supplied with nitrates, ammonia or asparagine (14).

Since these facts were also observed in *Azotobacter* and *root nodule bacterias*, it has been thought that blue-green algae posses the same enzymatic systems in N fixing process with them.

There is considerable liberation of soluble nitrogenous substances from the algal cells of healthy condition (15). The substances concerned are principally polypeptides and free amino acids are present only in minute amount, and the liberation of nitrogenous substances are not attributable to autolysis, as appeared invariably to accompany growth. Because they have the ability to form complexes with various ions, these substances have important effects on the growth of the blue-greens (16). The liberation of nitrogenous substances from the algal cells was estimated about 10–20 per cent of total N fixed in *Nostoc spongiaeforme AG*, but their constituents are not clear, however, a part of them have the coloration with nessler's reagent, therefore, further investigation may make it clear.

Although the new solution was obtained, it is still insufficient to bring about perfect satisfactory growth. Especially regarding the microelements, only Co and Mo was selected from the B group of Arnon (25), Ti, V, Cr, W and Ni were not contained. Whether V is replaceable for a part of Mo, many investigations have been cited.

As sometimes the addition of organic substances such as glucose brought about better results (10), the supply of organicmatter must be taken into consideration. Though succeeded in *Myco. tuberculosis*, further studies may be needed about the substances such as the surface activating reagent which increases the wetting ability and facilitates the passage of nutrients through the algal membranes (17). It is not yet solved in blue-greens about the growth factor such as thiamine, pyridoxal and IAA, as they are available on *Chlorella* as a growth factor. The extracts of soil, yeast and liver was reported as negative on blue-greens (19).

Final pH appears to be between 7.0–7.5 in overwhelming numbers especially in cultures of heavy growth. The alga seemed to have optimum range (7.0–8.5) (1) in all cultural stages. It must be investigated about pH of the above-mentioned optimum range, whether this may be variable by the differences or strains.

As the cultural equipment, there are questions about light, aeration and shaking. There are factors of intensity, wave length and time in relation to

light and optimum values of photosynthesis and growth are the respective different ones (19). The algal growth increased in proportion to the light intensity up to the 16000 lux (20) and became better in long wave length than short ones (21). Now, in this experiment the continuous illumination was adopted but it is said that 18 hours illumination per day *viz* with the dark period gives better results (9).

The most important problem is aeration on the present cultural method. CO_2 content in air, volume, humidity and temperature seems to be important factors as to the aerating conditions affecting cultures, and the cotton seal, liquid volume and degree of growth also kept their own effects. In the later stage of culture, aeration appears to be difficult and so the algal growth is limited. Then the viscosity of the algal suspension was increased by the absence of the aerating volume. It seems to occur by the increase of the slime excreted which covers the surface of the blue-greens (22). This viscous substances may be mucin or mucin-like substances contained in vacuole of the algae (23) though further works are needed. Aeration is absolutely needed in the initial stage of culture, but excessive aeration brought in the abnormal volatilization. 0.25 *l/l* media/min. is considered adequate and adopted. It appears to be insufficient for adding humidity to the air by the passage through the glass filter inserted in the 0.1 corresive sublimate water. The question of temperature might be important in winter because of the difference between the temperature of cultural solution and atomosphere. Though adequate concentration of CO_2 is said to be between 3–5 per cent, a small tardiness and ununiformity of growth is observed by air only. PH are affected by CO_2 concentration, but the final pH gave no obtained values below 6.0 being the limiting point of the algal growth. N_2, CO_2 and O_2 are supplied by aeration and it is said sometimes that N_2 is better than combined N as N source for amounts of fixed and growth. In $-$N cultures (N_2 only), the tardiness of initial stage was left over to the last extremity.

Concerning shaking, the shortage of stirring effect is considered with only reciprocating movement, but shaking is unnecessary as the gasses-liquid interfaces is large (20), and it will be dissolved easily when aeration is sufficient.

It is said that the optimum range of the cultural temperature is between 22–35°C. This range varies according to the species or strains of the algae, and *Nostoc spongiaeforme* AG. survives for a long time near the freezing point and some species are able to grow directly under boiling point (25). Generally, temperatures between room temperature and 32.5°C are used and good results are obtained in other plants by the presence of diurnal differences in the temperature. Sterilization, seems to be sufficient because the contamination and growth of the other microorganisms was never seen in room temperature after a half year from the end of the cultures.

Summary

Our first experiment was planned to obtain rapid and abundant growth of the N fixing blue-green alga, *Nostoc spongiaeforme AG.* through modifying the composition of conventional culture solution. The deficient culture of each macroelement was achieved using No. 101 cultural solution devised by Katagiri. From this result, it was confirmed that the algal growth was highly affected by the deficiency of each macroelement namely P, K, Ca, Mg and Na_2CO_3.

Next, sometimes deficiency phenomena of some elements were observed as to the conventional cultural solution, therefore the growth experiments were repeatedly performed in various concentrations of each macro nutrient and heavy growth was obtained in the two and a half fold concentration of No. 101 solution.

Besides, adopting the medium of this concentration as to the macroelements, the growth tests on the various concentrations of each microelement Mn, B, Mo, Cu and Zn were made by the same method. In this case, the alga grew vigorously in the medium from two to 20 fold concentrations of No. 101 solution, moreover Fe was supplied as EDTA-Fe and excellent growth was attained in 2.25 ppm. Fe. Co was found to produce vigorous growth at the concentration of 4 μg in 1 *l* cultural solution. Considering the above-mentioned results, the new cultural solution was made.

The vigorous growth of the alga was brought in the 20 mg of N fixation per 100 *ml* medium for about two weeks.

The reports to follow will describe the cultural conditions and physiological studies of the alga.

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