# 好塩性細菌Vibrio alginolticusの生長を促進するクロレラ抽出物中の活性物質


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# Growth-Stimulating Substances for *Vibrio alginolyticus* Contained in Chlorella Extract

Hideki MORISHITA*1, Toshihiko SANO*2, Norikiyo KAMIYA*2, and Masao OKUDA*2

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The effect of hot water extract (CE, I) of chlorella cells on the growth and protein synthesis of *Vibrio alginolyticus* was studied and compared with its effect on of *Vibrio parahaemolyticus*, a slightly halophilic bacterium, and *Escherichia coli*, a non-halophilic bacterium. A remarkable growth-promoting effect of CE was found for *V. alginolyticus* but not for *E. coli*. The CE was fractionated by Sephadex G-25 column chromatography into two fractions (II and III). Fraction II was composed of high molecular weight (above 5,000) compounds, and fraction III of low molecular weight (below 5,000) compounds. Fraction II contained amino acids in higher concentration than fraction III, but it contained a smaller amount of S-containing amino acids. Fraction III exhibited a marked stimulatory effect on the growth of *V. alginolyticus*; fraction II did not show such a remarkable effect. This effect of fraction III might be due to easily utilizable amino acids, especially the S-containing amino acids contained in the fraction. Fraction II was further fractionated into four fractions (IV, V, VI, and VII) by DEAE-cellulose column chromatography. Fractions IV, V, and VI contained neutral sugar and protein in relatively high concentrations, but nucleic acids in very low concentration. Fraction VII showed the highest concentration of nucleic acids among the four fractions. Fractions V and VII had a growth-stimulating effect, but fractions IV and VI did not. Fraction VII showed the most remarkable effect on the growth and protein synthesis of *V. alginolyticus*.

Based on these results, it is suggested that (1) high molecular weight fractions of CE, which contain thymine, adenine, guanine, cytosine and uracil as their main components, play an important role in the stimulation of growth and protein synthesis of *V. alginolyticus*, and that (2) amino acids in the low molecular weight fraction, especially S-containing amino acids, also stimulate the growth.

Chlorella extract (CE) has been used in the studies of growth and metabolism of various microorganisms, OKUDA *et al.*[1] has shown that CE had a growth-stimulating effect on *Saccharomyces cerevisiae* and *Tetrahymena pyriformis*. The CE also stimulated the cell division of *T. pyriformis*[2], and had a recovery effect on the growth inhibition by 4-nitroquinone-*N*-oxide and lauryl-pyridine chloride (LPC) in yeast[3], on the elimination of episome by acridine in *E. coli*[4], and on the inhibition by organophosphorous pesticides in human erythrocytes[5]. Furthermore, CE contributed to the stability of the structure of cellular ribosomes of *V. parahaemolyticus* in sodium-starvation[6].

It was reported as one of the CE activities that polysaccharide fraction of CE had stimulatory activity on phagocytosis in the reticuloendothelial system of the rat[7]. However, physiological mechanisms of the growth stimulation of CE are still unknown.

The purpose of the present work is to study on the mechanism involved in the growth-stimulating activity of CE on *V. alginolyticus*, a slightly halophilic bacterium.

## Materials and Methods

Two strains of slightly halophilic bacteria, *Vibrio alginolyticus* ATCC 17749 and *Vibrio parahaemolyticus* A-55, and a strain of non-halophilic bacterium, *Escherichia coli* B, were used. The stock cultures were maintained by the use of 1% polypeptone agar medium (pH 7.4). In the case of halophiles 0.5 M NaCl was added to the medium. Precultures were made in 1% polypeptone liquid medium (pH 7.4) at 37°C for

*1 Department of Microbiology, Osaka City Institute of Environmental Life Science, Osaka 543, Japan (森下日出旗: 大阪市立環境科学研究所微生物課).

*2 Research Laboratory, Chlorella Industry Co. Ltd., Chikugo, Fukuoka 833, Japan (佐野利彦・神谷典清・奥田正男: クロレラ工業株式会社研究部).

12 h, and cells were harvested by centrifugation at 2,000×*g* for 10 min. Effect of CE and its fractions on the growth of the bacteria were examined by using the synthetic medium containing 17.2 mM $K_2HPO_4$, 22.2 mM $KH_2PO_4$, 18.7 mM $(NH_4)_2SO_4$, 11.1 mM glucose, 1.0 mM $MgSO_4 \cdot 7H_2O$ and 500 mM NaCl in 0.1 M phosphate buffer (pH 7.4)[8]. Final concentration of CE was 0.2 mg dry weight per m*l* of culture medium. Growth was achieved by shaking at about 90 strokes per min on a reciprocal shaker at 37°C. Growth was estimated by the measurement of absorbance at 650 nm by a Bausch and Lomb Spectromic 20 Photometer.

The incorporation into cells of radioactive L-leucine was carried out by the methods of MANS and NOVELLI[9] and BYFIELD and SCHERBAUM[10] in which trichloroacetic acid (TCA) precipitation of macromolecules on membrane-filter disc was used. To the cell suspension added with CE or its fractions $^{14}$C-L-leucine was applied at 0.2 $\mu$C per $\mu$ mole per m*l*. Counting of radioactivity was performed by use of a liquid scintillation counter (Nuclear Chicago Corp.). A toluene scintillation fluid containing 0.3 g of 1,4-di-2-(5-phenyloxazole)benzene and 5.0 g of 2,5-diphenyloxazole per liter was used. The value obtained was corrected for background activity (average 30 cpm).

A strain of *Chlorella vulgaris* was grown in the medium of which composition was given in Table 1. This medium is modified Beijerinck medium[11]. The preculture was made for 5 days at a light intensity of 10,000 lx at 25°C by vigorous bubbling with filter-sterilized air and 5% $CO_2$ gas. A portion of the preculture was inoculated to the acetate medium in which acetate was added as a C-source at a final concentration of 1% and then cultivated in a pool at ca. 25°C. Light intensity was about 70,000 lx at the surface of the pool in the summer period. The cells grown were collected by centrifugation at the stationary phase and were dried with spray dryer at 100°C for 10 min after washing with distilled water. The extraction from chlorella cells was made as follows. Dried chlorella cells (100 g) were treated with 1,000 m*l* of deionized water at 100°C for 20 min, and then centrifuged at 10,000 rpm for 5 min. Supernatant fluid obtained was freeze-dried and was used as crude extracts of chlorella (CE, I).

**Table 1.** Medium for *Chlorella vulgaris*

| | |
|---|---|
| $(NH_2)_2CO$ | 1.0 g |
| $KH_2PO_4$ | 1.0 |
| $MgSO_4 \cdot 7H_2O$ | 0.5 |
| $FeSO_4$ | 0.01 |
| Dist. water | 1,000 m*l* |

Procedure of the column chromatography for the separation of CE was performed as shown in Fig. 1. The flow rate was 35 m*l* per h and each 10 m*l* fraction was collected. Total protein, neutral sugar, and nucleic acid were determined. The content of nucleic acid was spectrophotometrically determined at 260 nm. Protein content of each fraction was determined by the methods of LOWRY *et al.*[12] using bovine serum albumin as a standard. Neutral sugar content was determined by absorbance at 425 nm with orcinol-sulphuric

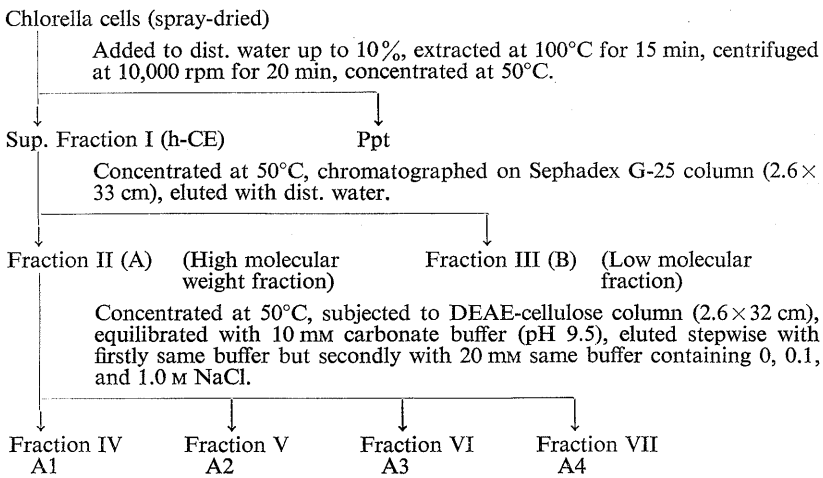


**Fig. 1.** Scheme for extraction and fractionation of chlorella extract.

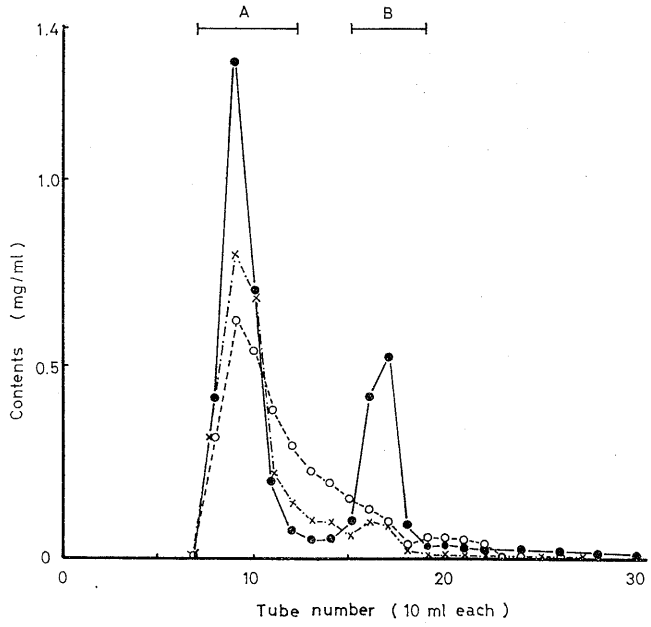


**Fig. 2.** Elution profiles for Sephadex G-25 column chromatography of CE. Assay conditions are described in Fig. 1, and **Materials and Methods**. protein, –○–; nucleic acid, –●–; neutral sugar, –×–.

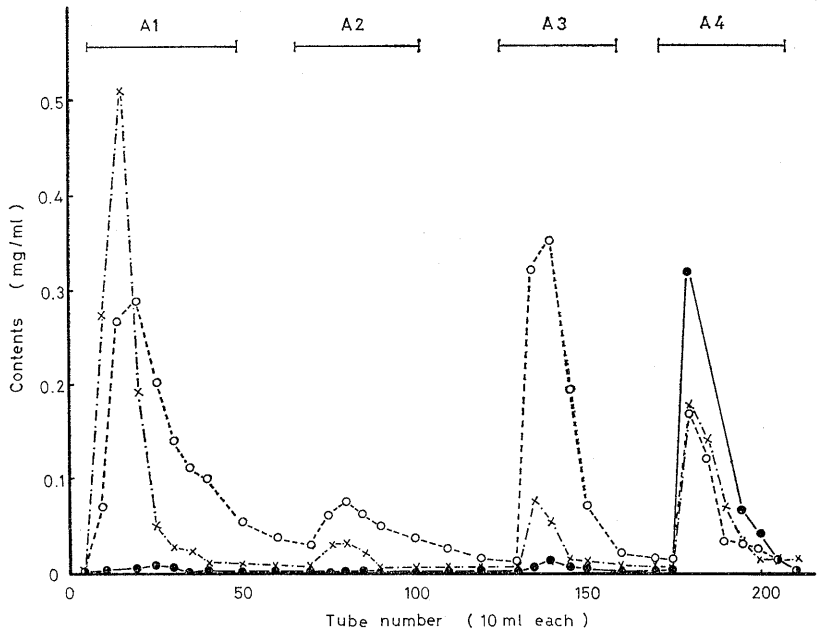


**Fig. 3.** Elution profiles for DEAE-cellulose column chromatography of fraction II. Assay conditions are described in Fig. 1, and **Materials and Methods**. protein, –○–; nucleic acid, –●–; neutral sugar, –×–.

acid method[13] as a standard of galactose. Purines and pyrimidines were determined by the difference of optical density in each base[14]. Amino acid composition was determined with a Shimadzu amino acid analyzer by the method of SPACKMAN *et al.*[15] after samples were hydrolyzed in 1 m*l* of 6 N HCl at 110°C for 24 h in sealed glass tubes.

## Results

In the fractionation of CE (I) with Sephadex G-25 column chromatography, two peaks (A and B) were found as shown in Fig. 2. The fractions corresponding to A and B peaks were detected by determination of protein, neutral sugar, and nucleic acid. Molecular weights of these fractions were above 5,000 and below 5,000, respectively. Fraction II (A) was rechromatographed using DEAE-cellulose column (Fig. 3). Elution was performed with bicarbonate buffer as described in Fig. 1. Four peaks were obtained with determination of protein, neutral sugar and nucleic acid in each fractionated tube. Fraction pooled in each peak was salted out and concentrated at 50°C.

Contents of protein, neutral sugar and nucleic acid in each fraction were shown in Table 2. Fraction II was 1.6 times larger in dry weight base than fraction III (B). Total protein and neutral sugar contents of fraction III were 25% to that of fraction II, but nucleic acid content of fraction III was 75%. Of fractions IV, V, VI and VII separated from fraction II, only fraction VII was specific in composition; contents of protein and neutral sugar were very low as compared with those of IV, V and VI, respectively, but nucleic acid content was very high, reaching to 50% of the total. The nucleic acid contents of fractions IV, V and VI were only 1 to 3%.

The effect of CE (I) and its fractions on the growth of *E. coli, V. alginolyticus* and *V. parahaemolyticus* was examined (Table 3); growth of the 3 strains was stimulated by addition of CE. The growth of *V. alginolyticus* increased proportionally with increase of the CE concentration (Fig. 4). There was a linear correlation between the growth and CE concentration within certain limit (0.1 to 1.5 mg per m*l*). When the CE concentration was below 0.1 mg, the growth decreased sharply. The growth increased approximately 3-fold at the CE concentration of 15 mg per m*l*. The minimum concentration of CE for the growth-promotion was 0.1 mg per m*l*.

In the subfractions of CE, fractions II and III showed the stimulating effect on growth of the 3

**Table 2.** Contents of protein, neutral sugar and nucleic acid in each fraction of CE and their yields during fractionation by column chromatography

| | Fraction | Total protein | Neutral sugar | Nucleic acid | Yield (%) |
|---|---|---|---|---|---|
| I | Crude extract, CE | 0.298 | 0.252 | 0.218 | 100 |
| II | Sephadex G-25, A | 0.443 | 0.395 | 0.200 | 59.3 |
| III | Sephadex G-25, B | 0.103 | 0.088 | 0.154 | 38.0 |
| IV | DEAE-cellulose, A1 | 0.276 | 0.714 | 0.012 | 14.0 |
| V | DEAE-cellulose, A2 | 0.514 | 0.451 | 0.017 | 3.3 |
| VI | DEAE-cellulose, A3 | 0.569 | 0.392 | 0.032 | 7.3 |
| VII | DEAE-cellulose, A4 | 0.170 | 0.370 | 0.513 | 13.3 |

Data were expressed as mg/mg dry wt.

**Table 3.** Effects of various fractions of CE on growth of *E. coli, V. alginolyticus* and *V. parahaemolyticus*

| | Fraction | *E. coli* | *V. alginolyticus* | *V. parahaemolyticus* |
|---|---|---|---|---|
| 0 | Control | 0.190 | 0.160 | 0.180 |
| I | Crude extract, CE | 0.230 | 0.501 | 0.360 |
| II | Sephadex G-25, A | 0.230 | 0.280 | 0.296 |
| III | Sephadex G-25, B | 0.270 | 0.600 | 0.380 |
| IV | DEAE-cellulose, A1 | 0.210 | 0.160 | 0.200 |
| V | DEAE-cellulose, A2 | 0.189 | 0.240 | 0.236 |
| VI | DEAE-cellulose, A3 | 0.190 | 0.160 | 0.176 |
| VII | DEAE-cellulose, A4 | 0.205 | 0.400 | 0.230 |

Final concentration of each fraction was 0.2 mg dry wt./m*l* in medium.
Data were expressed as increased optical density at 650 nm per h in logarithmic growth phase.

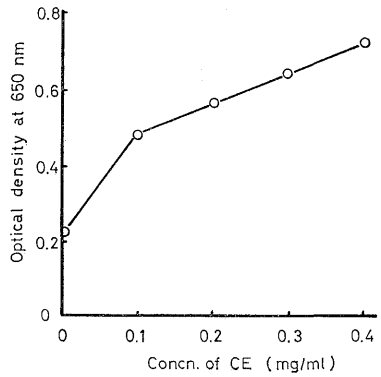


**Fig. 4.** Effect of CE concentration on growth of *V. alginolyticus*.

**Table 4.** Contents of nucleic acid components in fractions II and III

| Base | Fraction | | |
|---|---|---|---|
| | II | III | (III/II) |
| Adenine | 4.266 | 7.477 | (1.7) |
| Guanine | 6.947 | 11.852 | (1.7) |
| Uracil | 4.135 | 7.947 | (1.9) |
| Thymine | 6.375 | 10.928 | (1.7) |
| Cytosine | 3.708 | 5.052 | (1.4) |

Data were expressed as μg/mg dry wt.

**Table 5.** Contents of amino acids in fractions I, II and III

| Amino acid | Fraction | | |
|---|---|---|---|
| | I | II | III |
| Lysine | 21.719 | 23.379 | 8.965 |
| Histidine | 4.067 | 4.614 | 2.399 |
| Arginine | 45.275 | 45.191 | 44.200 |
| Aspartic acid | 35.326 | 37.834 | 24.954 |
| Threonine | 15.659 | 18.625 | 9.485 |
| Serine | 15.617 | 16.927 | 9.981 |
| Glutamic acid | 109.556 | 65.313 | 79.021 |
| Proline | 12.681 | 17.308 | 7.135 |
| Glycine | 22.869 | 26.491 | 15.931 |
| Alanine | 34.331 | 20.262 | 37.446 |
| Cystine | 1.164 | ± | 4.347 |
| Valine | 13.229 | 17.971 | 6.514 |
| Methionine | 2.329 | ± | 0.810 |
| Isoleucine | 6.794 | 11.829 | 0.193 |
| Leucine | 17.756 | 25.679 | 6.901 |
| Tyrosine | 10.602 | 16.931 | 1.901 |
| Phenylalanine | 9.186 | 13.742 | 3.166 |
| Total | 378.160 | 362.096 | 263.338 |

Data were expressed as mg/g dry wt.

strains. Fraction III was more stimulating for the growth than fraction II. This fact suggests that some compounds of low molecular weight were important for the stimulation of growth. The fraction III contained nucleic acid in relatively high concentration (44%, W/W). The nucleic acid content of fraction III was 1.7-fold higher than that of fraction II (Table 4). The growth-stimulation was 3.1-fold more active than fraction II. Therefore, it was considered that the stimulation was not only due to nucleic acid components. Amino acids in CE were analyzed with fractions II and III (Table 5). Chlorella cells contained various amino acids[16], and the CE also had similar amino acids components. Fractions III and CE contained S-containing amino acids but fraction II did not.

Furthermore, of fractions IV, V, VI and VII, fractions V and VII stimulated the growth. Fraction VII contained nucleic acids in large proportion. Absorption spectrum of fraction VII is shown in Fig. 5. Contents of nucleic acids of fraction VII was very large as compared with other neutral sugar and protein. As shown in Table 6, the contents of nucleic acid bases in fraction VII were 3-fold larger than those of CE except cytosine. Thymine content was the highest and the ratio of fraction VII to CE was 4.5.

With regard to the growth stimulation by frac-

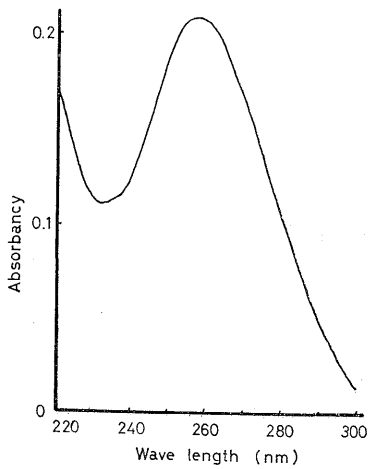


**Fig. 5.** Absorption spectra of fraction VII.

**Table 6.** Contents of nucleic acid components in fractions I and VII

| Base | I | VII | (VII/I) |
|---|---|---|---|
| Adenine | 4.000 | 12.376 | (3.1) |
| Guanine | 6.315 | 20.336 | (3.2) |
| Uracil | 3.966 | 10.948 | (2.8) |
| Thymine | 4.244 | 19.052 | (4.5) |
| Cytosine | 6.199 | 11.076 | (1.8) |

Data were expressed as μg/mg dry wt.

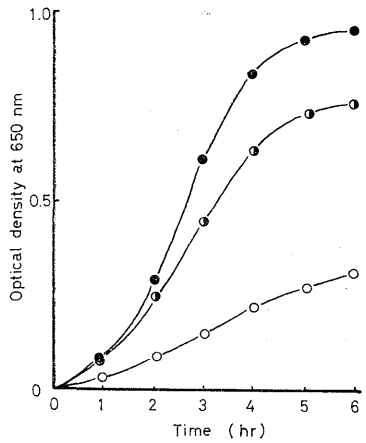


**Fig. 6.** Time course of growth of *V. alginolyticus* in medium containing CE or fraction VII. Assay conditions are described in **Materials and Methods**. control, –○–; CE, –●–; fraction VII, –◑–.

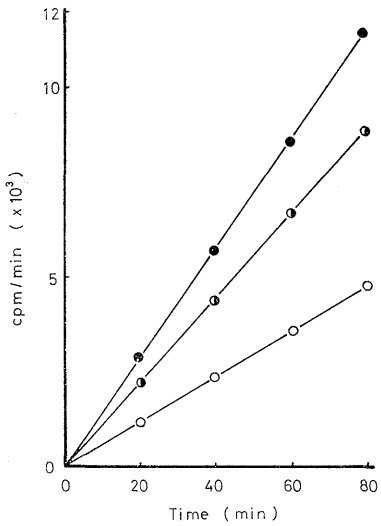


**Fig. 7.** Incorporation of $^{14}$C-L-leucine into TCA insoluble materials of *V. alginolyticus* when fraction VII or CE was added. Assay conditions are described in **Materials and Methods**. control, –○–; fraction VII, –◑–; CE, –●–.

tion VII, the time course of growth of *V. alginolyticus* was examined (Fig. 6). The growth was stimulated from initial stage by the addition of fraction VII, and the time of lag phase for growth was shortened. This means that fraction VII contains bases of nucleic acids and stimulates the synthetic activities of DNA and RNA for growth. Growth increased in proportion to the concentration of fraction VII but conspicuous decline of growth was observed under 1 mg per m*l*. Effect of fraction VII on protein synthesis in cells was examined with *V. alginolyticus*. Incorporation of $^{14}$C-L-leucine into cellular protein was stimulated by the addition of fractions VII and CE (Fig. 7). This was similar to the case of growth curve. It was recognized that the stimulation of growth was at least partly due to protein synthesis promoted by fraction VII. From the results it is suggested that purine and pyrimidine bases in CE enhanced the biosynthesis of DNA and RNA in cells.

## Discussion

Since the reason for the effect of extract of chlorella cells as a growth stimulant is not clear as yet, an attempt was performed on chemical analysis of CE and the stimulatory activity of the fractions obtained from CE was examined on growth and protein synthesis in *V. alginolyticus*, a slightly halophilic bacterium.

It was expected that CE contained various substances, i.e. protein, amino acids, nucleic acid components, carbohydrates and lipopolysaccharides etc., as cellular constituents of chlorella. From the analytical results, some of the above substances were found to be contained in CE (Table 2, 4, 5 and 6). Therefore, it was considered that those cellular constituent materials may be metabolically useful as nutrients for the growth of heterotrophic microorganisms. However, it was found that the same rule could not be applied in all microorganisms used since the difference was observed on growth of each microorganism when CE was present in the same concentration. And it was suggested that CE contained rather physiologically active substances for respective microorganisms.

On the growth of *V. alginolyticus*, the stimulation due to CE was 3.1-fold high to control, but only 1.2-fold for *E. coli* in the same concentration. The growth-stimulation of *V. alginolyticus* was remarkable in both fractions III and VII. Fraction III contained S-containing amino acids and this S-containing amino acids may be responsible for the stimulation of growth. Especially, it was interested that the considerable stimulation was found in *V. alginolyticus*. There is an information that sulfhydryl compounds such as L-cysteine have the stimulatory effect on growths of some slightly[17], modelately[18], and extremely[19] halophilic bacteria. Further, fraction VII consisted mostly of DNA and RNA bases and the contents of each base were 3-fold larger than that of CE on dry weight basis. In fraction VII, thymine content was remarkably high and was 1.7-fold to uracil.

A 25% of total base content was accounted for thymine originating from DNA in chlorella cells.

It seems then that the substances in CE were useful for the biosynthesis of cellular constituent. The CE showed stimulatory effect not only on the growth rate but also on the final cell yields of *V. alginolyticus* and *V. parahaemolyticus*. Growth stimulation due to the CE might be related to the DNA and RNA synthesis *in vivo* through purine and pyrimidine metabolism. The synthesis of DNA, RNA and protein is regulated in balanced mechanisms for growth, for which synthesis of individual nucleotide and peptide chanis from nucleic acid bases and amino acids is promoted from initiation of macromolecular synthetic system by addition of CE. This was suggested from results that growth rate was stimulated by relatively low concentration of CE.

As for the active effect of CE, it was reported that an addition of CE showed remarkable increase in the contents of cytochromes in *S. sllipsoides*[20], and also CE characteristic action on the promotion of filamentous cells under hypertonic condition with NaCl[21], and in chlorophyll formation in halophytes[22–24].

## References


1) M. Okuda, T. Sano, and H. Nakao: *Physiol. & Ecol.*, (Kyoto), **15**, 101–112 (1969).
2) T. Tabusa, S. Takada, and M. Okuda: *Proc. Agr. Chem. Jap.*, **49**, 197 (1974).
3) M. Okuda, N. Yanagishima, and H. Takada: *J. Biol. Osaka City Univ.*, **12**, 61–67 (1961).
4) K. Akiba, M. Hino, and N. Ikebe: *J. Agr. Chem. Soc. Jap.*, **45**, 201–206 (1971).
5) S. Matsueda, M. Nagaki, and N. Tsukada: *Sci. Rep. Hirosaki Univ.*, **20**, 14–22 (1974).
6) H. Morishita: *Proc. Ist Intersec. Cong. of IAMS.*, **2**, 421–432 (1974).
7) M. K. Kojima, T. Kasajima, Y. Imai, S. Kobayashi, M. Dobashi, and T. Uemura: *Rec. Adv. Res.*, **13**, 101–122 (1973).
8) H. Morishita and H. Takada: *Can. J. Microbiol.*, **22**, 1263–1268 (1976).
9) R. J. Mans and G. D. Novelli: *Arch. Biophys.*, **94**, 48–53 (1961).
10) J. E. Byfield and O. H. Scherbaum: *Anal. Biochem.*, **17**, 434–443 (1966).
11) H, Tamiya and A. Watanabe: in "Experimental Method for Algae". Nankodo, 1965, pp. 68.
12) O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall: *J. Biol. Chem.*, **193**, 265–275 (1951).
13) M. Sorensen and G. Hangaard: *Biochem. Z.*, **260**, 247–277 (1933).
14) S. Ukita: in "The Nuleic Acid—Nucleoside and Nucleotide", Organic Chemistry for Medicine and Biology, Vol., **7**, Asakurashoten, 1974, pp. 27–29.
15) D. H. Spackman, W. H. Stein, and S. Moore: *Anal. Chem.*,**i30**, 1190–1206 (1958).
16) H. Endo, K. Nakajima, R. Chino, and M. Shirota: *Agr. Biol. Chem.*, **38**, 9–18 (1974).
17) H. Morishita: *Proc. Ann. Meet. Bot. Soc. Jap.*, **33**, 43–44 (1968).
18) W. L. Flannery and D. M. Kennedy: *Can. J. Microbiol.*, **8**, 923–928 (1962).
19) H. Onishi, M. E. MacCance, and N. E. Gibbons: *Can. J. Microbiol.*, **11**, 363–473 (1965).
20) M. Okuda and H. Takada: *J. Antibact. Antifun. Agents, Jap.*, **3**, 160–165 (1975).
21) T. Yagi, H. Takada, and M. Okuda: *Physiol. & Ecol.*, (Kyoto), **13**, 75–80 (1965).
22) A. Yabe, M. Okuda, and H. Takada: *Int. Revue Ges. Hydrobiol.*, **59**, 423–435 (1974 a).
23) A. Yabe, M. Okuda, and H. Takada: *Int. Revue Ges. Hydrobiol.*, **59**, 557–567 (1974 b).
24) A. Yabe and H. Takada: *Int. Revue Ges. Hydrobiol.*, **59**, 801–808 (1974 c).