



Study on the practical application of halotolerant microalgae *Dunaliella*

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Kobe University
Doctoral Dissertation

Study on the practical application of halotolerant microalgae
Dunaliella

1999 August

Yoshiro Hatanaka

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耐塩性緑藻*Dunaliella* の実用的利用に関する研究

1999 August

Yoshiro Hatanaka

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CHAPTER I

GENERAL INTRODUCTION

The genus *Dunaliella* of the order Volvocales includes a variety of species of unicellular green algae. The genus was first described by Teodoresco (1905) with type species being *Dunaliella salina*, and named in honor of M. F. Dunal, who has first determined that the red color of certain hypersaline reservoirs was caused by this algae (Dunal, 1983). Since then, many species from a very wide range of habitats have been reported. Members of them are generally ovoid in shape, 4-10 μm wide and 6-15 μm long (Fig. 1-1, A). The cells are motile, due to the presence of two equal, long flagella in each cell, and contain one large chloroplast which occupies about half the cell volume (Fig. 1-1, B). The chloroplast contains a large pyrenoid surrounded by starch granules and they are the main storage products for the algae. *Dunaliella* is morphologically very similar to *Chlamydomonas*. The remarkable morphological characteristic of *Dunaliella*, in contrast to other members of the Chlorophyta, is the lack of a rigid polysaccharide cell wall and the cells were enclosed by only a thin elastic membrane. This lack of a rigid cell wall gives flexibility of the cell shape and volume to the algae. Since *Dunaliella* usually lives in saline environments and have to response to osmotic changes, flexibility of cell structure is very useful to adjust intracellular osmotic pressure by changing their cell volume. For this reason, *Dunaliella* demonstrate outstanding degree of environmental adaptation to salt and is widely distributed in natural habitats. The algae are very well adapted to propagate in media ranging from less than 0.1 M NaCl (sea water) to saturated salt solutions (NaCl > 5 M). The unique property of *Dunaliella* to grow in a wide range of salinity has made it favorite subject for the study of understanding the mechanism of osmoregulation in eucaryotic organisms.

The solute responsible for most of the intracellular osmotic pressure under high salinity is glycerol. Intracellular glycerol concentration is proportional to the salt concentration of surrounding solute. The intracellular glycerol concentration of the cells grown at 5 M NaCl is about 7 M. Since the algae has a unique property of changing their cell volume against rapid change of the salinity of media, relative concentration of intracellular glycerol can easily be changed responsible to the salt concentration of changed medium. The algae shrink or swell instantaneously under hypertonic or hypotonic conditions, respectively (Fig. 1-2). following

the rapid volume change, synthesis or elimination of glycerol begins minutes after transition to each medium and continues for a few hour until the cell volume returns to its original size (Fig. 1-2, metabolic conditions). Glycerol synthesis and elimination under hyper- or hypotonic condition are independent of protein synthesis and occur in the light or dark. Since little to no glycerol is found in the medium of all conditions, *Dunaliella* was thought to have an unusual cell membrane which can maintain high internal glycerol concentration. To obtain more knowledge about the cell membrane of *Dunaliella*, I investigated the cell surface structure of *Dunaliella parva* using several techniques (Chapter II).

Of the many strains of genus *Dunaliella*, some strains were known to accumulate massive amount of β -carotene in addition to glycerol. β -carotene content is best under appropriate conditions including high salinity and high light intensity (Table 1-1). The β -carotene occurs in a number of isomers (Table 1-2). From the fact that large amount of glycerol and β -carotene can be accumulated in their cells, algae of genus *Dunaliella* are among the microalgae most studied for mass culture. The property of the absence of cell wall makes many useful products in the algae easily be extracted and makes the algae easily be digested as food or feed. The amino acid composition of the protein rich material of *Dunaliella* after the extraction of β -carotene or glycerol is similar to soybean meal (Table 1-3) and have been used as feed for fish and fowl.

Starch is second abundant to glycerol and is main storage product for *Dunaliella*. The starch and its metabolism in *Dunaliella* has not been actively studied till today compare to other useful products such as β -carotene or glycerol. Since starch is the most important product of photosynthesis of plants, I consider the study about the starch in *Dunaliella* is also important to make the algae as food or feed resource. I have isolated starch from the algae and investigated some properties of the starch from *Dunaliella parva* (Chapter III). I also isolated an amylolytic enzyme to understand the mechanism of the starch metabolism in the algae (Chapter IV).

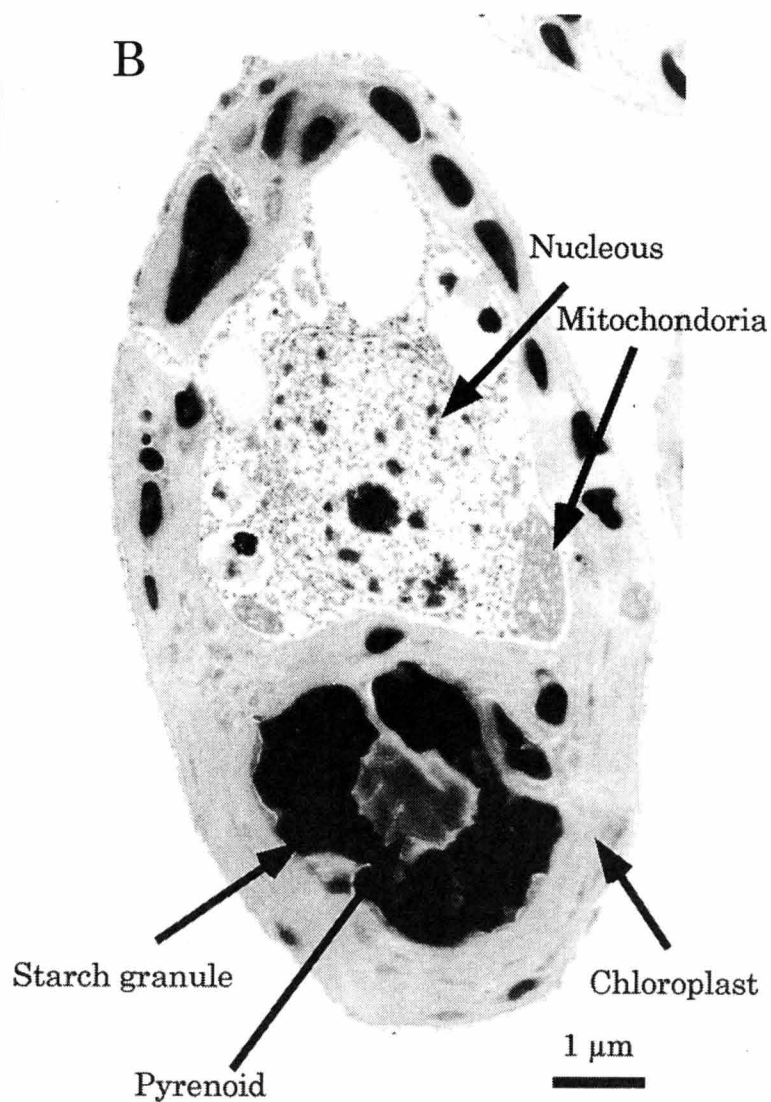
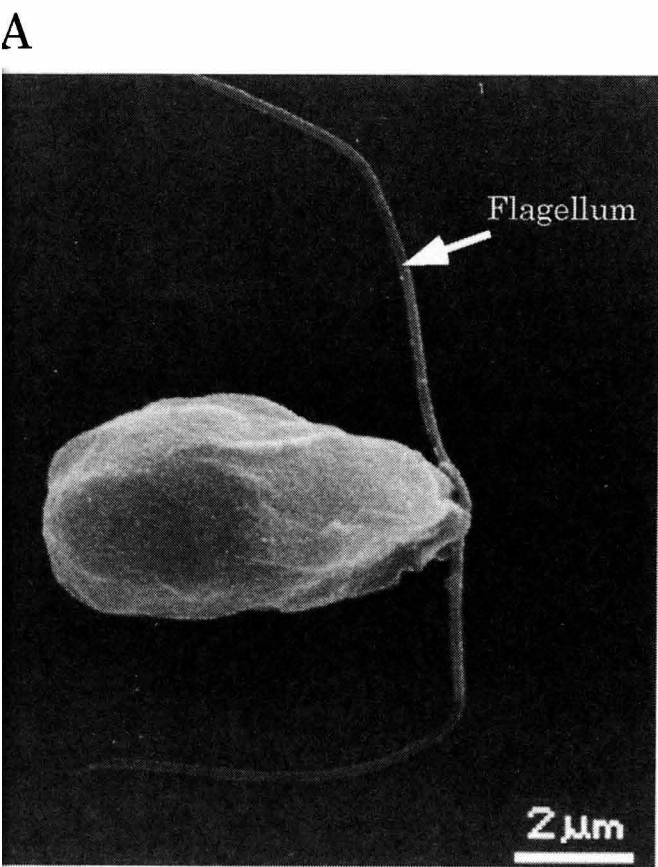


Fig. 1-1 Electron micrographs of *Dunaliella parva*.

A: SEM image, B: TEM image (ultra-thin section).

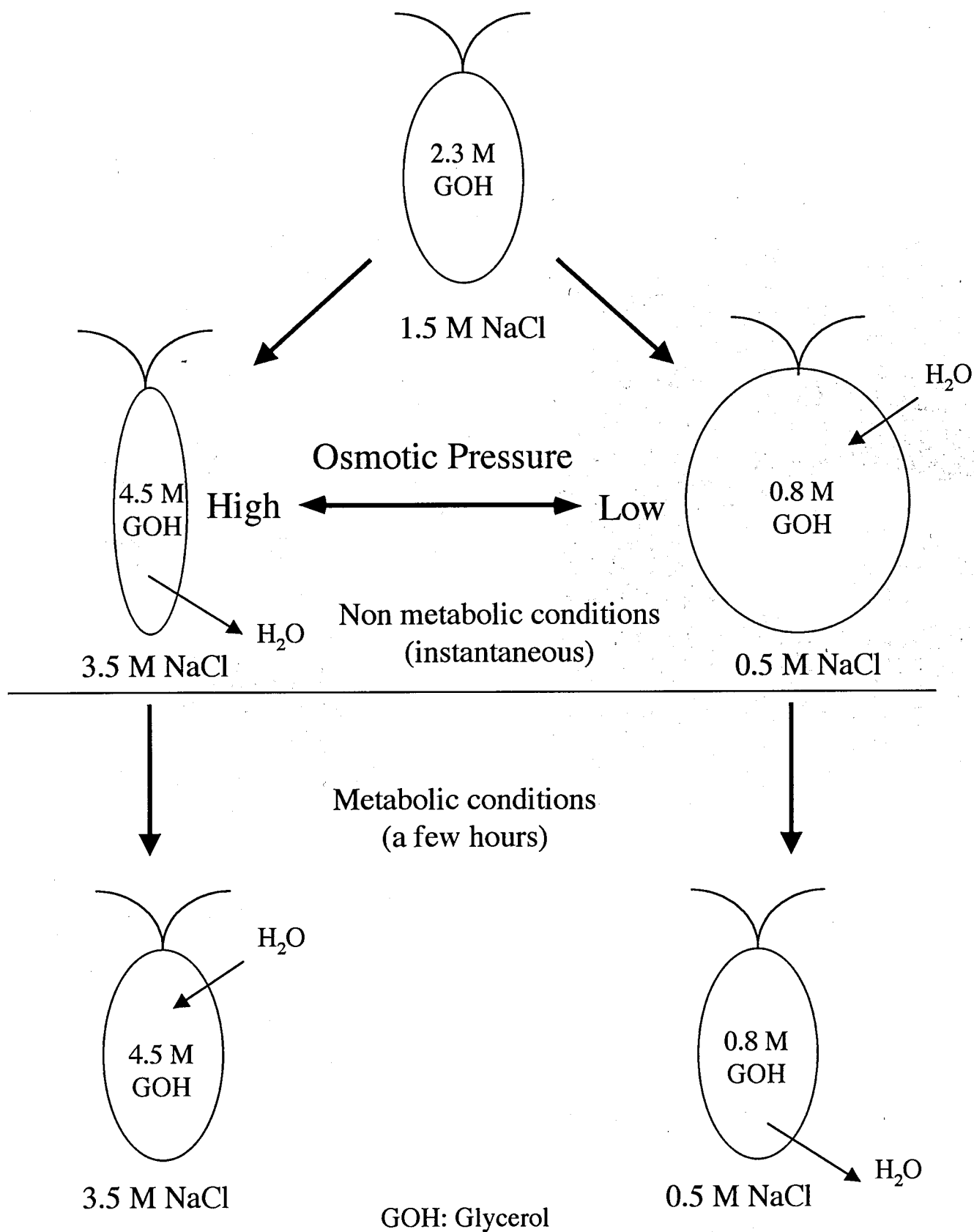


Fig. 1-2 Schematic representation of the volume change of *Dunaliella* in response to hypertonic or hypotonic conditions.
(Ben-Amotz and Avron, 1981)

Table 1-1. Amino acid composition of *Dunaliella*

Amino acid	g (100 g protein) ⁻¹		
	<i>D. tertiolecta</i> *	<i>D. bardawil</i> **	<i>D. salina</i> ***
Alanine	13	7.3	6.3-12.9
Arginine	4.3	7.3	0.9-5.2
Aspartic acid	10.4	10.4	2.5-10.7
Cysteine	-	1.2	-
Glutamic acid	12	12.7	7.9-11.9
Glycine	15.6	5.5	9.5-13.3
Histidine	1.6	1.8	1.2-8.8
Isoleucine	5.2	4.2	2.3-4.2
Leucine	11.3	11	5-17.6
Lysine	6.7	7	4.8-33.9
Methionine	-	2.3	1.4
Phenylalanine	-	5.8	1.4-5.4
Proline	6.9	3.3	4.2-8.8
Serine	3.1	4.6	6.3-8.6
Threonine	1.5	5.4	2.5-6.0
Tryptophan	-	0.7	-
Tyrosine	-	3.7	1.9
Valine	8.5	5.8	3.6-5.8

*Borowitzka. and Kivic 1988

**Ben-Amotz and Avron 1980

***Thomas and Dumas 1970

Table 1-2. Analysis of the stereo isomers of beta-carotene of *Dunaliella bardawil* .

Isomer	% of total beta-carotene
15-cis-beta-carotene	10
9-cis-beta-carotene	41
All-trans-beta-carotene	42
Unidetified isomer	6

(Ben-Amotz and Avron, 1982)

Table 1-3. Influence of various environmental factors on the biomass and beta-carotene content of *Dunaliella salina*.

Factor	Biomass	Beta-carotene
NaCl: 6% to 18%	-	+
N deficiency	-	+
P deficiency	-	+
Increase in (CO ₂) to 5% in air	+	0
White light to red light	0	0
White light to green light	0	0
Light: 1200 to 2200 lx	0	+
Light: 2200 to 500 lx	0	-
Temperature: 30°C to 20°C	0	-
Temperature: 30°C to 35°C	-	+
(O ₂): 12% to 15%	0	-
Combined effect:		
2200 lx at 27°C to 1200 lx at 34°C	--	++

(Mil'ko, 1963)

+: stimulating effect, -: inhibitory effect, 0: no effect

CHAPTER II

Sensitiveness of the surface coat of the halotolerant green alga *Dunaliella parva* (Volvocales, Chlorophyceae) to lysozyme

Abbreviations: DSC, differential scanning calorimetry; HPLC, high performance liquid chromatography; DP, degree of polymerization; GPC, gel permeation chromatography, TLC, thin layer chromatography, HEPES, 2-(4-(2-Hydroxyethyl)-1-piperazinyl) ethanesulfonic acid, DTT, Dithiothreitol, SDS, Sodium lauryl sulfate

INTRODUCTION

The halotolerant green alga *Dunaliella* is well known to be able to grow in extremely high salt concentration by using glycerol as an internal osmoregulatory solute (Ben-Amotz and Avron 1973., Brown 1976.). In addition to this high tolerance to saline environments, this alga also tolerates rapid changes in the environmental salt concentration. In order to adjust to the different osmotic pressures, this alga rapidly changes its cell volume. Accordingly, this alga can adjust the relative concentration of the internal osmoregulatory solute, glycerol, corresponding to the rapid change of the environmental salt concentration (Avron 1986). Since this alga lacks a rigid cell wall, change in the cell volume seems easy for this alga. To achieve such a flexibility of cell volume and accumulate high concentrations of glycerol, *Dunaliella* has been thought to have an unusual cell membrane. (Oliveira *et al.* 1980)

Einspahr *et al.* (1988) reported that the total surface area of the membrane of *Dunaliella salina* Teodoresco does not change during alteration of the cell volume against hyper- or hypoosmotic shock. This finding indicates that the cell membrane of *Dunaliella salina* has a somewhat rigid structure.

The ultrastructure of the surface coat of the cell membrane of *Dunaliella tertiolecta* Butcher was observed by Oliveira *et al.* (1980), who found that the surface coat was stained by cationic dyes, such as alcian blue and ruthenium red, and degraded by enzymes such as trypsin and neuraminidase. From these data, they assume that the surface coat contains a kind of glycoprotein.

Economic utilization of *Dunaliella* has been described many times (Ben-Amotz and

Avron 1983) and bioreactors using *Dunaliella* cells have been developed (Grizeau and Navarro 1986, Hatanaka *et al.* 1996). For a reactor using whole cells, cell membrane permeability of the substrates or the products is an important problem. To obtain more information about the cell membrane and the surface coat of *Dunaliella*, we have screened several enzymes that degrade the surface coat. Here we report the effects of lysozyme, which has been thought to degrade only the cell walls of procaryotes such as bacteria and certain cyanobacteria, on the surface coat of *Dunaliella parva* Lerche.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were obtained from commercial sources and were of reagent grade. Mouse anti-bacterial peptidoglycan monoclonal antibody was obtained from Chemicon International Inc. FITC labeled goat anti-mouse IgG1 was from The Binding Site Ltd. Glutaraldehyde and osmium tetroxide were obtained from TAAB Laboratory Equipment LTD. and were of electron microscopy grade.

Test organisms

Dunaliella parva, strain LB1983, was cultured in 500 ml or 250 ml culture vessel (Biopod, HARIO Co. Ltd., Tokyo) containing 400 ml or 200 ml of growth medium, respectively. The basal composition of the growth medium usually used was 0.5 M NaCl, 50 mM NaHCO₃, 5 mM KNO₃, 5 mM MgSO₄, 0.3 mM CaCl₂, 0.2 mM KH₂PO₄, 0.185 mM H₃BO₃, 7 μ M MnCl₂, 6 μ M Na₂EDTA, 1.5 μ M FeCl₃, 0.8 μ M ZnCl₂, 20 nM CoCl₂, and 0.2 nM CuCl₂, with an initial pH of 8.0 (Ben-Amotz *et al.* 1989).

Since high concentrations of NaCl disturb the measurement of zeta potentials and sometimes inactivate the cell lytic enzymes, 0.5 M NaCl was substituted by 0.5 M sorbitol in this experiment. Cultures were continuously illuminated with cool-white fluorescent lamps at an intensity of 20 μ E $\cdot$ sec⁻¹ $\cdot$ m⁻² at 25°C. Sorbitol concentration was changed to 1 M for the glycerol measurement.

Determination of the change in cell volume

Change in the volume of the cells was determined using the computer image analysis.

Cells cultured in 0.5 M sorbitol medium were harvested by centrifugation and suspended in various concentrations of sorbitol. Incubation was carried out at room temperature without illumination. To fix the changed cell volume, 10⁻¹ volume of 20% glutaraldehyde was added to the cell suspensions at 30, 60, 120, and 240 min after the start of the incubation. Images of the cells were obtained on a differential interference microscope (BX-50) equipped with U-VIS video system. Acquired images were analyzed on Macintosh personal computer using image analysis software (MacScope, MITANI Corporation, Fukui). Cell volume was calculated from the length of the major axis and minor axis of cells by the following equation.

$$V = \frac{4}{3}\pi ab^2$$

V: Cell volume (μm³)

a: Half length of major axis (μm)

b: Half length of minor axis (μm)

Twenty cells from the same conditions were analyzed at each experiment.

Determination of the cell lytic activity of the enzymes

Cell lytic activities of various enzymes were determined morphologically with a differential interference microscope (BX50, Olympus Co. Ltd.; Tokyo). Cells cultured in 0.5 M sorbitol medium were collected by centrifugation at 3,000xg for 10 min and suspended in 5 x 10² volume of 0.5 M sorbitol solution. This cell suspension usually contained 10⁸ cell•ml⁻¹. Various enzymes were added to this solution to make 5 mg•ml⁻¹ enzyme concentration and incubated at 30°C for 2 hours. Enzymes that could induce aggregation or change in the shape of the cells were judged to be effective.

Electron microscopy

For transmission electron microscopy (TEM), cells cultivated in 0.5 M sorbitol medium were concentrated by centrifugation and suspended in 0.5 M sorbitol solution. After various enzyme treatments, cells were collected by centrifugation and fixed by two different procedures.

For conventional fixation, cells were concentrated by centrifugation at 3,000xg for 10

min and fixed with 2% glutaraldehyde in 0.05 M Tris-HCl buffer (pH 8.0) containing 0.5 M sorbitol for 2 h at room temperature. Samples were then washed three times with 0.5 M sorbitol, post-fixed with 1% osmium tetroxide in 0.5 M sorbitol for 30 min at room temperature, and washed three times with distilled water. Next, samples were stained with saturated uranyl acetate for 10 min at room temperature, washed with distilled water (three times), dehydrated through an ethanol series, cleared with propylene oxide, and embedded in Spurr resin.

To emphasize the surface coat, ice-cold cells were mixed with the fixative, containing 2% glutaraldehyde, 1% osmium tetroxide, and 4.5% sucrose in 0.7 M sorbitol, and were kept ice-cold for 2 h. Samples were then washed with distilled water and stained with saturated uranyl acetate for 20 min at room temperature. After washing with distilled water (three times), samples were dehydrated through an acetone series and directly embedded in Spurr resin. Blocks prepared by the two different methods were polymerized at 70°C for 16 h. The blocks were sectioned on an ultramicrotome (OM U3, Richert, Vienna) and sections were post-stained with saturated uranyl acetate for 20 min followed by Reynolds' lead citrate (Reynolds 1963) for 10 min. Sections were observed and photographed on a electron microscope (1200 EX, JEOL, Tokyo).

For scanning electron microscopy (SEM), cell suspensions were filtered through microfiltration membranes (SEMPORE (pore size 0.6 μm), JEOL DATUMN, Tokyo). Filter-trapped cells were fixed with 2% glutaraldehyde in 0.5 M sorbitol and washed three times in 0.5 M sorbitol. After dehydration through an ethanol series, samples were dried further by *t*-butanol freeze-drying. The specimens were coated with gold in an ion sputtering apparatus and observed by SEM (JSM 5800 LV, JEOL).

Measurement of zeta potentials

Cells cultured in 0.5 M sorbitol medium were harvested by centrifugation and suspended in 0.5 M sorbitol containing 0.01% glutaraldehyde to stop cell motility. The electrophoretic mobility (μ) of cells was measured in a Particle Micro-electrophoresis Mark II apparatus (RANK BROTHERS, London). The cell suspension was diluted 10^{-1} with 0.5 M sorbitol and electrophoresis was done in the same solution. The zeta potential (ζ) of the cells was calculated from the electrophoretic mobility (μ) using the Smoluchowski equation (Gimmler

et al. 1989, 1991a):

$$\zeta = \frac{\mu\eta}{\epsilon\epsilon_0}$$

h: viscosity (cp)

e: relative dielectric constant

ϵ_0 : dielectric constant of vacuum

Determination of glycerol released from the cells

To determine the effects of the enzymes, the concentration of glycerol, which was released as the result of enzyme treatments, was measured by gas chromatography.

Cells cultured in 1 M sorbitol medium were collected by centrifugation and suspended in 5×10^2 volume of 1 M sorbitol solution. This cell suspension usually contained 10^8 cell $\cdot$ ml $^{-1}$. After the enzyme treatment, algal cells were removed by centrifugation and 10^{-1} volume of an aqueous 1,2-propanediol solution was added to the supernatant as an internal standard. The samples were injected directly into the gas chromatograph (GC-6A, Shimadzu Co., Tokyo) equipped with a flame ionization detector and a porous polymer column (Tenax-TA, 3mm $\varnothing$ x 2m, GL Sciences Co. Ltd., Tokyo) at 220 °C, and the concentration of glycerol was calculated from ratio of the the peak area of the component to that of the internal standard.

Detection of the peptidoglycan-like structure using anti-peptidoglycan monoclonal antibody

Dunaliella cells cultivated in 0.5 M sorbitol medium were harvested by centrifugation and washed three times with 0.5 M sorbitol. To expose the peptidoglycan embedded in the surface structure, washed cells were suspended in 0.05% SDS and incubated at 25°C for 30 min. After washing three times in 0.5 M sorbitol, cells were resuspended in 0.5 M sorbitol and 20 μ l of mouse anti-bacterial peptidoglycan monoclonal antibody was added to the cell suspension and incubated at 25°C for 3 h. After washing three times in 0.5 M sorbitol, cells were suspended in 0.5 M sorbitol containing FITC labeled anti-mouse IgG antibody and incubated at 25°C for 2 h. Washed cells were observed and photographed on a laser scanning microscope (FLUOVIEW, Olympus Co. Ltd.). As the positive and negative controls of this immnofluorescence, *Escherichia coli* IFO 3301 and *Chlorella pyrenoidosa* IAM C-106 were

treated as the same procedure as above, except 0.1% SDS and 0.1 M sorbitol were used.

RESULTS

Substitution of NaCl by sorbitol

For the study of the plasmalemma of *Dunaliella parva* about the electrical properties and the tolerance against the cell lytic enzymes, we had to use sorbitol as an osmoticum. Although the growth of this alga at sorbitol concentrations above 1 M was slightly inhibited, *Dunaliella parva* cultured in sorbitol medium also tolerates rapid change in the osmotic pressure of the sorbitol medium in the same way as in NaCl medium (Fig. 2-1). Figure 2-2A shows the time-course change in the cell volumes of the cells after hyper-osmotic shock. As reported previously with hyper-osmotic shock caused by NaCl (Ben-Amotz and Avron 1981), the changed volume of the cells was recovered to the original in about 2 h. From the observation of the changes in cell shapes, it seems more easy for this alga to change its volume toward horizontal direction than longitudinal direction (Fig. 2-2B).

All these experiments indicates that this alga has some kind of flexible surface structure even in the sorbitol medium.

Morphological changes induced by cell lytic enzymes

Effects of various cell lytic enzymes on *Dunaliella parva* are shown in Table 2-1. Proteolytic enzymes such as trypsin, chymotrypsin, and papain, which were reported to be effective in *Dunaliella tertiolecta* (Oliveira *et al.* 1980) also show cell lytic activity in *Dunaliella parva*. Although neuraminidase was also reported to degrade the cell surface coat of *Dunaliella tertiolecta* (Oliveira *et al.* 1980), no morphological change was observed when neuraminidase or other polysaccharide-degrading enzymes except lysozyme were used with *Dunaliella parva*.

Figure 2-3, 4, 5 show SEM observations of the morphological changes in *Dunaliella parva* that were induced by the enzymes. Cells treated with lysozyme (Fig. 2-4) or trypsin (Fig. 2-5) showed cell lysis and aggregation.

Ultra-thin sections of the cells treated with lysozyme (Fig. 2-7) showed strange shaped cells compare to the lysozyme untreated cell (Fig. 2-6). Figure 2-7 also showed cell adhesion and tight junction of the plasmalemma of oppressed cells.

From the observation of the specimens prepared using a conventional method (Fig. 2-6), no significant structure was observed around the cell surface. By using the mixed fixation method, the folded structure of certain membranes and high density materials around it were seen in Fig. 2-8 (arrows). Figure 2-8 also showed the fusion of the cell membrane.

Electrophoretic mobility

Changes in the electrophoretic mobility of the cells after the treatment with various enzymes were shown in Table 2-2. As reported before (Gimmler *et al.* 1991b), *Dunaliella* cells had the negative charge on their cell surface. After treatment with lysozyme or trypsin, the electrophoretic mobility was lowered and finally became reversed.

Determination of the change in permeability of the plasmalemma using gas chromatography

Measurements of released glycerol using gas chromatography showed that significant amounts of glycerol were released from the cells by the treatment with lysozyme or trypsin (Table 2-3). This leakage of glycerol may be caused either by cell disruption or by change in permeability of the cell membrane.

Detection of a peptidoglycan-like structure using monoclonal antibody

Anti-peptidoglycan monoclonal antibody was used to investigate the surface structure of *Dunaliella parva*, which is sensitive to lysozyme. Figure 2-9 shows the binding activity of this antibody to the bacterial (*E. coli*) cell wall which contains peptidoglycan. This antibody was also confirmed not to bind to the cell wall of the green alga *Chlorella pyrenoidosa*, which does not have any peptidoglycan (Fig. 2-10). Although almost all of *Dunaliella* cells were destroyed by SDS treatment which was used to expose the embedded peptidoglycan, some of the cellular components were found to react with the monoclonal antibody (Fig. 2-11).

DISCUSSION

The wall-less microalga *Dunaliella* has been thought to have certain specialized surface structures. The surface structure of *Dunaliella tertiolecta* was reported to contain some kinds

of glycoprotein (Oliveira *et al.* 1980). The present study showed that the surface coat of *Dunaliella parva* contains glycoprotein that is sensitive to lysozyme, the enzyme that principally degrades the cell wall of bacteria (Table 2-1, Figs. 2-1, -4). The effects of lysozyme on the surface charge and the leakage of glycerol (Table 2-2, 3) indicated that lysozyme also induces the damage of the plasmalemma of *Dunaliella parva*. The change in the surface charge may explain the cell aggregation after this enzyme treatment. Since lysozyme has a cationic charge, this neutralization of the anionic charge of the cell surface seems to be caused by the attachment of this enzyme to the surface of the cells or by the degradation of several anionic groups by the enzyme. Cells having negative charges may repulse each other, but the cells treated by the enzyme may aggregate.

The utilization of microalgae for the production of food, food additives, pharmaceuticals, and fine chemicals is growing in importance (Becker 1994). Besides using wild type strains of microalgae, improvement of some properties of microalgae by genetic engineering has been studied (Simon and Latorella 1986). In such study, technique for protoplast formation is much important for transformation or fusion of microalgal cells. Several studies on protoplast formation of green algae, such as *Chlorella ellipsoidea* (Yamada and Sakaguchi 1981) and *Chlamydomonas reinhardtii* (Robinson and Schlösser 1978) have been reported. To degrade cell wall and obtain protoplasts of *Chlamydomonas*, which is related to *Dunaliella*, a proteolytic enzyme such as autolysin (Matsuda *et al.* 1984, 1985) was used. From the results of the present study, lysozyme, which is a polysaccharide-degrading enzyme, also might be used to degrade certain surface structure of this alga.

In this report we demonstrated that the surface glycoprotein of *Dunaliella parva* is analogous to peptidoglycan (Figs 2-4, 7, 11), which is a characteristic cell wall component of bacteria, including cyanobacteria (Van Den Hoek *et al.* 1995). Observation of the specimen prepared by the mixed fixation method showed certain high density materials around the cell membrane (Fig. 2-8). These high density materials may contain the peptidoglycan-like glycoprotein.

As far as we know, peptidoglycan has not been detected on the cell surface of eucaryotic algae or higher plants.

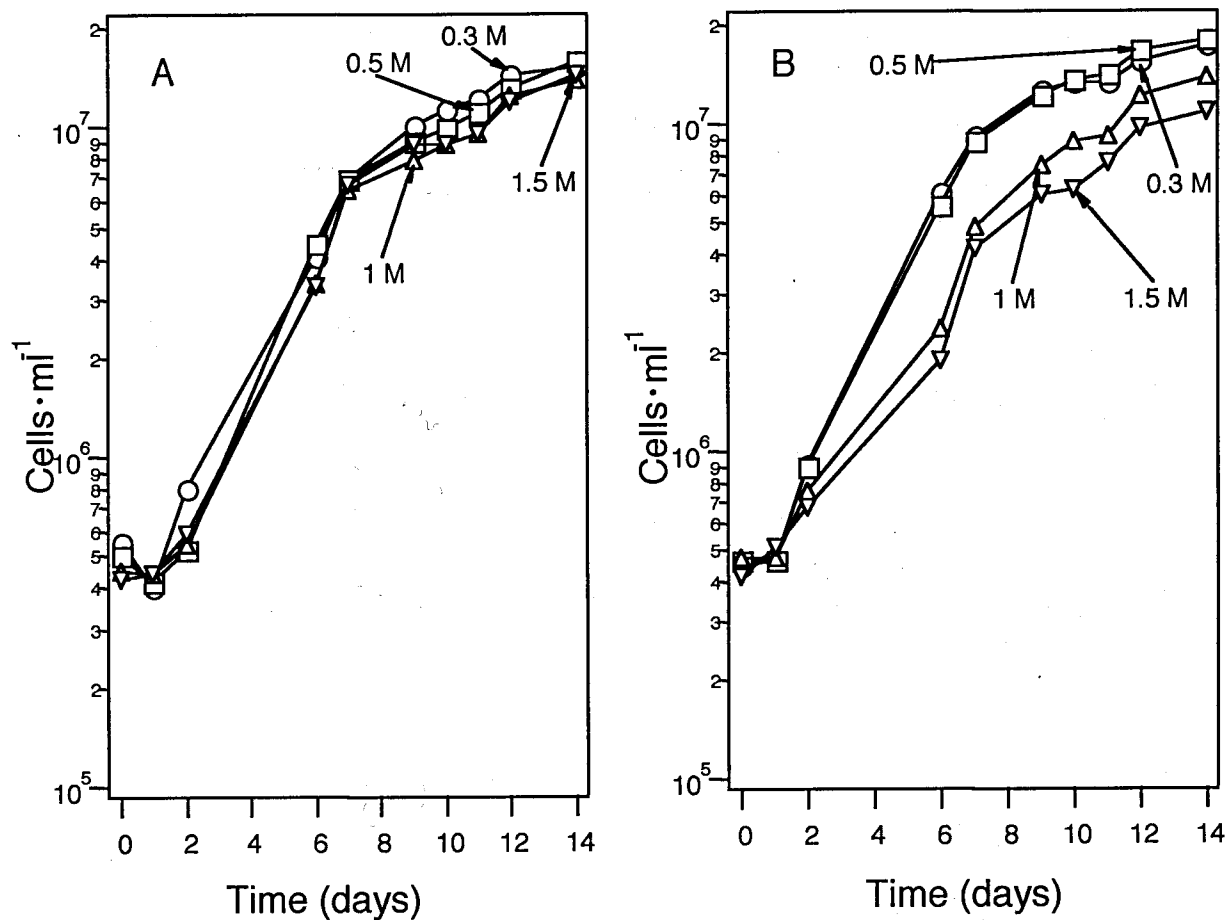


Fig. 2-1. Growth of *Dunaliella parva* at various concentrations of sorbitol and NaCl. Cells cultured in 0.5 M sorbitol medium were transferred to new media containing various concentrations of NaCl (A) or sorbitol (B).

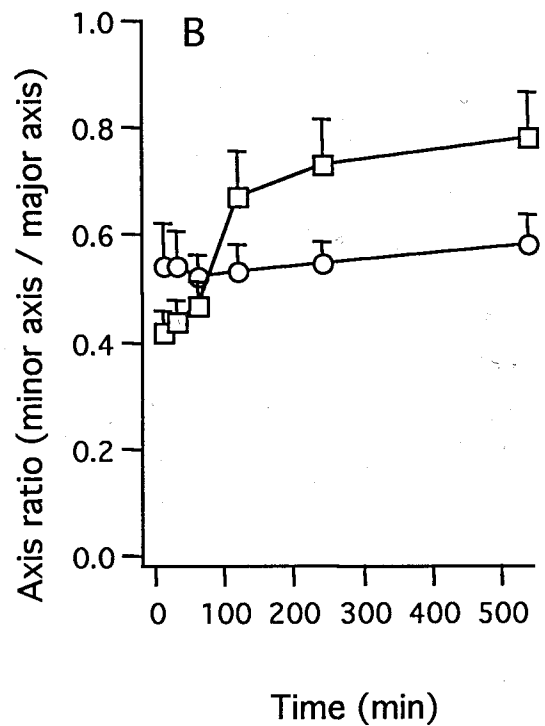
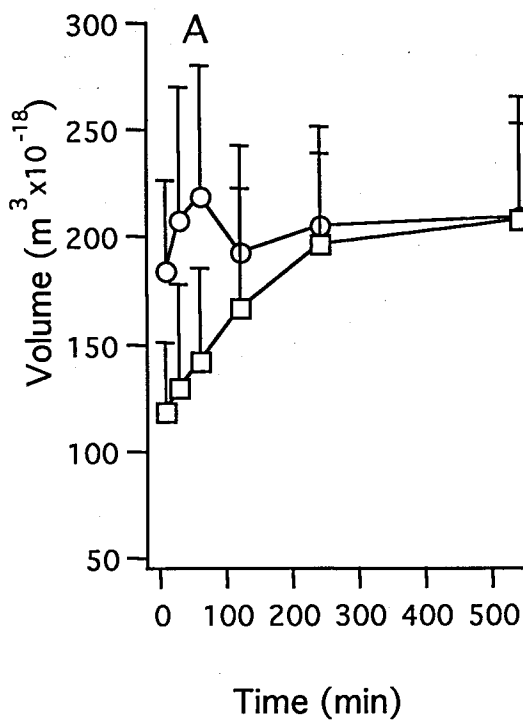
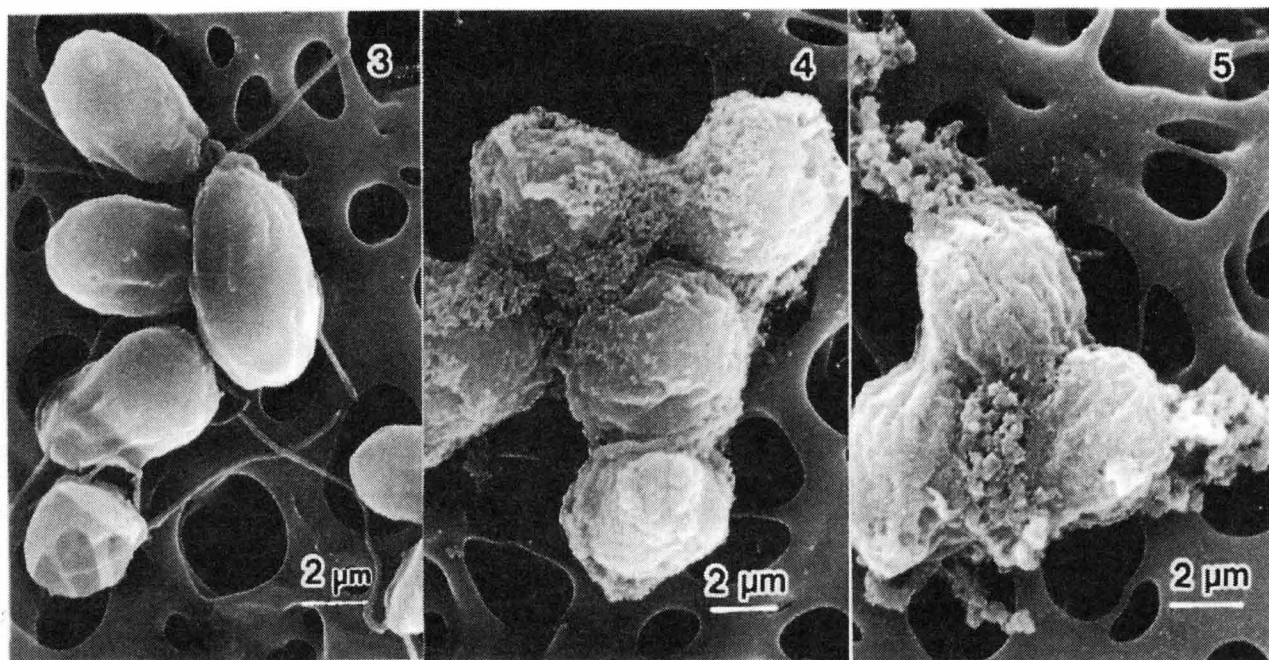
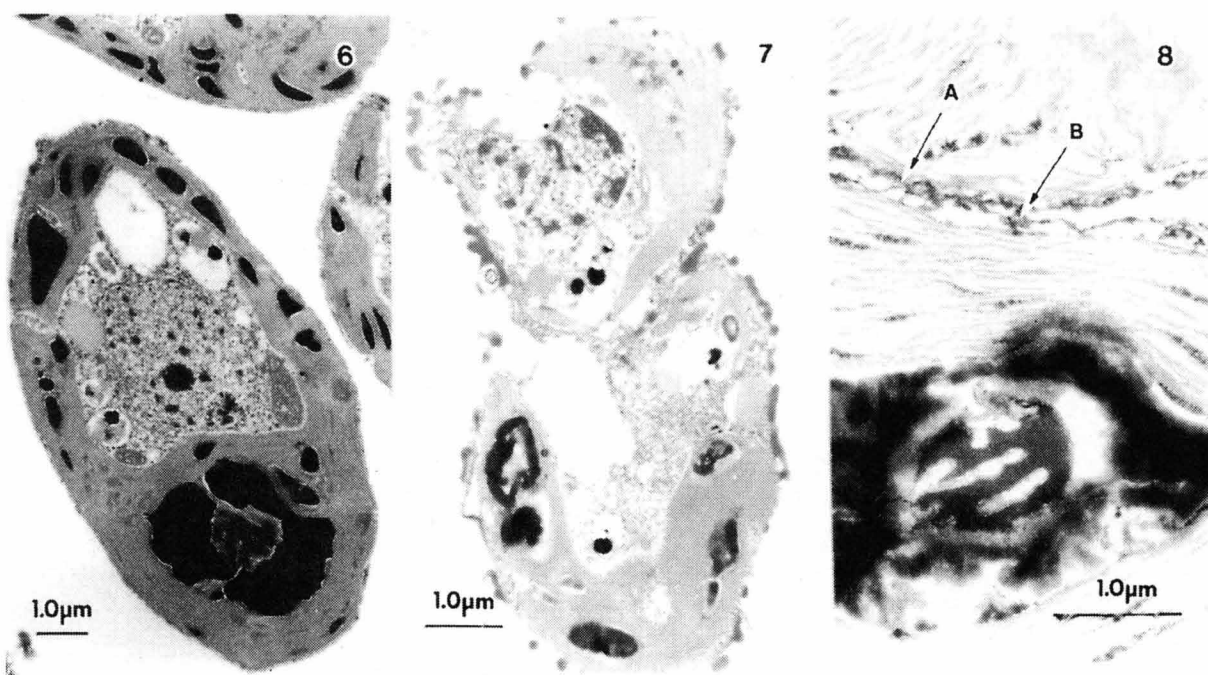


Fig. 2-2. Time course of change in the cell volume (A) and shape (B) of *Dunaliella parva* after hyper-osmotic shock. Cells cultured in 1 M sorbitol medium were transferred to 1.5 M sorbitol medium (□) or 1 M sorbitol medium (○). Mean and SD are shown (2-1 length, n=20 for each condition).



Figs 2- 3, -5. SEM observation of the morphological change in *Dunaliella parva* induced by trypsin and lysozyme treatment. Cells suspended in 0.5 M sorbitol were treated with enzymes at 30°C for 2 h. After filtration using SEMPORE, cells were fixed, washed, and dehydrated. Dehydrated cells were coated with gold and observed with SEM. 3. no enzyme, 4. trypsin treatment, 5. lysozyme treatment.



Figs 2-6, -8. Observation of ultra-thin sections of *Dunaliella parva* cells treated with lysozyme. Cells treated with lysozyme were fixed, dried, and embedded in Spurr resin following conventional or mixed fixation method. 6. Lysozyme untreated cells, 7. Longitudinal section of two cells fused by the treatment of lysozyme. 8. Observation of the plasmalemma of lysozyme treated *Dunaliella parva* cells using conventional fixation. Arrow in Fig. 8 shows the plasmalemma.

Table 2-1. Effect of several enzymes on cell lysis of *Dunaliella parva*

Enzymes	Cell lysis*
Control	-
Cellulase	-
Hemicellulase	-
β -1,3-glucanase	-
Neuraminidase	-
Chitosanase	-
Lysozyme	+
Trypsin	+
Chymotrypsin	+
Papain	+

*Cell lysis was observed by light microscope

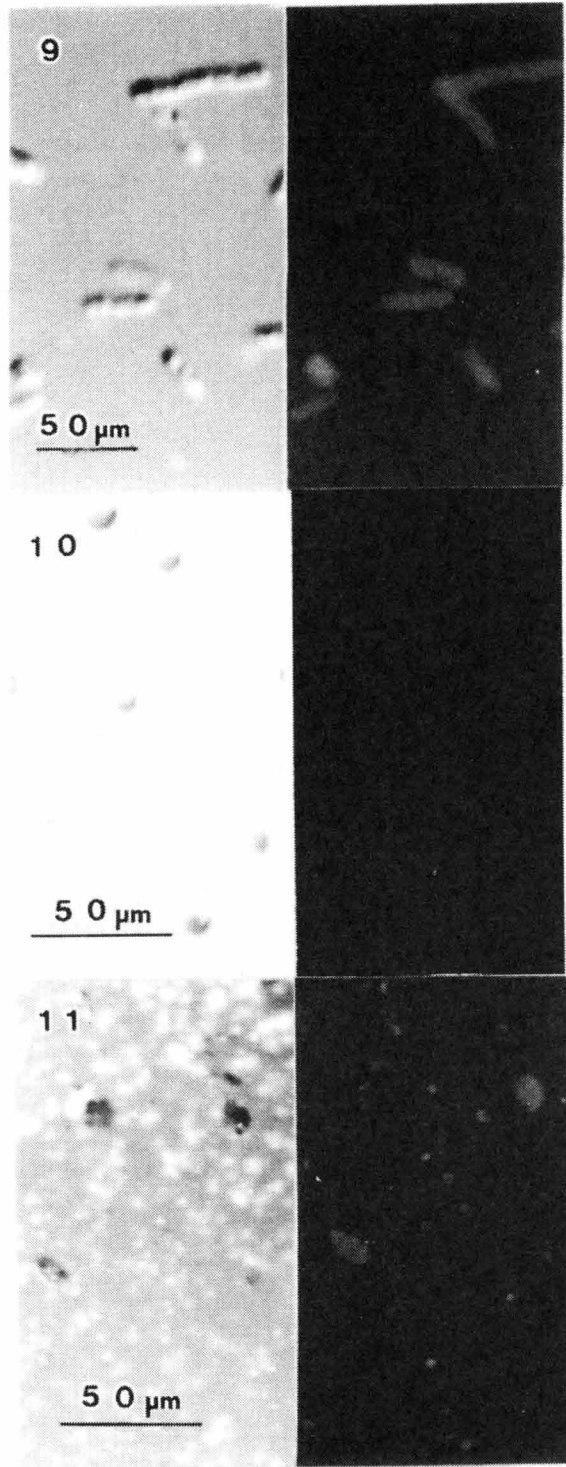
Table 2-2. Effect of several enzymes on the zeta potentials of *Dunaliella parva*

Enzymes	Zeta potential(ζ)
Control	-53.3
Cellulase	-59.6
Hemicellulase	-51.8
β -1,3-glucanase	-55.9
Neuraminidase	-46.1
Chitosanase	-61.2
Lysozyme	14.7
Trypsin	0

Table 2-3. Effect of lysozyme and trypsin on the permeability of the plasmalemma of *Dunaliella parva*

Treatment	Glycerol ($\times 10^{11}$ mol $\cdot$ cell $^{-1}$)
Control	0.18
*Cell destruction	1.61
Lysozyme	0.74
Trypsin	0.34

*Total amount of glycerol, measured after osmotic destruction of the cells.



Figs 2-9, -11. Immunochemical detection of peptidoglycan-like structure. Differential interference microscopy (left panel) and fluorescent microscopy (right panel) by laser scanning microscope at excitation wavelength of 488 nm. *E. coli* (Fig. 9), *Chlorella pyrenoidosa* (Fig. 10), and *Dunaliella parva* (Fig. 11). Small particles in Fig. 11 are thought to be cell residues (mainly starch granules) of *Dunaliella* destroyed by the detergent (SDS) treatment.

CHAPTER III

Study of Some Properties of Starch Isolated from Halotolerant Microalgae

Dunaliella

INTRODUCTION

Halotolerant microalgae *Dunaliella* is well known to accumulate massive amount of glycerol and β -carotene (Ben-Amotz and Avron 1983)(Ben-Amotz, Katz, and Avron 1982), the former for the purpose of osmoregulationA. (Ben-Amotz and Avron 1973) (Ben-Amotz 1975) and the latter for defence to high irradiation of light (Ben-Amotz and Avron 1989), respectively. Besides these useful chemicals, this algae also accumulate relatively high content of insoluble carbohydrate in its body (Borowitzka and Borowitzka 1988). Among the algae, there are two general types of carbohydrate reserves. One is α -1,4-glucans (starch) and another is β -1,3-glucans (leucosin, laminaran, or palamilon) (Sze 1986). In the members of the Division Chlorophyta (green algae), those are *Dunaliella*, *Chlorella*, *Chlamydomonas*, and so on, the reserved carbohydrate is starch. Since green algae have the most enzymes of the metabolic pathways of higher plants, it believed to be useful to study green algae as a model of higher plants. Because the cultivation of *Chlamydomonas reinhardtii* is very easy and the starch synthesis and degradation can easily be controlled by several stresses (Ball *et al.* 1990)(Harris 1989)(Cohen and Parnas 1976)(Levi and Gibbs 1984)(Peavy, Smith, and Latorella 1986), starch and its biogenesis was actively studied using this alga as a model of higher plants. As mentioned above, starch of an relative species of *Dunaliella* (*Chlamydomonas reinhardtii*) was studied very well, but the study about the starch of *Dunaliella* is very limited (Peavy, Smith, and Latorella 1986). In addition to the fact that the starch content is greater than that of *Chlamydomonas*, it is known that the starch biogenesis of *Dunaliella* is also regulated by the osmoregulation (Goyal, Brown, and Gimmmler 1987). Since the osmotic control of the starch synthesis and degradation in *Dunaliella* cells performed in the very short time (for several hour) and the starch preparation is very easy, starch of *Dunaliella* is thought to be a useful model compound to understand the starch of lower or higher plants in the high salinity environment. In this report, we show some

properties of the starch of *Dunaliella* and examine the value of the microalgal starch as the food resources.

MATERIALS and METHODS

Chemicals.

All the chemicals used in this study were obtained from commercial sources and were of reagent grade. Glutaraldehyde and osmium tetra oxide were obtained from TAAB LABORATORY EQUIPMENT LTD. and were of electron microscopic grade.

Pseudomonas amyloclavata isoamylase was from HAYASHIBARA BIOCHEMICAL LABORATORIES, INC. Glycogen from oyster was from NACALAI TESQUE INC.

Test organisms.

Dunaliella parva, strain LB1983, and other strains of *Dunaliella* were cultured in 500 ml or 250 ml culture vessels (Bio pod, HARIO Co. Ltd.) containing 400 ml or 200 ml of growth medium, respectively. The growth medium contained 1M NaCl, 50mM NaHCO₃, 5mM KNO₃, 5mM MgSO₄, 0.3 mM CaCl₂, 0.2 mM KH₂PO₄, 0.185 mM H₃BO₃, 7 μ M MnCl₂, 6 μ M Na₂EDTA, 1.5 μ M FeCl₃, 0.8 μ M ZnCl₂, 20 nM CoCl₂, and 0.2 nM CuCl₂, the initial pH was 8.0 (Ben-Amotz, Shaish, and Avron 1989). Cultures were continuously illuminated with cool-white fluorescent lamps at an intensity of 20 μ E•s⁻¹•m⁻².

Preparation of the starch from *Dunaliella*.

Dunaliella cells were harvested by centrifugation at 3000 g for 10 min and the cell debris were immediately lyophilized to estimate the dried cell weight. Dried cell debris was resuspended in 10 ml of 50 mM phosphate buffer, pH 7.0, containing 10 mg of trypsin and allowed to stay for 3 hour at 37°C. After centrifugation at 3000 g, cell extracts were further purified by detergent (0.1 % Triton x-100 in 0.1 M NaOH) treatment and dried by methanol washing.

Since the starches of *Chlamydomonas reinhardtii* and *Dunaliella* sp. could not be purified by the method mentioned above, those starches were purified by the density gradient centrifugation using percoll (Delure *et al.* 1992).

Starch samples were dissolved in 0.5 M KOH and the amounts of starch were

determined by the phenol-sulfuric acid method (Dubois *et al.* 1956).

Determination of the gelatinization temperature of the starch from *Dunaliella*.

Gelatinization temperature of various starch samples were determined using DSC (Stevence and Elton 1971). Aluminum vessels containing 2-8 mg starch samples and two volumes of distilled water were sealed and subjected to DSC (DSC/5200, Seiko Instrument Inc.). Samples were heated from 25 °C to 100 °C at the rate of 2 °C per min. In this experiment, the extrapolated onset temperature (Shiotsubo and Takahashi 1984) (the intersection point of the baseline and the extrapolated line of the maximum angle of inclination on the DSC curve) acquired in the measurement were defined as the gelatinization temperature.

X-ray diffraction measurements of starch samples.

About 100 mg of starch samples were suspended in a small amount of distilled water and subjected to X-ray diffraction measurements (MXP18, Mac Science Co. Ltd.). X-ray diffraction measurements were performed using monochromated Cu K_{α} radiation.

Electron microscopy.

For transmission electron microscopy, cells cultivated in various conditions were fixed by the addition 10⁻¹ volume of 20% glutaraldehyde and stayed for 2 h. Samples were subsequently washed 3 times with 1 M sorbitol. Washed samples were post-fixed with 1% osmium tetroxide in 1 M sorbitol for 1 h and washed 3 times with distilled water. Subsequently, samples were stained with saturated uranyl acetate for 20 min. After washing with distilled water (3 times), samples were dehydrated through an increasing ethanol series (30-100%), cleared with propylene oxide, and embedded in Spurr resin. All the procedure were performed at room temperature. The specimens were polymerized at 70 °C for 16 h. The blocks were sectioned on ultramicrotome (OM U3, Rihert) and sections were post-stained with saturated uranyl acetate for 20 min followed by Reynold's lead citrate (Reynoldz 1963) for 10 min. Sections were observed and photographed on a JEOL 1200 EX electron microscope.

Chlamydomonas reinhardtii was observed in the same way except 0.1 M sorbitol was

used for the osmoregulation.

Debranching analysis.

Debranching of starch by isoamylase was achieved as follows. 10-40 mg of starch samples were dissolved in 2 ml of 0.5 M KOH solution. After neutralization of pH by the addition of 1 M HCl, 300 units of isoamylase in 300 μ l of 1 M acetate buffer pH 4.0 were added to the starch solution. To avoid the contamination of microorganisms, one drop of toluene was added to the reaction mixtures. After 2 days incubation at 37 °C, the mixture was centrifuged at 1200 g for 10 min. The water-soluble materials were desalted by the chromatographic step on a TSK-HW40(F) column (20 mm ϕ x 500 mm) fed by 0.5% (v/v) acetic acid. The pooled polysaccharide fractions were dried and dissolved in 100 μ l of 60% acetonitrile (v/v). After centrifugation at 1200 g for 10 min, 60% acetonitrile soluble materials were subjected to HPLC (Asahipak NH2P-50 (asahi chemical industry Co. Ltd.), 0.6 ml flow, fed by 60% acetonitrile, RI detector).

Amperometric titration

Amylose contents of starches were measured by amperometric titration method. 100mg of starch samples were dissolved in 20 ml of 0.5 M KOH solution and diluted to 75 ml with distilled water. After the addition of 20 ml of 1 N HCl and 5 ml of 0.4 N KI, iodine affinity was measured using automatic recording titrator (ART-3, HIRAMA LABORATORIES). Amylose content (%) was calculated using the following equation.

$$\text{Amylose content(\%)} = \frac{\text{Iodine affinity of sample}}{\text{Iodine affinity of amylose}^*} \times 100$$

*From the preliminary measurement of the iodine affinity of purified potato amylose, the value used in this study was defined as 20 (mg I₂ / 100 mg of sample).

In this study, apparent amylose content was calculated by defining the iodine affinity of amylopectin as 0.

RESULTS

Starch content of several species of *Dunaliella*. Table 3-1 shows the starch content of several *Dunaliella*, which was cultured in our laboratory. Starch content of *Dunaliella* varied

4.8-14% of dried cell weight and this value was comparatively higher than that of *Chlamydomonas reinhardtii*, which is an relative species of *Dunaliella*. Electron micrographs (Fig. 3-1) of each alga also indicated that the *Dunaliella* cells had the larger starch granules.

Some properties of the starch from *Dunaliella*. To understand the characteristics of the starch from *Dunaliella*, some properties of this starch were compared to the starch from potato, maize or other sources.

1. Gelatinization temperature of starch from *Dunaliella*, potato, and maize. From the results acquired by the DSC analysis, the gelatinization temperatures of starch samples from several *Dunaliella* cultivated at 25 °C were at around 60 °C (Table 3-2). The starch from *Dunaliella salina* (cultivated at 30 °C) and *Dunaliella* sp. (cultivated at 5 °C) showed a little higher or lower gelatinization temperature compare to those of the starches from usually cultured cells.

2. X-ray diffraction measurements of the starch from potato, corn, and *Dunaliella parva*. The x-ray diffraction patterns of three starch samples (Fig. 3-2) indicated that the starch from *Dunaliella parva* cultured at 25 °C was similar to that of starch from maize.

The impurity peak appeared in the diffraction pattern of the starch from *Dunaliella* was identified with CaCO_3 (Joint Committee on Powder Diffraction Standards 1986).

3. Compositions of short chains of several carbohydrate samples. HPLC analysis of the short chains (up to 15 glucose residues long) of several debranched carbohydrate samples were shown in Fig. 3-3. Although the amount of short chains were not so much compare to that of glycogen, the chain distribution of *Dunaliella parva* inclined to the shorter chains than that of maize.

Starch accumulation induced by nitrogen starvation in *Dunaliella parva*. It was reported that the nitrogen starvation for the cells at logarithmic phase induces the starch accumulation in *Chlamydomonas* (Maddelein *et al.* 1994) and *Dunaliella* (Ben-Amotz and Tornabene

1985). Transfer to the nitrogen free medium increased the starch content of *Dunaliella parva* up to 45% of dried cell weight till 10 days of nitrogen starvation (Fig. 3-4).

Comparison of the electron micrographs of *Dunaliella parva* transferred to nitrogen sufficient (N+) or nitrogen deficient (N-) medium also shows marked increase in the number of starch granules in nitrogen starved cells (Fig. 3-5). Although the starch granules distributed only around the pyrenoid in the normally grown cell, the starch granules of the nitrogen starved cell were dispersed all around the cells and the shape of pyrenoid became unclear. Like as mentioned before about the nitrogen starved *Chlamydomonas reinhardtii* (Van den Koornhuysen, N. *et. al.* 1996), several lipid droplets were also observed in the nitrogen starved *Dunaliella parva*.

DISCUSSION

Algae of the genus *Dunaliella* are most studied microalgae for mass culture, because there are several strains which was known to accumulate massive amounts of useful compounds. The utilization of *Dunaliella* cells for a feed source in aquaculture (Spectrova *et al.* 1982) or for the source of β -carotene and glycerol (Ben-Amotz and Avron 1983) were well studied. In addition to these useful compounds, a large quantity of starch is also accumulated during the mass cultivation of *Dunaliella* (Becker 1988) (Table 3-1, Fig. 3-1).

From the x-ray diffraction measurements (Fig. 3-2), crystal structure of the starch of *Dunaliella* was similar to the starch of maize. DSC analysis revealed that the gelatinization temperatures of the starch of several strains of *Dunaliella* were closer to that of potato (Table 3-2). The starch from two strains of *Dunaliella* cultured at different temperatures were slightly different. The small shift of the gelatinization temperature of *Dunaliella* sp. and *Dunaliella salina* seems reasonable from the fact that the gelatinization temperature of certain plants are affected by the temperatures during the cultivation (Hizukuri, Fujii, and Nikuni 1965) (Hizukuri 1969) (Suzuki *et al.* 1987). The reason of these temperature shift is still not clear.

The apparent amylose contents of starch from *Dunaliella parva* and maize were measured in our laboratory by amperometric titration method and it was found that the apparent amylose content of the two starches were almost the same (20.6 % for *Dunaliella*

parva and 21.2 % for maize). The composition of the short chains of starch of this alga was, however, tended to distribute to shorter chains compare to the starch of maize. Although the debranching analysis was performed with the starch samples which had not been separated the amylose and amylopectin, composition of the short chains resembles that of amylopectin II fraction of *Chlamydomonas reinhardtii* (Van den Kooymhuyse *et al.* 1996).

From the experiment of nitrogen starvation, starch content of this alga can be increased to about 50% of dried cell weight (Fig. 3-4). Electron microscopic observation also indicated that nitrogen starved cells also accumulate some kind of lipid (Fig. 3-5). Nitrogen starvation seemed useful to improve *Dunaliella* cells to the high calory feed for aquaculture.

Since this alga has the extreme salinity tolerance, industrial cultivation of *Dunaliella* as a uni-algal culture, can easily be maintained (Borowitzka, Moulton, and Borowitzka 1985) and massive amounts of starch can be acquired. For these reasons, the starch of *Dunaliella* will be important for the agriculture of saline environment, where the most plants can not be cultured.

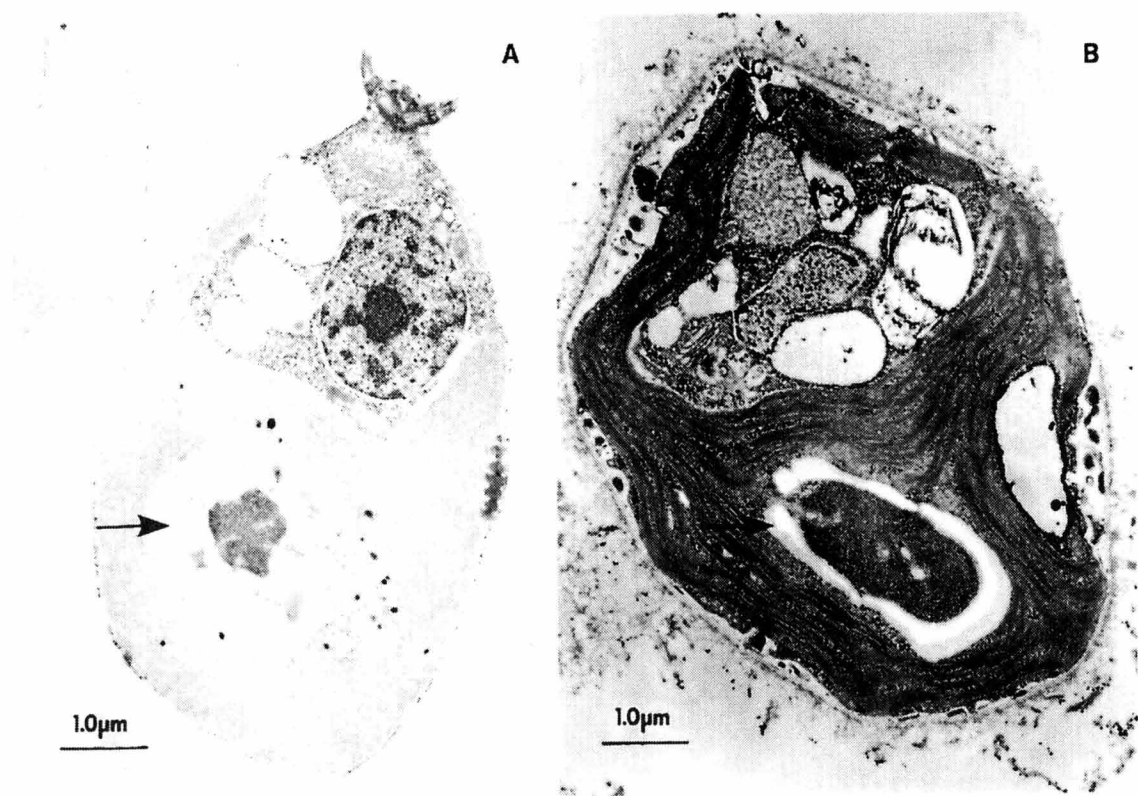


Fig. 3-1. Electron micrographs of *Dunaliella parva* and *Chlamidomonas reinhardtii*. Transmission electron micrographs of the ultra thin sections of *Dunaliella parva* (panel A) and *Chlamidomonas reinhardtii* (panel B). The starch granules are shown by arrows.

Table 3-1
Starch contents of *Chlamidomonas reinhardtii* and
several strains of *Dunaliella*

Strains	Starch (% of dry matter)
<i>Dunaliella bioculata</i>	4.8
<i>Dunaliella parva</i>	13.1
<i>Dunaliella plimolecta</i>	14.0
<i>Dunaliella peircei</i>	8.3
<i>Dunaliella tertiolecta</i>	7.9
<i>Dunaliella sp.*</i>	12.4
<i>Chlamidomonas reinhardtii</i>	2.1

*The strain isolated in the Antarctic Continent

Table 3-2
Gelatinization temperatures of starch from potato, maize,
and several strains of *Dunaliella*

Origin of starch	Gelatinization temperature (°C)
<i>Dunaliella bioculata</i>	58.2
<i>Dunaliella parva</i>	60.7
<i>Dunaliella primolecta</i>	62.5
<i>Dunaliella tertiolecta</i>	60.8
<i>Dunaliella sp. *</i>	60.3
<i>Dunaliella salina</i>	66.6
<i>Dunaliella sp. **</i>	55.6
Potato	57.8
Maize	68.6

*Strain isolated on the Antarctic Continent, cultivated at 15°C.

**The same strain cultivated at 5°C.

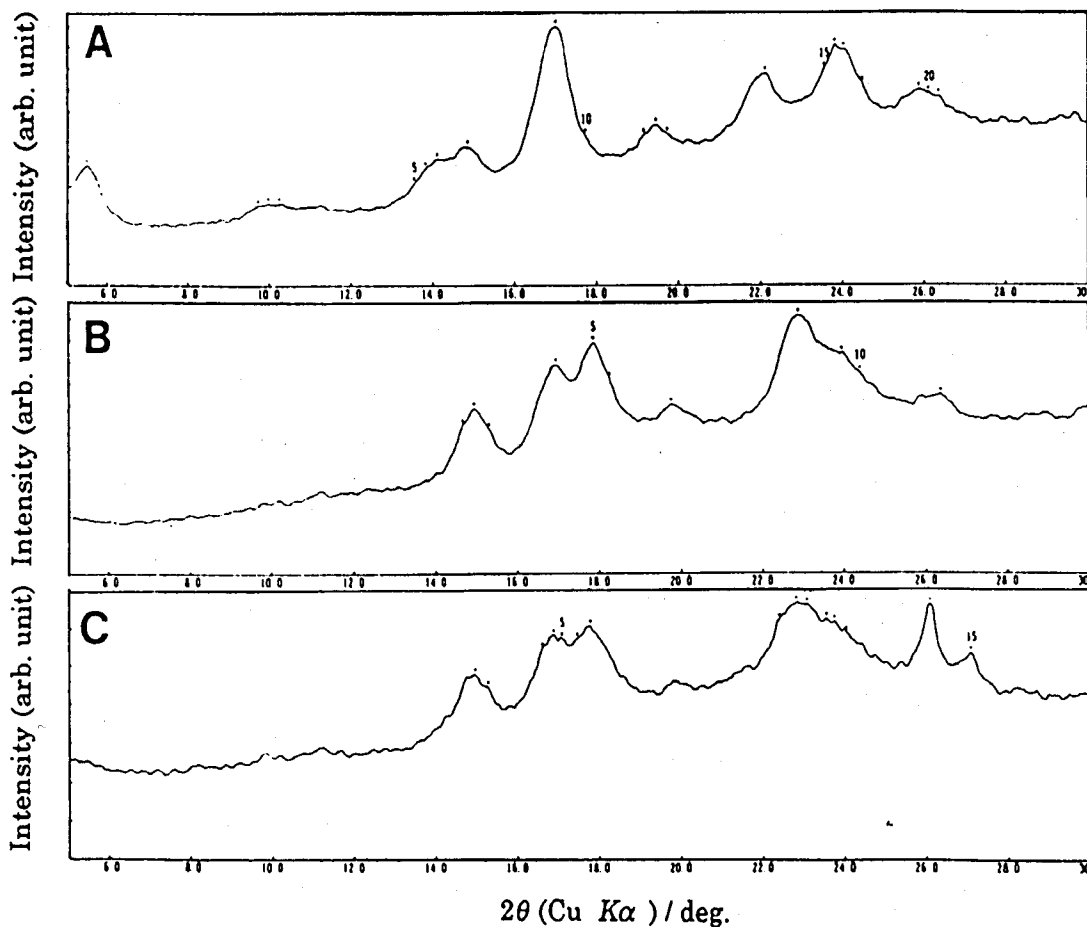


Fig. 3-2. Powder X-ray diffraction patterns of the starch samples isolated from potato, maize, and *Dunaliella parva*. Compare to the starch from potato (*panel A*), diffraction pattern of the starch of maize (*panel B*) seems similar to that of *Dunaliella parva* (*panel C*). Since the shape of the peak shown by the small arrow in the *panel C* was extremely sharp, it was thought to be an impurity peak.

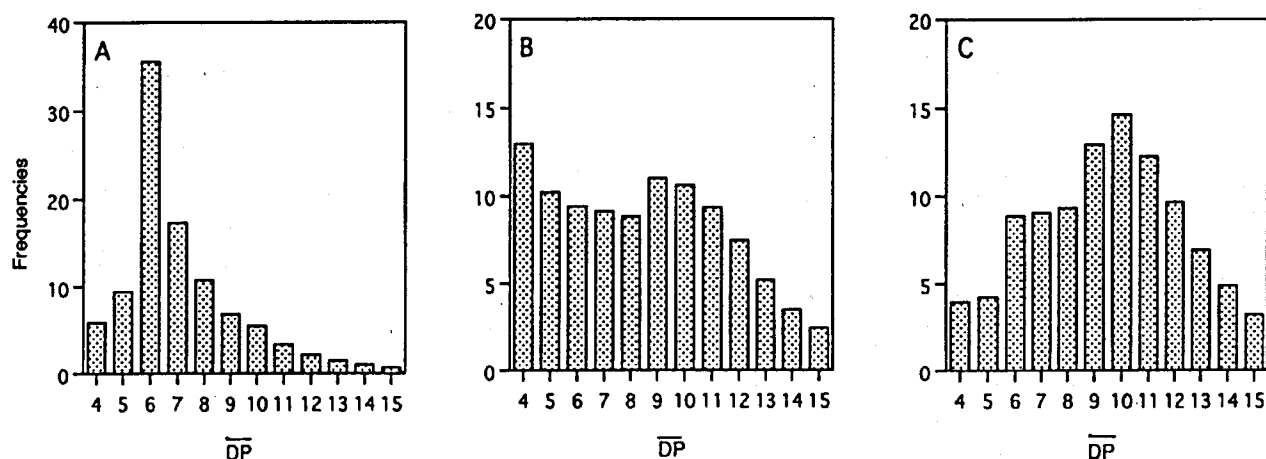


Fig. 3-3. Small glucan contents of debranched starch samples prepared from several origins. Results are given in relative frequencies histograms of chains ranging from DP 4 to 15. Since the detection procedure using RI detector is proportional to the amount of glucose present and not to the number of molecules, the chromatogram peak area had to be divided by the numbers of the each chain length. The histograms were computed from the processed chromatogram peak area with total amount of chains from 4 to 15 adjusted to 100 %. *Panel A*: glycogen (From oyster), *panel B*: *Dunaliella parva*, *panel C*: maize.

Table 3-3
Effect of nitrogen limitation on the cell multiplication and the
starch contents of *Dunaliella parva*

KNO ₃ (mM)	Cell dry wt. (mg)	Starch (mg)	Starch (% of dry matter)
0	11	3.06	27.8
0.1	19	7.54	39.7
1	41	6.97	17.0
10	65	7.21	11.1

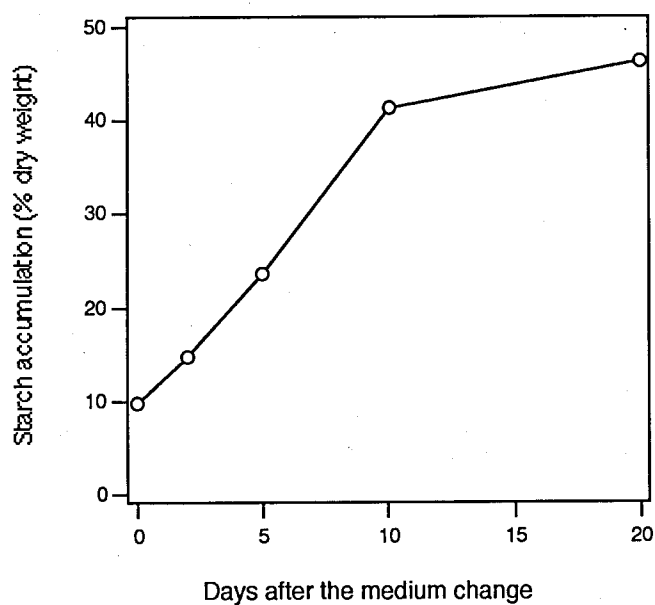


Fig. 3-4. Effect of nitrogen starvation on the starch content of *Dunaliella parva*. Time course change in the starch content was measured of the cells after the transfer to the N- medium.

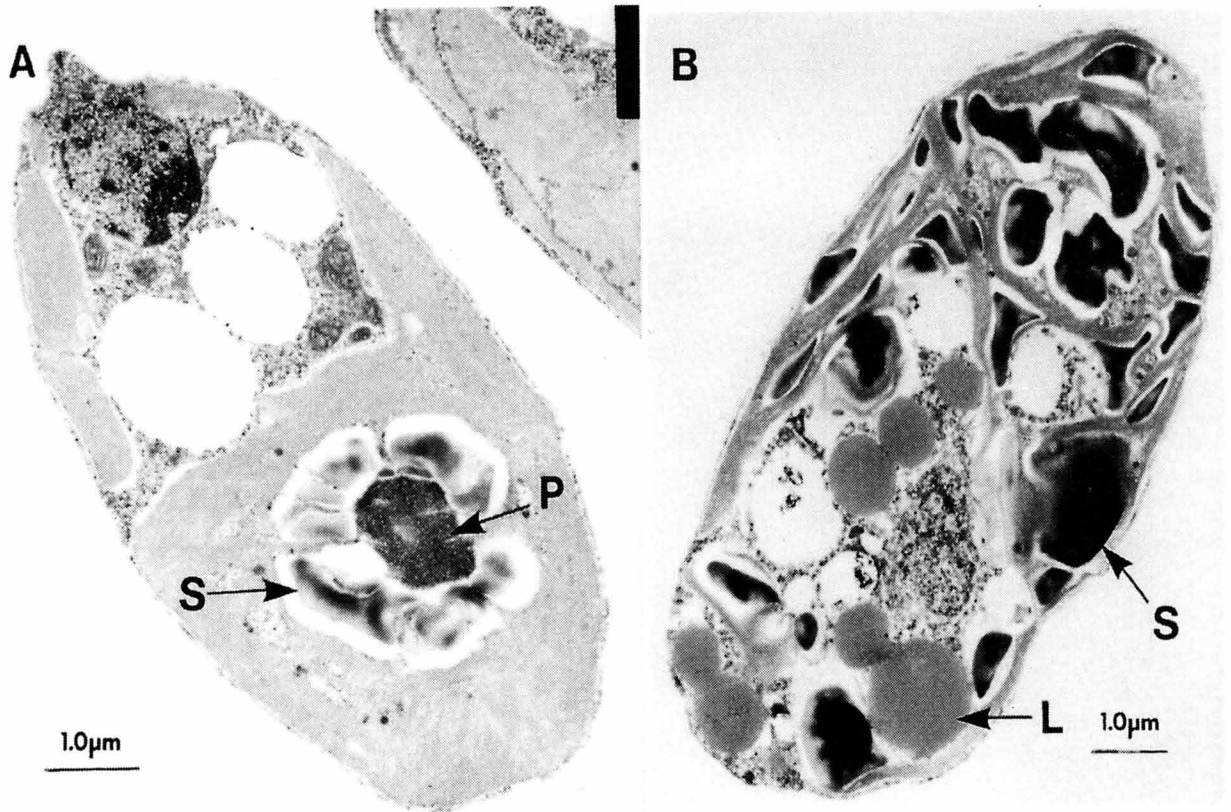


Fig. 3-5. Effect of nitrogen starvation on the number of the starch granules of *Dunaliella parva*. The ultra thin sections were prepared from the cells incubated in the N+ medium (*panel A*) or N- medium (*panel B*) for 10 days. Many starch granules (S) are shown to be dispersed in the cell of *panel B* and the shape of pyrenoid (P) became unclear. Some lipid droplets (L) are also seen in the cell of *panel B*.

CHAPTER IV

Isolation of Disproportionating Enzyme from Halotolerant Microalga *Dunaliella parva* (Volvocales, Chlorophyceae)

INTRODUCTION

Starch metabolism in microalgae such as *Chlamydomonas reinhardtii* Dangeard has been studied intensively (Ball 1998). Since microalgae can be cultured easily and the enzymes for starch metabolism are generally similar to the corresponding enzymes in higher plants, microalgae can be a suitable model organism for the study of the regulation of starch metabolism. Starch metabolism in the cells of microalgae has been studied in different culture conditions. For example, the effects of temperature on the starch degradation in *Chlorella vulgaris* 11h (Nakamura and Miyachi 1982) and the effects of the light/dark cycle on the starch degradation in *Chlamydomonas reinhardtii* 11-32/90 (Levi and Gibbs 1984) have been reported. The content and properties of starch in halotolerant microalgae *Dunaliella* have also been studied (Hatanaka *et al.* 1997). In *Dunaliella* cells starch is metabolized rapidly for the supply of glycerol as the osmoticum against hyperosmotic shock (Ben-Amotz and Avron. 1981). Thus the effects of salinity on the starch degradation in *Dunaliella tertiolecta* have been studied (Goyal, Brown, and Gimmler 1986). The activities of starch degrading enzymes such as amylase and phosphorylase have been studied using the cell extract without purification. Since many amylolytic enzymes including phosphorylases have been found in *Dunaliella marina* (Kombrink and Wober 1980), each amylolytic enzyme must be isolated to investigate the exact role of each enzyme in the metabolism of starch in *Dunaliella*.

In this study, we isolated an amylolytic enzyme and investigated the properties of the enzyme. We found the enzyme to be a disproportionating enzyme (D-enzyme, 4- α -D-glucanotransferase) from the specificity of the substrate and detected the same activity in the cells of other *Dunaliella* and *Chlamydomonas*.

MATERIALS AND METHODS

Chemicals. All chemicals used in this study were obtained from commercial sources and

were of reagent grade. Glutaraldehyde and osmium tetroxide were obtained from TAAB LABORATORY EQUIPMENT LTD. and were of electron microscopic grade. Soluble starch was obtained from Nacalai Tesque, Inc. and maltodextrins of degree of polymerization from G2 to G7 were from Hayashibara Biochemical Laboratories.

Test organisms

Dunaliella parva, strain LB1983, and *Dunaliella tertiolecta* Butcher were cultured in 500 ml culture vessels (Bio pod, HARIO Co. Ltd., Tokyo) containing 400 ml of growth medium. The growth medium contained 1M NaCl, 50mM NaHCO₃, 5mM KNO₃, 5mM MgSO₄, 0.3 mM CaCl₂, 0.2 mM KH₂PO₄, 0.185 mM H₃BO₃, 7 μ M MnCl₂, 6 μ M Na₂EDTA, 1.5 μ M FeCl₃, 0.8 μ M ZnCl₂, 20 nM CoCl₂, and 0.2 nM CuCl₂, the initial pH was 8.0 (Ben-Amotz *et al.* 1989). Cultures were continuously illuminated with cool-white fluorescent lamps at an intensity of 20 μ E $\cdot$ s⁻¹·m⁻².

For the purification of the starch degrading enzyme, cells were cultured in 2M NaCl medium in a 4L aeration culture bottle (HARIO Co. Ltd.,).

Chlamydomonas reinhardtii C-540 was cultured in TAP medium (Harris 1988).

Preparation of enzyme extract

Cell-free extracts of *Dunaliella* were obtained by osmotic bursting of the algae. Logarithmic phase algae were harvested by centrifugation at 16,000 x g for 10 min. The cell pellet was suspended in 100 mL of 10 mM HEPES buffer (pH 7) containing 0.1 mM DTT (Buffer A), and allowed to stand for 1 hour at 4 °C. After the dialysis against deionized water, broken cells were removed by centrifugation at 45,000 x g for 30 min.

Enzyme assays

The starch-degrading activity was determined by the measurement of the reducing sugars released after the digestion of soluble starch. For the reaction, 0.5 mL of the enzyme solution containing 0.1 mg soluble starch, 5 μ mol HEPES (pH 7), was incubated for 1 h at 30°C. Concentrations of the reducing sugars released from soluble starch were measured by the method of Somogyi and Nelson (Nelson 1944).

The phosphorylase activity was determined by spectrophotometrical measurement in the

direction of soluble starch degradation. The reaction was started by the addition of the enzyme solution to 1 mL of the reaction mixtures contained 40 μ mol HEPES buffer (pH 7), 20 μ mol K-phosphate, 13 μ mol $MgCl_2$, 0.1 μ mol EDTA, 0.36 μ mol NADP (KOJIN Co., Ltd, Tokyo), 4 pmol glucose-1,6-bis-phosphate (Boehringer Mannheim), 1 unit glucose-6-phosphate dehydrogenase (Nacalai Tesque, Kyoto), 0.8 unit phosphoglucomutase (Sigma, St Louis), and 1 mg soluble starch (Nacalai Tesque, Kyoto). One unit of activity is the amount of enzyme catalyzing the synthesis of 1 μ mol NADPH per min at 25°C.

For the assay of D-enzyme activity, 150 μ L of reaction mixture containing 0.45 μ mol HEPES (pH 7), 0.45 pmol DTT, 0.45 μ mol maltotriose, and enzyme was incubated at 37°C for 15 min. The reaction was terminated by immersing the reaction tubes in boiling water for 5 min. The released glucose was measured using a glucose measuring kit (Glucose B test, Wako). The enzyme activity resulting in the release of 1 mmol glucose from maltotriose per min was defined as 1 unit of enzyme activity.

An alpha amylase assay kit (DIACOLOR AMY rate, TOYOBO) was used to simplify the procedure of enzyme purification.

Protein determination

Protein was determined by the method of Lowry *et al.* (Lowry *et al.* 1951).

Purification of D-enzyme

Unless otherwise specified, all purification procedures were carried out at 15°C. After the addition of 20% saturation of ammonium sulfate to the cell-free extract, the coagulated material was removed by centrifugation. The supernatant, usually containing about 150 mg of protein, was applied to a Butyl-Toyopearl 650M column (1 x 10 cm, TOHCO, Tokyo) equilibrated with the same buffer. D-enzyme was not trapped by the column at 20% saturation of ammonium sulfate and the residual impurity was washed out with distilled water. After the adjustment of the ammonium sulfate concentration to 30% saturation, the eluent which contained D-enzyme activity was cleared by centrifugation at 45,000 x g for 30 min and applied to the same column which was equilibrated with buffer A containing 30% saturation of ammonium sulfate. The enzyme was eluted with a linearly decreasing concentration of ammonium sulfate in the buffer A from 30% saturation to 0%. The active

fractions were mixed and dialyzed through a Sephadex G-25 column (5 x 30 cm, Pharmacia, Uppsala) and purified further using an FPLC system (Pharmacia). After anion exchange chromatography from 0 to 0.5 M KCl using MonoQ (0.5 x 10 cm, Pharmacia), active fractions were mixed and concentrated by ultra-filtration (ARTOKISS micro-concentrator, ADVANTEC, Tokyo). The sample was finally purified by Superdex S-200 (1 x 30 cm, Pharmacia).

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis of native proteins was performed using a 7.5% slab gel with the buffer system described by Davis *et al.* (Davis *et al.* 1967). After electrophoresis, the gel was washed with distilled water and stored at room temperature for 10 min in buffer A. Amylolytic activities in the gel were identified by the treatment with buffer A containing 0.5 % soluble starch followed by KI₃ staining of soluble starch. Red or white bands of the starch digested by amylolytic enzymes were identified against purple colored starch in the background. Silver Stain II "DAIICHI" (DAIICHI Pure Chemicals Co., Ltd., Tokyo) was also used to determine the proteins in the gel.

The relative molecular mass of proteins was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1976), using a low molecular weight calibration kit (Electrophoresis Calibration Kit, Pharmacia LKB) as molecular weight markers.

Electron microscopy

For transmission electron microscopy, cells cultivated in various conditions were fixed by the addition of 10⁻¹ volume of 20% glutaraldehyde and left standing for 2 h. Samples were subsequently washed 3 times with 0.5 M sorbitol. Washed samples were post-fixed with 1% osmium tetroxide in 0.5 M sorbitol for 1 h and washed 3 times with distilled water. Then, samples were stained with saturated uranyl acetate for 20 min. After being washed with distilled water (3 times), samples were dehydrated through an ethanol series (30-100%), cleared with propylene oxide, and embedded in Spurr resin. All procedures were performed at room temperature. The specimens were polymerized at 70 °C for 16 h. The blocks were sectioned with ultramicrotome (ULTRACUT UCT, Leica, Vienna) and sections were post-

stained with saturated uranyl acetate for 20 min followed by Reynold's lead citrate (Reynolds 1963) for 10 min. Sections were observed by electron microscopy (1200 EX, JEOL, Tokyo) and photographed.

Determination of oligosaccharides by TLC

The reaction mixtures 2 μ L were spotted on Silica Gel 60 thin layer plates (Merck, Darmstadt) and developed three times in ethyl acetate/acetic acid/water (3:1:1). After chromatography, oligosaccharides were detected by spraying 6 M sulfuric acid in methanol and baking at 150 °C for 5 min.

Determination of oligosaccharides by HPLC

To 40 μ L of the reaction mixture after maltotriose digestion, was added 60 μ L of acetonitrile. After centrifugation at 1,200 g for 10 min, 60%-acetonitrile soluble materials were subjected to HPLC (Asahipak NH2P-50 (SHOWA DENKO, Tokyo), 0.6 mL flow, using 60% acetonitrile as eluent, RI detector).

Reaction mixtures after soluble starch digestion were filtrated and subjected to HPLC (SUGAR KS-802 (SHOWA DENKO, Tokyo), 0.5 mL flow, using water as eluent, RI detector).

RESULTS

Change of the starch content in *Dunaliella parva*

Table 4-1 shows the starch contents in *Dunaliella parva* after hyperosmotic shock. The starch content decreased markedly after a two-day incubation in 2 M NaCl. *Dunaliella* is known to consume starch to produce glycerol as the osmoticum against the hyperosmotic shock (Borowitzka and Borowitzka 1988). Ultra-thin sections of *Dunaliella parva* 2 h after hyperosmotic shock showed a shrunken appearance caused by the change of the osmolality of the surrounding solute (Fig. 4-1, B, first step) and those two days after osmotic shock recovery of cell volume (Fig 4-1, C, second step). Starch granules in cells 2 days after the osmotic shock showed marked reduction in size (Fig. 4-1, C, arrow).

Change in the activities of amylase and phosphorylase

Change in the amylolytic activity of total cell extract was determined as the activity of reducing sugar production from or phosphorylation of soluble starch (Table 4-2). Although the phosphorylase activity was tripled by hyperosmotic shock, amylase activity was half that in the control cells. There were five or more soluble starch degrading enzymes (Fig. 4-2). Phosphorylase activities could not be detected by electrophoresis in our experimental conditions (data not shown).

Purification of the enzyme

The major fraction of amylolytic activity was purified as summarized in Table 4-3. SDS PAGE gave two proteins with similar molecular weight (Fig 4-3, lane E). The similarity in amylolytic activity after the separation of the partially purified enzyme suggested that these two proteins were isozymes (Fig. 4-4). The mixture of these two inseparable isozymes was used as a purified enzyme solution in the following experiments.

Substrate specificity of the enzyme

The reaction products after the digestion of several oligosaccharides by the purified enzyme were separated on a TLC (Fig. 4-5). Although no reactant was detected in the reaction using maltose as a substrate, many transglycosylated oligosaccharides except maltose were observed after the reactions using other maltooligosaccharides. The time course of the reaction using maltotriose as a substrate was investigated using HPLC to determine the process of the reaction (Fig. 4-6). Maltopentaose and glucose were found at the early stage of the reaction (Fig. 4-6, 1h) and maltodextrins of G7 or higher degree of polymerization appeared later in the reaction (Fig. 4-6, 24h). This suggests that the enzymes mixture has the same reaction specificity as the D-enzyme.

Some properties of D-enzyme

Some properties of the purified isozyme were investigated.

D-enzyme activity was the highest at around pH 7 and the effects of buffer on the activity were small (Fig. 4-7). The optimum temperature of the reaction was around 40 °C (Fig. 4-8) and the heat stability was not high (>50% after 30 min incubation at 50 °C (Fig. 4-9)). Table 4 shows the effects of several metal ions. Mercury and copper ions at 1mM

inhibited D-enzyme activity by 60%.

Effects of glucose on the digestion of soluble starch

The effects of glucose on the degradation of soluble starch by D-enzyme from barley have been reported (Yoshio *et al.* 1986). Staining for amylolytic activity of partially purified *Dunaliella* D-enzyme separated by Native PAGE revealed that the activity for soluble starch degradation was also higher in the gel incubated with glucose than without glucose (Fig. 4-10). Maltodextrins with various degrees of polymerization were found in the reaction mixture with glucose, but only a trace amount of maltose was detected in the mixture (Fig. 4-11).

D-enzyme activity in the cells of other *Dunaliella* and *Chlamydomonas*

The D-enzyme activity in the crude extracts of *Dunaliella tertiolecta* and *Chlamydomonas reinhardtii* was investigated using TLC (Fig. 4-12). The reaction mixtures with the extracts of all other microalgae tested in this experiment showed similar separation patterns to those obtained using the extracts of *Dunaliella parva*.

DISCUSSION

The metabolism from glycerol to starch and vice versa has been reported previously (Borowitzka and Borowitzka 1988). Internal glycerol has been proposed to serve as the major solute osmotically balancing the external salt concentration (Borowitzka and Brown 1974). Both synthesis and metabolism of glycerol require ATP (Ben-Amotz and Avron 1973) but do not require protein synthesis (Borowitzka *et al.* 1977). Starch breakdown accounts for all glycerol synthesis in the dark and 70% of glycerol synthesis in the light following an increase in medium salinity (Brown and Borowitzka 1979). The glycerol content decreased following the decrease in the external solute concentration both in the light and dark, accompanied by an increase in starch content in the cells (Gimmler and Moller 1981). Thus, the starch content in *Dunaliella* is strongly affected by the osmolarity of the surrounding solutes.

The starch content in the cells of *Dunaliella parva* was also reduced after the increase in medium osmolarity (Table 4-1) and glycerol was utilized to reconstitute the cell shape (Fig. 4-1). Phosphorylase was found to play an important role in the adaptation against salinity in *Dunaliella parva* (Table 4-2), as suggested in *Dunaliella tertiolecta* (Goyal, Brown, and

Gimmler 1986).

Staining of the crude extract for amylolytic activity revealed several enzymes (Fig. 4-2) but no phosphorylase activity could be detected in our experimental conditions (data not shown). We purified the major amylolytic enzyme seemed (Fig. 4-3). Two proteins with similar properties could not be separated even at the final step of the purification, but the proteins assumed to be isozymes from the activity staining (Fig. 4-4). N terminal peptide sequence of the mixture of the two proteins was determined using an automatic peptide sequencer (Protein sequencer 471A, Applied Biosystems). All 20 N terminal amino acids of the two proteins were found to be similar (data not shown). This also strongly implied that the two enzymes are isozymes.

From the analysis of the products after the digestion of several oligosaccharides, these amylolytic enzymes were considered to be the D-enzyme (Figs. 4-5, 6). The D-enzyme was first reported in potato tubers (Peat, Whelan, and Rees 1956) and similar enzymes have been reported in spinach leaves (Linden, Tanner, and Kandler 1974), wheat germ (Edelmann and Keys 1961), tomato fruits (Manners and Rowe 1969), *Arabidopsis* leaves (Lin and Preiss 1988), and many other plants. Although the same type of enzymes has also been reported in *Bacillus subtilis* (Pazur and Okada 1969), *Streptococcus bovis* (Walker 1965) and *Escherichia coli* (Palmer, Ryman, and Whelan 1976), no such enzyme has been reported in algae. The D-enzyme acts on maltooligosaccharides as substrate or donor, to split out maltosyl or larger glucosidic chain units from the substrate and transfer these released glucosidic chains to another substrate molecule or glucose. One proposed function of the D-enzyme during starch breakdown is to convert small oligosaccharides into larger molecules to which phosphorylase can act (Lee, and Whelan 1974). The reason for the small activity of amylase (reducing sugar production) during starch breakdown after hyperosmotic shock can be explained by the fact that the conversion of oligosaccharides by the D-enzyme to larger molecules did not increase the reducing ends of oligosaccharide. Starch phosphorylase in pea can not utilize native starch granules or maltodextrins of G4 or lower degree of polymerization as a substrate but can utilize maltodextrins of G5 through G7 as substrates. Thus the oligosaccharides produced by the reaction with the D-enzyme were suitable substrates for phosphorylase of pea leaves (Kakefuda and Duke 1989). Oligosaccharides produced by the *Dunaliella* D-enzyme may also be digested to glucose-1-phosphate by the

increased activity of phosphorylase (Table 4-1).

The specific activity of the D-enzyme did not rise during the purification steps (Table 4-3), but this may be because the assay method can not detect the transglycosilation. Since only a small amount of glucose is produced by the reaction with the D-enzyme, the activity of the purified enzyme may be lowered by removing other glucose-producing amylases.

The substrate specificity of D-enzyme in plants is known to differ from that in microorganisms (generally called amylomaltase). Although amylomaltase can transfer the glucosyl residue to the D-enzyme can not. The D-enzyme of *Dunaliella parva* also could not transfer the glucosyl residue of maltose (Fig. 4-5, lane 2), as in higher plant. Several other properties of the enzyme (Figs. 4-7, -9, Table 4-4) were also similar to those in higher plants. For example, the inhibition by copper ion has also been reported in the D-enzyme in germinated barley seed (Yoshio *et al.* 1986).

Although glucose does not serve as a donor, it is known to serves as an acceptor. Digestion of soluble starch by *Dunaliella* D-enzyme is also enhanced by the addition of glucose (Figs. 4-10, 11). The time course of the reaction revealed that relatively short chain maltodextrins were produced in the reaction with glucose (Fig. 7, B-D) as against only longer chains can be found in the reaction without glucose (Fig. 7, E, arrow). Thus, the D-enzyme might have another function: it may change the structure of starch molecules and grain architecture by modifying the chain length.

Amylolytic enzymes of the same type as the D-enzyme were found in all the *Dunaliella* and *Chlamydomonas* cultured in our laboratory (Fig. 4-12). The TLC of the reaction mixtures with the extracts of *Dunaliella bioculata*, *Dunaliella primolecta*, and *Dunaliella salina* showed a similar chromatogram (data not shown). Thus, the D-enzyme appears to play a very important role in the starch metabolism in algae.

The D-enzyme gene of potato tuber has been isolated and expressed in *Escherichia coli* cells (Takaha *et al.* 1993). Although the molecular weight and other properties of the *Dunaliella* D-enzyme were similar to those of the potato D-enzyme, 20 amino acids of the N terminal sequence were different. To elucidate the exact role of the D-enzyme in the regulation of starch, we must isolate the gene of the D-enzyme in *Dunaliella*.

Table 4-1. Effect of osmotic shock on the starch contents of *Dunaliella parva*

NaCl (M) *	Starch (% dry matter) **
0.3	11.93
0.7	11.38
1	10.20
2	7.54
3	6.56

*Cells cultured in 1 M NaCl medium were transferred to the media containing various concentrations of NaCl.

**Starch contents of cells were measured 1 day after the transfer to the media.

Table 4-2. Change in the activities of starch degrading enzymes after hyper osmotic shock

NaCl conc. (M)*	Phosphorylase	Amylase
	U/mg protein	mU/mg protein
1	0.108	1.93
2	0.097	1.541
1-1	0.14	0.506
1-2	0.296	0.561

*Cells cultured in 1M NaCl medium were transferred to the media containing 1M (1-1) or 2M (1-2) NaCl. Activities of the enzymes were measured 1 day after the transfer to the media.

Table 4-3. Purification of D-enzyme from *Dunaliella parva*

	Total protein	Total activity	Specific activity
	mg	units	units/mg
Cell extract*	102.23	28.28	0.28
1st Butyl Toyopearl**	42.33	29.81	0.71
2nd Butyl Toyopearl	10.39	10.76	1.04
Sephadex G-25***	5.69	6.79	1.19
Mono Q	0.87	2.12	2.45
Superdex 200	0.72	0.44	0.62

*Cell extract after the osmotic bursting of the cells was cleared by centrifugation after the addition of 1.1M ammonium sulfate.

**The eluent which pass though the Butyl Toyopearl column equilibrated by the buffer A containing 1.1 M ammonium sulfate.

***The eluent of which conductivity under 50 μ mho was corrected.

Table 4-4. Effects of Chemicals on the Activity of D-enzyme

Chemicals (1mM)	Relative activities (%)
CaCl ₂	97.1
ZnCl ₂	96.2
CdCl ₂	93.9
CoCl ₂	92.5
CuCl ₂	38.6
FeCl ₃	84.8
SnCl ₂	92.8
PbCl ₂	88.7
NiCl ₂	89.9
MnCl ₂	90.6
HgCl ₂	41.9
EDTA	97.6

*200 μ L of the reaction mixtures containing 10 μ mol maltotriose, 0.1 munit enzyme, 20 mmol HEPES, and 1 mM of various chemicals were incubated at 37 °C for 15 min. After stopping the reaction by immersing the mixture in boiling water, glucose contents released from maltotriose were measured using Glucose B test.

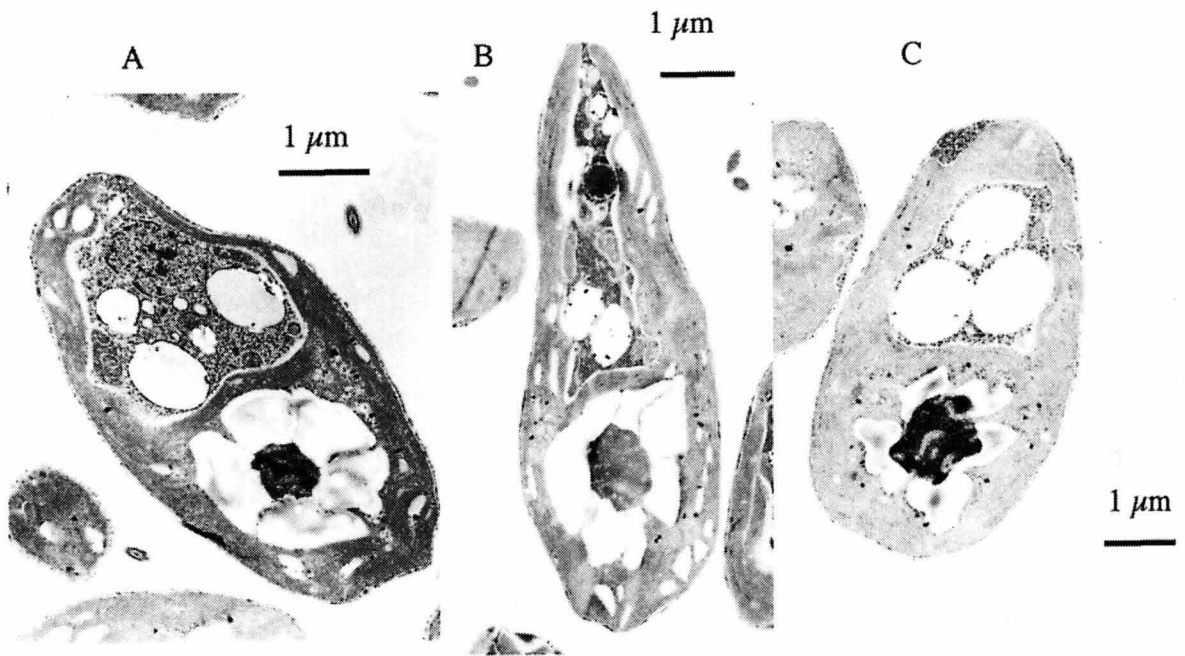


Fig. 4-1. Electron microscopic view of ultra-thin section of *Dunaliella parva* after hyper osmotic shock. Cells cultured in 1 M NaCl medium (A) were transferred to 2 M NaCl medium, and incubated for 1 h (B) and 2 h (C). Arrow heads show the starch granules.

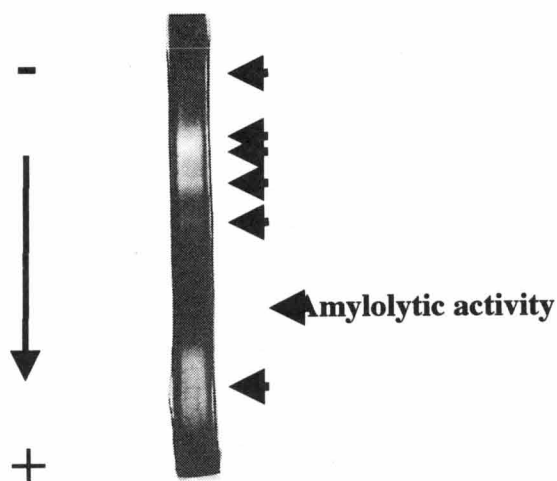


Fig. 4-2 Polyacrylamide electrophoresis of the crude extract. After the osmotic bursting of the cells, the crude extract was cleared by centrifugation and concentrated by ultrafiltration using an ARTKISS micro-concentrator. After the electrophoresis of the crude extract, the gel was washed with water and immersed in buffer A for 10 min. Amyolytic activity in the gel was, identified by KI3 staining of soluble starch after the treatment with buffer A containing 0.5% soluble starch. Arrows show the amyolytic activities.

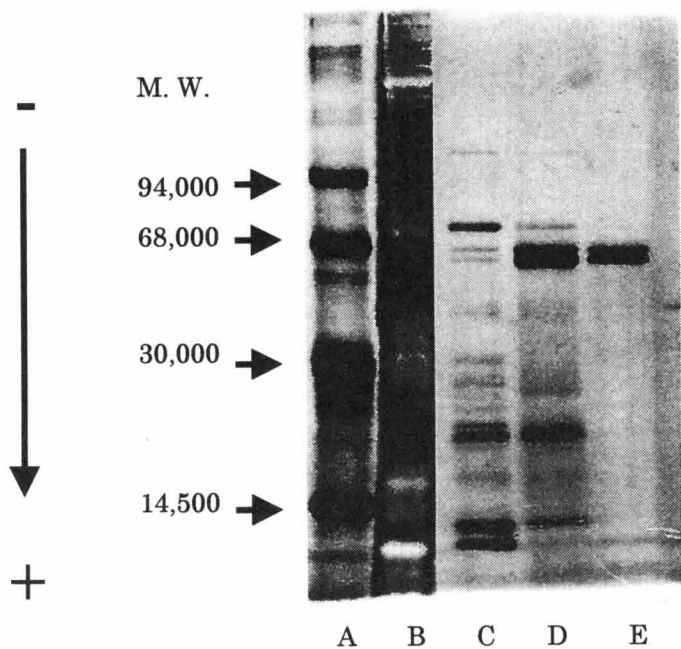


Fig. 4-3 SDS polyacrylamide electrophoresis of the purified enzyme. Samples at each purification step were concentrated by ultrafiltration using an ARTKISS micro-concentrator and subjected to the gel. After electrophoresis, proteins in the gel was stained by Silver Stain II "DAIICHI".

A: Molecular weight marker, B: Crude enzyme

C: Butyl Toyopearl, D: MonoQ, E: Superdex 200

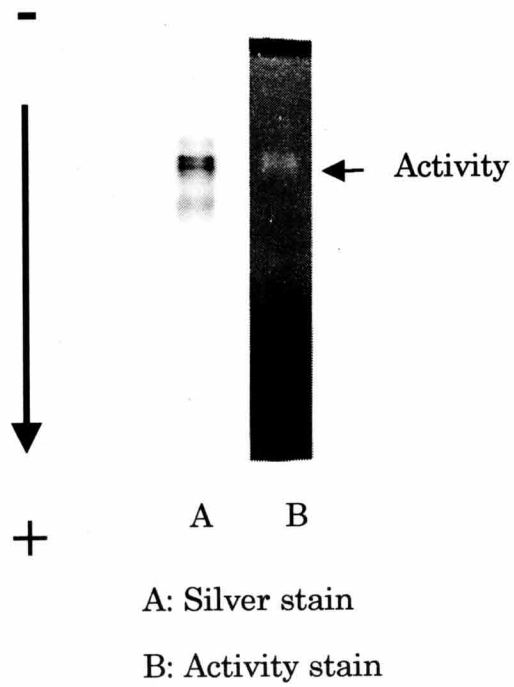


Fig. 4-4 Polyacrylamide electrophoresis of partially purified enzyme. Active fractions after MonoQ chromatography were concentrated and applied to 2 lanes of the gel. After electrophoresis, the gel was cut and stained for protein (A) or amylolytic activity (B).

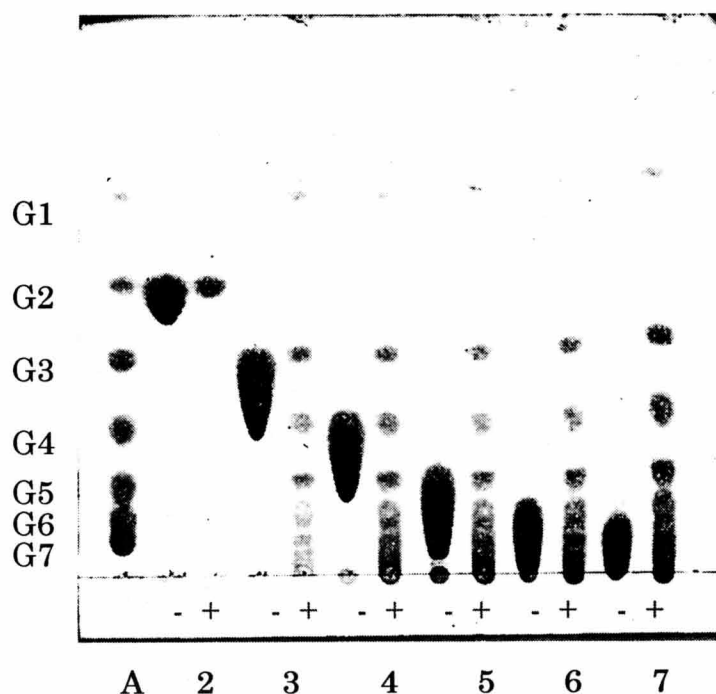


Fig. 4-5 Digestion of maltooligosaccharides by the purified enzyme. The reaction mixtures 200 μ L containing 20 μ mol HEPES, 0.4 munit enzyme, 2 μ mol of each maltooligosaccharide (G1 - G7), and 0.02% NaN₃ were incubated for 2 days at 37 °C. The reaction mixtures 2 μ L were spotted on Silica Gel 60 thin layer plates (Merck, Darmstadt) and developed three times in ethyl acetate/acetic acid/water (3:1:1). After chromatography, oligosaccharides were detected by spraying 6 M sulfuric acid in methanol and baking at 150 °C for 5 min. Lane A, standard oligosaccharide mixture from G1 to G7. Numbers in the lanes show the degree of polymerization of the substrate oligosaccharides with (+) or without (-) enzyme.

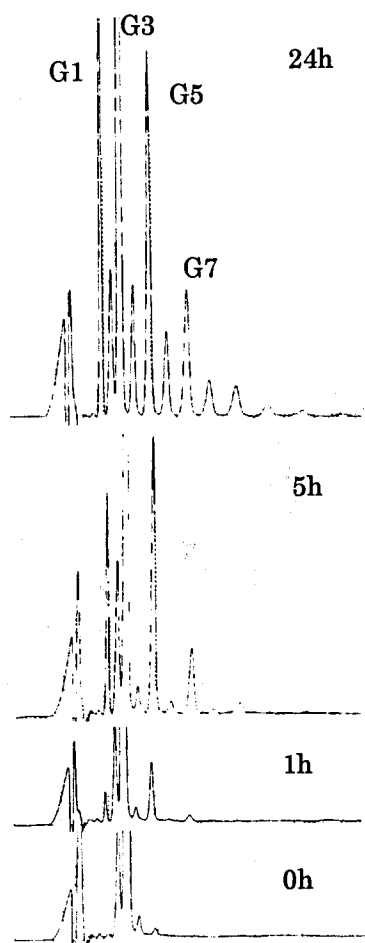


Fig. 4-6 Determination of the transglycosilation of maltotriose by the enzyme using HPLC. Maltotriose reaction mixtures 40 μ L of the same composition as in Fig. 5 for several reaction periods were mixed with 60 μ L of acetonitrile and subjected to HPLC (Asahipak NH2P-50 (SHOWA DENKO, Tokyo), 0.6 mL flow, fed by 60% acetonitrile, RI detector). Reaction periods and peaks of oligosaccharides are shown in the figure.

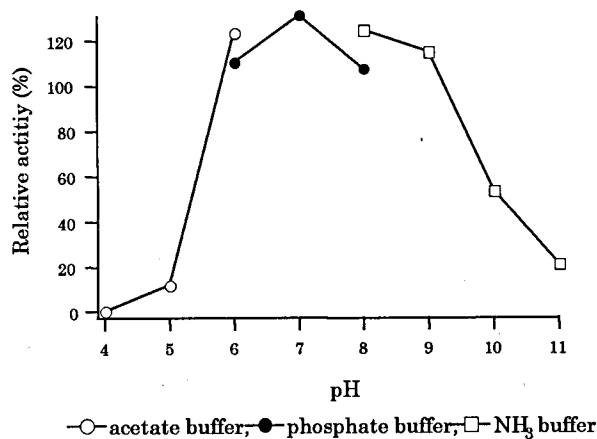


Fig. 4-7 Optimum pH of the enzyme. The reaction mixtures 200 μ L containing 10 μ mol maltotriose, 0.1 munit enzyme, and various buffers at 50 mmol were incubated at 37 °C for 15 min. After the reaction was stopped by immersion of the mixture in boiling water, glucose released from maltotriose was measured using Glucose B test. Acetate buffer (○), phosphate buffer (●), and NH₃ buffer (□) were used in the experiment.

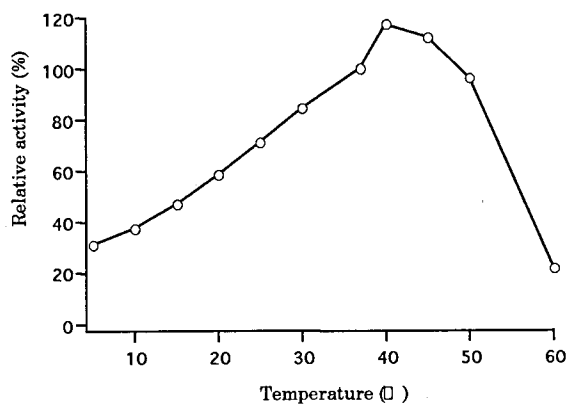


Fig. 4-8 Optimum temperature of the enzyme. The reaction mixture 200 μ L containing 10 μ mol HEPES, 10 μ mol maltotriose, and 0.1 munit enzyme was incubated at various temperatures for 15 min. Glucose contents were measured as in Fig. 7.

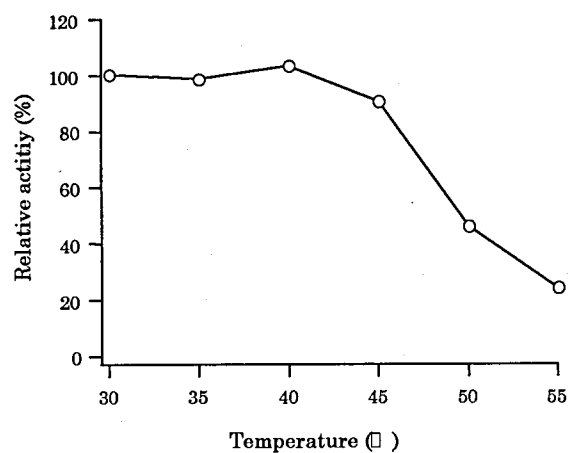


Fig. 4-9 Heat stability of the enzyme. The reaction mixture 100 μ L containing 10 μ mol HEPES and 0.1 munit enzyme were incubated at various temperature for 30 min. After the mixture was cooled on ice, the reactions were started by addition of 10 μ mol maltotriose to each mixture. Glucose contents were measured as in Fig. 7.

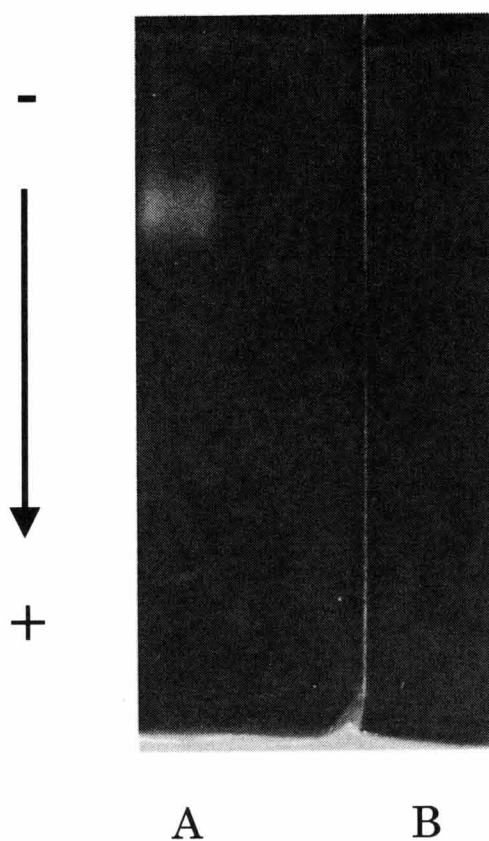


Fig. 4-10 Effect of glucose on the amylolytic activity of the enzyme. After polyacrylamide electrophoresis of the partially purified enzyme, gel was cut and stained for amylolytic activity with (A) or without (B) 2 mM glucose.

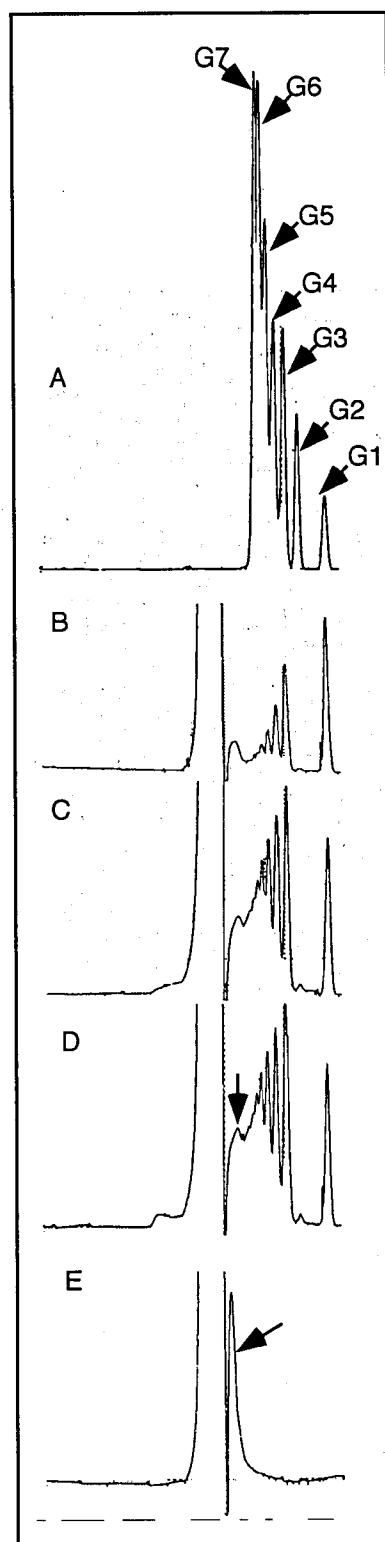


Fig. 4-11 Digestion of soluble starch with (B-D) or without glucose (E). The reaction mixtures 200 μ L containing 20 μ mol HEPES, 0.4 munit enzyme, 0.5 % soluble starch, and 0.02 % NaN_3 were incubated at 37°C for various reaction periods. After the reaction was stopped by immersion of the mixture in boiling water, each sample was filtered and subjected to HPLC (SUGAR KS-802 (SHOWA DENKO, Tokyo), 0.5 mL flow, eluted with water, RI detector). Standard maltooligosaccharides (degrees of polymerization are shown in the figure) (A); Reaction mixture with glucose for 10 min (B), 2 h (C), 8 h (D); Reaction without glucose (E).

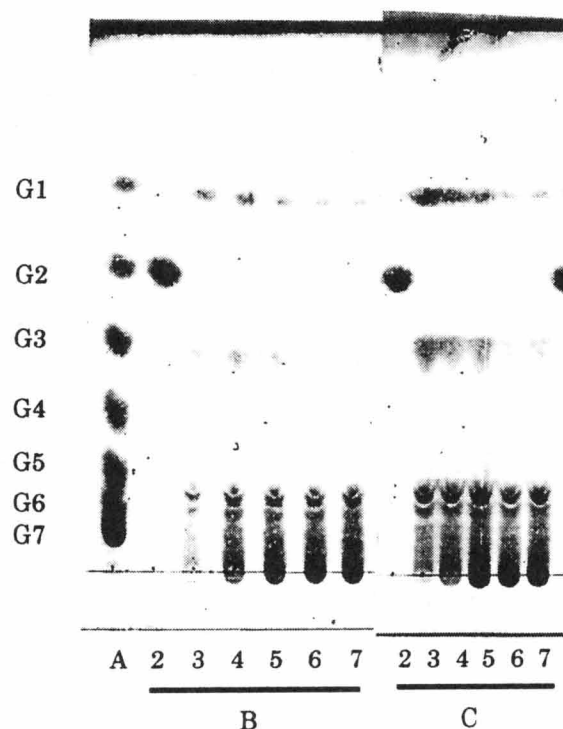


Fig. 4-12 Digestion of maltooligosaccharides by crude extracts of *Dunaliella tertiolecta* and *Chlamydomonas reinhardtii*. Crude extract of *Dunaliella* was obtained by osmotic bursting of the cells and the extract of *Chlamydomonas* was obtained by sonic bursting of the cells. The reaction mixtures 100 μ L containing 20 μ mol HEPES, 2 μ mol of each maltotriose, and 0.04 % NaN₃ were incubated for 2 days at 37 °C. Products were separated and detected by TLC by the same procedure as in Fig. 5. Lane A, standard oligosaccharide mixture from G1 to G7. Lanes B, reaction with *Dunaliella* extract, lanes C, reaction with *Chlamydomonas* extract. Numbers in the lanes B, C show the degree of polymerization of substrate oligosaccharides.

CHAPTER V

Reduction of Hydroxyacetone with NADPH Regenerated through Photosynthetic Pathway of the Halotolerant Alga *Dunaliella parva*

INTRODUCTION

In practical enzyme reactors in which cofactors are required for the desired reaction, regeneration and retention of the cofactor are very important, and thus many methods of cofactor regeneration and retention have been developed. Various methods of enhancing the retention of a relatively small cofactor in a reactor, such as binding the cofactor to an appropriate matrix (Yamazaki and Suzuki 1978) or polymerizing (Muramatsu et al. 1977)(LeGoffic, Sicsic, and Vincent 1980) a cofactor derivative, have been studied. However the methods are complicated and the modified cofactor is usually less effective than the native cofactor.

Whole cells are also used to enhance retention and regeneration of cofactors required in enzyme-catalyzed reactions in reactors. An advantage of such a reactor system is that the cofactor is supplied and regenerated by the cells. Though this method is attractive, many problems such as permeability of the substrates and products, metabolism of PD to undesirable products, and toxicity of the substrate were still remained.

The result of several studies on reactors for the asymmetric reduction of hydroxyacetone using yeast cells have been reported. In these reactors, NADH derived from the metabolic pathways of sugar (Levene and Walti 1943) or ethanol (Kometani et al. 1993)(Kometani et al. 1994) can be used for the reduction of HA. NADPH regeneration via a photosynthetic pathway was also been used for biological reduction reactions. It was reported that NADPH was regenerated by immobilized cells of the alga *Chlorella vulgaris* which can be successfully used for the hydrogen evolution with cooperative action of co-immobilized *Clostridium butyricum* cells (Kayano et al. 1981). NADPH regeneration using algal cells offers several advantages because the cells can use sunlight as the sole or major energy source for NADPH regeneration.

The utilization of unconventional microorganisms, particularly microalgae, for the production of feed, food, food additives, pharmaceuticals, or fine chemicals is growing in

importance (Becker 1994). As regards the use of algae for biotransformation or biosynthesis, various studies have shown the feasibility of algal systems for the production of useful biomolecules. Many techniques for immobilization of various species of algae have also been studied with the aim of applying algal cells as using biocatalysts for biotransformation and biosynthesis of useful products (Robinson, Mak, and Trevan 1986).

Among the various species of microalgae studied for mass culture, halotolerant unicellular *Dunaliella* species are thought to be very useful due to their ability to accumulate many different kinds of useful chemicals, such as β -carotene and glycerol (Ben-Amotz and Avron 1983). *Dunaliella salina* was the first microalga used to produce chemicals commercially, because its tolerance to extremely high salinity facilitates the maintenance of unialgal cultures free of competitors, pathogens, and predators (Borowitzka and Borowitzka 1988). Although a large number of studies to investigate possible uses of the alga have been undertaken, no studies on biotransformation using *Dunaliella* have been reported. The ability of *Dunaliella* to tolerate a high salt concentration environment as well as rapid changes in salt concentration is due to its accumulation of glycerol as an osmoregulatory solute (Brown 1976). Because many *Dunaliella* strains have been reported to leak considerable amounts of glycerol upon changes in salinity, *Dunaliella* is thought to be suitable for studies on the conversion of ketones to polyols using whole cells (Grizeau and Navarro 1986).

In this study we investigated the possibility of construction of a NADPH regeneration system for asymmetric reduction of hydroxyacetone to produce an optically active polyol, propanediol, using algal cells.

MATERIALS AND METHODS

Chemicals As a standard, (*R*)-PD was prepared according to the method reported elsewhere (4), using Baker's yeast IFO 1044 as a source of enzymes. HA was purchased from Aldrich Chemicals Co., Inc. and racemic 1,2-propanediol and DCMU were purchased from Nacalai Tesque, Inc. Other chemicals were obtained from commercial source and were of reagent grade.

Test organisms *Dunaliella parva*, strain LB1983, was cultured in a 4000 ml air-lift type culture bottle (HARIO Co., Ltd.) containing 4000 ml of growth medium. The growth medium contained 2 M NaCl, 50 mM NaHCO₃, 5 mM KNO₃, 5 mM MgSO₄, 0.3 mM CaCl₂, 0.2 mM

KH_2PO_4 , 0.185 mM H_3BO_3 , 7 μM MnCl_2 , 6 μM Na_2EDTA , 1.5 μM FeCl_3 , 0.8 μM ZnCl_2 , 20 nM CoCl_2 , and 0.2 nM CuCl_2 , and the initial pH was 8.0 (Ben-Amotz, Shaish, and Avron 1989). Cultures were illuminated with cool-white fluorescent lamps at an intensity of 20 $\mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ on a 16 - 8 h photoregime at 15 °C with continuous aeration (2000 $\text{ml}\cdot\text{min}^{-1}$) by bubbling.

Conditions of the reaction (*R*)-PD was produced as follows: Algal cells at the logarithmic phase were harvested by centrifugation at 3,000 $\times g$. After centrifugation the cells were suspended in 50 mM Tris-HCl buffer (pH 8.5) containing 2 M NaCl. This cell suspension usually contained 4×10^7 - 4×10^8 cells $\cdot\text{ml}^{-1}$. After addition of HA to the cell suspension to a final concentration of 30 to 500 mM, 20 ml of the reaction mixture in a plant cultivation tube (40 mm $\varnothing$ x 125 mm) was incubated at 15°C with illumination at 100 $\mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$.

Determination of substrate and product concentrations The concentrations of substrates and products were simultaneously determined using a gas chromatograph (GC-6A, Shimadzu Co.) with a flame ionization detector and a porous polymer column (Tenax-GC, 3 mm $\varnothing$ x 2 m, GL Sciences Co. Ltd.) at 160°C. Algal cells were removed by centrifugation at 3,000 $\times g$ and 10⁻¹ volume of an aqueous 1-butanol solution was added to the supernatant as an internal standard. The samples were injected directly into the column of the gas chromatograph and the concentrations were calculated from the peak area ratios of the components to that of the internal standard. The production rate was calculated as the reaction velocity and the productivity was represented as the amount of PD produced per cell per hour. The production rate and the productivity were calculated after the PD concentration reached approximately 20 mM (40% conversion of the added substrate) because a PD concentration higher than 20 mM allows accurate measurement using a gas chromatograph. In order to compare the initial rate of productivity, the productivities in Fig. 6c were exceptionally calculated using the values of 0 h to 41 h.

Analysis of optical isomers The type of optical isomer and the optical purity were determined using a capillary gas chromatograph (HP-5890 series II plus, Hewlett-Packard Co.) equipped with a CP-cyclodex B 236M capillary column (0.25 mm $\varnothing$ x 50 m, Chrompak). The chromatogram obtained using this capillary column for analysis of racemic propanediol shows two main peaks (former peak is (*S*)-(+)-1, 2-Propanediol ((*s*)-PD) and later peak is

(*R*)-PD) (Berthod, Li, and Armstrong 1992).

Determination of the cell number The number of algal cells was determined using a Thoma hemacytometer (E. Leitz Wetzlar).

Preparation of enzyme extract Cell-free extracts of *Dunaliella* were obtained by osmotic bursting of the algal cells. Cells were harvested by centrifugation at 3,000xg for 10 min, resuspended in 50 mM Tris-HCl buffer, pH 9.0, of approximately 30 times the volume of the precipitate and allowed to stand for 1 h at 4 °C. Cell debris was removed by centrifugation at 10,000xg for 10 min at 4 °C and the cell-free extract thus obtained was subjected to the DHAR assay.

DHAR assay The assay of DHAR was performed by monitoring of changes in the optical density at 340 nm using a Shimadzu UV-240 spectrophotometer with a recorder. The DHAR reaction was carried out in 10-mm light-path cuvettes, the temperature of which was controlled at 25 °C by circulating water through the holder. The standard assay mixture for DHAR contained 20 mM Tris-HCl pH 7.5, 0.1 mM NADPH, 5 mM dihydroxyacetone, and the enzyme solution in a total volume of 1.0 ml. The reaction was initiated by the addition of the enzyme solution. The enzyme activity resulting in oxidation of 1 μ mol of NADPH per min was defined as 1 unit of the enzyme.

RESULTS AND DISCUSSION

Optical isomer of diol from HA

DHAR was reported to play an important role in the glycerol biosynthesis pathway (Ben-Amotz, and Avron 1981) and due to its wide substrate specificity to be useful for producing several polyols including glycerol (Ben-Amotz, and Avron 1973)(Ben-Amotz, and Avron 1974). In our preliminary experiments using whole cells of *Dunaliella parva* the enzyme was confirmed to reduce HA to (*R*)-PD. Figure 5-1 shows the results of determination of the optical isomers using capillary gas chromatography. The retention time of the peak of the main product obtained using the *Dunaliella* cells coincides with that of (*R*)-PD prepared using yeast cells (the optical purity was approximately 98 %ee). Thus the DHAR of *Dunaliella parva* can catalyze the enantioselective reduction of ketones to the corresponding alcohol of R-isomer. (the optical purity was approximately 75 %ee).

Regeneration of NADPH

As reported previously (Berthod, Li, and Armstrong 1992) this enzyme reaction requires the same numbers of moles of NADPH as that of ketone as a proton donor. The regeneration of NADPH in the reduction of hydroxyacetone by *Dunaliella* cells was examined by use of DCMU which is well known to inhibit photosynthesis in plants and algae (Shneyour, and Avron 1975). Figure 5-2 shows that 10 μM DCMU strongly inhibits the production of PD. Therefore, NADPH regenerated by photosynthesis was expected to be very important for this reaction.

On the other hand, relatively low production of PD in the dark was also observed (Fig. 5-2, closed symbols). Thus, NADPH regenerated through use of energy from another source, such as stored starch, also seemed to contribute to the reaction. Therefore, conversion in the dark may be limited by the amount of stored starch.

Addition of NADPH to the reaction mixture did not result in an increase in PD productivity in the light or dark (data not shown). The reason for this is unclear. One explanation may be that the relatively large NADPH molecule cannot be incorporated into *Dunaliella* cells and another may be that added NADPH is consumed in other enzyme reactions.

Figure 5-2 also indicates that the magnitude of the PD concentration increase was almost the same as that of the HA concentration decrease. From our data, more than 95 % of the HA was confirmed to be converted to PD without turning into any by-products or being degraded.

The cell density did not vary markedly during this experiment (Fig. 5-2c). Since the final density of cells even after 20-d cultivation (in 1 M NaCl medium, at 25 °C, 50 $\mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$, without aeration) was at most 8×10^6 cells $\cdot\text{ml}^{-1}$, cell division seemed not to occur at cell densities above 10^8 cells $\cdot\text{ml}^{-1}$ used for the reaction.

Effect of the substrate concentration

Figure 5-3 shows the time course changes in the product concentration and the production rate when various substrate concentrations were used. A substrate concentration above 100 mM did not enhance the PD production rate (Fig. 5-3a) and the optimum substrate concentration ranged from 50 to 100 mM (Fig. 5-3c). The cell density did not vary markedly during the reaction period (Fig. 5-3b). A preliminary experiment revealed that the growth of *Dunaliella* inoculated into medium containing 10 mM HA was strongly inhibited by HA, but

no marked change in the level of DHAR activity in the cells was observed for a period of up to about 100 h from the start of the experiment (data not shown). As mentioned above, photosynthesis is very important for this reaction as an energy source for NADPH regeneration. The low productivity at a high substrate concentration might be caused by the shortage of NADPH regenerated through photosynthesis. Since the motility of this alga declined markedly at HA concentrations of over 100 mM and the cells were accumulated at the bottom of the vessel even in the light, very little photosynthesis seemed to occur at high HA concentrations. Accordingly, a 50 mM substrate solution was used for the following experiments.

Effect of temperature

The temperature dependence of the PD production rate showed optimum values around 25 °C (Fig. 5-4a, c). Though the magnitude of the cell density change at temperatures higher than 25 °C was apparently greater than those observed at low temperatures (Fig. 5-4b), it was thought that water evaporation from the medium occurring at high temperature or due to the humidity conditions in the incubators used in this experiment resulted in the increase in the cell density. Although the optimum temperature for DHAR activity was higher than 30 °C (about 45 °C, unpublished data), the maximum growth temperature for this alga was found to be about 30 °C in a preliminary experiment. The motility of the cells also declined at temperatures of over 30 °C, as it did at high HA concentrations. Accordingly, the reaction seemed to be uncompleted at temperatures above 30 °C because of the low level of DHAR activity in each algal cell.

Effect of light intensity

Figures 5-5a and 5-5b show the time course changes in the PD production and the cell density at various light intensities. The increase in light intensity caused an increase in PD production rate (Fig. 5-5a) but affected the cell density during the reaction period very little (Fig. 5-5b). The PD production rate increased with increasing light intensity up to 280 $\mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ (Fig. 5-5c). A light intensity above 280 $\mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ was not very effective for the production of PD in this reaction system. Since this alga tends to adhere to the inner surface of the reactor vessel, illumination was insufficient for photosynthesis at the center of the reactor. Internal illumination or use of a method for preventing adhesion of the cells to the inner surface of the reactor vessel would be effective for the PD production in this system.

Effect of initial cell density

Although the PD concentration apparently increased with increasing initial cell density up to $2 \times 10^8 \text{ cells} \cdot \text{ml}^{-1}$ (Fig. 5-6a), no significant effect of increase in initial cell density on the productivity was observed (Fig. 5-6c). When the initial cell density was lower than $2 \times 10^8 \text{ cells} \cdot \text{ml}^{-1}$ the cell density increased slightly during the experiment (Fig. 5-6b). However the increase in cell density showed no marked effect on the PD production rate. The rate of increase in PD concentration decreased during the reaction period (Fig. 5-6a). It is thought that in this batch reactor system, the PD productivity is influenced by a decrease in the substrate concentration and an increase in the product concentration. For this reaction system a continuous process may contribute to maintenance of high productivity during the operation period.

Effect of agitation

Although the level of intracellular DHAR activity was slightly decreased by agitation (Fig. 5-7a), no DHAR activity was detected in the medium during the experiment. However, agitation of the reaction mixture accelerates the PD production (Fig. 5-7b). This increase in the PD production seemed to be caused by an increased frequency of cell-substrate contact. Since the level of intracellular DHAR activity decreased rapidly during agitation (Fig. 5-7a) and the cell number decreased slightly during agitation (Fig. 5-7c), this alga seemed to be sensitive to agitation. Because of the structural weakness of this alga, use of a method to protect the cells, for example cell immobilization, may be necessary for long-term reactions. This may also enable the process to be used in a continuous operation system. By using a transparent material such as alginate for immobilization of this alga, the adhesion of the cells to the inner surface of the reactor vessel may also be prevented.

Comparison of PD productivities between *Dunaliella* and yeast

Though the cell density was lower and the total amount of product obtained was smaller when *Dunaliella* cells were used than when Baker's yeast cells were used, the PD productivity in the case of using *Dunaliella* cells was almost equal to that in the case of using Baker's yeast cells (Table 5-1). The highest productivity was obtained 2 d after the start of incubation for *Dunaliella* cells but 1 d after the start of incubation for the yeast cells. The reason for the delay which was observed in the case of the *Dunaliella* incubation before the maximum productivity was reached is unclear. However, it seems that the regeneration of

NADPH *via* photosynthetic pathway takes longer than that via the glycolysis occurring in the yeast.

Reaction system using *Dunaliella* cells has the advantage that the nicotinic cofactor, NADPH, which is required for the asymmetric reduction of HA is continuously supplied by photosynthesis without the need for any organic carbon sources (Figs. 5-2 and 5-5). Since glycerol was usually found to leak from the cells of several strains of *Dunaliella* (Enhauber, and Gimmler 1980), the degree of permeability of the cells to the substrate and the product seemed to be sufficient for use of whole cells of *Dunaliella* as a catalyst. Although stereoselectivity of the reduction is not strict and the optical purity of the product, (*R*)-PD, is 75%ee, the asymmetric reduction system using *Dunaliella* cells is still attractive because of their ability to regenerate NADPH via photosynthesis and produce many other useful products. Attempts to increase the optical purity of the products, such as by using a different substrate or species of *Dunaliella* or the modifying the substrate, are presently in progress.

The industrial utilization of algae, especially for biotransformation to prepare many useful products, is still in the primary stage and few reports have appeared on the use of algae as biocatalysts, e.g. photoconverters for biophotolysis of water (Ochiai *et al.* 1980) and formation of α -keto acids from amino acids (Wikström *et al.* 1982). This is the first report on a photobioreactor in which photosynthetically regenerated NADPH is continuously utilized for bioreduction.

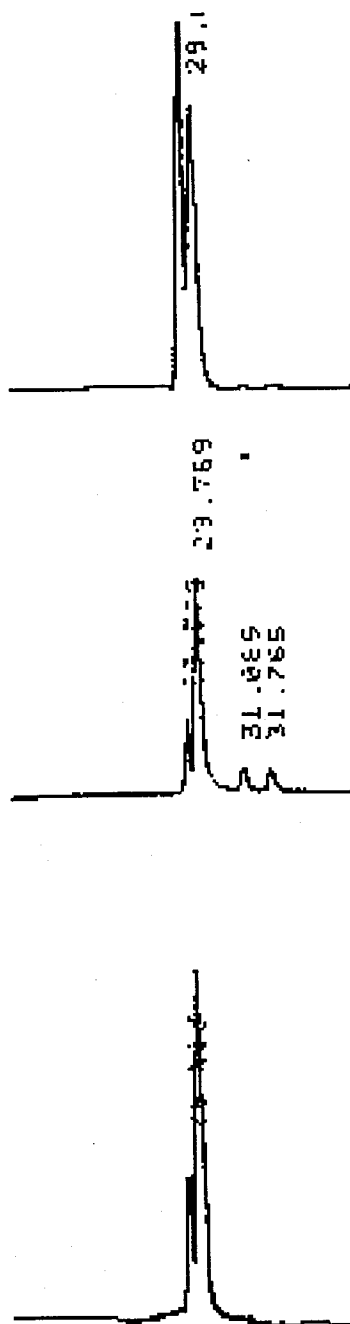


FIG. 5-1. Capillary gas chromatograms of racemic propanediol and propanediol produced using whole cells of *D. parva* and Baker's yeast. (a) Racemic propanediol; (b) R-PD produced using whole cells of baker's yeast; (c) product obtained using whole cells of *D. parva*.

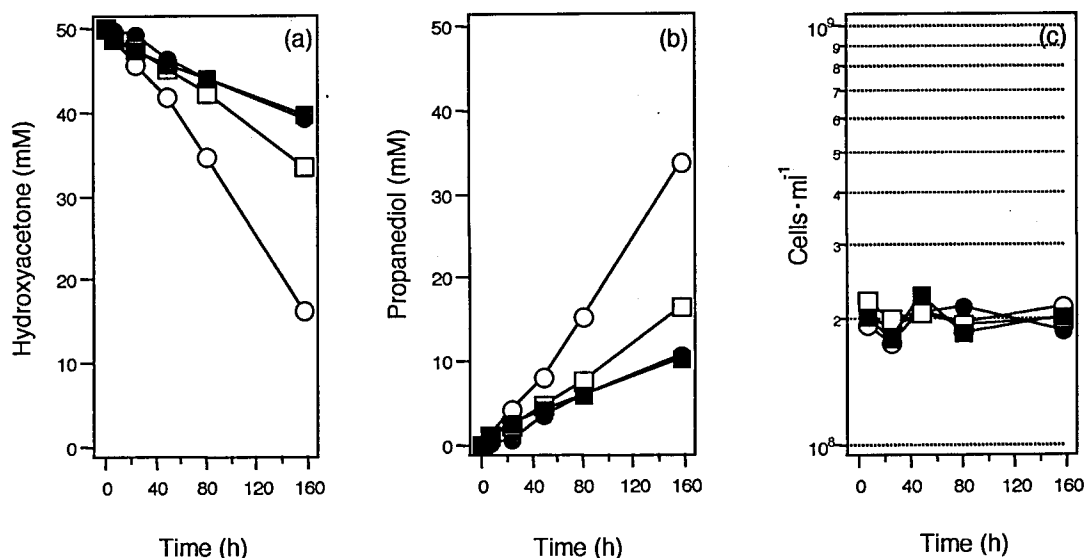


FIG. 5-2. Effect of DCMU on the conversion of HA to PD.

Ten micromoles of DCMU was added to the reaction mixture (2×10^8 cells·ml⁻¹ in 50 mM Tris-HCl (pH 8.0) containing 50 mM HA and 2 M NaCl). The reaction was performed under continuous irradiation with light ($100 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) at 15°C. (a) Time course change in remaining HA. (b) Time course change in the PD concentration. c: Time course change in cell density. Symbols: ○, -DCMU with light; ●, -DCMU without light; □, +DCMU with light; ■, +DCMU without light.

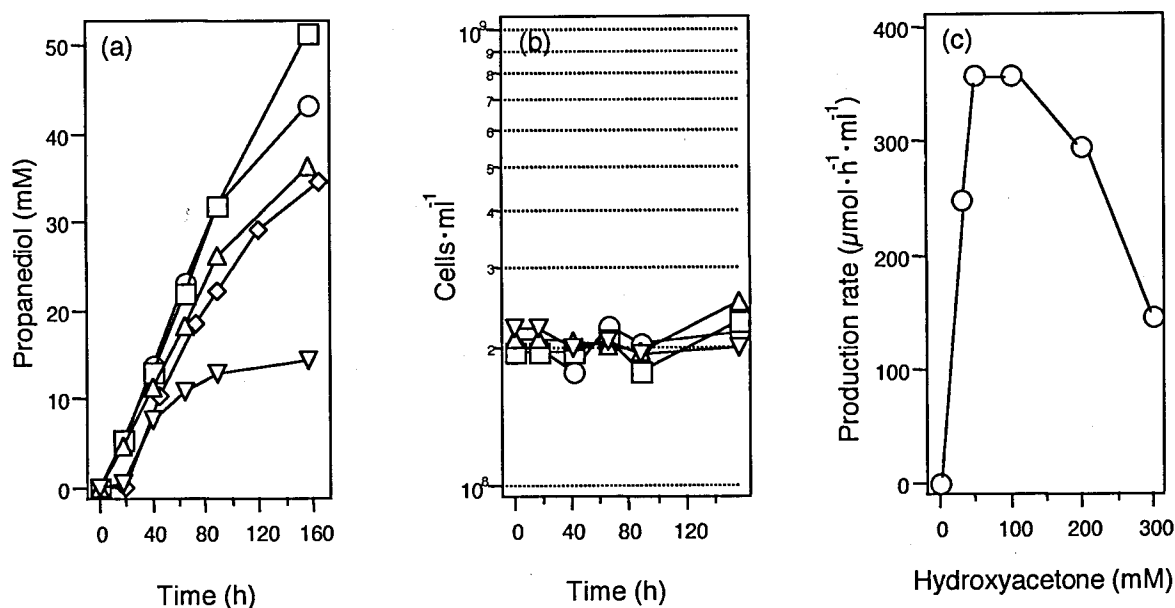


FIG. 5-3. Effect of substrate (HA) concentration on the PD production. (a) Time course change in PD concentration. The reaction was performed under continuous irradiation with light ($100 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) at 15°C. HA was added at various concentrations to the reaction mixture (2×10^8 cells·ml⁻¹ in 50 mM Tris-HCl (pH 8.0) containing 2 M NaCl). Symbols: ◇, 30 mM; ○, 50 mM; □, 100 mM; △, 200 mM; ▽, 300 mM. (b) Time course change in cell density. (c) Plot of production rate against the concentration of the substrate. Data were obtained at 90 h in the experiment in fig. 5-3a when the substrate conversion rate had exceeded over 40%.

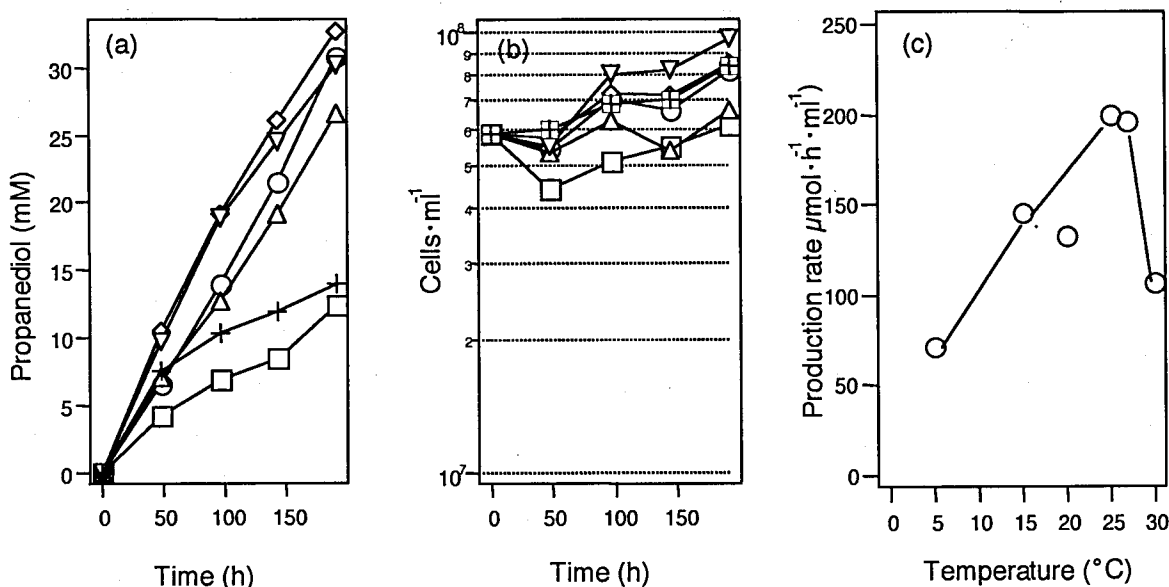


FIG. 5-4. Determination of the optimum temperature.

(a) Time course change in PD concentration. The reaction mixtures were incubated at various temperatures under continuous irradiation with light ($100 \mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$). Symbols: □, 5°C; ○, 15°C; △, 20°C; ◇, 25°C; ▽, 27°C; +, 30°C. (b) Time course change in cell density. (c) Production rate at various temperatures. Data were obtained at 144 h in the experiment in fig. 5-4a when the substrate conversion rate had exceeded 40%.

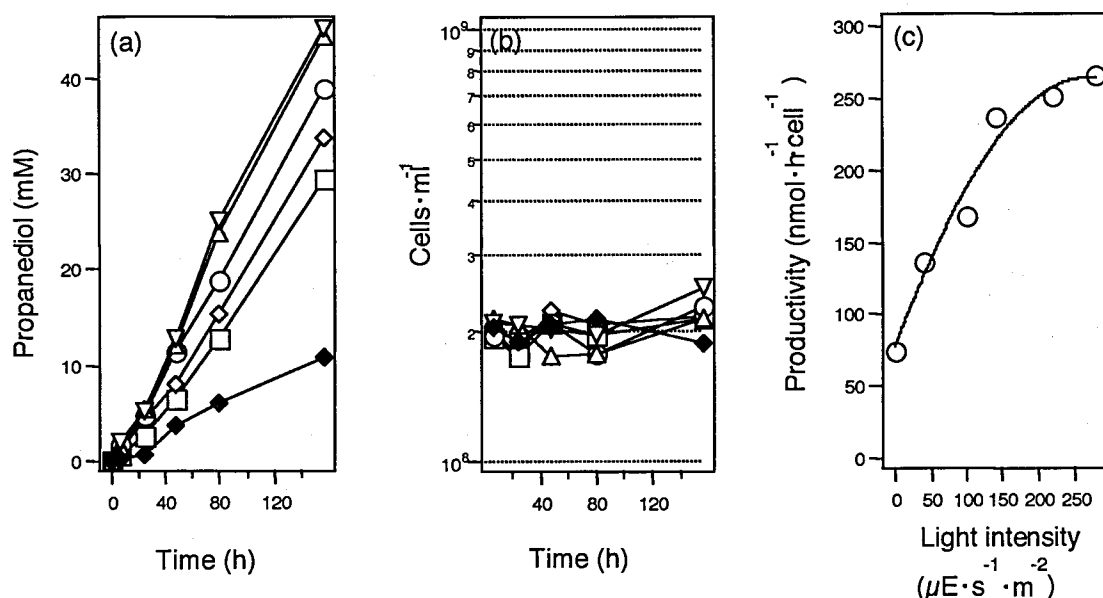


FIG. 5-5. Effect of irradiation on PD production.

Reaction mixtures were incubated under irradiation with light at various intensities. (a) Time course change in PD concentration. Symbols: ◆, 0; □, 40; ◇, 100; ○, 140; △, 220; ▽, 280 $\mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. (b) Time course change in cell density. (c) Plot of productivity against light intensity. Data were obtained at 80 h in the experiment in fig. 5-5a when the substrate conversion rate had exceeded 40%.

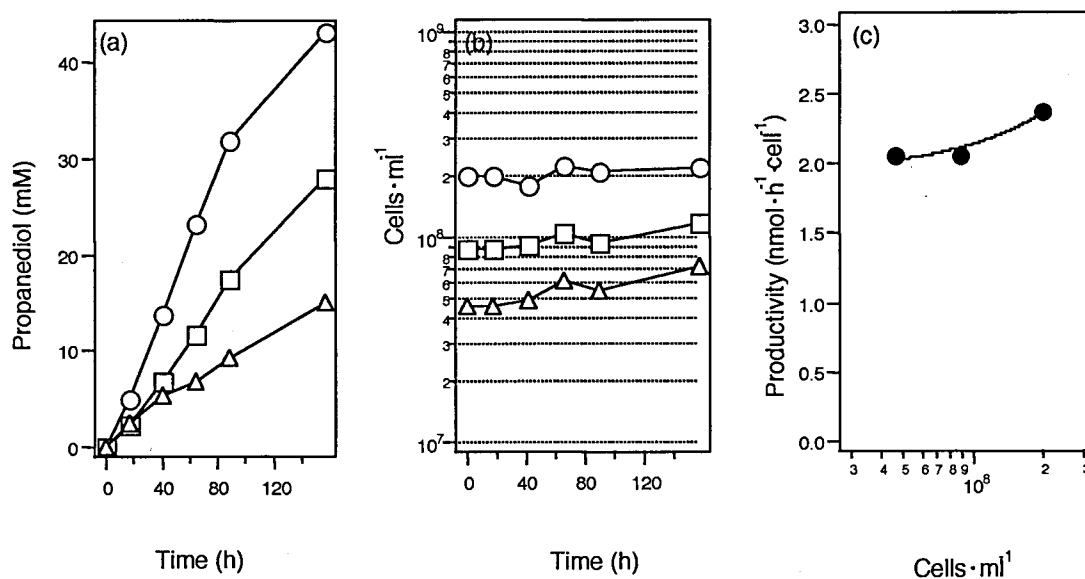


FIG. 5-6. Effect of initial cell density on the PD production.

The reaction mixture contained cells at various densities, 50 mM HA and 2 M NaCl in 50 mM Tris-HCl (pH 8.0). The reaction was performed under continuous irradiation with light ($100 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) at 15°C . (a) Time course change in PD concentration. (b) Time course change in cell density. Symbols: $\triangle$, 5×10^7 ; $\square$, 1×10^8 ; $\circ$, 2×10^8 cells · ml⁻¹. (c) Plot of productivity against cell density. Data were obtained at 41 h in the experiment in fig. 5-6a to calculate initial productivity.

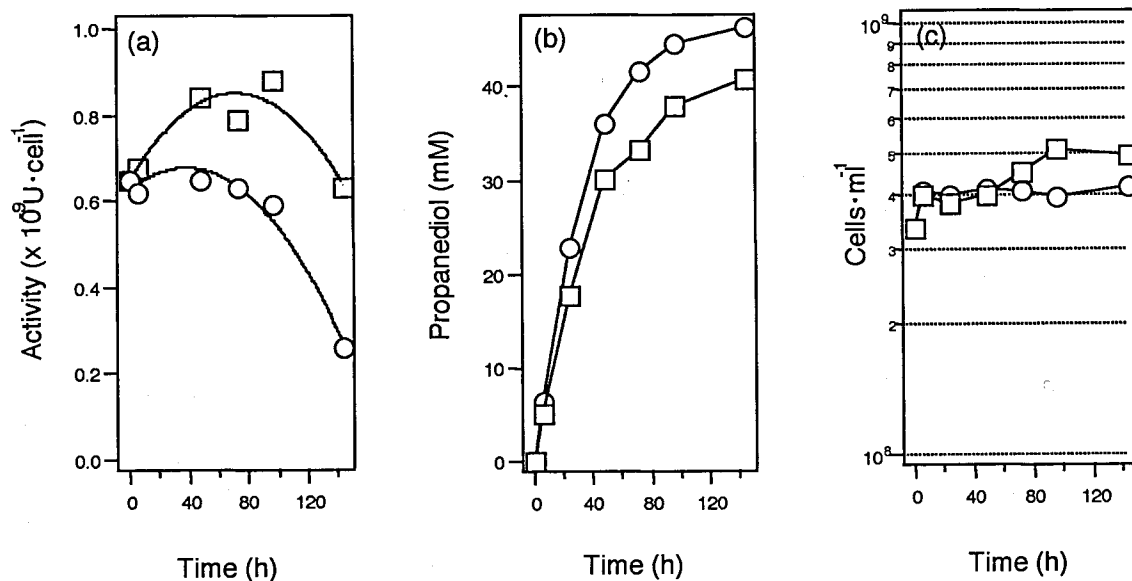


FIG. 5-7. Effect of agitation on PD production.

The reaction mixture was incubated with or without agitation under continuous irradiation with light ($100 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) at 15°C . (a) Time course change in DHAR activity during the reaction. (b) Time course change in PD concentration. (c) Time course change in cell density during the reaction period. Symbols: ○, with agitation at 200 rpm using a magnetic stirrer; □, without agitation.

TABLE 5-1. Comparison of PD productivities between
Dunaliella and Baker's yeast .

Hydroxyacetone (mM)	PD productivity (nmol · h ⁻¹ · cell ⁻¹) of		
	<i>Dunaliella</i>	<i>Dunaliella</i>	Yeast
	(with light)	(without light)	
50	3.1	1.4	3.9
100	3.2	1.7	3.8
300	2.7	0	3.2

The substrate, HA, was added to the reaction mixture to a final concentration of 50 mM, 100 mM, or 300 mM, respectively. The reaction mixture also contained *Dunaliella* cells (5×10^7 cells · ml⁻¹) and 2 M NaCl in 50 mM Tris-HCl (pH 8.0), or Baker's yeast cells (5×10^8 cells · ml⁻¹) and 0.1% glucose in 50 mM Tris-HCl (pH 7.5). Both experiments were performed under continuous irradiation with light ($100 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) at 15°C. The maximum productivities shown in this table were obtained one and two days after the start of incubation for the yeast cells, the *Dunaliella* cells, respectively.

CHAPTER VI

Effects of Immobilization on the Production of Propanediol using the Reducing Activity of Halotolerant Microalga *Dunaliella parva*

INTRODUCTION

Immobilization of algal cells has been mainly known to be applied to waste-water treatment, as in the form of percolating filters (Chevalier and de la Noüe 1985). Immobilized algal cells have recently been applied as biocatalysts performing biotransformation and *de novo* biosynthesis (Becker 1994). Use of microalgae as biocatalysts offers several prospects because algae can use sunlight as their sole, or major, energy source to make products from the substrates of photosynthesis. Many techniques for immobilization of various species of algae have also been studied with the aim of applying algal cells as biocatalysts for biotransformation and biosynthesis of useful products. For example, immobilized *Porphyridium* produces considerable amounts of sulfated capsular polysaccharides for over 17 months (Robinson, Mak, and Trevan 1986). Oleic acid can be transformed to various unspecified extracellular hydrocarbons by immobilized *Botryococcus* (Bailliez, Largeau, and Casadevall 1985).

Application of halotolerant microalgae, *Dunaliella* for the production of several useful chemicals has also been reported (Ben-Amotz, and Avron 1983)(Borowitzka, and Borowitzka 1988)(Ben-Amotz, and Avron 1983). *Dunaliella* can tolerate a high salt concentration environment as well as rapid changes in salt concentration because it accumulates glycerol as an osmoregulatory solute (Brown 1976). Since many *Dunaliella* strains have been reported to leak considerable amounts of glycerol upon changes in salinity, *Dunaliella* may be suitable for studies on the conversion of ketones to polyols using whole cells in immobilized form (Grizeau, and Navarro 1986). We have already reported that biotransformation of hydroxyacetone to optically active propanediol(75 %ee.) can be achieved by using whole cells of *Dunaliella parva*, and that the nicotine amide cofactor(NADPH) which is necessary for the reaction can be regenerated through the photosynthetic pathway itself (Hatanaka et al. 1996). In this study, we investigated the application of the immobilization technique to the reactor for asymmetric reduction by

Dunaliella parva cells, and the productivity of the column reactor using the immobilized cells at several conditions.

MATERIALS and METHODS

Chemicals

All chemicals used in this study were obtained from a commercial source and were of reagent grade. Sodium alginate (500 cp) was from Ishizu Seiyaku Ltd (Osaka, Japan). Glutaraldehyde and osmium tetroxide were obtained from TAAB Laboratory Equipment Ltd. (Aldermaston Berks, UK) and were of electron microscopic grade.

Test organisms

Dunaliella parva, strain LB1983, was cultured in a 4000 ml air-lift type culture bottle (HARIO Co. Ltd., Tokyo, Japan) containing 4000 ml of growth medium. The growth medium contained 2M NaCl, 50mM NaHCO₃, 5mM KNO₃, 5mM MgSO₄, 0.3 mM CaCl₂, 0.2 mM KH₂PO₄, 0.185 mM H₃BO₃, 7 μ M MnCl₂, 6 μ M Na₂EDTA, 1.5 μ M FeCl₃, 0.8 μ M ZnCl₂, 20 nM CoCl₂, and 0.2 nM CuCl₂, the initial pH was 8.0 (Ben-Amotz, Shaish, and Avron 1989). Cultures were illuminated with cool-white fluorescent lamps at an intensity of 20 μ E•s⁻¹•m⁻² on a 16h light-8h dark photoregime at 15°C.

Immobilization

Dunaliella cells at logarithmic phase were harvested by centrifugation at 3,000 x g. After centrifugation the cells were suspended in 50 ml of 50 mM Tris-HCl buffer (pH 8.5) containing 2 M NaCl. This cell suspension usually contained 4 x 10⁷ - 4 x 10⁸ cells•ml⁻¹. After the addition of the same volume of 4% sodium alginate to the cell suspension, the mixed solution was then dropped through the 2 mmØ nozzle into 1000 ml of buffer A which consisted of 50 mM Tris-HCl buffer (pH 8.5) containing 50 mM CaCl₂ and 1 M NaCl to make spherical calcium alginate beads. The beads were usually 3 mm in diameter, 0.05 g in wet weight and contained 6-10 μ g chlorophyll a per bead.

To determine the effect of the beads size, two different nozzle sizes (1 and 0.2 mmØ) were used to make smaller beads. Cell suspensions containing 2% sodium alginate were dropped through the nozzles into buffer A by using a peristaltic pump. The gel beads were

about 2 and 1 mm in diameter, 0.04 g and 0.014 g in wet weight and contained about 2.5 and 1 μg chlorophyll a in each bead, respectively.

Measurement of chlorophyll a

To extract chlorophyll a, three immobilized cell beads (about 0.15 g wet weight) were suspended in 5 ml of 90% acetone for 5h. Chlorophyll a was measured spectrophotometrically by the method of MacKinny (MacKinny 1941).

Chlorophyll a contents of smaller beads were determined in the same way by using the same weight of wet beads (4 2-mm $\varnothing$ beads and 10 1-mm $\varnothing$ beads).

Conditions of the reaction

Optically active (*R*)-propanediol ((*R*)-PD) was produced as follows:

1) Batch reactions using free living cells or immobilized cells.

Algal cells at the logarithmic phase were harvested by centrifugation at 3,000 x g. After centrifugation, the cells were suspended in 50 mM Tris-HCl buffer (pH 8.5) containing 2 M NaCl. This cell suspension usually contained 4×10^7 - 4×10^8 cells $\cdot\text{ml}^{-1}$. After addition of hydroxyacetone (HA) to the cell suspension to make the final concentrations of HA 30 to 500 mM, 20 ml of this reaction mixture in plant cultivation tubes (40 mm $\varnothing$ x 125 mm) were incubated at 25°C with illumination at $100 \mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. The batch reaction using immobilized cells was performed in the same way as above except that the same buffer containing 30 immobilized cell beads was used in place of the cell suspension. To maintain the hardness of immobilized cell beads, the reaction mixture always contains 50 mM of CaCl_2 (buffer A).

2) Continuous reaction using immobilized cells.

The chromatography column equipped with thermoregulating jacket (15 mm $\varnothing$ x 150 mm, HARIO Co. Ltd.) was filled with immobilized cell beads suspended in buffer A. Then buffer A containing various concentrations of HA was eluted using peristaltic pumps upward the column to start the reaction. Flow rates of each experiment were controlled to 1-1.5 mL $\cdot\text{h}^{-1}$ and calculated dilution rates were 0.1-0.15 h $^{-1}$ (about 10 mL void volume).

Columns were continuously illuminated with fluorescence lamps (330 mm, 15 W, TOSHIBA) and light intensities were controlled by changing the numbers of lamp. Light intensities on the surface of each column were 20-80 $\mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. Columns for the dark

conditions were shaded with aluminium foils.

When using the culture medium as the eluent, NaHCO_3 was subtracted from the medium to avoid the formation of CaCO_3 .

Determination of the substrate and the product

The concentrations of substrates and products were simultaneously analyzed using a gas chromatograph (GC-6A, Shimadzu Co.) with a flame ionization detector and a porous polymer column (Tenax-TA, 3mm $\varnothing$ x 2m, GL Sciences Co., Ltd.) at 160°C. Algal cells were removed by centrifugation and 10⁻¹ volume of an aqueous 1-butanol solution was added to the supernatant as an internal standard. The samples were injected directly into the gas chromatograph, and the concentrations were calculated from the peak area ratios of the components to that of the internal standard. The production rate was represented as the amount of PD produced per hour per bead.

Measurement of the photosynthetic activity

The photosynthetic activity of the immobilized algal cells was determined by the measurement of the oxygen partial pressure using Warburg's manometer specially equipped with illumination lamps (CP-9, TAITEC Co., Saitama, Japan). Thirty immobilized cell beads (1.5 g in wet weight) were suspended in 3 ml of 100 mM NaCO_3 buffer (pH 9.0) containing 1M NaCl and transferred into the measuring vessel. Measurement conditions were as follows: vessels were reciprocally shaken (60 rpm) at 15°C and illuminated at an intensity of 300 $\mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ at the surface of the vessel. To determine the respiration, the measurements for the first 30 min were performed without illumination. The activity of respiration was defined as the rate of the decrease of the oxygen partial pressure induced by one bead per min. Subsequently, the activity of photosynthesis was measured for 60 min with illumination. The activity of photosynthesis was defined as the rate of increase of the oxygen partial pressure induced by one bead per min. The value for respiration was subtracted to determine the net photosynthesis.

Photosynthesis was also measured in the same way by using smaller beads of same wet weight (about 40 2-mm $\varnothing$ beads and about 110 1-mm $\varnothing$ beads).

Electron microscopy

For transmission electron microscopy, immobilized cells were fixed in 1M sorbitol solution containing 2% glutaraldehyde and left for 2h. Samples were subsequently washed 3 times with 1 M sorbitol. Washed samples were post-fixed with 1% osmium tetroxide in 1 M sorbitol for 1 h and washed 3 times with distilled water. Then, samples were stained with saturated uranyl acetate for 20 min. After being washed with distilled water (3 times), samples were dehydrated through an ethanol series, cleared with propylene oxide, and embedded in Spurr resin. All the procedures mentioned above were performed at room temperature. The specimens were polymerized at 70°C for 16 h. The blocks were sectioned on a Richert ultramicrotome (OM U3) and sections were post-stained with saturated uranyl acetate for 20 min followed by Reynold's lead citrate (Reynoldz 1963) for 10 min. Sections were observed and photographed on a JEOL 1200 EX electron microscope.

Light microscopy

Semi-thin sections for light microscopy were prepared as follows: Immobilized cells were fixed by the same procedure as above. Samples were dehydrated through an ethanol series and embedded in Technovit 7100 resin. Polymerized specimens were sectioned on a Richert ultramicrotome (OM U3) and stained with Giemsa solution for 1 h. Sections were observed and photographed using an Olympus BX50 light microscope.

RESULTS

Viability of immobilized cells

The photomicrographs of the semi-thin section of the immobilized cells (Fig. 6-1) show that all the cells were completely entrapped in the calcium alginate gel matrix, and the cells resembled the free cells in shape. Deformed or disrupted cells were not observed in any of the gel beads. The measurement of photosynthesis of these immobilized cells also showed significant activity (Fig. 6-2).

Stability of the immobilized cells

To examine the stability of the immobilized cells, the time course change in the chlorophyll a contents and the activity of photosynthesis were measured under several

conditions by using the same system as the batch reaction (Fig. 6-2). Except for the slight reduction in the chlorophyll a content of the beads at 25°C, immobilized *Dunaliella* cells showed no drastic change in the chlorophyll a content for almost 2 weeks (Fig. 6-2A). The measurement of the photosynthesis revealed that about 80% of the activity of the photosynthesis remained at 5°C in the light for 2 weeks (Fig. 6-2B). The photosynthetic activity of the immobilized cells was reduced at higher temperatures, the activity being markedly lower in the dark. A transient increase in photosynthesis was sometimes observed after the exchange of the incubation buffer (fig. 6-2B, arrow).

Batch reactions using the immobilized *Dunaliella parva* cells

Figure 6-3 shows the PD production using free cells (Fig. 6-3A) and immobilized cells (Fig. 6-3B). Although the total cell number of each reaction was not the same (about 2×10^9 and 7×10^8 cells per vessel for free cells and immobilized cells, respectively), PD was produced by the immobilized cells similar to the free cells.

Optimum column reactor conditions using immobilized *Dunaliella parva* cells

1. Effect of cell densities

The production rate of PD was measured at the cell densities of 1.5×10^6 to 1×10^7 cells•bead⁻¹ (Table 6-1). The PD production rate was increased proportionally to the increase of the cell density.

2. Effect of light intensity

The effect of light intensity was investigated by changing the number of fluorescent lamps. The PD production rate was increased proportionally to the increase of light intensity from 20 to 80 $\mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ (Table 6-2).

The electron micrographs of the thin sections of the immobilized cells after a 10-day reaction showed that the size of the starch granules at lower light conditions had been decreased with the decrease of the light intensity (Fig. 6-4).

3. Effect of temperature

PD production rate of this reactor was best around 20°C (Table. 6-3).

4. Effect of the bead size

The measurements of photosynthesis with time revealed that the smaller beads had the

same stability as 3 mmø beads (data not shown). The PD productivity of the beads of each size was measured (Table 6-4). Although the 3 mmø beads exhibited higher productivity than the smaller beads (Table 6-4, productivity 1), the productivity of the beads packed in the same volume was similar.

5. Effect of the medium

Three different kind of eluent were tested to determine the appropriate eluent for the active state of the reduction and photosynthesis. Compared with buffer A (Fig. 6-5, A), the eluent consisting of the CaCO_3 -free medium and 5^1 diluted CaCO_3 -free medium markedly prolonged the active state of the reduction and photosynthesis (Fig. 6-5, B,C). Although the culture medium contained a small amount of KH_2PO_4 (0.2 mM), dissolution of the Ca-alginate gel matrix was not observed in our experimental condition.

DISCUSSION

Since the generation time of microalgae is long compared to bacteria, yeast or other microorganisms under consideration for industrial use, retention of the cells in the reactor using the methods of cell immobilization is an essential problem to develop a continuous flow reactor. Several polymers and gels have been tested for the adsorption or entrapment of the algal cells (Robinson, Mak, and Trevan 1986). Since synthetic polymers may be toxic to algal cells (Becker 1994), natural polymers, such as alginate, agar, carrageenan, and gelatin must be used for immobilization of the algal cells. Among these natural polymers, only alginate can be solidified at an ambient temperature and the resulted gel is transparent. Since algae or plant cells must conduct photosynthesis, the transparency of the entrapping material is also very important. For these reasons, alginate was selected to immobilize *Dunaliella* cells. Photo-microscopic observation of the semi-thin section (Fig. 6-1) and the measurement of photosynthesis of immobilized cells indicated that *Dunaliella* cells in calcium alginate were not seriously damaged by immobilization.

Although cell multiplication of microalgae in a gel matrix has been reported (Thepenier, and Gudin 1985), observation of thin sections of the beads and measurement of chlorophyll a content (Fig. 6-2A) revealed no cell multiplication of the immobilized *Dunaliella parva* in our experimental conditions. Since the alginate concentration in this experiment (2%) was lower than that in the previous experiments (3-4%) (Robinson, Mak, and Trevan

1986)(Grizeau and Navarro 1986), the effect of the diffusional constraint is smaller in this limited growth condition. Since the maximum cell density of the cultivation of *Dunaliella parva* in our culture condition is less than 10^7 cell•ml⁻¹, the cell density of the immobilized cell beads (2×10^7 - 2×10^8 cells•ml⁻¹ Na-alginate solution) was too high for this alga to multiply.

There have been few reports about the effects of immobilization on the physiology of viable algal cells (Jeanfils, and Collard 1983)(Robinson et al. 1985). Immobilization has also been reported to stabilize the activities of several enzymes and photosynthetic activity (Cocquempot et al. 1979)(Thomasset et al. 1982). The periodic measurement of photosynthesis (Fig. 6-2B) of immobilized *Dunaliella parva* revealed that immobilization did not affect the stability of photosynthetic activity of *Dunaliella*. The general cellular metabolic activity of *Chlorella emersonii* has been reported to be reduced when it was immobilized in calcium alginate gel matrix (Robinson et al. 1985), probably due to the difference in the activity of cells near the surface of the gel matrix and cells in the interior of the matrix. Since the reduced illuminance and the nutrient limitation occur in the interior of the matrix, cells embedded deeply in the gel were metabolically less active, the average cellular activity thereby being reduced. The reduced illuminance in the interior of the gel also seems fatal to *Dunaliella parva*, because this alga is strictly photoautotrophic. The photosynthetic activity of the immobilized cells in the dark was reduced, but transiently increased after the exchange of incubation buffer for an unknown reason (Fig. 6-2B, arrows).

Alginate-immobilized *Dunaliella* cells showed significant PD productivity as free cells (Fig. 6-3) Cytotoxicity of HA to *Dunaliella parva* was known problem for the reaction using free cells (Hatanaka et al. 1996). From the fact that productivities at higher HA concentrations over 50 mM showed no much increase in productivity (Fig. 6-3, B), resistance of immobilized cells to the cytotoxicity of HA was not increased by immobilization. As far as the reaction under 50 mM HA, these immobilized cells seem useful in the continuous flow reactors.

Since the increase in productivity was proportional to the number of the *Dunaliella* cells in our experimental condition (Table 6-1), it was found that the reaction was not saturated at cell density below 1×10^7 cells•bead⁻¹. In this experiment, more than 30% of the photosynthetic activity of the beads at the highest cell density remained after 10 days of

reaction (Table 6-1). The reason for the drastic decrease in the photosynthesis at the lowest cell density was unclear. It may be explained by the cytotoxicity of HA. Since the reduction of HA by the cells of low cell density was relatively slow, toxicity of HA may be kept higher.

At the low light intensity, since the cell suffered the shortage of illuminance for photosynthesis the starch granules were consumed to maintain the activity of the cell (Fig. 6-4). As reported previously (Hatanaka et al. 1996), regeneration of NADPH through the photosynthetic pathway is very important for PD production. This means that the shortage of light may also inhibited the regeneration of NADPH, and accordingly the PD production (Table. 6-2).

The optimum temperature for PD production was around 20°C (Table 6-3), which was lower than the optimum temperature of 45°C for the reducing enzyme (dihydroxy acetone reductase) or the optimum temperature of 25°C for the reduction using free cells (Hatanaka et al. 1996). The reason for this shift in the optimum temperature is still not clear.

The PD productivity of immobilized cells were not influenced by the size of beads but by the total packed volume of the beads (Table 6-4). Since the immobilized cells packed in the column reactor were not agitated in this experiment, all the gel beads in the reactor can be regarded to be one large gel. The reduction of illuminance may often be caused not by the size of gel but by the position of a beads in the column.

The CaCO_3 -free medium was found effective for prolonging the activity of reduction (Fig. 6-5, ●) and photosynthesis (Fig. 6-5, △) of the immobilized cells, even diluted five times (Fig 6-5, C). Certain minor minerals in the medium is thought to be important for the maintenance of the activity of the immobilized cells.

Table 6-1 Effect of cell density on the production of PD

Cell density ($\times 10^6$ cells $\cdot$ bead $^{-1}$)	PD productivity (nmol $\cdot$ h $^{-1}$ $\cdot$ bead $^{-1}$)	Relative photosynthesis* (%)
1.58	1.23	13
4.70	4.03	35
9.45	6.46	33

*after 10 days reaction

Columns containing about 350 immobilized cell beads of 3 mm diameter were illuminated continuously at $40 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ at 15 °C. For the reaction, buffer A containing 50 mM HA was eluted 1-2 mL per hour.

Table 6-2 Effect of light intensity on the production of PD

Light intensity ($\mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$)	PD productivity (nmol $\cdot$ h $^{-1}$ $\cdot$ bead $^{-1}$)	Relative photosynthesis (%)
20	3.28	2
40	6.90	30
80	9.47	28

*(Final photosynthesis (after 10 days))/(Initial photosynthesis) X 100

Columns containing about 350 immobilized cell beads of 3 mm diameter were illuminated continuously at each intensities at 15 °C. For the reaction, buffer A containing 50 mM HA was eluted 1-2 mL per hour.

Table 6-3 Effect of temperature on the production of PD

Temperature (°C)	PD productivity (nmol · h ⁻¹ · bead ⁻¹)	Relative photosynthesis* (%)
17	7.34	36
20	8.90	31
25	7.64	22

*after 10 days reaction

Columns containing about 350 immobilized cell beads of 3 mm diameter were illuminated continuously at $40 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ at each temperature. For the reaction, buffer A containing 50 mM HA was eluted 1-2 mL per hour.

Table 6-4 Effect of bead size on the production of PD

Beads size (mm ϕ)	PD productivity 1 (nmol · bead ⁻¹ · h ⁻¹)	PD productivity 2 (nmol · bead ⁻¹ (ml)* · h ⁻¹)	Relative photosynthesis (%)**
3	3.78	45.3	18
2	2.28	45.7	20
1	1.08	46.3	28

*Packed volume (ml) of each beads were measured using a measuring cylinder.

** (Final photosynthesis)/(Initial photosynthesis) X 100

Each column containing about 350, 600, and 1300 immobilized cell beads of 3, 2, and 1 mm diameter were illuminated continuously at $40 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ at 15 °C. For the reaction, buffer A containing 50 mM HA was eluted 1-2 ml per hour.

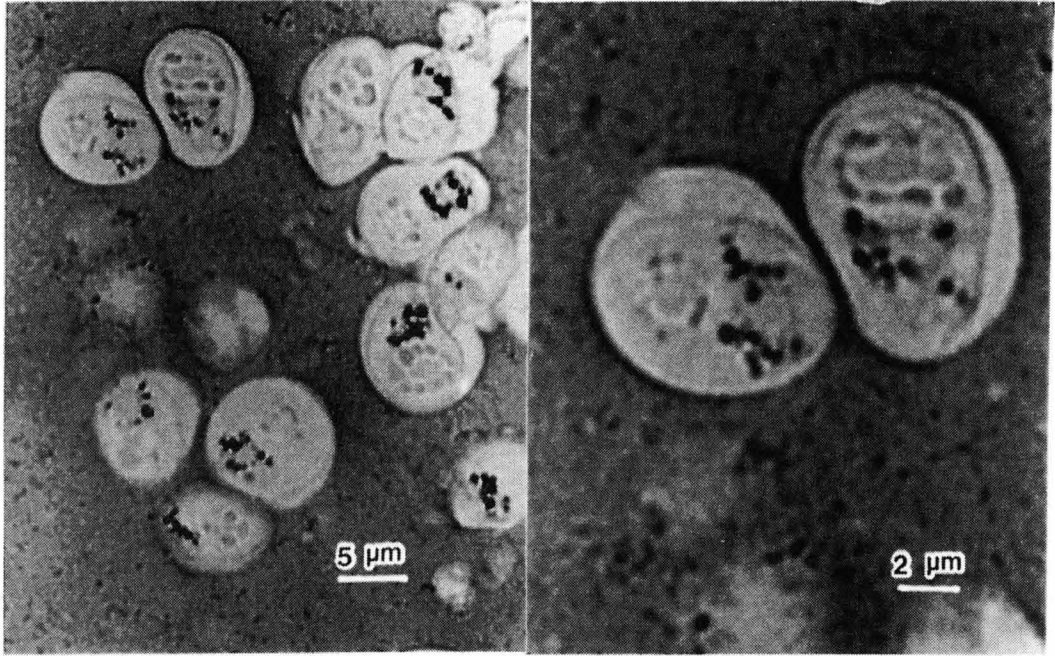


Fig. 6-1 Semi-thin sections of *Dunaliella Parva* immobilized in Ca-alginate gel.

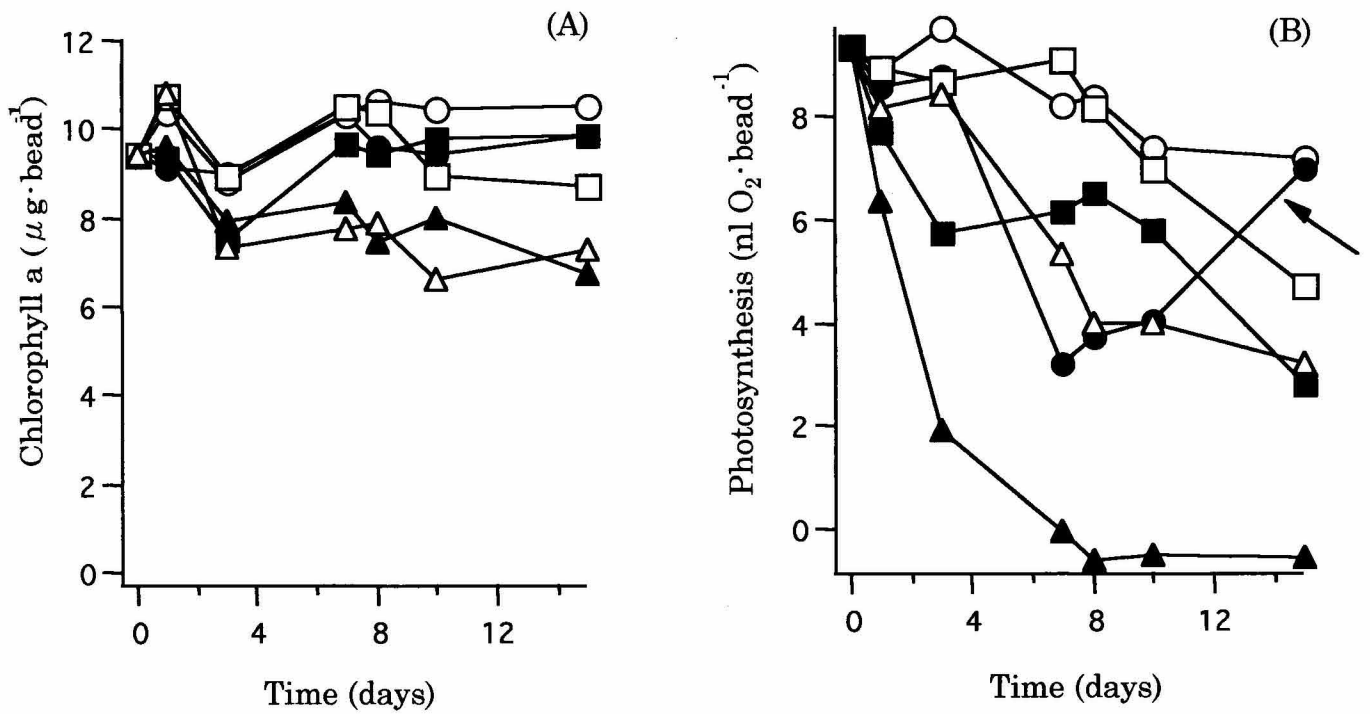


Fig. 6-2 Time course change in Chlorophyll a content (A) and photosynthesis (B) of immobilized *Dunaliella parva*.

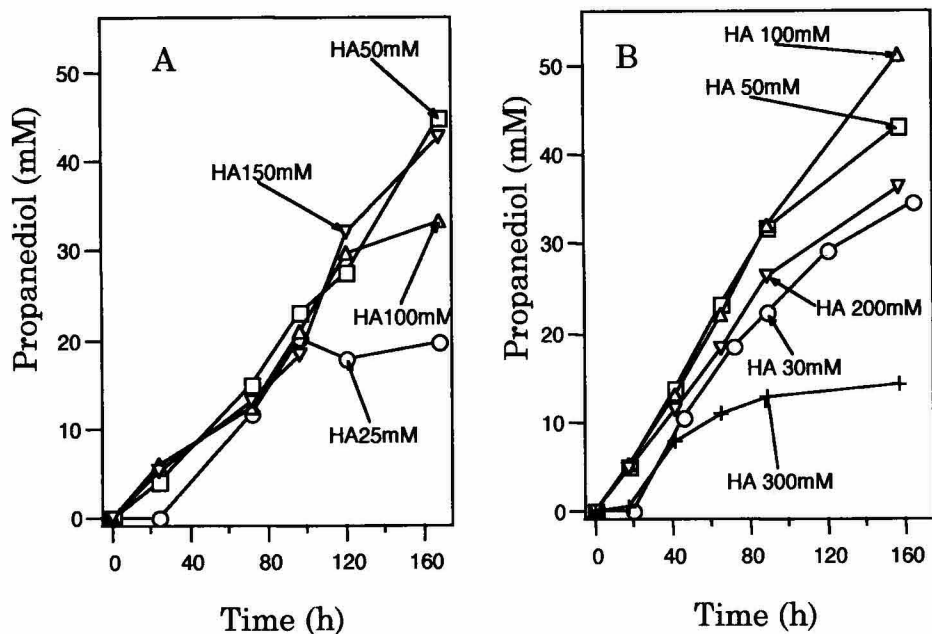


Fig. 6-3 Effect of the concentrations of HA on the productions of PD using free cells (A) or immobilized cells (B)

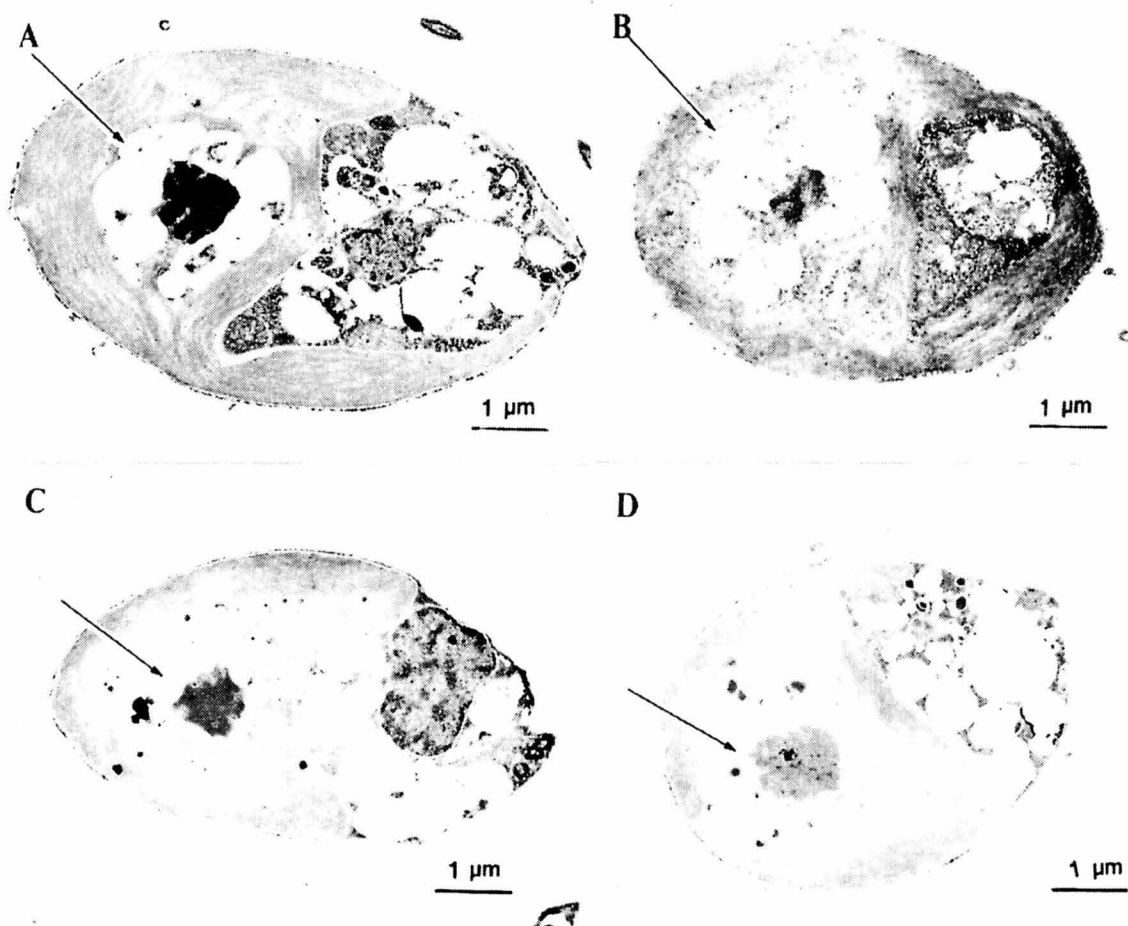


Fig. 6-4 Ultra-thin sections of immobilized *Dunaliella parva*.after the reaction under several light conditions. A: control, B-D: light intensities of 80 (B), 40 (C), 20(D) $\mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$.

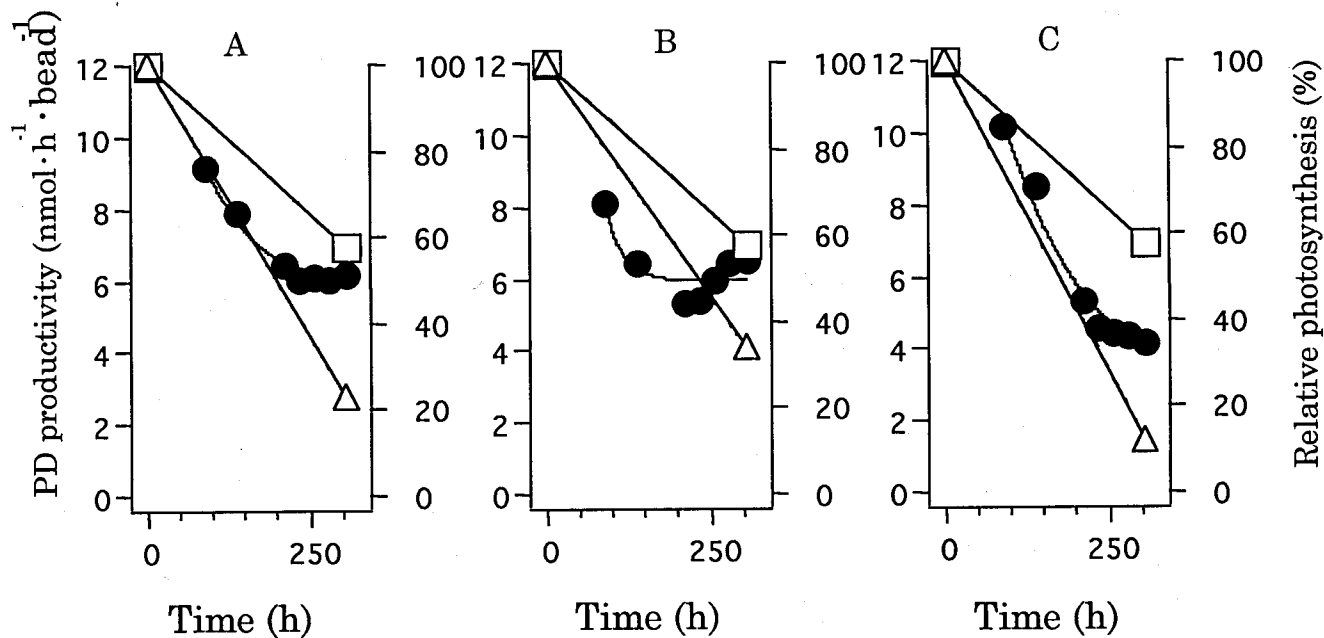


Fig. 6-5 Effect of the kind of eluent on the PD production by immobilized *Dunaliella*.
 A: buffer A with 50 mM HA, B: medium with HA, C: 5⁻¹ medium with HA.

CHAPTER VII

CONCLUSIONS

The economic utilization of microalgae has been explored for some time. The commercial utilization of microalgae gained impetus with the discovery that the extremely halophilic green alga *Dunaliella salina* was the best natural source of β -carotene. Since then, study about the practical application of *Dunaliella* had been focused on the accumulation of several useful chemicals, such as beta carotene and glycerol. Since the practical utilization of *Dunaliella* using technique of molecular biology has not been developed (Simon and Latorella), I investigated the surface structure of *Dunaliella parva* for the making of the protoplast for the aim of transformation or cell fusion. I also investigated the properties of starch in *Dunaliella* and an enzyme for the metabolism of starch, because the study about the starch in *Dunaliella* has been very limited. For another way of utilization, I made the asymmetric bioreactor using *Dunaliella* cells as free or immobilized form.

Study on the surface structure of *Dunaliella parva*

Production of useful chemicals such as glycerol and beta carotene using the whole cells of *Dunaliella* was studied sometimes. Bioreactors using the living cells of *Dunaliella* were also reported and the importance of the permeability of the cell membrane was suggested. The surface coat of *Dunaliella parva* was investigated by using several techniques. Degradation by several cell lytic enzymes and ultrastructural observation revealed that *Dunaliella parva* has a specialized cell surface structure containing a glycoprotein that is sensitive not only to proteinases but also to lysozyme. This sensitivity was also demonstrated by electrophoresis of the cells and measurement of released glycerol after enzyme treatment. Immunochemical labeling indicated that the surface glycoprotein of *Dunaliella parva* is analogous to peptidoglycan. Peptidoglycan is well known surface structure of bacteria and several cyanobacteria. This is the first report of the discovery of peptidoglycan like structure in eucaryotic cells and this finding may help us when making some bioreactor using free cells or immobilized cells of *Dunaliella*.

Study on the starch from *Dunaliella*

Besides glycerol and beta carotene, starch content is also abundant in *Dunaliella* cells. Although starch in plants is most important product from the conversion of solar energy, studies about the starch of *Dunaliella* is not enough. To determine the value of starch of *Dunaliella* as the source of food or feed, I isolated and investigated some properties of starch from *Dunaliella parva*.

The starch content of the algae reached about 10% after 10 days' cultivation. This starch content was larger than that of *Chlamydomonas reinhardtii*, which is a relative species of *Dunaliella*. Although the crystal structure of the starch seemed similar to the starch of maize, the gelatinization temperature of the starch was 10 °C lower. Gelatinization temperatures of the starch from *Dunaliella* cultured at different temperatures indicated that the change in gelatinization temperature depends on the temperature during cultivation. The same phenomenon was also reported about the gelatinization temperature of certain microalgae and plants.

Nitrogen starvation caused a significant increase in the starch content of *Dunaliella parva*. Since some kinds of lipid droplets were also observed in the starch-accumulating cells, nitrogen starvation may be useful to improve the values of *Dunaliella* cell for the biomass.

Isolation of a starch degrading enzyme from *Dunaliella*

To understand the effects of amylolytic enzymes on the contents and properties of starch in *Dunaliella*, I isolated an amylolytic enzyme and investigated some properties of the enzyme. Starch metabolism in *Dunaliella parva* was regulated by the osmolarity of the surrounding salute. Among many amylolytic enzymes, rise of the phosphorylase activity was observed after hyperosmotic shock. Purification of an amylolytic enzyme which easily could be stained after electrophoresis was attempted. Two isozymes have the same activity were purified after final gel filtration chromatography. Substrate specificity of the isozymes revealed that the enzymes have the disproportionating activity of maltooligosaccharides. Although many disproportionating enzymes (D-enzymes) were found in many plants and bacteria, D-enzyme of *Dunaliella parva* was first found and purified in algae. Properties of the enzyme indicated that the enzyme is similar to the enzymes of plants. Since D-enzyme

activity can be found in various *Dunaliella* and *Chlamydomonas*, function of D-enzyme was thought to be important in the starch metabolisms in those algae.

Construction of bioreactor for the asymmetric reduction using whole cells of *Dunaliella parva*

Regeneration of NADPH via photosynthetic pathway was applied to the development of a novel bioreactor in which NADPH is required for the desired reaction. In a preliminary experiment using dihydroxyacetone reductase (DHAR) from *Dunaliella parva*, hydroxyacetone (HA, Acetol) was reduced to yield (*R*)-(-)-1,2-propanediol ((*R*)-PD) with an optical purity of 75%ee. The bioconversion using *Dunaliella parva* whole cells in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) showed that the NADPH required for this reaction can be generated by photosynthesis. The optimum reactor conditions for propanediol (PD) production were 50 - 100 mM HA at 25 °C. The production rate of PD increased proportionally with increasing irradiation intensity up to $220 \mu\text{E}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. Although changes in cell density did not affect the productivity, agitation of the reaction mixture at 200 rpm raised the productivity. Since the amount of PD produced was almost the same as the amount of HA consumed, it was confirmed that more than 95 % of the HA was converted to PD without turning into any by-products or being degraded. The PD productivity in the photobioreactor system using *Dunaliella* cells was comparable to that in the yeast cell system.

Effects of immobilization on *Dunaliella parva* cells used in the asymmetric bioreactor

Halotolerant microalga *Dunaliella parva* was immobilized in calcium alginate gel. From the observation of the semi-thin section and the measurement of photosynthesis of *Dunaliella* cells in the alginate gel beads, the cells were found to be active like the free cells. Only a 20 % drop in the activity of photosynthesis was observed after 2 weeks of incubation at 5°C in the light. The asymmetric reduction of hydroxyacetone (HA) to make (*R*)-propanediol ((*R*)-PD) using free cells and immobilized cells was tested as batch reactions. Since the immobilized cells showed significant activity, the adequate conditions for the column reactor using immobilized cells were investigated. Since PD productivity of immobilized cells was

increased in proportion to light intensity during the reaction, regeneration of nicotine amide cofactor (NADPH) through photosynthesis seemed necessary for the asymmetric reduction. Observation of thin sections of immobilized cells after the reaction revealed that NADPH used for the asymmetric reduction in the dark seemed to be produced through the metabolism of the starch granules. The eluent for the column reactor consisting of CaCO_3 -free medium markedly prolong the active state of the reduction and photosynthesis.

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REFERENCES

- Avron, M.:** The osmotic components of halotolerant algae. *Trends. Biochem. Sci.*, **11**, 5-6 (1986).
- Bailliez, C., Largeau, C., and Casadevall E.:** Growth and hydrocarbon production of *Botryococcus braunii* immobilized in calcium alginate gel. *Appl. Microbiol. Biotechnol.*, **23**, 99 (1985).
- Ball, S. G., Diric, L., Decq, A., Martiat, J. C., and Matagne, R. F.:** Physiology of starch storage in the monocellular alga *Chlamydomonas reinhardtii*. *Plant Science*, **66**, 1-9 (1990).
- Ball, S. G.:** Regulation of starch biosynthesis. In *Molecular Biology of Chloroplast and Mitochondria in Chlamydomonas* **Rochaix, J-D., Goldschmidt-Clement, M. and Marchant, S. eds.** Kluwer Academic Publishers, Netherlands, pp. 549-567. (1998).
- Becker, E. W.:** Application of algae. In *Baddiley, Sir James, Carey, N. H., Higgins, I. J., and Potter, W. G. (ed.), Cambridge studies in biotechnology, vol. 10, Microalgae: Biotechnology and microbiology.* Cambridge University Press, New York, pp. 250-275 (1994).
- Ben-Amotz, A., and Avron, M.:** The role of glycerol in the osmotic regulation in the halophilic alga *Dunaliella parva*. *Plant Physiol.*, **51**, 875-878 (1973).
- Ben-Amotz, A. and Avron, M.:** NADP specific dihydroxyacetone reductase from *Dunaliella parva*. *FEBS Lett.*, **29**, 153-155 (1973).
- Ben-Amotz, A. and Avron, M.:** Isolation, characterization, and partial purification of reduced nicotinamide adenine dinucleotide phosphate-dependent dihydroxyacetone reductase from the halophilic alga *Dunaliella parva*. *Plant Physiol.*, **53**, 628-631 (1974).
- Ben-Amotz, A.:** Adaptation of the unicellular alga *Dunaliella parva* to a saline environment. *J. Phycol.*, **11**, 50-54 (1975).
- Ben-Amotz, A., and M. Avron.:** Glycerol and β -carotene metabolism in the halotolerant alga *Dunaliella*: a model system for biosolar energy conversion. *Trends Biochem. Sci.*, **6**, 297-299 (1981).
- Ben-Amotz, A., Katz, A., and Avron, M.:** Accumulation of β -carotene in

halotorelantalgae: purification and characterization of β -carotene-rich globules from *Dunaliella bardawil* (Chlorophyceae). *J. Phycol.*, **18**, 529-537 (1982).

Ben-Amotz, A. and Avron, M.: On factors which determine massive β -carotene accumulation in the halotolerant alga *Dunaliella bardawil*. *Plant Physiol.*, **72**, 593-597 (1983).

Ben-Amotz, A. and Avron, M.: Accumulation of metabolites by halotolerant algae and its industrial potential. *Ann. Rev. Microbiol.*, **37**, 95-119 (1983).

Ben-Amotz, A., and Tornabene, T. G.: Chemical profiles of selected species of microalgae with emphasis on lipid. *J. Phycol.* **21**, 72-81 (1985).

Ben-Amotz, A., Shaish, A., and Avron, M.: Mode of action of the massively accumulated β -carotene of *Dunaliella bardawil* in protecting the alga against damage by excess irradiation. *Plant Physiol.*, **91**, 1040-1043 (1989).

Berthod, A., Li, W., and Armstrong, D. W.: Multiple enantioselective retention mechanisms on derivatized cyclodextrin gas chromatographic chiral stationary phases. *Anal. Chem.*, **64**, 873-879 (1992).

Borowitzka, L. J. and Brown, A. D.: The salt relations of marine and halophilic strains of the unicellular green alga, *Dunaliella*. The role of glycerol as a compatible solute. *Arch. of Microbiol.*, **96**, 37-52 (1974).

Borowitzka, L. J., Kessly, D. S., and Brown, A. D.: The salt relations of *Dunaliella*. Further observation on glycerol production and its regulation. *Arch. of Microbiol.*, **113**, 131-138 (1977).

Borowitzka, L. J., Moulton, T. P. and Borowitzka, M. A. in Algal biomass: An Interdisciplinary perspective., W. Barclay and R. McIntosh eds J. CRAMER, Berlin-Stuttgart, pp217-222 (1985).

Borowitzka, M. A. and Borowitzka, L. J.: *Dunaliella*. In **Borowitzka, M. A. and Borowitzka, L. J. (ed.), Micro-algal biotechnology.** Cambridge University Press, New York, pp 27-58 (1988).

Brown, A. D.: Microbial water stress. *Bacteriol. Rev.*, **40**, 825-829 (1976).

Brown, A. D., and Borowitzka, L. J.: Halotolerance of *Dunaliella*. In *Biochemistry and Physiology of Protozoa*, vol 1, 2nd edn, Academic Press, New York, pp. 139-190 (1979).

- Chevalier, P. and de la Noüe, J.:** Waste water nutrient removal with microalgae immobilized in carrageenan. *Enzyme Microb. Technol.*, **7**, 621-624 (1985).
- Cocquempot, M. F., Thomas, D., Champigny, M. L., and Moyse, A.:** Immobilization of thylakoids in porous particles and stabilization of the photochemical process by glutaraldehyde at subzero temperature. *Eur. J. Appl. Microbiol. Biotechnol.*, **8**, 37-41 (1979).
- Cohen, D. and Parnas, H.:** An optimal policy for the metabolism of storage materials in unicellular algae. *J Theor. Biol.*, **56**, 1-18 (1976).
- Davis, C. H., Schliselfeld, L. H., Wolf, D. P., Leavitt, C. A., and Krebs, E. G.:** Interrelationships among glycogen phosphorylase isozymes. *J. Biol. Chem.*, **242**, 4824-4833 (1967).
- Delure, B., Fontain, T., Routier, F., Decq, A., Wierusezeski, J. M., Van Den Koornhuyse, N., Maddelein, M. L., Fournet, B., and Ball S.:** Waxy *Chlamidomonas reinhardtii*: Monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectine. *J. Bacteriol.* **174**, 3612-3620 (1992).
- Dubois, M., Gilles, K. A., Hamilton, J. K., Roberts, P. A., and Smith, F. :** *Anal. Chem.*, **28**, 350- (1956).
- Dunal, M. F.** Note sur les algues qui colorent en rouge certaines eaux des marais salants méditerranéens. *Comptes rendus de la Académie de Science, Paris*, **7**, 21-27 (1837).
- Edelmann, J., and Keys, A. J.:** A maltose-glucose transglucosylase from wheat germ. *Biochem. J.*, **70**, 12 (1961).
- Einspahr, K. J., Maeda, M. and Thompson, G. A., Jr.:** Concurrent change in *Dunaliella salina* ultrastructure and membrane phospholipid metabolism after hyperosmotic shock. *J. Cell Biol.*, **107**, 529-538 (1988).
- Enhauber, G. and Gimpler, H.:** The glycerol permeability of the plasmalemma of the halotolerant green alga *Dunaliella parva* (Volvocales). *J. Phycol.*, **16**, 524-532(1980).
- Gimpler, H., and Moller, E. M.:** Salinity-dependent regulation of starch and glycerol metabolism in *Dunaliella parva*. *Plant Cell and Environ.*, **4**, 367-375 (1981).
- Gimpler, H., Weiss, U., Weiss, C., Kugel, H., and Treffny Y.:** *Dunaliella acidophila*

- an alga with a positive membrane potential. *New Phytol.*, **113**, 175-184, (1989).

Gimmler, H., Treffny, B., Kowalski, M., and Zimmermann, U.: The resistance of *Dunaliella acidophila* against heavy metals: The importance of the zeta potential. *J. Plant Physiol.*, **138**, 708-716 (1991a).

Gimmler, H., Schieder, M., Kowalski, M., Zimmermann, U., and Pick, U.: *Dunaliella acidophila* - an alga with a positive zeta potential. *Plant. Cell and Environ.*, **14**, 261-269, (1991b).

Goyal, A., Brown, A. D., and Gimmler, H.: Regulation of salt-induced starch degradation in *Dunaliella tertiolecta*. *J. Plant Physiol.*, **127**, 77-96 (1986).

Grizeau, D. and Navarro, J. M.: Glycerol production by *Dunaliella tertiolecta* immobilized within Ca-alginate beads. *Biotechnol. Lett.*, **8**, 261-264 (1986).

Harris, E. H.: In *Chlamydomonas* Sourcebook Academic Press, New York, pp. 780 (1988).

Hatanaka, Y., Kobayashi, O., Higashihara, M., and Hiyama, K.: Reduction of hydroxyacetone with NADPH regenerated through photosynthetic pathway of the halotolerant alga *Dunaliella parva*.. *J. Ferment. Bioeng.*, **81**, 379-385 (1996).

Hatanaka, Y., Inaoka, K., Özboy, Ö., Demiate, I. M., Kobayashi, O. Higashihara, M., and Hiyama, K.: Study of Some Properties of Starch Isolated from Halotolerant Microalgae *Dunaliella*. *J. Appl. Glycosci.*, **44**, 515-522 (1997).

Hizukuri, S., Fujii, M., and Nikuni, Z. : Effect of temperature during germination on the crystal type of starch in soybean seedlings. *Stärke*, **17**, 40-43 (1965).

Hizukuri, S. : *Denpun Kogyo Gakkaishi*, **17**, 73-88 (1969).

Jeanfils, J. and Collard F.: Effect of immobilizing *Scenedesmus obliquus* cells in a matrix on oxygen evolution and fluorescence properties. *Eur. J. Appl. Microbiol. Biotechnol.*, **17**, 254-257 (1983).

Joint Committee on Powder Diffraction Standards, Set 5, No. 453, International Center for Diffraction Data, Swarthmore, PA (1986).

Kakefuda, G., and Duke, S. H.: Characterization of pea chloroplast D-enzyme (4- β -D-Glucanotransferase). *Plant Physiol.*, **91**, 136-143 (1989).

Kayano, H., Matsunaga, T., Karube, I., and Suzuki S.: Hydrogen evolution by

co-immobilized *Chlorella vulgaris* and *Clostridium butyricum* cells. *Biochim. Biophys. Acta.*, **638**, 80-85 (1981).

Kombrink, E., and Wober G.: Identification and subcellular localization of starch-metabolizing enzymes in green alga *Dunaliella marina*. *Planta.*, **149**, 130-137 (1980).

Kometani, T., Yoshii, H., Takeuchi, Y., and Matsuno, R.: Large-scale preparation of R-1,2-propanediol through baker's yeast-mediated bioreduction. *J. Ferment. Bioeng.*, **76**, 414-415 (1993).

Kometani, T., Morita, Y., Furui, H., Yoshii, H., and Matsuno R.: NAD(P)H regeneration using ethanol as an energy source in baker's yeast-mediated bioreduction. *J. Ferment. Bioeng.*, **77**, 13-16 (1994).

Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 211-214 (1976).

Lee, E. Y. C. and Whelan, W. J.: In *The Enzymes* (Boyer, P. D. ed) ed. 3, vol. 5, Academic Press, New York, pp.191-234 (1974).

LeGoffic, F., Sicsic, S., and Vincent, C.: A two-step synthesis of new water-soluble polymers of NAD⁺ and ADP. *Eur. J. Biochem.*, **108**, 143-148(1980).

Levene, P. A. and Walti, A.: (-)-Propylene glycol. *Org. Synth. Coll.*, **2**, 545-547 (1943).

Levi, C. and Gibbs, M.: Starch degradation in synchronously grown *Chlamydomonas reinhardtii* and characterization of the amylase. *Plant Physiol.*, **74**, 459-463 (1984).

Lin, T-P. and Preiss, J.: Characterization of D-enzyme (4- β -D-Glucanotransferase) in *Arabidopsis* leaf. *Plant Physiol.*, **86**, 260-265 (1988).

Linden, J. C., Tanner, W., and Kandler, O.: Properties of glucosyltransferase and glucan transferase from spinach. *Plant Physiol.*, **54**, 752-757 (1974).

Lowry, G.W., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275 (1951).

MacKinny, G.: Absorption of light by chlorophyll solutions. *J. Biol. Chem.*, **140**, 315-322 (1941).

Maddelein, M. L., Libessart, N., Bellanger, F., Delure, B., D'Hulst, C., Van den Koornhuyse, N. Fontain, T., Wierusezeski, J. M., Decq, A., and Ball, S.: Toward an

understanding of the biogenesis of the starch granule: Determination of granule-bound and soluble starch synthase functions in amylopectin synthesis. *J. Biol. Chem.*, **269**, 25150-25157 (1994).

Manners, D. J., and Rowe, K. L.: Studies on carbohydrate-metabolizing enzymes 21. The β -glucosidase and D-enzyme activity of extract of carrot and tomatoes. *Carbohydrate Res.*, **9**, 441-450 (1969).

Matsuda, Y., Yamasaki, A., Saito, T., and Yamaguchi, T.: Purification and characterization of cell wall lytic enzyme released by mating gametes of *Chlamydomonas reinhardtii* *FEBS Let.*, **166**, 294-297 (1984).

Matsuda, Y., Yamasaki, A., Yamaguchi, T., and Kawase, H.: Cell wall lytic enzyme released by gametes of *Chlamydomonas reinhardtii* is a metalloprotease and digests the sodium perchlorate-insoluble component of cell wall. *J. Biol. Chem.*, **260**, 6373-6377 (1985).

Muramatsu, M., Urabe, I., Yamada, Y., and Okada, H.: Synthesis and kinetic properties of a new NAD⁺ derivative carrying a vinyl group. *Eur. J. Biochem.*, **80**, 111-117 (1977).

Nakamura, Y. and Miyachi, S.: Effect of temperature on starch degradation in *Chlorella vulgaris* 11h cells. *Plant and Cell Physiol.*, **23**, 333-341 (1982).

Nelson, N. A photometric adaptation of Somogyi method for the determination of glucose. *J. Biol. Chem.* **153**, 375-380. (1944).

Ochiai, H., Shibata, H., Sawa, Y., and Katoh, T.: "Living electrode" as long-lived photoconverter for biophotolysis of water. *Proc. Natl. Acad. Sci. USA*, **77**, 2442-2444 (1980).

Oliveira, L., Bisalputra, T., and Antia, N. J.: Ultrastructural observation of the surface coat of *Dunaliella tertiolecta* from staining with cationic dyes and enzyme treatments. *New phytol.*, **85**, 385-392 (1980).

Palmer, T. N. Ryman, B. E. and Whelan, W. J.: The action pattern of amylomaltase from *Escherichia coli*. *Eur. J. Biochem.*, **69**, 105-115 (1976).

Pazur, J. H., and Okada, S.: The isolation and mode of action of a bacterial glucanosyltransferase. *J. Biol. Chem.*, **243**, 4732-4738 (1969).

Peat, S., Whelan, W. J., and Rees, W. R.: The enzymic synthesis and degradation of starch, part 20, the disproportionating enzyme of potato. *J. Chem. Soc.*, 44 (1956).

Peavy, D. G., Smith, R. A., and Latorella, A. H.: *In* Algal biomass technologies : An Interdisciplinary perspective., **Barclay, W. and McIntosh, R.** eds J. CRAMER, Berlin-Stuttgart, pp56-59 (1986).

Reynolds, E. S.: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.*, **17**, 208 (1963).

Robinson, D. G. and Schlösser, U. G.: Cell wall regeneration by protoplasts of *Chlamydomonas*. *Planta* , **141**, 83-92 (1978).

Robinson, P. K., Dainty, A. L., Goulding, K. H., Simpkins, I., and Trevan M. D.: Physiology of alginate-immobilized *Chlorella*. *Enzyme Microb. Technol.*, **7**, 212-216 (1985).

Robinson, P. K., Mak, A. L., and Trevan, M. D.: Immobilized algae: a review. *Proc. Biochem.*, 122-127 (1986).

Simon, R. and Latorella, A., H.: Approaches to the genetics of *Dunaliella*. *In* W. Barclay and R. McIntosh (Eds) Algal biomass technology: An interdisciplinary perspective. J. Cramer, Berlin-Stuttgart, pp 15-20 (1986).

Shiotsubo, T. and Takahashi, K.: *Agric. Biol. Chem.*, **48**, 9-17 (1984).

Shneyour, A. and Avron M.: A method for producing, selecting, and isolating photosynthetic mutants of *Euglena gracilis*. *Plant Physiol.*, **55**, 142-144 (1975).

Suzuki, A., Kaneyama, M., Abe, J., and Hizukuri, S. : Relationship between temperature for retrogradation and the gelatinization temperature of the retrograded soluble starch. *J. Jpn. Soc. Starch Sci.*, **34**, 286-291 (1987).

Sze, P.: *In* A biology of algae, pp2-16, Wm. C. Brown Publishers Dubuque, Iowa (1986).

Van den Koornhuysen, N. Libessart, N., Delure, B., Zabawinski, C., Decq, A., Iglesias, A., Carton, preiss, A. J., and Ball, S. : Control of starch composition and structure through substrate supply in the monocellular alga *Chlamidomonas reinhardtii*. *J. Biol. Chem.*, **271**, 16281-16287 (1996).

Van Den Hoek, C., Mann, D. G., and Jahns, H. M.: *In* Algae: An introduction to phycology. Cambridge University Press, New York, pp16-34 (1995).

Wikström, P., Szwajcer, E., Brodelius, P., Nilsson, K., and Mosbach, K.: Formation of α -keto acids from amino acids using immobilized bacteria and algae. *Biotechnol. Lett.*, **4**,

153-158 (1982).

Yamada, T. and Sakaguchi, K.: Protoplast induction of *Chlorella* species. *Agric. Biol. Chem.*, **45**, 1905-1909 (1981).

Yamazaki, Y. and Suzuki, H.: A new method of chemical modification of N⁶-amino group in adenine nucleotides with formaldehyde and a thiol and its application to preparing immobilized ADP and ATP. *Eur. J. Biochem.*, **92**, 197-207 (1978).

Takaha, T. Yanase, M. Okada, S. and Smith, S. M.: Disproportionating enzyme (4- β -Glucanotransferase; EC 2.4.1.25) of potato. *J. Biol. Chem.*, **268**, 1391-1396 (1993).

Teodoresco, E. C. Organisation de développement du *Dunalilla* nouveau genre de Volvocacée-Polyblepharidée. *Botanisches Zentralblatt, Beihefte*, **18**, 215-232 (1905).

Thepenier, C. and Guđin, C.: Immobilization of *Porphyridium cruentum* in polyurethane foams for production of polysaccaride. *Biomass*, **7**, 225-240 (1985).

Thomasset, B., Thomasset, Th., Vejux, A., Jeanfils, J., Barbotin, J. N., and Thomas, D.: Immobilized thylakoids in cross-linked albumin matrix. *Plant Physiol.* **70**, 714-723 (1982).

Walker, G. J.: A transglucosylase of *Streptococcus bovis*. *Biochem. J.*, **94**, 299-308 (1965).

Yoshio, N. Maeda, I. Taniguchi, H. and Nakamura, M.: Purification and properties of D-enzyme from malted barley (in Japanese). *J. Jpn. Soc. Starch Sci.*, **33**, 244-252. (1986).