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A Unique Feature of Hydrogen Recovery in Endogenous Starch-to-Alcohol Fermentation of the Marine Microalga, *Chlamydomonas perigranulata*

KOYU HON-NAMI

Engineering R&D Division, Tokyo Electric Power Co. Ltd., 1-1-3 Uchisaiwai-cho, Chiyoda-ku, Tokyo 100-8560, Japan, E-mail: koyu.honnami@tepco.co.jp

Abstract

A unicellular marine green alga, *Chlamydomonas perigranulata*, was demonstrated to synthesize starch through photosynthesis, store it in a cell, and ferment it under anaerobic conditions in the dark to produce ethanol, 2,3-butanediol (butanediol), acetic acid, and carbon dioxide (CO_2). Previous fermentation data of an algal biomass cultivated outdoors in a 50-L tubular photo-bioreactor showed good carbon (C) recovery in the fermentation balance, with a higher ratio to alcohols and, therefore, lower ratio to CO_2 in the C distribution of products than what would be expected from the Embden-Myerhof-Parnas pathway. These findings led to a proposed concept for a CO_2-ethanol conversion system (CDECS). The above data were evaluated in terms of hydrogen (H) recovery with the following results: C recovery at 105% was well balanced, although H recovery was as high as 139%, meaning an additional gain of H through fermentation. This finding was reproduced wholly in a set of experiments carried out in the same month of the following year, October, whereas another set of experiments was carried out in the following June provided ordinary fermentation results in terms of C and H recoveries with poor growth. Further analyses of these data revealed that butanediol is equal to ethanol as a product from a putative conversion system from CO_2 to the detected fermentation products, leading to the revision of the CDECS concept to a CO_2-alcohol conversion system (CDACS). The relevance of the CDACS will be discussed in relation to the cultivation conditions employed by chance.

Index Entries: Marine green microalga; *Chlamydomonas perigranulata*; endogenous starch fermentation; fuel alcohol; hydrogen recovery.

Introduction

Global warming and climate change are topics of world concern; one of their causes is recognized to be anthropogenic increases in the atmospheric levels of greenhouse gases, including carbon dioxide (CO_2) derived from fossil fuels through combustion. The reduction of such combustion and

expansion of the CO_2 sink are essential principal measures to take in addition to the direct separation and sequestration of CO_2 at emission sites. An effective approach to mitigating climate change is the subject of debate *(1)*; biological measures, including the utilization microalgae, have been proposed as possible options *(2–4)*, and efforts to discover new organisms *(5)*, even genetic materials *(6)*, that might be of value for carbon sequestration and the synthesis of renewable fuels are continuing.

Among renewable fuels, bio-ethanol produced by fermenting microorganisms such as yeast, followed by distillation and dehydration if necessary, has gained wide use as an alternative motor fuel and has recently been the subject of increased attention, mainly because of unstable oil prices and the conviction to produce a major movable hydrogen source for fuel cells in the future *(7)*. In general, ethanol fermentation by yeast results in a carbon loss of one-third, as carbohydrate is converted to ethanol through enzymatic processes known as the Embden-Meyerhof-Parnas pathway. We showed previously that *Chlamydomonas perigranulata*, a unicellular marine green alga *(8)*, converts endogenous carbohydrate, intracellular starch stored through photosynthesis to valuable substances including fuels and chemicals such as ethanol and 2,3-butanediol (butanediol) *(9–11)*. In this fermentation, it was found that the whole metabolic reaction provides about 40% more alcohols and results in a higher recovery of carbon than expected from the consumed starch, a result that led us to propose the concept of a CO_2-ethanol conversion system (CDECS) *(11)*.

This article describes an evaluation of the previous results in terms of hydrogen (H) recovery in the fermentation balance, and presents a revised proposal for a CO_2-alcohol conversion system (CDACS). Cultivation conditions will be discussed in relation to the CDACS and the resultant efficient carbon (C) recovery in alcohol and an unexpected gain in hydrogen.

Materials and Methods

Microalga

The microalga used in this study was *C. perigranulata (8)*, which was isolated from a water sample obtained at Yambu, Saudi Arabia, and on the Red Sea.

Microalga Cultivation

The culture medium and growth conditions were described previously *(10)*. F/2 medium *(12,13)* was used with a slight modification: the concentrations of nitrate and phosphate were enriched four times. The F/2 medium contained (/L) 75 mg $NaNO_3$; 5 mg $NaH_2PO4{\cdot}2H_2O$; 1 mL mineral solution; 1 mL vitamin solution; an artificial seawater agent, 16 g instant ocean; 22 g NaCl. The mineral solution concentrations (g/L) were

as follows: 0.18 $MnCl_2 \cdot 4H_2O$; 0.022 $ZnSO_4 \cdot 5H_2O$; 0.01 $CuSO_4 \cdot 5H_2O$; 0.006 $Na_2MoO_4 \cdot 2H_2O$; 0.01 $CoCl_2 \cdot 6H_2O$; 3.15 $FeCl_3(6H_2O$; 4.36 Na_2EDTA. The stock vitamin solution contained (mg/mL), 0.1 thiamine-HCl; 0.005 biotin; 0.0005 vitamin B12. The artificial seawater agent, Instant Ocean, was purchased from Aquarium Systems, Inc., Mentor, Ohio. Cells were pre-cultured in a 10-L carboy (working volume of 8 L) and supplied with 1% CO_2 in air at a rate of 300 mL/min. Illumination was provided by a fluorescence lamp, which surrounded the carboy and gave a light intensity of about 7 kLx at the outer surface of the vessel set in a room in which the temperature was controlled at 25°C. After about 7 d of culture, an algal concentration of 0.5 g/L was attained and used for inoculation.

Outdoor Cultivation in a 50-L Scale Tubular Bioreactor

Outdoor cultivations of the microalga were carried out between July and October, 1996 *(10)*, and between June and October, 1997, at the Hiroshima lab of Mitsubishi Heavy Industries, Ltd., in Hiroshima. Cultivation conditions were as described in previous papers *(10,11)*.

The F/2 medium was used with further modification for outdoor cultivation; the concentrations of nutrients, including nitrate, phosphate, metals, and vitamins, were enriched 8 times. A working volume of 50 L was used in a 53-L air-lifted tubular bioreactor. Tap water was used to prepare the medium after filtration through polypropylene-wound cartridge filters (TWC-1N-PPS, Advantec Tokyo Ltd., Tokyo). The reactor was sterilized with sodium hypochlorite solution (10 g/L) by overnight circulation before use *(14)*. Cultivation was started by the inoculation of 16 L of a preculture (about 30% of the working volume). Air containing 1.8% CO_2 was supplied at 1.7 L/min, which drove the medium circulation at a rate of 0.3 m/s. Illumination and temperature employed for cultivation were natural except for spraying water on the tubes if necessary to keep the temperature under 35°C. Illumination by sunlight was measured with a digital illuminometer (IM-3, Topkon Corporation, Tokyo). The reactor system consisted of a polyacryl tubular bioreactor, air compressor, flow meters for CO_2 and air, and separate temperature sensors for the culture medium and the atmosphere. The reactor specifications are as follows: two horizontal tubes (length of 2000 mm) and two vertical tubes (height of 3880 mm) with an inside diameter (ID) of 70 mm and an outside diameter of 76 mm, a tank (140 mm ID and 450 mm long), and a gas injection port. Cultures were harvested by centrifugation in a rotor (Qn rotor, Kokusan Co. Ltd., Tokyo) at 10 k rpm (9100*g*) and a flow rate of about 1 L/min. This system can yield about 100 g of cells in 50 L culture medium within 1 h. The packed cells were transferred immediately to a vessel as described later for fermentation.

Fermentation in the Dark

The fermentation procedures were described previously *(10,11)*. Cells were suspended in artificial sea water or an appropriate buffer to a suitable

concentration (150–200 g of cells/L). The air was removed by flushing with N_2 (1 L/min) at the start of fermentation. The microalgal slurry was maintained automatically at 25°C in a water bath and at pH 7.0 by intermittent additions of 2*M* NaOH solution coupled with a pH sensor and a controller that moved a U-shaped vane at a constant 60 rpm. The vane's shaft was made of polytetrafluor-ethylene (70-mm long and 10-mm wide for the vertical part and 70-mm long and 15-mm wide for the horizontal part, 5 mm in thickness). The fermentation apparatus consisted of a fermenter, a cooler for volatile substances, and a gas collector (Tedlar Bag for 1 L, Iuchi Seieido Ltd., Osaka). The fermentation vessel (a cylindrical glass flask with a flat bottom, No 0582-500, Shibata Scientific Technology Ltd., Tokyo) had an inner diameter of 85 mm and a height of 115 mm with a removable cover with four necks (No 0580-4), a mechanical-rotation drive and automatic pH-controlling systems.

Analyses

The analytical procedures undertaken and their conditions were described previously *(10,11)*. Starch in cells was measured by coupled methods with perchloric acid treatment *(15)*: glucose produced by hydrolysis by perchloric acid was determined using a Glucose CII-test kit (Wako Pure Chemical Industries Ltd., Osaka). Ethanol, butanediol, and gas concentrations were analyzed by gas chromatography on a Shimadzu (Shimadzu Corporation, Kyoto, Japan) GC-8A instrument equipped with a data processor (Chromatopac C-R1A), a flame ionization detector (FID) and a thermal conductivity detector. For alcohol determination, a glass column (ID 3.2 × 2.1 m) of PEG-20M 20% Uniport HP 60/80 (GL Sciences Ltd., Tokyo) was used. The column temperature was maintained at 200°C, the carrier gas was N_2, and the flow rate was 35 mL/min. For gas measurements, a SUS column (ID 1.4 × 1.8 m) of WG-100 (GL Sciences Ltd., Tokyo) was used at a column temperature of 50°C, with N_2 as the carrier gas at a flow rate of 35 mL/min. The concentrations of organic acids, including acetate and lactate, in the fermented microalgal slurry were measured by gas chromatography on a Shimadzu GC-9A instrument equipped with a data processor (Chromatopac C-R3A), a column (ID 3.2 × 2.6 m) of Thermon 3000 (Shinwa Chemical Industries Ltd., Kyoto) and a FID. The column temperature was maintained at 160°C, the carrier gas was N_2, and the flow rate was 35 mL/min. Inorganic carbon was determined by a total organic carbon analyzer *(16)* using a Shimadzu TOC-5000 instrument. The sample was treated at 150°C and CO_2 produced was measured. Nitrate concentration in the culture medium was determined by the brucine absorption method *(17)*.

Calculations

The calculations employed in this study were based on the literature *(18)*: carbon recovery is defined as the ratio of mol of C in the fermentative end products to the mol of C in the starch metabolized; hydrogen recovery

Table 1
Summary of the Outdoor Cultivation of *C. perigranulata* and the Subsequent Fermentation Volume

Cultivation series #	T33[a]	T35	T38
Cultivation date	Oct 18–30,1996	Jun 4–17,1997	Oct 7–20,1997
Concentration of biomass harvested (g/L)	1.53	0.55	1.55
Biomass harvested (g)	79	27	77
Starch content (%)	35	25	28
Fermentation volume (mL)	437	138	350

[a](*See* ref. *10*)

is defined as the ratio of molecule of available H in the fermentative end products to the molecule of available H in the starch metabolized, in which the molecule of available H in a compound with a general formula of CaHnOz is calculated as $4a + n - 2z$; the O/R index is defined as the ratio of the weighed sum of the O/R values of the oxidized end products to the weighed sum of the O/R values of the reduced end products, in which the O/R value of a compound with general formula of CaHnOz is calculated as $z - n/2$.

Chemicals

All chemicals used in this study were reagent grade unless otherwise stated. Ethanol and 2,3-butanediol were purchased from Wako Pure Chemical Industries, Ltd., Osaka.

Results and Discussion

Re-Evaluation of Previous Fermentation Results

A summary of the outdoor cultivation of *C. perigranulata* reported previously *(10)* is listed in Table 1 with the results of similar cultivations carried out the following year. The fermentation results as described in a previous paper *(10,11)* are evaluated in terms of H recovery in the fermentation balance in addition to C recovery, as listed in Table 2. The data show that C recovery was almost balanced (105%), while H recovery was significantly higher (139%), and leaning toward the product side, with more H gained during fermentation, which is also shown by the O/R index being as low as 0.23. These findings suggest that some reducing power was added to a metabolite(s) to lead to the production of more alcohols, and that, on the whole, the fermentation products recovered were more reduced than the substrate in the case of this alga.

Revision of the CO_2–Ethanol Conversion System

In the CDECS mentioned previously, only CO_2 derived from the original butanediol fermentation pathway was counted tentatively as source

Table 2
Endogenous Starch Fermentation of *C. perigranulata* and Recovery of Carbon and Hydrogen

Product	T33[a] mmol	T33[a] mmol C	T33[a] mmol H	T35 mmol	T35 mmol C	T35 mmol H	T38 mmol	T38 mmol C	T38 mmol H
Starch stored	170	1022		45	268		131	784	
Starch consumed	–134	–807(100)	–3216(100)	–41	–246(100)	–984(100)	–102	–614(100)	–2448(100)
Ethanol	209	418(52)	2508	52.5	105(43)	624	187	375(61)	2244
Butanediol	88	352(44)	1936	19.5	78(32)	418	48	191(31)	1056
Acetate	2	4(0)	16	2	4(2)	16	16	32(5)	128
CO_2	43	77[b](10)	–	88	88(36)	–	44	44(7)	–
Inorganic C	33	–	–	0	–	–	0	–	–
Total C analyzed		851(105)			275(112)			642(105)	
Total H analyzed		4460(139)			1058(108)			3428(140)	
O/R index		0.23			1.09			0.17	

[a]A part of data was reported previously (refs. *10, 11*).
[b]Including inorganic C.

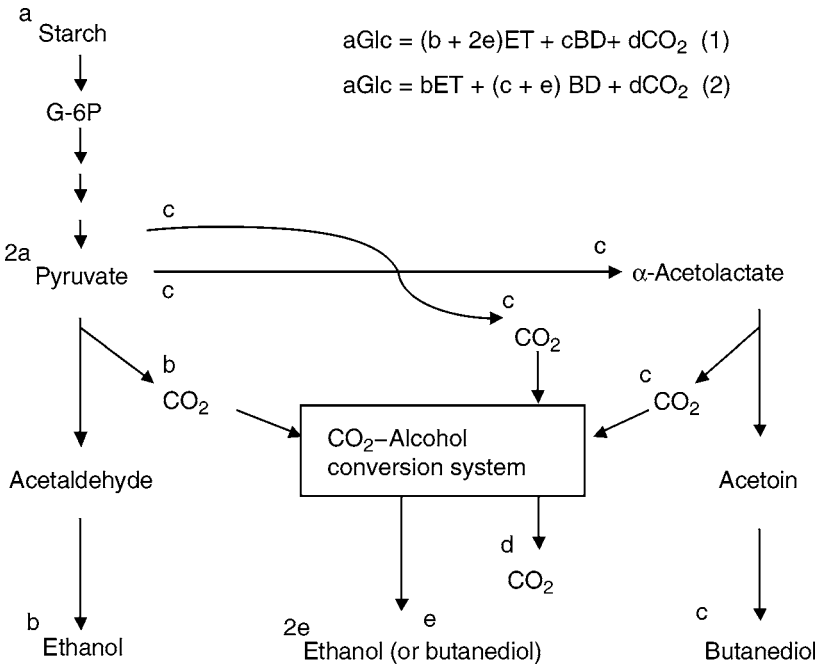

Fig. 1. A possible endogenous starch fermentation pathway in *C. perigranulata* and the CDACS. Molar amounts of substances in the pathway are presented as a–e for starch, ethanol, 2,3-butanediol, CO_2, and 2,3-butanediol produced through the CDACS, respectively. This figure indicates that alcohol obtainable from the CDACS is either ethanol or 2,3-butanediol. Equations 1 and 2 represent the stoichiometry for each case of ethanol and 2,3-butanediol as a product of the CDACS, respectively. Abbreviations: G-6P, glucose-6 phosphate; Glc, glucose; ET, ethanol; and BD, 2,3-butanediol. For other abbreviations *see* the text.

for the additional formation of ethanol through CDECS in order to explain the stoichiometry of the above novel fermentation, a higher ratio of alcohols to CO_2 than expected on the basis of the Embden-Meyerhof-Parnas pathway. In this article the CDECS is revised as follows along with its name. CO_2, which is formed at the decarboxylation step not only in the butanediol pathway, but also in the original ethanol pathway, is converted to ethanol and/or butanediol through a CDACS, as shown in Fig. 1. The revised system takes into account the fact that butanediol should not be excluded as an additional product produced through the CDACS, and that, therefore, this alcohol is equal to ethanol as a product through a putative CO_2 conversion system. CDECS is one type of such a putative CO_2 conversion system.

Considering a possible metabolic pathway from starch to alcohol via pyruvate and CDACS, acetic acid is assumed to be the same compound as ethanol, which was also a case with the previous CDECS. The basis of the above assumption is as follows: acetic acid (1) is a minor product in this

fermentation; (2) is equivalent to ethanol in C number as well as in basic chemical structure, although different in redox level; and (3) has the same metabolic pathway until just before the final step in a series of reactions in yeast fermentation, which is probably also the case in this alga, because pyruvate decarboxylase activity has been demonstrated in vitro under anaerobic and dark conditions *(9)*, and so the final step from acetaldehyde to ethanol would be catalyzed by this enzyme as in yeast.

Stoichiometry in the Fermentation of the Microalgae

Some features of the fermentation results, including product pattern and the distribution of C and H among products, are listed in Table 2. The conversion pathway in the CDACS is explained in the following three cases, the formation of ethanol only, the formation butanediol only, and the simultaneous formation of both ethanol and butanediol.

Possible Ethanol Formation in the CDACS

On the basis of the determined amount of substrate and the products listed in Table 3 and shown in Fig. 2A, the validity of the possible pathway and CDACS is described as follows: assuming that all of the 88 mmol of butanediol detected was formed through the original fermentation pathway from pyruvate to butanediol via α-acetolactate, the original ethanol pathway will yield as much as 93 (= 269 – 176) mmol ethanol, an amount that is estimated by subtracting the 176 mmol pyruvate used for butanediol formation from the 269 mmol pyruvate metabolized by glycolysis derived through starch consumption. As 211 mmol ethanol was detected, the additional amount of ethanol required is 118 (= 211 – 93) mmol, which is obtained by subtracting the above mentioned 93 mmol ethanol from the 211 mmol ethanol that was determined. On the other hand, if the CDACS is active and converts CO_2 to ethanol, the expected amount of ethanol will be as much as 96 mmol, which corresponds to 192 (= 269 – 77) mmol as carbon; the latter is estimated by subtracting the detected 77 mmol CO_2 from the 269 mmol CO_2 that is expected to be formed through both the original ethanol and butanediol fermentation pathways.

The above amounts of 118 and 96 mmol ethanol were obtained by subtracting the expected amount of ethanol, which is obtained if no more butanediol is formed through the CDACS, from the amount of ethanol actually determined, and by subtracting the amount of CO_2 actually determined from the expected amount of CO_2 derived through each original pathway. It should be mentioned that the former amount was derived through product side estimation, and the latter amount through substrate side estimation. The difference, 22 mmol ethanol, is as much as 44 mmol in carbon equivalents, which corresponds to the difference in the moles carbon in the total fermentation products and the starch consumed as substrate (Table 2). The extra ethanol equivalent to 44 mmol C probably arose

Table 3
Endogenous Starch Fermentation and Its Products Via the CDACS

	Assumed product from CO_2			
	(Cultivation T33)		(Cultivation T38)	
Product (mmol)	Ethanol	Butanediol[a]	Ethanol	Butanediol[a]
Starch consumed	–134	–134	–102	–102
Ethanol[b]				
Observed	211	211	203	203
Produced from acetaldehyde(A)	93	211	109	203
Produced from CO_2 (B)	96	N/A	80.5	N/A
Calculated (A + B)	189	N/A	189.5	N/A
Calculated and observed difference	22	N/A	13.5	N/A
Butanediol[a]				
Observed	88	88	48	48
Produced from acetoin(C)	88	29	48	1
Produced from CO_2 (D)	N/A	48	N/A	40
Calculated(C + D)	N/A	77	N/A	41
Calculated and observed difference	N/A	11	N/A	7
CO_2	77[c]	77[c]	44	44

[a]2,3-butanediol.
[b]Including acetic acid.
[c]Including inorganic C.

from starch derivatives, such as intermediates that comprise the glycolysis and fermentation pathway from glucose to alcohol already present before the start of the fermentation experiment, including starch measurement. It can be concluded, therefore, that the CDACS is active.

Possible Butanediol Formation Via the CDACS

In the same manner, the validity of the above pathway is described for butanediol as a product formed via the CDACS, as shown in Table 3 and Fig. 2B. In this case the amount of butanediol required is more 59 (= 88 –29) mmol more than that obtained by subtracting 29 mmol butanediol from 88 mmol butanediol. The expected amount of butanediol from the CDACS, on the other hand, is as much as 48 mmol. The difference, 11 mmol butanediol, is again equivalent to as much as 44 mmol C, which indicates that butanediol production provides an alternative situation to the case of ethanol production as discussed in the previous section. Therefore, CDACS is also demonstrated to be active, and butanediol can also be expected as a product of the CDACS.

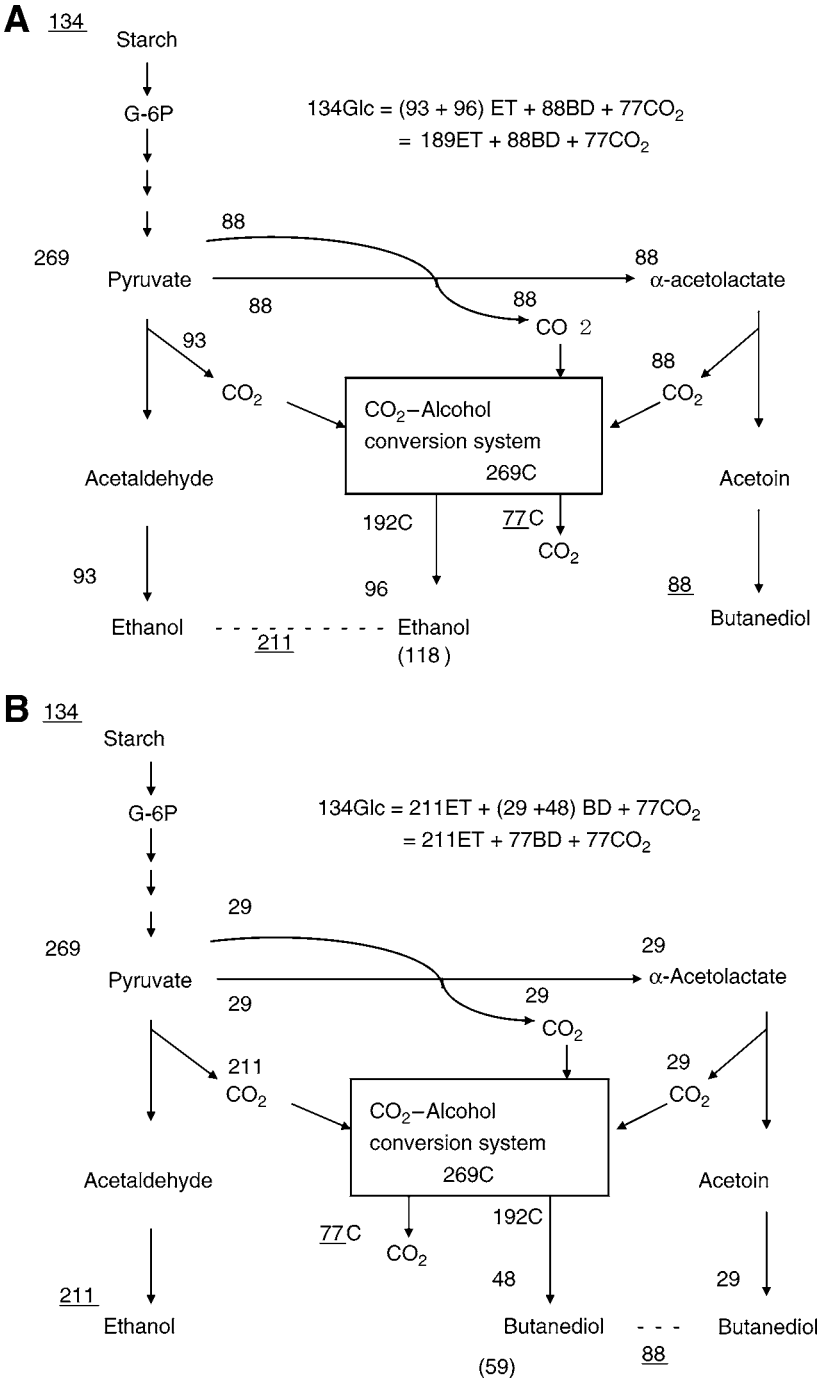

Fig. 2. Analysis of C flux in a possible endogenous starch fermentation pathway and CDACS in *C. perigranulata* cultivation T33. **(A)** All 2,3-butanediol analyzed is assumed to have been formed through the original 2,3-butanediol pathway shown in Fig. 1, and, therefore, extra CO_2 results in ethanol formation through CDACS. **(B)** All ethanol analyzed, in reverse, is also assumed to have been formed through the

Simultaneous Formation of Ethanol and 2,3-Butanediol via the CDACS

It should be mentioned that the simultaneous formation of ethanol and butanediol by the CDACS is highly probable. Ethanol formed via the CDACS is coupled with its formation in the original fermentation pathway as well as butanediol formation in both the original pathway and CDACS. Therefore, for example, a decrease in ethanol formed by the CDACS could be complemented by an increase in ethanol formed by the original pathway, which would lead to a decrease in the amount of butanediol formed by the original pathway and a compensating increase in the amount of butanediol formed in the CDACS. The above compensation would still range between 96 mmol and none for ethanol, and between none and 48 mmol for butanediol.

Reproduced Cultivation and Fermentation With Confirmation of the CDACS Concept

The following year, several sets of the experiments involving outdoor cultivation and subsequent fermentation were again carried out during the period from June to October, and their typical results are listed in Tables 1 and 2 and shown in Fig. 3. In the case of microalgal cells obtained in October (T38), the fermentation results reproduced almost completely the results obtained the previous year with a high C recovery and a significantly higher H recovery (140%), thus confirming the CDACS concept. The CDACS is assumed to account for 80.5 mmol ethanol or 40 mmol butanediol, a difference of as much as 13.5 mmol ethanol or 7 mmol butanediol from those analyzed on the product side (Table 3, Fig. 3).

Poor Cultivation and Resultant Inactive CDACS

Another set of experiments carried out in June, 1998 (T35), on the other hand, failed to reproduce the above results (Tables 1 and 2, and Fig. 4). In these experiments, the cultivation results were very poor: the biomass concentration at the stationary phase of growth was only about one-third that observed in the case of excellent growths, as in T33 and T38, and, therefore, the resultant biomass obtained was also only one-third of that obtained in T33 and T38 with a lower starch content (Table 1).

With respect to the fermentation results, as listed in Tables 2 and 4 and shown in Fig. 4, the C and H recoveries were 112% and 108%, respectively, values very close to each other. The O/R index was 1.09 (Table 2), and evolved CO_2 corresponded to one-third of the total mol C of the

Fig. 2. ***(Continued)*** original ethanol pathway shown in Fig. 1, and, therefore, extra CO_2 results in 2,3-butanediol formation through the CDACS. Numerical values represent the amount of each compound in mmol; values that are underlined and in parentheses represent analyzed values and the amounts required to conform with the analyzed values, respectively. For explanations, *see* Fig. 1.

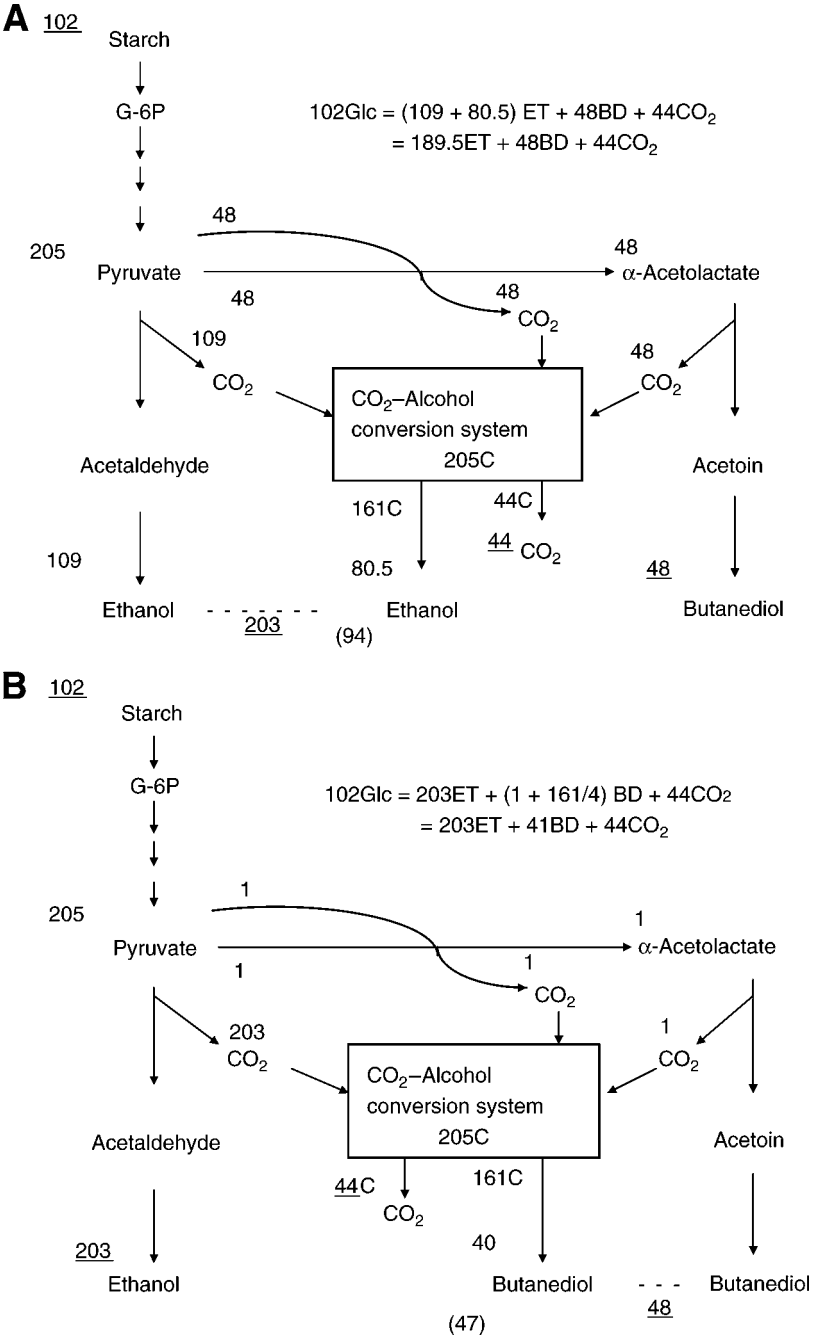

Fig. 3. Analysis of C flux in a possible endogenous starch fermentation pathway and CDACS in *C. perigranulata* cultivation T38. For explanations, *see* Figs. 1 and 2.

products. Taken together, although the C and H recoveries in this fermentation were increased as much as about 10% in stoichiometry, the fermentation itself in this case seems to have proceeded according to only the

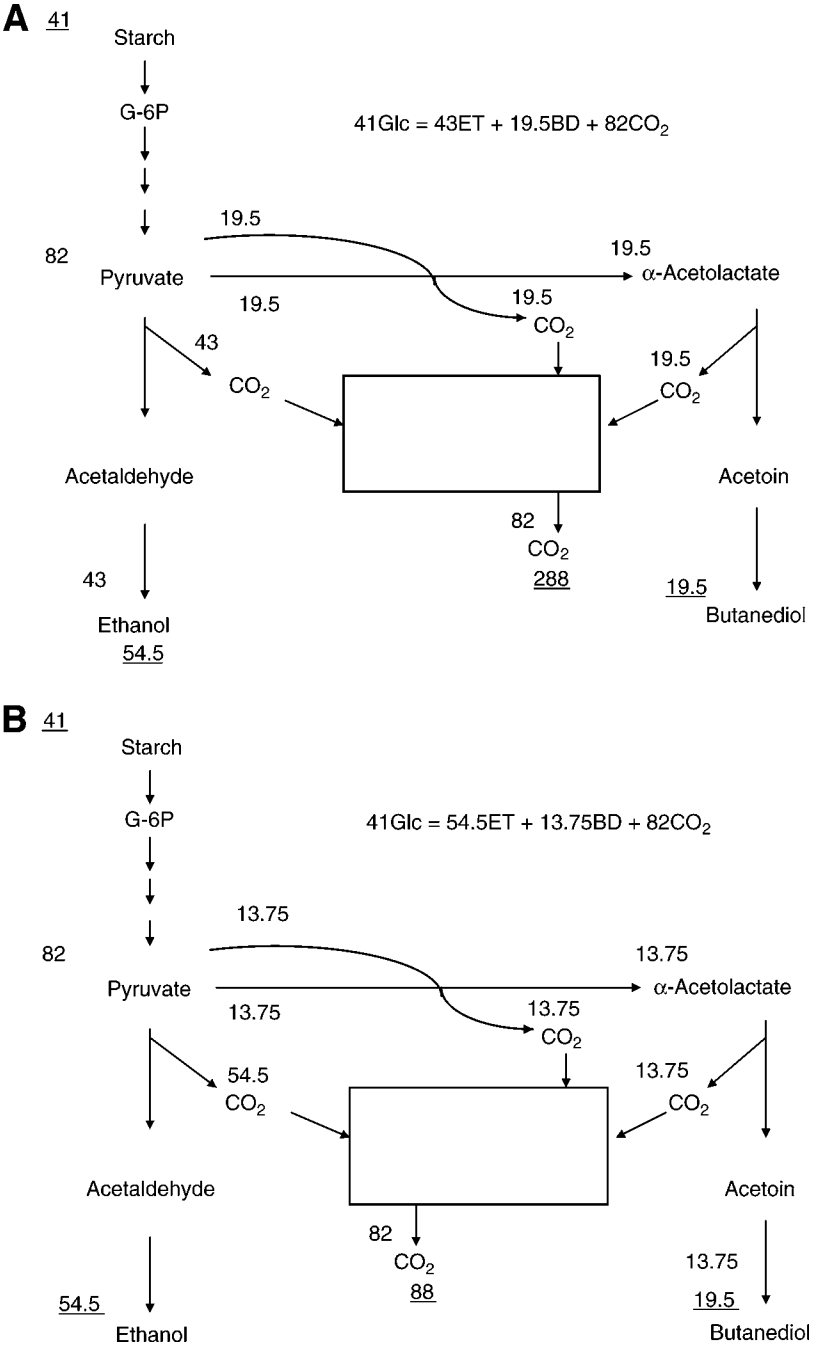

Fig. 4. Analysis of C flux in a possible endogenous starch fermentation pathway of *C. perigranulata* cultivation T35, a case where the CDACS was inactive and, therefore, no alcohol could come out through the CDACS. For explanations, *see* Figs. 1 and 2.

Embden-Meyerhof-Parnas pathway, which means the CDACS is not needed to explain the fermentation results.

Table 4
Endogenous Starch Fermentation and Its Products When the CDACS Is Inactive (Cultivation T35)

Product[a] (mmol)	Butanediol[b]	Ethanol
Starch consumed	−41	−41
Ethanol		
Observed	54.5[c]	54.5[c]
Produced from acetaldehyde	43	54.5
Calculated and observed difference	11.5	0
Butanediol[b]		
Observed	19.5	19.5
Produced from acetoin	19.5	13.75
Calculated and observed difference	0	5.75
CO_2		
Produced from pyruvate to acetaldehyde and from pyruvate to acetoin	82	82
Calculated and observed difference	6	6

[a]Product: Observed amount of the product was assumed to be obtained through its original metabolic pathway.
[b]2,3-butanediol.
[c]Including acetic acid.

On the basis of the data in Fig. 4A, the validity of the CDACS being inactive is described as follows. In this system, according to Embden-Meyerhof-Parnas pathway, 41 mmol glucose-equivalents of starch were metabolized to 82 mmol pyruvate as an intermediate and converted finally to ethanol, butanediol, and CO_2. The resultant CO_2 formed is expected to equal the amount of that of pyruvate metabolized and to be as much as 82 mmol. The measured amount of CO_2, however, was 88 mmol, just slightly more than expected. Therefore, no additional CO_2 needed to enter the CDACS to be turned into alcohol. With respect to the amount of alcohol produced, assuming all of the 19.5 mmol butanediol determined was formed through the original pathway, the original ethanol pathway will produce 43 (= 82 (19.5 × 2) mmol ethanol, which is the amount obtained by subtracting 19.5 mmol pyruvate twice, the amount of butanediol determined from the 82 mmol pyruvate formed through glycolysis of the starch consumed. As 54.5 mmol ethanol was found, there was an ethanol shortage of as much as 11.5 (= 54.5 (43) mmol.

Taken together, because 41 mmol starch was estimated to be consumed as the starting substrate and 19.5 mmol butanediol was formed, 11.5 mmol ethanol, and 6 mmol CO_2 were missing, which corresponds to 29 mmol C, the amount of the difference between the substrate and total products (Table 4). Therefore, owing to the shortage of CO_2 in the fermentation

system employing cultured T35, it is apparent that CO_2 did not enter the CDACS and be transformed into ethanol. The extra CO_2 and ethanol probably derived from metabolic intermediates that existed prior to the start of the fermentation experiments.

In a similar manner, the inactivity of the CDACS is confirmed by the other case in which the 54.5 mmol ethanol detected is assumed to have been formed through its original metabolic pathway (Fig. 4B). In this case, because 82 mmol pyruvate was considered to be metabolized and because 54.5 mmol ethanol was also considered to be formed, 5.75 mmol butanediol and 6 mmol CO_2, equivalent to 29 mmol C, were lacking as listed in Table 4. Because of a lack of CO_2 to enter the CDACS, the conversion of CO_2 to butanediol through the CDACS can be excluded. The extra CO_2 and butanediol probably derived from metabolic intermediates that existed prior to the start of the fermentation experiments. In conclusion, poor cultivation and subsequent fermentation resulted in the CDACS remaining inactive.

Conditions for CDACS to Be Active

In this section, conditions under which the CDACS becomes active are discussed in term of the cultivation and fermentation conditions. Typical profiles of the outdoor cultivation of *C. perigranulata* are shown in Fig. 5. Both the T33 and T38 cultivations were carried out in the same season in different years and resulted in excellent growth and fermentation. They showed the following features in common:

1. sufficient and stable illumination by sunlight;
2. biomass concentration reaching more than 1.5 g/L at the stationary phase of growth;
3. high-starch content near 30%;
4. relatively wide cultivation temperature from 30°C to 5°C; and
5. complete nitrate consumption and further cultivation for several days under nitrate starvation conditions.

The cultivation of T35, on the other hand, in which the CDACS was judged to be inactive, showed the following marked features:

1. biomass concentration of the medium at harvest as low as 0.5 g/L, about 1/3 of that observed for the excellent growth cases;
2. lower starch content of 25%; and
3. a narrow range of cultivation temperature relative to T33 and T38, which is owing to the temperature at night being higher at around 20°C.

The resultant failure of growth in the cultivation of T35 may be as a result of the strong illumination sunlight (over 200 Ly/d) during the initial phase of growth, although the illumination and cultivation temperature data for 3 d during the early stages of cultivation (5–7 June) could not be

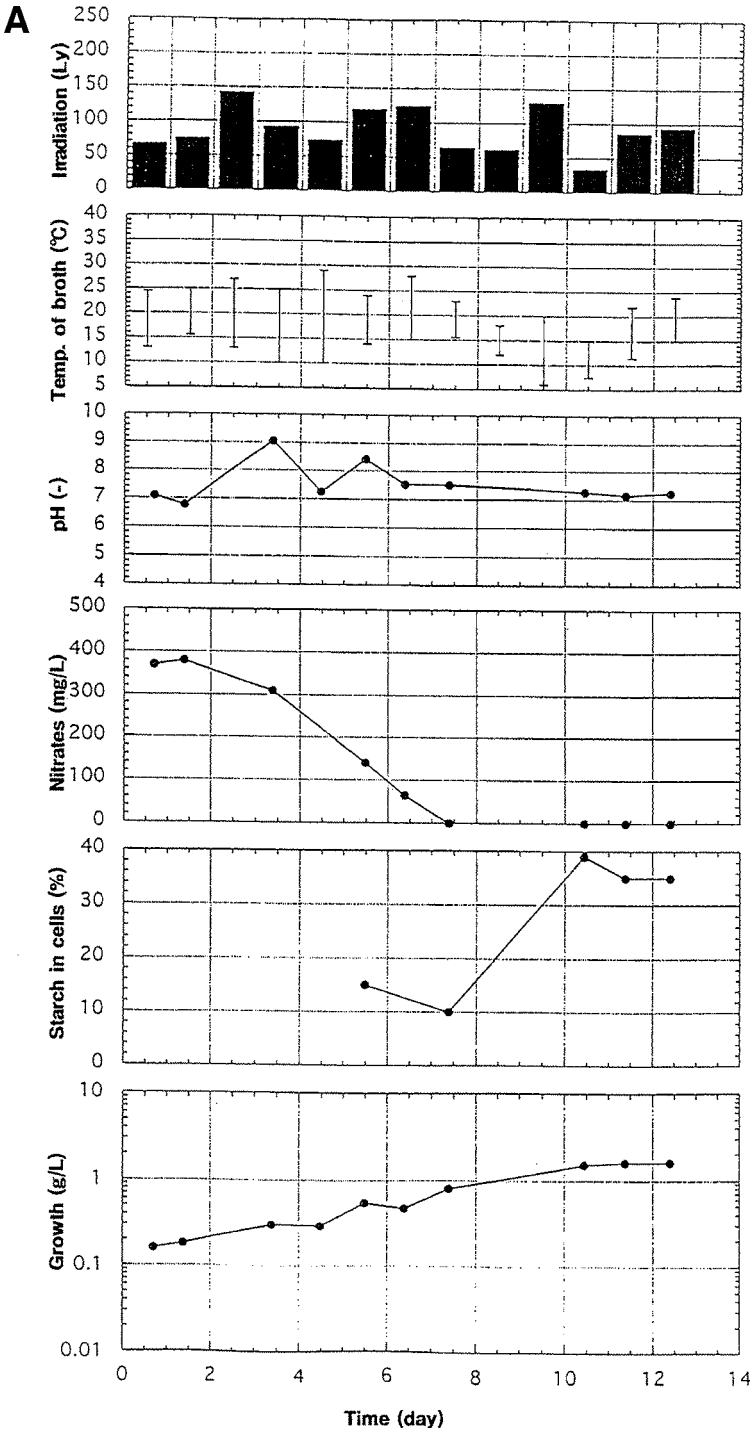

Fig. 5. Time courses of *C. perigranulata* growth and its parameters during outdoor cultivation. **(A)**, **(B)**, and **(C)** are cultivating series numbers T33, T35, and T38, respectively (*see* Table 1). Some results **(A)** have been reported previously *(10)*. In **(B)**, data for the illumination and cultivation temperature for 3 d in the early stages of cultivation (June 5–7) could not be obtained as a result of technical circumstances.

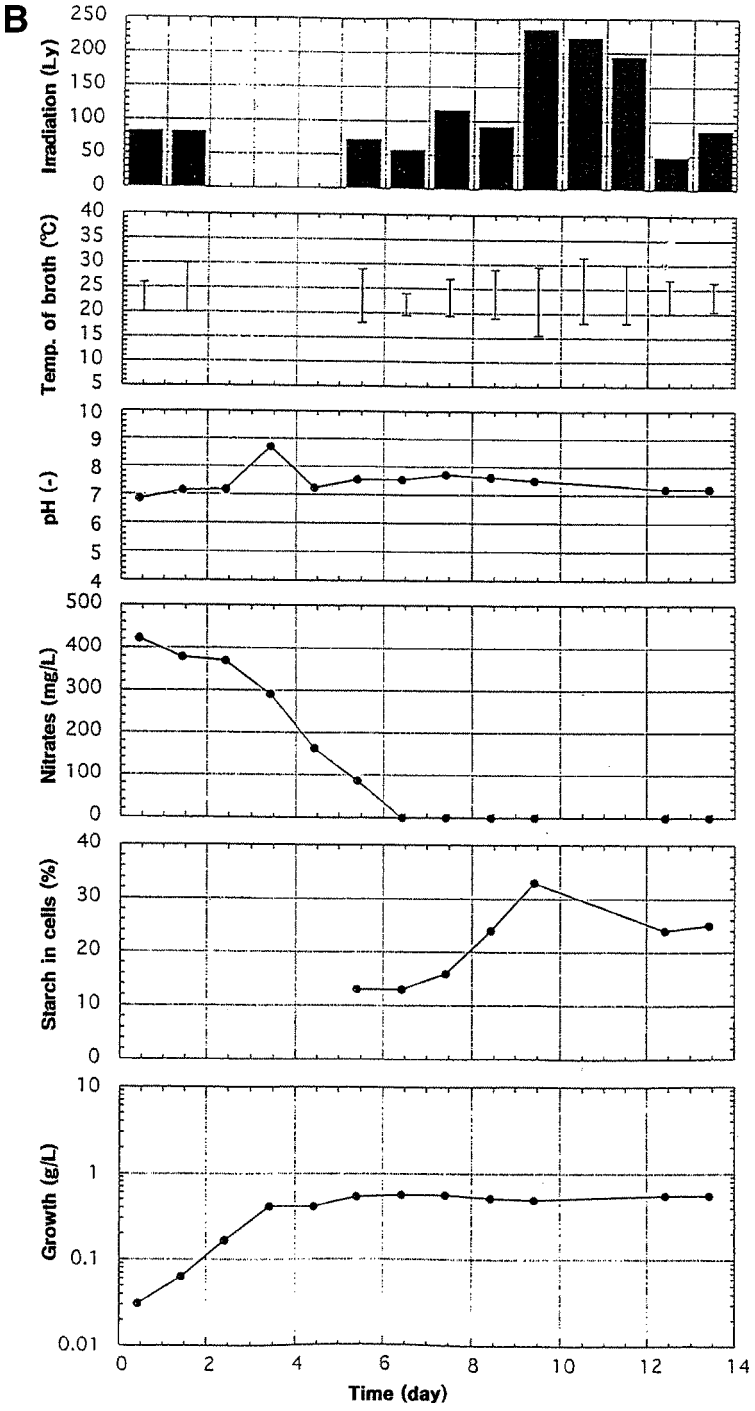

Fig. 5 ***(Continued)***

obtained due to some technical difficulties. The former data were estimated to be 220, 120, and 250 Ly/d, respectively, on the basis of the values observed and reported at the Hiroshima Meteorological Observatory.

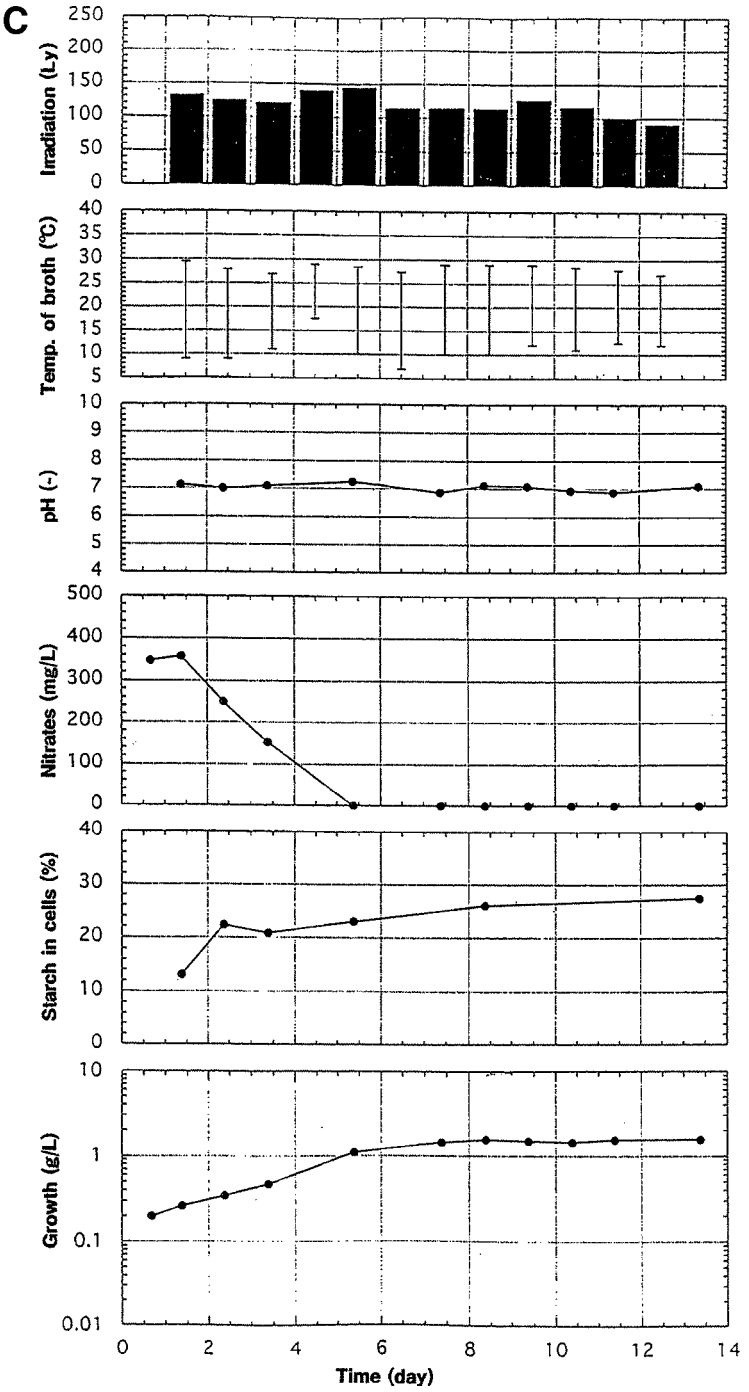

Fig. 5 ***(Continued)***

With respect to the fermentation conditions, on the other hand, the algal slurry concentration employed for all three lots was adjusted to about 20%,

and the volume of each fermentation mixture varied depending on the biomass obtained (Table 1) with no significant effects on the CDACS activity.

Taken together, some cultivation conditions are considered to be essential for the CDACS to be active. The remarkable differences between the excellent cases (T33 and T38) and the poor case (T35) offer suggestions as to favorable cultivation conditions:

1. stable sunlight illumination and excellent growth; and
2. a temperature range of the medium during cultivation varying from 30°C to 5°C.

Therefore, potential conditions under which the CDACS is active are considered to be as follows:

1. excellent growth under stable illumination; and
2. a culturing temperature providing suitable level of warmth during the daytime accompanied by cool at nights; the latter, especially, should be required during the stationary phase of growth under nitrate starvation.

It is well known that during the stationary phase of growth, continued cultivation and starvation of some nutrients leads to an increase in starch content *(19)*. If the night temperature is high enough to consume the accumulated starch, algal cells will begin to degrade it without any reducing power being accumulated. The CDACS is, therefore, considered to be no longer active. In conclusion, the accumulation of sufficient amounts of starch and reducing power are needed for the CDACS to be active. In order to maintain these accumulations in the culture medium during periods of low illumination, the temperatures should be maintained as low as possible.

Possible Metabolic Pathway of Alcohol Fermentation Via the CDACS

As described previously, the refixation of CO_2 formed through alcohol fermentation and its further conversion to alcohol are proposed in Fig. 1. In the previous proposal of the CDECS, a formic acid derivative was presented as a candidate for the initial product of CO_2 fixation *(11)*; here, another possibility using the existing photosynthetic system may be pointed out, a part of this photosynthetic system, especially the CO_2 fixation process including Rubisco, would be expected to work even under anaerobic and dark conditions. In addition, a possibility using the existing central carbon metabolic pathway and alcohol fermentation pathways should be also pointed out for the CDACS, after its fixation, CO_2 would be introduced into the central carbon metabolic pathway as an intermediate, and, finally, some of it would be converted to alcohol.

CO_2 fixation by photosynthesis stands always on a background of enriched energy by ATP storage and reducing power such as NADPH, reduced Fd and reduced a state of the Fd/thioredoxin system *(20,21)*. These substances or states in this microalga could also be produced and

stored by photosynthesis during cultivation, especially during nitrate starvation where no proliferation can proceed *(22)*.

Recently, the refixation and utilization of formed CO_2 through lipid formation have been reported in green rape seeds under conditions of some illumination *(23)*. The CDACS that is active in this microalga is also a novel example of the re-fixation and utilization of formed CO_2, and it should be pointed out that this alga performs them under anaerobic and dark conditions. It is an important model system with which to elucidate the mechanisms of effective C and H utilization because it involves single cells and the reaction occurs after photosynthesis and can be separated from the light reaction of photosynthesis. Finally, it should be noted that this algal fermentation system was demonstrated to produce lactic acid as an additional product under some conditions of cultivation and fermentation *(24)*.

In conclusion, the potential for microalgae to contribute to CO2 mitigation might be higher than currently expected. Recently, a large report of a microalgae utilization R&D project undertaken over almost two decades has appeared *(25)*. In this report, it is noted that the use of some microalgal species results in the production of biodiesel, an alternative fossil fuel has the potential to become competitive depending on current petroleum costs. This present study is similar in that, although biodiesel was not produced, the bio-alcohol products are renewable. Many issues, including the physiology and applicability to mitigation remain to be elucidated *(3)*.

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References

1 Claussen, E. (2004), *Science*, **306,** 816.
2 Benemann, J. R. (2001), In: *Photosynthetic Microorganisms in Environmental Biotechnoloy*, Kojima, H. and Lee, Y. K., eds., Springer-Verlag, Hong Kong, pp. 1–10.
3 Pedroni, P., Davison, J., Beckert, H., Bergman, P., and Benemann, J. (2001), *J. Energy Environ. Res.* **1,** 136–150.
4 Hon-Nami, K. (2001), In: *Photosynthetic Microorganisms in Environmental Biotechnoloy.* Kojima, H. and Lee, Y. K., eds., Springer-Verlag, Hong Kong, pp. 291–310.
5 Smith, H. O., Friedman, R., and Venter, J. C. (2003), *The BRIDGE*, Summer, pp. 36–40; available at http://www.princeton.edu/~seasplan/lifesciences/NAE%20Bridge.pdf. Accessed date: Feb. 23, 2004.
6 Venter, J. C., Remington, K., Heidelberg, J., (2004), *Science*, **304,** 66–77.
7 Deluga, G. A., Salge, J. R., Schmidt, L. D., and Verykios, X. E. (2004), *Science* **303,** 993–997.

8 Hirayama, S., Ueda, R Nakayama, T., and Inou1ye, I. (2001), *Botanica Marina* **44,** 41–46.
9 Hirayama, S., Ueda, R., Ogushi, Y., Hirano, A., Hon-Nami, K., and Kunito, S. (1997), In: *Proceedings of the Annual Meeting of the Japanese Society for Marine Biotechnology*, Tokyo, June, pp. 78 (in Japanese).
10 Hirano, A., Samejima, Y., Hon-Nami, K., et al. (1997), In: *Making Business from Biomass in Energy, Environment, Chemicals, Fibers and Materials*, Overend, R. P. and Chornet E., eds., Pergamon, New York, pp. 1069–1076.
11 Hon-Nami, K., Hirano, A., Samejima, Y., et al. (1998), In: *Biomass for Energy and Industry*, Kopetz, H., Weber, T., Palz, W., Chartier, P., and Ferrero, G. L., eds., C.A.R.M.E.M., Rimpar, pp 602–605.
12 Ong, L. J., Glazer, A. N., and Waterbury, J. B. (1984), *Science* **224,** 80–83.
13 Castenholz, R. W. (1988), *Methods Enzymol.* **167,** 68–92.
14 Tatewaki, M. (1979), In: *Sourui-Kenkyu-Hou*, Nishizawa, K. and Chihara, M. eds., Kyoritsu Shuppan Ltd., Tokyo, pp. 69–87 (in Japanese).
15 Ohta, S., Miyamoto, K., and Miura, Y. (1987), *Plant Physiol.* **83,** 1022–1026.
16 JIS K0102 22 (1996), In: *JIS Handobukku Kankyousokutei*, Japanese Standard Association, Tokyo (in Japanese), pp. 1083–1085.
17 JIS K0102 43. 2. 4 (1996), In: *JIS Handobukku Kankyousokutei*, Japanese Standard Association, Tokyo (in Japanese), pp. 159.
18 Gfeller, R. P. and Gibbs, M. (1984), *Plant Physiol.* **75,** 212–218.
19 Martin, M. C. and Goodenough, U. W. (1975), *J. Cell Biol.* **67,** 587–605.
20 Buchanan, B. B. (1991), *Arch. Biochem. Biophys.* **288,** 1–9.
21 Huppe, H. C., Farr, T. J., and Turpin, D. H. (1994), *Plant Physiol.* **105,** 1043–1048.
22 Poolman, M. G., Fell, D. A., and Raines, C. A. (2003), *Eur. J. Biochem.* **270,** 430–439.
23 Schwender, J., Goffman, F., Ohlrogge, J. B., and Shachar-Hill, Y. (2004), *Nature* **432,** 779–782.
24 Hon-Nami, K. (2004), In: *Biotechnoloy of Lignocellulose Degradation and Biomass Utilization*, Ohmiya, K., Sakka, K., Karita, S., Kimura, T., Sakka, M., and Ohnishi, Y., eds., Uni Publishers Co., Ltd., Tokyo, pp. 746–754.
25 Sheehan, J., Dunahay, T., Benemann, J., and Roessler, P. (1998), *A look back at the US Department of Energy's aquatic species program-biodiesel from algae.* Prepared by the National Renewable Energy Laboratory, A national laboratory of the U.S. Department of Energy operated by Midwest Research Institute, Under Contract No. DE-AC36-83CH10093. http://205.168.79.26/docs/legosti/fy98/24190.pdf. Accessed date: Oct. 21, 2005.