

Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions

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Abstract

Chlorella pyrenoidosa was cultivated under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. The influence of light on the carbon and energy metabolism of microalgae was investigated by the use of metabolic flux analysis. The respiratory activity of microalgae in the light was assessed from the autotrophic flux distribution. Results showed that the glycolytic pathway, tricarboxylic acid cycle and mitochondrial oxidative phosphorylation maintained high activities during illumination, indicating little effect of light on these pathways, while the flux through the pentose phosphate pathway during illumination was very small due to the light-mediated regulation. The theoretical yields of biomass on ATP decreased in the following order: heterotrophic culture > mixotrophic culture > autotrophic culture, and a significant amount of the available ATP was required for maintenance processes in microalgal cells. The energy conversion efficiency between the supplied energy to culture, the absorbed energy by cells and the free energy conserved in ATP were analyzed for the different cultures. Analysis showed that the heterotrophic culture generated more ATP from the supplied energy than the autotrophic and mixotrophic cultures. The maximum thermodynamic efficiency of ATP production from the absorbed energy, which was calculated from the metabolic fluxes at zero growth rate, was the highest in the heterotrophic culture and as low as 16% in the autotrophic culture. By evaluating the energy economy through the energy utilization efficiency, it was found that the biomass yield on the supplied energy was the lowest in the autotrophic cultivation, and the cyclic culture gave the most efficient utilization of energy for biomass production. © 2000 Elsevier Science S.A. All rights reserved.

Keywords: Microalgal cells; Metabolic flux; Central metabolism; Energetics

1. Introduction

Microalgal cultures have received much attention in recent years because of their potential application for industrial CO₂ removal, production of many valuable metabolites, and life support in space [1–3]. There have been extensive studies on the effect of medium composition, illumination technique and various photobioreactors on the growth and photosynthetic rates of microalgae [4–6]. However, little is known about the metabolism of cultured microalgal cells. In order to improve the performance of microalgal cultures, a good understanding of the carbon and energy metabolism in microalgal cells is needed. In addition, a deeper understanding of the metabolic mechanism of microalgal cells may also help to investigate the metabolism inside the plant

cells, because there are many similarities between microalgae and plant cells. In fact, there are many distinct features about the microalgal cell metabolism comparing with other microorganisms. Microalgae can perform oxygenic photosynthesis and fix carbon dioxide through Calvin cycle like plant cells. That is, microalgal cells can trap light energy as the energy source and assimilate CO₂ as the carbon source. Moreover, organic substrates can also be utilized as the carbon and energy sources by many microalgae [7]. Therefore, by varying the nature of carbon and energy sources, the different underlying metabolic status of cells, especially the influence of light on the carbon and energy metabolism, can be elucidated.

Some models have been proposed for the description of the growth of algal and plant cells, but in these models the cells were seen as a black box for which only consumption rates and production rates are considered [8,9]. Since this is too limited an approach for a deeper understanding of

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Nomenclature	
C_b	biomass concentration (g/l)
E_s	total light energy supplied to the reactor (W)
F_{vol}	photon flux absorbed by the culture ($\mu\text{mol}/\text{m}^3/\text{s}$)
I_{av}	average irradiance inside the culture ($\mu\text{mol}/\text{m}^2/\text{s}$)
I_0	irradiance in the center of photobioreactor filled with water ($\mu\text{mol}/\text{m}^2/\text{s}$)
I_s	incident light intensity on the surface of reactor ($\mu\text{mol}/\text{m}^2/\text{s}$)
K_a	biomass absorption coefficient (m^2/kg)
R	reactor radius (m)
S_A	illuminated surface area (m^2)
X_p	total pigment content (mass fraction)
Y_{SE}	biomass yield on the supplied energy to culture (g/kJ)
$Y_{ATP/AE}^{\max}$	maximum thermodynamic efficiency of ATP formation from the absorbed energy by cells

metabolism, metabolic flux analysis was applied in this study to quantitatively assess intracellular fluxes through the main metabolic pathways of microalgal cells [10,11].

The performance of microalgal culture systems can be evaluated and compared through the efficiency with which the supplied energy to the culture can be utilized for biomass production [12]. In order to improve the energy utilization efficiency of the culture, it is desired to obtain a fundamental understanding of the energy conversion from the supplied energy to biomass formation, i.e. how the microalgal cells harvest light or chemical energy from the environment and then convert them into ATP, the universal currency of free energy for cell growth. However, so far there have been few studies on this subject. The elucidation of the carbon and energy metabolism of microalgae from the metabolic flux distributions can provide a basis for the investigation of the energy utilization efficiency when cells are grown on different energy sources.

In the present study, *Chlorella pyrenoidosa* C-212 was cultivated under autotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. Metabolic flux analysis was applied to elucidate the metabolism of microalgae grown on different carbon and energy sources. Of particular interest is the effect of light on the carbon and energy metabolism. Based on the results of flux distribution, the energy conversion from the supplied energy to biomass formation were investigated in autotrophic, heterotrophic and mixotrophic cultures. The energy economy of the autotrophic, mixotrophic and cyclic autotrophic/heterotrophic cultivations were also evaluated through the energy utilization efficiency.

2. Materials and methods

2.1. Microorganism and culture conditions

The microalga *Chlorella pyrenoidosa* C-212 (renamed as *Chlorella sorokiniana* recently) was obtained from the collection of the Institute of Applied Microbiology, University of Tokyo, Japan. The mineral culture medium used was the M-8 medium according to Mandalam and Palsson [4]. Glucose was added to the culture medium under heterotrophic and mixotrophic conditions, and glucose concentration in the medium was controlled at about 5 g/l, because it has been suggested that this concentration did not exert any effect on the photosynthetic activity of *Chlorella* cultured mixotrophically [13].

Cells were activated by inoculating a loop of a slant culture into 100 ml of M-8 medium and pre-cultivating them under continuous illumination at 5% CO_2 balanced with air at 37°C for 24 h. A circular cool white 30 W fluorescent lamp (National FCL32EX-D/30, Osaka) was used as the light source. Agitation was provided by a suspended magnetic stirbar.

The main cultivation was performed in 21 jar fermentor (M-100, Rikakikai, Tokyo) with a working volume of 1.51 and a diameter of 0.135 m. The cells were grown at a constant temperature of 37°C. Aeration was achieved by sparging air enriched with 5% CO_2 through a ring sparger at 0.4 vvm. The culture was agitated at a speed of 200 rpm. The pH value of the medium was monitored using a pH sensor (Y31J-PH2-1, Tokyo Rikakikai Co., Ltd, Tokyo). A CO_2/O_2 gas analyzer (LX-750, Iijima Electronics MFC Co. Ltd, Tokyo) was used to measure the carbon dioxide/oxygen concentrations in the exhaust gas. The cells were illuminated by four circular cool white 32 W fluorescent lamps (a light intensity of 500 $\mu\text{mol}/\text{m}^2/\text{s}$ at the surface of fermentor). For cyclic light-autotrophic/dark-heterotrophic cultivation, cells were cultured under continuous illumination for 48 h and then subjected to 12 h of darkness followed by 12 h of continuous illumination (a light intensity of 500 $\mu\text{mol}/\text{m}^2/\text{s}$ at the surface of fermentor). The fermentor was wrapped with aluminum foil during the night. The total light energy supplied to the reactor, E_s , was estimated by Eq. (1), as suggested by Ogbonna et al. [14].

$$E_s = 0.2176 I_s S_A \quad (1)$$

where I_s is the incident light intensity and S_A is the illuminated surface area.

2.2. Analytical methods

For the determination of cell dry weight, duplicate samples of the culture were washed with 0.5 M HCl, rinsed with distilled water and dried overnight at 85°C. The amounts of glucose and nitrate in the culture medium were determined with a glucose kit (Wako Pure Chemicals, Osaka)

and a nitrate kit (Boehringer Mannheim Chemicals, Tokyo), respectively.

The pigment content of total lipids was obtained by summing chlorophyll (a and b) and the carotenoid mass fractions. Chlorophylls and carotenoid were determined after extraction with 100% methanol at 4°C for 30 min, and the extracts analyzed spectrophotometrically [15]. Total lipids were extracted with chloroform/methanol and weighed according to Piorreck et al. [16].

To determine the protein content, cells were washed and dried as described for the biomass dry weight determination and ground with a mortar and pestle, then extracted with 0.1 N NaOH for 60 min at 80°C. The amount of protein in cell extracts was determined according to Lowry et al. [17] using bovine serum albumin as a standard.

For determination of intracellular carbohydrate and starch concentration, the biomass was washed, dried and ground as described above, then extracted with distilled water. The total carbohydrate content in the suspension of cell extracts was measured using phenol sulfuric acid method [18]. One part of the cell extracts was centrifuged for starch determination. The supernatant was decanted and the pellet was washed, resuspended in distilled water, heated in boiling water for 30 min in order to solubilize the starch. The suspension was cooled down to room temperature and the starch content was analyzed with an enzyme kit (Boehringer Mannheim Chemicals, Tokyo).

2.3. Metabolic flux analysis

Fig. 1 shows the metabolic networks for autotrophic, heterotrophic and mixotrophic cultures. The relevant reactions are listed in Appendix A. The manner in which the networks have been obtained is described below.

Algae cells can use light as the energy source. Light quanta absorbed by pigments drives the photosynthetic electron transport, which results in the reduction of NADP⁺ and couples to formation of ATP. It has been concluded that the P/2e⁻ ratio (the number of ATP molecules formed per pair of electrons moving through the photosynthetic electron transport chain) is about 1.3 [19]. According to the widely accepted two-step model of photosynthesis, 8 mol quanta of light is required to evolve 1 mol O₂. Therefore, the photosynthetic O₂ evolution rate can be determined from the photon flux absorbed throughout the reactor volume, F_{vol} , which was estimated using the following equations proposed by Molina Grima et al. [20].

$$F_{vol} = I_{av} K_a C_b \quad (2)$$

$$I_{av} = \frac{2I_0}{RK_a C_b \pi} \times \left(1 - \int_0^{\pi/2} \cos(\phi) \exp[-2RK_a C_b \cos(\phi)] d\phi \right) \quad (3)$$

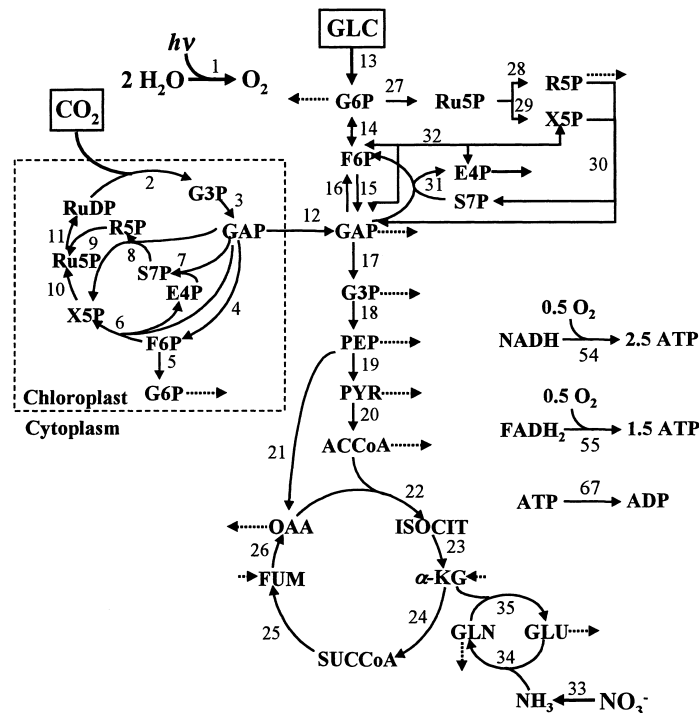


Fig. 1. Central reaction network for microalgae metabolism. Numbers correspond to the reactions shown in Appendix A. The dotted lines represent fluxes for cell mass synthesis.

where I_{av} is the average irradiance inside the culture, I_0 the irradiance measured in the center of the photobioreactor filled with water, C_b the biomass concentration, R the reactor radius. The biomass absorption coefficient, K_a , of the culture, as a function of the total pigment content, X_p , was determined through the following relation [21].

$$K_a = 10.5 + 2990X_p \quad (4)$$

NADPH and ATP formed by the action of light then reduce CO_2 by a series of dark reactions called the Calvin cycle. The first step in the Calvin cycle, photosynthetic CO_2 fixation, is catalyzed by ribulose 1,5-bisphosphate carboxylase. This enzyme is also an oxygenase, which can react with O_2 and lead to a different pathway called photorespiration. Algae have the photorespiration pathway, and photosynthesis is inhibited by high O_2 concentration. However, under normal conditions CO_2 loss by algal photorespiration is minimal and not readily measured [22]. Because the experiments in this study were carried out in a high level of CO_2 (5%), the photorespiration pathway was not included in the metabolic network.

Photosynthesis reactions, including the light reactions, Calvin cycle and starch synthesis, are located in chloroplast. Glyceraldehyde phosphate (GAP) is withdrawn from Calvin cycle and exported to cytoplasm for consumption. Although the alga and plant cells have a high degree of subcellular compartmentation of metabolism, compartmentation of most metabolites between chloroplast, mitochondria, and cytoplasm was not taken into account in this study because the extent to which biosynthetic reactions are localized in chloroplast in algae cells is not understood or fully established until now [23,24].

After the export of GAP from chloroplast to cytoplasm, the flow of carbon is divided to synthesis of sugars or oxidation through the glycolytic pathway to pyruvate. Sugars including sucrose are the major storage products in the cytoplasm of plant cells. In addition, the structural carbohydrates, such as cell wall components, are considered to be synthesized in cytoplasm. Some researchers found that alga cell fed [$1-^{14}C$] glucose yielded starch in which glucose was still predominantly labeled in position 1 and the amount of glucose that remained unchanged was about 70% [25]. This suggested that glucose could be directly converted to starch without prior conversion to GAP and then uptake by the chloroplast. Therefore, in the network of mixotrophic metabolism, one part of the exogenous glucose was directly converted to starch, and the remainder was oxidized through glycolytic pathway.

In plant cells, replenishment of carbon to maintain the operation of the TCA cycle is achieved by anaplerotic reactions involving CO_2 fixation by PEP carboxylase [26]. Pentose phosphate (PP) pathway has been reported to operate in the cytoplasm at the same time as the Calvin cycle is functioning in the chloroplast [27,28]. In the metabolic networks for autotrophic and mixotrophic cultures, only the PP pathway was considered to supply pentose phosphate for

nucleic acid synthesis and produce erythrose-4-phosphate (E4P) for the synthesis of shikimi acid, because the production of aromatic amino acids occurs in the cytoplasm and the transport of pentose phosphate out of the chloroplast is not possible. On the other hand, there has been evidence to prove that PP pathway may function in both cytoplasm and chloroplast in the dark in plant cells [29]. Therefore, both PP pathways located in different compartments were combined in the network of heterotrophic metabolism.

Nitrate is the predominant form of nitrogen available to most plants. Nitrate is reduced to ammonia before being available for assimilation. The reduction is divided into two steps: nitrate is reduced by a cytoplasmic NADH-dependent nitrate reductase to nitrite, which is further reduced to ammonia by a chloroplast-located NADPH-linked nitrite reductase [30]. Glutamate dehydrogenase (GDH) and glutamine synthetase (GS) are considered as the important entries of ammonia into organic form. If both reactions are included in the metabolic network, a singularity arises. However, more and more labeling experiments produced results consistent with the predominant operation of GS in alga cells [31]. Hence, only GS was included in this study, and the formed glutamine provided the nitrogen donor to α -ketoglutarate by the action of glutamate synthetase. GS/glutamate synthetase were considered as the reactions of ammonia assimilation in the metabolic networks.

There are three different mechanisms of electron transport in plant respiratory chain [32]. De Gucht and van der Plas [33] determined the activities of these pathways and calculated the P/O values for ATP production, which is about 2.5. Therefore, here it was assumed that the P/O ratios were 2.5 and 1.5 for NADH and $FADH_2$, respectively. It is not clear until now whether the enzyme nicotinamide nucleotide transhydrogenase, which catalyzes the reversible transfer of hydride ion between NAD and NADP, is present in plant cells. Some experiments demonstrated the presence of this enzyme but with a very low activity, so this reaction is not included in the metabolic networks [34].

Of all the pigments, *chlorophyll a* takes a major fraction, thus only the *chlorophyll a* formation is considered here. δ -Aminolevulinic acid (δ -ALA) is the key chlorophyll precursor molecular. δ -ALA may be formed from different routes. The classical succinate-glycine pathway is the condensation of glycine and succinyl-CoA catalyzed by δ -ALA synthetase. In addition, 5-carbon compounds, glutamate and α -ketoglutarate were found to be incorporated into δ -ALA much more efficiently than were glycine and succinate in many green cells. Since the incorporation of all the synthesis reactions of δ -ALA in the metabolic network leads to a singularity and C5 route was found to make a major contribution, C5 route is considered to be the only contributor for δ -ALA formation [35].

Eukaryotic algae and higher plants have a great variety of lipids. Since these cells contain chloroplast and have the biological function of photosynthesis, they have some unique lipids which are responsible for the characteristic features

Table 1
Reactions in the networks of three different metabolism

Autotrophic network	Heterotrophic network	Mixotrophic network
1, 2–11, 12, 14, 16–26, 27–32, 33–35, 36–53, 54–55, 56, 57, 60–62, 63, 66, 67	13–15, 17–26, 27–32, 33–35, 36–53, 54–55, 57, 60–62, 64, 66, 67	1, 2–11, 12, 13–15, 17–26, 27–32, 33–35, 36–53, 54–55, 57, 58, 59, 60–62, 65, 66, 67

of chloroplast membrane [36]. For the metabolic flux analysis all lipids but pigments were lumped into diacylglycerol (DG), which is the key precursor in the synthesis of triglyceride, phospholipids, galactolipids, and perhaps sulfolipids. Although most of the synthesis reactions of fatty acids occur in the chloroplast, the source of acetyl-CoA (AcCoA) derives from its synthesis in the mitochondria. It should be pointed out that the fatty acid composition of the lipids isolated from *Chlorella* cells grown under different conditions varies considerably, particularly for the α -linolenic acid (C18:3) content [37]. This leads to the difference in the synthetic reactions of DG in the three metabolic networks (see Appendix A).

The protein composition has been obtained from Yanagi et al. [38], and the composition of RNA and DNA have been given by Wanka et al. [39]. The amino acid compositions of protein are assumed to be constant under different cultivation conditions, since the reported composition values for different cultivation conditions are similar [40,41]. The nucleotide compositions of DNA and RNA are also assumed to be maintained under different growth conditions.

Three matrixes of stoichiometric coefficients were constructed using the reactions listed in Appendix A (Tables 1 and 2) and applied to calculate the metabolic fluxes under different culture conditions. The software, Fluxmap [42], was used for flux calculation.

3. Results and discussion

3.1. Cultivation of *C. pyrenoidosa* under different conditions

C. pyrenoidosa cells were growing in autotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic cultures. Figs. 2–4 show the cell growth, the cellular composition, the consumption of glucose and nitrate, and the rates of CO₂ production and O₂ uptake for these experiments. It was assumed that the contents of RNA and DNA were maintained at 5.5 and 0.4% for all cultures [43]. The experimental results for these cultures were summarized in Table 3. The autotrophic culture converted about 0.15 mol of CO₂ into biomass and evolved as much as 0.21 mol of O₂. Nevertheless, the increase in biomass resulted in a rapid reduction of cell growth rate due to light limitation, hence the final cell concentration achieved by autotrophic growth is very low. With the increase in cell density, the contents of protein, lipids and chlorophylls increased up to

maximum values, then declined gradually in the stationary growth phase, while the amount of carbohydrates decreased to a minimum value, and then accumulated to a high level. This result is in agreement with the variation of cellular ultrastructure reported by Hu et al. [44], who suggested this modification is characteristic of photoadaptation so as to optimize light harvesting and light utilization.

As expected for the mixotrophic and cyclic cultures, a significant improvement was observed in the biomass concentration, because the ability of *Chlorella* to grow on organic carbon sources was exploited in both cultures. On the other hand, the uptake of glucose also resulted in the consumption of O₂ and evolution of CO₂. The growth yields on glucose in the mixotrophic and cyclic cultures were close to each other. Provided light and organic carbon simultaneously as energy sources, the mixotrophic culture reached the maximum final cell concentration, but formed a much less content of chlorophyll in comparison to the autotrophic culture. From the time profiles of the cyclic culture, it was found that the subsequent light autotrophic cell growth was not adversely affected by the carbon source addition during the night, showing that *Chlorella* cells can swiftly switch from autotrophic to heterotrophic metabolism and vice versa.

3.2. Flux analysis

By cultivating cells under autotrophic, mixotrophic and cyclic autotrophic-heterotrophic cultures, it is possible to create different conditions for analyzing cell metabolism. Here metabolic flux analysis was applied to elucidate three different metabolism, autotrophic, heterotrophic and mixotrophic metabolism. Before flux analysis was carried out, the data were analyzed for the presence of measurement errors using elemental balances [45]. It was shown that the elemental balances for C and N could be closed and that no gross measurement errors were present.

Therefore, a flux analysis could be carried out using the data given in Figs. 2–4. Fig. 5 shows the estimated fluxes for autotrophic, heterotrophic and mixotrophic cultures. These values represent the flux distributions of the exponential growth phase during the autotrophic and mixotrophic cultivations and the first dark period during the cyclic autotrophic/heterotrophic cultivation. The specific growth rates were almost the same for all three systems. The flux values are expressed in mmol produced metabolites per gram cell per unit time (mmol/g/h).

Table 2
Metabolite vector^a

(1)	AcCoA	Acetyl-coenzyme A
(2)	AKG	α -Ketoglutarate
(3)	ALA	Alanine
(4)	APF	Absorbed light photon flux
(5)	ARG	Arginine
(6)	ASN	Asparagine
(7)	ATP	Adenosine-5'-triphosphate
(8)*	CAR	Carbohydrates
(9)	CYS	Cysteine
(10)	CHLO	Chlorophyll a
(11)	DNA	Deoxyribonucleic acid
(12)*	E4P	Erythrose-4-phosphate
(13)	E4P _{chl}	Erythrose-4-phosphate, chloroplast
(14)*	F6P	Fructose-6-phosphate
(15)	F6P _{chl}	Fructose-6-phosphate, chloroplast
(16)	FADH ₂	Flavin adenine dinucleotide, reduced
(17)	FUM	Fumarate
(18)*	G3P	3-Phosphoglycerate
(19)	G3P _{chl}	3-Phosphoglycerate, chloroplast
(20)*	G6P	Glucose-6-phosphate
(21)	G6P _{chl}	Glucose-6-phosphate, chloroplast
(22)*	GAP	Glyceraldehyde-3-phosphate
(23)	GAP _{chl}	Glyceraldehyde-3-phosphate, chloroplast
(24)	GLC	Glucose
(25)	GLN	Glutamine
(26)	GLU	Glutamate
(27)	GLY	Glycine
(28)	HIS	Histidine
(29)	ILE	Isoleucine
(30)	ISOCIT	Isocitrate
(31)	LEU	Leucine
(32)	LYS	Lysine
(33)	DG	1,2-Diacylglycerol
(34)	MET	Methionine
(35)	NADH	Nicotinamide adenine dinucleotide, reduced
(36)	NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
(37)	NH ₃	Ammonium
(38)	NO ₃	Nitrate
(39)	OAA	Oxaloacetate
(40)	PEP	Phosphoenolpyruvate
(41)	PHE	Phenylalanine
(42)	PRO	Proline
(43)	PROT	Protein
(44)*	R5P	Ribose-5-phosphate
(45)	R5P _{chl}	Ribose-5-phosphate, chloroplast
(46)	RNA	Ribonucleic acid
(47)*	Ru5P	Ribulose-5-phosphate
(48)	Ru5P _{chl}	Ribulose-5-phosphate, chloroplast
(49)	RuDP	Ribulose-1,5-bisphosphate
(50)	SER	Serine
(51)*	S7P	Sedoheptulose-7-phosphate
(52)	S7P _{chl}	Sedoheptulose-7-phosphate, chloroplast
(53)	STA	Starch
(54)	SUCCoA	Succinyl-coenzyme A
(56)	THR	Threonine
(57)	TRP	Tryptophan
(58)	TYR	Tyrosine
(59)	VAL	Valine
(60)*	X5P	Xylulose-5-phosphate
(61)	X5P _{chl}	Xylulose-5-phosphate, chloroplast

^a Metabolite vectors indexed with an asterisk (*) are the cytoplasmic metabolites in the networks of autotrophic and mixotrophic metabolism, while in the heterotrophic networks, they refer to the total pool of these metabolites. Measured fluxes include the absorbed photon flux by cells, the uptake rates of glucose and nitrate, and the formation rates of cellular carbohydrates, protein, lipids, chlorophylls, DNA and RNA.

3.3. Effect of light on respiratory metabolism

It is well known that cells can obtain substrates, reducing power and ATP for biosynthesis through the respiratory pathways, i.e. glycolysis, PP pathway, and TCA cycle linked to mitochondrial oxidative electron transport pathways. However, since the light reactions of photosynthesis can provide much reducing power and ATP, respiratory metabolism is unlikely to be essential for these normal functions. It has been proposed by some researchers that the respiratory activity of plants is inhibited in the light. On the other hand, evidence can be cited that indicates little or no effect of light on the respiratory rate [27]. Thus the physiological evidence for the effect of light on respiratory metabolism in algae and plant cells is conflicting. Here the autotrophic flux distribution estimated from metabolic flux analysis can assess the respiratory activity of microalgae in the light.

3.3.1. Glycolysis and TCA cycle

As shown in Fig. 5, the autotrophic culture showed comparable activities of glycolytic pathway and TCA cycle with the heterotrophic culture. This result is consistent with the labeling experiments by Gibbs' laboratory using the alga *Scenedesmus*, which showed that the rates of equilibration of ¹⁴C through the intermediates of the TCA cycle were essentially equivalent in light and dark [27]. In addition, a significant flux through PEP carboxylase was observed for all cultures, suggesting an important role for this enzyme to maintain the operation of TCA cycle.

3.3.2. Pentose phosphate pathway

It was found in different plant cells that the percentage of glucose metabolism via the pentose phosphate pathway is relatively small, i.e. between 5 and 15% from the total glycolytic flux [46]. However, using mass balance to determine the flux distribution of algal cells in heterotrophic culture, we found a much higher activity in the PP pathway than was found by others: about 90% of the glucose proceeds via glucose-6-phosphate dehydrogenase. This difference is probably a result of different cultivation methods. In all earlier studies, cells were cultured with a very limited addition of [¹⁴C] glucose. The labeling method was used to assess the contribution of the PP pathway to the total metabolism of glucose when plant cells metabolized the storage carbohydrate (sucrose and starch). The energy metabolism of these cells was, therefore, focused on maintenance rather than growth. However, the requirement for NADPH, which is consumed in biosynthetic reactions, is much higher in rapidly growing cells than in resting cells.

In the autotrophic flux distribution, the flux through the PP pathway was very small. Since the function of NADPH synthesis is provided by photosynthetic electron transport, the main synthetic functions of the PP pathway in the light appear to be only the supply of precursors for synthesis of nucleic acid and amino acids. Glucose-6-phosphate dehydrogenase is known as the major site for regulation in PP

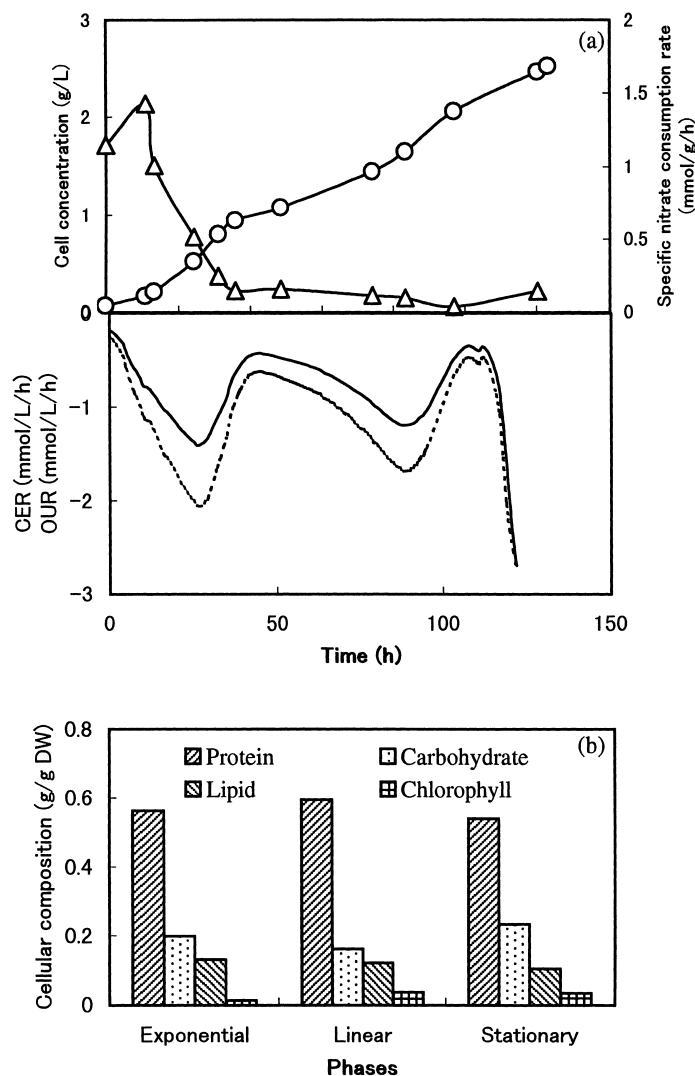


Fig. 2. Cultivation results of *C. pyrenoidosa* under autotrophic condition. (a) Time profiles of cell growth (○), consumption of nitrate (△), and the rates of CO₂ production (solid line) and O₂ uptake (dotted line). (b) Contents of carbohydrates, protein, lipids and chlorophylls in cells during the various growth phases. The incident light intensity was 500 μmol/m²/s. The various growth phases were identified from the curve of cell growth: exponential phase (0–48 h), linear phase (48–120 h) and stationary phase (120 h–end).

pathway, and its activity is strongly inhibited by NADPH [47]. It has been already proposed that the light-modulated regulation of glucose-6-phosphate dehydrogenase may be due to the change in the ratio of NADPH/NADP⁺ [48]. The low activity of PP pathway in the autotrophic culture is therefore probably a result of light-mediated control on glucose-6-phosphate dehydrogenase.

3.3.3. Oxidative phosphorylation

It has been suggested that the mitochondrial electron transport would be light-inhibited because of the high ATP/ADP ratios in the light [27]. However, more recent data indicates that any increases in the cytoplasmic ATP/ADP ratios observed in photosynthetic cells upon light to dark transitions are only transient and that steady-state cytoplasmic ATP/ADP ratios are similar under both light and dark conditions [49]. Furthermore, it has been shown that light

has no direct effect on the activity of the respiratory chain in green alga and plant cells [50]. This is consistent with our results because we found that a substantial activity of the respiratory chain do persist in the light. Therefore, it seems that the function of mitochondrial electron transport might have been underestimated and that oxidative phosphorylation could provide a significant fraction of energy for cell growth.

3.4. Energetics

3.4.1. Energy metabolism for autotrophically, heterotrophically and mixotrophically grown microalgae

From the estimated flux distribution, the fluxes involved in the generation and utilization of ATP can be obtained as shown in Table 4. In the autotrophic culture a significant fraction (40%) of the ATP was formed from the mito-

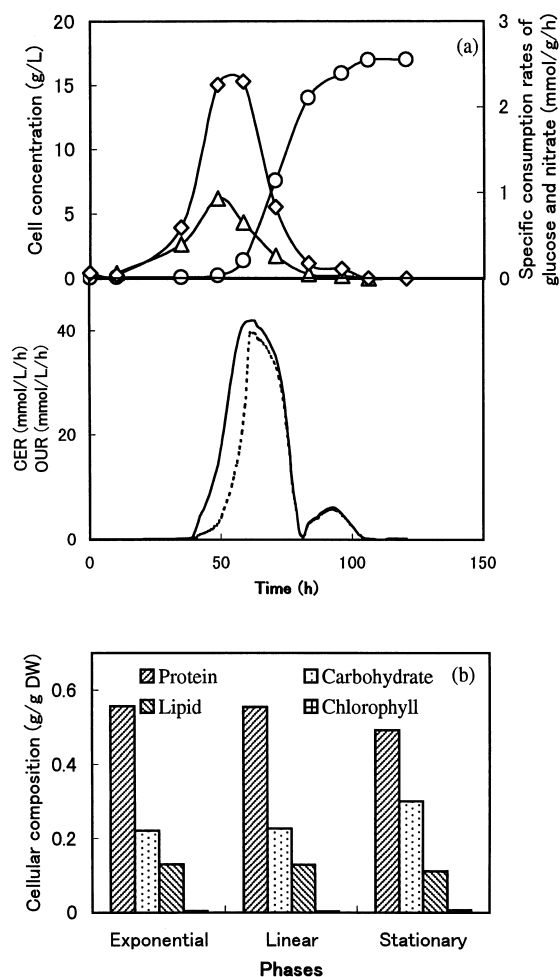


Fig. 3. Cultivation results of *C. pyrenoidosa* under mixotrophic condition. (a) Time profiles of cell growth (○), consumption of glucose (◇) and nitrate (△), and the rates of CO₂ production (solid line) and O₂ uptake (dotted line). (b) Contents of carbohydrates, protein, lipids and chlorophylls in cells during the various growth phases. The light intensity was 500 μmol/m²/s. Glucose was added to the culture and glucose concentration in the medium was controlled at 5 g/l. The various growth phases were identified from the curve of cell growth: exponential phase (0–58 h), linear phase (58–83 h) and stationary phase (83 h–end).

chondrial oxidative phosphorylation, suggesting its important role in ATP production. The Calvin cycle was the main ATP sink in the autotrophic culture, and the ATP demand for the assimilation of CO₂ accounted for about 77% of the total. From Table 4, the theoretical yields of biomass on ATP for three different cultures could be calculated. The results are listed in Table 5. These values were comparable to the yield found in plant and microbial systems [33,51]. Obviously, the ATP yield decayed in the following order: heterotroph>mixotroph>autotroph. Since Calvin cycle requires a large amount of ATP, the difference in the contribution of Calvin cycle to the total carbon metabolism leads to the different ATP yields. From the flux of excess ATP, the growth-related maintenance ATP requirements were estimated for three cultures as shown in Table 5. It was found that a significant amount of the available ATP was required

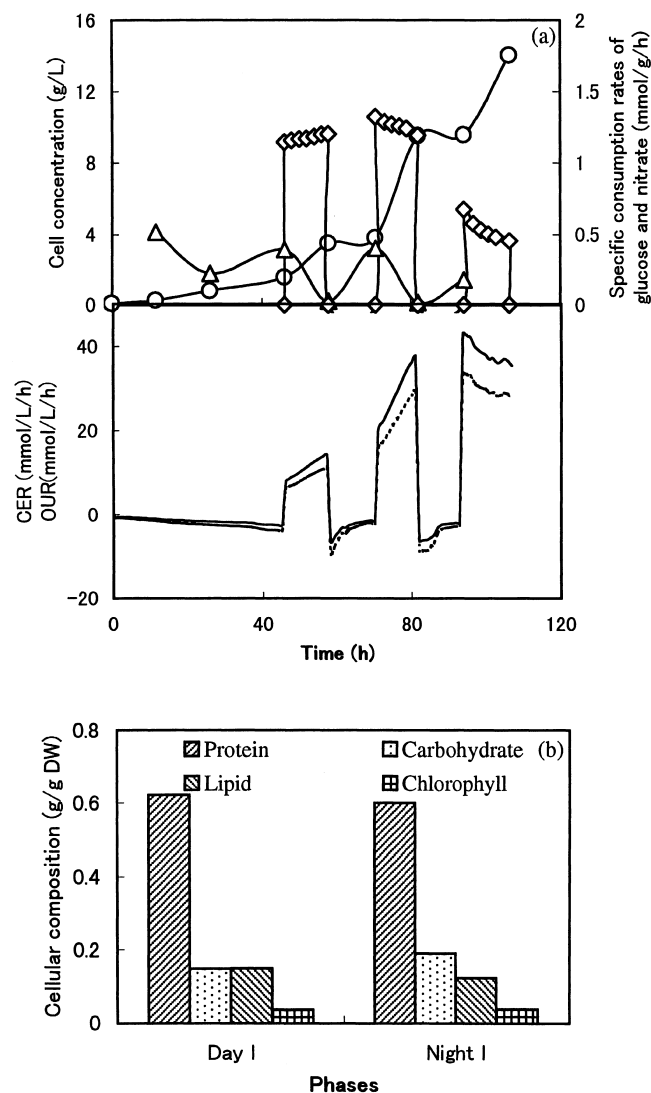


Fig. 4. Cultivation results of *C. pyrenoidosa* under cyclic light-autotrophic/dark-heterotrophic condition. (a) Time profiles of cell growth (○), consumption of glucose (◇) and nitrate (△), and the rates of CO₂ production (solid line) and O₂ uptake (dotted line). (b) Contents of carbohydrates, protein, lipids and chlorophylls in cells during the first light/dark cycle. Cells were cultivated autotrophically for 24 h and then subjected to 12 h-light/12 h-dark cycles. The light intensity during the light period was 500 μmol/m²/s. Glucose was added during the dark period and glucose concentration in the medium was controlled at 5 g/l.

for maintenance. Maintenance processes took up as much as 45–82% of the total ATP produced. This result is consistent with the data reported by de Gucht and van der Plas [33], who found that maintenance requires 50–75% of the available ATP in the continuous cultures of plant cells. This maintenance energy includes ATP requirements for transport, translocation, futile cycles and so on. Especially for algae and plant cells, which have a high degree of subcellular compartmentation of metabolism, various transport reactions are involved in the metabolic pathways [24]. These transport processes may consume a large amount of energy.

Table 3

The consumption of glucose, CO₂ production and O₂ uptake of *C. pyrenoidosa* under different cultivation conditions^a

Experiment	$Y_{GLC/X}$ (mol/g)	$Y_{CO_2/X}$ (mol/g)	$Y_{O_2/X}$ (mol/g)
Autotrophic cultivation	–	–0.0398	–0.0556
Mixotrophic cultivation	0.0179	0.0653	0.0478
Cyclic cultivation	0.0170	0.0585	0.0384

^a $Y_{GLC/X}$, $Y_{CO_2/X}$ and $Y_{O_2/X}$ were calculated from the division of glucose consumed, CO₂ evolved and O₂ uptake throughout the cultivations by the formed biomass. Negative values indicate CO₂ uptake and O₂ evolution.

In the autotrophic and heterotrophic cultures there are only one energy contributor: light or glucose. However, both light and glucose are sources for the ATP production in the mixotrophic culture. Since glyceraldehyde-3-phosphate generated by photosynthesis partly enters the glycolytic pathway and TCA cycle, both light and glucose account for the production of NADH and FADH₂ in the TCA cycle. Therefore, the ATP production from light includes not only the ATP produced from photophosphorylation but also the ATP provided by the oxidative phosphorylation of NADH and FADH₂ derived from photosynthesis. The contribution of light and glucose to NADH and FADH₂ can be calculated using the fractional contribution model [52]. With this in-

formation, the contribution of light and glucose to the ATP production could be obtained as shown in Table 6. The amount of ATP produced from photosynthesis was about 63%. Hence, light was the major source for ATP production in the early phase of mixotrophic cultivation.

3.4.2. Energy conversion in autotrophic, heterotrophic and mixotrophic cultures

In all three cases, the energy provided by light and/or glucose is absorbed by the microalgal cells, then transformed into ATP for various energy demands inside the cells. Thus the conversion of energy involves three energy forms, the energy supplied to the culture, the energy absorbed by the cells and the high free energy stored in the phosphoanhydride bonds of ATP. Fig. 6 shows the conversion efficiencies among the three energy forms in the autotrophic, heterotrophic and mixotrophic cultures. Since the concentration of the organic substrate was maintained constant, the energy provided by the addition of glucose were completely utilized by the cells of heterotrophic and mixotrophic cultures. The capture of light energy in the autotrophic culture was found to be as low as 14% of the total supplied light energy, while in the mixotrophic culture for which both light and glucose provided energy, the conversion efficiency of the supplied energy to the absorbed energy was even much lower than that of the autotrophic culture. The low energy availability

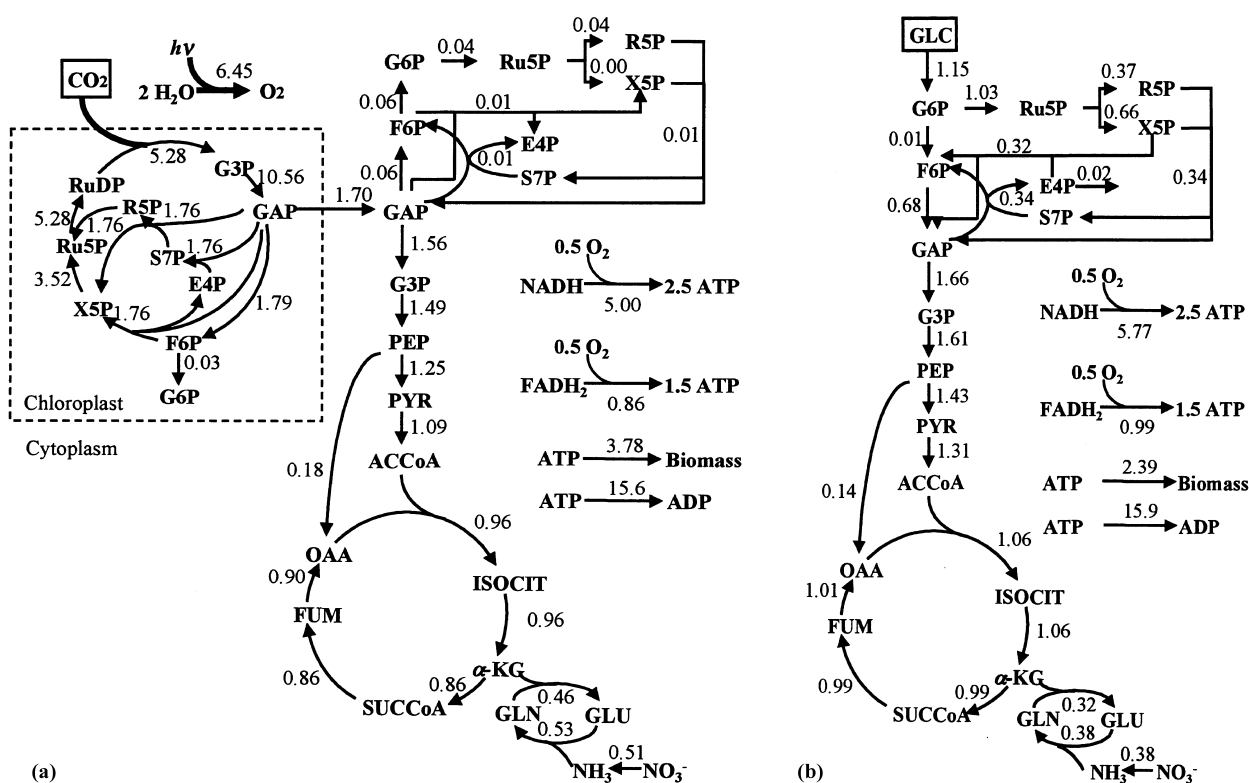


Fig. 5. Metabolic flux distribution of *Chlorella* cells in (a) autotrophic, (b) heterotrophic and (c) mixotrophic cultures. The flux values are expressed in mmol/g/h. These values represent the flux distributions of the exponential growth phase during the autotrophic (11 h) and mixotrophic (35 h) cultivations and the first dark period during the cyclic autotrophic/heterotrophic cultivation (48 h). The specific growth rates for all three systems are approximately 0.066 h⁻¹.

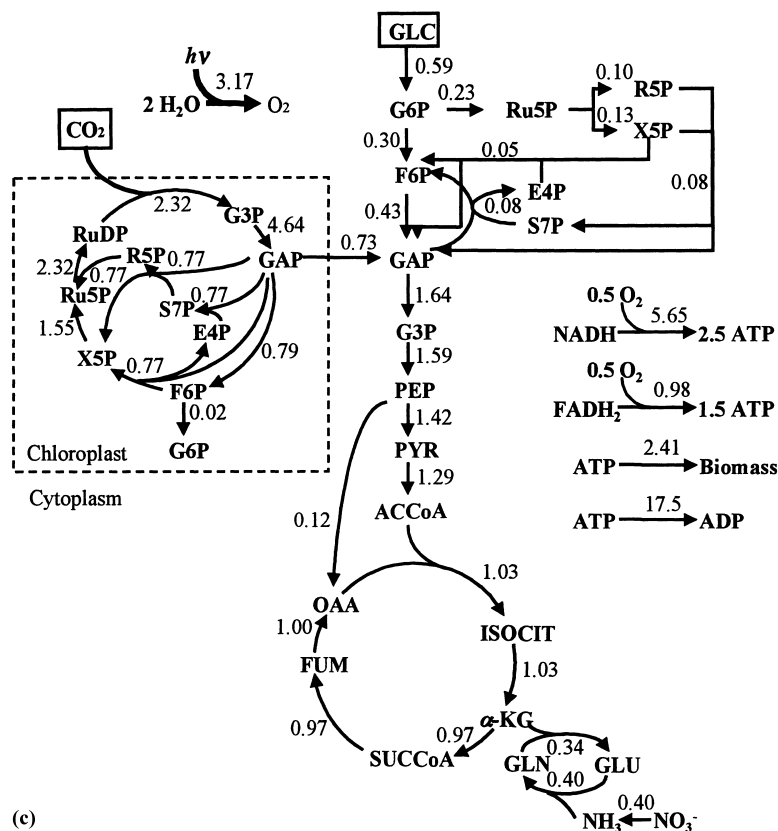


Fig. 5. (Continued).

in the mixotrophic culture was the result of the less content of pigments in the cells. In addition to the pigments content, the light trapping efficiency also relates to the cell density and reactor type, and so far many efforts are focused on how to improve the harvesting efficiency of light energy [14,53].

From Fig. 6 it was found that the microalgal cells transferred 10, 18, 12% of the absorbed energy into ATP in the autotrophic, heterotrophic and mixotrophic cultures, respectively. The maximum thermodynamic efficiency of ATP formation from the absorbed energy in the three cultures can be calculated from the fluxes through the relevant metabolic networks at zero growth rate. The result is shown in Fig. 7. It can be seen from this figure that the theoretical yield of

ATP on the absorbed energy, $Y_{ATP/AE}^{\max}$, was the highest in the heterotrophic culture, while the maximum ATP production in the autotrophic culture was only 16% of the harvested light energy. In the mixotrophic culture, $Y_{ATP/AE}^{\max}$ was a linear function of the fraction of the absorbed light energy of the total. Since the mitochondrial oxidative phosphorylation is a more efficient energy-producing pathway than the photophosphorylation, the conversion efficiency of ATP from the absorbed energy depends on the contributions of both phosphorylation systems to the total ATP production [54]. If the energy conversion efficiency through the photosynthetic electron transport could be improved, a higher availability of ATP from the absorbed energy would be expected.

Table 4

The generation and utilization of ATP in the autotrophic, heterotrophic and mixotrophic cultures^a

Autotrophic culture		Heterotrophic culture		Mixotrophic culture	
ATP production					
Direct ATP	3.83	Direct ATP	3.54	Direct ATP	3.70
Oxidative phosphorylation	13.8	Oxidative phosphorylation	15.9	Oxidative phosphorylation	15.5
Photo-phosphorylation	16.8			Photo-phosphorylation	8.24
ATP consumption					
Calvin cycle	15.8	Glucose uptake	1.15	Glucose uptake	0.59
Synthesis of cell mass	3.78	Synthesis of cell mass	2.39	Synthesis of cell mass	2.41
				Calvin cycle	6.96

^a Data were calculated from the flux distributions shown in Fig. 5. Values are expressed in mmol/g/h.

Table 5
Theoretical yields of biomass on ATP and ATP maintenance requirements in the autotrophic, heterotrophic and mixotrophic cultures^a

Culture	Theoretical ATP yield (g/mol)	ATP maintenance demand (mmol/g/h)
Autotrophic culture	3.11	15.6
Heterotrophic culture	19.3	15.9
Mixotrophic culture	6.64	17.5

^a Data were calculated from the flux distributions shown in Fig. 5.

Table 6
Contributions of light energy and glucose to ATP production in the exponential phase of mixotrophic culture^a

Energy source	ATP production	
	mmol/g/h	%
Light	17.3	63.1
Glucose	10.1	36.9

^a Data were calculated from the flux distribution shown in Fig. 5.

Recently an alga which lacks of photosystem I and requires a single photon rather than two in the process of photosynthesis has been discovered [55]. Therefore, the conversion efficiency of light energy into chemical energy can be potentially doubled.

From the above analysis, it was found that about 18% of the supplied energy was transformed to ATP in the heterotrophic culture, while the percentages decreased to 1.5 and 1.1% in the autotrophic and mixotrophic cultures, respectively. Apparently, the difference was caused by the different energy sources supplied to the cultures. It seems

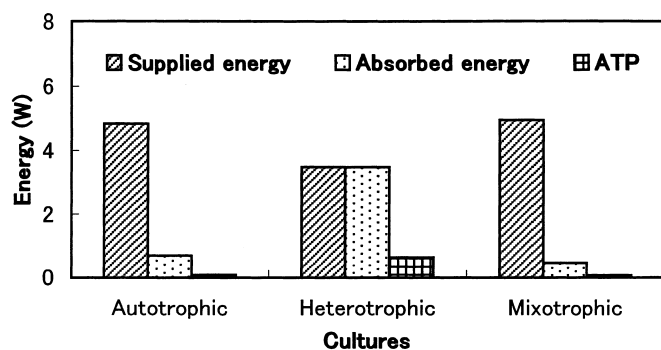


Fig. 6. Energy conversion efficiency between the energy supplied to the culture, the energy absorbed by the cells and the high free energy stored in ATP. These values represent the energy conversion efficiency of the exponential growth phase during the autotrophic and mixotrophic cultivations and the first dark period during the cyclic autotrophic/heterotrophic cultivation. The supplied and absorbed light energy were calculated from Eqs. (1)–(3), and the estimation of the total ATP produced was based on the flux distribution shown in Fig. 5. Assuming that the wavelength of fluorescent light is 600 nm, 1 mol of photons has an energy content of 200.8 kJ. The supplied glucose energy was calculated by multiplying the glucose consumption rate by the free energy change in the reaction of glucose oxidation (2868.852 J/mol). The free energy stored in 1 mol ATP is 30.5 kJ.

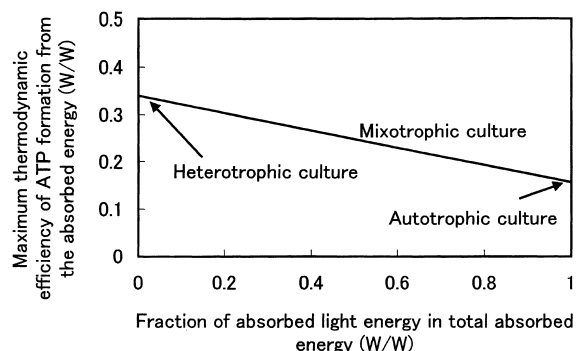


Fig. 7. Theoretical thermodynamic efficiency of ATP formation from the absorbed energy ($Y_{ATP/AE}^{\max}$) as a linear function of the fraction of the absorbed light energy of the total. $Y_{ATP/AE}^{\max}$ was calculated from the fluxes through the relevant metabolic network at zero growth rate. For the fraction of the light energy of the total absorbed energy, zero represents heterotrophic culture, and one represents autotrophic culture.

that comparing with the organic substrate, the light energy is difficult to be trapped and converted to ATP by the cells.

3.4.3. Energy economy in autotrophic, mixotrophic and cyclic autotrophic/heterotrophic cultivations

The energy economy of the microalgae cultures can be evaluated through the efficiency of energy utilization. Table 7 shows the overall yield of biomass on the supplied energy for the autotrophic, mixotrophic and cyclic autotrophic/heterotrophic cultivations. Not surprisingly, the energetic growth yield in the autotrophic culture was the lowest due to the inefficient conversion of light energy into biomass as discussed above. For the mixotrophic and cyclic autotrophic/heterotrophic cultures, both light and glucose are the energy sources. However, the difference in the way of energy supply resulted in the different bioenergetic yields. The biomass energetic yield in the mixotrophic culture was lower than that in the cyclic culture.

The conversion efficiencies of total energy and light energy were calculated for different growth phases during the mixotrophic cultivation as shown in Fig. 8. The total ATP production and ATP derived from light were estimated from the results of metabolic flux analysis. From Fig. 8, it can be seen that in the exponential phases, which light played a major role in ATP production as discussed above, only 0.73% of the supplied light energy was transferred into ATP. This value was much lower than that in the autotrophic culture because of the less pigment content in the mixotrophic cells. The formation of photosynthetic apparatus was disturbed due to the presence of organic substrate. With the age of culture and the increase in the cell concentration, the contribution of light energy to ATP production was decreased according to flux analysis. In the linear phase, nearly all the production of ATP originated from glucose, and the supplied light energy to the culture could not be efficiently utilized for cell growth due to the high cell density. Therefore, the energy was not utilized efficiently in the mixotrophic

Table 7

Biomass yields on the supplied energy ($Y_{X/SE}$) in the autotrophic, mixotrophic and cyclic autotrophic/heterotrophic culture experiments^a

Experiment	Cell produced (g)	Glucose supplied (kJ)	Light supplied (kJ)	$Y_{X/SE}$ (g/kJ)
Autotrophic culture	3.78	–	2144.6	0.00177
Mixotrophic culture	25.5	1307.6	2099.4	0.00749
Cyclic culture	21.1	1041.1	1238.6	0.00924

^a The supplied light energy was calculated from Eq. (1). The supplied glucose was calculated by multiplying the totally consumed glucose by the free energy change in the reaction of glucose oxidation.

cultivation. This result was unexpected since the mixotrophic culture is often applied for commercial algal production. It has been expected before that the two processes of photosynthesis and glucose catabolism proceed independently and are not interacted with each other [13]. However, from the above analysis it was found that the photosynthetic capacity of microalgal cells was reduced significantly due to the uptake of glucose. Consequently, the result presented here is meaningful for the improvement of industrial microalgae production.

Fig. 9 shows the energy conversion efficiency between the three energy forms during the first light/dark cycle of the cyclic autotrophic/heterotrophic culture. In the cyclic culture, cells were cultivated autotrophically for the first two days, and then subjected to dark/light cycles. From Fig. 9, it was found that during the first two days of autotrophic growth, the absorbed light energy by the cells has reached the maximum value ($F_{vol} = 2I_0/R\pi$), indicating that the autotrophic growth had undergone a rapid growth phase and the availability of light to the culture had begun to be limited. In the subsequent dark period, the addition of glucose

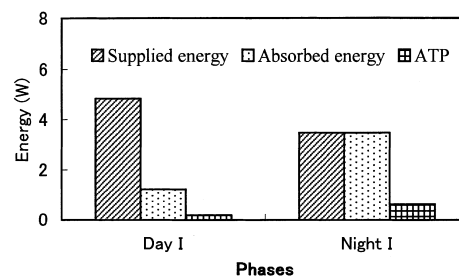


Fig. 9. Energy conversion efficiency between the three energy forms during the first light/dark cycle of the cyclic autotrophic/heterotrophic cultivation. The total ATP production and ATP derived from light were estimated from the results of metabolic flux analysis. The calculation was the same as for Fig. 6.

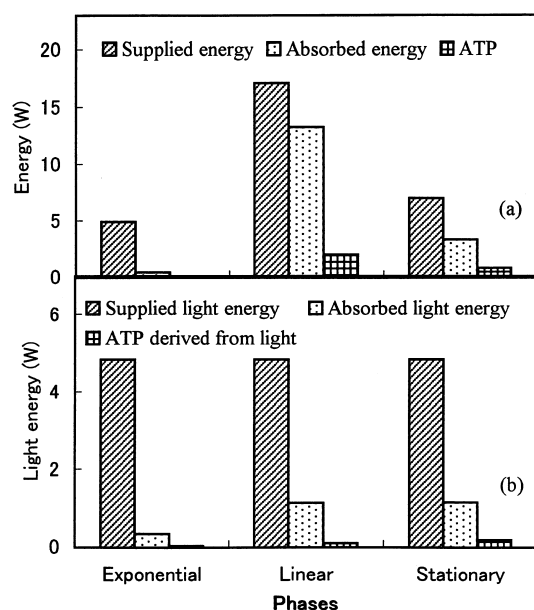


Fig. 8. Conversion efficiency of (a) total energy and (b) light energy during the various growth phases of the mixotrophic cultivation. The total ATP production and ATP derived from light were estimated from the results of metabolic flux analysis. The calculation was the same as for Fig. 6.

to the culture inspired the cell growth and resulted in a significant increase in the cell concentration. Therefore, the advantage of the cyclic culture was that the autotrophic cell growth and photosynthetic process were not adversely affected by the addition of organic substrate during the night. In the late light period of the cyclic culture, although the growth rates were very low, the autotrophic growth could improve the contents of some important components such as pigments and linolenic acid. Thus the nutritional value and product quality of the microalgae would be better than that in the simple heterotrophic culture. More importantly, the cyclic culture can be easily employed for the utilization of solar energy. As well known, utilization of solar energy is very desirable, but the solar light supply is not continuous due to diurnal and seasonal changes [56]. For the autotrophic and mixotrophic cultures which require continuous illumination, it is necessary to capture and concentrate the solar energy for the light supply during the night, while for the illumination of the cyclic culture, solar light energy can be used directly. Therefore, from the viewpoint of economy, the cyclic light-autotrophic/dark-heterotrophic culture can be employed for efficient production of microalgae.

4. Conclusion

The metabolic flux distribution of microalgal cells for autotrophic, heterotrophic and mixotrophic cultures has been determined by the use of mass balances. The effect of light on the carbon and energy metabolism in microalgal cell culture systems has been thoroughly studied. The respi-

ratory activity of microalgae in the light has been assessed from the autotrophic flux distribution. Results showed that the glycolytic pathway, TCA cycle and mitochondrial oxidative phosphorylation maintained high activities during illumination, indicating little effect of light on these pathways. However, the flux through the pentose phosphate pathway during illumination was very small due to the light-mediated regulation.

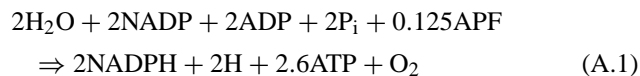
The theoretical yields of biomass on ATP and the maintenance ATP requirements were estimated for autotrophic, heterotrophic and mixotrophic cultures. The results showed that the difference in the contribution of Calvin cycle to the total carbon metabolism led to the different ATP yields of the three cultures, and a significant amount of the available ATP was required for maintenance processes in microalgal cells.

The energy conversion efficiency between the supplied energy to culture, the absorbed energy by cells and the free energy conserved in ATP were calculated for the autotrophic, heterotrophic and mixotrophic cultures. It was found that the heterotrophic culture generated more ATP from the supplied energy than the autotrophic and mixotrophic cultures. The maximum thermodynamic efficiency of ATP production from the absorbed energy ($Y_{ATP/AE}^{max}$), which was calculated from the metabolic fluxes at zero growth rate, was the highest in the heterotrophic culture and as low as 16% in the autotrophic culture. In the mixotrophic culture, $Y_{ATP/AE}^{max}$ was a linear function of the fraction of the absorbed light energy of the total.

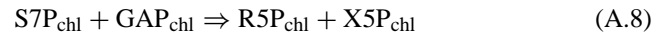
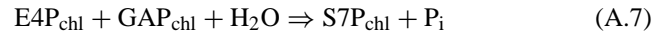
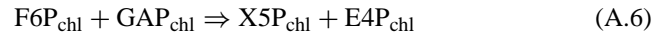
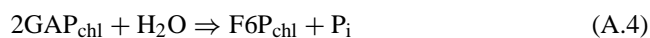
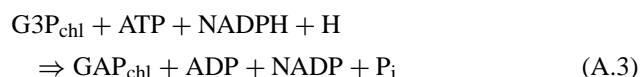
The energy economy of the autotrophic, mixotrophic, cyclic autotrophic/ heterotrophic cultivations were evaluated through the energy utilization efficiency. The biomass yield on the supplied energy was the lowest in the autotrophic cultivation, and the cyclic culture displayed the most efficient utilization of energy for biomass production. The analysis of the energy utilization efficiency has been shown to be very useful for providing information concerning cell energetics and guidance to improve the microalgal cell culture performance.

Appendix A. Biochemical reactions

Light reactions



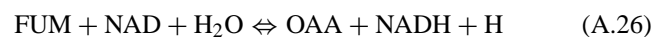
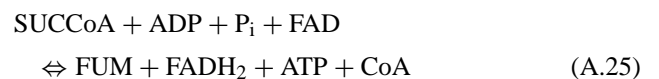
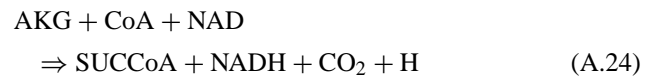
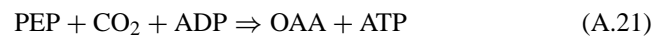
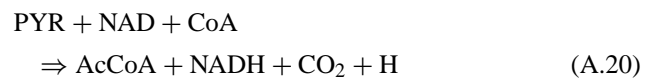
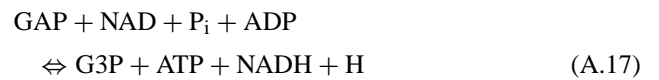
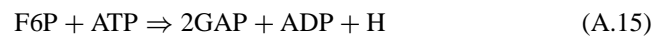
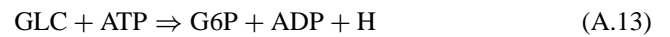
Calvin cycle



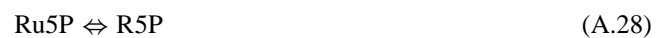
Transport of triose phosphate from chloroplast to cytoplasm

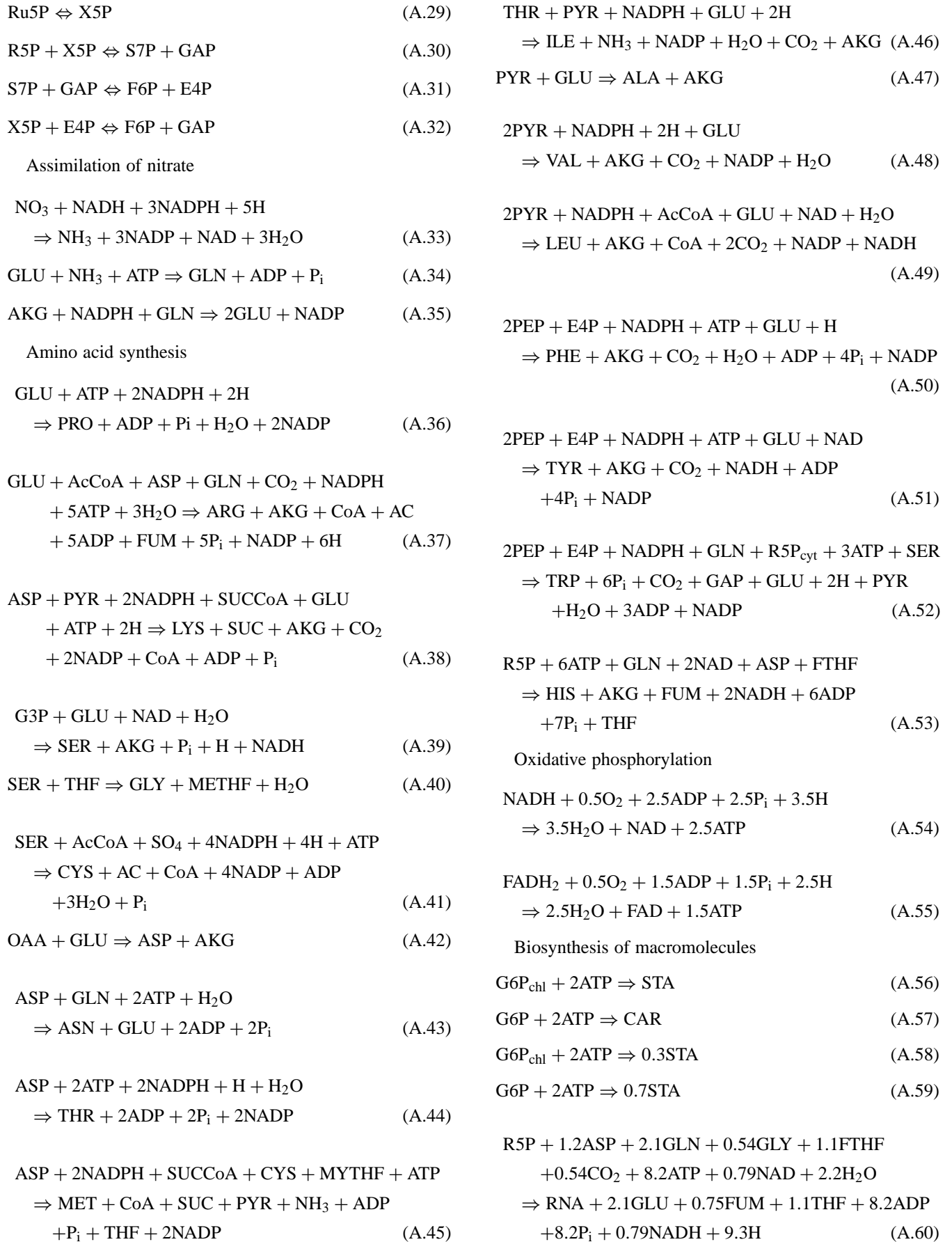


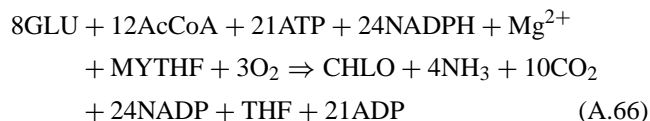
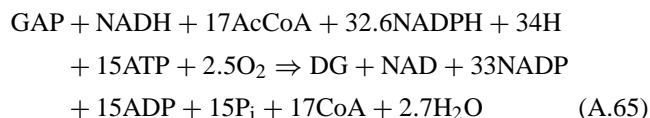
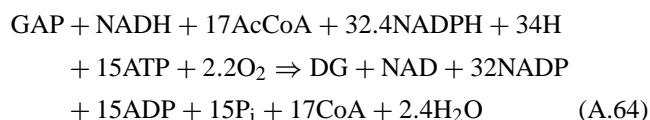
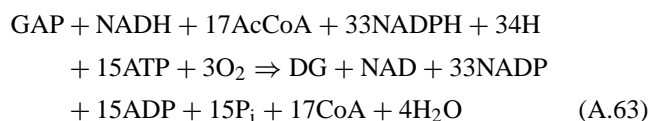
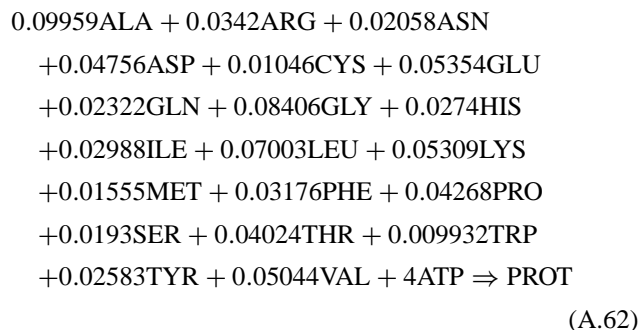
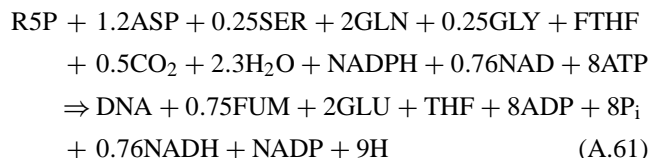
Glycolytic pathway and tricarboxylic acid cycle



Pentose phosphate pathway







Miscellaneous reaction



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