Appl Microbiol Biotechnol (2000) 54: 112–117

ORIGINAL PAPER

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Limited feeding of potassium nitrate for intracellular lipid and triglyceride accumulation of *Nannochloris* sp. UTEX LB1999

Received: 27 October 1999 / Received revision: 26 November 1999 / Accepted: 24 December 1999

Abstract Limited feeding of nitrate during culture of *Nannochloris* sp. UTEX LB1999 for intracellular lipid and triglyceride accumulation was investigated with the aim of obtaining cells superior for liquefaction into a fuel oil. The intracellular lipid contents and the percentage of triglycerides in the lipids of cells grown in a nitrogen-limited medium (0.9 mM KNO_3) were 1.3 times as high as those grown in a modified NORO medium containing 2.0–9.9 mM KNO_3. However, the cell concentration was too low for the practical production of fuel oil by high-pressure liquefaction of the cell mass. A single feeding of 0.9 mM nitrate after nitrate depletion during cultivation in a nitrate-limited medium increased the cell concentration to twice that obtained without such feeding, and the lipid content was maintained at a high level. The timing of nitrate feeding, i.e., whether it was given during the log phase (before nitrate depletion), the constant growth phase (just after the depletion), or the stationary phase (after the depletion), had negligible effect on the intracellular lipid content and percentage of triglycerides in the lipids. When 0.9 mM nitrate was intermittently fed ten times during the log phase in addition to the initial nitrate feed (0.9 mM), the cell concentration reached almost the same (2.16 g/l) and the intracellular lipid content and the percentage of triglycerides in the lipids increased from 31.0 to 50.9% and 26.0 to 47.6%, respectively, compared with those of cells cultured in a modified NORO medium containing 9.9 mM KNO_3 without additional nitrate feeding.

Introduction

Since atmospheric CO_2 accumulation has a serious effect on the global environment, the control of total CO_2 emission into the atmosphere is an important issue related to the biosphere. Marine microalgae are expected to play an important role in resolving this problem because they have a high capability for photosynthesis and grow well in the sea, which solubilizes a high amount of CO_2 and accounts for 70% of the surface area of the earth.

There are two major approaches to large-scale CO_2 fixation by marine microalgae. One is the production of noncarbon energy sources such as hydrogen gas from the algal biomass. The other is the conversion of algal biomass into liquid fuel by thermochemical liquefaction reactions at high temperature and pressure (Suzuki et al. 1988; Kishimoto et al. 1994). The latter process is expected to stimulate CO_2 circulation on earth, and is considered to be a simpler and more appropriate process for worldwide implementation than the former.

One of the most important criteria in assessing the performance of thermochemical liquefaction is the oil yield from the organic materials in the algal biomass. A report on liquefaction using various model substrates revealed that a high liquefaction yield was obtained from cell components containing hydrophobic compounds such as lipids, fatty acids, and fatty acid esters (Evans and Felbeck 1983). Among marine microalgal species, cells of the genus *Nannochloris* are known to contain a large amount of intracellular lipids (Ben-Amotz and Tornabene 1985; Negoro et al. 1991). It was reported that the intracellular triglyceride content increased after nitrate depletion during a culture of *Nannochloris* sp. UTEX LB1999 cells in modified NORO medium containing 9.9 mM KNO_3, even though the intracellular lipid content did not increase, and the oil yield following liquefaction of the cells increased in proportion to the intracellular triglyceride content (Yamaberi et al. 1998).

Nitrogen-limited cultivation in a medium containing nitrogen sources at low concentrations was reported to result in an increase in the lipid content of the algal cells (Shifrin and Chisholm 1981; Piorreck et al. 1984; Ben-Amotz et al. 1985; Sriharan et al. 1989). The nitrogen concentration might be an important operational variable for increasing the lipid content in practical large-scale cultivation, because it is easy to manipulate

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and is low-cost compared with other factors influencing the intracellular lipid content, such as light intensity (Albitskaya et al. 1974; Orcutt and Patterson 1975; Renaud et al. 1991), temperature (Kleinschmidt and McMahon 1970; Aaronson 1973; Renaud et al. 1995), high salt concentration (Dubinsky et al. 1978; Elenkov et al. 1996), and heavy metal concentration (Constantopoulos 1970; MacCarthy and Patterson 1974; Riches et al. 1996). However, the cell concentration in the nitrogen-limited culture was generally too low for the practical production of fuel oil by liquefaction of the cell mass. Moreover, a high concentration of cells with a high lipid content should be obtained without a decrease in the percentage of triglycerides in the lipids.

This study investigated the effects of low-concentration nitrate feeding on cell concentration, intracellular lipid contents, and percentage of triglycerides in the lipids during cultivation of *Nannochloris* sp. UTEX LB1999 cells.

Materials and methods

Algal strain and media

Nannochloris sp. UTEX LB1999 was used in this study as a marine CO_2-fixing microalgal strain and grown in a marine medium. It was subcultured every month in a modified GUI medium which had the following composition (per liter): $NaNO_3$, 75 mg; $NaH_2PO_4 \cdot H_2O$, 5.65 mg; $Na_2 \cdot EDTA$, 4.36 mg; $FeCl_3 \cdot 6H_2O$, 3.15 mg; $MnCl_2 \cdot 4H_2O$, 180 μg; $CoCl_2 \cdot 6H_2O$, 10 μg; $CuSO_4 \cdot 5H_2O$, 10 μg; $ZnSO_4 \cdot 7H_2O$, 22 μg; Na_2MoO_4, 6 μg; vitamin B1, 0.1 mg; vitamin B12, 0.5 μg; biotin, 0.5 μg; and Aquamarine S (Yashima Pure Chemicals Co. Ltd., Osaka, Japan), 4 g as a source of NaCl and trace elements. The basal medium for further cultivation was a modified NORO medium which had the following composition (per liter): NaCl, 29.22 g; KNO_3, 1.0 g (9.9 mM); $MgCl_2 \cdot 6H_2O$, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.2 g; $CaCl_2$, 0.2 g; K_2HPO_4, 0.045 g; tris(hydroxymethyl)aminomethane, 2.45 g; $EDTA \cdot 2Na$, 1.89 mg; $ZnSO_4 \cdot 7H_2O$, 0.087 mg; H_3BO_3, 0.61 mg; $CoCl_2 \cdot 6H_2O$, 0.015 mg; $CuSO_4 \cdot 5H_2O$, 0.06 mg; $MnCl_2$, 0.23 mg; $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$, 0.38 mg; $Fe(III) \cdot EDTA$, 3.64 mg; vitamin B1, 0.1 mg; vitamin B12, 0.5 μg; and biotin, 0.5 μg; the pH was adjusted to 8.0 with 1 N HCl.

Cultivations

As a seed culture, 20 ml of the basal medium was placed in a 100-ml Erlenmeyer flask, inoculated with the cells to an OD_{680} of 0.05, and incubated at 28 °C with reciprocal shaking (60 spm) for 6 days. The light intensity on the surface of the flask was adjusted to 65 μmol/s m^{-2} (5000 lx) using fluorescent lamps. The seed culture was transferred into a Roux bottle containing 500 ml of the same medium, again to obtain an adjusted OD_{680} of 0.05. The temperature, light intensity, and aeration conditions were 30 °C, 150 μmol/s m^{-2} (10,000 lx) and 500 ml/min CO_2-enriched air (3% CO_2), respectively. All cultivation experiments were performed twice independently and similar results were obtained from each experiment.

Determination of cell and nitrate concentrations

The cell concentration was determined by measuring the OD_{680} and converted to ash-free dry cell weight where necessary using a calibration curve. The precipitate obtained by centrifugation (3,000 rpm, 15 min) of a 10-ml culture was heated overnight at 105 °C and weighed (α g). After carbonization of the precipitate for 3 min on a Bunsen burner, it was weighed again (β g). The ash-free dry cell weight (g/l) was determined by dividing the difference between α and β by the culture volume (0.01 l). The conversion coefficient of OD_{680} to cell concentration was 0.10 (g/l UOD^{-1}). The culture supernatant was diluted with the medium without KNO_3 and the nitrate concentration was determined from the difference between its absorbance values at 221.4 and 232.0 nm.

Determination of the contents of lipids and triglycerides

A culture sample containing about 30 mg cells was centrifuged and resulting precipitate was washed with 5 ml 1% NaCl. After extraction of lipids from the precipitate with methanol-chloroform (2:1), chloroform and 1% NaCl solution were added to adjust the ratio of methanol, chloroform, and water to 2:2:1. All the chloroform layers, collected three times, were evaporated, dried in a desiccator, and weighed as the total lipid (Ben-Amotz and Tornabene 1985). The percentage of triglyceride in the total lipids solubilized in isopropylalcohol was determined by the glycerol-3-phosphate-oxidase-*p*-chlorophenol method (triglyceride G-test Wako, Wako Pure Chemicals, Osaka, Japan), with triolein as the standard.

Results

Effect of initial nitrate concentration on the cell composition of *Nannochloris* sp. UTEX LB1999

To clarify the effects of the initial nitrate concentration on the cell composition, cultivations were carried out in Roux bottles containing modified NORO medium with nitrate at concentrations of 9.9, 8.0, 6.0, 4.0, 2.0, or 0.9 mM. Although cell concentration in the stationary phase decreased monotonously with a decrease in initial nitrate concentration from 9.9 to 0.9 mM (Table 1), there was no marked difference in the intracellular lipid content or the percentage of triglycerides in the lipids in cultures with initial nitrate concentration in the range of 2.0 to 9.9 mM and 6.0 to 9.9 mM, respectively. However, the lipid content in cultures with an initial nitrate concentration of 0.9 mM (40%) was markedly higher than that in cultures with higher initial nitrate concentrations, while the percentage of triglycerides increased over a wider range of initial nitrate concentrations, from 0.9 to 4.0 mM.

The measurements shown in Table 1 were obtained from cells harvested at the stationary phase after nitrate depletion in each culture, and the duration from nitrate

Table 1 Effect of initial nitrate concentration on the intracellular lipid contents and percentage of triglycerides in the total lipids at the stationary phase

Initial nitrate conc. (mM)	Cell (g/l)	Lipids (%)	Triglyceride content (%)
9.9	2.86	31	26
8.0	2.18	32	30
6.0	1.73	30	29
4.0	1.44	30	40
2.0	0.79	30	37
0.9	0.24	40	54

depletion to cell harvest was longest in the culture with an initial nitrate concentration of 0.9 mM. There were reports that cells growing after nitrogen depletion have increased the intracellular lipid content (Shifrin and Chisholm 1981; Piorreck et al. 1984). Therefore, we harvested and analyzed cells at different time-points from the time of nitrate depletion in the cultures with an initial nitrate concentration of 0.9 or 9.9 mM, in order to confirm whether continuation of culture after nitrate depletion could affect the intracellular lipid content. After nitrate depletion (50 or 124 h), cells were harvested at 101, 192 and 143, 192 h, respectively, in cultures with an initial nitrogen concentration of 0.9 or 9.9 mM, as shown in Fig. 1. There was no marked difference in intracellular lipid content among cells harvested at different time-points after nitrate depletion in cultures with an initial nitrate concentration of 0.9 and 9.9 mM (Table 2).

Influence of 0.9 mM nitrate feeding on cell growth and composition

To confirm the possibility that the concentration of cells with a high lipid content could be increased by nitrate feeding at a concentration as low as 0.9 mM, 0.9 mM nitrate was fed during the stationary phase after nitrate depletion in the culture with an initial nitrate concentration of 0.9 mM. The intracellular lipid contents and the percentage of triglycerides in the lipids were then measured for cells harvested at the end of cultivation. As shown in Fig. 2, the cell concentration increased following nitrate feeding and reached twice that obtained without nitrate feeding. Almost the same intracellular lipid content and percentage of triglycerides in the lipids as those obtained in the case of cells cultured without nitrate feeding were obtained in the culture with nitrate feeding (Fig. 2).

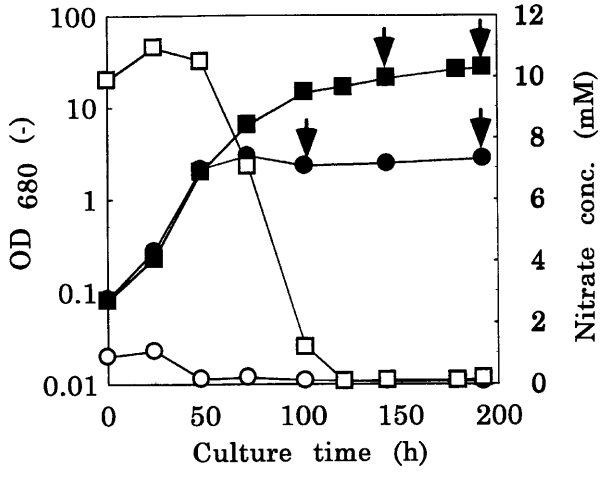

Fig. 1 Effect of initial nitrate concentration on algal cell growth. Cells were cultivated with an initial nitrate concentration of 0.9 (○, ●) or 9.9 mM (□, ■). Nitrate concentration (*open symbols*) and OD_{680} (*closed symbols*) as an index of cell concentration, were measured during the cultivation. *Arrows* indicate the time of cell harvest for the measurement of intracellular lipid content as shown in Table 2

Table 2 Influence of duration after nitrate depletion on intracellular lipid content

Initial nitrate conc. (mM)	Culture time (h)	Duration after nitrate depletion (h)	Cell (g/l)	Lipids (%)
0.9	101	51	0.24	38.6
	192	142	0.31	40.3
9.9	143	19	2.1	31.0
	192	68	2.7	29.9

Effect of timing of nitrate feeding on cell growth and composition

To investigate the effect of timing of nitrate feeding on the cell growth and composition, 0.9 mM nitrate was fed

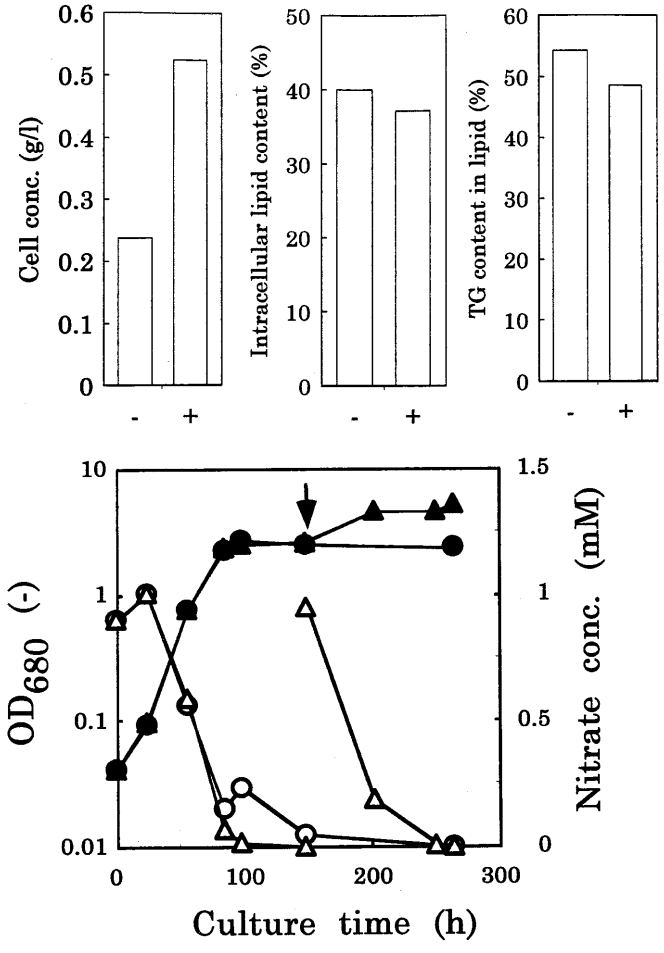

Fig. 2 Effect of nitrate feeding on algal cell growth, intracellular lipid content, and triglyceride content in lipids. Cells were cultivated with an initial nitrate concentration of 0.9 mM with a single nitrate feeding (△, ▲), at the time indicated with an *arrow*, or without (○, ●) nitrate feeding (0.9 mM). Nitrate concentration (*open symbols*) and OD_{680} (*closed symbols*) were measured during the cultivation. Cell concentration, intracellular lipid content, and triglyceride content in lipid for the cells harvested at the end of cultivation with (+) or without (−) nitrate feeding are shown in the *bar graphs*

to the culture containing an initial nitrate concentration of 0.9 mM at one of three phases during the culture: either (1) the late log phase (before nitrate depletion), or (2) the linear growth phase (after nitrate depletion), or (3) the stationary phase (after nitrate depletion) (Fig. 3). The intracellular lipid content and percentage of triglycerides in the lipids were measured at the end of cultivation. Cultures with nitrate feeding at any phase exhibited an increase in cell concentration compared with those without feeding (Figs. 2, 3), although the cell concentration tended to be higher in the cultures with nitrate feeding at a later phase. Lipid contents were not significantly different from each other, although a slightly higher content of triglycerides in the lipids was obtained in the culture with feeding at the third (last) phase.

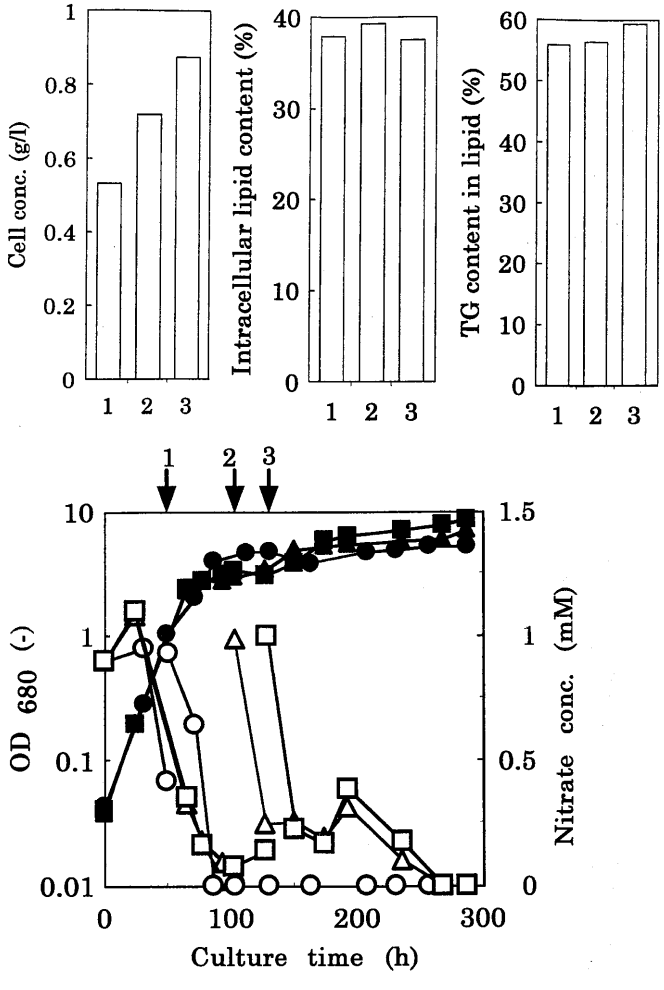

Fig. 3 Nitrate feeding at various phases in the culture with an initial nitrate concentration of 0.9 mM. Cells were cultivated using medium with an initial nitrate concentration of 0.9 mM, and nitrate (0.9 mM) was fed once during either the log phase before nitrate depletion (○, ●), or the linear growth phase after the depletion (△, ▲), or the stationary phase after the depletion (□, ■), which times are indicated by numbered *arrows 1*, *2*, and *3*, respectively. Nitrate concentration (*open symbols*) and OD_{680} (*closed symbols*) were measured during the cultivation. Cell concentration, intracellular lipid content, and triglyceride content in lipids for the cells harvested at the end of cultivation are shown in the *bar graphs*, where *1*, *2*, and *3* correspond to the nitrate feeding times shown in the *lower graph*

Effect of intermittent nitrate feeding on the cell growth and contents of intracellular lipids and triglycerides

To confirm the applicability of the nitrate feeding method to obtaining high concentrations of algal cells having high intracellular lipid contents and a high percentage of triglycerides in the lipids, cultures with an initial nitrate concentration of 0.9 mM were intermittently fed with 0.9 mM nitrate. Some nitrate concentrations higher than 0.9 mM were observed during the culture, because in some cases 0.9 mM nitrate was fed before the nitrate in the culture was completely depleted. The results were compared with those of cultures with an initial nitrate concentration of 9.9 mM without nitrate feeding in Table 1. Nitrate (0.9 mM) was fed ten times during the log phase or four times during the stationary phase after nitrate depletion, and the total nitrate concentrations supplied during the 490-h culture were 9.9 and 4.5 mM, respectively.

While cells continued to grow during almost the entire period of culture with both methods of feeding, growth with later feeding was delayed compared with that with earlier feeding (Fig. 4). The cell concentration obtained at the end of cultivation with feeding during the log phase (2.16 g/l) was similar to that obtained in the batch culture with the medium containing 9.9 mM nitrate, and markedly higher than that (1.33 g/l) with feeding during the stationary phase (Fig. 4). The intracellular lipid contents of cells harvested at the end of cultivation with intermittent nitrate feeding during the log and stationary phases were 50.9 and 51.5%, respectively. The percentage of triglycerides in the lipids of cells cultivated with nitrate feeding during the log and stationary phases were 47.6 and 46.3%, respectively. Moreover, there was no apparent difference in these contents between two cultures with different timing for nitrate feeding, that is, feeding during the log and stationary phases.

Discussion

Table 1 shows that the critical concentration of nitrate for intracellular lipid accumulation is around 0.9 mM. The lipid content (40%) of cells cultured with an initial nitrate concentration of 0.9 mM was markedly higher than those (approximately 30%) of cells cultured with higher nitrate concentrations. Nitrogen limitation was reported to decrease the cellular content of the thylakoid membrane, the glycolipid content of which is more than 90% (Wettern 1980), activate acyl hydrolase which could degrade glycolipid (Cases et al. 1998), and stimulate the hydrolysis of phospholipid (Muro-Pastor et al. 1996). These changes brought about by nitrogen limitation may increase the intracellular content of fatty acid acyl-CoA. Moreover, the nitrogen depletion was

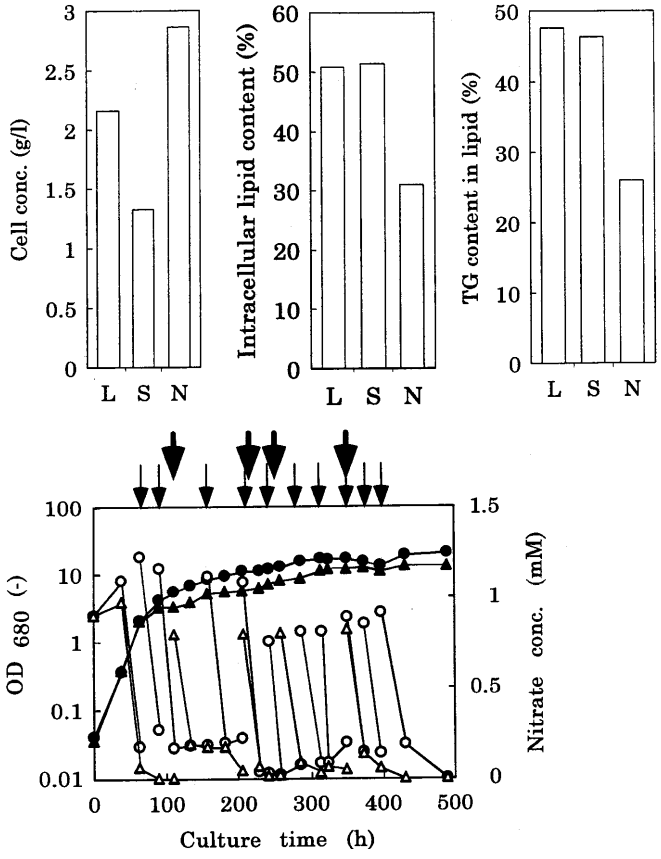

Fig. 4 Algal cell growth with intermittent feeding of 0.9 mM nitrate. Cells were cultivated employing medium with an initial nitrate concentration of 0.9 mM, and nitrate (0.9 mM) was fed intermittently during the log phase before nitrate depletion (○, ●) or the stationary phase after the depletion (△, ▲). The feeding times are indicated by *thin* and *thick arrows*, respectively. Nitrate concentration (*open symbols*) and OD_{680} (*closed symbols*) were measured during the cultivation. Cell concentration, intracellular lipid content, and triglyceride content in lipids for the cells harvested at the end of cultivation are shown in the *bar graphs*, where *L* and *S* indicate cultures with intermittent nitrate feeding during the log and stationary phases, respectively, and *N* indicates cultures with an initial nitrate concentration of 9.9 mM and no feeding

reported to activate diacylglycerol acyltransferase, which converts fatty acid acyl-CoA to triglyceride (Sukenik and Livne 1991). These could be the reasons why low nitrate concentrations increased not only the intracellular lipid content, as reported previously, but also the triglyceride content in the lipids. It was also considered that the critical nitrate concentration (approximately 4.0 mM) to increase the triglyceride content in the lipids should be higher than that for lipid accumulation (approximately 0.9 mM). The initial nitrate concentration of 0.9 mM was finally confirmed to be appropriate for obtaining cells with higher intracellular lipid contents and a higher percentage of triglycerides in the lipids, because there was no apparent increase in the intracellular lipid content during cultivation after nitrate depletion (Fig. 1).

However, the maximum cell concentration in the culture with 0.9 mM nitrate was limited to 11% of that with 9.9 mM, as shown in Fig. 1. Feeding 0.9 mM nitrate during the stationary phase after nitrate depletion in the culture with an initial nitrate concentration of 0.9 mM was shown to solve this problem, namely, to increase the cell concentration as well as maintaining the high contents of intracellular lipids and the percentage of triglycerides in the lipids (Fig. 2). Although the reason for the difference in cell concentrations between the cultures fed with nitrate at the two different phases was not clear, nitrate feeding at the culture phase was shown to have no marked influence on the intracellular lipid contents or the percentage of triglycerides in the lipids (Fig. 3). This might be related to the observation that residual nitrate concentration was no higher than approximately 1 mM during the entire period of cultivation, independent of the culture phase for nitrate feeding.

The reason why cell growth in cultures with four intermittent feedings of nitrate during the stationary phase after nitrate depletion was delayed compared with that in cultures with ten feedings during the log phase might be the time loss due to the extension of the stationary phase (Fig. 4). The intracellular lipid contents in cells harvested at the end of culture with intermittent nitrate feeding during the log and stationary phases (50.9 and 51.5%) were surprisingly higher than those (31.0%) in the culture with an initial nitrate concentration of 9.9 mM without nitrate feeding. The triglyceride contents (47.6 and 46.3%) were also markedly higher than those (26.0%) in the culture with an initial nitrate concentration of 9.9 mM without nitrate feeding. Therefore, nitrate feeding during the log phase might have an advantage compared with that during the stationary phase because the former could produce a greater numbers of cells having the same quality as that of the latter within a limited time. The reason why intracellular lipid contents in the culture with intermittent feeding (approximately 50%; Fig. 4) were higher than those (approximately 40%; Table 2, Figs. 2 and 3) with single or no feeding was not clear. This study proved that intermittent feeding of 0.9 mM nitrate to cultures with an initial nitrate concentration of 0.9 mM could produce almost the same concentration of cells with markedly higher intracellular lipid contents and percentage of triglycerides in the lipids as those in a conventional batch culture with an initial nitrate concentration of 9.9 mM.

In conclusion, algal cells having high intracellular lipid contents and a high percentage of triglycerides in the lipids can be harvested at high concentrations if they are cultivated with an initial nitrate concentration of 0.9 mM and intermittently fed with 0.9 mM nitrate during the log phase.

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