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Isolation of high-CO_2-acclimated *Micractinium* sp. strains from eutrophic reservoir water

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ABSTRACT

CO_2-tolerant microalgae have received much attention for their potential application in the fixation of CO_2 from industrial flue gas. Consequently, there have been extensive efforts to isolate and characterize these microalgae. However, microalgae acclimated to high levels of CO_2, which optimally grow at CO_2 concentrations close to that of industrial flue gas, can be more useful for CO_2 fixation. Several microalgal strains have been reported to be tolerant to high levels of CO_2, but the optimal CO_2 concentrations for their growth is generally lower than their maximum tolerable CO_2 level. In the present study, we isolated several microalgal strains from freshwater reservoirs. Among the isolates, two *Micractinium* sp. strains were found to grow preferably at high levels of CO_2. The strains grew best at around 30% CO_2, and were capable of growing even at 80% CO_2. As a combustion gas from coal-fired power plants, the largest CO_2 source, contains around 15% of CO_2, it can be a suitable carbon source for the strains in photoautotrophic cultivation. Interestingly, the strains were found to contain high amounts of vitamin B6 and γ-aminobutyric acid. Among the identified fatty acids, the C18 unsaturated and C16 saturated fatty acids were the most dominant. Based on these results, the strains appear to be promising candidates for application in nutraceutical production using CO_2 in industrial flue gas as a substrate.

1. Introduction

Microalgal CO_2 conversion is thought to be a preferable strategy to reduce CO_2 emission from industries, one of the main causes of global warming [1,2], because of its advantages, such as low production cost, its ability to fix CO_2 directly from industrial flue gas, and the lack of a need for catalysts [3]. Several microalgal strains have been employed in the commercial production of raw materials used for aquaculture feeds and nutraceuticals [4], and many others are under investigation for future commercialization for application in the manufacture of value-added products. Because industrial flue gas is rich in CO_2 (for instance, flue gas from coal-fired power plants, the largest CO_2 source, contains around 15% of CO_2 [5,6]), it can be used as a substrate for microalgae that grow at high levels CO_2. However, microalgal mass cultures are usually cultivated under atmospheric air, which contains only 0.04% CO_2, because acidification of the culture medium caused by excess CO_2 inhibits algal growth [7]. Therefore, isolation of cyanobacteria and eukaryotic microalgae that are tolerant to high concentrations of CO_2 might lead to development of efficient CO_2 utilization technology.

There have been many reports on CO_2-tolerant cyanobacteria and eukaryotic microalgae [8]. However, the optimal CO_2 concentration for microalgal growth should be regarded as a more important indicator than the maximum tolerable CO_2 concentration in evaluating the feasibility of culturing algal strains at high levels of CO_2. To the best of our knowledge, there are a limited number of microalgal strains that have been reported to grow optimally at > 15% CO_2 (Table 1). For freshwater microalgae, only a few strains belonging to the genus *Chlorella* or *Scenedesmus* are known to show the best growth at 15–20% CO_2. Recently, isolation of marine microalgal strains (*Picochlorum* sp.) that can grow well at around 30% CO_2 were reported [9]. Thus, it is attractive to isolate freshwater microalgae capable of rapid growth at such high CO_2 concentration. In the present study, we isolated and characterized high-CO_2-acclimated freshwater microalgal strains, and evaluated their potential in biotechnological applications.

2. Materials and method

2.1. Chemicals

Salts, vitamins, and organic solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA), Wako Pure Chemicals (Osaka, Japan), and Nakarai Tesque (Kyoto, Japan). Reagents for molecular biology

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Table 1
CO_2-tolerant microalgal strains whose reported optimal CO_2 levels for growth are > 15%.

Strain	Taxonomy	Origin	Max CO_2 tolerated (v/v %)	Optimal CO_2 for growth (v/v %)	Reference
Chroococcidiopsis sp.	Cyanobacterium	Hot spring	60	20	[26]
Chlorella sp.	Eukaryotic alga	Freshwater	> 40	20	[27]
Chlorella kessleri			> 18	18	[14]
Chlorella sp.			80	15	[28]
Scenedesmus obliquus			> 18	18	[14]
Picochlorum sp.		Seawater	40	30	[9]

Table 2
Characteristics of the samples used in this study.

Sample no.	Location of reservoir for sampling		Water temperature	pH	Nitrogen content (ppm)	Phosphorus content (ppm)	Total organic carbon (ppm)
	Latitude	Longitude					
1	34° 9′ 2″	131° 28′ 6″	19	7.01	0.40	0.18	10.5
2	34° 55′ 9″	131° 30′ 14″	18	7.00	0.20	0.26	14.4
3	34° 12′ 13″	131° 28′ 6″	18	6.95	0.55	0.55	18.0

techniques, including a KOD FX Neo DNA polymerase, were obtained from Toyobo (Osaka, Japan). Glass vials of 50 mL and 15 mL were purchased from Nichiden-Rika (Kobe, Japan) and Maruemu (Osaka, Japan), respectively. Other chemicals and materials were obtained from commercial sources.

2.2. Enrichment of high-CO_2-acclimated microalgae

In order to isolate high-CO_2-acclimated microalgal strains, freshwater samples were collected from three points in Yamaguchi City, Yamaguchi Prefecture, Japan (Table 2). Microbial cells were harvested by centrifuging 1 L of each sample at 6000 × *g* for 20 min at 4 °C and were then suspended in 10 mL of sterile water. The resulting suspension was inoculated into 50-mL glass vials containing 10 mL of culture medium (MBM or MBM-N) supplemented with or without cycloheximide and bubbled with 0.04%, 20%, 40%, or 100% CO_2. The vial numbers and their corresponding culture conditions are summarized in Table 3. The MBM medium consisted of the following (in $mg{\cdot}L^{-1}$ unless otherwise stated): KNO_3, 250; KH_2PO_4, 410; $MgSO_4{\cdot}7H_2O$, 75; K_2HPO_4, 75; NaCl, 25; $CaCl_2{\cdot}2H_2O$, 10; H_3BO_4, 2.86; $MnSO_4{\cdot}7H_2O$, 2.5; $ZnSO_4{\cdot}7H_2O$, 0.222; $CuSO_4{\cdot}5H_2O$, 0.079; $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, 0.702; $Na_2EDTA{\cdot}2H_2O$, 0.66; and $Na_2MoO_4{\cdot}2H_2O$, 0.0247. The composition of MBM-N medium is nearly identical to MBM, except for the lack of KNO_3, which enriches the culture for microalgae or cyanobacteria that possess nitrogen-fixing activity. The pH of the media was adjusted to 6.0 by adding 1 M KH_2PO_4 and 1 M K_2HPO_4 solutions. The suspension was bubbled with CO_2 at a flow rate of $2\ mL{\cdot}min^{-1}$ for 1 min and sealed immediately with a butyl rubber stopper and an aluminum crimp. The vials were then incubated on a reciprocal shaker (120 rpm) for 4 weeks at 25 °C and with illumination provided by fluorescent lights ($30\ \mu mol\ photon{\cdot}m^{-2}{\cdot}s^{-1}$) to enrich the cultures for high-CO_2-acclimated microalgae.

2.3. Isolation of algal strains

To isolate microalgae, enriched cultures in which microalgal growth was detected (indicated by green turbidity) were subcultured five times (2 weeks each). Subculturing was performed by inoculating 100 µL aliquots of the culture into 10 mL of fresh medium, after which 20 µL of the subcultured sample was inoculated onto MBM medium solidified by the addition of 1.5% agar. The samples were then incubated at 25 °C under an irradiance of $30\ \mu mol\ photons{\cdot}m^{-2}{\cdot}sec^{-1}$. Emerging colonies were purified three times on fresh agar plates.

2.4. Growth test for microalgae under varying CO_2 concentrations

Algal colony of the isolated strains were suspended in 100 mL of fresh MBM medium to obtain a cell density of $1.0 \times 10^5\ cells{\cdot}mL^{-1}$. Subsequently, 10 mL of the cell suspension was dispensed into each of nine 50-mL glass vials. The vials were then bubbled with different concentrations of CO_2 (0.04%, 10%, 20%, 30%, 40%, 50%, 60%, 80%, and 100% v/v) at a flow rate of $2\ mL{\cdot}min^{-1}$ for 1 min and sealed with a butyl rubber stopper and an aluminum crimp. The vials were shaken on a reciprocal shaker (120 rpm) at 25 °C with illumination ($30\ \mu mol\ photon{\cdot}m^{-2}{\cdot}s^{-1}$). Further, 10 µL of the algal culture were collected from the glass vials every 3 days using a microsyringe, and its cell density was determined using a Thoma hemocytometer.

2.5. Vitamin content analysis

Grown 1-week-old preculture (10 mL) of the isolates strains was inoculated and cultured in 5.0 L of MBM medium bubbled continuously with 0.2-µm-filtered 5% CO_2 at a rate of $6.0\ L{\cdot}min^{-1}$ and illuminated with fluorescent light ($30\ \mu mol\ photon{\cdot}m^{-2}{\cdot}s^{-1}$) at 25 °C. Once the culture reached the stationary growth phase (10 days), the cells were harvested via centrifugation at 8000 × *g* for 5 min at 4 °C. The resultant cell pellet was washed three times with 30 mL of deionized water and then freeze-dried at −50 °C for 48 h using FD1000 Freeze Dryer (Eyela, Tokyo, Japan).

To extract water-soluble vitamins, 300 mg of the freeze-dried cells, 500 mg of 0.5-mm glass beads (Biospec, Oklahoma, USA), and 500 mg of 0.1-mm zirconia silica beads (Biospec) were suspended in 5 mL of MilliQ water and dispensed equally into 10 screw cap microtubes (Sarstedt, Nümbrecht, Germany). The cell suspensions were ground for 30 s using a Mini Bead Beater-8 (Biospec). The resultant cell homogenates were then microfuged at 14,500 rpm for 5 min at 4 °C, and the supernatants were pooled in a 15-mL glass vial. The extraction procedure was repeated three times, and the collected supernatants were subsequently lyophilized in FD1000 Freeze Dryer. The lyophilized supernatants were dissolved in 1 mL of 50 mM KH_2PO_4 and analyzed using an HPLC system (Shimadzu, Kyoto, Japan) equipped with a Luna 5 µm C18 column (Phenomenex, CA, USA). The solvent system consisted of 50 mM KH_2PO_4 (pH 4.5, solvent A) and methanol (solvent B), and vitamins were separated using gradient elution at 42 °C. The initial proportions of the solvents were 96% A and 4% B; subsequently, three linear gradients were used (85% A and 15% B at 4 min, 77% A and 23% B at 10 min, and 65% A and 35% B at 18 min), followed by isocratic elution using 65% A and 35% B for an additional 2 min. The flow rate was $1.0\ mL{\cdot}min^{-1}$ during the entire run. Vitamins B and C were