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Short Communication

A new Arctic *Chlorella* species for biodiesel production

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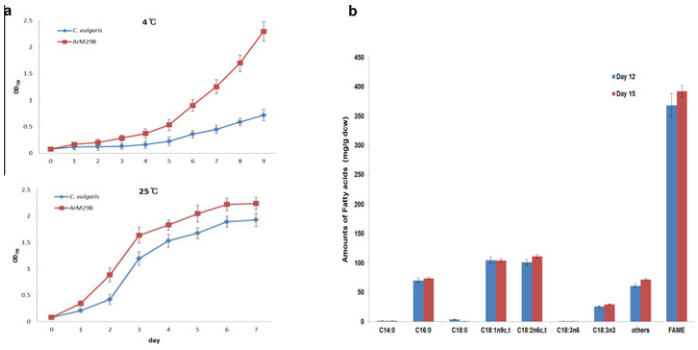
HIGHLIGHTS

- We provide specific characters of Arctic *Chlorella* ArM0029B as a biodiesel source.
- ArM0029B displayed fast growth at a wide temperature range of 4–32 °C.
- ArM0029B accumulated high levels of total fatty acids, reaching 39% of dry weight.
- In ArM0029B, 54% of total fatty acids was oleic acid (18:1) and linoleic acid (18:2).
- These results suggest that ArM0029B may be a suitable source for biodiesel.

GRAPHICAL ABSTRACT



(A) Growth rate of ArM0029B at various temperatures. *Chlorella vulgaris* served as a control. Data are expressed as ± SD (n = 3). (B) Analysis of fatty acid composition in ArM0029B. The amounts of each fatty acid and total fatty acids were presented as mg/dry cell weight.

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ABSTRACT

Microalgae are a potential resource for biodiesel production. A green alga, *Chlorella* sp., was isolated from Arctic sea ice, which was named ArM0029B. These algae displayed faster growth at a wide temperature range of 4–32 °C compared to *Chlorella vulgaris*. ArM0029B also accumulated high levels of total fatty acids under nitrogen starvation conditions, reaching 39% of dry cell weight, with the proportion of oleic acid (18:1) and linoleic acid (18:2) reaching 54% of total fatty acids. Taken together, these results indicate that the newly identified *Chlorella* species, ArM0029B, is a promising candidate for biodiesel production.

1. Introduction

Biodiesels are derived from biomass, a renewable energy source because the energy contained comes from the sun. Biodiesel is mostly derived from crop oils, animal fats, and waste cooking oils (Chisti, 2007; Taufiqurrahmi et al., 2011; Zhang et al., 2003). Microalgae are considered an attractive source for producing biodiesel due to their potential for high oil content (Chisti, 2007; Ugwu et al., 2008). Biodiesel production using microalgae provides several advantages over oil crops (Li et al., 2008); it requires less water than crop plants and does not compete for agricultural land use.

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Furthermore, different types of bioenergy, such as methane and hydrogen, can be derived from microalgae (Ghirardi et al., 2000; Spolaore et al., 2006).

Most research for commercialization of biodiesel from microalgae has focused on cost-effective production such as biomass harvesting, photobioreactor engineering, and genetic engineering of microalgae (Chisti, 2007; Li et al., 2008). These efforts require algal species that can provide high productivity and quality of the algal oils. Diverse microalgal species provide different types of lipids and hydrocarbons (Chisti, 2007; Guschina and Harwood, 2006; Metzger and Largeau, 2005), and the composition of fatty acids in algal oils differs from that of crop oils. Many algal oils contain abundant saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) (Belarbi et al., 2000; Chisti, 2007; Knothe, 2011); SFA affect the flow properties of biodiesel in cold climates and PUFA lead to low oxidative stability during storage (Belarbi et al., 2000; Knothe, 2011).

In the present study, the Arctic green alga ArM0029B was identified as a *Chlorella* sp. The potential of ArM0029B as a source of biodiesel was evaluated in terms of fatty acid content and growth temperature range. This Arctic *Chlorella* sp. displayed a wide temperature range for growth and a high total fatty acid content, with a high proportion of both 18:1 and 18:2 fatty acids. These data suggest that ArM0029B may be a useful algal species for biodiesel production.

2. Methods

2.1. Isolation and identification of arctic algae ArM0029B

Samples were collected from sea ice near the Dasan Station located in NyÅlesund, Spitsbergen, Norway (78° 55′ N, 11°56′ E) in 2008. The algae were purified by serial dilution followed by plating on agar. Individual green colonies were isolated and inoculated into liquid Tris–acetate–phosphate (TAP) medium (Harris, 1989). One of the pure cultures was examined by microscopy and was named ArM0029B. For sequence analysis of the *rbc*L gene, the genomic DNA fragment was amplified by PCR, sequenced, and analyzed according to Hoham et al. (2002). Sequence comparison of the *rbc*L genes between ArM0029B and related species was performed using the NCBI databases with BLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the ClustalW2-Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.2. Culture conditions

ArM0029B and *Chlorella vulgaris* UTEX 395 were used for this study. The ArM0029B cells grew in TAP medium containing 300 mM NaCl, but growth was better in medium without NaCl. For growth rate measurements, cells ($\sim 2 \times 10^5$) were inoculated in 50 ml of TAP media and cultured at 4, 15, and 25 °C with 200 rpm shaking under constant white light (40 μmol m^{-2} s^{-1}). The OD_{750} was measured every 24 h for 9 days. To identify the fatty acid profile, ArM0029B cells ($\sim 2 \times 10^5$) were incubated in 50 ml of nitrogen-deficient TAP (NH_4Cl was omitted from TAP salt) medium at 25 °C under the same conditions as mentioned above. Samples were collected at12 h and 15 h.

2.3. Fatty acid methyl ester (FAME) analysis by gas chromatography (GC)

Total lipids were extracted from 20 mg of freeze dried sample according to the method of Sasser (1990). Briefly, saponification was carried out with 2 ml of saponification reagent (7.5 M NaOH:CH_3OH, 1:1 v/v) at 100 °C for 30 min. For generation of FAMEs, 4 ml of methylation reagent (CH_3OH: 6 N HCl, 1:1 v/v) was added to the saponified sample and incubated at 80 °C for 10 min. After the reaction, 2.5 ml of extraction reagent (hexane:methyl tert-butyl ether, 1:1 v/v) was added and incubated for 10 min. The upper phase was recovered by centrifugation. Washing was performed with 6 ml of 0.5 M NaOH. The FAMEs were analyzed by GC (YL-6100GC, Young Lin Science, Korea) with a flame ionized detector (FID) and INNOWAX capillary column (Agilent, USA, 30 m × 0.32 mm × 0.5 μm). Each FAME component was identified and quantified using the Supelco® 37 Component FAME Mix (Sigma, USA).

2.4. Transmission electron microscopy (TEM)

Seven-day-old cells grown at 25 °C in nitrogen-deficient TAP media were rinsed twice with 250 mM phosphate buffer (pH 7.0)

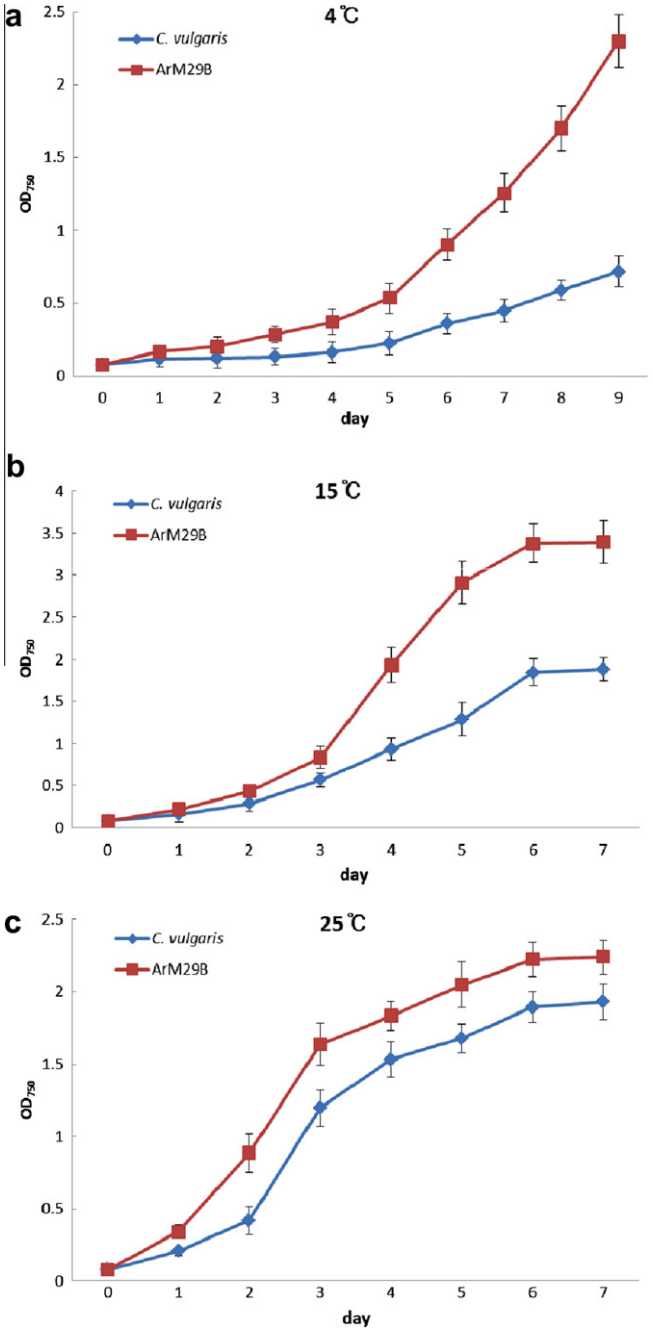

Fig. 1. Growth rate of ArM0029B at various temperatures. *C. vulgaris* served as a control. Data are expressed as ±SD ($n = 3$).

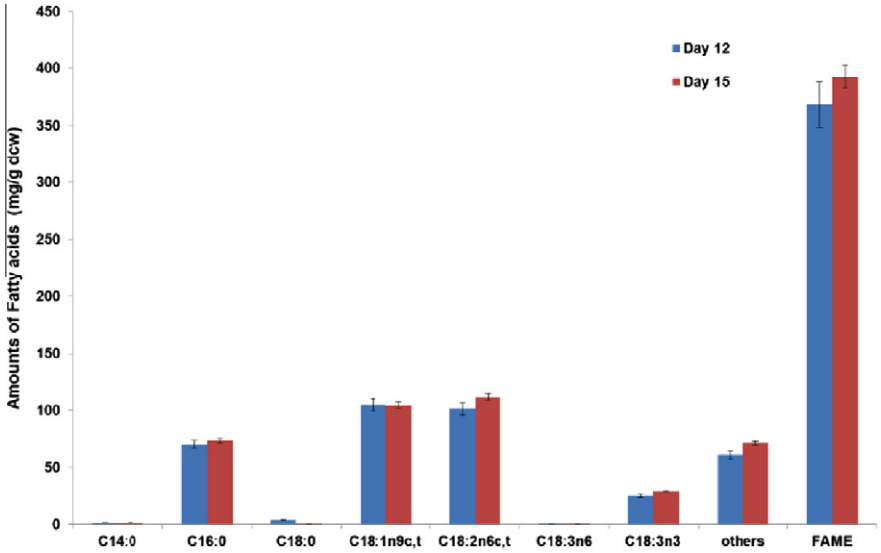

Fig. 2. Analysis of fatty acid composition in ArM0029B. Twelve- and fifteen-day-old cultures grown in nitrogen-deficient TAP media were used. Data are expressed as ±SD ($n = 3$). The amounts of each fatty acid and total fatty acids were presented as mg/dry cell weight.

and then fixed with 2.5% (v/v) glutaraldehyde and 1% (v/v) osmium tetraoxide. Dehydration was carried out using a graded ethanol series (50–100%). Specimens were embedded in Spurr's resin. Thin sections were prepared using an ultramicrotome (Ultracut UCT, LEICA, Germany) and stained with uranyl acetate. Specimens were observed using a transmission electron microscope (Tecnai, FEI, Netherlands).

2.5. Detection of lipid droplet using Nile red staining

To detect lipid droplets, cells were stained with Nile red (1 μg/ml, 9-diethylamino-5H-benzo [a]phenoxazine-5-one; Sigma–Aldrich, USA) in acetone and then incubated for 30 min. The stained cells were observed using a Zeiss LSM 5 PASCAL confocal microscope system with PASCAL version 4.0 software (Jena, Germany). The microscope was fitted with LASOS HeNe 543 nm and Argon 488 nm scanning lasers (Jena, Germany).

3. Results

The *rbc*L sequence of ArM0029B displayed high identity with those of *Chlorella* sp.; the sequence showed 96% and 94% identity with *Chlorella pyrenoidosa* and *C. vulgaris*, respectively (Supplemental Fig. 1). However, less than 90% identities of *rbc*L genes were detected between ArM0029B and other microalgae, including *Chlamydomonas* and *Senedesmus* (data not shown). These data indicate that ArM0029B would be classified as belonging to the genus *Chlorella* sp. To identify the optimal growth temperature, the growth rate of cells was determined under various temperatures (Fig. 1). Arctic *Chlorella* sp. ArM0029B displayed significantly more rapid growth in TAP media at 4 °C compared to the *C. vulgaris* control (Fig. 1a); at day 9, optical density (OD) value of ArM0029B was reached to 2.29 ($\sim 4.2 \times 10^7$ cells/ml^{-1}), whereas the control showed relatively low OD value of 0.72 ($\sim 9.5 \times 10^6$ cells ml^{-1}). Growth of ArM0029B at 15 °C was faster than the control (Fig. 1b); OD values of ArM0029B and the control at day 6 were 3.37 ($\sim 6.7 \times 10^7$ cells/ml^{-1}) and 1.84 ($\sim 2.9 \times 10^7$ cells ml^{-1}), respectively. Interestingly, ArM0029B was able to grow at 25 °C (Fig. 1c), although the natural habitat of this *Chlorella* sp. is in the extreme cold. Furthermore, outdoor culture of ArM0029B was successful, with the maximum temperature for culture ~32 °C under sunlight (data not shown).

The ArM0029B cells are spherical in shape and 4–8 μm in diameter without flagella (Supplemental Fig. 2a). To determine lipid accumulation in ArM0029B cells under nitrogen-deficient conditions, analyses of TEM and Nile red staining were performed. High levels of accumulation of both starch granules and lipid droplets were found in ArM0029B cells (Supplemental Fig. 2b); Supplemental Fig. 2c also shows abundant accumulation of lipid droplets by Nile red staining. Using GC, the profile and content of fatty acids were analyzed. The total amount of fatty acids was ~39% of dry cell weight (Fig. 2), with the major components 16:0, 18:1, and 18:2. The proportion of 18:2 was ~28% of total fatty acids, and 18:1 and 16:0 constituted ~26% and ~17% of total fatty acids, respectively. Interestingly, PUFA (three or more double bonds) were detected as only ~7% of total fatty acids (Fig. 2).

4. Discussion

ArM0029B displayed growth over a broad range of temperatures from 4 to 32 °C (Fig. 1, data not shown). A previous study reported two *Chlorella* sp. isolated from the Antarctic region that grew at temperatures ranging from 4 to 30 °C (Teoh et al., 2004), and Seaburg et al. (1981) also reported Antarctic algae that could grow at high temperatures. These previous studies support the eurythermal character of Arctic *Chlorella* sp. ArM0029B. It is known that the growth temperature of algae is one of the most important parameters in algae culture (Andersen, 2005). Thus, the eurythermal property of ArM0029B suggests that these algae can be cultured cost-effectively for biodiesel production without temperature control, especially in a cold climate.

A technical issue of biodiesel derived from microalgae is the SFA and PUFA that are found simultaneously in algal oils, which affect the cold flow properties and oxidative stability of biodiesel, respectively (Knothe, 2011). In contrast, vegetable oils from soybean and sunflower are enriched in both 18:1 (one-double bond) and 18:2 (two-double bonds). In this study, total fatty acid content in ArM0029B was ~39% of dry cell weight when grown in nitrogen-deficient TAP medium, with the proportion of 18:1 and 18:2 reaching ~26% and ~28% of total fatty acids, respectively (Fig. 2). Furthermore, PUFA were only ~7% of total fatty acids from ArM0029B, indicating that the oil is less susceptible to oxidation. *Chlorella* sp., including *C. vulgaris*, contain approximately 23–34% PUFA of total fatty acids (Matucha et al., 1972; Zhukova and

Aizdaicher, 1995). These results suggest that ArM0029B may be a suitable source for biodiesel, and can minimize the problems of cold flow properties and oxidative stability of the algal oil.

5. Conclusions

Algal oil composition and productivity are key parameters in determining commercial use of algae for biodiesel production. The psychrotrophic character of ArM0029B may offer year-around culture without temperature control, suggesting economic feasibility for biodiesel production. In addition, high amounts of 18:1 and 18:2 in ArM0029B may increase kinematic viscosity of biodiesel, and relatively low levels of PUFA can enhance oxidative stability. Thus, Arctic *Chlorella* sp. ArM0029B may be a valuable candidate for biodiesel production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.09.026.

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