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Growth and lipid content at low temperature of Arctic alga *Chlamydomonas* sp. KNM0029C

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Abstract Biodiesel produced from microalgae is a promising source of alternative energy. In winter, however, outdoor mass cultivation for biodiesel production is hampered by poor growth. Here, we report that Arctic *Chlamydomonas* sp. KNM0029C exhibits optimal growth at 4 °C and reaches densities up to 1.4×10^7 cells mL^{-1}. Lipid body formation in the alga was visualized through BODIPY 505/515 staining and fluorescence microscopy. The fatty acid methyl ester (FAME) production level of KNM0029C was 178.6 mg L^{-1} culture and 2.3-fold higher than that of *C. reinhardtii* CC-125 at 4 °C. Analysis of the FAME content showed a predominance of polyunsaturated fatty acids such as C16:3, C18:2, C18:3, and C20:2. C18:3 fatty acids comprised the largest fraction (20.7 %), and the content of polyunsaturated fatty acids (39.6 %) was higher than that of saturated fatty acids (6.8 %) at 4 °C. These results indicate that *Chlamydomonas* sp. KNM0029C, as a psychrophilic microalga, might represent a favorable source for biodiesel production in cold environments.

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Introduction

Fossil fuels such as petroleum, coal, and natural gas dominate global energy consumption, but fossil fuel reserves are predicted to be exhausted within a few years due to rapid, worldwide industrialization [1], and thus other sources of fuel are in high demand. For the production of alternative biofuels, microalgae represent a promising source as a first-generation feedstock owing to their high growth rate, excellent CO_2-fixation ability, and high biomass productivity in comparison to terrestrial crops [2]. The fatty acids produced by microalgae can be converted into renewable, biodegradable biodiesels that exhibit low toxicity [1]. Microalgae are also attractive because of their high commercial potential for use in functional foods, pharmaceuticals, and livestock feed.

The Arctic is characterized by the lowest temperatures on earth, but despite the prevalent harsh conditions, the polar region possesses a rich diversity of microorganisms [3]. To survive extremely cold environments, microalgae produce unique biochemical substances such as antifreeze proteins, ultraviolet radiation-screening compounds, and antioxidants [4, 5]. Therefore, Arctic microalgae might provide a favorable resource for alternative energy and wastewater treatment.

Large-scale cultivation studies have shown that biomass productivity is influenced substantially by culture conditions such as temperature and light intensity [6]. Outdoor cultivation at low temperatures presents the particular drawback of increasing the cost of maintaining optimal growth temperatures. Cultivation of polar psychrophilic

strains can reduce the costs of mass production at low temperature; however, few reports have been published on mass-scale culture of psychrophilic microalgae. Production of highly valuable compounds and bioenergy requires the development of culture systems for psychrophilic microalgae.

We screened a collection of polar microalgae to identify producers of valuable lipids and identified *Chlamydomonas* sp. KNM0029C from the Arctic Ocean. In this study, we evaluated the potential utility of *Chlamydomonas* sp. KNM0029C as a source for biofuel production in the context of fatty acid content and growth at low temperatures.

Materials and methods

Isolation and purification of microalgal strains

The Arctic microalga *Chlamydomonas* sp. KNM0029C (formerly KOPRI-ArM0029C) was isolated from sea ice near the Dasan station in Ny-Ålesund, Spitsbergen, Norway (78°55′N, 11°56′E). Samples were inoculated in autoclaved Bold's basal medium (BBM) [7] at 2–3 °C under white light and purified through serial dilution followed by plating on BBM agar, and then single green colonies were picked and cultured in BBM broth. *Chlamydomonas reinhardtii* CC-125 (UTEX 2244) was obtained from the UTEX Culture Collection of Algae (Austin, TX, USA) and maintained on Tris–acetate-phosphate (TAP) medium.

Morphological and molecular identification

Morphological characteristics of KNM0029C were investigated under a light microscope (Axio Imager.A2, Zeiss Co., Germany) equipped with Nomarski differential interference optics. Images were captured using a camera (AxioCam HRc, Zeiss Co.), and the sizes of cells were calculated using an image analyzer (AxioVision SE64 Rel. 4.8, Zeiss Co.).

Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, MD, USA), and the nuclear SSU rDNA (Small Subunit ribosomal DNA) was amplified with primers G01 (5′-CACCTGGTTGATCCTGCCAG-3′) and G07 (5′-AGCTTGATCCTTCTGCAGGTTCACCTAC-3′) and ExTaq polymerase (Takara, Japan) and then sequenced (Macrogen, Seoul, Korea). The sequences obtained using an ABI 3730xl DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) were aligned using the Genetic Data Environment (GDE 2.2) program [8], and MODELTEST ver. 3.7 [9] was used to determine the optimal model for the data. The molecular identification was performed using Bayesian [10] and Randomized Axelerated Maximum Likelihood (RAxML) [11] methods.

Culture conditions

To determine the optimal medium and temperature for growth, samples were cultured in F/2 medium [12, 13], BBM, TAP medium [14], and Jaworski's medium (JM) [15]. Approximately 2.5×10^5 cells mL^{-1} were inoculated in each medium at 8 °C and cultured for 2 weeks. Growth was attempted under static conditions at 4, 8, 12, and 20 °C under cool white fluorescent lamps (40 μmol photon m^{-2} s^{-1}) on a 16:8 h light–dark cycle, and the growth of KNM0029C was compared with that of the reference strain *C. reinhardtii* CC-125 at 4 and 20 °C. Cells were counted using a hemocytometer and an optical microscope (Zeiss Axio Imager.A2). For the nitrogen-starvation study, cultures were centrifuged at 4000 rpm for 5 min at 4 °C, and the cell pellets were retained and washed twice in TAP medium without nitrogen (TAP-N). The pellets were then resuspended in TAP-N for further growth.

BODIPY 505/515 staining

Cultures were stained with BODIPY 505/515 (0.2 μg mL^{-1}, 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene, Invitrogen, USA) dissolved in anhydrous dimethyl sulfoxide and incubated for 10 min at room temperature. The lipid bodies and cell morphology were examined using a laser-scanning confocal microscope (LSM 510 META) and analyzed using LSM 510 software version 4.2 (Zeiss Co., Oberkochen, Germany). The BODIPY 505/515 signal and chlorophyll fluorescence were captured using laser excitation at 488 nm and 633 nm, respectively.

Fatty acid methyl ester (FAME) analysis

Total lipids were extracted from 20 mg of freeze-dried samples, as described by Sasser [16]. Saponification was performed with 2 mL of a saponification reagent (7.5 M NaOH:CH_3OH, 1:1 v/v) at 100 °C for 30 min, and after the samples were cooled, a methylation reagent was added (4 mL; CH_3OH:6 N HCl, 1:1 v/v) and incubated at 80 °C for 10 min. The reactions were then treated with 2.5 mL of extraction reagent (hexane:methyl tert-butyl ether, 1:1 v/v) and incubated for 10 min. The lower aqueous phase from the samples was removed by pipetting and 6 mL of 0.5 M NaOH was added to the organic phase. Fatty acids in the organic phase were analyzed by using a gas chromatography system (YL-6100GC, Young Lin Science, Korea) equipped with a flame-ionization detector (FID) and HP-

FFAP capillary column (30 m × 0.32 mm × 0.25 μm, Agilent, USA). FAME analysis was performed under the following conditions: (1) constant flow mode, 3 mL min^{-1}; (2) temperature, 100 °C for 5 min, then 4 °C min^{-1} to 240 °C, at which the program was held for 20 min; and (3) detector temperature, 250 °C. Each FAME component was identified and quantified using the Supelco 37 Component FAME Mix (Sigma, St. Louis, MO, USA). Additional components and molecular structures were identified by using a mass spectrometer (PerkinElmer Clarus 600T, Turku, Finland) with TurboMass ver. 5.2.0. (Perkin-Elmer, USA). The major methyl ester was purified by means of thin-layer chromatography and its molecular weight was estimated using LC/MS/MS (AB SCIEX Triple TOF 5600). Fatty acids were identified by comparing their molecular mass and quantified against the internal standard (1 mg of the fatty acid C16:0 in hexane), and the values were summed and expressed as milligrams FAME per gram dry cell weight.

Results

Cell morphology and phylogenetic relationship of the Arctic green microalga KNM0029C

The vegetative cells of KNM0029C were solitary, biflagellate, and ovoid in shape, and 10–14 μm long and 8–11 μm wide. The cells contained a single cup- or urn-shaped chloroplast, a prominent eyespot, a pair of flagella, and pyrenoids (Suppl. Figure 1). These distinct characteristics indicate that KNM0029C could belong, morphologically, to the group Euchlamydomonas [17].

The final SSU rDNA sequence of KNM0029C was aligned with those of 91 taxa including 84 species representing 39 genera in Chlamydomonales. A tree generated by means of Bayesian inference for the SSU data is presented with the bootstrap results (maximum likelihood; ML) from ML analysis and posterior probabilities (B) appended (Suppl. Figure 2). KNM0029C was allied to *Chlamydomonas coccoides* NIES-1021 from Osaka Bay, Japan, with strong support (ML = 82 %; $B = 0.93$), and these two taxa were grouped with *Borodinellopsis texensis*, *Chlamydomonas parkeae*, *Chlorococcum brevispina*, *Oophila amblystomatis*, *Radiosphaera negevensis*, *Spongiococcum tetrasporum*, and *Tetracystis aeria* with strong support (ML = 88 %; $B = 1.00$).

Numerous molecular phylogenetic analyses have shown that the genus *Chlamydomonas* is highly polyphyletic. In our molecular data, KNM0029C was not grouped with *C. reinhardtii*, the type species of the genus in the "*Reinhardtii*" clade. Nevertheless, KNM0029C does not differ from *Chlamydomonas* spp. in morphological characteristics, and therefore we identified KNM0029C as a species of *Chlamydomonas* in this study.

Determination of optimal media and growth temperature

The growth of the Arctic microalga was measured by counting cells after culturing in 4 selective media: F/2, representing a seawater medium; and JM, TAP, and BBM, representing freshwater media. KNM0029C grew in both freshwater and seawater media, but yielded the highest cell density in TAP medium (Fig. 1a). When KNM0029C was cultured for 60 days at 4, 8, 12, and 20 °C in TAP medium, the maximum yield ($\sim 1.4 \times 10^7$ cells mL^{-1}) was obtained at 4 °C (Fig. 1b); by contrast, no KNM0029C growth was observed at 20 °C after 7 days (Fig. 1b).

Figure 2 shows the microalgal growth of KNM0029C and *C. reinhardtii* CC-125 at 4 and 20 °C. The cell density of KNM0029C was sevenfold higher than that of *C. reinhardtii* CC-125 at 4 °C, and the cell number of KNM0029C increased even under nitrogen-deficient conditions (TAP-N).

Fatty acid production and composition

Microalgae accumulate starch and synthesize neutral lipids in an N-depleted medium [18]. Here, lipid accumulation was examined using BODIPY 505/515 staining and fluorescence microscopy. In KNM0029C and *C. reinhardtii* CC-125, lipid accumulation was detected at 4 °C after 2 weeks under N-starvation (Fig. 3). However, KNM0029C contained more intracellular lipid bodies than *C. reinhardtii* CC-125 at 4 °C (Fig. 3), and larger lipid droplets were observed in KNM0029C after cultivation at 8 °C than at 4 °C (Fig. 4).

Supplementary Fig. 3 shows the FAME concentration in weight (mg) per dry cell weight (DCW, g) or production per unit volume of culture (L) at 4 °C. The FAME contents of KNM0029C and *C. reinhardtii* CC-125 were 165.4 and 153.7 mg g^{-1} DCW, respectively, and their respective FAME production levels were 178.6 and 78.4 mg L^{-1} culture. The FAME production level of KNM0029C was 2.3-fold higher than that of *C. reinhardtii* CC-125 at 4 °C.

N-starvation of KNM0029C yielded a fatty acid composition that was dominated by C18:3. Fatty acids in 4 and 8 °C cultures were rich in polyunsaturated fatty acids (PUFAs) such as C16:3, C18:2, C18:3, and C20:2 (Suppl. Figure 4). Conversely, at 4 and 8 °C, the levels of monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) were lower than those in cultures grown at 12 °C. Specifically, the C18:3 proportion reached 20.7 and 20.6 % at 4 and 8 °C, respectively. PUFAs were accumulated at 4 °C and reached 39.6 % in psychrophilic KNM0029C cultured at 4 °C (Suppl. Figure 4).

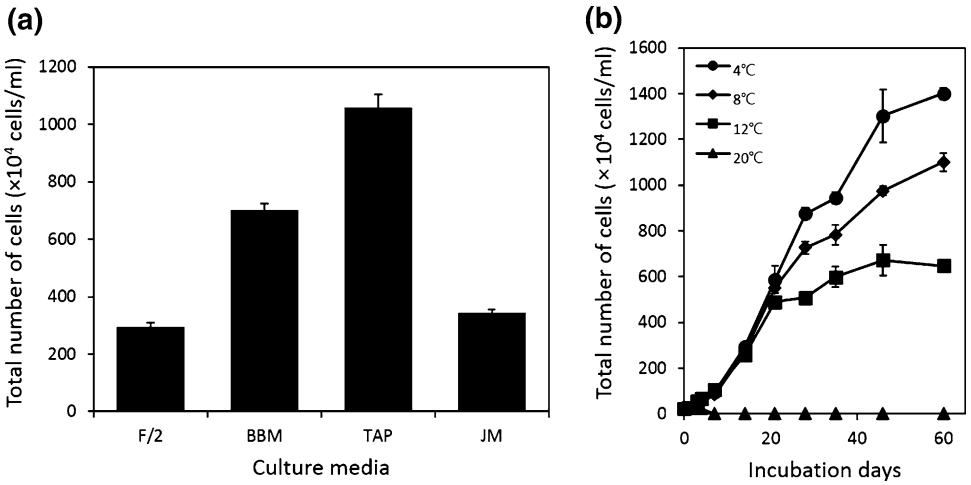

Fig. 1 Effects of medium and temperature on the growth of *Chlamydomonas* sp. KNM0029C. **a** Total number of cells in different growth media: F/2, as a seawater medium; and BBM, TAP, and JM, as freshwater media. Approximately 2.5×10^5 cells mL^{-1} were inoculated in each medium at 8 °C for 2 weeks. **b** Growth curves of microalgae at various temperatures. KNM0029C cells were cultured for 60 days at 4, 8, 12, and 20 °C in TAP. Data shown are the average cell densities ± standard deviation (SD) from replicates

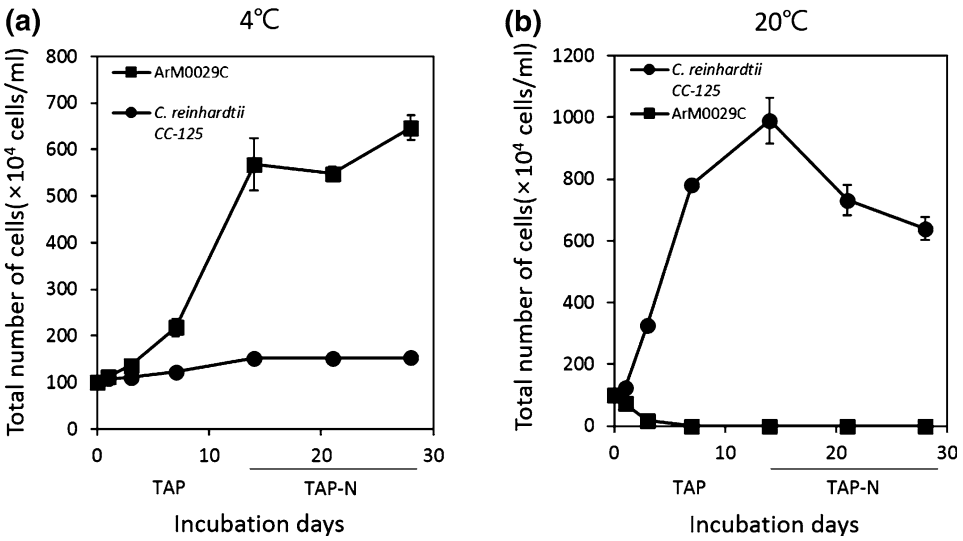

Fig. 2 Effects of temperature on the growth of KNM0029C and *Chlamydomonas reinhardtii* CC-125. **a** 4 °C; **b** 20 °C. Approximately 1×10^6 cells mL^{-1} were inoculated and grown in TAP for 2 weeks, and the cells were then cultivated in TAP-N for an additional 2 weeks. Data shown are the average cell densities ± SD from replicates

Discussion

Biodiesel is used as an alternative for diesel fuel in compression ignition engines. Biodiesel offers several advantages as compared to regular diesel, such as a higher cetane number, reduced emissions of particulates (NO_x, SO_x), and low toxicity [19]. Microalgae can produce considerable amount of lipids that can be converted into biodiesel. However, regions where the temperature is low (<12 °C) are not suitable for microalgal culture preparation with mesophilic strains. Moreover, only a few studies have been conducted on biofuel production by using psychrophilic microalgal strains to overcome the disadvantage of poor growth of mesophilic microalgae under cold conditions [20, 21].

The Arctic green microalga KNM0029C was identified here as a *Chlamydomonas* sp. based on SSU data. Interestingly, this marine strain showed a higher growth rate in freshwater media than in a seawater medium, and maximal growth and yield of the strain were obtained at 4 °C. These results indicate that KNM0029C can be categorized as a psychrophilic microorganism.

C. reinhardtii is a widely distributed microalgal species, and it has emerged as a model organism for studying the regulation of metabolic pathways or cultivation strategies aimed at enhancing lipid production. Although *C. reinhardtii* has not been considered an oleaginous microalga, the study of lipid biosynthesis in this species might provide information for large-scale biofuel production [22–24]. *C. reinhardtii* CC-125 was selected as the control strain for

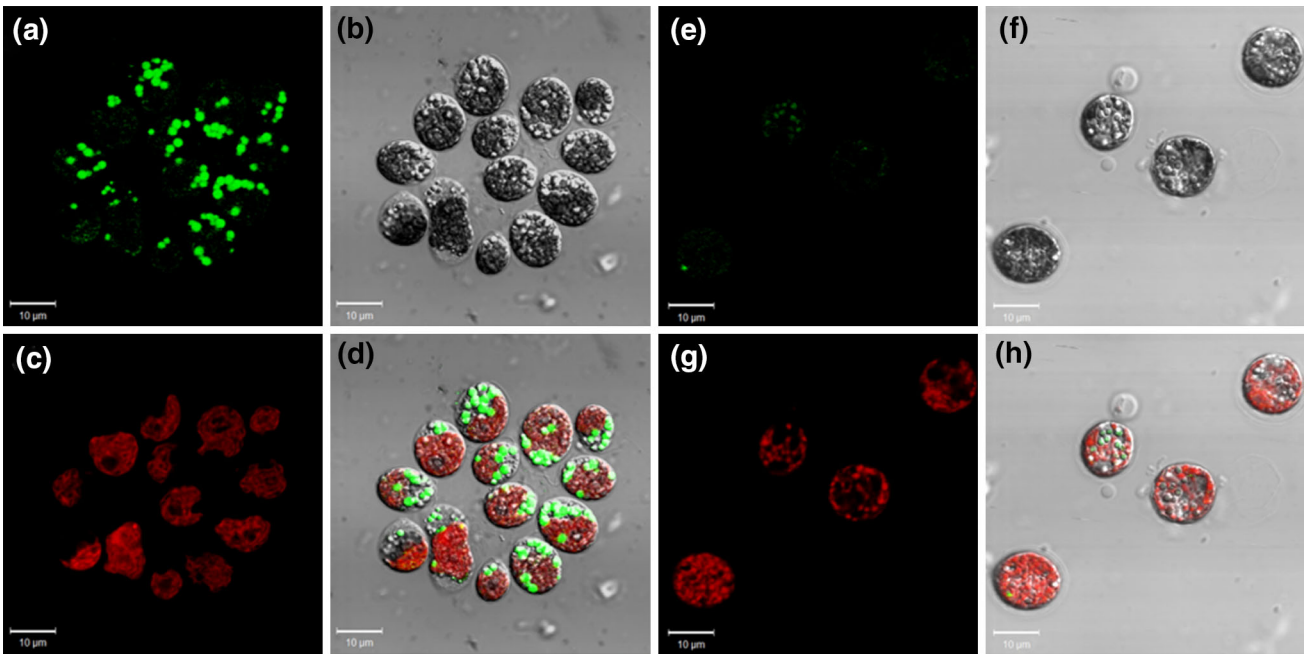

Fig. 3 Confocal microscopy images of BODIPY 505/515-stained *green* microalgae. **a**–**d** KNM0029C, **e**–**h** *C. reinhardtii* CC-125. **a**, **e** BODIPY 505/515 channel; *false-colored green*. **b**, **f** *Black-and-white* DIC microscopy images. **c**, **g** Chlorophyll autofluorescence channel. **d**, **h** Merged images of chlorophyll autofluorescence and BODIPY 505/515 images. The two microalgae were cultured in TAP-N for 2 weeks at 4 °C. *Scale bar* 10 μm (color figure online)

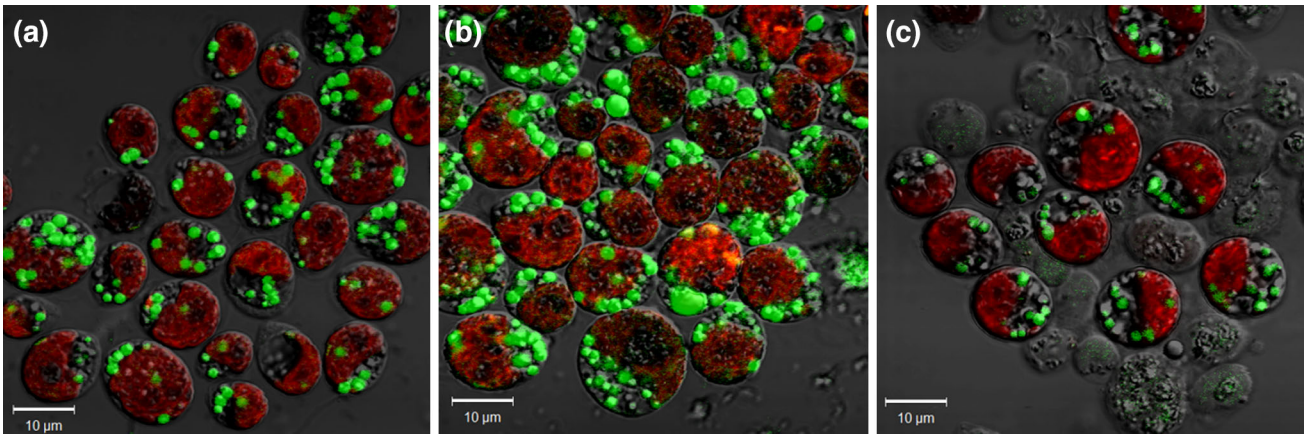

Fig. 4 Effect of cultivation temperature on the morphological changes of lipid bodies of KNM0029C. **a** 4 °C; **b** 8 °C; **c** 12 °C. KNM0029C was cultured in TAP-N for 2 weeks. *Green* BODIPY 505/515 fluorescence, *red* chlorophyll autofluorescence. *Scale bar* 10 μm (color figure online)

this study; KNM0029C and *C. reinhardtii* CC-125 belong to the same genus, and thus numerous genetic technologies developed in *C. reinhardtii* can be applied to KNM0029C for increasing lipid production.

KNM0029C was found to contain larger intracellular lipid bodies than *C. reinhardtii* CC-125 at 4 °C, and the lipid bodies in KNM0029C were larger at 8 °C than at 4 °C. The results of these morphological examinations suggest that KNM0029C could increase its total lipid content: temperatures above 4 °C were estimated to increase fatty acid synthesis. Recent studies on *C. reinhardtii* CC-125 (wild-type) revealed that the total fatty acid concentration of this microalga varied from approximately 100 to 150 mg g^{-1} DCW at 25 °C [22–24]. In this study, KNM0029C showed a biomass productivity of 0.97 g L^{-1} and a total fatty acid content of 165.4 mg g^{-1} DCW at 4 °C. By comparison, *C. reinhardtii* CC-125 showed a lower biomass productivity of 0.46 g L^{-1} and total fatty acid content of 153.7 mg g^{-1} DCW, as in previous studies [22–24]. These results suggest that KNM0029C can produce lipids at low temperature, even below 12 °C.

KNM0029C produced mainly C18:1, C18:3, and C20:2 fatty acids. The percentages of C18:3 and C20:2 were higher at 4 and 8 °C than at 12 °C. This increase in unsaturated fatty acids at low temperature is a common characteristic of microalgae. For example, PUFA levels in *Chaetoceros*, *Rhodomonas*, *Isochrysis*, and *Cryptomonas* are elevated in cultures grown at low temperatures [25]. Microalgae might survive at low temperatures due to the predominance of PUFAs, which increase membrane fluidity [26]. Fatty acid carbon-chain length and degree of unsaturation largely determine biodiesel properties such as viscosity, oxidative stability, cold flow, and cetane number [27]. High-quality biodiesels require sources that provide high lipid content and desirable fatty acids such as C14:0, C16:0, C16:1, C18:0, C18:1, and C18:2 [28]. In cold climates, an increased content of unsaturated fatty acids is required to enable fuels to perform at the prevailing low temperature [28]. In a cold region or in winter, it could be favorable to use biodiesel from KNM0029C, which contains a high level of unsaturated fatty acids such as C18:1, C18:3, and C20:2. A high level of unsaturation of fatty acids contributes toward lowering the cold filter plugging point (CFPP), which is used for evaluating the flow of biodiesel at low temperatures. In cold-climate countries, the use of CFPP values is crucial when selecting fuels because the clogging phenomenon can result in mechanical damage to vehicle engines [22]. The fatty acid composition of KNM0029C makes it a promising candidate for the production of biofuels in cold environments.

In conclusion, an Arctic microalgal strain identified as a *Chlamydomonas* sp. showed a growth temperature range from 4 to 12 °C and produced high concentrations of intracellular oils. Therefore, KNM0029C could represent a highly favorable candidate for producing biofuels in cold environments.

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