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Isolation, Identification, and Biochemical Characteristics of a Cold-Tolerant *Chlorella vulgaris* KNUA007 Isolated from King George Island, Antarctica

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Abstract: A cold-tolerant unicellular green alga was isolated from a meltwater stream on King George Island, Antarctica. Morphological, molecular, and biochemical analyses revealed that the isolate belonged to the species *Chlorella vulgaris*. We tentatively named this algal strain *C. vulgaris* KNUA007 and investigated its growth and lipid composition. We found that the strain was able to thrive in a wide range of temperatures, from 5 to 30 °C; however, it did not survive at 35 °C. Ultimate analysis confirmed high gross calorific values only at low temperatures (10 °C), with comparable values to land plants for biomass fuel. Gas chromatography/mass spectrometry analysis revealed that the isolate was rich in nutritionally important polyunsaturated fatty acids (PUFAs). The major fatty acid components were hexadecatrienoic acid (C16:3 ω3, 17.31%), linoleic acid (C18:2 ω6, 8.52%), and α-linolenic acid (C18:3 ω3, 43.35%) at 10 °C. The microalga was tolerant to low temperatures, making it an attractive candidate for the production of biochemicals under cold weather conditions. Therefore, this Antarctic microalga may have potential as an alternative to fish and/or plant oils as a source of omega-3 PUFA. The temperature tolerance and composition of *C. vulgaris* KNUA007 also make the isolate desirable for commercial applications in the pharmaceutical industry.

Keywords: *Chlorella vulgaris*; cold-tolerant; PUFAs (polyunsaturated fatty acids); calorific value

1. Introduction

Green microalgae (Chlorophyta) can be found in almost every conceivable environment from polar regions to deserts; they play a pivotal role in global carbon, nitrogen, and phosphorus cycles [1–3]. Due to their remarkable tolerance to harsh conditions combined with rapid growth rates, phototrophs are often dominant in the cryosphere, representing a large portion of the total ecosystem biomass [4–6].

Microalgae have unique characteristics that enable them to succeed in frozen environments, including a high tolerance to adverse weather conditions and a lack of unfrozen water.

Accordingly, microalgae that inhabit the cryosphere, including cyanobacteria, have received considerable interest. Recent studies have assessed algal diversity in Antarctica using polymerase chain reaction-based methods [7–9]. Molecular phylogenetic techniques have been successfully applied to investigate the hidden microbial communities within Antarctic ecosystems; however, the isolation and characterization of individual algal strains remain important for many areas of research and various applications [10–12].

Chlorella (Chlorophyta) was first isolated by Dutch microbiologist Beijerinck in 1890. The genus is widely distributed and can be found thriving in freshwater, marine, and soil environments. There are currently 37 taxonomically acknowledged species in the genus *Chlorella*; these single-celled organisms consist of a spherical or oval cell, containing a single cup-shaped chloroplast with pyrenoids. *Chlorella vulgaris* range from 2 to 10 µm in diameter, lack flagella, and reproduce asexually, forming daughter cells within the parental cells through mitosis [13–15]. *Chlorella* species are regarded as promising biological resources due to their high photosynthetic abilities and rapid growth rates. In particular, *C. vulgaris* is commercially produced for applications in the food industry due to its high protein content, as well as to create specialty oils used in the cosmetic and nutraceutical industries [16]. Due to the increasing demand for the bioengineering of microalgae in recent years, whole-genome and transcriptome studies have been extensively conducted using next generation technologies in order to reveals potential genes for better utilization of high-value compounds from commercially important microalgae [15,17–20].

However, members of the genus *C. vulgaris* can be difficult to differentiate from the genus *Micractinium* because they share similar morphological traits. The characteristic bristles that are important species-specific characteristics of the genus *Micractinium* can be easily missed since some *Micractinium* species do not produce bristles when they are not exposed to grazing zooplankton pressure or harsh conditions [21]. The similarities of these taxa can cause confusion for those attempting to identify the two genera using morphology alone. Despite the ecological and economic importance of *Chlorella*, the relevant research has been shown little attention. In addition to the commonly used region for molecular identification, recent studies have discovered that a secondary structure of internal transcribed spacer 2 (ITS-2) can be used to define precisely between the two morphologically ambiguous genera [22]. Precise identification based on characteristics studied in various aspects provides concrete evidence for further research and potential applicability. As such, polyphasic studies to delineate culturable cold-tolerant Antarctic microalgae will provide a better understanding of algal diversity and physiology as well as biotechnological potential.

In this paper, we describe the isolation of a unicellular microalga from temporal meltwater on King George Island and the determination of its phylogenetic position using several molecular markers and morphological traits. The phylogenetic position was investigated using small subunit (SSU) rRNA sequence analysis and ITS-2 secondary structure prediction. Physicochemical and chemotaxonomic characteristics were also analyzed to determine the isolate's potential for biotechnological applications.

2. Materials and Methods

2.1. Sample Collection and Isolation

Antarctic freshwater bloom samples were collected in January 2010 from temporal water runoff near King Sejong Station (62° 13'S, 58° 47'W) located on the Barton Peninsula, King George Island, South Shetland Islands, West Antarctica. Samples were then transported to the laboratory and 1 mL of each sample was inoculated into 100 mL BG-11 medium [23]. The broad-spectrum antibiotic imipenem (JW Pharmaceutical, Seoul, Korea) was added to the medium at a concentration of 100 µg mL^{-1} to prevent bacterial growth. The inoculated flasks were incubated on an orbital shaker (Vision Scientific, Bucheon, Korea) at 160 rpm and 15 °C under cool fluorescent light (approximately 70 µmol photon

m^{-2} s^{-1}) under a 16:8 light/dark cycle until algal growth was apparent. Well-developed algal cultures (1.5 mL) were centrifuged at 3000× *g* for 15 min to harvest the algal biomass. The resulting pellets were streaked onto BG-11 agar supplemented with imipenem (20 µg mL^{-1}) and incubated under the aforementioned conditions. A single colony was then aseptically re-streaked onto a fresh BG-11 plate to obtain an axenic culture.

2.2. Morphological and Molecular Identification

The isolate was grown in BG-11 medium for 20 days. Live cells were harvested by centrifugation at 3000× *g* for 5 min, washed twice with sterile distilled water, and examined at 1000 × magnification using a Zeiss Axioskop 2 light microscope (Carl Zeiss, Standort Göttingen, Vertrieb, Germany) equipped with differential interference contrast optics.

For scanning electron microscope (SEM) analysis, 10 mL of cells were fixed in osmium tetroxide (OsO_4; Electron Microscopy Sciences, EMS hereafter, Hatfield, PA, USA) for 10 min at a final concentration of 2% (*v*/*v*) in distilled water. Fixed cells were collected on a 3 µm pore polycarbonate membrane filter without additional pressure, then rinsed with distilled water to remove residual salts. Cells were dehydrated in an ethanol series (Merck, Darmstadt, Germany) and dried using a critical point dryer (CPD 300, Bal-Tec, Balzers, Liechtenstein). The dried filters were mounted on a stub and coated with gold–palladium using a sputter coater (SCD 005; Bal-Tec). Cell images were analyzed with a field emission (FE)-SEM (S-4800; Hitachi, Hitachinaka, Japan).

For molecular analysis, genomic DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Hilden, Germany). The universal primers NS1 and NS8 described by White et al. [24] were used for 18S rRNA sequence analysis. The D1–D2 region of the large subunit (28S) rRNA gene was amplified using NL1 and NL4 primers [25], and the internal transcribed spacer (ITS) region was amplified using ITS1 and ITS4 primers [24]. The DNA sequences obtained in this study were deposited in the National Center for Biotechnology Information database under accession numbers KJ148623 to KJ148625 (Table 1). Phylogenetic analysis was performed with the 18S rDNA string sequences using the software package MEGA version X [26]. The combined datasets of *C. vulgaris* strains and *Neochloris* sp. (as an outgroup) were aligned with those of the 20 chlorellacean microalgae strains using ClustalW in MEGA X. The 18S rDNA sequence lengths ranged from 1697 bp to 1732 bp. The best-fit nucleotide substitution model K2 was selected using MEGA X based on the Bayesian information criterion. This model was used to build a maximum likelihood (ML) phylogenetic tree with 1000 bootstrap replicates [27]. The ITS-2 secondary structures were constructed using Mfold [28] according to Germond et al. [29].

Table 1. Results from BLAST searches using sequences of small subunit rRNA (18S rRNA), internal transcribed spacer (ITS), and large subunit rRNA (LSU) genes from strain KNUA007.

Marker Gene	Accession no.	Product Size (bp)	Closet Match (GenBank Accession no.)	Query Cover (%)	Identification (%)
18S rRNA	KJ148623	1771	*C. vulgaris* CCAP 211/19 (MK541792)	100	99.89
ITS	KJ148624	783	*C. vulgaris* ATFG2 (MT137382)	100	99.87
LSU	KJ148625	612	*C. vulgaris* NIES:227 (AB237642)	100	99.84

2.3. Temperature Testing

Late exponential-phase cultures of *C. vulgaris* KNUA007 (1 mL each) were inoculated into BG-11 medium in triplicate and incubated for 20 days. The survival and growth of KNUA007 cells maintained at temperatures ranging from 5 to 35 °C (at intervals of 5 °C) were examined to determine the optimum culture temperature. Algal cell density was determined by measuring the optical density (OD) of the cultures at 680 nm with an Optimizer 2120UV spectrophotometer (Mecasys, Daejeon, Korea). We also incubated samples in BG-11 medium in a PhotoBiobox (Shinhwa Science, Daejeon, Korea) at

temperatures ranging from 6 to 28 °C and light intensity ranging from 50–400 μmol photon m^{-2} s^{-1} with cycles of 24 h light for 3 days to monitor the optimal conditions. OD at 680 nm was measured using a SpectraMax i3x microplate reader (Molecular Devices, San Jose, CA, USA) and OD values were visualized on a heat map by SigmaPlot 13 software (Systat Software, San Jose, CA, USA) [30].

2.4. Biomass Characterization

The isolates were autotrophically grown in BG-11 medium for 20 days and cells were harvested by centrifugation at 2063 *g* (1580R, Labogene, Daejeon, Korea). The freeze-dried biomass samples were pulverized with a mortar and pestle and sieved through ASTM No. 230 mesh (63 μm holes). Ultimate analysis was conducted to determine the carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) contents using a Flash 2000 elemental analyzer (Thermo Fisher Scientific, Milan, Italy). Gross calorific value (GCV) was estimated using the following equation developed by Friedl et al. [31], and protein content was calculated from the N content using a conversion factor of 6.25 ×.

$$GCV = 3.55C^2 - 232C - 2230H + 51.2C \times H + 131N + 20{,}600 \text{ (MJ kg}^{-1}\text{)}$$

2.5. Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

Extracted fatty acid methyl ester (FAME) composition was analyzed using a gas chromatograph–mass spectrometer (GC/MS 7890A; Agilent, Santa Clara, CA, USA) equipped with a mass selective detector (5975C; Agilent) and DB-FFAP column (30 m, 250 μm ID, 0.25 μm film thickness; Agilent). The initial temperature of 50 °C was maintained for 1 min. The temperature was then increased to 200 °C at a rate of 10 °C min^{-1} for 30 min, then increased to 240 °C at a rate of 10 °C min^{-1} and held for 20 min. The injection volume was 1 μL, with a 20:1 split injection ratio. Helium was supplied as the carrier gas at a constant flow rate of 1 mL min^{-1}. For the mass spectrometer parameters, the injector and source temperatures were 250 and 230 °C, respectively, and the electron impact mode was used for sample ionization at an acceleration voltage of 70 eV, with an acquisition range of 50–550 mass to charge ratio [32]. All compounds were identified by analyzing their mass spectra with the Wiley/NBS libraries.

3. Results

3.1. Identification of Strain KNUA007

The cells were solitary, non-motile, and spherical, with a diameter of approximately 4–6 μm (Figure 1) and a prominent cup-shaped chloroplast. The cell walls were composed of a single smooth layer. The strain KNUA007 exhibited morphology typical of the genus *Chlorella*.

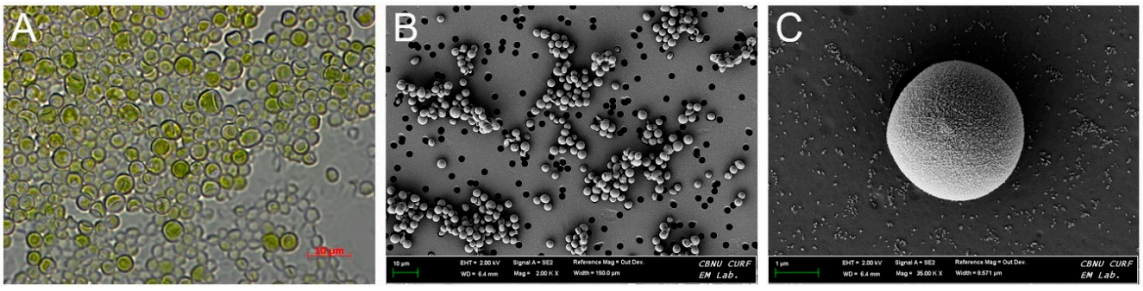

Figure 1. Light microscopy and FE-SEM images of *C. vulgaris* KNUA007.

Molecular characterization inferred from sequence analyses of the genes for 18S rRNA, 28S rRNA, and the ITS region confirmed that the isolate belonged to the *C. vulgaris* group (Table 1). As illustrated in Figure 2, strain KNUA007 was clustered with *C. vulgaris* in a different clade from *Micractinium* and *Parachlorella*. Therefore, the isolate was tentatively identified as *C. vulgaris* KNUA007.

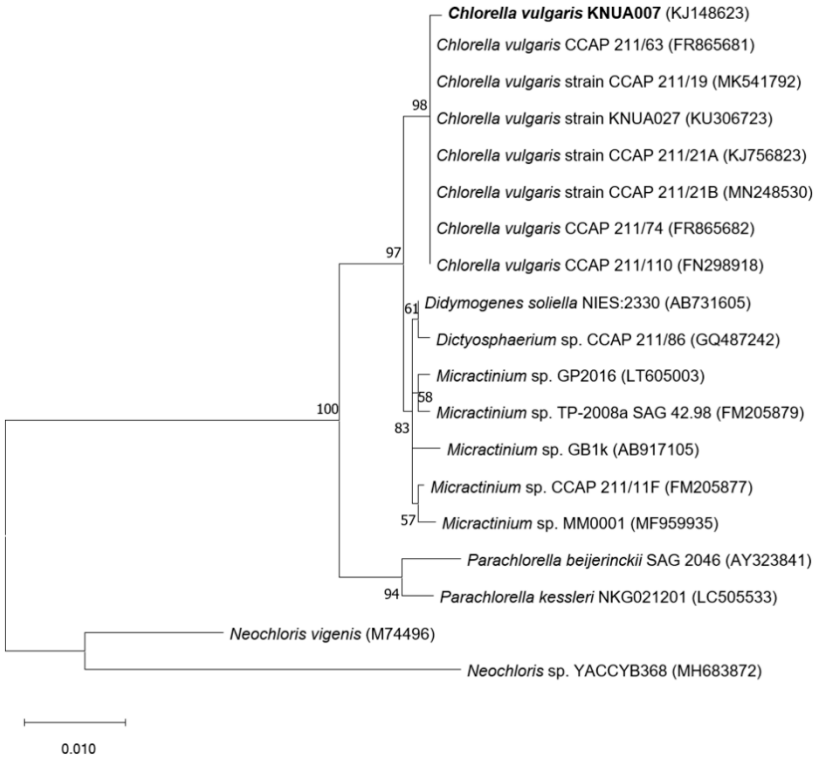

Figure 2. Maximum likelihood (ML) tree of small subunit rRNA genes. The numbers at the nodes indicate bootstrap probabilities (>50%) of ML analyses (1000 replicates). The scale bar represents a 0.1% difference.

To accurately differentiate between the morphologically ambiguous genera *Micractinium* and *Chlorella*, the secondary structure of ITS-2 was examined. Helix 3 of ITS-2 has a distinct molecular signature that separates *Chlorella* and *Micractinium*. Specifically, in Figure 3, the paired nucleotides indicated in the orange box are a synapomorphy of *Micractinium* [22]. Therefore, strain KNUA007 was tentatively identified as *C. vulgaris* KNUA007.

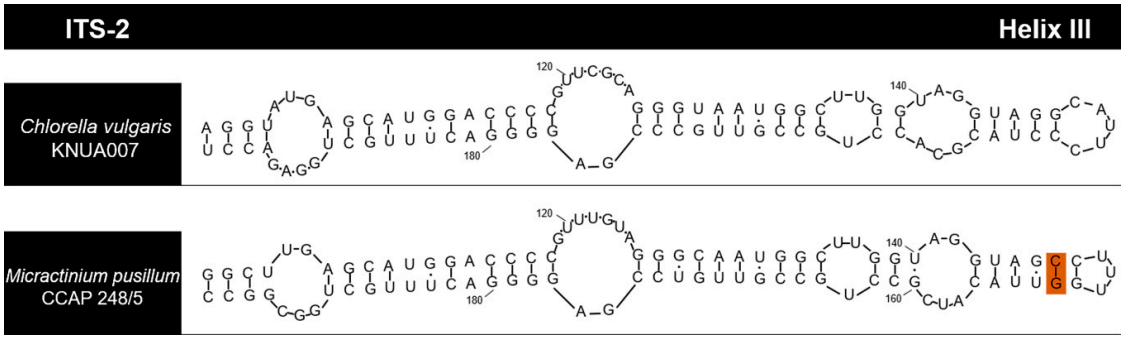

Figure 3. Comparison of the Helix 3 of ITS-2 rRNA secondary structures.

3.2. Cold Tolerance of Strain KNUA007

C. vulgaris KNUA007 could grow at temperatures ranging from 5 to 30 °C; maximum growth was observed at 20 °C. The cells grew and survived at 5, 10, 15, and 25 °C, but relatively slow growth was observed (Figure 4). However, the isolate did not survive at 35 °C. This strain also grew at a wide range of light intensity, from 50 to 400 µmol photon m^{-2} s^{-1}; maximum growth was observed at 150 µmol photon m^{-2} s^{-1}, and the strain preferred relatively low light intensity.

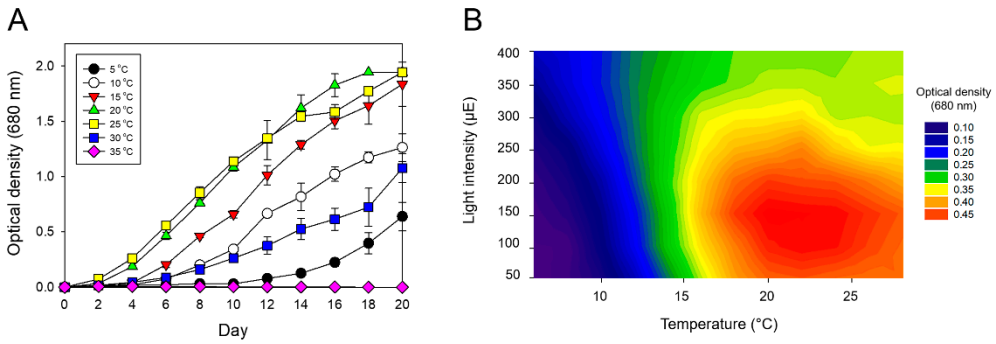

Figure 4. (**A**) Growth curves for KNUA007 cells maintained at various temperatures. (**B**) Optimal light intensity and temperature of KNUA007.

3.3. Biomass Properties

The C, H, N, and S contents were determined by elemental analysis (Table 2). C and N decreased in inverse proportion to the temperature increase, S increased with temperature, and H content was independent of the temperature gradient. This result affected the GCV values determined based on elemental analysis, and consequently, the GCV decreased with increasing temperature.

Table 2. Ultimate analysis and gross calorific value of *C. vulgaris* KNUA007.

	Contents (wt%)		
	10 °C	**20 °C**	**30 °C**
C	45.36 ± 0.06	42.94 ± 0.15	42.72 ± 0.26
H	6.64 ± 0.03	6.42 ± 0.05	6.54 ± 0.07
N	6.17 ± 0.00	5.84 ± 0.03	5.25 ± 0.02
S	0.76 ± 0.07	1.10 ± 0.03	1.14 ± 0.02
Gross calorific value (MJ kg^{-1})	18.7 ± 0.02	17.7 ± 0.06	17.5 ± 0.11

3.4. GC/MS Analysis of Strain KNUA007

The FAME profile of *C. vulgaris* KNUA007 is summarized in Table 3; values are presented as the average ± standard deviation of three determinations. The major cellular fatty acids of the microalgae were C16:3 ω3, C16:0, C18:2 ω6, and C18:3 ω3. The C18:3 composition was approximately 43% at 10 °C, decreasing to 36 and 20% with increasing temperatures. C16:3 also decreased more than five-fold with the temperature increase, similar to C18:3. Meanwhile, C16:2 and C18:2, which have two unsaturated chains, increased two and three times, respectively, and the other fatty acid contents did not change. The protein contents calculated using ultimate analysis were 38.56, 36.5, and 32.81% at 10, 20, and 30 °C, respectively.

Table 3. FAME profiles of *C. vulgaris* KNUA007.

	Contents (wt%)		
	10 °C	**20 °C**	**30 °C**
C15:0 (Pentadecanoic acid)	0.12 ± 0.01	0.15 ± 0.03	N.D
C16:0 (Palmitic acid)	18.55 ± 0.02	22.35 ± 0.48	25.02 ± 0.03
C16:1 (Palmitoleic acid)	0.51 ± 0.02	0.53 ± 0.08	0.55 ± 0.04
C16:2 (Hexadecadienoic acid)	2.29 ± 0.07	4.25 ± 0.33	5.16 ± 0.04
C16:3 (Hexadecatrienoic acid)	17.31 ± 0.19	8.08 ± 0.31	2.93 ± 0.11
C18:0 (Stearic acid)	1.30 ± 0.10	1.52 ± 0.05	2.36 ± 0.28
C18:1 (Oleic acid)	8.05 ± 0.16	9.00 ± 0.12	13.78 ± 0.46
C18:2 (Linoleic acid)	8.52 ± 0.08	17.97 ± 0.84	29.54 ± 0.17
C18:3 (α-Linolenic acid)	43.35 ± 0.05	36.14 ± 2.01	20.67 ± 0.42

N.D: Not detected.

4. Discussion

In this study, a cold-tolerant *C. vulgaris* strain, KNUA007, was axenically isolated using a combination of imipenem treatment and physical separation techniques. We analyzed the morphological, molecular, physiological, and biochemical characteristics of the isolates to identify the strain. The small and rounded cells lacked flagella and possessed the morphological features typical of *C. vulgaris*, including smooth cell wall layers and the presence of chloroplast pyrenoids (Figure 1). In addition, the phylogenetic position of the isolate was confirmed through sequence analysis of the SSU, ITS, and LSU regions (Table 1). As shown in Figure 2, the isolated strain was clearly separated from the genus *Micractinium* and was closely related to *C. vulgaris*. However, *Micractinium* has similar morphological features to the isolated strain [22], so the Helix 3 structure of the ITS-2 region was compared for further classification (Figure 3). Our analysis of the Helix 3 structure revealed that the strain used in this experiment did not possess the characteristic paired-nucleotide synapomorphy of *Micractinium* [22,29,33]. The isolate had a 100% sequence homology with *C. vulgaris* CCAP 211/63 (accession no. FR865681) (Figure 2) and the same ITS-2 overall sequence (data not shown); therefore, the strain used in this experiment was named *C. vulgaris* KNUA007.

The GCV was calculated to estimate the potential of this microalga as a biofuel feedstock. The results indicated that the GCV was similar to terrestrial energy crops and cellulosic biomass (17.0–20.0 MJ kg^{-1}) (Table 2). A number of previous studies have reported the GCVs of *Chlorella* strains under various autotrophic growth conditions, and the biomass samples were characterized in the range of 20.0–30.0 MJ kg^{-1} [34,35]. However, given the high photosynthetic efficiency and growth rate of this *C. vulgaris* strain, it could be a superior bioenergy source for biofuel production. We also measured protein using ultimate analysis; the protein content was over 30% at all temperature ranges and was highest at 10 °C. These results demonstrate the potential of *C. vulgaris* KNUA007 microalgal biomass for producing animal feedstock or bio-fertilizer.

Antarctica is one of the most extreme environments on the planet. Microalgae in Antarctic ecosystems are continuously exposed to direct sunlight during the summer months and to freezing temperatures during the winter months. Thus, Antarctic microalgae have developed a number of protection strategies to survive under these harsh conditions. In general, microalgae protect themselves from excessive light and oxidative damage by dissipating excess energy as heat, which has been termed non-photochemical quenching of chlorophyll fluorescence [36,37]. Moreover, some *Chlorella* species have been reported to express anti-freezing genes in response to low-temperature stress, enhancing their tolerance to freezing temperatures [38–40]. One of the most common self-defense mechanisms observed in microalgae to acquire cold tolerance is to increase the fluidity of lipid membrane, carried out by fatty acid desaturation, which increases the production of unsaturated fatty acids with lower chain lengths that remain in a liquid state at low temperatures [41,42]. The majority of fatty acids in *C. vulgaris* are C16 and C18, and the sequential desaturation of these fatty acids usually involves the following steps: C16:0 → C18:0 → C18:1 → C18:2 → C18:3 [43,44]. Analysis of the cellular fatty acid composition of the strain KNUA007 revealed that it was rich in C16:3, C18:2, and C18:3 unsaturated fatty acids (Table 3). In particular, C16:3 and C18:3, which have three unsaturated chains, increased in inverse proportion to the temperature; the C16:3 and C18:3 contents were more than twice as high at 10 °C than at 30 °C. As mentioned above, the content of polyunsaturated fatty acids is thought to increase as a defense mechanism to cope with low-temperature stress. Meanwhile, decreases in C18:1 and C18:2 were observed in the 10 °C culture of KNUA007 compared to those of the 20 °C culture of KNUA007. We presume that these slight decreases in C18:1 and C18:2 accounted for the degree of unsaturation, since C18:1 and C18:2 are intermediate forms of desaturation in the biosynthetic pathway of the major fatty acids by desaturases. On the other hand, the most unsaturated form of the major C18 fatty acids, C18:3, showed a significant increase in response to the low temperature. We assume C18:3 plays an important role in the fluidity modification capacity of *C. vulgaris* KNUA007. This kind of trend was also observed in *Scenedesmus acutus* [45]. Its fatty acid profile changed in response to temperature changes and the same pattern (increase in C18:3 composition in lower temperatures and increase

in C18:1 and C18:2 compositions in higher temperatures, respectively) as our study with *C. vulgaris* was also observed in their study. Numerous studies demonstrated that these essential PUFAs also have many beneficial health effects [46] and various commercial products are available worldwide. Omega-3 PUFAs are typically derived from fish oils and omega-6 PUFAs are primarily obtained from plant sources such as sunflower, corn, and soybean oils. Therefore, this isolate may have the potential to be used as an alternative to fish- and/or plant-based oil sources. In addition, the biomass itself may serve as an excellent animal feed because of its adequate protein content.

Lastly, it should be stated that most polar microalgae are known to be psychrotolerant; however, truly psychrophilic microalgae are relatively rare [47,48], and temperature flexibility is more common than psychrophily [49,50]. Likewise, strain KNUA007 was shown to be a psychrotolerant and mesophilic microalga that could grow at temperatures as low as 5 °C; optimum growth was observed at 20 °C (Figure 4). Furthermore, the strain grew over a wide temperature range (5–30 °C). This wide temperature tolerance is similar to those previously reported for Antarctic *Chlorella* strains [38,51]. The eurythermal properties of *Chlorella* may be advantageous for outdoor mass cultivation. The *C. vulgaris* KNUA007 isolate may be used for the production of value-added products under unfavorable weather conditions, including the cold temperatures that occur in autumn and winter seasons since ambient temperatures (approximately 20–35 °C) were the optimal growth conditions for most of *C. vulgaris* cultures for PUFA production [52]. In general, cell growth is suppressed at lower temperatures and the metabolic rate is accelerated at higher temperatures, but it is reported that temperatures above 35 °C usually inhibit growth [13].

In conclusion, this Antarctic microalga could serve as a potential biological resource to produce compounds of biochemical interest. The real potential of the isolate described in this paper should be evaluated through further cultivation studies at molecular, laboratory, and field scales.

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References

1. Komárek, J.; Nedbalová, L. Green cryosestic algae. In *Algae and Cyanobacteria in Extreme Envionments*; Seckbach, J., Ed.; Springer: Dordrecht, The Netherlands, 2007; pp. 323–344.
2. Lewis, L.A.; Lewis, P.O. Unearthing the molecular phylodiversity of desert soil green algae (Chlorophyta). *Syst. Biol.* **2005**, *54*, 936–947. [CrossRef] [PubMed]
3. Vincent, W.F. Cyanobacterial dominance in the polar regions. In *The Ecology of Cyanobacteria: Their Diversity in Time and Space*; Whitton, B.A., Potts, M., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; pp. 321–340.
4. Broady, P.A. Diversity, distribution and dispersal of Antarctic terrestrial algae. *Biodivers. Conserv.* **1996**, *5*, 1307–1335. [CrossRef]
5. Vincent, W.F.; James, M.R. Biodiversity in extreme aquatic environments: Lakes, ponds and streams of the Ross Sea sector, Antarctica. *Biodivers. Conserv.* **1996**, *5*, 1451–1471. [CrossRef]
6. Wynn-Williams, D.D. Antarctic microbial diversity: The basis of polar ecosystem processes. *Biodivers. Conserv.* **1996**, *5*, 1271–1293. [CrossRef]
7. De Wever, A.; Leliaert, F.; Verleyen, E.; Vanormelingen, P.; Van Der Gucht, K.; Hodgson, D.A.; Sabbe, K.; Vyverman, W. Hidden levels of phylodiversity in Antarctic green algae: Further evidence for the existence of glacial refugia. *Proc. R. Soc. B Biol. Sci.* **2009**, *276*, 3591–3599. [CrossRef]

8. Jungblut, A.D.; Vincent, W.F.; Lovejoy, C. Eukaryotes in Arctic and Antarctic cyanobacterial mats. *FEMS Microbiol. Ecol.* **2012**, *82*, 416–428. [CrossRef]
9. Wood, S.A.; Rueckert, A.; Cowan, D.A.; Cary, S.C. Sources of edaphic cyanobacterial diversity in the Dry Valleys of Eastern Antarctica. *ISME J.* **2008**, *2*, 308–320. [CrossRef]
10. Ferrara, M.; Guerriero, G.; Cardi, M.; Esposito, S. Purification and biochemical characterisation of a glucose-6-phosphate dehydrogenase from the psychrophilic green alga *Koliella antarctica*. *Extremophiles* **2013**, *17*, 53–62. [CrossRef]
11. Pulz, O.; Gross, W. Valuable products from biotechnology of microalgae. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 635–648. [CrossRef]
12. Řezanka, T.; Nedbalová, L.; Sigler, K. Unusual medium-chain polyunsaturated fatty acids from the snow alga *Chloromonas brevispina*. *Microbiol. Res.* **2008**, *163*, 373–379. [CrossRef]
13. Ru, I.T.K.; Sung, Y.Y.; Jusoh, M.; Wahid, M.E.A.; Nagappan, T. *Chlorella vulgaris*: A perspective on its potential for combining high biomass with high value bioproducts. *Appl. Phycol.* **2020**, *1*, 2–11. [CrossRef]
14. Safi, C.; Zebib, B.; Merah, O.; Pontalier, P.Y.; Vaca-Garcia, C. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. *Renew. Sustain. Energy Rev.* **2014**, *35*, 265–278. [CrossRef]
15. Cecchin, M.; Marcolungo, L.; Rossato, M.; Girolomoni, L.; Cosentino, E.; Cuine, S.; Li-Beisson, Y.; Delledonne, M.; Ballottari, M. *Chlorella vulgaris* genome assembly and annotation reveals the molecular basis for metabolic acclimation to high light conditions. *Plant J.* **2019**, *100*, 1289–1305. [CrossRef] [PubMed]
16. Ahmad, M.T.; Shariff, M.; Md. Yusoff, F.; Goh, Y.M.; Banerjee, S. Applications of microalga *Chlorella vulgaris* in aquaculture. *Rev. Aquac.* **2020**, *12*, 328–346. [CrossRef]
17. Merchant, S.S.; Prochnik, S.E.; Vallon, O.; Harris, E.H.; Karpowicz, S.A.; Witman, G.B.; Terry, A.; Salamov, A.; Fritz-Laylin, L.K.; Maréchal-Drouard, L.; et al. The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Natl. Inst. Heal.* **2007**, *318*, 245–250. [CrossRef]
18. Blanc, G.; Duncan, G.; Agarkova, I.; Borodovsky, M.; Gurnon, J.; Kuo, A.; Lindquist, E.; Lucas, S.; Pangilinan, J.; Polle, J.; et al. The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex. *Plant Cell* **2010**, *22*, 2943–2955. [CrossRef]
19. Vieler, A.; Wu, G.; Tsai, C.H.; Bullard, B.; Cornish, A.J.; Harvey, C.; Reca, I.B.; Thornburg, C.; Achawanantakun, R.; Buehl, C.J.; et al. Correction: Genome, functional gene annotation, and nuclear transformation of the Heterokont oleaginous alga *Nannochloropsis oceanica* CCMP1779. *PLoS Genet.* **2017**, *13*, e1006802. [CrossRef]
20. Arriola, M.B.; Velmurugan, N.; Zhang, Y.; Plunkett, M.H.; Hondzo, H.; Barney, B.M. Genome sequences of *Chlorella sorokiniana* UTEX 1602 and *Micractinium conductrix* SAG 241.80: Implications to maltose excretion by a green alga. *Plant J.* **2018**, *93*, 566–586. [CrossRef]
21. Luo, W.; Pflugmacher, S.; Pröschold, T.; Walz, N.; Krienitz, L. Genotype versus phenotype variability in *Chlorella* and *Micractinium* (Chlorophyta, Trebouxiophyceae). *Protist* **2006**, *157*, 315–333. [CrossRef]
22. Luo, W.; Krienitz, L.; Pflugmacher, S.; Walz, N. Genus and species concept in *Chlorella* and *Micractinium* (Chlorophyta, Chlorellaceae): Genotype versus phenotypical variability under ecosystem conditions. *SIL Proc.* **2005**, *29*, 170–173. [CrossRef]
23. Rippka, R.; Deruelles, J.; Waterbury, J.B.; Herdman, M.; Stanier, R.Y. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **1979**, *111*, 1–61. [CrossRef]
24. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: A Guide to Method and Applications*; Academic Press: Cambridge, MA, USA, 1990; pp. 315–322.
25. Marshall, M.N.; Cocolin, L.; Mills, D.A.; VanderGheynst, J.S. Evaluation of PCR primers for denaturing gradient gel electrophoresis analysis of fungal communities in compost. *J. Appl. Microbiol.* **2003**, *95*, 934–948. [CrossRef] [PubMed]
26. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
27. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783–791. [CrossRef] [PubMed]
28. Zuker, M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* **2003**, *31*, 3406–3415. [CrossRef]

29. Germond, A.; Hata, H.; Fujikawa, Y.; Nakajima, T. The phylogenetic position and phenotypic changes of a *Chlorella*-like alga during 5-year microcosm culture. *Eur. J. Phycol.* **2013**, *48*, 485–496. [CrossRef]
30. Heo, J.; Cho, D.-H.; Ramanan, R.; Oh, H.-M.; Kim, H.-S. PhotoBiobox: A tablet sized, low-cost, high throughput photobioreactor for microalgal screening and culture optimization for growth, lipid content and CO_2 sequestration. *Biochem. Eng. J.* **2015**, *103*, 193–197. [CrossRef]
31. Friedl, A.; Padouvas, E.; Rotter, H.; Varmuza, K. Prediction of heating values of biomass fuel from elemental composition. *Anal. Chim. Acta* **2005**, *544*, 191–198. [CrossRef]
32. Aussant, J.; Guihéneuf, F.; Stengel, D.B. Impact of temperature on fatty acid composition and nutritional value in eight species of microalgae. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 5279–5297. [CrossRef]
33. Chae, H.; Lim, S.; Kim, H.S.; Choi, H.-G.; Kim, J.H. Morphology and phylogenetic relationships of *Micractinium* (Chlorellaceae, trebouxiophyceae) taxa, including three new species from antarctica. *Algae* **2019**, *34*, 267–275. [CrossRef]
34. Illman, A.M.; Scragg, A.H.; Shales, S.W. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. *Enzyme Microb. Technol.* **2000**, *27*, 631–635. [CrossRef]
35. Do, J.-M.; Jo, S.-W.; Kim, I.-S.; Na, H.; Lee, J.H.; Kim, H.S.; Yoon, H.-S. A feasibility study of wastewater treatment using domestic microalgae and analysis of biomass for potential applications. *Water* **2019**, *11*, 2294. [CrossRef]
36. Lunch, C.K.; Lafountain, A.M.; Thomas, S.; Frank, H.A.; Lewis, L.A.; Cardon, Z.G. The xanthophyll cycle and NPQ in diverse desert and aquatic green algae. *Photosynth. Res.* **2013**, *115*, 139–151. [CrossRef] [PubMed]
37. Müller, P.; Li, X.-P.; Niyogi, K.K. Non-photochemical quenching. A response to excess light energy. *Plant Physiol.* **2001**, *125*, 1558–1566. [CrossRef] [PubMed]
38. Chong, G.-L.; Chu, W.-L.; Othman, R.Y.; Phang, S.-M. Differential gene expression of an Antarctic *Chlorella* in response to temperature stress. *Polar Biol.* **2011**, *34*, 637–645. [CrossRef]
39. Li, H.; Liu, X.; Wang, Y.; Hu, H.; Xu, X. Enhanced expression of antifreeze protein genes drives the development of freeze tolerance in an Antarctica isolate of *Chlorella vulgaris*. *Prog. Nat. Sci.* **2009**, *19*, 1059–1062. [CrossRef]
40. Machida, T.; Murase, H.; Kato, E.; Honjoh, K.; Matsumoto, K.; Miyamoto, T.; Iio, M. Isolation of cDNAs for hardening-induced genes from *Chlorella vulgaris* by suppression subtractive hybridization. *Plant Sci.* **2008**, *175*, 238–246. [CrossRef]
41. Spijkerman, E.; Wacker, A.; Weithoff, G.; Leya, T. Elemental and fatty acid composition of snow algae in Arctic habitats. *Front. Microbiol.* **2012**, *3*, 1–15. [CrossRef] [PubMed]
42. Lyon, B.R.; Mock, T. Polar microalgae: New approaches towards understanding adaptations to an extreme and changing environment. *Biology* **2014**, *3*, 56–80. [CrossRef]
43. Harris, P.; James, A.T. The effect of low temperatures on fatty acid biosynthesis in plants. *Biochem. J.* **1969**, *112*, 325–330. [CrossRef]
44. Suga, K.; Honjoh, K.I.; Furuya, N.; Shimizu, H.; Nishi, K.; Shinohara, F.; Hirabaru, Y.; Maruyama, I.; Miyamoto, T.; Hatano, S.; et al. Two low-temperature-inducible *Chlorella* genes for Δ12 and *ω*-3 fatty acid desaturase (FAD): Isolation of *Δ12* and *ω-3 fad* cDNA clones, expression of *Δ12 fad* in *Saccharomyces cerevisiae*, and expression of *ω-3 fad* in *Nicotiana tabacum*. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 1314–1327. [CrossRef] [PubMed]
45. El-sheekh, M.; Abomohra, A.E.; El-azim, M.A.; Abou-shanab, R. Effect of temperature on growth and fatty acids profile of the biodiesel producing microalga *Scenedesmus acutus*. *Biotechno. Agron. Soc. Environ.* **2017**, *21*, 233–239.
46. Mehta, L.R.; Dworkin, R.H.; Schwid, S.R. Polyunsaturated fatty acids and their potential therapeutic role in multiple sclerosis. *Nat. Clin. Pract. Neurol.* **2009**, *5*, 82–92. [CrossRef]
47. Nadeau, T.-L.; Castenholz, R.W. Characterization of psychrophilic Oscillatorians (cyanobacteria) from antarctic meltwater ponds. *J. Phycol.* **2000**, *36*, 914–923. [CrossRef]
48. Tang, E.P.Y.; Vincent, W.F. Strategies of thermal adaptation by high-latitude cyanobacteria. *New Phytol.* **1999**, *142*, 315–323. [CrossRef]
49. Chevalier, P.; Proulx, D.; Lessard, P.; Vincent, W.F.; De La Noüe, J. Nitrogen and phosphorus removal by high latitude mat-forming cyanobacteria for potential use in tertiary wastewater treatment. *J. Appl. Phycol.* **2000**, *12*, 105–112. [CrossRef]

50. Tang, E.P.Y.; Tremblay, R.; Vincent, W.F. Cyanobacterial dominance of polar freshwater ecosystems: Are high-latitude mat-formers adapted to low temperature? *J. Phycol.* **1997**, *33*, 171–181. [CrossRef]
51. Hu, H.; Li, H.; Xu, X. Alternative cold response modes in *Chlorella* (Chlorophyta, Trebouxiophyceae) from Antarctica. *Phycologia* **2008**, *47*, 28–34. [CrossRef]
52. Hong, J.W.; Kim, O.H.; Jo, S.-W.; Kim, H.; Jeong, M.R.; Park, K.M.; Lee, K.I.; Yoon, H.-S. Biochemical composition of a Korean domestic microalga *Chlorella vulgaris* KNUA027. *Microbiol. Biotechnol. Lett.* **2016**, *44*, 400–407. [CrossRef]