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Isolation and Characterization of the Indigenous Microalgae *Chlamydomonas reinhardtii* K01 as a Potential Resource for Lipid Production and Genetic Modification

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The green alga *Chlamydomonas reinhardtii*, a unicellular haploid eukaryote, has long been used by researchers and industries as a cell factory to produce high value-added microalgae substances using genetic modification. Microalga K01, presumed to be *Chlamydomonas*, was isolated from 12 freshwater samples from the Chungcheong and Jeolla regions to replace *C. reinhardtii*, an introduced species currently used in most basic and industrial research. The isolated K01 strain was identified as *C. reinhardtii* through morphological and phylogenetic studies of the 18S rDNA gene sequence (NCBI accession number KC166137). The growth and lipid content of the isolated *C. reinhardtii* K01 were compared with three wild and four mutant strains in TAP medium, and it was found that the K01 strain could produce 1.74×10^7 cells/ml by the third day of culture. The growth rate of *C. reinhardtii* K01 was 1.5 times faster than UTEX2244, which showed the highest number of cells (1.20×10^7 cells/ml) among the compared strains. The lipid content of the isolated *C. reinhardtii* K01 (20.67%) was similar to those of the wild strains, although the fatty acid oleate C18:1 was not detected in the isolated strain but was identified in the seven others. The cell density of the isolated strain increased to 0.87 g/l during a six-day culture in BG11 medium, where nitrate ($NaNO_3$) was introduced as a nitrogen source, while the seven acquired strains showed almost no cell proliferation.

Key words : *Chlamydomonas reinhardtii*, cell growth, FAME composition, lipid contents, microalgae

Introduction

The increase in carbon dioxide emission due to industrialization and population growth causes global warming. Therefore, environmental, food, and energy problems are emerging as immediate challenges needing to be solved. Microalgae, having a higher photosynthetic efficiency per unit area compared to plants, are being considered as a biological method to reduce carbon dioxide, and methods for converting carbon dioxide into microalgal biomass are also being studied [5, 18].

The biomass of microalgae, which is converted from carbon dioxide through a photoautotrophic mechanism, contains lipids, proteins, photosynthetic pigments, and various physiologically active substances. These materials are being used as biofuels, ingredients for cosmetics, raw materials for protein foods, and ingredients for health foods using photosynthetic pigments [4, 13]. Furthermore, research is underway to use them more as industrial materials [10, 17]. However, economic feasibility is an issue continuously raised due to the low productivity of these materials. Therefore, various studies have been conducted with the objective of increasing biomass productivity and producing high value-added substances with methods such as separation of high-efficiency microalgae, modification of microalgae, development of microalgae culture apparatuses, and optimization of culture methods for carbon dioxide fixation [3, 14-16].

Chlamydomonas reinhardtii is one of the most representative green microalgae having been the subject of active molecular genetic research for about 50 years, and it is very promising as a cell factory to produce synthetic biology-based high value-added substances [9]. In addition, many studies are also conducted in Korea because high value- added beneficial substances can be produced from *Chlamydomonas reinhardtii* by utilizing carbon dioxide [1, 7, 11, 12]. However, *C. reinhardtii* is an introduced strain so it is not

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suitable for industrial application. This is because biomaterials became resources through the regulation of Access and Benefit-sharing (ABS) in the Nagoya Protocol, so the profit generated from biomaterials must be shared with the source provider.

This study attempted to isolate an indigenous strain of *C. renhardtii* that can be utilized in industries. The growth rate, culture characteristics, lipid content, and fatty acid composition of the isolated *C. reinhardtii* K01 were analyzed. The analysis results were compared with those characteristics of three introduced *C. reinhardtii* strains and four transformed mutant *C. reinhardtii* strains to review its potential as a photosynthetic cell factory.

Materials and Methods

Isolation and culture of microalgae

Samples collected from a total of 12 freshwaters in the Chungcheong and Jeolla regions were centrifuged at 3,000 rpm for 20 min. and concentrated to select for indigenous microalgae *Chlamydomonas*, which have a fast growth rate and high lipid productivity. Agglomerated cells were dispersed with a sonicator, and the pure strain of microalgae was isolated through micro-pipetting. The isolated microalgae were transferred to sterilized Tris–acetate phosphate (TAP) medium (Tris base, 2.42 g/l; NH_4Cl, 0.375 g/l; $MgSO_4 \cdot 7H_2O$, 0.1 g/l; $CaCl_2 \cdot 2H_2O$, 0.05 g/l; K_2HPO_4, 0.288 g/l and KH_2PO_4, 0.144 g/l; glacial acetic acid 1.0 ml/l) and suspension culture was performed at 25°C and a light source of 100 $\mu mol/m^2/s$ [6].

Identification of isolated microalgae

The isolated microalgae were identified by examining their morphological and genetic characteristics. A morphological study was conducted using an optical microscope (Nikon Eclipse 80i, Nikon, Japan), and genetic identification was carried out by amplifying the gene sequence of 18S rDNA through polymerase chain reaction (PCR). For PCR, GO Taq DNA polymerase (GoTaq Green Master Mix 2×, Promega, USA) was introduced, and genomic DNA extracted with a DNA extraction kit (Wizard® Genomic DNA Purification Kit, Promega, USA) was used as a template. 18S-chl65F (5′-CGACTTCTGGAAGGGACGTA-3′) and 18S-chl200R (5′-GAGTCAAATTAAGCCGCAGG-3′), which are primers for Chlorophyta identification, were used [8]. As conditions for PCR, the isolated algae were denatured at 94℃ for 5 min. and the reaction was repeated 35 times at 94℃ for 45 sec at 58℃ for 45 sec, and at 72℃ for 45 sec, and then finally at 72℃ for 5 min. Gene sequencing was conducted by Solgent Co., Ltd. by request, and the gene sequences of the analyzed microalgae were identified using the NCBI BLAST system.

Comparison strain *Chlamydomonas*

In order to compare the culture characteristics and lipid productivity of the isolated strain *C. reinhardtii* K01 with previously isolated *Chlamydomonas*, three wild strains (UTEX 2243, UTEX2244, and W14) were acquired from UTEX (Culture Collection of Algae at the University of Texas in Austin, Texas), a microalgae distribution institution, and four mutant strains (Sta6-parent, Sta6, BAFJ5C2, and I7) were acquired from the Chlamycollection (Chlamydomonas Resource Center, University of Minnesota), and a comparative experiment was performed (Table 1).

Culture and measurement of cell density

Isolated *C. reinhardtii* K01 and the acquired strains of *Chlamydomonas* were inoculated in TAP medium and batch

Table 1. *Chlamydomonas reinhardtii* strains used for this study

Class	Strains	Sources	Genotype
WT	K01	-	Isolated in this study
WT	2243	UTEX	*mt⁻ nit1 nit2*
WT	2244	UTEX	*mt⁺ nit1 nit2*
WT	W14	UTEX	*mt⁺ nit1 nit2*
MT	Sta6-p	CRS	*mt⁺ nit1 nit2 cw15 arg7-7*
MT	Sta6	CRS	*mt⁺ nit1 nit2 cw15 arg7-7 sta6-1*::ARG7
MT	Bafj5C2	CRS	*mt⁺ nit1 nit2 cw15 arg7-7 sta6-1*::ARG7 pSL18-STA6
MT	I7	CRS	*mt- nit1 nit2 sta1-1*

WT; wild type, MT; mutant type, UTEX; Culture Collection of Algae at the University of Texas at Austin, CRS; Chlamydomonas Resource Center (Siaut et al. BMC Biotechnology 2011, 11:7 http://www.biomedcentral.com/1472-6750/11/7)

culture was performed at 25℃ while stirring at 100 rpm and irradiation with light at 100 μmol/m^2/s for six days. Cell counting was performed by using a hematocytometer.

Comparison of lipid content of microalgae

Total lipid contents were determined for *C. reinhardtii* K01 and the acquired strains of *Chlamydomonas* by modifying the Bligh and Dryer method [2]. A culture solution volume of 10 ml was centrifuged at 5,000x *g* for 30 min. and then the strain colonies were harvested. A total of 15 ml of extraction solvent (chloroform: methanol= 2: 1, v/v) was poured into the harvested strains and the cells were crushed using an ultrasonicator (Vibra Cell, Sonics & materials Inc., USA) for 5 min. Then 10 ml of distilled water was added into the extracted solution and it was centrifuged at 5,000x *g* for 15 min to divide into an organic solvent and water layer. The organic solvent was separated and filtered with a PTFE filter (0.22 μm, Whatman, UK). The sample, devoid of cell debris, was concentrated with a rotary vacuum evaporator (Buchi, Switzerland) to remove the extraction solvent. The remaining sample was dried and weighed to determine the lipid weight. From the measured weight, the lipid weight was determined per dried weight.

Analysis of fatty acid composition

In order to analyze the fatty acid composition of *C. reinhardtii* K01 and the acquired strains of *Chlamydomonas*, a total of 5 ml of the culture solution was centrifuged at 5,000x *g* for 20 min to harvest the strain colonies, and then the fatty acids were extracted [32]. The harvested strain colonies were transferred to a glass tube and mixed with 1 ml of saponification solution (NaOH 45 g, methanol 150 ml, and distilled water 150 ml). The mixed sample was reacted at 100℃ for 5 min and then mixed again, followed by another reaction at 100℃ for 25 min. When the temperature of the reacted mixture became room temperature, 2 ml of methylation solution (6 N HCl 325 ml, methanol 275 ml) was added and stirred, followed by reaction at 80℃ for 10 min. The mixture was taken, rapidly cooled down, and 1.25 ml of extraction solvent (hexane 200 ml, methyl tert-butyl ether 200 ml) was added, stirred for 10 min. and the fatty acids were extracted. Then 3 ml of base wash solution (NaOH 10.8 g, distilled water 900 ml) was added to the extracted fatty acid solution, stirred, and the layers were separated. The organic solvent layer was taken, filtered with a PTFE filter (0.2 μm, Watman, UK), and 1 ml was taken. Then 50 ul of C17 internal standard (Fluka) was added to prepare the sample for fatty acid analysis. The fatty acids were analyzed through a gas chromatograph (Shimadzu GC-200, Japan) equipped with a Wax column (Zebron, ZB-WAX, max. temp.: 250℃, Phonomenex, USA) and a flame ionization detector (FID, maximum temperature: 300℃). A sample quantity of 1 μl was injected into the gas chromatograph for fatty acid detection and the total detection time was set to 25 min. FAME mix 18918 (C8-C24) from Supelco was used as a standard for fatty acid detection. The detection results and standard were relatively compared, and then the fatty acid contents were determined.

Growth characteristics of the strains

The isolated strain *C. reinhardtii* K01 and the acquired strains of *Chlamydomonas* were inoculated into TAP medium containing ammonium (NH_4Cl) and into BG11 medium ($NaNO_3$ 1.5 g/l, $MgSO_4$ $7H_2O$ 74.9 mg/l, $CaCl_2$ $2H_2O$ 36 mg/l, citric acid 6 mg/l, 0.25 M Na-EDTA (pH 8.0) 11.2 ul/l, H_3BO_3 2.860 mg/l, $MnCl_2$ $4H_2O$ 1.810 mg/l, $ZnSO_4$ $7H_2O$ 0.222 mg/l, Na_2MoO_4 $2H_2O$ 0.390 mg/l, $CuSO_4$ $5H_2O$ 0.079 g/l, $Co(NO3)_2$ $6H_2O$ 0.0494 mg/l, ammonium iron (III) citrate 6 mg/l, $NaCO_3$ 20 mg/l, and K_2HPO_4 30.5 mg) containing nitrate ($NaNO_3$) as a nitrogen source. Batch culture was carried out while stirring the media at 25℃ and 100 rpm with light irradiation of 100 μmol/m^2/s for 6 days [6]. The dry weights of the cells were measured during culture and the availabilities of ammonium (NH_4Cl) and nitrate ($NaNO_3$) were analyzed. The dry weight of the cell was measured after drying the filtered cells at 105℃ for 3 hr using Glassmicrofiber filters (Whatman,UK).

Results and Discussion

Isolation and identification of the strains

A total of 12 microalgae were isolated through micropipetting to find indigenous microalgae. One of the isolated algae was the microalga K01, which was presumed to be *Chlamydomonas* due to its mobility. The observation results of the isolated microalga K01 under optical microscope revealed that it was a green alga, and its morphology was similar to that of *Chlamydomonas* (Fig. 1). For the molecular genetic identification of the isolated strain K01, the genome was extracted and the 18S rDNA sequence was amplified to obtain a sequence of 1,480 bp, and its homologous features were checked through the NCBI BLAST system. Phylogenetic

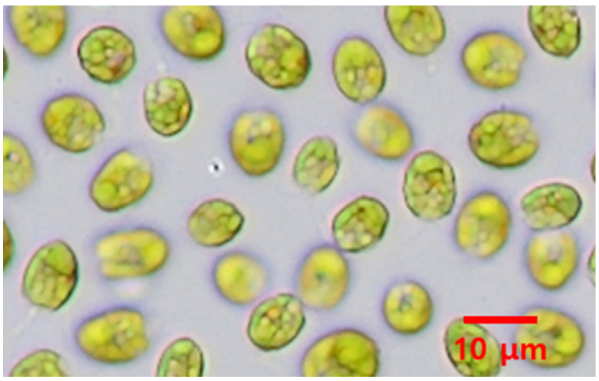

Fig. 1. Light microscopy image of isolated strain K01.

analysis results showed that the 18S rDNA sequence of the isolated K01 was 99% similar to that of *C. reinhardtii*. In addition, the stain was compared using Clustal X and MEGA 4.0, and it was quite similar to *Chlamydomonas reinhardtii*. Therefore, the isolated strain K01 was named *Chlamydomonas reinhardtii* K01 (Fig. 2).

Growth of the Isolated Strain

The growth pattens of the isolated strain *C. reinhardtii* K01, the three acquired wild strains (2243, 2244, and W14), and the four acquired mutant strains (Sta6-parant, Sta6, BAFJ5, and I7) of *Chlamydomonas* were examined (Fig. 3). The isolated and acquired strains were cultured in Tris–acetate phosphate (TAP) medium at a temperature of 25℃, a light source of 100 μmol/m^2/s, and in a 10 l glass photobioreactor at a stirring speed of 100 rpm for 6 days.

The isolated strain *C. reinhardtii* K01 had a cell number of 1.74×10^7 cells/ml on the 3rd day of culture. This was a 1.5-fold higher cell number compared to the 1.20×10^7 cells/ml of UTEX2244, the highest cell number on the 3rd day of the comparison strains. Furthermore, *C. reinhardtii* K01 showed a faster growth rate than the 7 comparison strains. In addition, the maximum cell numbers of *C. reinhardtii* K01 and mutant strain I7 were 2.50×10^7 cells/ml on the 5th day and 2.3×10^7 cells/ml on the 6th day of culture, respectively. Therefore, *C. reinhardtii* K01 showed the highest maximum cell number at the fastest rate. In the case of the mutant strains sta6 and sta6-P, slow growths of 6.8×10^6 cells/ml and 6.3×10^6 cells/ml were observed in the same culture conditions.

Analysis of biomass of the strains

The growth of the microalgae and their lipid contents were compared after culturing the isolated *C. reinhardtii* K01 and the 7 acquired *Chlamydomonas* in TAP medium for 6 days (Fig. 4). The dry cell weight and the lipid content by

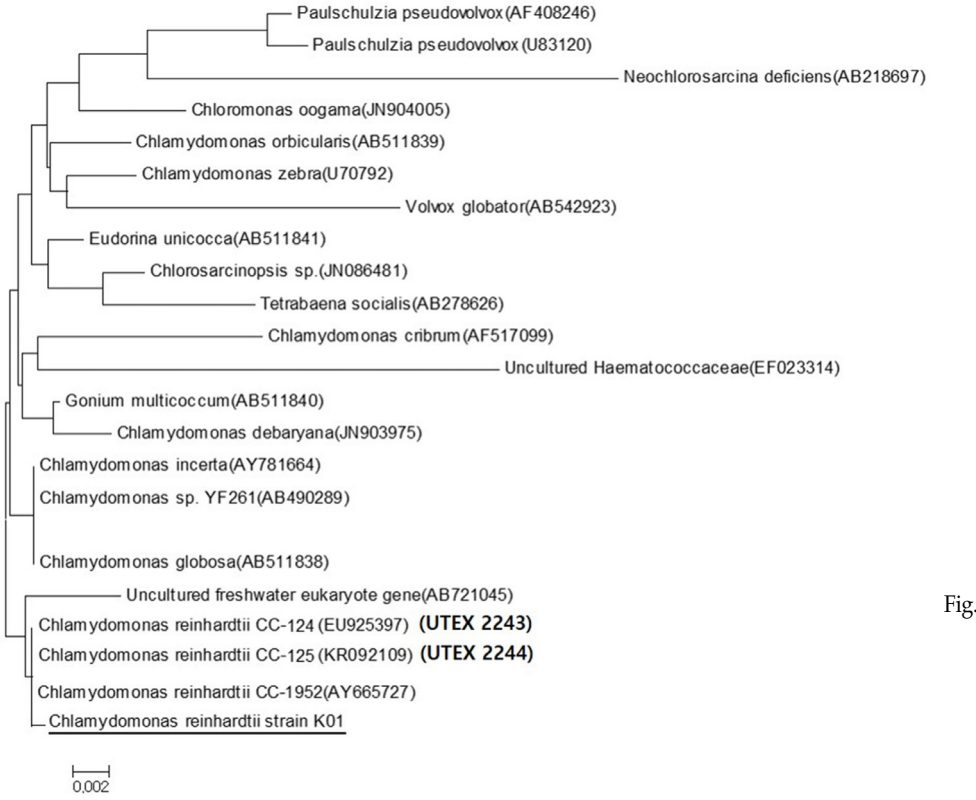

Fig. 2. The phylogenetic tree of isolated strain K01 18SrDNA gene sequence. the scale bar denotes nucleotide substitutions per site.

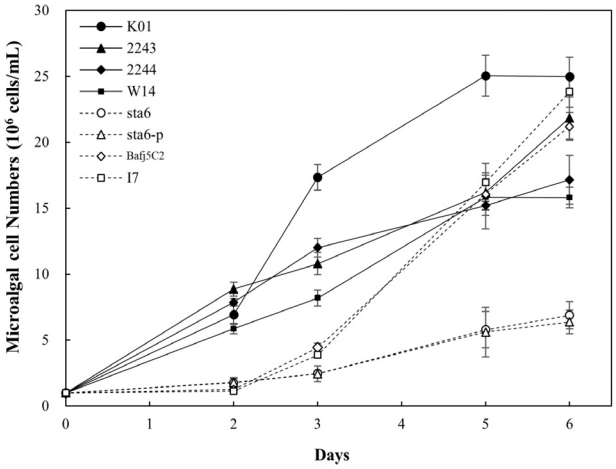

Fig. 3. Comparison of cell growth of the isolated strain *Chlamydomonas. reinhardtii* K01 and the 7 types of acquired strains *Chlamydomonas* in TAP medium. The solid line is the wild-type strains and the dotted line is the mutant strains. Symbols : (-●-) K01; isolated strain, (-▲-) 2243; UTEX 2243, (-◆-)2244; UTEX 2244, (-■-) W14; UTEX W14, (-○-) sta6-p, (-△-) sta-6; (-◇-) BAFJ5C2; (-□-) I7.

dry cell weight of the isolated strain *C. reinhardtii* K01 were 1.49 g/l and 21%, respectively. In the case of the UTEX2243 strain, the cell growth and the lipid content by dry cell weight were 1.53 g/l and 20%, almost the same as the isolated strain K01. On the other hand, the mutant strain Sta6 had a dry cell weight of 0.6 g/l, indicating low cell proliferation, but the lipid content by dry cell weight was very high (37%). Therefore, cell growth was not found to be active in the mutant stains compared to the *C. reinhardtii* K01 and UTEX strain.

The biomass concentration and lipid production measurements results (Fig. 5) showed that the biomass concentration of the isolated strain *C. reinhardtii* K01 was 0.25 g/l/day, and the lipid production was 0.05 g/l/day. In the case of UTEX2243, the biomass concentration and lipid production were 0.26 g/l/day and 0.05 g/l/day, respectively, showing that the biomass concentration and lipid production were similar to those of the isolated *C. reinhardtii* K01. In addition, the biomass concentration and lipid production of all the four mutant strains were lower compared to those of the isolated strain *C. reinhardtii* K01.

Lipid accumulation and fatty acid composition of the strains

The intracellular neutral lipid accumulation statuses of the isolated strain *C. reinhardtii* K01 and the 7 acquired strains of *Chlamydomonas* were confirmed by Nile red staining. When each microalga was cultured in TAP medium for 6 days, the neutral lipids accumulation pattern of the isolated *C. reinhardtii* K01 was observed to be similar to the wild strains rather than the mutant strains, which accumulated neutral lipids (Fig. 6A).

The fatty acid compositions of the isolated strain *C. reinhardtii* K01 and the 7 acquired strains of *Chlamydomonas* were investigated after detecting the fatty acids through GC (Fig. 6B). It was found that the fatty acids in the FAME extracted from the 7 acquired strains commonly contained caprylate (C8:0), laurate (C12:0), palmitate (C16:0), hexadecadienoate (C16:2), hexadecadienoate (C16:3), stearate (C18:0), oleate (C18:1), linoleate (C18:2), and linoleate (C18:3). However, oleate (C18:1) was not detected in the isolated strain *C. reinhardtii* K01. The compositional analysis of the FAME extracted from the isolated strain *C. reinhardtii* K01 showed that C18 and C16 made up 26% and 24% of the total fatty acids, and other fatty acids made up smaller proportions with C10 at 10%, C12 at 4%, and C8 at 3%.

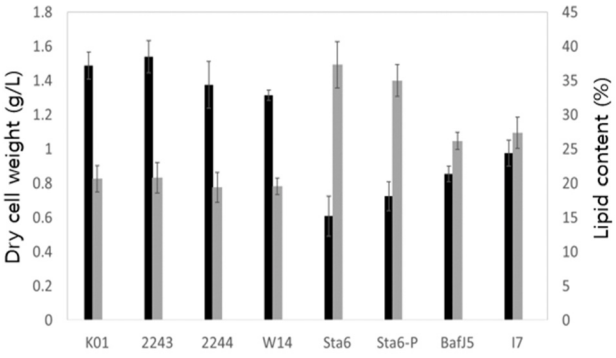

Fig. 4. Comparison of dry cell weight and lipid content of the isolated strain *Chlamydomonas.reinhardtii* K01 and the 7 types of acquired strains *Chlamydomonas* in TAP medium.

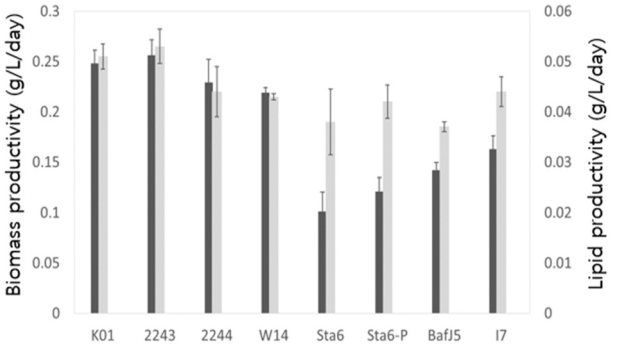

Fig. 5. Comparison of biomass and lipid productivity of the isolated strain *Chlamydomonas.reinhardtii* K01 and the 7 types of acquired strains *Chlamydomonas* in TAP medium.

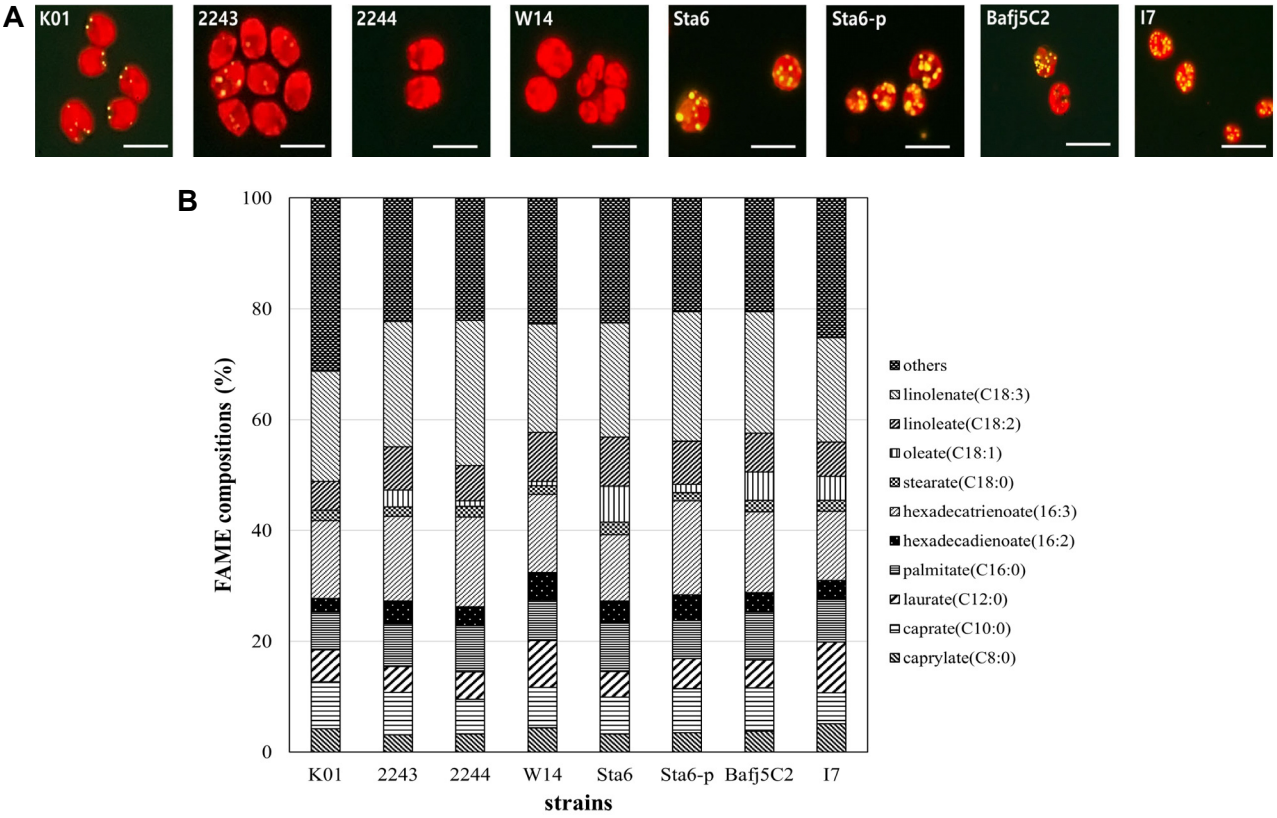

Fig. 6. Comparison of (A) microscopic comparison of intracellular neutral lipid accumulation with nile red staining. Neutral lipids are observed as yellow droplets in the cells and red color is due to the self- autofluorescence of chlorophyll. The size bar shown 10 micrometers. (B) FAME composition of the isolated strain *Chlamydomonas reinhardtii* K01 and the 7 types of acquired strains *Chlamydomonas*.

Growth characteristics of the strains

The isolated strain *C. reinhardtii* K01 and the acquired strains of *Chlamydomonas* were cultured under light for 6 days in TAP medium containing ammonium (NH_4Cl) and BG11 medium containing nitrate ($NaNO_3$) as a nitrogen source, respectively, and then the dry cell weight was measured to determine the availability of ammonium (NH_4Cl) and nitrate ($NaNO_3$) (Fig. 7). The dry cell weights of the isolated *C. reinhardtii* K01 and UTEX2243 in the TAP medium containing ammonium (NH_4Cl) were similar at 1.49 g/l and 1.53 g/l, respectively, and the dry cell weight of the mutant strain was the lowest at 0.61 g/l. Such results implied that both the isolated strain *C. reinhardtii* K01 and the 7 acquired strains of *Chlamydomonas* well utilized the nitrogen source ammonium (NH_4Cl). In the BG11 medium containing nitrate ($NaNO_3$), the dry cell weight of the isolated strain *C. reinhardtii* K01 was 0.87 g/l, which indicated that the isolated strain *C. reinhardtii* K01 utilized nitrate. On the other hand, the dry cell weights of all the 7 acquired *Chlamydomonas* measured lower than 0.1 g/l, indicating that nitrate was hardly utilized, causing almost no strain growth.

The poor cell growth of the 7 acquired strains could confirm that no nitrate reductase genes (nit1, nit2) in the genotypes of the acquired wild UTEX type strains were found.

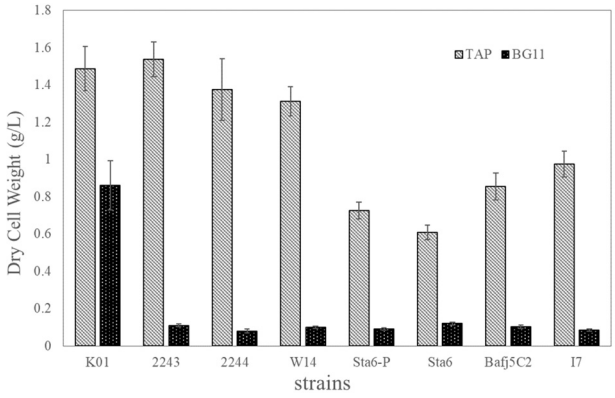

Fig. 7. Comparison of cell growth of the isolated strain *C. reinhardtii* K01 and the 7 acquired strains of *Chlamydomonas* in TAP medium containing ammonium (NH_4Cl) and BG11 medium containing nitrate ($NaNO_3$) as nitrogen sources. Symbols : (▨) TAP medium, (■) BG11 medium.

It was assumed that these strains could not utilize nitrate as a nitrogen source or their utilization efficiency was very low. Nitrate reductase was preserved in the isolated strain *C. reinhardtii* K01, confirmed by its growth characteristics made capable by utilizing nitrate unlike the 7 acquired strains. The results also confirmed that the indigenous isolated strain *C. reinhardtii* K01 could grow in more diverse media compared to the existing *Chlamydomonas*.

The obtained results through this study confirmed that the strain *C. reinhardtii* K01 isolated from the domestic indigenous environment could be well utilized in the academic field as well as in industries due to its fast growth rate and utilization of various nitrogen sources.

Acknowledgement

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 지질생산 및 유전자 조작의 잠재적 자원으로서의 토착 미세조류 *Chlamydomonas reinhardtii* K01의 분리 및 특성

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반수체 단세포 진핵생물인 녹조류 *Chlamydomonas reinhardtii*는 미세조류와 유전자 변형을 이용하여 고부가가치 물질을 생산하는 세포 공장으로 연구자와 산업계에서 오랫동안 사용되어 왔다. 현재 대부분의 기초 및 산업 연구에 사용되는 외래종인 *C. reinhardtii*를 대체하기 위하여 충청과 전라지역 12개의 담수에서 채취한 시료에서 *Chlamydomonas*로 추정되는 미세조류 K01을 분리하였다. 분리균주 K01의 형태학적 분석과 18S rDNA 유전자 서열(NCBI 수탁번호 KC166137)의 계통발생학적 분석을 통하여 분리균주 K01는 *Chlamydomonas reinhardtii*로 동정되었다. 분리균주 *C. reinhardtii* K01의 성장 및 지질 함량은 TAP 배지에서 3개의 야생 균주 및 4개의 돌연변이 균주와 비교하였다. 이들 균주 중 *C. reinhardtii* K01 균주가 배양 3일째 1.74×10^7 cells/ml개의 세포수가 관찰되었다. 이는 비교 균주 중 가장 높은 세포 수(1.20×10^7 cells/ml)를 나타내어 UTEX2244와 비교했을 때 1.5배 빠른 성장속도를 보였다. 분리균주 *C. reinhardtii* K01의 지질함량(20.67%)은 야생형 균주의 지질함량과 유사하였다. 분리균주 *Chlamydomonas reinhardtii* K01의 지방산 성분은 7종의 분양 균주에서 확인된 지방산 중 oleate (C18:1)가 검출되지 않았다. 분리균주 *C. reinhardtii* K01은 nitrate ($NaNO_3$)를 질소원으로 포함하는 BG11배지에서 배양 6일 동안 0.87 g/l까지 세포밀도가 증가하였으나, 분양균주인 7종의 *Chlamydomonas*의 경우 모든 균주에서 세포증식이 거의 일어나지 않았다.