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Isolation and description of a Korean microalga, *Asterarcys quadricellulare* KNUA020, and analysis of its biotechnological potential

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A eukaryotic microalga, *Asterarcys quadricellulare* KNUA020, was isolated from garden soil at Kyungpook National University in Daegu, South Korea and its biotechnological potential was assessed. Optimal growth was obtained when the culture was incubated at 25°C and around pH 7.0. The total lipid content of the isolate was 15.5% of dry weight and its most abundant fatty acid was nutritionally important C18:3 ω3 (α-linolenic acid, ALA). In addition, a high-value fatty alcohol, hexadecenol ($C_{20}H_{40}O$), was also identified in this photosynthetic microorganism. Hence, *A. quadricellulare* KNUA020 appears to be promising for use in the production of microalgae-based biochemicals.

Key Words: algae-based biochemicals; fatty acids; fatty alcohol; Korean domestic microalga

INTRODUCTION

Microalgae are photosynthetic organisms that convert carbon dioxide (CO_2) into a wide spectrum of organic compounds including chlorophyll, carotenoid, vitamin and lipid. They are known as the primary producers of a variety of long-chain polyunsaturated fatty acids (PUFAs) in aquatic environments and numerous studies have shown that nutraceutically important omega-3 (ω3) and omega-6 (ω6) PUFAs are quite abundant in microalgae (Otleş and Pire 2001, Bigogno et al. 2002, Ikawa 2004, Patil et al. 2007, Khozin-Goldberg et al. 2011). As the global ω3 and ω6 PUFAs market is expanding at a remarkable rate, microalgae have gained considerable attention as an alternative source to fish oil due to their fast growth rate and high PUFA contents (Seto et al. 1984, Benemann et al. 1987). Therefore, the commercial cultivation of *Chlorella* and *Spirulina* in the U. S. and Far East countries is increasing rapidly. In the current study, a Korean domestic microalga, *Asterarcys quadricellulare*, was isolated from garden soil and its physiological properties were investigated to determine the optimal growth conditions. The lipid content of the isolate was then analyzed to see whether this eukaryotic microalga could be used as a candidate for biotechnological applications.

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MATERIALS AND METHODS

Sample collection and isolation

Samples of garden soil were taken near the Biological Sciences Building at Kyungpook National University (35°53′ N, 128°36′ E) in Daegu, South Korea in May 2010. A few micrograms of fresh soil were used to inoculate 100 mL BG-11(+) medium containing nitrate (Rippka et al. 1979) with 100 μg mL^{-1} of meropenem (Yuhan Pharmaceuticals, Ochang, Korea). The flasks were incubated on an orbital shaker (Vision Scientific, Bucheon, Korea) at 160 rpm and 25°C until algal growth was observed. Well-established algal cultures (1.5 mL) were centrifuged at 3,000 ×g for 15 min. The resulting pellets were streaked onto BG-11 agar supplemented with meropenem and incubated at 25°C with a light : dark cycle (16 : 8 h). A single colony was then aseptically re-streaked onto a fresh BG-11 plate containing meropenem (20 μg mL^{-1}) to obtain an axenic culture.

Morphological identification

The isolate was grown in BG-11(+) medium for 28 days. Live cells were harvested by centrifugation at 3,000 ×g for 5 min, washed with sterile distilled water, and inspected at 400× magnification with a Zeiss Axioskop 2 light microscope (Carl Zeiss, Standort Göttingen, Vertrieb, Germany) equipped with differential interference contrast (DIC) optics.

Molecular identification

Genomic DNA was extracted by using a DNeasy plant mini kit (Qiagen, Hilden, Germany). The PCR conditions and primer sets NS1 (5′-GTA GTC ATA TGC TTG TCT C-3′) and NS8 (5′-TCC GCA GGT TCA CCT ACG GA-3′) used for 18S ribosomal RNA (rRNA) sequence analysis were previously described by White et al. (1990). Amplification was performed in 25 μL reaction volume containing approximately 10 ng of template DNA, 0.5 μM of each primer (Macrogen, Seoul, Korea), 0.5 U of *Ex Taq* DNA polymerase, 200 μM of each dNTP, 2.5 mM $MgCl_2$ and 1× *Ex Taq* PCR buffer (Takara, Otsu, Japan). Amplification was carried out in a thermocycler (Dice Model TP600; Takara) under the following conditions: 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 45°C, and 1 min 30 s at 72°C with a final extension of 5 min at 72°C. The internal transcribed spacer (ITS) region was also amplified using the primers Al1500bf (5′-GAT GCA TTC AAC GAG CCT A-3′) (Helms et al. 2001) and LR3 (5′-CCG TGT TTC AAG ACG GG-3′) (Friedl and Rokitta 1997). The PCR conditions for amplifying this fragment were as follows: an initial denaturing step for 10 min at 95°C followed by 35 cycles of 1 min at 95°C, 40 s at 51°C, and 1 min at 72°C with a final extension of 10 min at 72°C.

The PCR products were purified using a LaboPass PCR purification kit (Cosmo Genetech, Seoul, Korea). The purified PCR amplicons were ligated into a pGEM Easy vector system (Promega, Madison, WI, USA) and used to transform *Escherichia coli* DH5α cells. Recombinant plasmid was extracted from transformed *E. coli* using a plasmid mini prep kit (SolGent, Daejeon, Korea) according to the manufacturer's instructions. The identity of plasmids containing the correct inserts were verified by agarose gel electrophoresis and sequencing at the Macrogen facility (Macrogen). DNA sequences obtained in this study were submitted to the NCBI database under the accession nos. JQ043183 and JQ043184 (Table 1).

Physiological testing

A 30-day-old seed culture of *A. quadricellulare* KNUA020 (1 mL) was inoculated into BG-11(+) medium in triplicate and incubated for 30 days. Survival and growth of KNUA020 cells maintained at 15, 20, 25, and 30°C were examined to determine the optimum temperature for culturing. An acidity tolerance test was per-

Table 1. Results from BLAST searches using the 18S rRNA and ITS sequences of *Asterarcys quadricellulare* KNUA020

Marker gene	Accession No.	Length (bp)	Closest match (GenBank accession No.)	Overlap (%)	Sequence similarity (%)	Taxonomic affinity
18S rRNA	JQ043183	1,729	*Asterarcys quadricellulare* Comas 1977/75 (AF388375)[a]	100	100	*Asterarcys quadricellulare*
ITS	JQ043184	695	*Graesiella emersonii* CCAP 211/8P (FR865687)[b]	100	91	-

[a]Renamed *Asterarcys quadricellulare* (Behre) E. Hegewald and A. Schmidt (Hegewald et al. 2010).
[b]The closest cultured organism to the KNUA020 strain.

formed at 25°C over a pH range from 3.0 to 13.0. Algal cell density was determined by measuring the optical density (OD) of a culture at 680 nm with an Optimizer 2120UV spectrophotometer (Mecasys, Daejeon, Korea).

Lipid extraction and gas chromatography/mass spectrometry (GC/MS) analysis

To simulate commercial production of microalgae-based biochemicals and obtain enough biomass for analysis, each seed culture was used to inoculate 16 L of a commercial liquid fertilizer (1 : 1,000 dilution; BioNex; 5.1% N, 10% P_2O_5, and 5% K_2O; Biosangsa, Busan, Korea) in an 18-L transparent polycarbonate bottle in triplicate. The cultures were autotrophically grown at 25°C with a flow of air bubbles at rate of approximately 2 L min^{-1} under cool fluorescent lighting (approximately 70 μmole m^{-2} s^{-1}) with a light : dark cycle of 16 : 8 h. After incubating for 30 days, algal cells were harvested by centrifugation at 3,220 ×g (Centrifuge 5810R; Eppendorf, Hamburg, Germany) for 10 min. The harvested algal biomass was freeze-dried and mixed with chloroform-methanol (2 : 1) overnight according to the method described by Yeo et al. (2011). The chloroform extract was isolated and dried in a rotary evaporator (RV10; IKA, Wilmington, NC, USA). The crude lipid was weighed and treated with a pre-made solution of methanol and potassium hydroxide Hexane was added to the reaction and the entire mixture was heated to 30°C and stirred for 10 h. The mixture was then cooled and the methanol and hexane layers were separated. The yellow hexane layer was isolated for further analysis. The fatty acid composition of the culture was determined by GC/MS (Jeol JMS700 mass spectrometer equipped with an Agilent 6890N GC; Agilent Technologies, Palo Alto, CA, USA) at the Daegu Center, Korea Basic Science Institute (KBSI). Peak identification and compound assignment were performed based on the electron impact mass spectrum (EI-MS). The National Institute of Standards and Technology (NIST) mass spectral libraries were used as reference databases.

RESULTS AND DISCUSSION

Identification of the isolated microalga

The algal isolate was non-motile with three-dimensionally arranged coenobia. Its diameter varied from 3-4 μm up to 20 μm depending on the growth stage (Fig. 1). The coenobia consisted of randomly distributed (1-, 2-, 4-, or more) cells within a spherical mucilage envelope. However, it was very difficult to accurately classify the isolate by comparing morphological characteristics shared with other algae because only one species in this genus [*Asterarcys quadricellulare* (Behre) E. Hegewald and A. Schmidt] has been recorded (Hegewald et al. 2010).

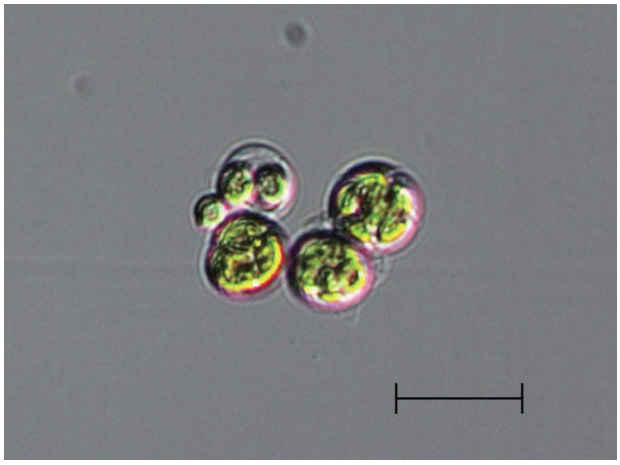

Fig. 1. Light microscope image of *Asterarcys quadricellulare* KNUA020. Scale bar represents: 20 μm.

According to the 18S rRNA sequencing data (Table 1), the isolate had a 100% sequence homology with *A. quadricellulare* (Behre) E. Hegewald and A. Schmidt (formerly known as *A. quadricellulare* Comas 1977/75). However, no identification could be made based on ITS sequence comparison because of the low sequence homology (Table 1). This is due to the lack of sequence data for the genus *Asterarcys* in public databases.

A. quadricellulare was first described by Hegewald and Schmidt (1992). Hegewald et al. (2010) suggested that the genus *Asterarcys* is a member of the family Scenedesmaceae along with other 28 genera based on ITS2 rRNA data. However, the ITS2 rRNA sequence of *A. quadricellulare* (Behre) E. Hegewald and A. Schmidt (GQ375088) was removed by the submitter because the sequence was found to be incorrect. The phylogenetic position of the genus *Asterarcys* therefore needs clarification. Nevertheless, the 18S rRNA sequencing results and some of morphological characteristics observed in the present study suggested that strain KNUA020 is a member of the species *A. quadricellulare*.

Physiological properties of *Asterarcys quadricellulare* KNUA020

As shown in Fig. 2, optimal growth temperature for

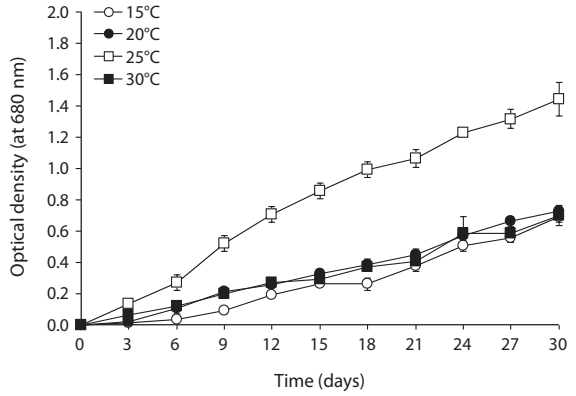

Fig. 2. Growth curves for KNUA020 cells maintained at various temperatures.

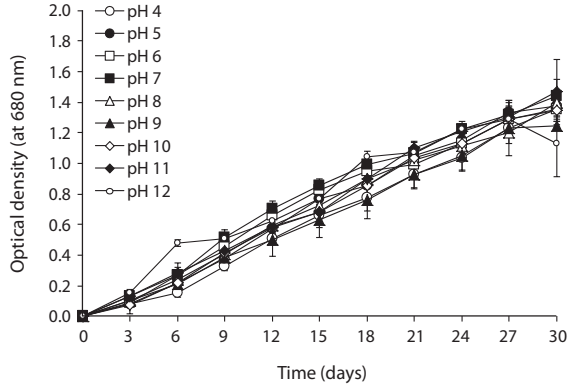

Fig. 3. Growth curves for KNUA020 cells maintained in different pH levels.

strain KNUA020 was 25°C. These cells were able to grow and survive at 15, 20, and 30°C, but delayed growth was observed when they were incubated at these temperatures. The microalga also grew well across a wide pH range (pH 4.0-12.0) (Fig. 3) while maximal growth was obtained around pH 7.0 at 25°C. However, the autotrophic growth rate of strain KNUA020 was relatively low. To develop cost-effective algal biomass production, microalgae can be cultured in heterotrophic conditions where organic carbons such as sugars, sugar alcohols or organic acids serve as less expensive carbon sources (Pyle et al. 2008, Liang et al. 2009). As heterotrophic growth of strain KNUA020 in complex media such as LB and R2A was also observed in this study (data not shown) and a number of reports have demonstrated that heterotrophically grown microalgae accumulate high proportions of lipids (Miao and Wu 2006, Xiong et al. 2008, Gao et al. 2010), future work should be carried out to determine whether mixotrophic cultivation of strain KNUA020 results in both faster growth rate and increased lipid productivity.

Lipid extraction and GC/MS analysis

The total lipid content of the isolate was 15.5 ± 0.2% of dry weight (DW). The GC/MS results showed that the KNUA020 strain produced palmitic acid ($C_{16:0}$, 15.3 ± 0.7%, 23.7 mg g^{-1} DW), hexadecatetraenoic acid ($C_{16:4}$, 14.8 ± 0.4%, 22.9 mg g^{-1} DW) and α-linolenic acid ($C_{18:3}$ ω3, 41.2 ± 8.3%, 63.7 mg g^{-1} DW) as major fatty acids (Table 2, Fig. 4). It was shown that the most abundant fatty acid produced by the KNUA020 strain was α-linolenic acid ($C_{18:3}$ ω3, ALA, molecular weight = 292) which is a nutritionally important ω3 fatty acid (Table 2, Fig. 5). This isolate may thus have potential as an alternative ω3 PUFA source for fish oil. In addition, it was discovered that a significant amount of hexadecenol ($C_{20}H_{40}O$, molecular weight = 296, 24.9 mg g^{-1} DW) was also produced by this photosynthetic microorganism (Table 2, Fig. 6). Long-chain fatty alcohols have been widely used in the cosmetics and soap industries and have recently gained particular interest as biofuels to replace petroleum-derived compounds (Kalscheuer et al. 2006, Fortman et al. 2008, Röttig et al. 2010, Steen et al. 2010). Therefore, this algae-derived hexadecenol also has potential to be used as a petroleum additive.

Asterarcys quadricellulare KNUA020 as PUFA feedstock

As mentioned above, little is known about the microalga isolated in the present study since there is only one species in the genus *Asterarcys* has been accepted taxonomically. Consequently, studies on *A. quadricellulare* are quite rare. It therefore is worth studying the physio-

Table 2. List of fatty acids and fatty alcohol present in *Asterarcys quadricellulare* KNUA020

Peak No.	Component	Content (%)	Yield (mg g^{-1} DW)
1	Hexadecatetraenoic acid ($C_{16:4}$)[a]	14.8 ± 0.4	22.9
2	Palmitoleic acid ($C_{16:1}$ ω7)[b]	6.8 ± 0.8	10.5
3	Palmitic acid ($C_{16:0}$)	15.3 ± 0.7	23.7
4	Unknown[c]	2.2 ± 0.2	3.4
5	Linoleic acid ($C_{18:2}$ ω6)[b]	3.6 ± 0.4	5.6
6	α-Linolenic acid ($C_{18:3}$ ω3)[b]	41.2 ± 8.3	63.7
7	Hexadecenol ($C_{20}H_{40}O$)	16.1 ± 8.5	24.9

[a]Double-bond positions were unknown.
[b]Unsaturated positions were assigned by the gas chromatography/mass spectrometry (GC/MS) library.
[c]The unknown fatty acid had no name listed in the GC/MS library.

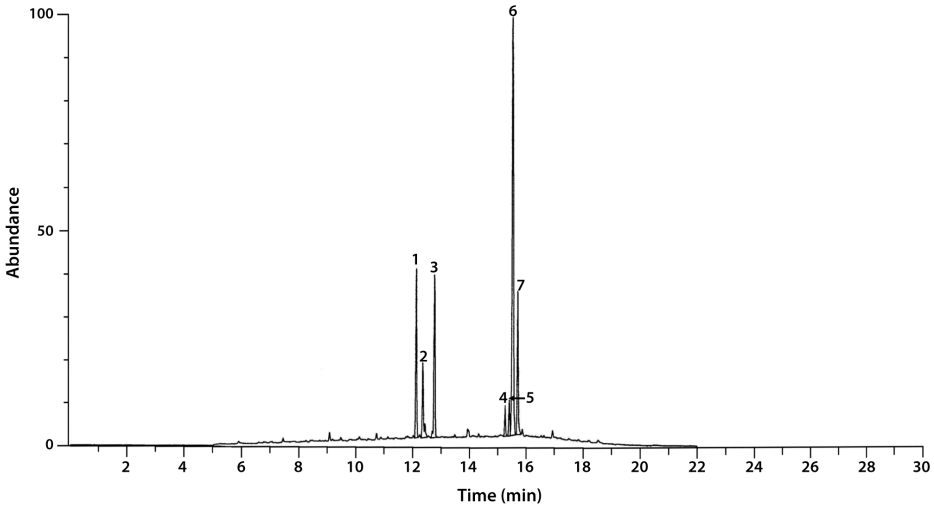

Fig. 4. Gas chromatography/mass spectrometry (GC/MS) profile of lipids extracted from *Asterarcys quadricellulare* KNUA020. 1, hexadecatetraenoic acid (C16:4); 2, palmitoleic acid (C16:1 ω7); 3, palmitic acid (C16:0); 4, unknown substrate; 5, linoleic acid (C18:2 ω6); 6, α-linolenic acid ($C_{18:3}$ ω3); 7, hexadecenol ($C_{20}H_{40}O$).

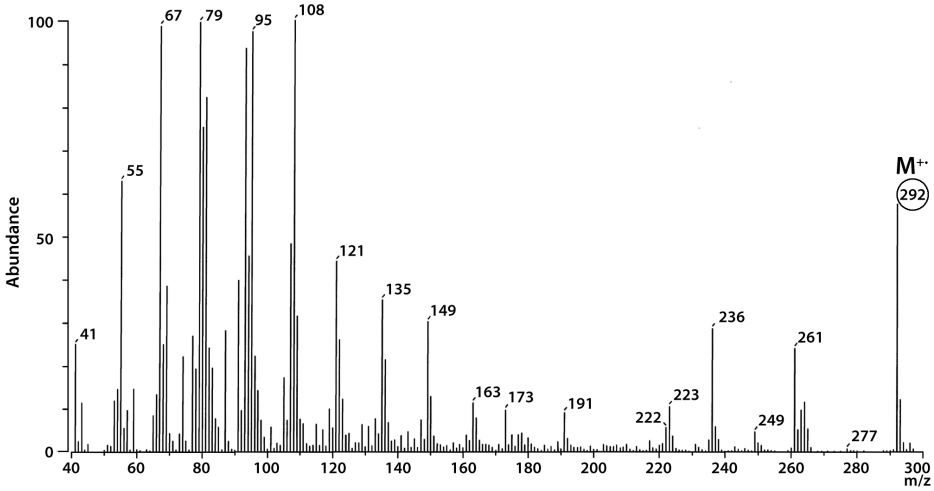

Fig. 5. Electron impact mass spectrum (EI-MS) of α-linolenic acid ($C_{18:3}$ ω3) produced by *Asterarcys quadricellulare* KNUA020.

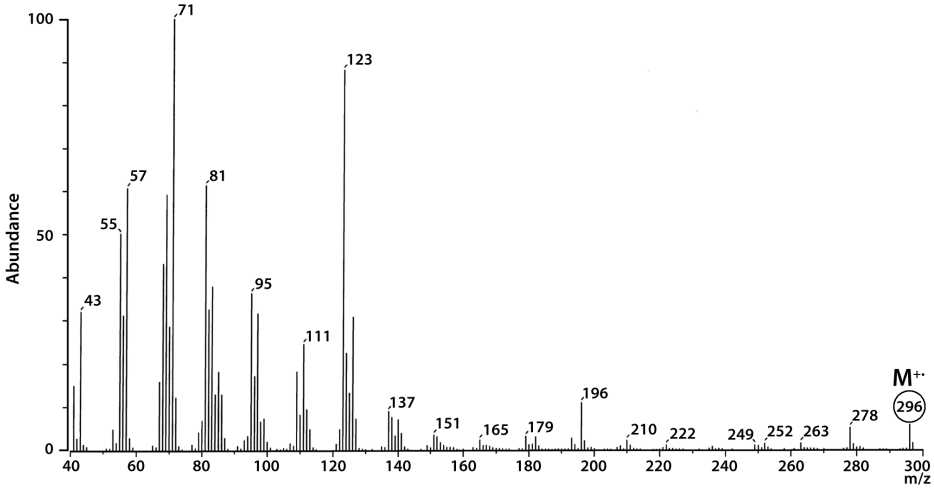

Fig. 6. Electron impact mass spectrum (EI-MS) of hexadecenol ($C_{20}H_{40}O$) produced by *Asterarcys quadricellulare* KNUA020.

logical characteristics and potential of *A. quadricellulare* KNUA020 as PUFA feedstock because it is a microorganism indigenous to South Korea. Furthermore, it can be easily cultivated in fresh water given the proper sunlight, temperature, and inorganic salts. The full potential of the isolate should be evaluated by further cultivation and molecular studies in the laboratory and field using various approaches.

CONCLUSION

In the present study, a South Korean domestic microalga, *Asterarcys quadricellulare* KNUA020, was isolated and its optimal growth conditions (at 25°C and pH 7.0) were determined. GC/MS results suggested that the KNUA020 strain has the fatty acid profile ($C_{18:3}$ ω3) desirable for the ω3 PUFA production. This microalga was also able to autotrophically produce hexadecenol ($C_{20}H_{40}O$), a high value, long-chain fatty alcohol. In conclusion, the *A. quadricellulare* KNUA020 strain appears to be a promising algae-based biochemical feedstock.

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REFERENCES

Benemann, J. R., Tillett, D. M. & Weissman, J. C. 1987. Microalgae biotechnology. Trends Biotechnol. 5:47-53.

Bigogno, C., Khozin-Goldberg, I., Boussiba, S., Vonshak, A. & Cohen, Z. 2002. Lipid and fatty acid composition of the green oleaginous alga *Parietochloris incisa*, the richest plant source of arachidonic acid. Phytochemistry 60:497-503.

Fortman, J. L., Chhabra, S., Mukhopadhyay, A., Chou, H., Lee, T. S., Steen, E. & Keasling, J. D. 2008. Biofuel alternatives to ethanol: pumping the microbial well. Trends Biotechnol. 26:375-381.

Friedl, T. & Rokitta, C. 1997. Species relationships in the lichen alga *Trebouxia* (Chlorophyta, Trebouxiaceae): molecular phylogenetic analyses of nuclear-encoded large subunit rRNA gene sequences. Symbiosis 23:125-148.

Gao, C., Zhai, Y., Ding, Y. & Wu, Q. 2010. Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Appl. Energ. 87:756-761.

Hegewald, E. & Schmidt, A. 1992. *Asterarcys Comas*, eine weit verbreitete tropische Grünalgengattung. Algol. Stud. 66:25-30.

Hegewald, E., Wolf, M., Keller, A., Friedl, T. & Krienitz, L. 2010. ITS2 sequence-structure phylogeny in the Scenedesmaceae with special reference on *Coelastrum* (Chlorophyta, Chlorophyceae), including the new genera *Comasiella* and *Pectinodesmus*. Phycologia 49:325-335.

Helms, G., Friedl, T., Rambold, G. & Mayrhofer, H. 2001. Identification of photobionts from the lichen family Physciaceae using algal-specific ITS rDNA sequencing. Lichenologist 33:73-86.

Ikawa, M. 2004. Algal polyunsaturated fatty acids and effects on plankton ecology and other organisms. UNH Cent. Freshw. Biol. Res. 6:17-44.

Kalscheuer, R., Stölting, T. & Steinbüchel, A. 2006. Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology 152:2529-2536.

Khozin-Goldberg, I., Iskandarov, U. & Cohen, Z. 2011. LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. Appl. Microbiol. Biotechnol. 91:905-915.

Liang, Y., Sarkany, N. & Cui, Y. 2009. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol. Lett. 31:1043-1049.

Miao, X. & Wu, Q. 2006. Biodiesel production from heterotrophic microalgal oil. Bioresour. Technol. 97:841-846.

Otleş, S. & Pire, R. 2001. Fatty acid composition of *Chlorella* and *Spirulina* microalgae species. J. AOAC Int. 84:1708-1714.

Patil, V., Källqvist, T., Olsen, E., Vogt, G. & Gislerød, H. R. 2007. Fatty acid composition of 12 microalgae for possible use in aquaculture feed. Aquac. Int. 15:1-9.

Pyle, D. J., Garcia, R. A. & Wen, Z. 2008. Producing docosahexaenoic acid (DHA)-rich algae from blodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition. J. Agric. Food Chem. 56:3933-3939.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111:1-61.

Röttig, A., Wenning, L., Bröker, D. & Steinbüchel, A. 2010. Fatty acid alkyl esters: perspectives for production of al-

ternative biofuels. Appl. Microbiol. Biotechnol. 85:1713-1733.

Seto, A., Wang, H. L. & Hesseltine, C. W. 1984. Culture conditions affect eicosapentaenoic acid content of *Chlorella minutissima*. J. Am. Oil Chem. Soc. 61:892-894.

Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., del Cardayre, S. B. & Keasling, J. D. 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature 463:559-562.

White, T. J., Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (Eds.) *PCR Protocols: A Guide to Methods and Applications*. Academic Press, San Diego, CA, pp. 315-322.

Xiong, W., Li, X., Xiang, J. & Wu, Q. 2008. High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Appl. Microbiol. Biotechnol. 78:29-36.

Yeo, I., Jeong, J., Cho, Y., Hong, J., Yoon, H. -S., Kim, S. H. & Kim, S. 2011. Characterization and comparison of biodiesels made from Korean freshwater algae. Bull. Korean Chem. Soc. 32:2830-2832.