J. Mar. Biosci. Biotechnol. 2017, p. 22 - 29 Vol. 9, No. 2 [Research Paper]

https://doi.org/10.15433/ksmb.2017.9.2.022 ISSN 2383-5400 (Online)

Characterization of *Heterochlorella luteoviridis* (Trebouxiaceae, Trebouxiophyceae) isolated from the Port of Jeongja in Ulsan, Korea

Kyeong Mi Kim[1], Nam Seon Kang[2], Hyeong Seok Jang[1], Joon Sang Park[1], Byung Hee Jeon[1], Ji Won Hong[1]*

[1]Department of Taxonomy and Systematics, National Marine Biodiversity Institute of Korea, Seocheon 33662, Republic of Korea
[2]Marine Life-resources Management Department, National Marine Biodiversity Institute of Korea, Seocheon 33662, Republic of Korea

(Received 14 November 2017, Revised 4 December 2017, Accepted 6 December 2017)

Abstract A unicellular green alga was axenically isolated from the Port of Jeongja, Ulsan, Korea. Morphological, molecular, and biochemical analyses revealed that the isolate belonged to *Heterochlorella luteoviridis*. This is the first report of this species in Korea. The microalgal strain was named as *H. luteoviridis* MM0014 and its growth, lipid composition, and biomass properties were investigated. The strain thrived over a wide range of temperatures (5–30°C) and withstood up to 0.5 M NaCl. The results of gas chromatography/mass spectrometry analysis showed that the isolate was rich in nutritionally important polyunsaturated fatty acids. Its major fatty acids were linoleic acid (35.6%) and α-linolenic acid (16.2%). Thus, this indigenous marine microalga is a potential alternative source of ω3 and ω6 polyunsaturated fatty acids, which are currently obtained from fish and plant oils. Ultimate analysis indicated that the gross calorific value was 19.7 MJ kg^{-1}. In addition, the biomass may serve as an excellent animal feed because of its high protein content (51.5%). Therefore, *H. luteoviridis* MM0014 shows promise for applications in the production of microalgae-based biochemicals and biomass feedstock.

Keywords : Elemental analysis, Fatty acids, First record, *Heterochlorella luteoviridis*, Marine microalga

Introduction

Green microalgae (Chlorophyta) are the primary producers in the marine ecosystem and play pivotal roles in global carbon, nitrogen, and phosphorus cycles [1, 7, 14]. Because of their ability to convert carbon dioxide into a variety of valuable organic compounds via photosynthesis [8, 17, 23], microalgae have gained considerable attention and numerous studies have assessed their potential as biotechnological resources [6, 27, 28].

In this study, we evaluated a unicellular marine microalga, *Heterochlorella luteoviridis* MM0014 from the Port of Jeongja, Ulsan, Korea. *Heterochlorella luteoviridis* was formerly known as *Chlorella luteoviridis*, but was revised by Neustupa *et al*. in 2009 [19], and was originally isolated from a pool in a forest of Oisquercq in Belgium [19]. Although this species has been commercialized in Europe for a long time, few studies have examined its biomass composition [9, 15]. This report provides information on the first record of this species

* Corresponding author
Phone: +82-41-950-0743 Fax: +82-41-950-0727
E-mail: jwhong@mabik.re.kr

in Korea and its morphological, molecular, and chemotaxonomic features.

Materials and Methods

Sample collection and isolation

Seawater samples were collected in October 2016 from the Port of Jeongja (35°36' 54.8"N, 129°27' 00.0"E), Jeongja-dong, Buk-gu, Ulsan, Korea. The temperature of the seawater was 22.7℃ and its salinity was 27.3 PSU. Samples were transported to the laboratory and 1 mL of each sample was inoculated into 100 mL BG-11 medium [25]. Imipenem (JW Pharmaceutical, Seoul, Korea), a broad-spectrum antibiotic, was added to the medium at a concentration of 100 μg mL^{-1} to suppress bacterial growth. The flasks were incubated under static conditions at 20℃ in a growth chamber (FLI-2010A, Eyela, Tokyo, Japan) under cool fluorescent light (approximately 40 μmole m^{-2} s^{-1}) with a light:dark cycle (16:8 h) until algal growth was apparent. Well-grown algal cultures (1.5 mL) were centrifuged at 3000 ×*g* for 3 min to separate contaminating bacteria from the algal cells and harvest the algal biomass. The resulting pellets were streaked onto R2A agar (BD Biosciences, San Jose, CA, USA) supplemented with imipenem (20 μg mL^{-1}) and incubated as described above. A single colony was aseptically re-streaked onto a fresh R2A agar plate to obtain an axenic culture.

Morphological identification

The isolate was grown in R2A medium for 2 weeks. Live cells were harvested by centrifugation at 3000 ×*g* for 3 min, washed thrice with sterile distilled water, and examined at 1000× magnification under a Zeiss Axio Imager A2 light microscope (Carl Zeiss, Göttingen, Germany). For scanning electron microscopy (SEM), 10 mL aliquots of cultures at approximately 1000 cells mL^{-1} were fixed for 1 h in osmium tetroxide (OsO_4, Electron Microscopy Sciences, Hatfield, PA, USA) at a final concentration of 2% (v/v). The fixed cells were collected on a 3-μm pore size, polycarbonate membrane filter (Whatman, Kent, UK) and washed thrice with distilled water to remove residual media components. The membranes were dehydrated in an ethanol series (10, 30, 50, 70, 90, and 100% ethanol, followed by two changes in 100% ethanol, Merck, Darmstadt, Germany) and immediately dried using an automated critical point dryer (EM CPD300, Leica, Wetzlar, Germany). The dried filters were mounted on an aluminum stub (Electron Microscopy Sciences) using copper conductive doubled-side tape (Ted Pella, Redding, CA, USA) and coated with gold in an ion sputter (MC1000, Hitachi, Tokyo, Japan). Surface morphology was observed by SEM (SU3500, Hitachi).

Molecular identification

For molecular analysis, genomic DNA was extracted using a DokDo-Prep Genomic DNA kit (Elpis, Daejoen, Korea) and further purified using the Wizard Genomic DNA Clean-Up System (Promega, Madison, WI, USA). The universal primers NS1/NS8 and ITS1/ITS4 described by White *et al*. [32] were used to amplify the 18S rRNA sequence and internal transcribed spacer (ITS) region, respectively. Because of the highly conserved nature of rRNA, the region RuBisCO *rbc*L was also amplified with the primers *rbc*L 7F and *rbc*L 1391R, described by Verbruggen *et al*. [30]. Phylogenetic analyses were performed with the 18S rRNA and *rbc*L sequences of strain MM0014 using the software package MEGA ver. 6.0 [29]. Closely related sequences were downloaded and aligned using MEGA software with the ClustalW tool. The best-fit nucleotide substitution models (Kimura 2-parameter + Gamma distributed for 18S rRNA and General Time Reversible + Gamma distributed for *rbc*L, respectively) were selected using MEGA 6.0 based on the Bayesian information criterion. These models were used to build a maximum likelihood phylogenetic trees with 1000 bootstrap replicates. *Auxenochlorella protothecoides* MM0011 sequences (MF040300 and MF043910) were used as an outgroup in both trees. The phylogenetic tree for ITS sequence was not built because of the lack

of sequence data for the species in the public database. All analyses were carried out in triplicate unless otherwise stated. DNA sequences obtained in this study were deposited in the database of the National Center for Biotechnology Information under accession numbers MG491519, MG491520, and MG495094 (Table 1).

Table 1. Results from BLAST searches using the sequences of the 18S rRNA, ITS, and *rbc*L genes of strain MM0014.

Marker Gene	Accession No.	Size (bp)	Closest match (GenBank accession No.)	Overlap (%)	Similarity (%)
18S rRNA	MG491519	1773	*Heterochlorella luteoviridis* SAG 2213 (KM116462)	100	100
ITS	MG491520	706	*Heterochlorella luteoviridis* SAG 2213 (KM116462)	100	99
*rbc*L	MG495094	1385	*Heterochlorella luteoviridis* (HE984580)	94	93

Temperature and NaCl tolerance testing

Routine serial subculturing on R2A agar slant was performed to maintain the pure culture of *H. luteoviridis*. A single colony of strain MM0014 was streaked onto R2A agar plates in triplicate and incubated for 21 days. Survival and growth of MM0014 cells maintained at temperatures ranging from 5℃ to 35℃ (at intervals of 5℃) were examined to determine the optimum culture temperature as described by Jang *et al*. [16]. An NaCl tolerance test was conducted at 20℃ using R2A agar supplemented with 0.0, 0.5, 1.0, 1.5, and 2.0 M NaCl.

Gas chromatography/mass spectrometry (GC/MS) analysis

The isolate was heterotrophically grown in R2A medium for 18 days at 20℃ with shaking at 160 rpm on an orbital shaker (SH30, Fine PCR, Gunpo, Korea) and cells were harvested by centrifugation at 2063 ×*g* (1580R, Labogene, Daejeon, Korea) for lipid analysis. The samples were freeze-dried and pulverized to enhance the extraction efficiency. Lipid extraction was performed as described by Breuer *et al*. [2]. The FAME composition was analyzed using a 7890A gas chromatograph equipped with a 5975C mass selective detector (Agilent Technologies, Santa Clara, CA, USA). GC runs were performed on a DB-FFAP column (30 m, 250 μm ID, 0.25 μm film thickness; Agilent Technologies). The initial oven temperature of the gas chromatograph was 50℃ and maintained for 1 min. The temperature was increased to 200℃ at a rate of 10℃ min^{-1} for 30 min and then increased to 240℃ at a rate of 10℃ min^{-1}, which was held for 20 min. The injection volume was 1 μL with a split ratio of 20:1. Helium was used as a carrier gas at a constant flow rate of 1 mL min^{-1}. The mass spectrometer parameters were as follows: injector and source temperatures were 250℃ and 230℃, respectively, and electron impact mode at an acceleration voltage of 70 eV was used for sample ionization, with an acquisition range of 50 – 550 m z^{-1}. Compound identification was performed by matching the mass spectra with those in the Wiley/NBS libraries. Searches showing a match value higher than 90% were considered valid.

Biomass characterization

The freeze-dried biomass samples were pulverized with a mortar and pestle and sieved through ASTM No. 230 mesh (opening = 63 μm). 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, Waltham, MA, USA) in duplicate. Gross calorific value (GCV) was estimated using the following equation developed by Friedl *et al*. [10]: [$GCV = 3.55C^2 - 232C - 2230H + 51.2C \times H + 131N + 20600$ (MJ kg^{-1})]. Protein content was calculated from the N content in the ultimate analysis by using a conversion factor (×6.25).

Results

Identification of the strain MM0014

The cells were solitary, non-motile, and round-shaped with diameters ranging from approximately 5 µm (young cells) to 10 µm (old cells) (Fig. 1, Fig. 2). A prominent cup-shaped chloroplast was present (Fig. 1). Overall, strain MM0014 showed typical morphology of the species *H. luteoviridis*. Molecular characterization inferred from sequence analyses of the 18S rRNA, ITS region, and *rbc*L also showed that the isolate belonged to the *H. luteoviridis* group, and all results were in agreement (Table 1, Fig. 3, Fig. 4). Therefore, this marine microalga was identified as *H. luteoviridis* MM0014. The isolate was deposited at the Korean Collection for Type Cultures under accession number KCTC13395BP.

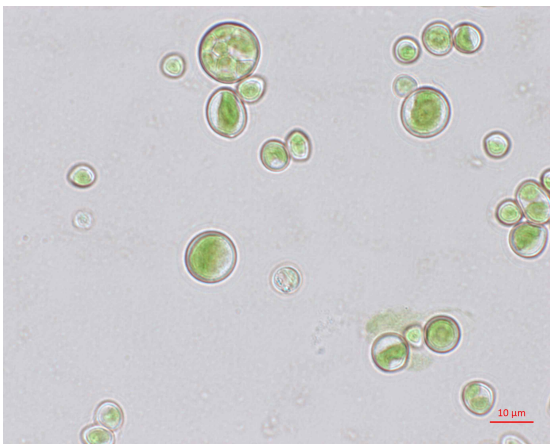

Figure 1. Light microscopy image of *H. luteoviridis* MM0014.

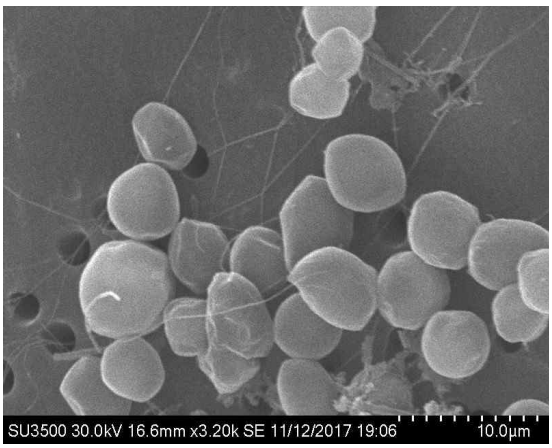

Figure 2. SEM image of *H. luteoviridis* MM0014.

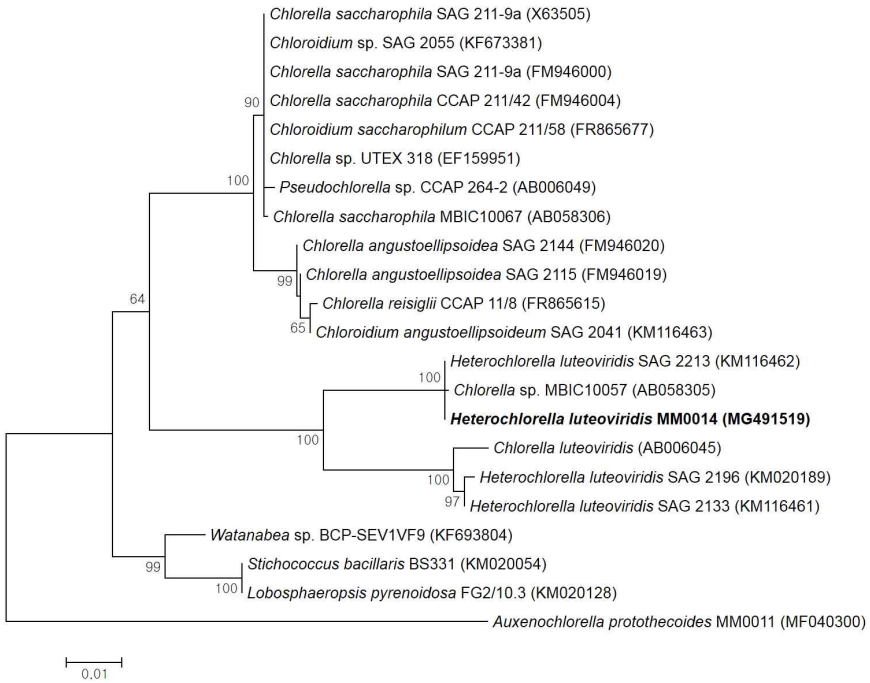

Figure 3. Phylogenetic relationship of strain MM0014 and its closely related species inferred from the 18S rRNA sequence data. The tree was generated by the maximum likelihood method with 1000 bootstrap replicates. The scale bar represents a 1% difference in nucleotide sequences.

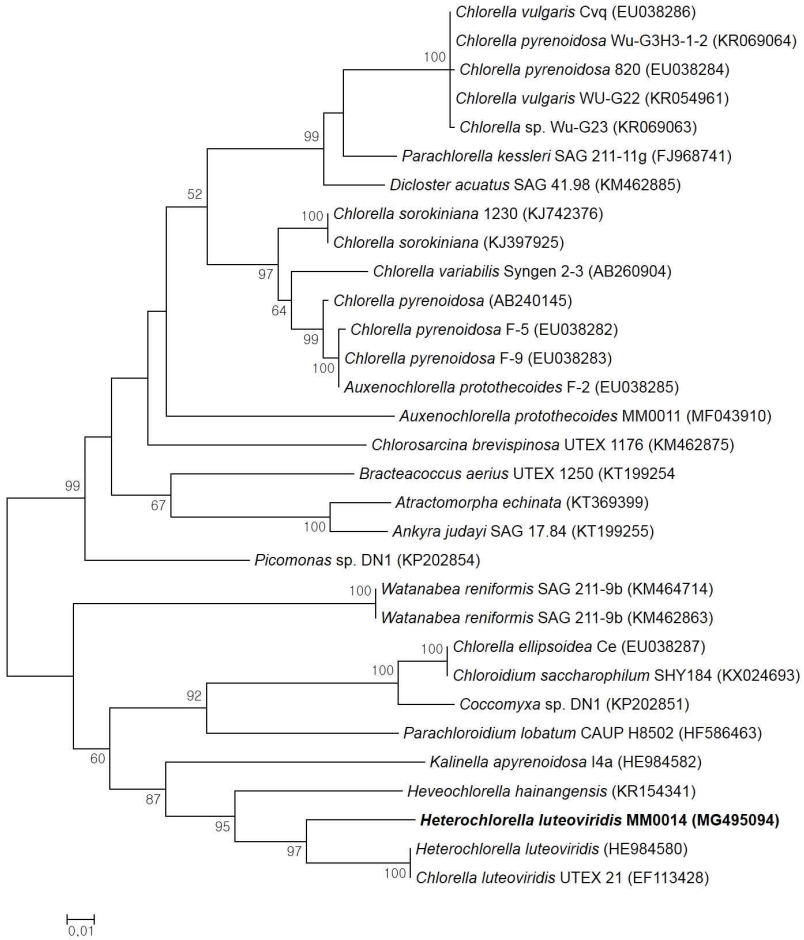

Figure 4. Phylogenetic relationship of strain MM0014 and its closely related species inferred from the *rbc*L sequence data. The tree was generated by the maximum likelihood method with 1000 bootstrap replicates. The scale bar represents a 1% difference in nucleotide sequences.

Optimal growth conditions for strain MM0014

H. luteoviridis MM0014 grew at temperatures of 5–30℃; maximum growth was observed at ambient temperatures (Table 2). It did not grow at 35℃ and growth was suppressed at 5℃. Additionally, the isolate withstood up to 0.5 M NaCl and did not survive at 1.0 M NaCl.

Table 2. Growth of strain MM0014 at various temperatures and NaCl concentrations.

Temp.	5℃	10℃	15℃	20℃	25℃	30℃	35℃
Growth	+	+++	+++	+++	+++	+	-
NaCl	0.0 M	0.5 M	1.0 M	1.5 M	2.0 M		
Growth	+++	+++	-	-	-		

+++ : good growth, + : poor growth, - : no growth

Fatty acid composition of strain MM0011

The FAME profile of *H. luteoviridis* MM0014 based on the average ± standard deviation of three determinations is summarized in Table 3. The major cellular fatty acids of the isolate were $C_{16:0}$ (20.7% ± 0.3%), $C_{18:2}$ (35.6% ± 1.1%), and $C_{18:3}$ (16.2% ± 0.7%). In addition, trace amounts of unsaturated fatty acids such as $C_{16:2}$ (2.5% ± 0.1%), $C_{16:3}$ (2.8% ± 0.1%), and $C_{18:1}$ (7.1% ± 6.0%) were detected in this photosynthetic microorganism.

Biomass characterization of strain MM0011

Ultimate analysis results are shown in Table 4. The GCV and protein content based on ultimate analysis were 19.7 MJ kg^{-1} and 51.5%, respectively.

Table 3. Lipid profile of strain MM0014.

No.	Component	Content	Note
1	6,9-Heptadecadiene ($C_{17}H_{32}$)	0.6 ± 0.0	-
2	Palmitic acid ($C_{16:0}$)	20.7 ± 0.3	SFA
3	Hexadecadienoic acid ($C_{16:2}$)	2.5 ± 0.1	-
4	Hexadecatrienoic acid ($C_{16:3}$)	2.8 ± 0.1	-
5	Stearic acid ($C_{18:0}$)	1.4 ± 0.2	-
6	Oleic acid ($C_{18:1}$)	7.1 ± 6.0	ω9 PUFA
7	Linoleic acid ($C_{18:2}$)	35.6 ± 1.1	ω6 PUFA
8	α-Linolenic acid ($C_{18:3}$)	16.2 ± 0.7	ω3 PUFA

Table 4. Ultimate analysis results of *H. luteoviridis* MM0014.

Elemental composition	Ultimate analysis (wt%)
C	8.2 ± 0.0
H	46.8 ± 0.1
N	7.2 ± 0.0
S	0.3 ± 0.1
Protein	51.5 ± 0.1
GCV (MK kg^{-1})	19.7 ± 0.0

Discussion

Because of its extremely simple morphology, *H. luteoviridis* was previously regarded as *Chlorella luteoviridis*, but it was later was ranked as a new genus [19] and *H. luteoviridis* is currently the only taxonomically accepted species [13]. Light and electron microscopic analyses suggested that the isolate shared very similar morphological characteristics with *H. luteoviridis*, including its cup-shaped chloroplast and round-shaped cells (Fig. 1, Fig. 2). Molecular identification results also confirmed that MM0014 belonged to the species. *H. luteoviridis* is known as a terrestrial species [19], but this manuscript describes the first record of the species isolated from seawater in Korea.

Analysis of the cellular fatty acid composition of strain MM0014 revealed that it is rich in $C_{16:0}$ (20.7%) saturated fatty acid and the $C_{18:2}$ ω6 (35.6%) and $C_{18:3}$ ω3 (16.2%) unsaturated fatty acids. The results are similar to those of a previous study by Jaeschke *et al.* [15]. Numerous studies have emphasized that these essential PUFAs have many beneficial health effects [18] including antibacterial activities [4, 12] and anti-oxidant properties [22]. PUFAs are known to play important roles for human metabolism as they are the major components of cell membrane phospholipids and cellular storage oils [3, 11]. In addition, signaling molecules that regulate mediate inflammation, cardiac function, and tumor growth are biosynthesized by PUFAs [21, 24, 31]. These properties are indicative of the potential of PUFA for nutraceutical and pharmaceutical purposes and a variety of commercial products containing these PUFAs are available worldwide [20]. Omega-3 PUFAs are typically derived from fish oils, while omega-6 PUFAs are primarily obtained from plant sources such as sunflower, corn, and soybean oils. Therefore, the isolate may be useful as an alternative to fish-based sources for vegetarians. In addition, the 16-carbon saturated palmitic acid, which is suitable for biodiesel production, was also biosynthesized by strain MM0014 as one of the major fatty acids. However, improved productivity may be achieved by evaluating the effects of various culture conditions including media components on stain MM0014.

The GCV was also calculated to understand the potential of microalgal biomass as a biofuel feedstock (Table 4). The results demonstrated that the GCV was within the range of terrestrial energy crops (17.0–20.0 MJ kg^{-1}) [26]. Considering the fast growth rate of the microalgae, microalgal production of biomass is advantageous compared to plants. In addition, the biomass may serve as an excellent animal feed because of its high protein content (51.5%).

Because *H. luteoviridis* along with *C. vulgaris* and *C. pyrenoidosa* were consumed as foods in the European Union prior to 1997, it is not subjected to Novel Food Regulation (EC) No. 258/97 [5]. Consequently, pre-market authorization is not required,

which will aid future commercial applications in European countries.

Conclusions

In this study, we report the first record of *H. luteoviridis* in Korea and its living cultures were added to the public culture collections. In conclusion, this indigenous microalga may serve as biological resource for producing compounds of biochemical interest. The potential of this maritime microalga should be evaluated through further cultivation studies at the molecular, laboratory, and field scales.

Acknowledgments

This work was supported by the Securement, Analysis, and Evaluation of Marine Plant Bioresources (2017M00700), funded by the National Marine Biodiversity Institute of Korea (MABIK).

References

1. Arrigo, K. R. 2005. Marine microorganisms and global nutrient cycles. *Nature* **437**, 349-355.
2. Breuer, G., Evers, W. A. C., de Vree, J. H., Kleinegris, D. M. M., Martens, D. E., Wijffels, R. H. and Lamers, P. P. 2013 Analysis of fatty acid content and composition in microalgae. *J. Vis. Exp.* **80**, e50628.
3. Calder, P. C. 2010 Omega-3 fatty acids and inflammatory processes. *Nutrients* **2**, 355-374.
4. Desbois, A. P. and Lawlor, K. C. 2013. Antibacterial activity of long-chain polyunsaturated fatty acids against *Propionibacterium acnes* and *Staphylococcus aureus*. *Mar. Drugs* **11**, 4544-4557.
5. Champenois, J, Marfaing, H. and Pierre, R. 2015 Review of the taxonomic revision of *Chlorella* and consequences for its food uses in Europe. *J. Appl. Phycol.* **27**, 1845-1851.
6. Chew, K. W., Yap, J. Y., Show, P. L., Suan, N. H., Juan, J. C., Ling, T. C., Lee, D. J. and Chang, J. S. 2017. Microalgae biorefinery: High value products perspectives. *Bioresour. Technol.* **229**, 53-62.
7. Cloern, J. E., Foster, S. Q. and Kleckner, A. E. 2014. Phytoplankton primary production in the world's estuarine-coastal ecosystems. *Biogeosciences* **11**, 2477-2501.
8. Cuellar-Bermudez, S. P., Aguilar-Hernandez, I., Cardenas-Chavez, D. L., Ornelas-Soto, N., Romero-Ogawa, M. A. and Parra-Saldivar, R. 2015. Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. *Microb. Biotechnol.* **8**, 190- 209.
9. Diprat, A. B., Menegol, T., Boelter, J. F., Zmozinski, A., Rodrigues Vale, M. G., Rodrigues, E., and Rech, R. 2017. Chemical composition of microalgae *Heterochlorella luteoviridis* and *Dunaliella tertiolecta* with emphasis on carotenoids. *J. Sci. Food Agric.* **97**, 3463-3468.
10. Friedl, A., Padouvas, E., Rotter, H. and Varmuza, K. 2005. Prediction of heating values of biomass fuel from elemental composition. *Anal. Chim. Acta* **544**, 191-198.
11. Gill, I. and Valivety, R. 1997. Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications. *Trends Biotechnol.* **15**, 401-409.
12. Guedes, A. C., Amaro, H. M. and Malcata F. X. 2011. Microalgae as sources of high added-value compounds—a brief review of recent work. Biotechnol. Progr. **27**, 597-613.
13. Guiry, M. D. and Guiry, G. M. 2017. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org (searched on 12 November 2017)
14. Hutchins, D. A., Mulholland, M. R. and Fu, F. 2009. Nutrient cycles and marine microbes in a CO_2-enriched ocean. *Oceanography* **22**, 128-145.
15. Jaeschke, D. P., Menegol, T., Rech, R., Mercali, G. D. and Marczak, L. D. F. 2016. Carotenoid and lipid extraction from *Heterochlorella luteoviridis* using moderate electric field and ethanol. *Process Biochem.* **51**, 1636-1643.
16. Jang, H. S., Kang, N. S., Kim, K. M., Jeon, B. H., Park, J. S. and Hong, J. W. 2017. Description and application of a marine microalga *Auxenochlorella protothecoides* isolated from Ulleung-do. *J. Life Sci.* **27**, 1152-1160.
17. Leu, S. and Boussiba, S. 2014. Advances in the production of high-value products by microalgae. *Ind. Biotechnol.* **10**, 169-183.
18. Mehta, L. R. Dworkin, R. H. and Schwid, S. R. 2009. Polyunsaturated fatty acids and their potential therapeutic role in multiple sclerosis. *Nat. Clin. Pract. Neurol.* **5**, 82-92.
19. Neustupa, J., Němcová, Y., Eliáš, M. and Škaloud, P. 2009. *Kalinella bambusicola* gen. et sp. nov. (Trebouxiophyceae, Chlorophyta), a novel coccoid *Chlorella* like subaerial alga from Southeast Asia. *Phycol. Res.* **57**, 159-169.
20. Packaged Facts. 2012. The Global Market for EPA/DHA Omega-3 Products. Published online at: https://www.packagedfacts.com/Global-EPA-DHA-7145087/ (accessed on 12 November 2017).
21. Pereira, H., Barreira, L., Figueiredo, F., Custódio, L., Vizetto-Duarte, C., Polo, C., Rešek, E., Engelen, A. and

Varela, J. 2012. Polyunsaturated fatty acids of marine macroalgae: potential for nutritional and pharmaceutical applications. *Mar. Drugs* **10**, 1920-1935.
22. Plaza, M., Herrero, M., Cifuentes, A. and Ibáñez, E. 2009. Innovative natural functional ingredients from microalgae. *J. Agric. Food Chem.* **57**, 7159-7170.
23. Pulz, O. and Gross, W. 2004. Valuable products from biotechnology of microalgae. *Appl. Microbiol. Biotechnol.* **65**, 635-648.
24. Radwan, S. S. 1991. Sources of C_{20}-polyunsaturated fatty acids for biotechnological use. *Appl. Microbiol. Biot.* **35**, 421-430.
25. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. and Stanier, R. 1979. Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **111**, 1-61.
26. Ross, A. B., Jones, J. M., Kubacki, M. L. and Bridgeman, T. 2008. Classification of macroalgae as fuel and its thermochemical behaviour. *Bioresour. Technol.* **99**, 6494-6504.
27. Schirmer, A., Rude, M. A., Li, X., Popova, E. and Del Cardayre, S. B. 2010. Microbial biosynthesis of alkanes. *Science* **329**, 559-562.
28. Spolaore, P., Joannis-Cassan, C., Duran, E. and Isambert, A. 2006. Commercial applications of microalgae. *J. Biosci. Bioeng.* **101**, 87-96.
29. Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725-2729.
30. Verbruggen, H., Ashworth, M., LoDuca, S. T., Vlaeminck, C., Cocquyt, E., Sauvage, T., Zechman, F. W., Littler, D. S., Littler, M. M., Leliaert, F. and DeClecrk, O. 2009. A multi-locus time-calibrated phylogeny of the siphonous green algae. *Mol. Phylogenet. Evol.* **50**, 642-653.
31. Vrablik, T. L. and Watts, J. L. 2013. Polyunsaturated fatty acid derived signaling in reproduction and development: insights from *Caenorhabditis elegans* and *Drosophila melanogaster*. *Mol. Reprod. Dev.* **80**, 244-259.
32. White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (eds), PCR protocols: a guide to methods and applications. Academic Press, San Diego, California, pp. 315-322.