THESES OF DOCTORAL (PhD) DISSERTATION

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PHYLOGENETIC RE-EVALUATION OF PREVIOUSLY IDENTIFIED *CHLAMYDOMONAS* (CHLOROPHYTA, CHLAMYDOMONADACEAE) STRAINS FROM THE MOSONMAGYARÓVÁR ALGAL CULTURE COLLECTION, HUNGARY, USING MOLECULAR DATA, AND CHARACTERIZATION OF EXTRACELLULAR POLYSACCHARIDE (EPS) PRODUCTION IN SOME *CHLAMYDOMONAS* STRAINS.

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1. INTRODUCTION

Chlamydomonas is one of the biggest green algal genera with more than 800 described species. Approximately 400 strains are available in collections and applicable for research purposes. Referring to the versatility of genus *Chlamydomonas*, it is applied on scientific fields such as genetics, photosynthesis research, UV resistance issues, possibilities of hydrogen, biogas and biodiesel production, hormone research, agriculture and medicine.

Since the 1990s, the use of molecular markers for phylogenetic analysis demonstrated that the morphological approach for taxonomic identification is appropriate neither for most green algae, nor for the genus *Chlamydomonas*. Most green alga genera are polyphyletic, so their status and species number require further revision. The latest trend is the polyphasic approach that combines different methods like morphology, cytology, ultrastructural and molecular biological studies.

Systematic studies on 70 MACC (Mosonmagyaróvár Algal Culture Collection, Hungary) isolates previously identified as '*Chlamydomonas*', a unicellular flagellate, were carried out based on the selected gene section of the 18S rRNA gene.

The other aim of this dissertation was to find some valuable extracellular polysaccharides (EPS) producing *Chlamydomonas* strains for agriculture. Some species of the genus *Chlamydomonas* Ehrenberg nom. cons. (1833) can secrete EPS under specific conditions. Up to now, no results were available for EPS production of MACC strains. There are valuable EPS producers among the MACC *Chlamydomonas* strains. These may be, for example, raw materials for soil conditioning products.

Objectives:

- Updating the taxonomic classification of MACC *Chlamydomonas* strains, putting them into phylogenetic groups. The latter will show the present position of the MACC *Chlamydomonas* strains on a section of the algae tree of life.

- Extraction and characterization of extracellular polysaccharides from selected strains. This may form the basis for future soil conditioning experiments.

2. MATERIALS AND METHODS

2.1. Taxonomic re-evaluation using molecular data

Laboratory cultivation of microalgae

Seventy strains were selected from MACC, Széchenyi István University (Mosonmagyaróvár, Hungary), which were previously identified as *Chlamydomonas* using morphological data. Forty-six of the 70 MACC *Chlamydomonas* strains were from Brazil, 11 from Hungary, 9 from the Czech Republic, 2 from Ukraine, one from Slovenia and one from Russia. The author propagated single-cell cultures of *Chlamydomonas* in a microalgae farming equipment as described earlier by Ördög (1982):

Stock cultures were inoculated into 500 mL Erlenmeyer flasks containing 250 mL modified Z8 medium at 24-26 °C under a light intensity of 130 μM m^{-2} s^{-1} provided by Lumoflor and cool white fluorescent tubes (14 h/10 h light/dark cycle). Cultures were aerated with 20 L h^{-1} (=1.33 L air L^{-1} nutrient medium per minute) sterile humidified air enriched with 1.5 % CO_2 during the light period. Cultures were further inoculated for growth analysis. The analyzed cultures always contained 10 mg/L of algae biomass.

Based on the characteristic of showing jelly-like units when grown in a Petri dish, twenty green algae strains were selected from the Mosonmagyaróvár Algal Culture Collection (MACC) for extracellular polysaccharides (EPS) analysis. The strains were cultivated as described above. Harvesting was done at regular time intervals (every 5^{th} day) until

the 30th day. 10 mL was used for the dry weight and 10 mL for the EPS analysis.

Algal growth was measured by dry weight (DW). Whatman GF/C glass fiber filters (5 cm diameter) were dried for 2 h at 105 °C and then cooled in a desiccator and weighed. After this, 10 mL algal sample was filtered. Each filter was dried for 2 h at 105 °C again, cooled in desiccator, and weighed. The density of the suspension was calculated as g L^{-1} DW. The DW was used to construct growth curves. There were three replicates for each sample.

Analysis of Cell Morphology

The author examined the cells of strain cultures and their morphological characteristics with an Olympus BX60 microscope and a digital camera (Olympus DP 70, 400x) connected to it. Cell size was determined by image analysis software (Olympus DP Soft 3.2). The author enhanced the contrast of the photos with Adobe Lightroom software, while the photo collages were created with Fotor 2.0.3. The morphological examination of the strains was based on the work of Ettl (1983).

Extraction of genomic DNA, PCR method, sequencing

One mL of algae cell suspension was centrifuged at 14000 rpm for 2 minutes. After centrifugation, the supernatant was removed with a pipette and a 10 mg pellet was resuspended in 500 µL of 10% Chelex-100 diluted in ddH2O. The suspension was vortexed for 30 seconds and

incubated at 95 °C for 10 minutes (Eppendorf ThermoMixer). The sample was cooled to 20 °C and vortexed again for 10 seconds and centrifuged at 14000 rpm for 2 minutes. One µL of the supernatant was used in each 19 µL PCR reaction. A certain section of the 18S rRNA gene was amplified with primers EUK528F (5-'CCGCGGTAATTCCAGCTC-3') and Chlo02R (5'-CTTCGAGCCCCCAACTTTC-3') (Bio Basic Canada Inc.). The PCR mixture contained 10µL Phusion Flash High-Fidelity PCR Master Mix, 7 µL dH2O, 1 µL of each primer (0.5 µm final concentration per primer) and 1 µL purified DNA (50-100 ng) to give 20 µL final volume of PCR reaction. The binding position of the Euk528f forward primer is 573-590, while the binding position of the Chlo02R reverse primer is 996-979. The binding positions of the primers were determined based on the 18S rRNA sequence of *Chlorella vulgaris* strain SAG 211-11b.

For PCR amplification, an initial denaturation was carried out at 98°C for 30 secs, followed by denaturation at 98°C for 10 secs, annealing at 58°C for 20 secs, extension at 72°C for 30 secs, and the final extension at 72°C for 1 min over 40 cycles. After DNA amplification, the products were run for 45 minutes at 120V in 0.5 % TBE buffer containing 0.1 µg/mL ethidium bromide, and visualized on a 1.5% agarose gel under 254 nm UV light. The length of the PCR products ranged from 380 to 400 base pairs. The PCR products were purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR products were sequenced using a LifeTech 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) capillary sequencer at the Biological Research Centre of the Hungarian Academy of Sciences (BRC) (Szeged, Hungary). Sequencing was done from one direction by the forward primer. Only a few, qualitatively poor basepairs, were deleted from the beginning

of the sequences. Since the MACC sequences did not extend the longer reference sequences, it was not necessary to trim them for this reason.

Sequence alignment and phylogenetic analyses

Reference sequences of *Chlamydomonadaceae* strains were retrieved from GenBank (NCBI). The sequences were aligned using MUSCLE method by MEGA 7 software. The jModelTest 2 test was confirmed by the TIM2+G+I model of substitution. The maximum likelihood (ML) analysis was run on a dataset of 265 sequences using RAxML program with 1000 bootstraps and *Ulothrix zonata* UTEX 745 was used to root the tree. The final phylogenetic tree was edited using Adobe Illustrator CC Version 2014.01. The similarity matrix for MACC strains (similarities in percentage between the 18S rRNA partial sequences) was calculated by Geneious 10.2.3.

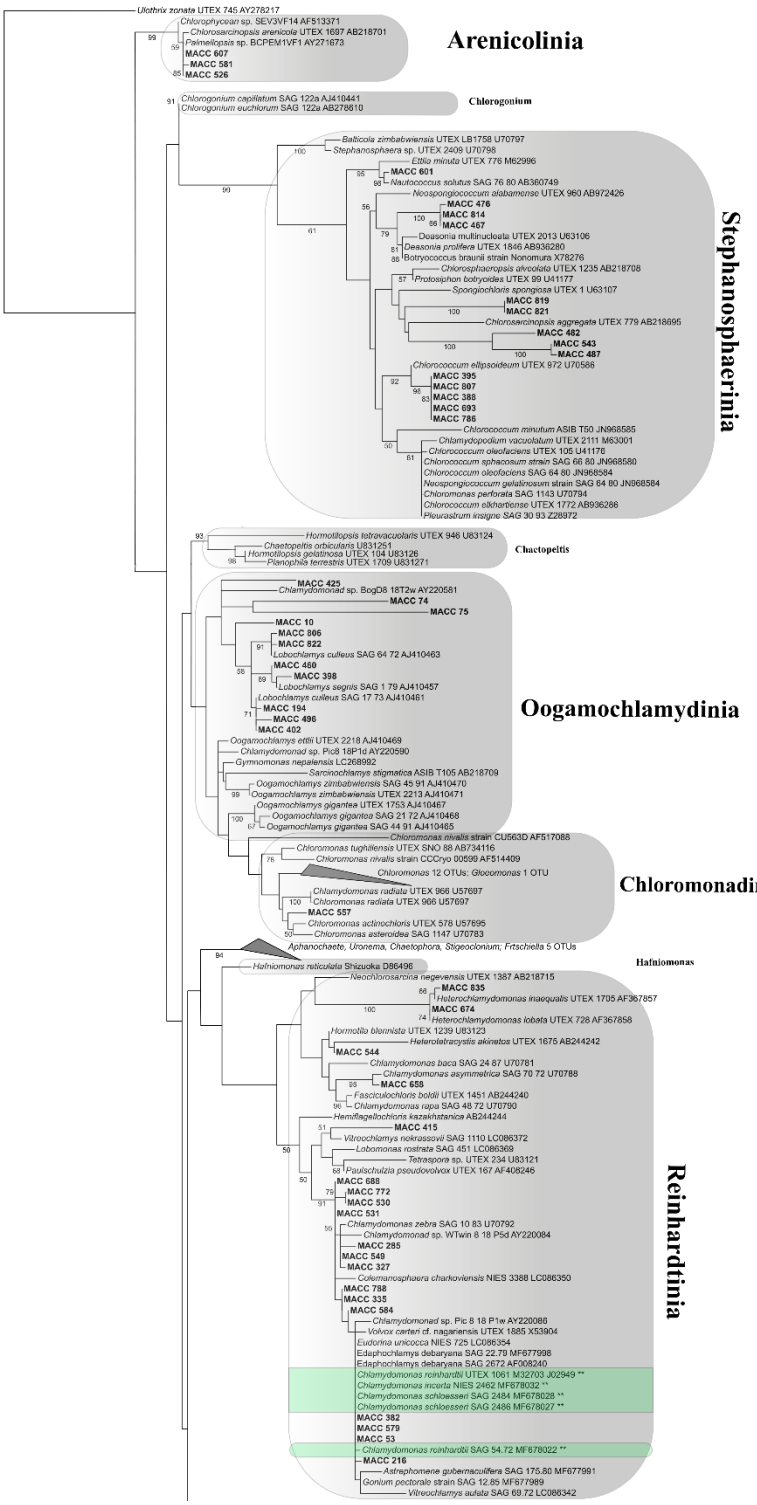
2.2. Analysis of EPS

The author measured carbohydrates in two different fractions (soluble and bound EPS) by the phenolic sulfuric acid method. The author prepared a glucose stock solution at a concentration of 200 μg/mL and diluted 5 further concentrations in the 0-200 μg/mL range. Thereafter, 400 μL of glucose solution was mixed with 400 μL of 5% phenol and 2 mL of concentrated sulfuric acid. The absorbance was measured at 485 nm (Varian CARY 50 spectrophotometer). Soluble and bound EPS were expressed as glucose equivalents using the standard glucose curve. Measurements were made three times per sample.

3. RESULTS AND CONCLUSION

3.1. Phylogenetic and morphological analysis

The author placed the 70 strains on the green algae tree with the help of 279 strains previously studied in the literature. As a result, the author distinguished nine different phylogenetic groups, which were classified into three orders. The MACC strains can be found throughout the tree, forming different phylogenetic groups. MACC strains are highlighted in bold on the tree. The bootstrap value (≥50%) determined by the maximum likelihood (ML) method is shown next to the nodes. *Ulothrix zonata* was used as an outgroup (Figure 1).


Arenicolinia
Chlorogonium
Stephanosphaerinia
Chaetopeltis
Oogamochlamydinia
Chloromonadinia
Hafniomonas
Reinhardtinia

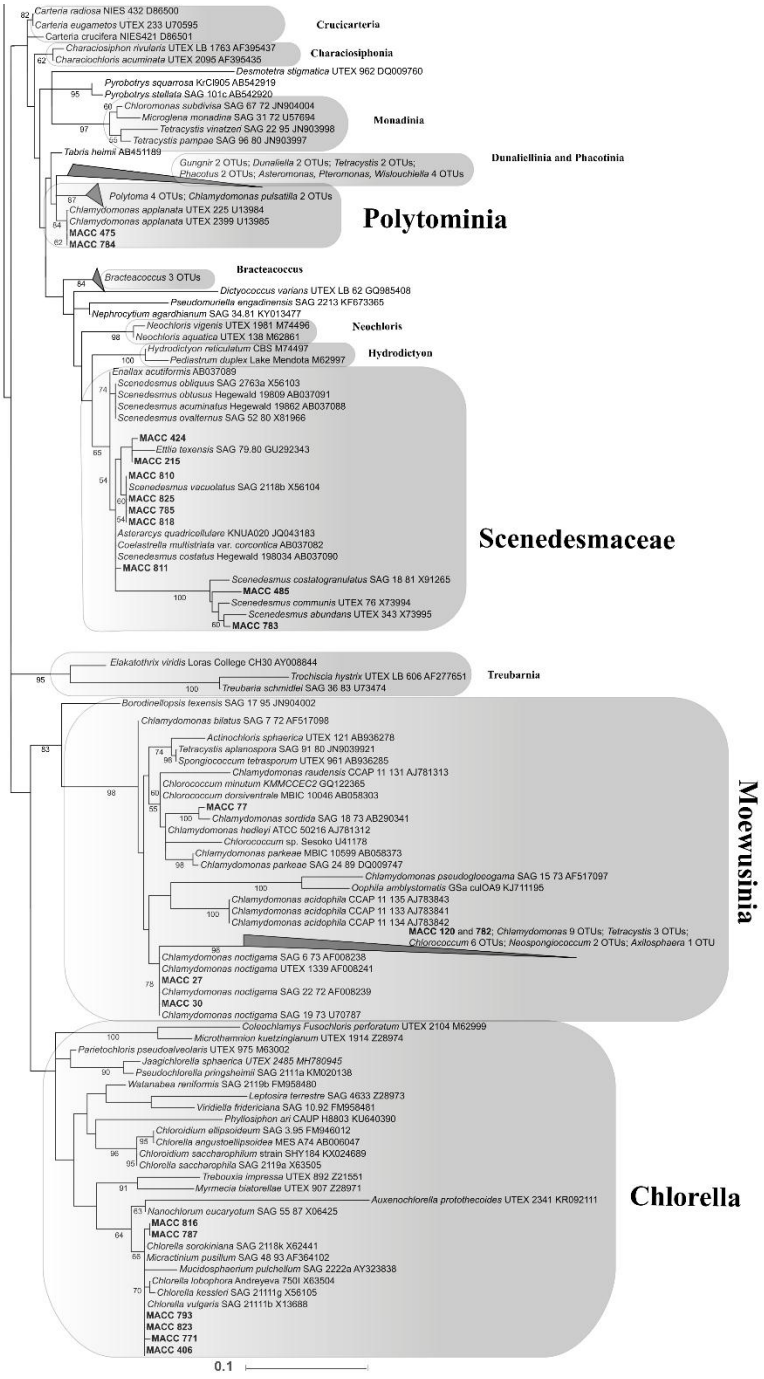


Figure 1. Phylogenetic analysis of 18S rRNA.

During the analysis, most of the phylogenetic groups, 7 in total, were classified into the *Chlamydomonadal* order (Figure 2). Namely, they are the Moewusinia phylogenetic group with 5 MACC strains, the Reinhardtinia 19, the Arenicolinia 3, the Stephanosphaerinia 14, the Chloromonadinia 1, the Oogamochlamydinia 11 and the Polytominia phylogenetic group 2, that is, with a total of 55 MACC strains. The molecular analysis of the author in this order also made it possible to reclassify the strains previously classified as *Chlamydomonas* into genera such as *Chlorococcum*, *Chloromonas*, *Chlorosarcinopsis*, *Deasonia*, *Heterochlamydomonas*, *Lobochlamys*, *Nautococcus* and *Tetraspora*. This also confirms the fact that the use of molecular tools, from a taxonomic point of view, is essential for the classification of algae.

Figure 2. Microscopic image at 400x magnification of the MACC 285 *Chlamydomonas sp.*, a typical strain of the Reinhardtinia group.

Numerous molecular phylogenetic analyzes have shown that *Chlamydomonas* is highly polyphyletic. This fact is exacerbated by the fact that Pröschold *et al.* (2018), after complex comparative studies it was found that only 3 species, namely *Chlamydomonas incerta*, *Chlamydomonas reinhardtii* and *Chlamydomonas schloesseri*, are

currently considered to be members of the genus. For the purposes of this dissertation, this means that, is *sensu stricto*, only the MACC strains close to the reference sequences of the above-mentioned 3 species on the phylogenetic tree can be considered members of the genus *Chlamydomonas*. In practice, this reduces the original number of 70 MACC strains - believed to be *Chlamydomonas* - to 4 (MACC 53, 216, 382 and 579) strains. The magnitude of the change is a good illustration of the seriousness of the processes that are currently taking place in algae taxonomy.

One phylogenetic group, Scenedesmaceae, with 9 strains of MACC belonged to the order of *Sphaeropleales*. Most of these strains were isolated from soils, from Brazil (Figure 3). MACC 485 and MACC 783 had the highest bootstrap support within this phylogenetic group (100%). As for the morphological characteristics, the cell characteristics were the same, except that the cell size of the lake-originated strain (MACC 485) was different from the other eight MACC strains.

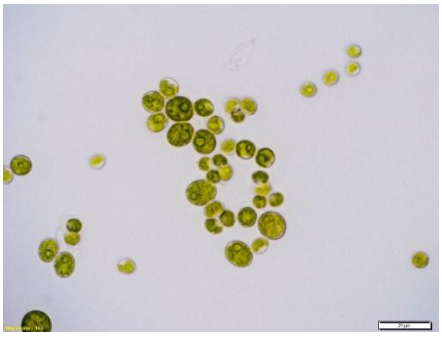


Figure 3. Microscopic image at 400x magnification of the MACC 424 *Scenedesmus sp.*, a typical strain of the Scenedesmaceae group.

The *Chlorellales* order had one phylogenetic group, Chlorella, with 6 MACC strains (Figure 4). Molecular analysis modified the earlier determination of the above mentioned six MACC strains based on morphology. Light microscopy images showed spherical cells with a diameter of 2 to 9 μm. The cells contained a single cup-shaped parietal chloroplast with a pyrenoid and without contractile vacuole, eyespot and flagella.

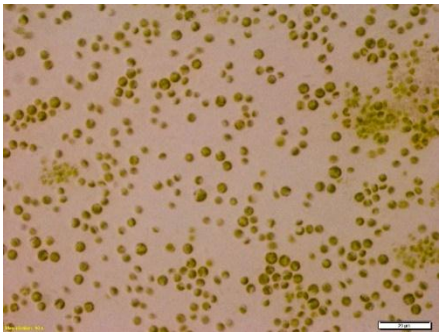


Figure 4. Microscopic image at 400x magnification of the MACC 823 *Chlorella sp.*, a typical strain of the Chlorella group.

3.2. Analysis of EPS

The author divided the strains into 3 groups according to their soluble extracellular polysaccharides (sEPS) production: high (A), medium (B) and low (C) producers. Three strains of Group A produced soluble extracellular polysaccharides above 2000 mg L^{-1}. Group B contained 7 strains with sEPS content between 1000 and 2000 mg L^{-1}. The author placed the remaining 10 strains in group C because their sEPS content was below 1000 mg L^{-1}. The literature supports the fact that bound extracellular polysaccharides (bEPS) content coheres to sEPS production,

but the amount of bEPS is significantly lower. This was also detectable in the present experiment. These are strain-specific traits that need further investigation as they can play a significant role in differentiating strains as part of the so-called polyphasic approach.

4. NEW SCIENTIFIC RESULTS

1. For first time in the history of the Mosonmagyaróvár Algae Culture Collection (MACC), the author isolated, amplified by PCR, then sequenced and systematically analyzed the genomic DNA of 70 MACC green algal strains previously identified as members of the *Chlamydomonas* genus. A certain section of the 18S rRNA gene was amplified by PCR with EUK528F (5-'CCGCGGTAATTCCAGCTC-3 ') and Chlo02R (5'-CTTCGAGCCCCCAACTTTC-3'), short, single-stranded DNA segments, and then sequenced and systematically analyzed. The length of the PCR product ranged from 380 to 400 base pairs.
2. The author pointed out that most of the strains, due to the significant changes in green algal taxonomy in recent years, should be classified into new genera. In addition, it was found that the majority of strains can be classified into 9 phylogenetic groups on the green algal phylogenetic tree. These are: Arenicolinia, Chlorella, Chloromonadinia, Moewusinia, Oogamochlamydinia, Polytominia, Reinhardtinia, Scenedesmaceae, Stephanosphaerinia. Molecular data revealed that only 22 of the 70 strains were *Chlamydomonas*, 9 of the other strains were members of the genus *Scenedesmus*, 8 *Chlorosarcinopsis*, 8 *Lobochlamys*, 6 *Chlorella*, 5

Chlorococcum, 3 *Deasonia*, 2 *Heterochlamydomonas*, 1-1 *Chloromonas*, *Nautococcus* , while the classification of 4 strains remains unresolved.

3. The author performed microscopic analysis on seventy strains. It was pointed out that the morphological definition should be strengthened with molecular biological results, since in the age of rapidly generated phylogenetic data, even the genus-level identification cannot be assured at the morphological level.

4. The author was the first to investigate the presence of soluble and bound extracellular polysaccharides (EPS) in the Mosonmagyaróvár Algae Culture Collection (MACC). In case of seven strains more than 1 g L^{-1} soluble EPS was measured, whereas soluble EPS content exceeded 2 g L^{-1} in three strains. The most EPS was produced by MACC 398 (2763 mg L^{-1}) during the 30-day incubation period. The results confirmed that there are valuable EPS producers among MACC *Chlamydomonas* strains, which can be, for example, raw materials for soil conditioning products.

5. REFERENCES

1. Ettl, H. 1983: Chlorophyta I- Phytomonadina. In Ettl H, Gerloff J, Heynig H, Mollenhauer D (eds) Süßwasserflora von Mitteleuropa, Bd. 9. Gustav Fischer, Stuttgart, New York.
2. Ördög V. 1982: Apparatus for laboratory algal bioassays. Internationale Revue der Gesamten Hydrobiologie 67, 127-136.
3. Pröschold T., Darienko T., Krienitz L., Coleman A.W. 2018: *Chlamydomonas schloesseri* sp. nov. (Chlamydophyceae, Chlorophyta) revealed by morphology, autolysin cross experiments, and multiple gene analyses. Phytotaxa 362, 021-038.

6. PUBLICATIONS RELATED TO THE SUBJECT OF THE PRESENT DISSERTATION

Publications in an international scientific journal with impact factor:

1. **S. Katona**, N. Horváth, D.E. Berthold, Z. Molnár, P. Bálint, V. Ördög, B. Pap, G. Maróti, F. Bánáti, K. Szenthe, L. Vörös, C. Kilgore, H.D. Laughinghouse 2019: Phylogenetic re-evaluation of previously identified *Chlamydomonas* (Chlorophyta, Chlamydomonadaceae) strains from The Mosonmagyaróvár Algal Culture Collection, Hungary, using molecular data. South African Journal of Botany. 125, 16-23. Q2, **IF: 1.792**.

2. N. Horváth, **S. Katona**, D.E. Berthold, Z. Molnár, P. Bálint, V. Ördög, B. Pap, G. Maróti, F. Bánáti, K. Szenthe, L. Vörös, C. Kilgore, H.D. Laughinghouse 2019: The reclassification of 37 strains from The Mosonmagyaróvár Algal Culture Collection, Hungary, which were previously identified as *Anabaena* (Cyanobacteria, Nostocaceae). South African Journal of Botany. 123, 333-340. Q2, **IF: 1.792**.

Publications in a Hungarian scientific journal:

3. **S. Katona**, N. Horváth, Z. Molnár, V. Ördög 2018: Extracellular polysaccharides in twenty *Chlamydomonas* strains of the Mosonmagyaróvár Algal Culture Collection. Acta agronomica Óváriensis. 59, 62-81.
4. **S. Katona**, Z. Molnár, V. Ördög 2016: A *Chlamydomonas* zöldalga nemzetség algáinak szerepe a biotechnológiában és helyük a zöldalgák rendszerében. Botanikai közlemények. 103, 153-171.

Foreign language presentation at an international conference:

5. N. Horváth, **S. Katona**, Z. Molnár, V. Ördög 2019: Extracellular polysaccharides in twenty *Chlamydomonas* strains of the Mosonmagyaróvár Algal Culture Collection. 9th Symposium on Microalgae and Seaweed Products. Mosonmagyaróvár, 25-26 June, 2019.

Posters presented at an international conference:

6. **S. Katona**, N. Horváth, D.E. Berthold, Z. Molnár, P. Bálint, V. Ördög, B. Pap, G. Maróti, F. Bánáti, K. Szenthe, L. Vörös, C. Kilgore, IV H.D. Laughinghouse 2019: The reclassification of 37 strains from The Mosonmagyaróvár Algal Culture Collection, Hungary, which were previously identified as *Anabaena* (Cyanobacteria, Nostocaceae). 9th Symposium on Microalgae and Seaweed Products. Mosonmagyaróvár, 25-26 June, 2019.
7. **S. Katona**, N. Horváth, Z. Molnár, V. Ördög 2015: Review of the biotechnological research results of the genus *Chlamydomonas*. 7th Symposium on Microalgae and Seaweed Products. Mosonmagyaróvár, 29-30 June, 2015.
8. N. Horváth, **S. Katona**, Z. Molnár, V. Ördög 2015: Review of the possible ways to enhance the akinete germination of the genus *Anabaena*. 7th Symposium on Microalgae and Seaweed Products. Mosonmagyaróvár, 29-30 June, 2015.
9. **S. Katona**, N. Horváth, Z. Molnár, V. Ördög 2014: Taxonomic and phylogenetic analysis of *Chlamydomonas* green alga strains. XXXV. Óvári Tudományos Nap. Mosonmagyaróvár, 13 November, 2014.
10. N. Horváth, **S. Katona**, Z. Molnár, V. Ördög 2014: Taxonomic and phylogenetic analysis of *Anabaena* cyanobacterium strains. XXXV. Óvári Tudományos Nap. Mosonmagyaróvár, 13 November, 2014.
11. **S. Katona**, N. Horváth, Z. Molnár, V. Ördög 2014: Phylogenetic and taxonomic review of the *Chlamydomonas* (Volvocales,

Chlorophyta) green algae strains. 11th Congress of the Hungarian Society of Plant Biology. Szeged, 27-29 August, 2014.

12. N. Horváth, **S. Katona**, Z. Molnár, V. Ördög 2014: Phylogenetic and taxonomic review of the *Anabaena* (Nostocales, Cyanobacteria) cyanobacteria strains. 11th Congress of the Hungarian Society of Plant Biology. Szeged, 27-29 August, 2014.
13. N. Horváth, **S. Katona**, N. Makra, Z. Molnár, V. Ördög 2013: Application of PCR methods on the algae strains of Mosonmagyaróvár Algae Culture Collection (MACC). 6th Symposium on "Microalgae and seaweed products in plant/soil-systems". Mosonmagyaróvár, 24-25 June, 2013.
14. **S. Katona**, N. Horváth, Z. Molnár, V. Ördög 2012: Gibberellinsav és mikroalga kezelés hatása néhány fűszernövény magjának csírázására. XXXIV. Óvári Tudományos Nap. Mosonmagyaróvár, 2012. október 5.