University of Pannonia, Department of Limnology
Doctoral School of Chemistry and Environmental Sciences

Ph.D. Thesis

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PHYSIOLOGICAL AND BIOTECHNOLOGICAL INVESTIGATION OF THE FILAMENTOUS GREEN ALGA *KLEBSORMIDIUM FLACCIDUM* – PHOTOSYNTHESIS, PLANT HORMONE PRODUCTION, AND SOIL-AMELIORATING POTENTIAL

Supervisors

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1. Background

At my workplace, Albitech Biotechnológiai Kft., I have been working over the past 10 years with a *Klebsormidium* green alga species isolated from a cave. It has been proven that the isolate can be cultivated not only in flasks but also at pilot-scale, despite its filamentous organization, providing an excellent foundation for the potential biotechnological applications of the species. We conducted numerous preliminary experiments to understand the properties of the algal strain, during which we investigated, among other things:

1. its ability to colonize soil,
2. its UV resistance,
3. its effect on the moisture content of different soil types, and
4. its plant growth-promoting effects.

Within the framework of my doctoral (PhD) studies, I continued my research based on the experiences gained and the information collected during these preliminary experiments.

2. Objectives

2.1. Ecophysiological Investigation of a Cave-Isolated Klebsormidium Strain

The aim of this chapter is to characterize, from ecological and biotechnological perspectives, a previously unstudied *Klebsormidium* strain of cave origin. To achieve this, I investigated the growth dynamics and photosynthetic activity of the cave-isolated strain under different temperature conditions and analyzed the temperature dependence of brassinosteroid-type plant hormones present in the cultures.

2.2. Development of a Rice Leaf Bending Bioassay for Determining Brassinosteroid (BR) Content in Algal Cultures

The aim of this study was to develop a simple and cost-effective method to estimate the approximate amount of biologically active BRs present in *Klebsormidium* cultures without using expensive analytical instruments.

2.3. Effect of Alga-Dominated Biological Soil Crust on Soil

The objective of this research was to (i) examine whether a biological soil crust can form upon field application of the investigated *Klebsormidium* strain, (ii) investigate the effect of the biological soil crust on soil moisture and porosity, and (iii) assess whether algal inoculation influences erosion losses under simulated artificial rainfall conditions.

3. Material and Methods

3.1. Ecophysiological Investigation of a Cave-Isolated Klebsormidium Strain

3.1.1. Isolation and Algal Cultivation Conditions

The biofilm containing the investigated algal strain was collected from the wall of a cave in northern Hungary (GPS coordinates: 47.90067, 20.37917). The culture was maintained in modified BBM medium (Bold's Basal Medium, Starr and Zeikus 1993) at 20 ± 2°C under 35 µmol photons m^2 s^{-1} light intensity on a shelf equipped with illumination.

3.1.2. Identification of the Algal Strain – DNA Sequence Analysis

PCR amplification of the 18S rRNA gene and ITS region was performed using the primers Euk328f–Chlo02R (Moon-Van der Staay et al., 2000; Zhu et al., 2005) and ITS_f–ITS_r (Liu et al. 2014), following protocols by Somogyi et al. (2013) and Greipel et al. (2023). Sequences were identified using BLAST (GenBank; Sayers et al. 2022), SINA (Pruesse et al. 2012), and ClustalW (MEGA 11; Tamura et al. 2021). Phylogenetic trees were reconstructed based on 817 bp (18S, K2 model) and 757 bp (ITS, K2P+G model).

3.1.3. Oxygen Yield Measurements

Experiments were conducted in a 9-cell photosynthetron. Measurements were performed at nine different light intensities (0, 5, 18, 40, 100, 200, 450, 900, and 1200 µmol photons m^{-2} s^{-1} PAR) and ten different temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 °C). Dissolved oxygen was measured with an LDO sensor. Chlorophyll-a content of algal samples was determined spectrophotometrically (Caesar et al. 2018). Photosynthetic and respiration rates were calculated per biomass according to Wetzel and Likens (2000).

3.1.4. Determination of Photosynthetic Activity by Chlorophyll Fluorescence Measurements

After homogenization, the *Klebsormidium* culture was transferred to Karlsruhe flasks and incubated in darkness at 5–50 °C for 1 hour. Rapid light and fluorescence induction curves were then recorded using a DUAL-PAM-100 device. Rapid light curves were recorded over 11–830 µmol photons m^{-2} s^{-1}, and induction curves at 131 µmol photons m^{-2} s^{-1}. Both measurements used 600 ms saturating light pulses of 10,000 µmol photons m^{-2} s^{-1}. Key photosynthetic parameters were calculated by the instrument software.

3.1.5. Determination of Growth Rates

Growth rates of the *Klebsormidium* culture were studied at 10–40 °C in a growth chamber using 150 mL batch cultures (n=4). Cultures were maintained for four days under 85 µmol photons m^{-2} s^{-1} cool white light with a 12:12 h photoperiod. Optical density (OD750) was measured daily, and specific growth rates (µ) were calculated according to Krzemińska et al. (2014).

3.1.6. Brassinolide Determination

For LC–MS analysis, 600 mL of culture was filtered through Whatman GF/F filters, followed by solid-phase extraction (SPE) on polymer weak cation-exchange cartridges using an AutoTrace 280 device (Maász et al. 2019). Samples were resuspended in acetonitrile. Brassinolide (BL) standards were prepared in a calibration range of 5–1000 ng L^{-1}. Separation was performed on an Agilent HPLC system with a Phenomenex Kinetex Polar C18 column using an ACN/water (0.1% formic acid, 10 mM ammonium formate) gradient. BL detection was carried out on a Bruker amaZon SL ion trap mass spectrometer (ESI+, MS/MS) based on m/z 315.56 and 479.68 ions. Data were processed using Bruker Compass DataAnalysis 4.4 SR1 software.

3.2. Development of a Rice Leaf Bending Bioassay to Determine Brassinosteroid Content in Algal Cultures

3.2.1. Rice Leaf Bending Bioassay (RLIA) and Applied Rice Varieties

Four rice varieties were used: 'Arsenal', 'Hoshinoyume', 'M 60', and 'Koshihikari'. BR content in prepared samples was determined following Han et al. (1997). Rice seeds were soaked in tap water for 48 hours and germinated on distilled water agar in darkness at 30°C. Seven-day-old seedlings of uniform height were cut into 4 cm segments from shoot tips and incubated in distilled water at 30°C for 24 hours. Segments were treated, and after incubation (30°C, dark, 48 hours), the angle (α) between the second leaf blade and the stem was measured using ImageJ.

3.2.2. Sample Preparation and UHPLC-MS/MS Analysis

Eighteen liters of *Klebsormidium* culture were used. After centrifugation, the supernatant was removed, and cell mass was extracted overnight at 5°C with four times its volume of 80% ice-cold methanol. The extract was centrifuged again, supernatant stored cold, and extraction repeated. The collected supernatant was purified on an SPE column, evaporated to dryness under rotary vacuum, and dissolved in 20 mL water. Prior to analysis, samples were derivatized with 3-(dansylamino)phenylboronic acid (Gamoh et al. 1990). UHPLC-MS/MS was performed using a Dionex Ultimate3000 UHPLC system coupled with an Orbitrap Q Exactive Focus mass spectrometer (positive ESI). Column: Kinetex C18. Eluents: A: 0.1% formic acid, B: acetonitrile:0.1% formic acid (80:20). Gradient: 0 min: 80% B → 5 min: 100% B → 10 min: 100% B. ESI parameters: 3500 V (+), capillary temperature 256°C, N_2 gases: 47.5 / 11.25 / 2.25 units. MS/MS: 70,000 resolution, 50 eV collision energy; precursor: m/z 815, product ion: 174 (quantification).

3.3. Effect of Alga-Dominated Biological Soil Crust on Soil

3.3.1. Study Site and Experimental Design

The experimental site was a no-till maize field near Esztergályhorváti (GPS Coordinates: 46.68200, 17.10556, 133 m a.s.l.). Soil was slightly eroded, loamy, clay-illuvial brown forest soil. Maize was sown on April 21, and algal culture applied on May 11. Sampling and artificial rainfall experiments were conducted 4.5 months later.

3.3.2. *Klebsormidium Re-Isolation*

Enrichment cultures were prepared from topsoil samples and incubated at 20°C for two weeks. Samples were examined using a light microscope.

3.3.3. Community DNA Isolation and Sequencing

Prokaryotic and eukaryotic 16S and 18S rRNA genes were amplified with fusion primers (Teske & Sørensen, 2008; Herlemann et al. 2011; Apprill et al. 2015). PCR products were quantified using a Qubit dsDNA HS assay, followed by Illumina MiSeq v2 2×250 bp paired-end sequencing. Sequence analysis was performed with mothur v1.44.3 using ARB-SILVA SSU Ref NR 138 and PR2 v4.10 databases, and OTUs were defined at a 97% threshold (Kozich et al. 2013).

3.3.4. Artificial Rainfall simulation and Soil Analyses

Infiltration and erosion were measured using a Meersmans-type rainfall simulator at 40 mm h^{-1} (Centeri et al. 2011; Jakab et al. 2019). Differential soil porosity was determined in 0–6 cm layers using 100 cm^3 samples (Rowell 2014). Water-stable aggregate (WSA) ratio was measured by wet sieving (Kemper & Koch 1966). Crust structure after rainfall was examined via thin sections using scanning electron microscopy (Jakab et al. 2013).

Statistical Analyses

Data from all experiments were analyzed using R software (4.2.1 – R Core Team, 2021; 4.4.3 – R Core Team, 2024) with appropriate packages: vegan (Oksanen et al. 2022), ggplot2 (Wickham, 2016), circlize (Gu et al. 2014), and nlme (Pinheiro et al. 2022).

4. Key Results in Thesis Points

4.1. Ecophysiological Investigation of a Cave-Isolated *Klebsormidium* Strain

The investigated cave-origin *Klebsormidium* strain is capable of photosynthesis over a wide temperature range (5–45°C) and growth between 10–40°C. Its photosynthetic optimum [30–40°C (P_{max}) and 35–40°C ($rETR_{max}$)] is higher than its growth optimum (20–25°C). The strain exhibits high light-use efficiency (α) and a low light adaptation parameter (I_k). A negative correlation was observed between the optimal cultivation temperature and the intracellular brassinosteroid content of the cultures.

4.2. Development of Rice lamina inclination Test for Detecting Brassinosteroid Content in *Klebsormidium* Cultures

Among the rice varieties tested for the rice lamina inclination bioassay (RLIA) – 'Arsenal', 'Hoshinoyume', 'M 60', and 'Koshihikari' – 'Koshihikari' proved to be the most suitable for the 0.001–0.1 mg L^{-1} BL range. Methanolic samples extracted from algal biomass can be applied in the RLIA after a methanol-water solvent exchange. Using the developed method, the brassinosteroid content of the investigated *Klebsormidium* cultures was estimated. I demonstrated a precise and reliable correlation between RLIA and BR concentrations [R^2: 0.947; RSE: 6.8% (Residual Standard Error) and RMSE: 16.8% (Root Mean Square Error)].

4.3. Effect of Alga-Dominated Biological Soil Crust on Soil

In the experiment, the applied *Klebsormidium* culture remained viable even under initially dry conditions and formed a biological soil crust on weakly eroded, loamy, clay-illuvial brown forest soil commonly used in agricultural production. The resulting *Klebsormidium*-dominated crust significantly improved the structure of the eroded soil, as indicated by increases in porosity (+17%), macropores (+52%), and the proportion of stable aggregates (+44%), along with a decrease in bulk density (-8%). The inoculated *Klebsormidium* culture was also able to reduce erosion losses by 43%.

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6. References

Apprill A, McNally S, Parsons R, Weber L (2015) Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquat Microb Ecol 75:129–137. https://doi.org/10.3354/ame01753

Caesar J, Tamm A, Ruckteschler N, et al. (2018) Revisiting chlorophyll extraction methods in biological soil crusts – methodology for determination of chlorophyll *a* and chlorophyll *a* + *b* as compared to previous methods. Biogeosciences 15:1415–1424. https://doi.org/10.5194/bg-15-1415-2018

Centeri C, Jakab G, Szalai Z, et al. (2011) Rainfall simulation studies in Hungary. In: Fournier AJ (ed) Soil Erosion: Causes, Processes and Effects. Nova Science, New York, NY, pp 177–217

Gamoh K, Okamoto N, Takatsuto S, Tejima I (1990) Determination of traces of natural brassinosteroids as dansylaminophenylboronates by liquid chromatography with fluorimetric detection. Anal Chim Acta 228:101–105. https://doi.org/10.1016/s0003-2670(00)80484-5

Greipel E, Kutasi J, Solymosi K, et al. (2023) Hidden diversity of *Chlorococcum* (Chlorophyta) in a shallow temporary freshwater lake: description of *Chlorococcum szentendrense* sp. nov. Eur J Phycol 58:110–120. https://doi.org/10.1080/09670262.2022.2076291

Gu Z, Gu L, Eils R, et al. (2014) circlize Implements and enhances circular visualization in R. Bioinformatics 30:2811–2812. https://doi.org/10.1093/bioinformatics/btu393

Han K-S, Ko K-W, Nam S-J, et al. (1997) Optimization of a rice lamina inclination assay for detection of brassinosteroids: I. effect of phytohormones on the inclination activity. J Plant Biol 40:240–244. https://doi.org/10.1007/bf03030454

Herlemann DP, Labrenz M, Jürgens K, et al. (2011) Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. ISME J 5:1571–1579. https://doi.org/10.1038/ismej.2011.41

Jakab G, Dobos E, Madarász B, et al. (2019) Spatial and temporal changes in infiltration and aggregate stability: A case study of a subhumid irrigated cropland. Water (Basel) 11:876. https://doi.org/10.3390/w11050876

Jakab G, Német T, Csepinszky B (2013) The influence of short term soil sealing and crusting on hydrology and erosion at Balaton uplands. Carpathian 147–155

Kemper WD, Koch EJ (1966) Aggregate stability of soils from western portions of the United States and Canada. US dep agric 1355

Kozich JJ, Westcott SL, Baxter NT, et al. (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq

Illumina sequencing platform. Appl Environ Microbiol 79:5112–5120. https://doi.org/10.1128/AEM.01043-13

Krzemińska I, Pawlik-Skowrońska B, Trzcińska M, Tys J (2014) Influence of photoperiods on the growth rate and biomass productivity of green microalgae. Bioprocess Biosyst Eng 37:735–741. https://doi.org/10.1007/s00449-013-1044-x

Liu J, Gerken H, Li Y (2014) Single-tube colony PCR for DNA amplification and transformant screening of oleaginous microalgae. J Appl Phycol 26:1719–1726. https://doi.org/10.1007/s10811-013-0220-3

Maász, G, Mayer M, Zrinyi Z, et al. (2019) Spatiotemporal variations of pharmacologically active compounds in surface waters of a summer holiday destination. Sci Tot Environ 677:545–555.

Moon-van der Staay SY, van der Staay GWM, Guillou L, et al. (2000) Abundance and diversity of prymnesiophytes in the picoplankton coμmunity from the equatorial Pacific Ocean inferred from 18S rDNA sequences. Limnol Oceanogr 45:98–109. https://doi.org/10.4319/lo.2000.45.1.0098

Oksanen J, Simpson GL, Blanchet FG, et al. (2022) Vegan: community ecology package

Pinheiro J, Bates D, DebRoy S, et al. (2022) Package 'nlme': Linear and Nonlinear Mixed Effects Models

Pruesse E, Peplies J, Glöckner FO (2012) SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics 28:1823–1829. https://doi.org/10.1093/bioinformatics/bts252

R Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, 4.2.1-es verzió Vienna, Austria. URL https://www.R-project.org/.

R Core Team (2024) R: A language and environment for statistical computing. R Foundation for Statistical Computing, 4.4.3-as verzió Vienna, Austria. URL https://www.R-project.org/.

Rowell DL (2014) Soil science Methods & Applications. Routledge, New York

Sayers EW, Bolton EE, Brister JR, et al. (2022) Database resources of the national center for biotechnology information. Nucleic Acids Res 50:D20–D26. https://doi.org/10.1093/nar/gkab1112

Somogyi B, Felföldi T, Solymosi K, et al. (2013) One step closer to eliminating the nomenclatural problems of minute coccoid green algae: *Pseudochloris wilhelmii*, gen. et sp. nov. (Trebouxiophyceae, Chlorophyta). Eur J Phycol 48:427–436. https://doi.org/10.1080/09670262.2013.854411

Starr RC, Zeikus JA (1993) UTEX-The culture collection of algae at the University of Texas at Austin. Journal of phycology 23:1–47. https://doi.org/10.1111/j.0022-3646.1993.00001.x

Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis version 11. Mol Biol Evol 38:3022–3027. https://doi.org/10.1093/molbev/msab120

Teske A, Sørensen KB (2008) Uncultured archaea in deep marine subsurface sediments: have we caught them all? ISME J 2:3–18. https://doi.org/10.1038/ismej.2007.90

Wetzel RG, Likens GE (2000) Limnological Analyses. Springer-Verlag, New York

Wickham H (2016) Ggplot2: Elegant graphics for data analysis. Biometrics 67:678–679. https://doi.org/10.1111/j.1541-0420.2011.01616.x

Zhu F, Massana R, Not F, et al. (2005) Mapping of picoeukaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. FEMS microbiol 52:79–92. https://doi.org/10.1016/j.femsec.2004.10.006

7. Publication List

7.1. Summary

Number of presentations at international conferences: 4
Number of posters presented at international conferences: 0
Number of presentations at domestic conferences: 5
Number of posters presented at domestic conferences: 1
Number of international publications: 2
Number of domestic publications: 3
Impact factor: 5.2
Number of independent citations: 2

7.2. Publications Related to the PhD Thesis

1. **Futó Péter**, Lengyel Edina, Futó Máté, Németh Zoltán, Pirger Zsolt, Komáromy András, Padisák Judit, Felföldi Tamás, Kutasi József, Bernát Gábor: Ecophysiological characterisation of a *Klebsormidium* strain isolated from a cave environment Journal of Applied Phycology 36, 1197–1208 (2024) **IF: 3.0, SJR: Q2**.
2. **Futó Péter**, Kutasi József, Lengyel Edina, Futó Máté, Murvai Nikoletta, Jancsó Mihály, Bernát Gábor: Straightforward method for brassinosteroid detection in microalgae. Acta Physiologiae Plantarum: 46, 25 (2024). https://doi.org/10.1007/s11738-024-03649-5 **IF: 2.2, SJR: Q2**.
3. **Futó Péter**, Kutasi József; Lengyel Edina; Bernát Gábor: *Klebsormidium* sp. BEA_IDA_0061B fonalas zöldalga fotoszintetikus aktivitásának fény és hőmérsékleti optimuma. HIDROLÓGIAI KÖZLÖNY (0018-1323): 102 1 pp 43-47 (2022)
4. Zsigrai György, **Futó Péter**, Kovács Tibor, Daoda Zoltán, Kutasi József: Egy talajalga-készítmény erózióvédelmi potenciáljának vizsgálata a Tokaji borvidéken BORÁSZATI FÜZETEK 32 : 3 pp. 31-36. , 6 p. (2022)
5. **Futó Péter**, Kutasi József, Zsigrai György, Daoda Zoltán: A biológiai talajbevonatok jelentősége és talajvédelmi szerepe. SZŐLŐ-LEVÉL 10: 36-44. (2020)

7.3. Conferences Related to the PhD Thesis

Domestic Conferences

1. **Futó Péter**, Lengyel Edina, Futó Máté, Németh Zoltán, Pirger Zsolt, Komáromy András, Padisák Judit, Felföldi Tamás, Kutasi József, Bernát Gábor: Egy biotechnológiai jelentőségű *Klebsormidium* törzs ökofiziológiai vizsgálata, Magyar Mikrobiológiai Társaság 2024. évi Nagygyűlése, Siófok 2024. október 16-18.
2. **Futó Péter**, Lengyel Edina, Futó Máté, Németh Zoltán, Pirger Zsolt, Komáromy András, Padisák Judit, Felföldi Tamás, Kutasi József, Bernát Gábor: Ecophysiological characterisation of a *Klebsormidium* strain isolated from a cave environment, poszterelőadás, FIBOK 2024 - Fiatal Biotechnológusok VI. Országos Konferenciája, Martonvásár, 2024. április 4-5.
3. **Futó Péter**, Kutasi József, Lengyel Edina, Bernát Gábor *Klebsormidium* sp. fonalas zöldalga fény-, és hőmérsékleti optimumának meghatározása LXII. Hidrobiológus Napok, Tihany 2021. október 6-8.
4. **Futó Péter**, Madarász Balázs, Bernát Gábor, Futó Máté, Jakab Gergely, Kutasi József: A talajpusztulás és talajszerkezet összehasonlító vizsgálata biológiai talajkérget képző alga készítménnyel kezelt erózió és talajszáradás sújtotta termőterületeken. Első Országos Interdiszciplináris Éghajlatváltozási Tudományos Konferencia, online, 2021. április 12-15.
5. **Futó Péter**: *Klebsormidium* sp. BEA IDA_0061B algatenyészet brasszinoszteroid tartalmának kimutatása HPLC fluorimetriás eljárással, rizslevéllemez elhajlási bioteszt optimálása. Tavaszi Szél Konferencia, online, 2020. október 16.

International Conferences

1. **Futó Péter**, Madarász Balázs, Zsigrai György, Bernát Gábor, Futó Máté, Jakab Gergely, Daoda Zoltán, Kutasi József: The importance of biological soil crust forming microalgal cultures on soil in croplands and vineyards vulnerable to erosion, Budapest Soil Health Forum, Budapest 2024. december 4.
2. **Futó Péter**, Madarász Balázs, Zsigrai György, Bernát Gábor, Futó Máté, Jakab Gergely, Daoda Zoltán, Kutasi József: Investigating the effect of biological soil crust forming microalgal cultures on soil in erosion-prone croplands and vineyards, International Conference and TUdi Workshop on Alternatives to Reduce Soil Degradation, Budapest 2024. május 7.

3. **Futó Péter**, Madarász Balázs, Bernát Gábor, Futó Máté, Jakab Gergely, Kutasi József: Comparative analysis of soil degradation and soil structure in croplands affected by erosion and soil dehydration treated with a biological soil crust forming algal culture, AlgaEurope 2022, Róma, Olaszország 2022. december 13-15.
4. **Futó Péter**, Madarász Balázs, Bernát Gábor, Futó Máté, Jakab Gergely, Kutasi József Comparative analysis of soil degradation and soil structure in croplands affected by erosion and soil dehydration treated with a biological soil crust forming algal culture: A talajpusztulás és talajszerkezet összehasonlító vizsgálata biológiai talajkérget képző alga tenyészettel kezelt erózió és talajszáradás sújtotta termőterületeken, V. Kriptogám Konferencia, Eger 2021. november 25 – 26.