Summary of Ph.D. thesis

A sweet and sour relationship:

Algal-bacterial interactions and the interplay of carbohydrate accumulation and fermentation on biohydrogen production

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1.0 BACKGROUND

For the past few decades, green algae have been exploited as agents to enhance the bioremediation of wastewater effluents with high nutrient levels. Under specific circumstances, green algae belonging to the taxon Chlorophyta have also demonstrated to be effective biohydrogen producers. Biohydrogen production is possible due to an enzyme called hydrogenases. Hydrogenases are only active under anaerobic conditions.

Several strategies have been explored to ensure high activity of hydrogenase. These include nutrient deprivation to inactivate oxygen evolving PSII, genetic transformation of different parts of the photosynthetic electron chain and commonly available oxygen absorbers.

Algal-bacterial co-cultures have recently been the subject of several investigations aimed at biohydrogen generation. Co-cultures provide several advantages over monocultures, primarily in an ecological context. In natural ecosystems, green algae live in association with a multitude of different organisms collectively termed the microbiome. The associated microbiome is generally found surrounding or attached to the algal host in a region called the phycosphere. Algal hosts release dissolved organic matter or signaling molecules to promote certain bacterial communities in the phycosphere. The bacterial partners, in turn, improve the growth of algal hosts by providing additional major essential nutrients (N, P), vitamins, phytohormones, and siderophores. Bacterial reparation can also mitigate problems arising from the high dissolved oxygen concentrations and prevent algal photo-oxidative culture death. Consumption of O_2 by bacterial respiration raises photosynthetic efficiency by allowing Rubisco to preferentially bind CO_2 rather than O_2.

In this project, we investigated the utility of bacterial partners in maintaining a microoxic environment, enabling green algae to thrive in high-nutrient wastewater, and treating wastewater while producing copious amounts of biohydrogen.

2.0 GOALS AND OBJECTIVES

Part A - High-throughput co-culture assays to investigate algal-bacterial interactions

1) Presence of phylogenetic trait conservation in algal biohydrogen and biomass production
    a) Do members of the same bacterial genera increase algal biohydrogen production?
    b) Do members of the same bacterial genera increase algal biomass?
2) Mechanistic nature of improvement in biohydrogen production
    a) Do members of the same bacterial genera increase algal biohydrogen production across different species of algae?
3) Impact of algal-bacterial co-cultivation on algal biomolecule concentration
    a) Does bacterial co-cultivation change algal chlorophyll content?
    b) Does bacterial co-cultivation change accumulated algal lipid and carbohydrate content?
4) Deeper understanding of expressed genes under algal-bacterial co-cultivation
    a) Do members of the same genus show similar gene expression patterns?
    b) Is there clear evidence of which pathways lead to improved biohydrogen production?

Part B - Algal-Bacterial Consortia in Combined Biohydrogen Generation and Wastewater Treatment

1) Impact of wastewater concentration
    a) Does wastewater concentration linearly improve algal growth?
    b) Does wastewater concentration linearly improve hydrogen production?
2) Impact of pretreatment of wastewater
    a) Does pretreatment (Filter-sterilization and heat treatment) of wastewater have an impact on algal biohydrogen production?
3) Metagenome studies
    a) Is there evidence for algal selection on wastewater microbiomes?
    b) What bacterial members are enriched in wastewater effluent samples?

3.0 MATERIALS AND METHODS

Part A - High-throughput co-culture assays to investigate algal-bacterial interactions

Description of algal strains used in this study

Three different algal strains are used in this study. All pairwise combinations were screened using *C. reinhardtii* cc124 strain. 2 other strains: *Micractinium* MACC-360 and *Parachlorella* MACC38 were used for additional tests against select bacterial strains. All algal strains were obtained from Mosonmagyaróvár Algal Culture Collection (MACC; Institute of Plant Biology, University of West-Hungary, Hungary) and maintained on mixed media plates.

Preparation of Hypo-vials for hydrogen measurements

Algae used for hydrogen measurements are first grown in mixed media and incubated at 25ºC under 50 µmol $m^{-2}s^{-1}$. The algal culture was grown for 4 days with continuous stirring at 180 rpm. Algae were then harvested on the 4^{th} day and algal stock inoculum was prepared by standardizing counts at $1x10^7$ cells ml^{-1}. Bacteria strains used for pairwise co-cultivation were cultured overnight in mixed media at 30ºC. Bacterial stock inoculum was prepared by standardizing cell counts of overnight cultures to $1x10^5$ cell ml^{-1}. All samples were cultured in 40ml sterile glass vials.

Cell counts

Both the algal and bacterial cells were counted using automated cell counters. The LUNA-FL dual fluorescence cell counter was used with the red fluorescence channel. When chlorophyll molecules are excited, they emit red fluorescence. This can be used as a proxy for automated counted of live algal cells. Bacterial cells were enumerated using the QUANTOM Tx microbial cell counter using live cell staining.

Lipid estimation and visualization under a confocal microscope

Sulfo-phosphovanillin (SPV) method was used to estimate lipid concentration. A 4 mM solution for BODIPY was prepared by dissolving 1 g of BODIPY dye in 1ml of absolute methanol. Samples from 3-day old culture were collected and 1µl of 4mM BODIPY dye was added to 100 µl of the respective sample. Samples were observed under Olympus Fluoview FV1000 confocal laser scanning microscope. The emission range was fixed between 500 nm to 515 nm to visualize BODIPY fluorescence. Images were taken under 60X oil immersion objective at 6X zoom.

Transcriptome analysis

2-day old samples were used to extract total RNA. 4 different conditions were used: Axenic, *Bacillus cereus*, *Bacillus thuringiensis,* and *Methylobacterium* sp., with 3 replicates per condition.

The quality of extracted RNA was assessed using an RNA ScreenTape on a TapeStation 4150 (Agilent Technologies) and the quantity of extracted RNA was determined with Qubit RNA Assay and sequenced on an Illumina NextSeq550 NGS platform to generate 150nt of paired end reads.

Part B - Algal-Bacterial Consortia in Combined Biohydrogen Generation and Wastewater Treatment

Preparation of Microbial Inoculum

Samples were collected from a full-scale methane bioreactor using sludge generated by the wastewater pre-treatment process of a beer brewing factory.

Algae Inoculum

For the photoheterotrophic degradation experiments axenic green algae *Micractinium* MACC360 was added to the dark fermentation effluents.

Photoheterotrophic Bioreactor Operation

The batch-mode experiments were conducted in 40 ml serum vials with 20 ml of dark fermentation effluent (either 3x diluted or non-diluted effluents), inoculated with 2 ml freshly grown *Micractinium* MACC-360 algae ($5.54 * 10^8$ algae cell). For certain samples, 1 ml (5 %) of enriched microbial inoculum (EMI) was added. Photoheterotrophic fermentation was performed at 24°C in batch mode for a period of 72 h under continuous illumination with 50 μmol $m^{-2}s^{-1}$ light intensity, the vials were shaken at 120 rpm.

Analytical Methods

Biomass in each culture was estimated by daily measurement of optical density (OD_{600}) with a Jenway 6320D Spectrophotometer. The algae cell numbers were counted on Tris-Phosphate solid media using serial dilutions. All experiments were repeated three times.

Hydrogen was directly measured by gas chromatography using an Agilent Technologies 7890A GC system equipped with a thermal conductivity detector and argon as a carrier gas.

BOD measurements were performed according to the instructions of Hach-Lange Cuvette Test LCK555 at the start of the photo-fermentation experiment and 72 h. Total nitrogen and total phosphorous were measured according to the instructions of Hach-Lange Cuvette Tests LCK138 and LCK349, respectively, at the start of the photo-fermentation experiment and 72 h.

Total DNA Extraction from Samples

Total DNA from selected samples was prepared for high-throughput next-generation sequencing analysis performed on the Ion Torrent PGM platform

4.0 RESULTS

This thesis was written based on two projects conducted at the Biological Research Center, Hungary. The results are broken down into two separate parts. In part A of the study, we focused on the impact of pairwise algal-bacterial co-cultures on microalgal biohydrogen production and algal bioproduct accumulation such as lipids and carbohydrates. We also used transcriptome analysis to study the microalgal gene expression pattern under bacterial association. In part B of the study, we investigated the feasibility of using green algae to bioremediate brewery effluent and to produce biohydrogen. We also studied what impact the microbes natively present in the effluent had on microalgal growth and bioremediation capacity.

The following points summarize the primary findings from part-A of my research.

1. Initial biohydrogen studies identified the importance of Bacillus species in improving biohydrogen production.
2. No relationship between bacterial phylogeny and microalgal growth was observed.
3. Bacillus species improved microalgal hydrogen production across multiple algal species, making it a general mechanism of improvement.
4. Co-cultivation with bacterial partners increased microalgal Chlorophyll a/b ratio, accumulated lipids, and accumulated carbohydrates.
5. Co-cultivation with *Methylobacterium* sp. significantly greater accumulation of accumulated carbohydrates compared to co-cultivation with both Bacillus species.
6. Bacillus co-cultivation upregulated genes involved in Photofermentation and Dark fermentation. Improved biohydrogen generation may result from upregulation of genes involved in fermentation processes.
7. *Methylobacterium* sp. co-cultivation upregulates FNR1, theoretically leaching electrons away from hydrogense and resulting in low biohydrogen production.
8. We also observed the *hydA2* gene being upregulated only in samples co-cultivated with *Methylobacterium* sp. The *hydA2* isoform of exhibits a greater predilection for consuming hydrogen. This could be another reason for low hydrogen production.
9. Genes upregulated under bacterial co-cultivation revealed enrichment of Salicylic acid biosynthetic and metabolic process and glycoprotein biosynthesis.

10. Genes upregulated under axenic conditions revealed enrichment of amino acid catabolic process, indicating that under axenic cultivation *C. reinhardtii* cc124 is starved for nitrogen.

The following points summarize the primary findings from part-B of my research.

1. Higher rate of change in Total N and P was observed in concentrated samples, despite having lower cell numbers in concentrated samples.
2. Presence of algae drove Total N and Total P changes.
3. Enriched microbial inoculum amended samples produced the highest amount of biohydrogen. Samples without enriched microbial inoculum produced the lowest amount of biohydrogen.
4. Heat treatment changed the microbiome community structure in enriched microbial inoculum. Bacteria from the *Lactobacillus* genus reduced in abundance while bacteria from *Clostridium* genus increased in abundance.
5. 3-day photofermentation led to a significant increase in bacteria from the genus *Prevotella*, *Clostridium* and *Veillonella*.
6. Whole genome assembled bins from *Prevotella* and *Veillonella* identified complete pathways for biosynthesis of vitamins.

5.0 LIST OF PUBLICATIONS

LIST OF PUBLICATIONS RELATED TO THIS THESIS

MTMT Author ID: 10072189

Cumulative impact factor: 48.552

1. **Shetty, Prateek**, Attila Farkas, Bernadett Pap, Bettina Hupp, Vince Ördög, Tibor Bíró, Torda Varga, and Gergely Maróti. "Comparative and phylogenomic analysis of nuclear and organelle genes in cryptic *Coelastrella vacuolata* MACC-549 microalgae." *Algal Research* 58 (2021): 102380. (IF_{2021}: 5.276)
2. **Shetty, Prateek**, Iulian Z. Boboescu, Bernadett Pap, Roland Wirth, Kornél L. Kovács, Tibor Bíró, Zoltán Futó, Richard Allen White III, and Gergely Maróti. "Exploitation of algal-bacterial consortia in combined biohydrogen generation and wastewater treatment." *Frontiers in Energy Research* 7 (2019): 52. (IF_{2021}: 3.858)
3. **Shetty, Prateek**, Margaret Mukami Gitau, and Gergely Maróti. "Salinity stress responses and adaptation mechanisms in eukaryotic green microalgae." *Cells* 8, no. 12 (2019): 1657. (IF_{2021}:7.666)

OTHER PUBLICATIONS

1. Wirth, Roland, Bernadett Pap, Dénes Dudits, Balázs Kakuk, Zoltán Bagi, **Prateek Shetty**, Kornél L. Kovács, and Gergely Maróti. "Genome-centric investigation of anaerobic digestion using sustainable second and third generation substrates." *Journal of Biotechnology* 339 (2021): 53-64. (IF_{2021}:3.595)
2. Knisz, Judit, **Prateek Shetty**, Roland Wirth, Gergely Maróti, Tamás Karches, Ilona Dalkó, M. Bálint, E. Vadkerti, and T. Bíró. "Genome-level insights into the operation of an on-site biological wastewater treatment unit reveal the importance of storage time." *Science of the Total Environment* 766 (2021): 144425. (IF_{2021}:10.753)
3. Soós, Vilmos, **Prateek Shetty**, Gergely Maróti, Norbert Incze, Eszter Badics, Péter Bálint, Vince Ördög, and Ervin Balázs. "Biomolecule composition and draft genome of a novel, high-lipid producing Scenedesmaceae microalga." *Algal Research* 54 (2021): 102181. (IF_{2021}:5.276)
4. Valappil, Sarshad Koderi, **Prateek Shetty**, Zoltán Deim, Gabriella Terhes, Edit Urbán, Sándor Váczi, Roland Patai et al. "Survival Comes at a Cost: A Coevolution of Phage and Its Host Leads

to Phage Resistance and Antibiotic Sensitivity of *Pseudomonas aeruginosa* Multidrug Resistant Strains." *Frontiers in microbiology* 12 (2021). (IF_{2021}:6.064)

5. Wirth, Roland, Bernadett Pap, Tamás Böjti, **Prateek Shetty**, Gergely Lakatos, Zoltán Bagi, Kornél L. Kovács, and Gergely Maróti. "*Chlorella vulgaris* and its phycosphere in wastewater: microalgae-bacteria interactions during nutrient removal." *Frontiers in bioengineering and biotechnology* 8 (2020): 557572. (IF_{2021}: 6.064)

6.0 CO-AUTHOR WAIVER

Hereby, I certify that I am familiar with the thesis of the PhD. candidate, Prateek Shetty. Regarding our jointly published results used for this PhD. desertion, I declare the applicant's contribution was prominent in obtaining the results, and these papers were not used for PhD defense and will be used for at most one PhD dissertation in the future. Furthermore, the Shetty *et al*., Frontiers in energy research (DOI: 10.3389/fenrg.2019.00052) article will not be used as first but at the most as a co-author paper by anyone of the authors.

List of publications:

1. **Shetty, Prateek**, Attila Farkas, Bernadett Pap, Bettina Hupp, Vince Ördög, Tibor Bíró, Torda Varga, and Gergely Maróti. "Comparative and phylogenomic analysis of nuclear and organelle genes in cryptic *Coelastrella vacuolata* MACC-549 microalgae." *Algal Research* 58 (2021): 102380. (IF_{2021}: 5.276)
2. **Shetty, Prateek**, Iulian Z. Boboescu, Bernadett Pap, Roland Wirth, Kornél L. Kovács, Tibor Bíró, Zoltán Futó, Richard Allen White III, and Gergely Maróti. "Exploitation of algal-bacterial consortia in combined biohydrogen generation and wastewater treatment." *Frontiers in Energy Research* 7 (2019): 52. (IF_{2021}: 3.858)
3. **Shetty, Prateek**, Margaret Mukami Gitau, and Gergely Maróti. "Salinity stress responses and adaptation mechanisms in eukaryotic green microalgae." *Cells* 8, no. 12 (2019): 1657. (IF_{2021}:7.666)

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