Summary of PhD Thesis

Understanding nitrate assimilation by eukaryotic green microalgae

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1 INTRODUCTION

Increasing anthropogenic pressure on water bodies has resulted in the worldwide problem of eutrophication, in which nitrate has emerged as one of the principal pollutants. The widespread use of fertilizers in agricultural fields and the improper disposal of wastewater in water bodies are the primary causes of eutrophication. This eutrophication, resulting from nutrient enrichment of nitrogen and phosphorus, poses a major threat to the aquatic ecosystem. Multiple nations have associated rising levels of nitrogen and phosphorus in water with significant environmental problems. The primary inorganic nitrogen compounds are nitrate, nitrite, and ammonia. Nitrite and ammonia are unstable, whereas nitrate is extremely stable, making it one of the most prevalent water contaminants. The consequences of eutrophication are a decrease in macrophyte abundance, an increase in the growth of algae and plankton, algal blooms, and deoxygenation. Various technologies are being utilized to eliminate nitrate from water based on scientific developments.

There are two main methods for nitrate removal: physicochemical and biological. The major disadvantage of physicochemical methods is the presence of a large amount of salt or nitrate in the disposal stream, whereas the major disadvantage of biological methods is the generation of an excessive biomass of bacterial cells and residual carbon sources that must be carefully removed from the treated water. Therefore, the use of microalgae has become a viable approach for the treatment of wastewater throughout time as the microalgal biomass obtained after the wastewater treatment can be used as feedstock in biorefineries or other applications as microalgae require inorganic nutrients like nitrogen and phosphorus for their growth.

Nitrate assimilation pathway in microalgae consists of two transport steps (nitrate and nitrite transport) and two reduction steps. First, nitrate is transported into the cell, where a cytosolic nitrate reductase (NR) catalyzes the reduction of nitrate into nitrite, which is subsequently transported into the chloroplast. Nitrite is further reduced to ammonium by the action of the enzyme nitrite reductase (NiR) in the chloroplast. Chloroplast is the main site for ammonium incorporation into carbon skeletons by glutamine synthetase/glutamine oxoglutarate aminotransferase (GS/GOGAT), or glutamate synthase cycle.

The ability of microalgae to uptake nitrate from a certain medium can be influenced by multiple factors, including nitrate concentration, light conditions, pH, temperature, salinity, etc. Light is crucial to the life cycle of cyanobacteria, algae, and higher plants; the colour or wavelength of the light has a significant impact on their growth. Light is essential to microalgae because it facilitates the synthesis of important molecules needed for their growth via the production of adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) through photosynthesis. Both light colour/wavelength and intensity have been shown to affect the growth of microalgae. As the absorption bands of photosynthetic pigments chlorophyll-a and chlorophyll-b lie in the blue and red ranges of visible light, numerous studies have been conducted to characterize the effects of these two colours on the growth of microalgae. Still, there is a lack of data regarding the effect of the blue + red light combination on the nitrate removal efficiency of microalgae.

Therefore, the work in my PhD thesis was carried out to study the effect of various concentrations of nitrate and light conditions on microalgae to figure out a suitable candidate for nitrate removal.

2 AIM

The main aim behind my PhD thesis work was to identify a microalga with great potential for nitrate removal studies. For this aim, studies were carried out with three main objectives, which are as follows:

- **Objective 1:** To assess the nitrate removal capacity of two eukaryotic green microalgae, *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360.
- **Objective 2:** To assess the effect of various light conditions on the nitrate removal capacity of *Chlamydomonas* sp. MACC-216.
- **Objective 3:** To analyze differential gene expression in *Chlamydomonas* sp. MACC-216 and *Chlamydomonas reinhardtii* cc124 under nitrate-replete and nitrate-deplete conditions.

3 MATERIALS AND METHODS

3.1 Objective 1

- *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 were cultivated in TAP (tris-acetate-phosphate), TAP-N5, TAP-N10, and TAP-N15 media. TAP-N5, TAP-N10, and TAP-N15 media were prepared by substituting 5 mM, 10 mM, and 15 mM concentrations of sodium nitrate, respectively, instead of ammonium chloride (nitrogen source in TAP).
- Growth, cell count, and nitrate removal efficiency were determined.
- Reactive oxygen species (ROS) production was measured by 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) dye.
- Lipids were extracted using the Folch method (chloroform: methanol → 2:1). Total lipids were quantified using sulpho-phospho-vanillin reagent assay. For the localization of lipids inside microalgae cells, BODIPY dye was used, and then microalgae were observed under an Olympus Fluoview FV1000 confocal laser scanning microscope.
- *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360 were cultivated in synthetic wastewater (SWW) prepared with various concentrations of nitrate, i.e., 5 mM, 10 mM, 25 mM, and 50 mM. Growth and nitrate removal efficiency were determined.

3.2 Objective 2

- *Chlamydomonas* sp. MACC-216 was cultivated in TAP-N5 and TAP-N10 media under 12 light conditions made up of combinations of three light colours (blue, red, and white) and three light intensities (50 µmol m^{-2} s^{-1}, 100 µmol m^{-2} s^{-1}, and 250 µmol m^{-2} s^{-1}). Growth and nitrate removal efficiency were determined.
- Blue 250, Blue 125 + Red 125, Red 250, and White 250 light conditions were selected for the cultivation of *Chlamydomonas*

sp. MACC-216 in SWW. Growth and nitrate removal efficiency were determined.

- Nitrate reductase activity was determined using Griess reagent after cultivation of *Chlamydomonas* sp. MACC-216 under Blue 250, Blue 125 + Red 125, Red 250, and White 250 light conditions in SWW.
- Quantification of the expression of five genes (*NRT1*, *NRT2.1*, *NRT2.2*, *MCP1*, and *NIA1*) involved in nitrate transport and reduction in *Chlamydomonas* sp. MACC-216 grown under Blue 250, Blue 125 + Red 125, Red 250, and White 250 light conditions in SWW was done using quantitative reverse transcription polymerase chain reaction.

3.3 Objective 3

- 3-day-old cultures of *Chlamydomonas sp.* MACC-216 and *Chlamydomonas reinhardtii* cc124 cultivated in TAP medium were transferred to TAP-N0 (medium without any nitrogen source) and TAP-N15 media to a final cell density of 6×10^6 cells mL^{-1} for 6 hours. After 6 hours, samples were collected from each culture for the estimation of nitrate removal efficiency and RNA isolation.
- Isolation of total RNA was performed, followed by quantification using the Qubit RNA Assay. Illumina Nextseq550 was used to generate 150 bp paired-end reads. Sequenced reads were trimmed and then mapped to reference *Chlamydomonas reinhardtii* v5.6 transcripts downloaded from the Phytozome database. Genes with a log2 fold change of ±1 and an adjusted p-value < 0.05 were identified as differentially expressed genes.

4 RESULTS

4.1 Objective 1

Through the first objective, the ability of two eukaryotic green microalgae, *Chlamydomonas* sp. MACC-216 and *Chlorella* sp. MACC-360, to grow and remove nitrate at varied nitrate concentrations was determined. Three concentrations of nitrate were selected for this purpose, i.e., 5 mM, 10 mM, and 15 mM. Both microalgae were capable of removing 100% nitrate when grown in TAP medium supplied with 5 mM nitrate. However, at higher nitrate concentrations, *Chlamydomonas* sp. MACC-216 removed more nitrate in comparison to *Chlorella* sp. MACC-360. Furthermore, to observe whether the presence of nitrate is causing any stress on both microalgae, ROS production was determined. No major ROS production was observed in *Chlamydomonas* sp. MACC-216, but *Chlorella* sp. MACC-360 showed high ROS production at 15 mM nitrate concentration, signifying the stress caused by this particular nitrate concentration. Additionally, *Chlamydomonas* sp. MACC-216 showed enhanced lipid accumulation with increasing nitrate concentration. Furthermore, after observing the nitrate removal capacity of both microalgae in TAP-N5, TAP-N10, and TAP-N15 media, the nitrate removal capacity of both microalgae was determined in SWW. Both microalgae could grow well in SWW supplied with different nitrate concentrations; *Chlamydomonas* sp. MACC-216 showed better growth in SWW supplied with either 5 mM or 10 mM nitrate, and *Chlorella* sp. MACC-360 showed better growth in SWW supplied with higher nitrate concentration, i.e., 25 mM and 50 mM nitrate. However, even in SWW, *Chlamydomonas* sp. MACC-216 showed better nitrate removal than *Chlorella* sp. MACC-360.

4.2 Objective 2

The effect of 12 light conditions made up of combinations of three light colours (blue, red, and white) and three light intensities (50 µmol m^{-2} s^{-1}, 100 µmol m^{-2} s^{-1}, and 250 µmol m^{-2} s^{-1}) on the nitrate removal efficiency of *Chlamydomonas* sp. MACC-216 was determined through the second objective. When *Chlamydomonas* sp. MACC-216 was cultivated in TAP-N5 and TAP-N10 media, no major effect of different light colours was observed on the growth of the microalgae. However, in comparison to monochromatic light colours (blue, red, and white), *Chlamydomonas* sp. MACC-216 showed better nitrate removal efficiency under the combination of blue + red light colour. An increase in the light intensity from 50 µmol m^{-2} s^{-1} to 250 µmol m^{-2} s^{-1} led to significantly higher nitrate removal efficiency. Therefore, the nitrate removal efficiency was found to be both light colour and light intensity dependent. Furthermore, *Chlamydomonas* sp. MACC-216 was cultivated under Blue 250, Red 250, Blue 125 + Red 125, and White 250 light conditions in SWW. The nitrate removal efficiency of *Chlamydomonas* sp. MACC-216 was observed to be highest under Blue 125 + Red 125 light condition in SWW; similar results were observed for nitrate reductase activity too. Expression of five genes (*NRT1*, *NRT2.1*, *NRT2.2*, *NIA1*, and *MCP1*) participating in nitrate transport and reduction was thoroughly analyzed, and all these genes showed the highest expression under the Blue 125 + Red 125 light condition. Overall, blue + red light combination with high light intensity represented an optimal light condition for efficient nitrate removal from synthetic wastewater.

4.3 Objective 3

As *Chlamydomonas* sp. MACC-216 showed efficient nitrate removal in the first two objectives, it was decided to compare the transcriptome of microalgae (*Chlamydomonas* sp. MACC-216), which can remove nitrate efficiently, to the transcriptome of microalgae (*C. reinhardtii* cc124), which cannot even grow well in

the presence of nitrate. Therefore, for the third objective, transcriptome analysis was performed to compare the transcriptional changes occurring in the presence and absence of nitrate in *Chlamydomonas* sp. MACC-216 and *C. reinhardtii* cc124, which revealed interesting results. Differential expression analysis of *C. reinhardtii* cc124 grown in the presence of nitrate revealed that only 45 genes were differentially regulated, where 23 genes were upregulated, and 22 genes were downregulated. In *Chlamydomonas* sp. MACC-216, differential expression analysis revealed that 3143 genes were differentially regulated, where 1604 genes were upregulated, and 1539 genes were downregulated in the presence of nitrate. In *Chlamydomonas* sp. MACC-216, upregulation of *NRT2.1*, *NRT2.2*, *NAR2*, *NIA1*, *NAR1.5*, *NAR1.6*, and *NII1* genes playing roles in nitrate metabolism was observed in the presence of nitrate. However, in *C. reinhardtii* cc124, upregulation of genes related to urea transport (*DUR3A*, *DUR3B*, and *DUR3C*) was observed, which is interesting because no such nitrogen source was provided to this microalga in the growth medium. This objective demonstrated a clear picture of species-specific regulation of nitrate metabolism in *Chlamydomonas*.

5 CONCLUSIONS

Through the studies carried out for my PhD thesis, the following conclusions can be drawn:

- *Chlamydomonas* sp. MACC-216 performs better in nitrate removal than *Chlorella* sp. MACC-360.
- Increasing concentrations of nitrate lead to lipid accumulation in *Chlamydomonas* sp. MACC-216.
- *Chlamydomonas* sp. MACC-216 shows highest nitrate removal efficiency under the blue + red light combination in comparison to monochromatic blue, red, or white light colour.
- Nitrate removal efficiency of *Chlamydomonas* sp. MACC-216 increases as the light intensity is increased from 50 µmol m^{-2} s^{-1} to 250 µmol m^{-2} s^{-1}.
- Blue + red light combination with high light intensity represents an optimal light condition for efficient nitrate removal from wastewater.
- In the presence of nitrate, the transcriptome of *Chlamydomonas* sp. MACC-216 goes through a major transcriptional reorganization, where upregulation of genes related to nitrate transport and reduction can be observed. No such major transcriptional reorganization takes place in *C. reinhardtii* cc124 in the presence of nitrate.
- Nitrate metabolism is a species-specific complex pathway.

6 ÖSSZEFOGLALÓ

A gyakran nitrát formájában megjelenő nitrogénszennyezés, világszerte a víztestek eutrofizációjának egyik fő oka. Az eutrofizáció hátterében álló fő tényezők legfőképpen a műtrágyák kiterjedt használata a mezőgazdasági területeken, az ipari szennyvizek nem megfelelő elhelyezése és az emberi szennyvizek víztestekbe történő kibocsátása. A mikroalgákat hagyományosan a nitrogén- és foszforfelhasználási képességük miatt alkalmazzák a szennyvíz tisztítására. Ez a környezetbarát kezelés kevesebb energiát fogyaszt, jelentősen csökkenti a szén-dioxid-kibocsátást, és bioüzemanyagok, olcsó növényi biostimulánsok, állati takarmányok előállításához vezethet. Ezen dolgozat célja a mikroalgák nitráteltávolító képességének vizsgálata és a nitrátasszimiláció megértése volt, mivel a nitrát az egyik legfontosabb eutrofizációt okozó szennyezőanyag.

Az első célkitűzés révén két eukarióta zöld mikroalga, a *Chlamydomonas* sp. MACC-216 és a *Chlorella* sp. MACC-360 növekedési és nitráteltávolítási képességét határoztuk meg különböző nitrátkoncentrációk mellett. Három nitrátkoncentrációt; 5 mM, 10 mM és 15 mM választottunk ki erre a célra. 5 mM nitrátot tartalmazó TAP tápoldatban növesztve mindkét mikroalga képes volt a nitrát 100%-os eltávolítására. Emellett a *Chlamydomonas* sp. MACC-216 fokozott lipidfelhalmozódást is mutatott a nitrátkoncentráció növekedésével. Mindkét mikroalga nitráteltávolító képességét tovább vizsgáltuk szintetikus szennyvízben (SWW) is, ahol a *Chlamydomonas* sp. MACC-216 jobb nitráteltávolítást mutatott, mint a *Chlorella* sp. MACC-360.

A második célkitűzéssel a különböző fényintenzitások és színek (vagy hullámhosszok) kombinációinak a *Chlamydomonas* sp. MACC-216 alga nitráteltávolítási hatékonyságára gyakorolt hatását kívántuk meghatározni. A monokromatikus fényszínekkel (kék, vörös és fehér) összehasonlítva a *Chlamydomonas* sp. MACC-216

jobb nitráteltávolítási hatékonyságot mutatott a kék + vörös fényszín kombinációja mellett, amikor TAP-N5 és TAP-N10 tápoldatban termesztettük. A fényintenzitás 50 µmol m^{-2} s^{-1}-ről 250 µmol m^{-2} s^{-1}-re történő növelése is szignifikánsan magasabb nitráteltávolítási hatékonyságot eredményezett. A *Chlamydomonas* sp. MACC-216 nitráteltávolítási hatékonysága még az SWW-ben is a kék + vörös fény színkombinációban volt a legmagasabb; hasonló eredményeket figyeltünk meg a nitrátreduktáz-aktivitás tekintetében is. A nitrát szállításában és redukciójában részt vevő öt gén (*NRT1*, *NRT2.1*, *NRT2.2*, *NIA1* és *MCP1*) expresszióját alaposan megvizsgáltuk, és ezen gének mindegyike a kék + piros fény színkombináció mellett mutatta a legmagasabb expressziót. Összességében a kék + vörös fény kombinációja nagy fényintenzitással optimális fényviszonyokat jelentett a szintetikus szennyvízből történő hatékony nitráteltávolításhoz.

A harmadik célkitűzés vizsgálatára transzkriptom-elemzést végeztünk, hogy összehasonlítsuk a transzkripciós változásokat nitrát jelenlétében és hiányában a *Chlamydomonas* sp. MACC-216 és a *Chlamydomonas reinhardtii* cc124 algákban, ami érdekes eredményeket hozott. A nitrát jelenlétében növesztett *C. reinhardtii* cc124 differenciális expressziós elemzése kimutatta, hogy csak 45 gén volt differenciálisan szabályozott, ebből 23 gén felülszabályozott, illetve 22 gén alulszabályozott volt. A *Chlamydomonas sp.* MACC-216-ban a differenciális expressziós elemzés kimutatta, hogy 3143 gén volt differenciálisan szabályozott, 1604 gén felülszabályozott, továbbá 1539 gén alulszabályozott nitrát jelenlétében. A *Chlamydomonas* sp. MACC-216-ban nitrát jelenlétében a nitrát-anyagcserében szerepet játszó gének szabályozódását figyeltük meg, míg a *C. reinhardtii* cc124 esetében nem tapasztaltunk ilyen eredményeket. A harmadik célkitűzéssel ezek a vizsgálatok világos képet adtak a nitrát-anyagcsere fajspecifikus szabályozásáról *Chlamydomonas* algákban.

7 LIST OF PUBLICATIONS

MTMT Author ID: 10084705

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Following are the publications related to the PhD thesis:

1. Rani, V., Maróti, G. 2021. Assessment of Nitrate Removal Capacity of Two Selected Eukaryotic Green Microalgae. Cells. 10:2490. **IF_{2021}: 7.666 (Q1)**
2. Rani, V., Maróti, G. 2023. Light-Dependent Nitrate Removal Capacity of Green Microalgae. International Journal of Molecular Sciences. 24:77. **IF_{2023}: 6.208 (Q1)**

8 CO-AUTHOR WAIVER

I certify that I am familiar with the PhD thesis work of Vaishali Rani. As the corresponding author of the two peer-reviewed articles listed below that were published as a result of the research carried out for her PhD thesis, I hereby attest that I have no conflict of interest. In addition, I confirm that PhD candidate Vaishali Rani worked under my supervision and significantly contributed to the findings presented in her PhD thesis.

List of publications:

1. Rani, V., Maróti, G. 2021. Assessment of Nitrate Removal Capacity of Two Selected Eukaryotic Green Microalgae. Cells. 10:2490. **IF_{2021}: 7.666 (Q1)**
2. Rani, V., Maróti, G. 2023. Light-Dependent Nitrate Removal Capacity of Green Microalgae. International Journal of Molecular Sciences. 24:77. **IF_{2023}: 6.208 (Q1)**

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