Potential production of polyhydroxybutyrate PHB in cyanobacteria isolated from microbial mats from James Ross Archipelago

Conventional plastics are almost entirely made from fossil fuels, such as polyethylene (PE), polypropylene (PP), polyvinylchloride (PVC) and polystyrene (PS). Regarding these problems, significant effort has been inverted in the development and production of "biopolymers". Degradation of bioplastics takes much lesser time as compared to petroleum-based plastics. In this sense, Poly-β-hydroxybutyrate (PHB) is a wide spread intracellular storage compound typically found in prokaryotic organisms. The properties of pure poly-β-hydroxybutyrate includes thermoplastic process ability, absolute resistance to water and complete biodegradability, suggesting that PHB could be an attractive plastic alternative and would fit well with new waste management strategies [10]. Many cyanobacteria have developed the ability to produce a variety of biopolymers to store macro-nutrients, such as carbon (in the form of glycogen), phosphate (polyphosphate), or nitrogen (cyanophycin). When nutrients (e.g. nitrogen or phosphorus) are limited some cyanobacteria start producing intracellular storage products (e.g. PHB) which can be extracted and converted to biofuels or biopolymers. It has been hypothesized that PHB serves as an additional carbon and energy storage, similarly to glycogen, which could help to survive environmental stress conditions. However, until today, the true physiological function remains unknown [14]. During nitrogen starvation, which triggers a process called chlorosis, switch starts with the degradation of photosynthetic pigments. PHB slowly accumulates during the course of several weeks, depending on the cyanobacterial strain, in addition to glycogen granules. When nitrogen deficiency is prolonged, cells degrade the bulk of cellular proteins and the photosynthetic apparatus until they reach a final chlorotic stage. At this stage, they maintain a residual level of photosynthesis, which allows them to preserve full viability over at least 6 months.

Results

After several months of isolation, six strains were obtained, four of which belong to cyanobacteria and two to eukaryotic cells. These strains were isolated from microbial mats on Clear Water Mesa Island. Dichotomous and Sanger sequencing approaches revealed that the cyanobacterial strains belong to the genera *Phormidium*, *Pseudoanabaena*, *Nodosilinea*, and *Leptolyngbya*, while the eukaryotic strains correspond to the genera *Coccomyxa* and *Chlorella* (Figure 1).

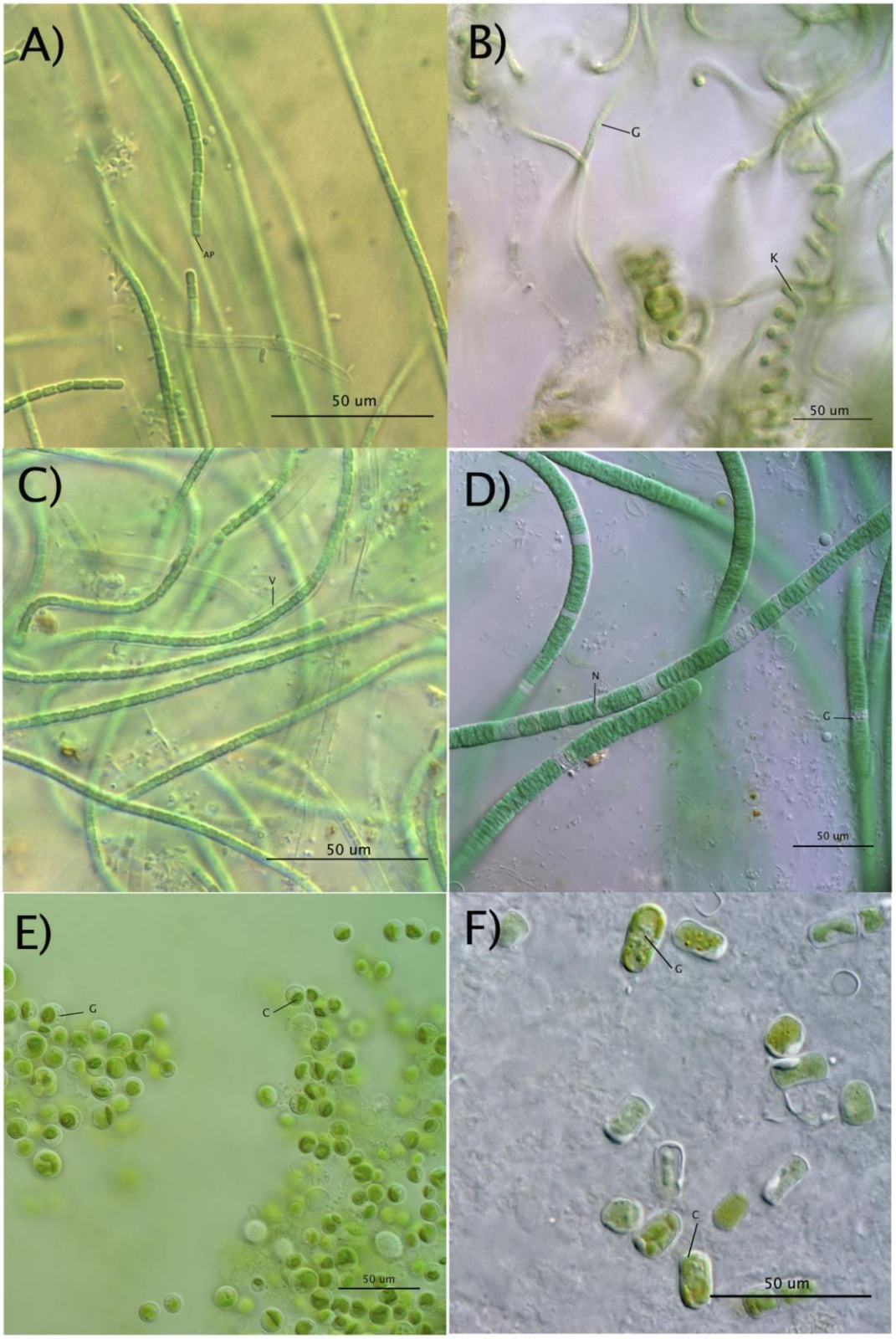
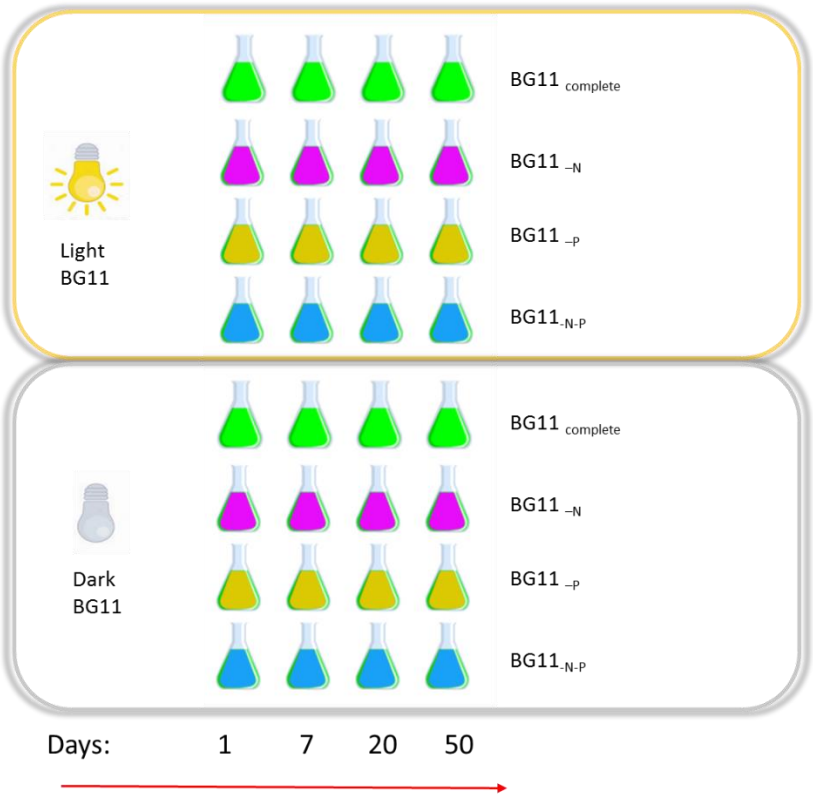


Figure 1: Optical microscopy images showing the different morphologies corresponding to the isolated strains. A) *Pseudoanabaena*, B) *Nodosilinea*, C) *Leptolyngbya*, D) *Phormidium*, E) *Chlorella* and F) *Coccomyxa*.

For the first batch of experiments, one cyanobacterial strain from the *Nodosilinea* genus and one eukaryotic strain (*Chlorella*) were subjected to chlorosis using the protocol outlined below:



No substantial or significant differences were found under light treatment. After 50 days of nitrogen (N) and phosphorus (P) deprivation, a tendency toward chlorosis was observed, but there were no significant changes in morphology or chlorophyll *a* content compared to the control in both strains.

Based on the results of the light experiment, we decided to focus on the dark condition experiment. As in the light condition, colonies were observed at 1, 7, 20, and 50 days using epifluorescence microscopy to measure chlorophyll a and potential morphological changes. The results showed that the autofluorescence of photosynthetic pigments responded similarly to chlorophyll a performance ([$F(3,46) = 9.838$; $p < 0.01$] for *Nodosilinea* and [$F(3,40) = 16.893$; $p < 0.01$] for *Chlorella*). The autofluorescence of photosynthetic pigments in both strains was significantly reduced compared to the control (BG11 complete). The decrease in autofluorescence intensity was most pronounced at 50 days and particularly notable under the BG11 $_{-N\text{-}P}$ condition (Figure 2). Moreover, this result was corroborated by spectrophotometry, which showed the same pattern of chlorophyll decrease.

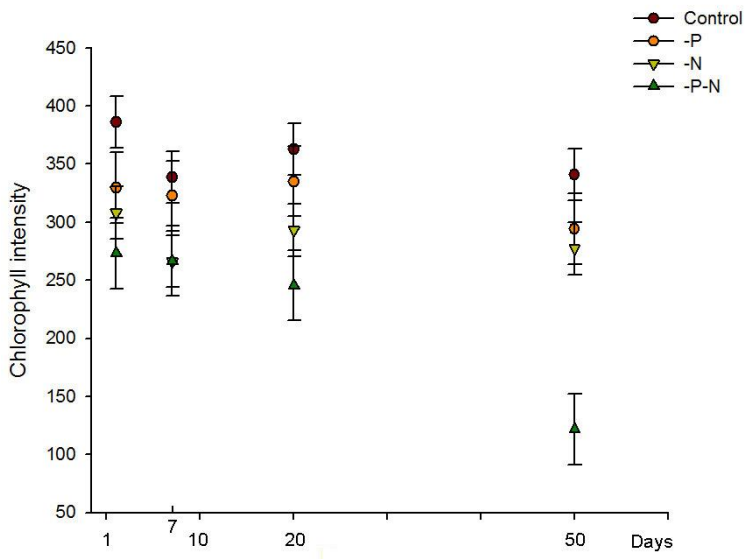


Figure 2: Chlorophyll *a* intensity under dark condition and different nutritional treatments.

PHB accumulated by *Nodosilinea* and *Chlorella* was visualized microscopically after staining with two different dyes, Sudan Black B and Nile Red. For Sudan Black B staining, cells were heat-fixed onto clean, grease-free glass slides, and a few drops of Sudan Black B staining solution (0.3% in 70% ethanol) were added. After 5–10 minutes, the slides were immersed in xylene until complete decolorization. The slides were allowed to dry and then examined with an oil immersion lens.

Nile Red (1 mg) was dissolved in dimethyl sulfoxide (1 ml) to prepare the staining solution. Two drops of this solution were added to approximately 200 µl of sterile culture, which was then incubated at 55°C for 10 minutes. The cells were transferred to a glass slide and viewed using a fluorescent microscope (Nikon, USA) with a Cyto3 filter at 1000× magnification.

During microscopic visualization, PHB appeared as black granules with Sudan Black, while it appeared as brightly fluorescent granules with Nile Red staining. Pixel information collected with the Cyto3 filter corresponding to Nile Red staining was pseudocolored green to co-localize in the same image with Chlorophyll *a*, which was collected using the Cyto5 filter (Figure 3).

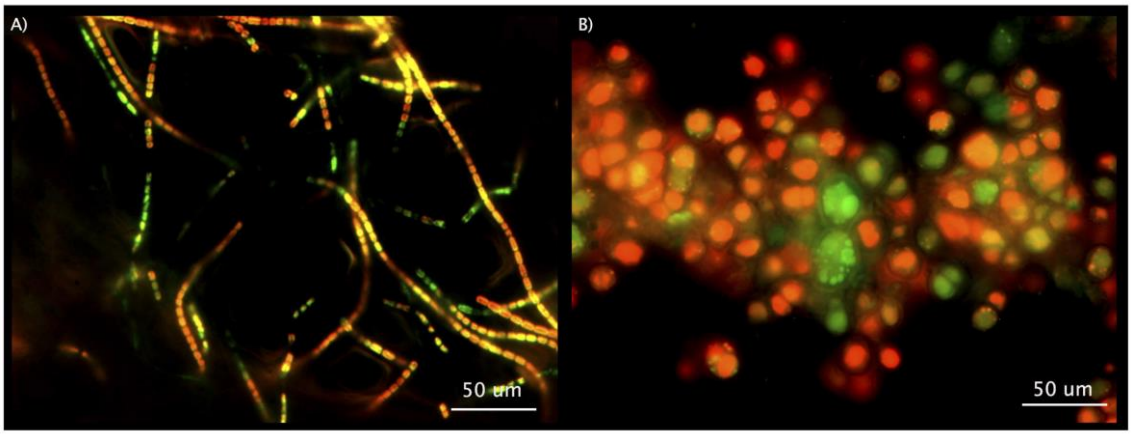


Figure 3: A) Epifluorescence microscopy image of cyanobacteria showing the intracellular granules using the Cyto3 filter and chlorophyll A with the Cyto5 filter. The Nile Red signal was pseudocolored green. B) Epifluorescence microscopy image of eukaryotic cells showing the intracellular granules using the Cyto3 filter and chlorophyll with the Cyto5 filter. The Nile Red signal was pseudocolored green.

Both microalgal strains isolated showed PHB accumulation, proving that *Nodosilinea* and *Chlorella* are good sources of PHB. Since PHB accumulation is indirectly related to the decrease in photosynthesis, we decided to measure the ratio of PHB intensity to chlorophyll intensity (Figure 4). This further demonstrates that nitrogen and phosphorus deprivation is crucial for the increase in PHB production and accumulation, with the highest values observed after 50 days of deprivation.

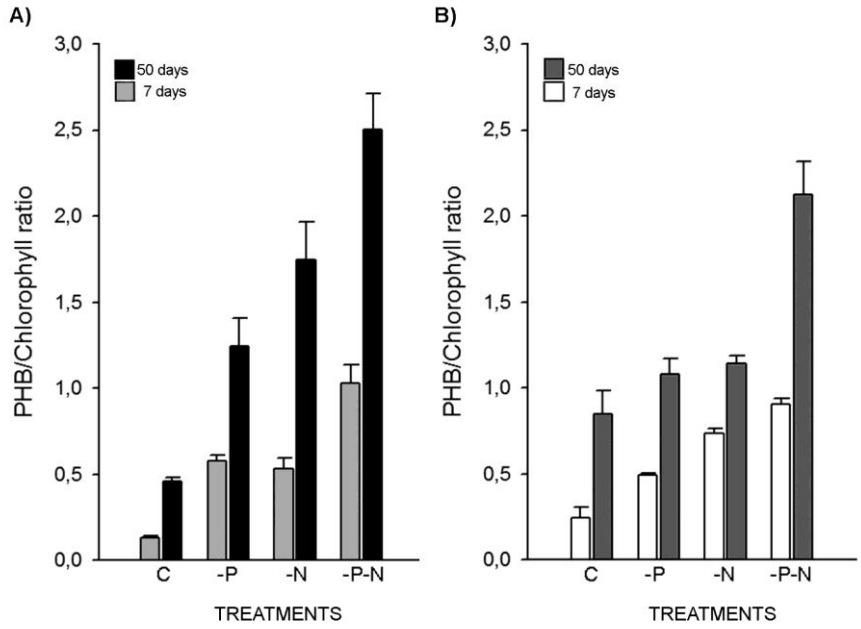


Figure 4: PHB/Chloropyll ratio in A) *Chlorella* and B) *Nodosilinea*.

Also, Fourier transform infrared spectroscopy (FTIR) was conducted between 4000 and 600 cm-1 using an ATR accessory with a zinc selenide crystal to analyze PHB isolated after 50 days of nitrogen and phosphorus deprivation. Figure 5 shows the spectra obtained from the PHA samples of both strains. In the figures, it is possible to observe that the

transmittance bands at 1735 and 1720 cm-1 are attributed to the strong vibration of the carbonyl ester group (C=O). Another characteristic feature observed in the spectra is the presence of elongations of the –OH group within the carboxyl group, ranging between 3400-3200 cm-1. All these indicate that both strains have the ability to synthesize biopolymers capable of forming bioplastics.

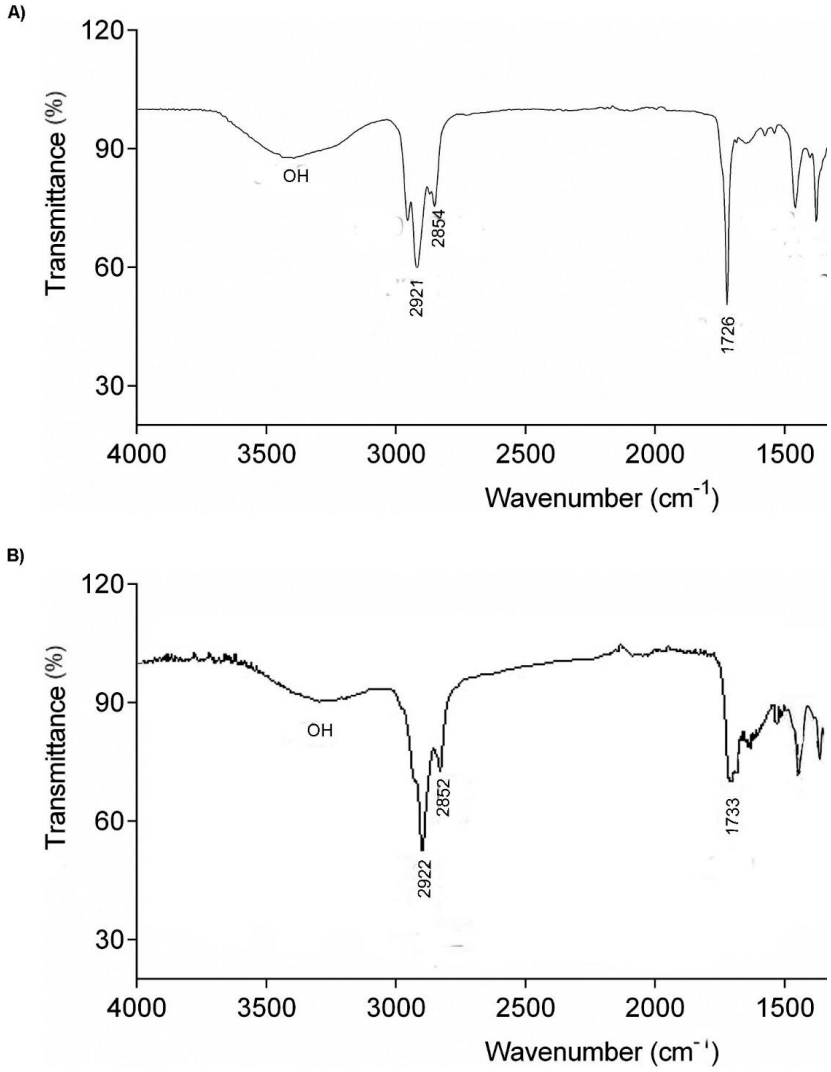


Figure 5: FTIR/ATR spectrum of A) *Nodosilinea* and B) *Chlorella.* The spectrum was recorded ranging from 4000-600 cm-1

With these preliminary results, I will continue investigating more properties related to PHB and will analyze the RNA-seq sequencing data already sent to NOVOGENE. For the bioinformatic analysis, I will be assisted by Dr. Daniel Kurth from the University of Tucumán (Argentina), who is involved in the development and advising of several courses related to this type of analysis. Moreover, it is worth mentioning that part of these results were presented at the last Argentine Microbiology Meeting (October 2024), with the participation of an undergraduate student in this project.