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Modulating Glycogen Metabolism for improved PHB Yield in *Synechocystis* sp. B12

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Abstract

The use of fossil fuels and plastic products pollutes and damages our planet, our land, our water and our entire lives. The common objective is to find strategies to solve this problem and build a better world. A possible strategy is to use cyanobacteria capable of producing biopolymers as an interesting source of environmentally friendly and sustainable plastics, without using fossil fuels. In fact, some cyanobacterial species can synthesize bioplastics like PHB (polyhydroxybutyrate). Moreover, as cyanobacteria are photosynthetic microorganisms, fixing atmospheric carbon dioxide to convert it into biomass, they have the potential, in the meantime to reduce greenhouse gases (GHG) emissions in the atmosphere. A particular species, *Synechocystis sp.* B12, isolated in a polluted area of Brazil, showed to be particularly advantages in high light and to produce a discrete amount of PHB. So *Synechocystis* sp. B12 grown in different growth curves, in Nitrogen starvation and in phosphorus starvation and then these two stresses were combined, some parameters like OD, PHB accumulation and glycogen trends were monitored. Moreover, to manipulate glycogen metabolism nucleotides and amino acids sequences were aligned for genes GlgP1 and GlgP2 between reference strain PCC6803 and B12 to enhance differences. Then some molecular biology experiments were done with the aim to overexpress gene GlgP2 involved in glycogen metabolism and especially in glycogen degradation the ways of stable recombination and transient transformation were attempted.

Glossary

aa (amino acids)

bp (base pairs)

BMC (bacteria micro-compartments)

CCS (Carbon concentration storage)

CCU (Carbon concentration usage)

CWD (cell weight dry)

ED (Entner Doudoroff pathway)

ELMs (engineered living materials)

EMP (Embden Meyerhof Parnas pathway)

FDCA (2,5-Furandicarboxylic acid)

GAP (glyceraldehyde phosphate)

GFP (Green fluorescent protein)

HEPES (4-(2-hydroxyethyl)1-piperazineethansulfonic acid buffer)

HMF (5-(hydroxymethyl)furfural)

Kbp (Kilo base pairs)

MCCS (multiple cloning site)

N (Nitrogen)

Nsc (no-stop-codon)

OPP (oxidative pentose phosphate pathway)

P (Phosphorus)

PBAT (polybuthylene adipate co-terephthalate)

PBR (photobioreactor)

PHA (Polyhydroxyalkanoate)

PHB (Polyhydroxybutyrate)

PHV (Polyhydroxy valerate)

PLA (Poly lactic acid)

PUFA (Poly unsaturated fatty acids)

1 Introduction

1.1 Perspective and market of circular economy

The process of circular economy involves all nodes of the market from production, to processing of products. The demand of biobased products had a robust growth in the last years and in future it is expecting to further increase. Through circular economy, wastes coming from the industry can be used as a source for new products. So circular economy can be seen as a set of markets that exchange resources and wastes to produce many new products, like a web of interaction between different sectors (Siderius and Zink, 2023).

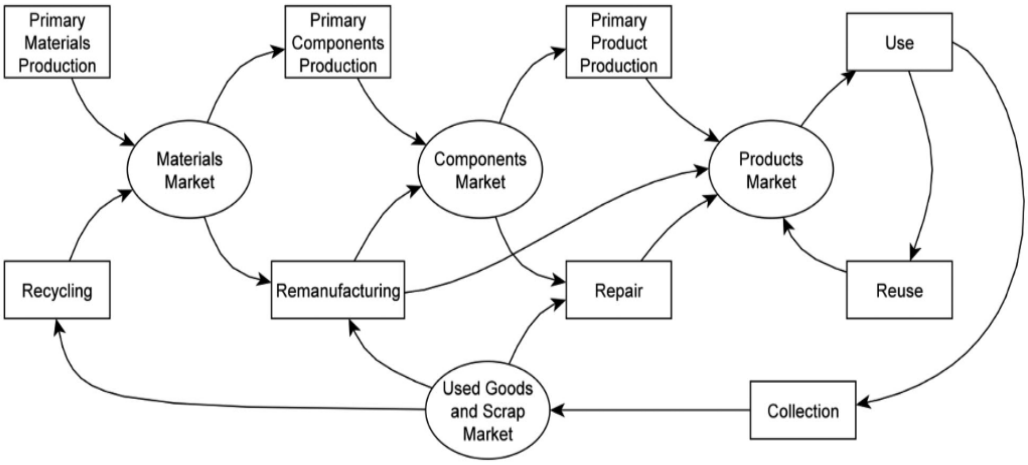


Figure 1. Different types of markets and the web of circular economy that bound all of them (Zink et al.2017).

As can be seen in Fig 1. resources flow from materials market to products market, through processing of raw materials, following different paths, involving recycling, remanufacturing, repair. General market is in this case divided in materials markets (market of raw materials), components market, and product market. There are some concepts about circular economy that need to be considered: Life cycle assessment (LCA), and Sustainability. LCA can be defined as the analysis of all negative and positive effects on environmental and human health associated with a good or a service. While Sustainability involves solutions to protect nature and its components from pollution, environmental degradation and heavy industrial activities.

1.2 Carbon capture methods

Extending the vision of damage caused by greenhouse gasses like CO_2 and CH_4 are arising in atmosphere increasing causing negative effects to the planet. These gasses are responsible of greenhouse effect, and consequences in environment and human health are evident, because greenhouse gasses released in atmosphere trap sun beams. They are negative because heat trapped in atmosphere increase global temperature, accelerate ice melting and consequently increase the sea level. To plan actions to help environment It is useful to analyse methods that allow carbon capture and storage (CCS) and carbon capture utilization (CCU). According to research conducted by Goodchild-Michelman et al. 2023, some possibilities to use cyanobacteria in CCS were explored, among which:

- Bio-composites
- Bioconcrete
- Biophotovoltaics

Biocomposites are developed using Loofah, the fruit of the plant *Luffa cylindrica* (family of Cucurbitaceae), used as plant base sponge. It is used as scaffold for cyanobacteria, because it is highly porous and allow a good aeration. By this way CO_2 and O_2 can spread through pores, and carbon capture can be intensified thanks to cyanobacteria CO_2 fixation. Bioconcrete is a block made by biomineralizing cyanobacteria and sand, developed as another strategy to maximize Carbon storage which exploits cyanobacteria in a sand-hydrogel matrix. The last case deals with Biophotovoltaics, which consider the fact that cyanobacteria in some biofilms are able to transfer electrons. This method have to be optimized to assure high scale efficiency (Goodchild-Michelman et al. 2023).

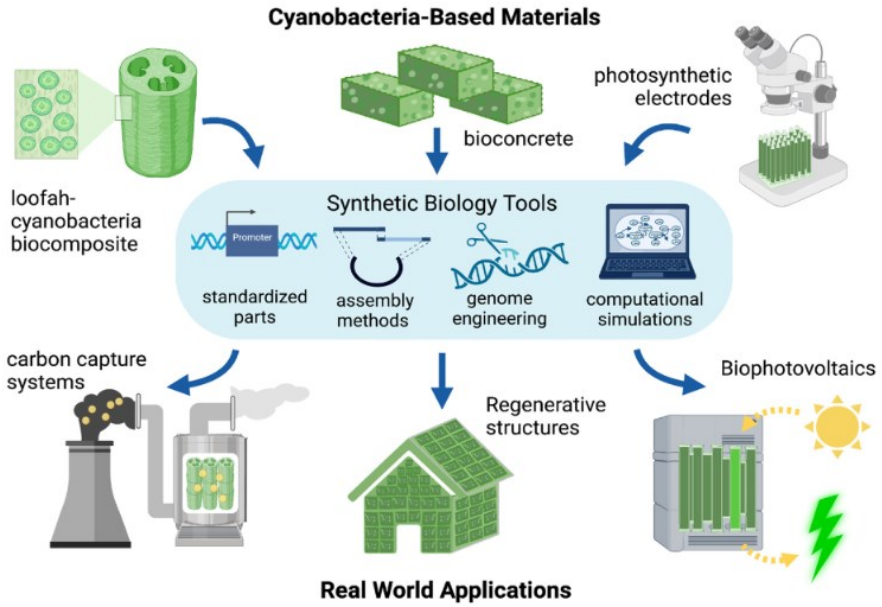


Figure 2. Representation of principal approaches and effective possibility to implement cyanobacteria in sustainable development.

In the last decade they were introduced method of Carbon Capture Utilization (CCU). In fact, CO_2, the main and most abundant greenhouse gas, can be not only stored, as for CCS, but can be also used or converted to reach ecologic and sustainable process, both for environment and economy. CCU are essential to look at the potential of implementation to reach carbon neutrality also known as C-net zero, so that some solutions were proposed to employ directly CO_2 and reduce drastically emissions. (Chauvy and De Weireld, 2020):

- Convert CO_2 into fuel, chemical, and durable materials.
- Make CO_2 mineral carbonation, to develop sustainable construction materials.
- Use CO_2 as substrate for algae cultivation and enzymatic conversion.

This means that both products obtained in case of CCS, and processes in case of CCU, can be adopted to manage innovative plants, to achieve solids results facing climate change and global warming.

1.3 Production of biobased plastics

Bioplastics are polymeric substances deriving by biogenic synthesis, they can be biodegradable and compostable. The main difference that characterizes production of common plastics and bioplastics is the fact that production of biobased plastics follows the process of circular economy, and work as a close circle, while traditional plastic is produced using fossil fuels derivates and in this case there are no circle of resources. There are 2 aspects to consider: the first is that production of bioplastics does not involve fossil fuels and the second is that biodegradation ideally does not produce waste. The production of biobased products is rising in these years, as also EU (European Union) has continued to finance many programs to find concrete solutions on the problem of conventional plastics. The use of microalgae and cyanobacteria in the production of bioplastics has many advantages: they contain carbon through photosynthesis, thereby reducing greenhouse gas emissions, reducing environmental impact and producing at the same time sustainable products. In fact, photosynthesis and fixation of inorganic carbon enable cleaner production without the use of organic carbon. There are distinct categories of bioplastics:

- PHA (polyhydroxyalkanoate)
- PLA (polylactic acid)
- Starch blends bioplastics
- FDCA from HMF (2,5 furandycarboxylic acid) (hydroxy methyl furfural)
- PBAT (polybutylene adipate co-terephthalate)

In this list, the first two are biobased polyester, the third is typically a polymer formed by many units of lactate, usually produced by microorganisms through fermentation. All of them have interesting properties because they are biodegradable and compostable, in particular PLA is successfully employed in biomedicine due to its antimicrobial properties. A good property that has to be discuss is the capability of a bioplastic to be extruded, and to realize objects with 3D print system. In additive manufacturing, a material to be printable should be fluid in the moment of extrusion and maintain a shape after extrusion. It has to pass mechanical tests to be characterized and determine mechanical properties. Examining all types of bioplastics, it is possible to note that some blends, like PHA/PLA hybrids, have good potentiality to be employed in additive manufacturing, because these filaments show improved mechanical properties, and in few cases better behaviour during extrusion (Torabi et al., 2023).

To better understand the market of bioplastic, it is necessary to look some trends, types and capacities of bioplastic products. This diagram, under reported, (Fig 3.) deals with the global bioplastics' capacities, so how many

tonnes of bioplastics can be produced by companies worldwide. In this graph perspectives of production for 2025 and 2026 are shown, observing a constant increase in the next future.

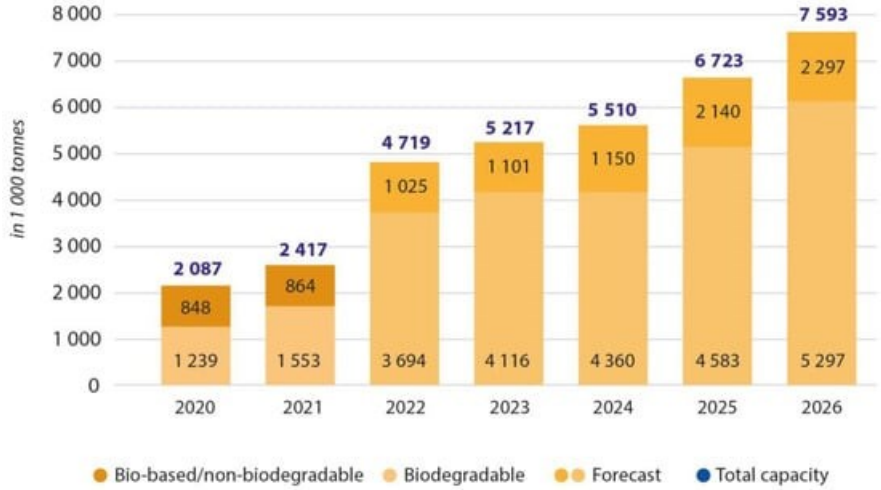


Figure 3. global bioplastic capacity from 2020 to 2026.By A. Costa et al. (2023).

Considering industrial applications of bioplastics, the most important, and the most involved ones, are packaging, both flexible and rigid and fibres. Bioplastics are also used in agriculture and biomedical field (Costa et al., 2023). Market is dominated by some industrial companies like BASF (Ludwigshafen am Rhein Germany) and Corbion (Gornichem Netherlands) that produce bioplastics, and have plants based on exploitation of biomass, involving the cultivation of bacteria. BASF develops biobased products employable in several sectors of industries and human activities, like building materials, pharmaceutical, bioplastics, eco-friendly catalysts. Recently they have explored the field of biobased volatile compound to make naturals flavours and food additivities. Corbion is more active on production of PLA blends, food additives, antioxidants, coating and adhesives and personal care products.

This second diagram (Fig4.) describes all market sector where bioplastics are applied, most of the employment concern about packaging both rigid and flexibles, but also consumer goods, fibres raw and woven, and products for agriculture are relevant. Examples of products are boxes, films, tubes, small plastic containers, web for crop support, bags.

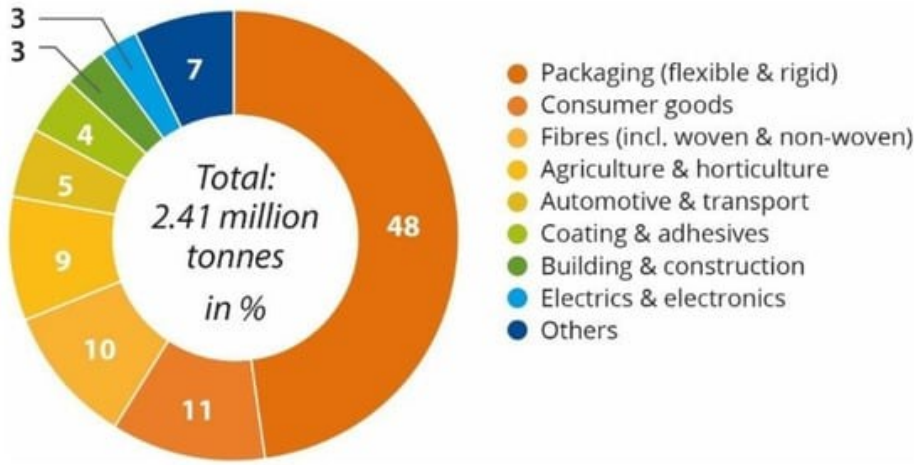


Figure 4. diagram that describes different segments of the market involved in bioplastics by A. Costa et al. (2023).

1.4 PHB

Polyhydroxybutyrate (PHB) is a type of PHA (poly hydroxy alkanoates), it is a biobased polyester synthesized as substance of reserve by some species of bacteria, it is biodegradable and compostable. It has some interesting mechanical properties like: high melting temperature, low glass transition temperature, middle grade of crystallinity (McAdam et al., 2020). These properties are useful for industry to develop competitive materials, as they are similar to fossil polyester polypropylene, but can reduce carbon footprint. PHB can be shaped and functionalized with chemical or physical treatments, to add new properties and to improve his stability. It can then be shaped in sheet, nanoparticles, or coupled with chitosan (Fernandez-Bunster and Pavez, 2022).

This polymer finds many using sectors as biomedical materials. In this case, composites with high content in PHB are developed to build several devices as heart valves, drug delivery systems and bone tissue engineering (Fernandez-Bunster and Pavez, 2022). PHB is a versatile material because, it is possible to employ in addition to other biomaterials. For example composites were developed to treat bones fractures made by hydroxyapatite and PHB: this composite has excellent properties of osteointegration and biodegradation and, despite some composite fragments were present in the site treated, material degraded to consent an excellent wound repair (Reis et al., 2010). Also, a cell-seeded scaffold to help wound healing was developed using PHB, assuring a good vascularization and low content of inflammatory cytokines (Guo et al., 2022). Another using sector is agriculture, where PHB is used to make mulch films (Menossi et al., 2021).

1.5 Organisms producing bioplastics

There are many organisms able to produce PHB. To be an efficient PHB producer an organism have to detain some essentials features such as: high rate of conversion of substrate into PHB, not being pathogenic or toxic, being able to grow to different conditions of temperature and pH, having an high PHA accumulation, using different sources of carbon (Wang and Chen, 2017).

Bioplastics are usually produced by exploitation of plant biomass, derived as waste from agriculture, dairy products, glycerol, or food waste. So using different biowaste is possible to produce several types of bioplastics, at different level of purity, and using various pretreatments methods like: (Lorini et al., 2021) wet acidification, thermal drying or freeze drying.

There are also microorganisms that can produce PHB in strong amounts. An example is *Cupriavidus necator*, a chemolitotrophic bacterium that can store a lot of PHB in granules inside cells, exploiting sugars as source of carbon. It can also fix CO_2 in presence of hydrogen and low oxygen. Another example of organism used for PHB synthesis is *Haloferax mediterranei*: it is cultivated using alternatives biomass like waste of food industries. Also in this case, bioplastics is accumulated in intracellular granules (Parroquin-Gonzalez and Winterburn, 2023). This organism is particularly interesting as it grows typically in environment with high salinity, a condition that is highly stressful for other organisms. Another interesting microorganism that produces bioplastics exploiting biomasses is *Priestia megaterium*, a large bacterium found in many substrates like soil and seawater. It is documented that *P. megaterium* is able to grow efficiently with biomass derived from food industry, and has the ability to exploit rice husk to produce an high amount of PHB (Sehgal et al., 2023).

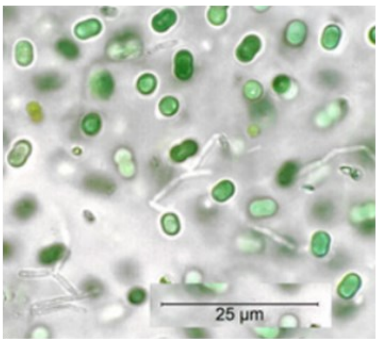
However, the use of cyanobacteria for biotechnology purpose has many advantages, as they overcome strongest limitations of heterotrophic bacteria. In fact, the main problem with non-photosynthetic bacteria is that they have to use only organic carbon that sometimes is expensive and not environmentally friendly. Yet, cyanobacteria have the capability to use inorganic carbon as CO_2 that is less expensive, clean and at the same time can be applied to limit greenhouse gas emissions.

Focusing on cyanobacteria as producer of PHB, a study handled by (Koller, 2020) describes many species of cyanobacteria cultivated a various conditions, like:

- *Nostoc sp.* (produces more PHB when cultures are supplied with different carbon source),
- *Anabaena cylindrica* 10C (can produce more PHB under heterotrophic cultivation conditions, adding acetate),
- *Synechococcus enlongatus.*

Nostoc sp., image from Roger Burks.

Anabaena cylindrica, image from Robin Matthews.



Synechococcus enlongatus, image from Algaebase.

Figure 5. Examples of cyanobacteria considered for interesting biotechnological properties.

1.6 Cyanobacteria as cell-factories

Extending this concept of cyanobacteria as PHB producers, it is useful to consider the role of cyanobacteria in the production of biofuels and bioplastics, as well as their role in the bioremediation of polluted areas.

So microalgae and cyanobacteria can be consider as producers of many others important metabolites such as (Vigani, 2020):

- Polyunsaturated fatty acids (PUFA's) ex: oleic acid
- Vitamins B, E
- Phycobiliproteins like: phycoerythrin
- Polysaccharides
- Sterols like: brassicasterol, sitosterol
- Phenolic and volatile compound

Due to potentiality of cyanobacteria in numerous applications, in future there is the hope that more industrial scale plants will be implemented, using cyanobacteria as cell factories. Thus, the environmental effects, potential toxicity and effectiveness of plants can be evaluated. Kilograms of absorbed and fixed CO2 can be calculated per kilogram of polymer produced by monitoring the conditions of the culture. (Frehner et al. 2022). Then cyanobacteria can also be used for wastewater treatments, to obtain sustainable product and reduce environmental impact of human activities, like GHG emissions and land exploitation and pollution. This process imply two advantages: the reduction of the toxicity of waste water and the possibility to synthesize new products as polysaccharides, which can be used in pharmaceutical industry to treat dangerous diseases (Franco-Morgado et al., 2023).

The idea is quite simple: the mains ingredients are sun light, CO_2, nutrients, and water that could be seawater or fresh water, then cyanobacteria can produce a large spectrum of useful substances (Zahra et al. 2020).

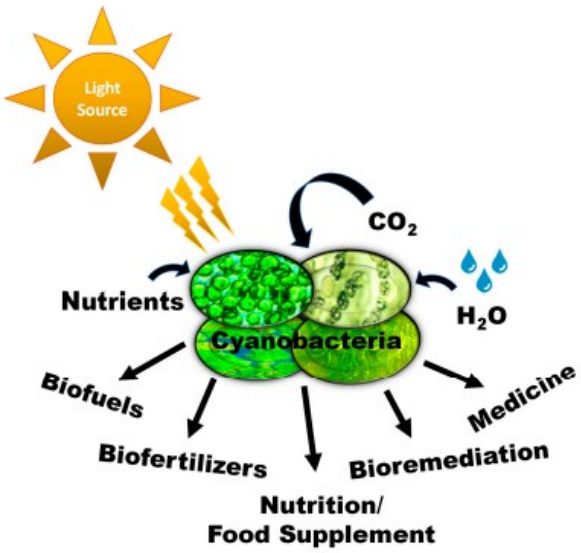


Figure 6. possible applications of cyanobacteria. By Zahra et al. 2020.

1.7 *Synechocystis* sp. B12

An impressive organism for PHB production is *Synechocystis* sp. B12, recently isolated in a polluted area, contaminated with heavy metals (copper, cadmium) and aromatics organics compounds, in Brazil (Gracioso et al., 2021). This strain has shown to be highly resistant to pollutants and to high light than reference strain (PCC 6803). It is reported that Synechocystis sp. B12 can easily tolerate high light intensities around 300 µmol photons m^{-2} s^{-1}, and it is able to grow until 700 µmol photons m^{-2} s^{-1} (Verdesca, 2024). Moreover, it has an ability to accumulate more PHB, opening the perspective of employing *Synechocystis sp.* B12 in industrial fields.

1.8 Metabolic pathways and genetic engineering

In *Synechocystis sp.* PHB production is guided by a specific metabolic pathway. Direct way starts from different molecules like acetate, glucose and glucose-3-P given as organic carbon feedstocks. They are converted in Acetyl-CoA the precursor for PHB production. It is interesting to note that in cyanobacteria such as Synechocystis sp., the main carbon source, CO_2, is fixed by photosynthesis (P R. Yashavanth et al., 2021). Afterwards, CO_2 is converted to 3PG (3-phosphoglycerate) and consequently, to GAP (phosphate Glyceraldehyde), after GAP is converted to Pyruvate and, finally, Pyruvate is converted to Acetyl CoA, the precursor of PHB. The synthesis of PHB is mediated by the presence of three specific enzymes: acetyl-CoA acetyltransferase (PhaA), acetyl-CoA reductase (PhaB), and acetyl-CoA synthase (PhaE-C). Thus, PHB is produced by AcetylCoA through this reaction:

$$AcetylCoA \rightarrow \text{AcetoAcetylCoA} \rightarrow \text{R 3HydroxybutirrylCoA} \rightarrow \text{PHB}$$

Besides the classical way to synthesize PHB, there are other different and parallel ones which result in different final polymers. For example, in *Haloferax mediterranei*, there is another pathway to produce PHV (polyhydroxyvalerate), starting from serine. This strain has the capability to produce both PHB and PHV, while *Synechocystis* can produce only PHB but has the great advantage that can fix inorganic carbon, while *Haloferax* cannot (Pacholak et al., 2021).

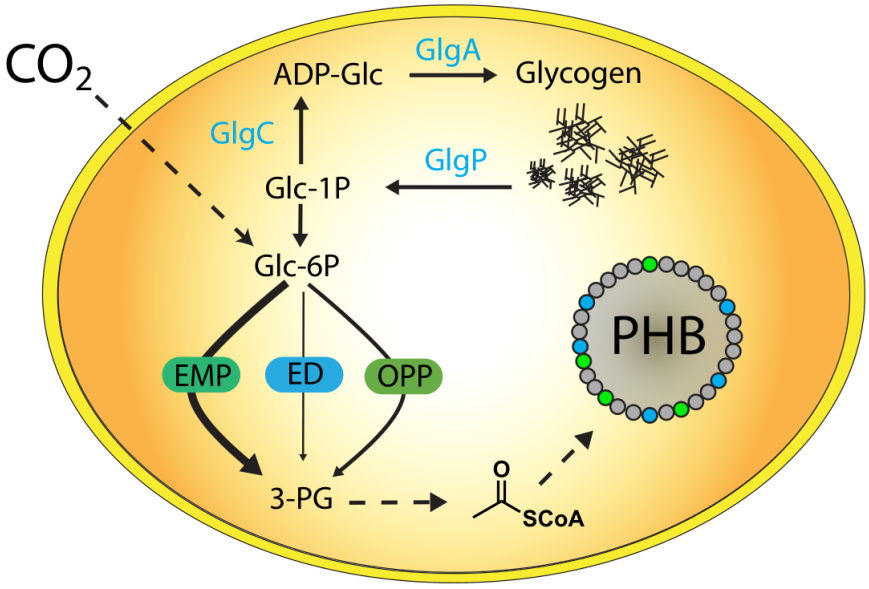


Figure 7. This image reports principal metabolic pathways to synthesizes substrate for PHB. In light blue colour genes of glycogen metabolism are indicated, all metabolic reactions occur in cytosol shown in orange colour, in this image mains intermediates for PHB synthesis are shown.

From G6P (glucose 6 phosphate) three pathways are possible: EMP (Embden Meyerhof Parnas), ED (Entner Doudoroff), OPP (oxidative pentose phosphate), they generate a direct precursor of Acetyl CoA, 3PG (3 phosphoglycerate). The 3PG is converted into acetyl CoA and follows reactions that lead to PHB. It also shows mains genes involved in glycogen anabolism and catabolism. ATP is necessary to cells to survive and complete metabolic reactions (Testa et al., 2022), a study reported that EMP pathway also known as glycolysis generates ATP from glucose and it is essential to cells growth, while ED pathway is less efficient because it convert one molecule of glucose in one molecule of ATP (Veaudor et al., 2020)

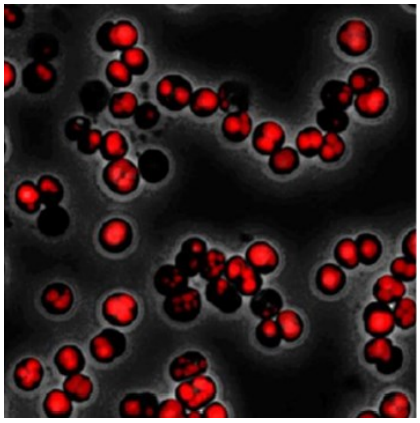


Figure 8. *Synechocystis sp.* Treated with Nile Red under confocal microscope. PHB granules are well evidenced, visible in red colour.

From a metabolic point of view, *Synechocystis* needs both an optimal carbon and nitrogen metabolism and a good redox balance to be able to fix carbon and synthesize all useful metabolites.

Under different stress conditions *Synechocystis* could accumulate reserve substances that help cells to manage stress and survive, examples of these substances are: cyanophycin, PHB, terpenes, succinate and carotenoids (Mills et al., 2020). An important metabolite is glycogen, a reserve source of carbon that is accumulated in cytosol. At the same time, nutrient stress induces an increase in PHB synthesis and accumulation. In *Synechocystis sp.* also glycogen helps cells to survive in environmental stress situations, it is accumulated and then degraded to build up other molecules like PHB (Koch et al., 2020a). Other molecules can be produced under different stress situations like: fatty acids, glucosilglycerol, sucrose (Noh et al., 2021).

In recent time researchers and companies have investigated and developed strategies to enhance the content of PHB using cyanobacteria. An example is given by a study conducted by Koch et al.(2020b). In this study genetic engineering was performed on *Synechocystis sp.* PCC 6803, knocking out the *PirC* gene, encoding for a regulatory protein, the mutated strain showed a strong activity of phosphoglycerate mutase, which allowed to increase PHB production almost to 81% CWD (cell weight dry) in starvation of nitrogen and phosphorus. The deletion of this gene had also strongly increase glycogen catabolism, so the function of this gene is essential both for augmentation of PHB content and for glycogen catabolism.

Therefore, in specific conditions, carbon flux inside cyanobacteria can flow from fixed CO_2 to storage molecules like glycogen. *Synechocystis sp.* can efficiently modulate glycogen metabolism to produce PHB as carbon reserve. There are some genes involved in the regulation of glycogen content, such as the gene *apcE*, which codifies for a glycogen transferase. Gene *apcE* has many effects on phenotype: it regulates cells growth rhythm, glycogen content, it controls the glycogen distribution inside the cell (Joseph et al., 2014).

In another recent study, impact of stress factors in biomass and glycogen content was evaluated. Experiments conducted in photobioreactors (PBR) reported that in nutrient starvation, glycogen and PHB have a great variation during mid-long period of time, while glycogen content shown a sensitive decrease during time PHB shown only a slight decrease (Rueda et al., 2022).

To manipulate glycogen catabolism can be another interesting strategy to enhance PHB production. Glycogen is a polymer, branched polysaccharide made by many units of glucose, bound each other with mostly α1-4 glycosidic bounds. Glycogen in cyanobacteria has several functions, beyond carbon reserve, it has the function of regulate growth rate during rapid dark-light cycle (Bishé et al., 2023), it has also the role to increase photosynthetic productivity in outdoor conditions (Cantrell et al., 2023).

Investigations reported that variation of light and dark affect glycogen metabolism in cyanobacteria, because glycogen biosynthetic pathways are more active during day, and degradative pathways are more active during night (Johnson and Rust, 2021). Especially regarding to glycogen metabolism, it is important to consider genes GlgP1 sll1356 and GlgP2 slr1367 are different, and the second, in particular, plays a major role in glycogen catabolism, mainly under stress conditions. So, carbon backbones of glycogen can be mobilized and potentially PHB synthesis could be increased. *GlgP* genes, encoding for two glycogen-phosphorylase, play a key role in in glycogen breakdown, especially gene *GlgP2,* as it is more expressed in stress conditions (Koch et al., 2019). The role of these genes is essential for glycogen degradation and consequently for the production of PHB, the cell's redox balance can regulate the activation of these genes, so the gene GlgP1 is suppressed under reduced conditions, and its activity can be restored by oxidation, another function of this gene is to resist long-term chlorosis, instead the gene GlgX1 increases the activity of the genes GlgP1 sll1356 and GlgP2 slr1367. Analysing the activity of these two genes, the first is more active at night, the second is more active during the day, and it is reported that there is strong cooperation between the GlgX1 and GlgP2 genes (Neumann et al., 2022).

Another study (Koch et al., 2019) had investigated the role of genes *GlgA1* (sll0945), and *GlgA2* (sll1393), involved in Glycogen synthesis. Expression level of these genes expressed in nitrogen starvation, an experiment was conducted on *Synechocystis* mutant for glycogen synthase, they found that gene *GlgA1* was impaired in PHB production, instead gene *GlgA2* was not impaired.

Others genes can be considered as targets to increase indirectly the content of PHB, like gene *MalQ (*sll1656), this gene codify for a protein, 4-α-glucotransferase, essentially it enhance Glycogen degradation when cooperates with genes *GlgP* (Nguyen et al., 2019).

2 Thesis aim

The principal purpose of this thesis is to monitor PHB production and glycogen degradation under different conditions of starvation. To do so, growth curves of *Synnechocystis sp.* B12 were made in Nitrogen starvation and Phosphorous starvation and then the results were quantified by assays both for glycogen and PHB, to investigate the rate of PHB synthesis and glycogen catabolism.

Then, genes to overexpress were identified, namely genes *GlgP1* and *GlgP2*. In particular, gene *GlgP2* was considered due its relation in glycogen catabolism to redirect carbon flux in PHB production, to induce this cyanobacterium to produce more PHB.

Before molecular biology experiments, some investigations on target genes *GlgP1* and *GlgP2* and related proteins were performed. So, nucleotides and amino acids sequences were aligned between reference strain PCC6803 and B12, to highlight differences and similarities.

Others important aims were the set up the transformation of *Synechocystis sp.* B12 and in parallel to generate the plasmids for the overexpression of the genes of interest, related to glycogen catabolism. Transformations were done through different approaches: natural transformation and electroporation for stable recombination, and bacterial conjugation for transient transformation.

3 Methods and materials

3.1 Curves in nutrient starvation

Synechocystis sp. B*12* usually was grown at 30°C in agitation with a light intensity of 500 µmol photons m^{-2} s^{-1}. *Synechocystis sp.* B*12* was grown in BG11 liquid medium (Ripkka 1979).

3.1.1Curves in Nitrogen starvation

Cells were cultured in BG11 medium with and without sodium nitrate ($NaNO_3$) to investigate trends in Glycogen and PHB. Four flasks were prepared, two of them with normal BG11 and two of them with modified medium. OD_{750} (optical density measured at 750 nm) was measured by using a plate reader Tecan SPARK for 200 µl of sample to understand the rate of cellular growth. Then the concentration of glycogen and PHB was monitored in all samples.

Component	Concentration mg/L
Na_2 MG EDTA	1
ferric ammonium citrate	6
citric acid · H_2O	6
$CaCl2 \cdot 2 H_2O$	36
$MgSO_4$ · H_2O	75
K_2HPO_4	30,5
H_3BO_3	2,86
$MnCl_2 \cdot 4 H_2O$	1,81
$ZnSO4 \cdot 7 H_2O$	0,222
$CuSO4 \cdot 5 H_2O$	0,079
$COCl2 \cdot 6 H_2O$	0,05
$Na_2MoO_4 \cdot 2 H_2O$	0,391
$NaHCO_3$	20
$NaNO_3$	1500

Table 1. Composition of BG11 medium for growth of *Synechocystis sp.* B12.

Cultures were monitored one, three, seven and nine days after the beginning of the experiment.

Curves in Phosphorous starvation

Then the role of phosphorous on OD, glycogen and PHB production was investigated. To develop this experiment cultures were made in four flasks, a couple with BG11 standard, and a couple with modified BG11 without Phosphate. Then, to understand better the behaviour under Phosphorous starvation, another experiment was set up where *Synechocystis sp.* B12

was grown under lack of phosphorus for five days to acclimate cells for a longer time period. After the acclimatation curves was started with two flasks, control and P starvation.

Also in this case, PHB trend was monitored over time.

Combined stresses

Then the two mains stress factors, nitrogen and phosphorous starvation, were combined. And the trend of OD_{750}, glycogen and PHB was monitored.

3.2 Glycogen assay

Measurement of glycogen was taken with Glycogen Assay kit MAK016 (Sigma-Aldrich), following manufacturer's instructions.

Appropriate volumes of cultures were centrifuged at 5000 g for 10 minutes at room temperature and then diluted in deionized water to reach final OD_{750} of 1 in 200 µl total. After that, cells were transferred into a screw cap Eppendorf with some glass beads to increase cells fragmentation and put for 10 seconds into Bullet blender® (Next Advance). Samples were then conserved in ice for 1 minute and boiled at 100°C for 10 minutes in a thermoblock Accublock™ digital dry bath Labnet International®. At the end samples were centrifugated at 15000 g for 10 minutes at 20°C.

To correctly quantify glycogen a standard curve was used, with glycogen concentrations ranging from 0 µg/well to 2 µg/well. Then, to make colorimetric assay, this protocol was applied: in order, 48 µl of supernatant, 2 µl of Hydrolysis buffer, and 2 µl of hydrolysis enzyme were added. In samples used to measure the background concentration of glucose, hydrolysis enzyme was not added. Then, all samples were left for 30 minutes in dark at room temperature. 46 µl of developmental buffer, 2 µl of development enzyme and 2 µl of peroxidase were added, in this order, to the samples, and left again for 30 minutes in the dark.

Measurements were taken by reading the absorbance at 570 nm of 200 ul of samples loaded on a transparent plate, through a Tecan Spark plate-reader.

Obtained fluorescence values were then normalized using a standard curve method: equation of standard curve was calculated, and the formula was exploited to calculate glycogen concentration based on absorbance measurements. Finally values obtained were normalized on the growth and expressed in $\mu g/OD_{750}$.

3.3 PHB levels monitoring

To monitor the trend of PHB, samples were first normalized to OD_{750} 0,2 using sterilized water to a volume of 400 µl. Then samples were treated with Nile Red (Sigma Aldritch) at final concentration of 0,5 mg/ml, and incubated in agitation at 600 rpm for 1 hour at 54°C. Then samples were duplicated in order to have Nile Red in half samples and the other half without as control. Blank was done with sterile water. Nile Red fluorescence was then measured by using a black multi-well plate and by reading the fluorescence through a Tecan Spark plate-reader, exciting samples at wavelength of 485 nm and detecting the fluorescence at wavelength of 580 nm.

3.4 Sequence alignments

sequences alignment was performed of nucleotides and amino acid sequences. Two nucleotides sequence of gene *GlgP1* (sll1356), one for the reference strain PCC 6803 and the other for B12 were compared. The same was done for the gene *GlgP2* (slr1367) and for the corresponding protein sequences of both genes. MultAalins open-source online tool was used (http://multalin.toulouse.inra.fr) (Corpet, 1988). Then was applied AlphaFold (Jumper et al. 2021) (www.Alphafold2.com) to predict 3D protein structure of GlgP2.

3.5 DNA extraction

To extract DNA of *Synechocystis* sp. B12, samples were treated following this protocol: 1 ml of liquid culture was centrifuged at 8000 g for 5 minutes, then supernatant was removed, and 100 µl of sterile water were added to the pellet. At this point, samples were treated at 98°C for 5 minutes and 1 minute in ice, for three times, then samples were centrifuged for 1 minute at 12000 g. At the end debris was removed and supernatant containing extracted DNA was conserved.

3.6 Gene amplification

To amplify gene *GlgP2* from *Synechocystis sp. B12,* it was made PCR reaction, and this protocol was followed:

Reagents protocol	
H_2O	12 µl
Phusion Buffer HF	4 µl
dNTPs	0,5 µl
Primer for	1 µl
Primer rev	1 µl
Taq Phusion HF	0,5 µl
Template (DNA)	50 ng
Total volume	20 µl

Thermal protocol	
98 °C	30''
98°C	10''
60°C	30''
72°C	4'
72°C	10'

To do PCR reactions these primers were used:

- GlgP2 B12 for

TAAGGAATTATAACCATATGGTGATGTCACCCCGACTTTTTC Ta=63°C

- GlgP2 B12 rev

AGACCTAGGCCTTAAGATCTTAAATCGGCGTATTCGGG Ta=63°C

- pAI glgP2 back for: ATTCCTCGAGCAGTAATACAAGGGGT Ta=65°C
- pAI glgP2 back rev: TGATAGATCTTAAATCGGCGTATTCGGGA Ta=65°C

BglII GFP for: TCATAGATCTATGGTGAGCAAGGGC Ta= 65°C

XhoI GFP rev: CTATCTCGAGTTACTTGTACAGCTCGT Ta= 65°C

- GlgP2_B12_nsc_fwd: TAAGGAATTATAACCATATGGTGATGTCACCCCGACTTTTTC Ta= 63°C
- GlgP2_B12_nsc_rev: AGACCTAGGCCTTAAGATCTTAAATCGGCGTATTCGGG Ta= 63°C

- psbAII_prom_for: CTCGTAACCCGGGCCCGCGGTCGCGACGTACGCGTATC Ta= 64°C
- psbAII_prom_rev: GTTATAATTCCTTATGTATTTGTCGATGTTCAG Ta= 64°C

3.7 Agarose gel

To visualize PCR products, an agarose gel was made as following: in a flask 50 ml of TAE 1X, it derived by a dilution of TAE 50X (Tab 2), and 0,5 g (to make gel at 1%) of agarose powder were added, then solution was heated in microwave oven and then 0,25 µl of SERVA DNA stain g were added. At the end of this procedure, gel was put into a support with wells and was left cooling. To visualize each PCR reaction on the agarose gel, it was run with high tension 100 V for 20 minutes. There was a stock of TAE 50X but for agarose gel it is needed TAE 1X.

Components TAE 50X	amount
Trizma base powder	242g
Acetic Acid	57,1 ml
EDTA 0,5 M	100ml
Deionized water	Until 1l

Table 2. Composition of TAE 50x. To prepare TAE 1x, 20 ml of TAE 50x was added to 980ml of deionized water.

3.8 Purification of DNA

To purify DNA, Gene JET PCR purification Kit (Thermo Scientific) was used, and this protocol was adopted: first, Binding Buffer in 1:1 ratio was added to samples, then they were mixed, and solution became yellow (optimal condition). Consequently, samples were transferred into a GeneJET purification column. They were centrifuged at 12000 g for 1 minute, and the flow-through was discarded. Thus, 700 µl of Wash buffer was added, it was repeated centrifugation, and the flow-through was discarded. Then GeneJET columns were centrifuged again empty, to remove completely wash buffer. At the end purification columns were transferred into Eppendorf tubes of 1,5 ml and 20 µl of sterile water were added at the centre of column. Finally, another centrifugation was done as previously reported, and then DNA was conserved. The amount of DNA in ng was determined using NanoDrop® ND-1000.

3.9 Digestion of Plasmid

Then digestion of plasmid, to obtain both backbones and fragments to be inserted in, was performed following the protocol described in Tab 3 as followed:

Digestion protocol	
NdeI	1 µl
XhoI	1µl
Buffer O	2µl
DNA fragments	60-200 ng
H_2O	up to 20 µl

Table 3. representation of digestion reaction of 2 DNA fragments, for digestion can be used, different restriction enzymes that cut DNA fragments in specific recognition sites. Plasmid pAI and gene *GlgP2* were digested with these restriction enzymes.

Then pAI expression plasmid for stable integration in the genome (Carmel et al., 2013), containing recombination site flanking psbAI gene and PsbAII strong promoter, was digested to obtain backbone.

Then subsequent step was to insert gene of interest in this case GlgP2 into expression plasmid pAI-psBAII, fragments were cut with two restriction enzymes to avoid pairing of sticky ends, so this way literally digests DNA.

Digestion protocol	
NdeI	1 µl
BglII	1 µl
Buffer O	2 µl
DNA fragments	60-200 ng
H_2O	up to 20 µl

Table 4. Digestion protocol for pAI-psbAII and *GlgP2,* they were digested with restriction enzymes NdeI and BglII.

Then samples were treated with alkaline Antarctic phosphatase to dephosphorylate plasmid to avoid closing on itself, using this protocol: in an Eppendorf tube was added, in the order, 60-20000 ng of digested DNA, 2 µl of buffer APR, and 1 µl of alkaline Antarctic phosphatase.

3.10 DNA ligation

This protocol was followed to carry out ligase reaction:

Components	amount
Vector DNA	100 ng
Insert DNA	20 ng in 1:3 molar ratio
Ligase Buffer 10X	1 µl
Ligase T4	1 unit = 0,2 µl
Sterile water	Up to 10 µl

Table 5. Description of general protocol to do ligation of two DNA fragments.

Then samples were incubated at 16°C overnight.

3.11 Transformation of E. coli DH5α

After ligation, the product was transformed in *E. coli* DH5α cells. 10 ul of ligation mix were added to cells were and left in ice for 30 minutes, then were put in thermoblock at 42°C for 30 seconds and at the end again 2 minutes in ice. Then, was added 800 µl of LB medium, and cells were incubated at 37°C in agitation at 180 rpm for 1 hour.

When Petri dishes with solid LB together with 100 µg/ml ampicillin were seeded, cells were spread on them and incubated overnight at 37°C. The day after it was done screening of colonies to find what colonies presented plasmid with insert.

components	Concentration g/L
Yeast extract	5
Tryptone	10
NaCl	10

Table 6. Composition of LB liquid medium to grow *E. coli* DH5α. To make solid medium was added agar 1%. (w/v).

3.12 Screening of bacterial colonies by PCR

The day after transformation, bacterial colonies appeared on the selective plates, they were deposited in Eppendorf tubes with 50µl of sterile water and 6µl was taken. Then they were screened by PCR, in order to select the ones that correctly inserted the gene of interest

Reagents protocol	
H_2O	3 µl
Orange Taq	5 µl
Primer for	0,5 µl
Primer rev	0,5 µl
template	6 µl
total	20 µl

Thermal protocol	
95°C	7 minutes
95°C	30 seconds
49 °C	30seconds
72°C	4 minutes
72°C	5 minutes

Table 7. Reagents protocol and thermal protocol used to do PCR, in thermal protocol the passages of 95°C 30s, 49°C 30s, and 72°C 4 minutes were repeated for 35 cycles. Template indicates colonies picked from petri dishes and suspended in 50µl of sterile water.

To make PCR these primers were used:

- psbAII_prom_seq_for: CATCGACAAATACATAAGGA Ta: 49°C
- Km_seq_rev: TCCCATACAATCGATAGATT Ta: 49°C

- pJET_back_for: ACTTGTGCCTGAACACCATATCCA Ta: 55°C
- pJET_back_rev: ACTTGCAAAGCAAAAAATGGATGCT Ta: 55°C

3.13 Miniprep

To perform miniprep, Gene JET plasmid miniprep Kit (Thermoscientific) was used. This protocol was followed: at the beginning cells were centrifuged and supernatant was removed, then cells were resuspended in 250 µl of Resuspension Solution, subsequently 250 µl of Lysis Solution was added, and samples were mixed by inverting until solution became viscous. Then 350 µl of Neutralization solution was added and also in this case samples were mixed by inverting 6 times. Then, cells were centrifuged at 12000 g for

5minutes. At this point, supernatant was transferred into a GeneJET spin column. Samples were centrifuged for 1 minute at 12000 g, and flow-through was discarded. Thus, 500 µl of Wash solution was added, samples were centrifuged for 1 minute, and flow-through was discarded; this step was repeated twice. Then empty column was centrifuged another time to remove residual Wash solution. Consequently, GeneJET spin column was transferred into Eppendorf tube and 20 µl of sterile water were added at the centre of the column, it was incubated at room temperature for 2 minutes, and then, column was centrifuged last time for 2 minutes. At the end column was discarded and DNA was conserved at -20°C.

As it had been seen previously, DNA was quantified in concentration ng/µl, using Nanodrop.

3.14 Gibson Assembly

Another strategy for cloning, which do not involve restriction enzymes and ligations is represented by Gibson Assembly (Gibson D et al. 2009). this was applied in this context to clone fragments in complicated backbones in which too many restriction sites where present.

DNA of pJET modified backbone and psbAII promoter were before amplified (paragraph 3.6) with the following primers:

- pJET_MCSmod_for(for cloning plasmid pJET)
  AATACATAAGGAATTATAACCATATGATCAGATCTTAAGGCCTAG
  GTCTAGACGTCGTACG Ta= 72°C
- pJET_MCSmod_rev (for cloning plasmid pJET)
  CCGCGGGCCCGGGTTACG Ta= 72°C
- psbAII_prom_for:
  CTCGTAACCCGGGCCCGCGGTCGCGACGTACGCGTATC
  Ta=64°C
- psbAII_prom_rev:
  GTTATAATTCCTTATGTATTTGTCGATGTTCAG
  Ta=64°C

Then Gibson Assembly reaction was carried out as following:

Reagents	amount
PCR fragments+ linearized vector in molar ratio 1:3	0,02 pmol of vector and 0,006 pmol of insert
Gibson Assembly Master mix 10X	10 µl
Sterile Water	up to 20 µl

Table 8. This tab describes reagents needed to do GA (Gibson Assembly)

Consequently, samples were incubated at 50°C for 15 minutes, then chemically competent cells were used (as seen in 3.9 paragraph). Products of reaction were added directly to chemically competent cells (paragraph 3.9), samples were mixed gently by pipetting up and down.

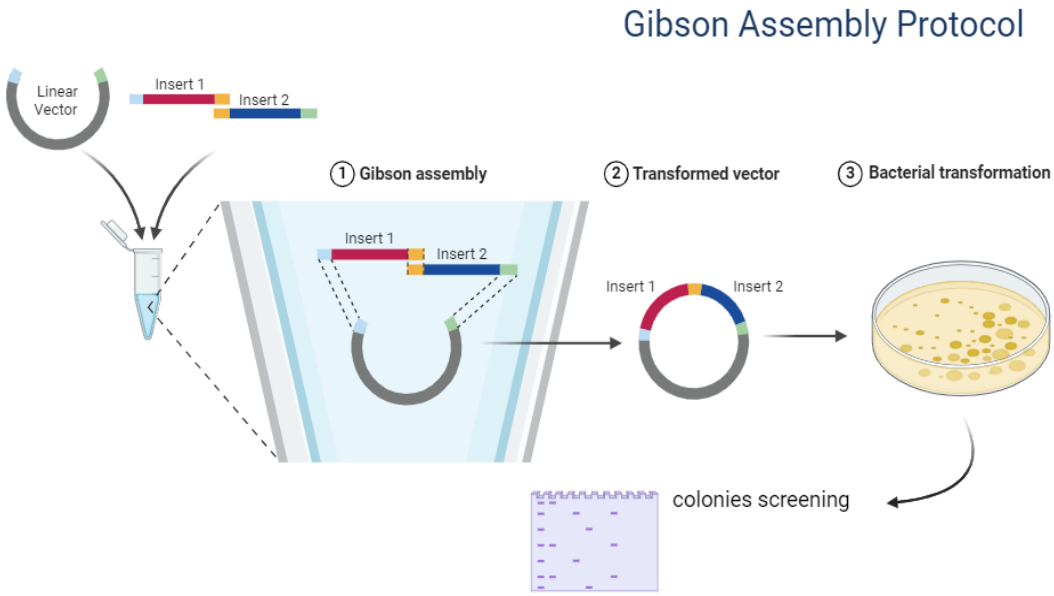


Figure 9. Schematic representation of Gibson Assembly reaction sometimes inserts are treated with restriction enzymes to avoid the pairing on themselves.

Colony screening was made and, Orange Taq protocol was used (paragraph 3.12), length of fragments expected were for backbone 3385 bp, for gene 2265 bp, and these primers were used:

3.15 *Synechocystis* sp. Transformations

Principal approaches adopted to transform *Synechocystis sp.* B12 were:

- Bacterial conjugation
- Stable recombination in the genome

Through conjugation, expression plasmid is generally inserted into cyanobacteria but not integrated into bacteria genome but it is necessary to have gene of antibiotic resistance to select cells that have plasmid, by this way it avoids damage against key regions of genome (Zurbriggen, 2022). Instead, to achieve stable recombination in the genome, two ways were tried: natural transformation and electroporation.

3.15.1 Natural transformation

For natural transformation (Pope et al.,2020), this protocol was followed: first, *Synechocystis* sp. B12 cells were grown in normal BG11 medium to reach OD_{750} of 1. Then 50 ml of cells were harvested and centrifuged at 2500 g, 30°C for 10 minutes, then washed using 5 ml sterile water to remove salts traces. Centrifugation was repeated and supernatant was discarded. At the end of this step, cells were resuspended in 1 ml of BG11 medium, and 5 µg of plasmid were added. Then cells were incubated in agitation, dark, at 180 rpm, at 35° for 6 hr. At this point 50 µl of cells were spread on a 0,45 mm nitrocellulose filter membrane, and placed onto a normal BG11 agar plate for recovery phase. Thus, plates were covered with three layers of paper towels, and incubate them under constant illumination at 100 µmol photons m^{-2} s^{-1}, at 30°C, for two days. After that, membranes were transferred onto normal BG11 agar plate containing kanamycin, at concentration of 25 µg/mL. Then, plates were covered again with three layers of paper towels, and they were incubated under light illumination at 100 µmol photons m^{-2} s^{-1}, at 30°C, until colonies appear. At the end, colonies were re-streak onto normal BG11 agar plate containing kanamycin at concentration of 10 µg/mL, and this step is needed until complete gene segregation is achieved.

3.15.2. Electroporation

For electroporation, this protocol was adopted (Zurbriggen, 2022): *Synechocystis sp. B12* was grown at 25°C under continuous light to reach OD_{750} around 0,5. So cells were harvested by centrifugation at 4000 g, for 10 minutes. Then cells were washed three times with 10 ml of sorbitol and mannitol 0,4M, thus cells were resuspended in 1 ml of HEPES at concentration of 1mM. After that, 60 µl of suspension were mixed with 1 µg of DNA, digested and purified with NaAc 3M. So, 1 ml of cells of *Synechocystis sp.* were inserted in special cuvettes and put into Electro Cell Manipulator® ECM 630. They were electroporated at a capacitor of 25 µF, the resistor used was 400 Ω, with an electric field of 12000 V/cm. Time constant required was 9 ms. Quickly, after electric pulse, cells were resuspended in 1 ml of BG11 and mixed with 10 ml of fresh BG11 medium in falcon tubes of 15 ml. At the end of this step, cells were incubated for 24 hr at 25°, under continuous low light (20 µmol photons m^{-2} s^{-1}). Then cells were harvested by centrifugation at 4000 g, for 10 minutes, and resuspended in 500 µl of BG11 medium. At the end cells were spread onto Immobilon-NC membranes (0,45µm pore size, 82mm, Millipore), resting on solid BG11 plates, supplemented with kanamycin at concentration of 10 µg/ml, at 25°C under light 100 µmol photons m^{-2} s^{-1}.

3.16 *Synechocystis* colonies screening

To select mutants, *Synechocystis sp.* B12 and reference strain PCC 6803 were grown in a selective solid cultivation medium with kanamycin as agent of selection. Then grown colonies were deposited in Eppendorf tubes in water and then they were heated at 98°C for 5 minutes and after conserved in ice for 5 minutes, these two passage were repeated for three times and at the end conserved at -20°C. A screening PCR was performed to check promoter+gene+resistance, with a length of 5373 bp.

At this point DNA of *Synechocystis sp.* is extracted as protocol previously seen (paragraph 3.5), then was done PCR with screening protocol (paragraph 3.12) the length of fragment was 2565 bp, and these primers were used:

- psbAI up seq for: AAAATCAAATCCAAGCTTAA Ta: 58°C
- glgP2 seq rev: CTTCAATTGTGATAGTGGGT Ta: 58°C

Then also *rps* primers were used to verify presence or absence of DNA:

- RPS for: AGAATTGAAGGACTGCGGGG Ta: 55°C
- RPS rev: ATTGAAGACGCTGTGGGGAG Ta: 55°C

4 Results

It is useful to find new stress sources or new feedstock nutrient and to observe variation and increase yield of bioplastic production, to pursue the mission of sustainability, to face the problem of conventional plastic and its dangerous bond with fossil fuels, global warming and greenhouse effect. Investigations have to be done, to find better conditions for cells proliferation and maximization of PHB content. Replicates for each growth curve were prepared to measure OD_{750}, PHB content and glycogen content. Then sequence alignments were performed to understand better target gene and related protein. So, molecular experiments were done to prepare plasmids for manipulation of glycogen metabolism. it is appropriate to reflect on the importance of sustainability because, the possibility to live in a better world starts by actions of every one of us, if our actions are in equilibrium with environment, we made our duty and we can live better but if our actions are oriented to damage and to exploit wild we were in disequilibrium leading huge damage to our own home.

4.1 Nitrogen starvation

Figure 12 shows growth trends both for curves grown in nitrogen starvation and in normal BG11 medium, where OD_{750} was measured over days. It is possible to see that there were almost no variations of OD_{750} between N starvation and normal medium (Fig 12).

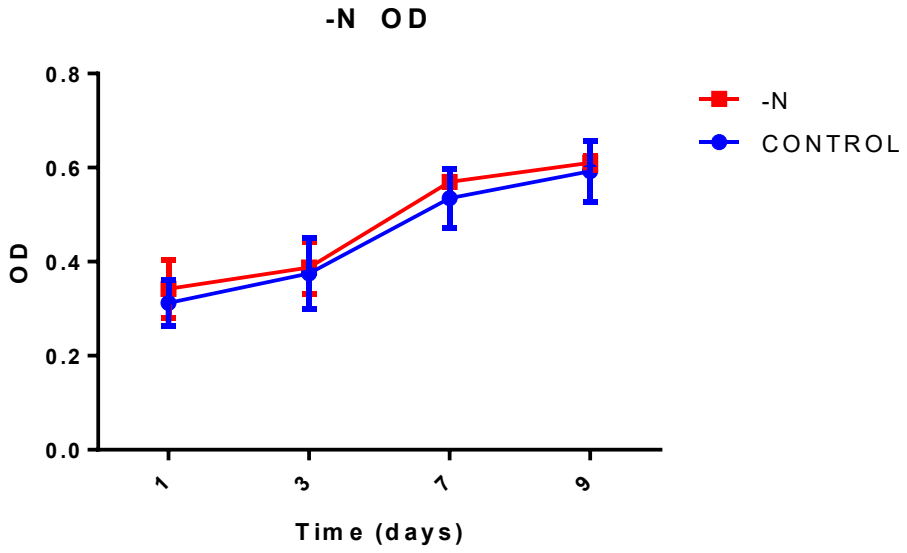


Figure 10. In x axis time in days is indicated instead in y axis OD_{750} is indicated, different colours describe curve in N starvation shown in red colour and control curve without stress in blue colour. Asterisk * indicates $p<0.05$, ** for $p<0.01$, *** for $p<0.001$ and **** for $p<0.0001$.

Source of variation	P value	Significancy	Degree of freedom
Interaction	0,974	No significant	3
Time	<0,001	*** (Yes)	3
Nitrate	0,2252	No significant	1

Table 9. Statistical analysis of Fig. 10 where *p*-value, significancy and degree of freedom are shown for time and nitrate availability.

Then glycogen content was measured through days, both in control curves and N starvation. Glycogen content reached its maximum at day 7 and then it started to decrease. Cells cultures in Nitrogen starvation demonstrate a glycogen content higher than cultures growth in normal medium BG11. Quantification of glycogen was expressed in µg/OD, and it was done with standard curve method. Higher values of glycogen were recorded in curve without Nitrate so N deprivation could increase glycogen content.

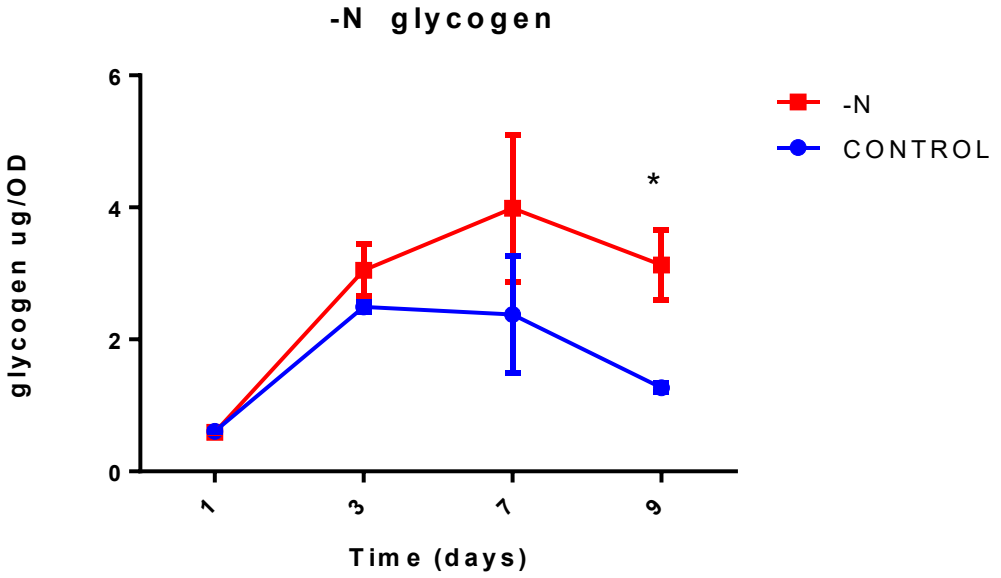


Fig 11. In x axis times in days is shown instead in y axis concentration of glycogen measured in µg/OD is shown. Red curve means cultures in N starvation instead blue curve is control. Asterisk * indicates p<0.05, ** for p<0.01, *** for p<0.001 and **** for p<0.0001.

Source of variation	P value	Significancy	Degree of freedom
Interaction	0,1310	No significancy	3
Time	0,0008	*** (Yes)	3
Nitrate	0,0069	** (Yes)	1

Tab 10. Statistical analysis of Fig. 11 *p*-value, significancy and degree of freedom are reported stars highlight intensity of significancy of results.

Tab 10 shows differences in Glycogen content were determined both by time and depletion of nitrate, because both *p*-value were significative to

determine glycogen content, as it is possible to see time impact heavily than nitrate, on glycogen synthesis.

Diagram of PHB content pointed out a slight increase even if not significant in PHB production in Nitrogen starvation than non-stress conditions (Fig 14).

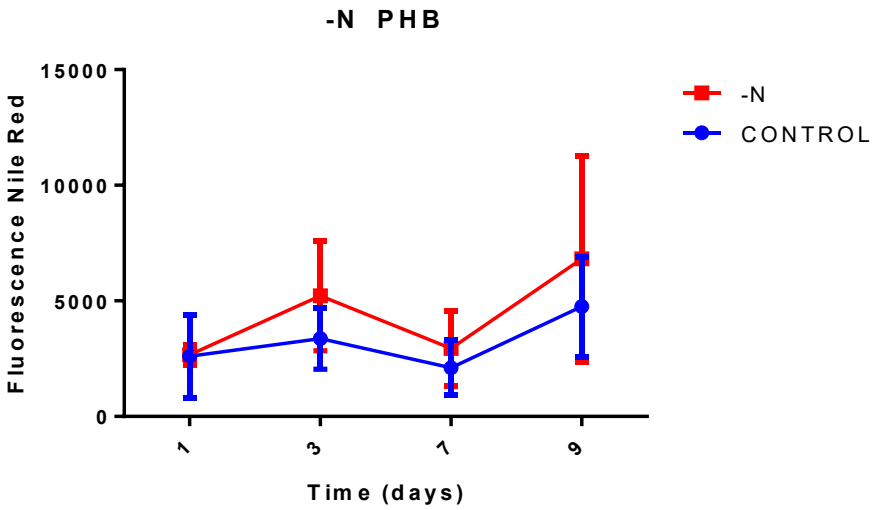


Fig 12. X axis indicates time of curve in days, y axis indicates Fluorescence measured with Nile Red normalizing sample ant OD 0,2. Red line indicates N starvation and blue line indicates control. Asterisk * indicates p<0.05, ** for p<0.01, *** for p<0.001 and **** for p<0.0001

Source of variation	P value	Significancy	Degree of freedom
Interaction	0,8071	No significant	3
Time	0,0370	* (Yes)	3
Nitrate	0,1746	No significant	1

Tab 11. Statistical analysis of Fig 12 *p*-value, significancy and degree of freedom are indicated. Asterisk * indicates p<0.05, ** for p<0.01, *** for p<0.001 and **** for p<0.0001.

Then photos were taken at confocal microscopy, there was slight difference between control and N starved cells, as previously observed from PHB measurements (fig 15.).

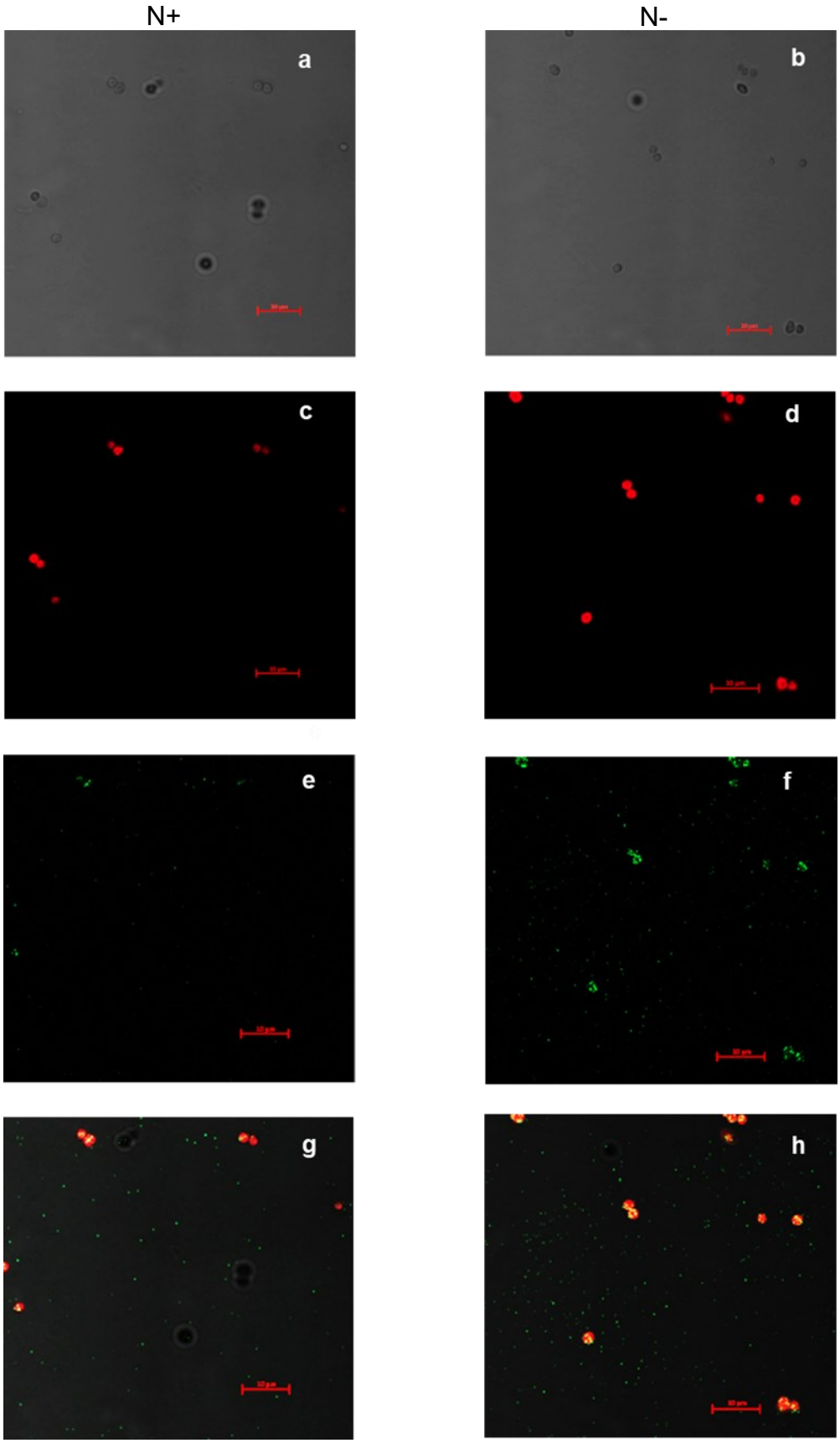


Fig 13. In this series of images are indicated control condition N+ and N- for Nstarvation, letters a, b report Bright field, c,d mean chlorophyll, e,f indicate Nile Red fluorescence of PHB granules and g,h indicate merged between

chlorophyll and Nile Red. Letters BF indicate bright field, Ch indicate chlorophyll, NR means Nile Red and Mg means Merged.

4.2 Phosphorus starvation

Experiments carried out reveal that unexpectedly cells growth was higher in Phosphorus starvation than in control culture. It means that cells seem to manage efficiently this stress factor, and they seem to be able to grow efficiently. Then several generations are needed to accuse starvation.

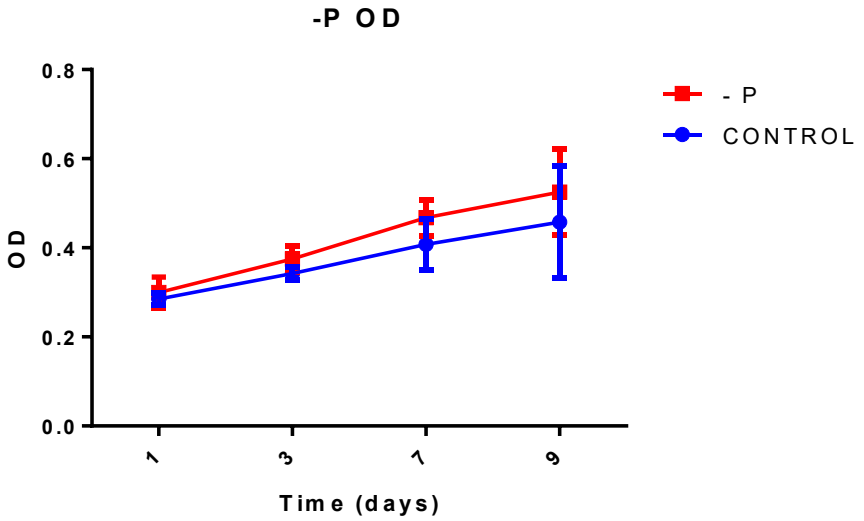


Fig 14. In axes OD and time are disposed, red line indicate curve in P starvation and blue line means control curve OD grow constantly during day as indication of good growth of cells. Asterisk * indicates p<0.05, ** for p<0.01, *** for p<0.001 and **** for p<0.0001.

Source of variation	P value	Significancy	Degree of freedom
Interaction	0,8307	No significant	3
Time	<0,0001	*** (Yes)	3
Phosphate	0,0642	No significant	1

Tab 12. Statistical analysis of Fig 14 *p*-value, significancy and degree of freedom are indicated, asterisks indicate the grade of significancy, many asterisks mean higher significancy.

Glycogen levels have a different trend because control cultures show a slight decrease in day 3, instead glycogen in cell with P starvation remain almost stationary in day 3. But then glycogen levels start increment and reach maximum levels at the end of measurement in day 9 (Fig. 17).

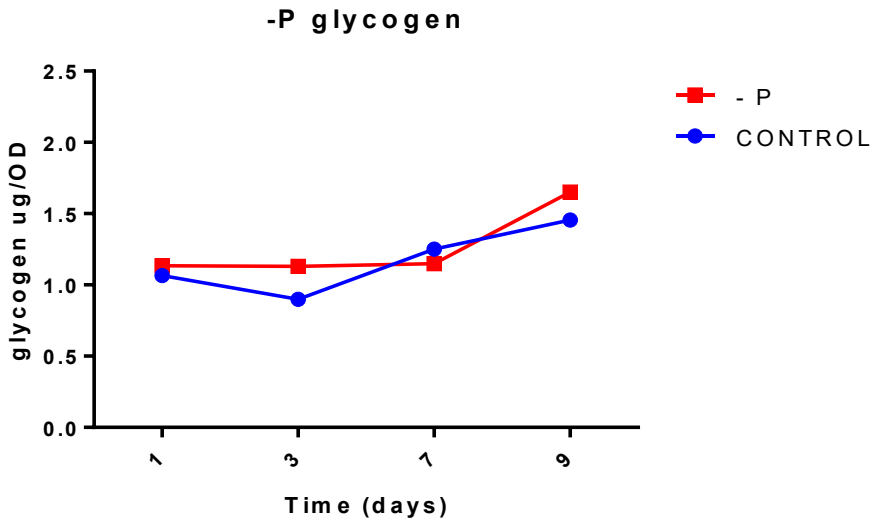


Fig 15. In this graph concentration of glycogen and time in days are reported, respectively in y axis and x axis, red line indicates glycogen content of curve under P starvation while blue line shows glycogen content of control curve. Asterisk * Indicates p<0.05, ** for p<0.01, *** for p<0.001 and **** for p<0.0001

Source of variation	P value	Significancy	Degree of freedom
Interaction	0,7239	No significant	3
Time	0,0392	*(Yes)	3
Phosphate	0,4003	No significant	1

Tab 13. Statistical analysis of Fig 15 here *p*-value, significancy and degree of freedom are shown, it is reported that time had a weak effect on variation of glycogen content.

Regarding at PHB diagram Fig 18, during phosphorous starvation cell shown globally an increment in Nile Red fluorescence, and consequently in PHB content, for all the duration of curve compared with control curve (Fig 18). PHB content remains almost stationary form day 3 to day 7 and in the sequent measurement it continues to increase. This means that P stress impact heavily in PHB accumulation. In control cultures, Nile Red fluorescence remains much lower than fluorescence recorded under stress level.

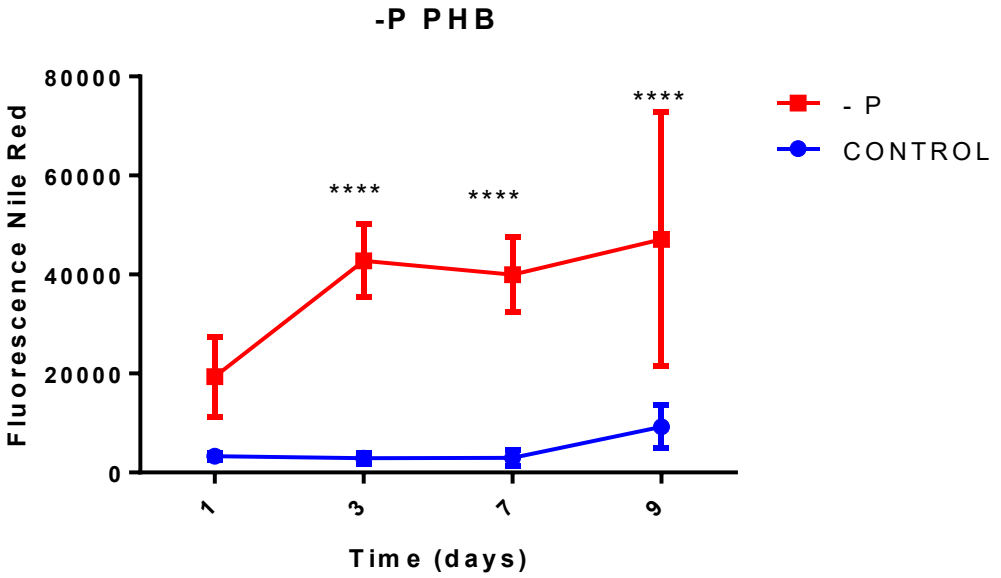


Fig 16. In this graph line red represent PHB trend during P starvation, blue line represents PHB trend during days in control curve, asterisks point out the significancy of high values in PHB accumulation, and fluorescence maintains high in P starvation curve number of asterisks indicate results of two-way ANOVA and indicate comparisons with means of results for control and starved curve. Asterisk * indicates p<0.05, ** for p<0.01, *** for p<0.001 and **** for p<0.0001

Source of variation	P value	Significancy	Degree of freedom
Interaction	0,1015	No significant	3
Time	0,0256	*(Yes)	3
Phosphate	<0,0001	*** (Yes)	1

Tab 14. Statistical analysis of Fig 16 in this table *p*-value, significancy and degree of freedom are pointed out, asterisks denote grade of significancy of results.

Table here reported confirmed that lack of Phosphate has a strong role in increase PHB production, without impacting heavily in growth of the cells, if graph 4 was considered. Because this table compared with table of OD_{750} underline that there are cells growth is not impacted and cells grow in constant way.

These following Figure 19 shown in next table, were realized with confocal microscopy, express some quantitative and qualitative aspects about PHB storage. In Phosphorus starvation cells accumulate high content of PHB stored in few big granules.

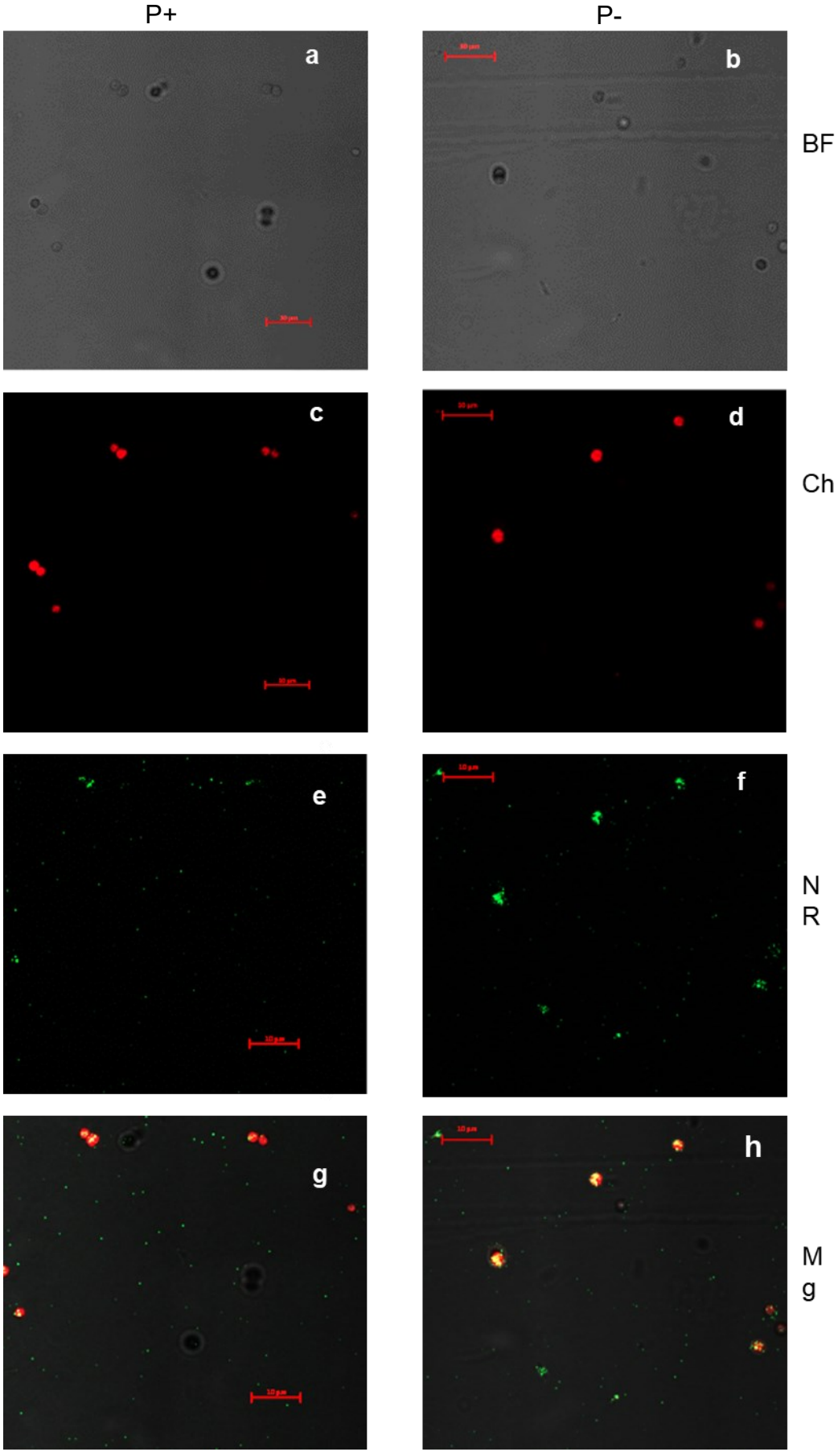

P+
P-
a
b
BF
c
d
Ch
e
f
N
R
g
h
M
g

Fig 17. Images taken at confocal microscopy are reported, letters a, b means bright field, letters c, d means chlorophyll enhanced in red, letters e, f indicate PHB granules in green, letters g,h indicate merged image between chlorophyll and PHB in these image PHB granules are visible in yellow colour. Letters BF indicate bright field, Ch means chlorophyll, NR point out Nile Red and Mg signify merged.

4.3 Acclimation to Phosphorous starvation

Another important consideration is how change PHB content in a prolonged period of P starvation. Acclimation was tried because previous results are different each other. So, cells were acclimated in phosphorus starvation for five days, and after measurement were started.

Fig 18 shows that cells cultured in P starvation appeared to grow a little bit better than control. This could mean that cells were adapted during acclimation.

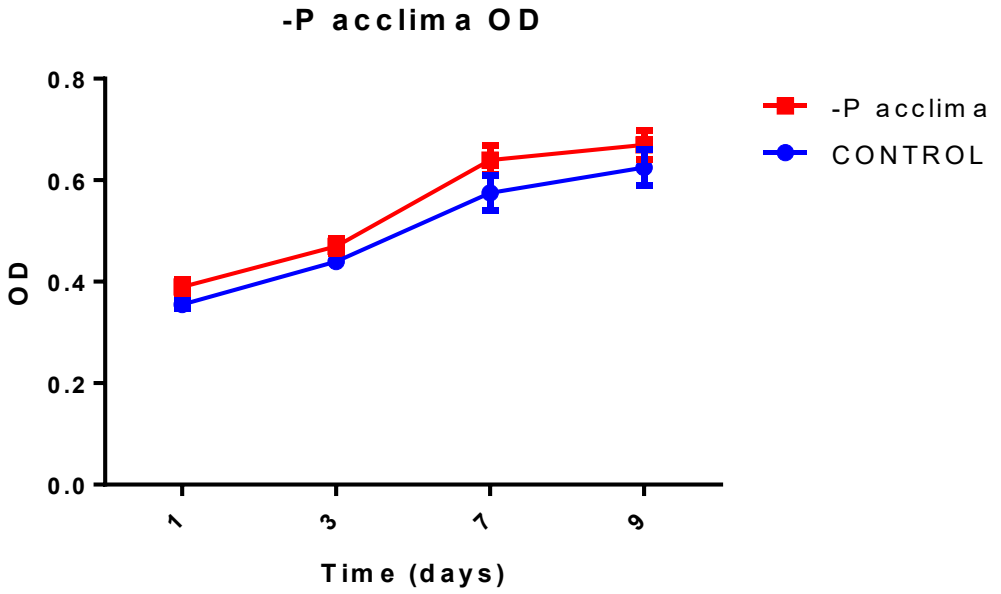


Fig 18. In this graph variations of OD_{750} are reported during curve conducted in acclimation of P starvation, red line indicates growth rate measured in variation of OD_{750}, blue line means control curve and time is shown in days in x axis. Asterisk * indicates $p<0.05$, ** for $p<0.01$, *** for $p<0.001$ and **** for $p<0.0001$

Source of variation	P value	Significancy	Degree of freedom
Interaction	0,7429	No significant	3
Time	<0,0001	*** (Yes)	3
Phosphate acclimation	0,0063	** (Yes)	1

Tab 15. Statistical analysis of Fig 18 here *p*-value, significancy, and degree of freedom are pointed out, asterisks identify strength of significance.

This table report, as expected, that time had great significancy in explanation of results, instead lacking phosphate in medium was less significant for an increase of OD. However, both factors have significancy in variation of OD

Measurement of PHB content demonstrate that, also, in acclimation without phosphorus, strongly increase between day 1 and day 3, but it remains almost stationary, for all the duration of growth curve. It is possible to say that PHB content is sensitive higher than control curve.

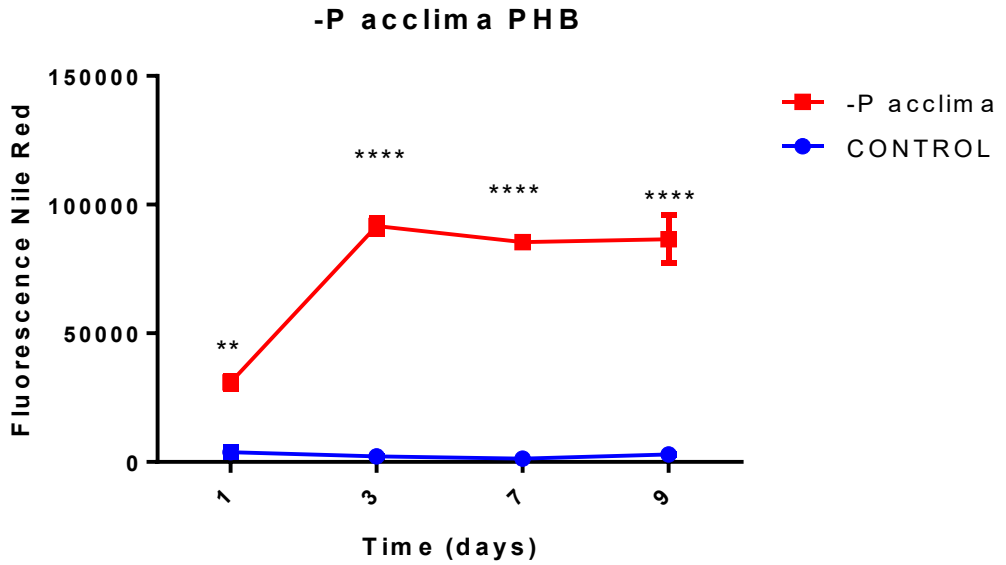


Fig 19. In this diagram x axis indicates time spent in days and y axis indicates Nile Red fluorescence, with red line condition of acclimation in P starvation is indicated while in blue line fluorescence of control is reported. Asterisks * indicates p<0.05, ** for p<0.01, *** for p<0.001 and **** for p<0.0001.

Source of variation	P value	Significancy	Degree of freedom
Interaction	<0,0001	*** (Yes)	3
Time	<0,0001	*** (Yes)	3
Phosphate	<0,0001	*** (Yes)	1

Tab 16. Statistical analysis of Fig 19 here it is reported *p*-value, significancy and degree of freedom, asterisks indicate high significancy of results.

So, Fig 22 and tab 15, showed that there was a strong interaction between stress factor, and time spent. Both variables (Time) and lack of Phosphorus impact strongly in PHB accumulation. In this experiment cells were acclimated for 5 days before starting the growth curve. It is possible to see

on the graph that cells cultured and acclimated in P starvation have a sensitive higher PHB content than cells cultured in normal BG11 medium. And statistics highlighted and confirmed that Time, P starvation and interaction between these two factors is highly significative.

4.4 Combined stress

Experiments carried out combining two main stress factor Nitrogen and Phosphorous starvation, were useful to increase the stress of cells, and their possibility to produce PHB. This diagram shows how OD_{750} varied during curves: as expected cells under stress had a lower OD_{750} than control cultures, so it suggested that cells have some difficulties to grow under these two-stress factors combined.

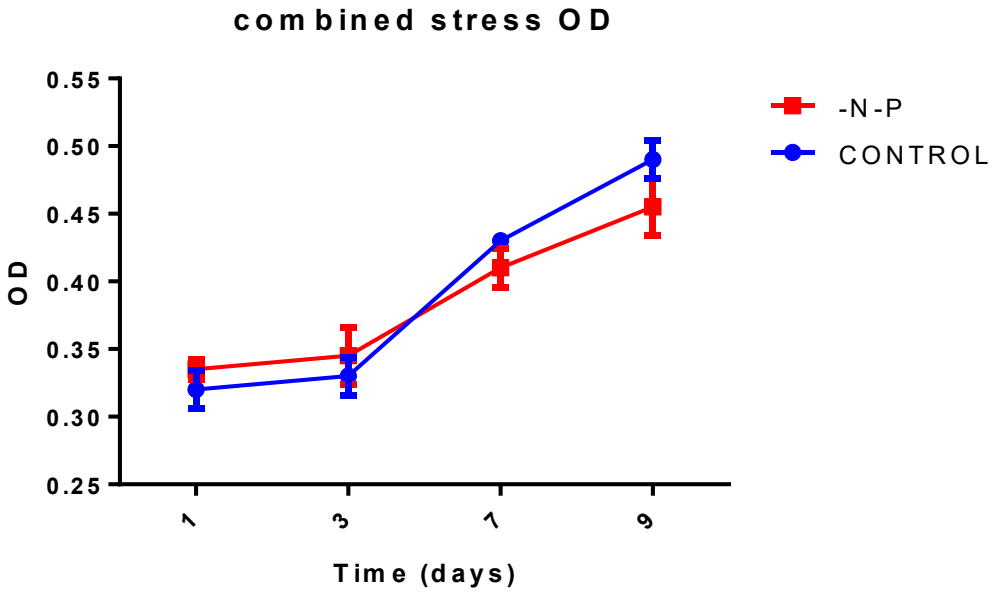


Fig 20. In this figure variation of OD for curve under combined stress in red line and control curve in blue line is shown, OD_{750} is reported on y axis, while Time in days is reported in x axis. Asterisk * indicates $p<0.05$, ** for $p<0.01$, *** for $p<0.001$ and **** for $p<0.0001$ if not indicated results are not significative.

Source of variation	P value	Significancy	Degree of freedom
Interaction	0,10	No significant	3
Time	<0,0001	*** (Yes)	3
Nitrate & Phosphate	0,4226	No significant	1

Tab17. Statistical analysis of Fig 20 this table reports *p*-value, significancy and degree of freedom asterisks indicate strength of significance.

Table 17, point out that there are not significancy on stress factor in variation of OD_{750}, but as expected, it indicates that time spent has a significant

impact on OD_{750} variation. By this way it is possible to sustain that nutrient starvation decreases weakly cells growth, but main agent in variation of OD_{750} is time (in days).

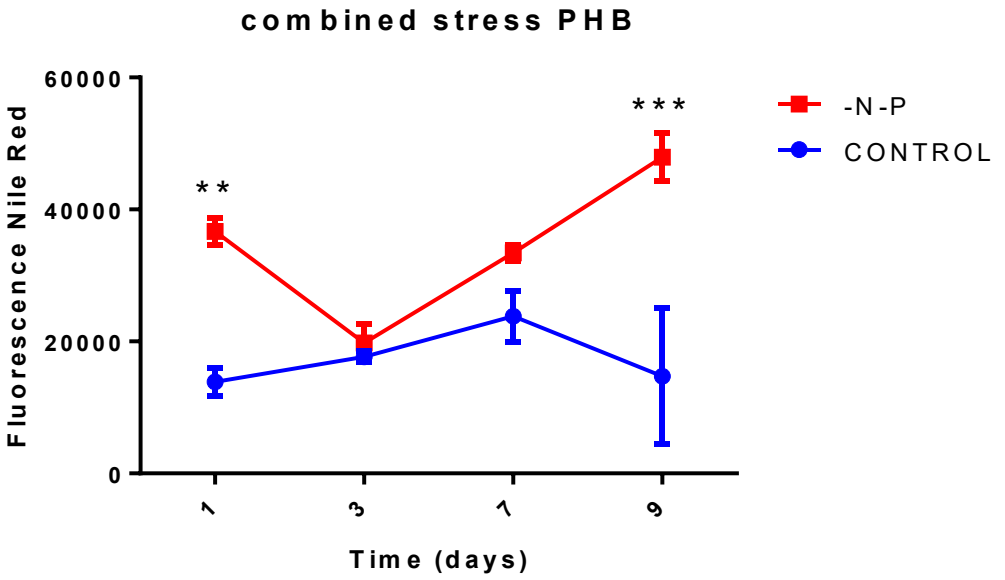


Fig 21. This graph shows trend in PHB accumulation in control curve (blue line) and combined starvation in red line, time in days is represented in x axis and Nile Red fluorescence is represented in y axis. Asterisk * indicates p<0.05, ** for p<0.01, *** for p<0.001 and **** for p<0.0001 if not indicated results are not significative. Low value in day 3 indicates a measurement error.

Source of variation	P value	Significancy	Degree of freedom
Interaction	0,0045	** (Yes)	3
Time	0,0177	*(Yes)	3
Nitrate & Phosphate	<0,0001	*** (Yes)	1

Tab18. Statistical analysis of Fig 21 in this table *p*-value, significancy and degree of freedom are reported number of asterisks define significancy of results.

Fig 24 shows a different behaviour in stressed cells than control. In control curve PHB content was quite constant. Instead, cells cultured in combined stress show at first a decrease of PHB content, but then PHB content increases constantly. Measure took in day 3 were so low and it could mean an error. However, there are interaction between time spent and stress factor. Yet as reported in previous table, *p*-value indicate that stress factor, is more significant than time spent, in PHB accumulation. In combined

stress it was not possible measure glycogen content because of occurring technical issues.

4.5 Sequences alignments

Sequence alignments were performed for nucleotides and then for amino acids from two genes GlgP1 sll1356 and GlgP2 slr1367. Alignment of sequences reference strain PCC6803 and B12. Gene GlgP2 is reported to have a key role in glycogen metabolism (Fu and Xu, 2006).

Nucleotides sequences of *GlgP2* were:

>srl1367_Synechocystsis_sp_PCC6803

gtgatgtcaccccgacttttttcatcaggaacaatcaatgattgatcaatctaccctaaacacttcccctcaac
ccactatcacaattgaagatgaccgcaccggcatgagtgtggccactttgaagcgggccttttttggataaccct
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>srl1367_Synechocystsis_sp_ B12

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cgccgatttatag

Alignment showed few differences, sequences of *Synechocystis sp.* PCC6803 and B12 was compared. The Percentage of similarity was 99,6% there were only 11 mismatches all of them involve single nucleotides there were no insertion or deletions.

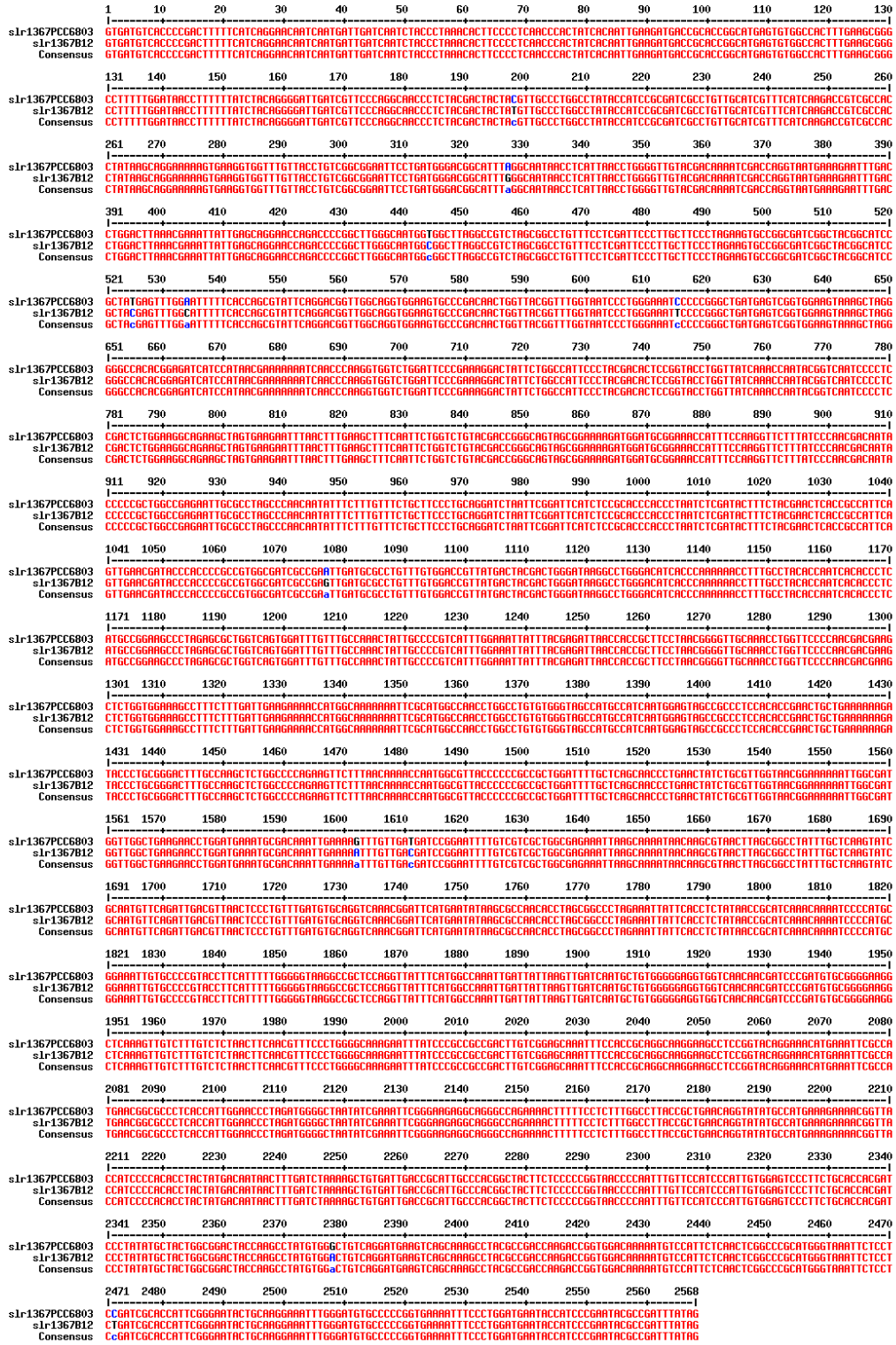

Fig 22. Image of nucleotide sequences alignment of GlgP2. There were only few substitutions.

Amino acid sequence of srl1367, for amino acid sequence a single letter code has been used.

>aa slr1357 PCC6803

MMSPRLFHQEQSMIDQSTLNTSPQPTITIEDDRTGMSVATLKRAFLDNLFYLQGIDRSQATLYDYYVALAYTIRDRLLHRFI
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IHLRTHPNLDTFYELTAIQLNDTHPAVAIAELMRLFVDRYDYDWDKAWDITQKTFAYTNHTLMPEALERWSVDLFAKLLPRH
LEIIYEINHRFLTGLQTWFPNDEALVESLSLIEENHGKKIRMANLACVGSHAINGVAALHTELLKKDTLRDFAKLWPQKFFN
KTNGVTPRRWILLSNPELSALVTEKIGDGWLKNLDEMRQIEKFVDDPEFCRRWREIKQNNKRNLAAYLLKYRNVQIDVNSLF
DVQVKRIHEYKRQHLAALEIIHLYNRIKQNPHAEIVPRTFIFGGKAAPGYFMAKLIIKLINAVGEVVNNDPDVRGRLKVVFV
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KFSSDRTIREYCKEIWDVPPVKISLDEYHPEYADL

>aa srl1367 B12

MSPRLFHQEQSMIDQSTLNTSPQPTITIEDDRTGMSVATLKRAFLDNLFYLQGIDRSQATLYDYYVALAYTIRDRLLHRFIK
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HLRTHPNLDTFYELTAIQLNDTHPAVAIAELMRLFVDRYDYDWDKAWDITQKTFAYTNHTLMPEALERWSVDLFAKLLPRHL
EIIYEINHRFLTGLQTWFPNDEALVESLSLIEENHGKKIRMANLACVGSHAINGVAALHTELLKKDTLRDFAKLWPQKFFNK
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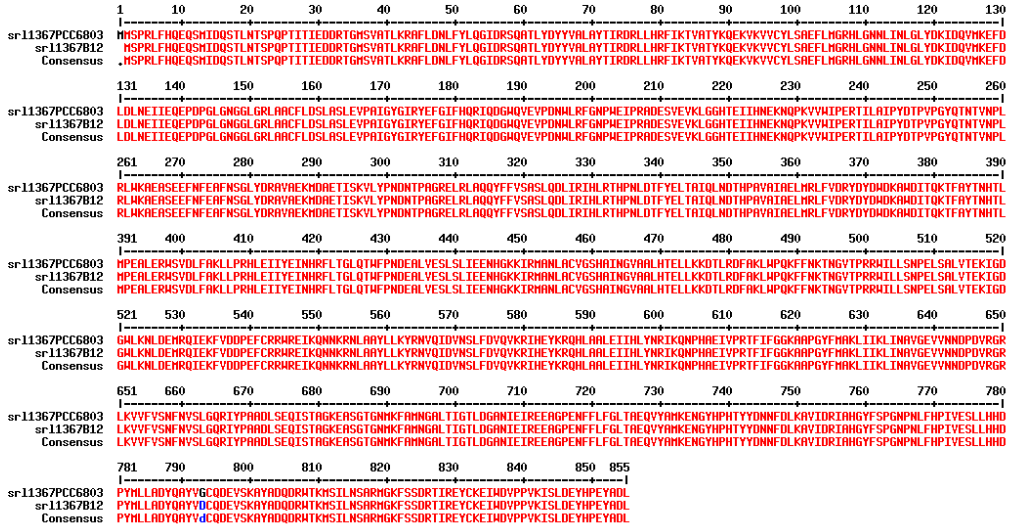

Fig 23. This figure shows alignment of amino acids sequence only one difference is detected between reference strain and B12

Two sequences of *GlgP2* were very similar between PCC6803 and B12, there is only one amino acid substitution, B12 has aspartic acid instead PCC6803 in the same position has a glycine. This substitution may have effect on intramolecular bonds or in fold of protein itself. However, even if nucleotides sequences have some changes alignment of amino acids show only one change so amino acids sequences are really conserved.

Then it was done a 3D structure prediction using AlphaFold (Jumper et al. 2021), for amino acids sequence of slr1367 of *Synechocystis* sp. B12. Model was done to highlight active sites, subunits, molecular features like hydrophobicity and disposition of chains in 3D space. So, AlphaFold prediction reported different colours blue colour of chains indicates high prediction score, instead terminal chains coloured in yellow, and orange have medium or low prediction score, this means that results with high score are confident with sequence modelized, instead low score means that results are not so confident with data analysed. Dominant pattern of these protein structure is α helix.

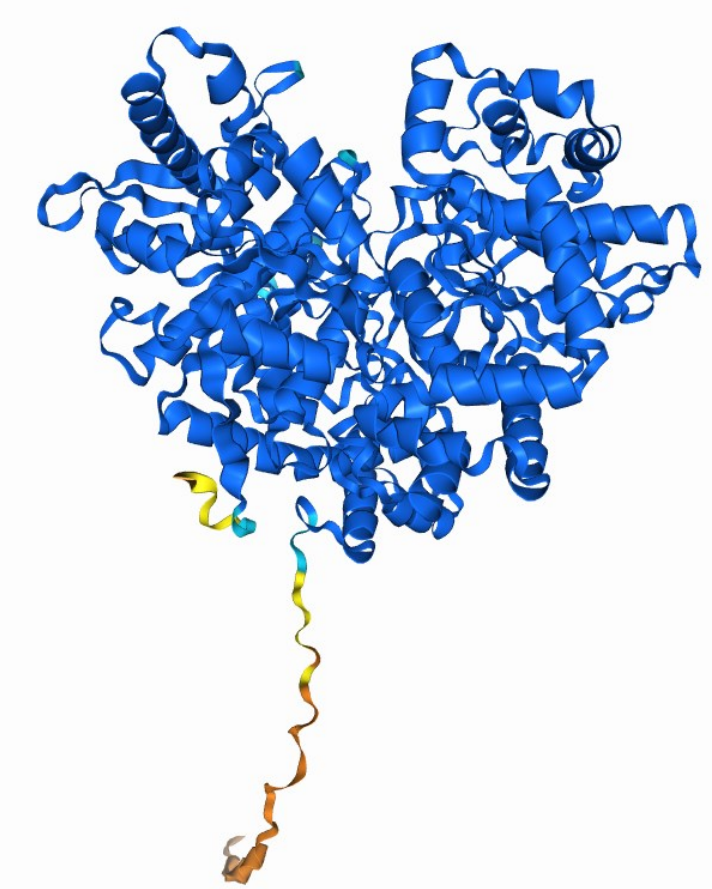

Figure 24. This figure reports 3D model of protein sequence of slr1367 that has a key role in glycogen metabolism. This 3D model was made using protein sequence of *Synechocystis sp.* B12 orange colour has low prediction score while blue colour has high prediction score.

Nucleotides sequences of sll1356 are:

>GelgP1_Synechocystsis_sp_PCC6803

atggagcaccttcccatggcttttaacaccaccaatcccctcattcaggtggaggatgaccgcactggtctga
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>GlgP1 Synechocystsis_sp_B12

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aataacctgattaatttagggctatgggagccggtgcaacaggcggtggaagaatcggggttgagcttggatg
agttaattgatattgaagaagaaccggggttgggcaacggtggtttaggtcgtttggcccgcctgctttatgg
attccctggctacgttggaaattccggcgatcggctacggcatccgctacgaattcggcatttttgaccagga
aattaaagatggttggcaggtggaaatcaccgataaatggctacaactgggcaatccttgggaaattgcccgc
cctgaatccgctgtgctagtcaaattgggggccatacggaatcctatcaagatgaaaagggcaattatcgag
tgcgctgggttcctggcaatttagttaagggcattccctatgacacccccattttgggctacaaagtcagcac
cgccaataatttacgcctgtggaaatcggaagcggcggaggattttgattttcaacggtttaacattggggac
tattacggagccgtgcagggccaaggtaacgtcggaaaatttcactaaggtgctttatcccaatgatgaacaa
ctccagggtaaggaactgaggctagcccaacagtacttttttgtttcctgttctttgcaggacatgatccgca
ttcattttgccgataatcctagtctggacaatttccacgaacactttgcggtgcaattaaacgatacccatcc
ttccattggcgtagcggagttgatgcgattgttggtggatgagtaccattacgagtgggaaaaagcctgggcc
atcacccatgccacctttggttacaccaatcacaccctcttaccagaagccctggaaaaatggtctttgcctc
tgtttgagcggatgttaccccgccatttggaaattatttacgaaatcaaccaacgtttcctagatcaagtacg
gatgaaatatcccaacgacggcgatcgcctggcccggttatcaatcattgacgaagcgggggaaaagtcggtg
cggatggcctatctggccacggtgggctcccatgcagtcaatggggtggcggcgttgcacagtcaattggtca
aagaaacaattctcaaagacttttacgaactctggccagaaaaatttagcaataaaaccaatggagtcacccc
ccgccgctggctggtgcgcagtaatccccgcctcagtgatttaattacttcccgcattggcgatggttggatt
aaaaacttggatgagttgaaacaattagaaccctttgctgatctagcgggttttcggcaggattggcagcaaa

tgaaacgggcagtgaaagaggagttggccgactacatcaagactgccaataatttaattgttaaccccgattc
cctgttcgatgtgcaggtgaaacgtattcatgaatataaacggcaacagttaaatattctccatgttattcat
ctttatttgcagttaaaaaataatcccaatttggatatgcctccccgtacttttatctacggaggcaaagcgg
cccccggttatttcctggccaaattaattattaaattaatcaactccgtggccgaggtagttaataatgaccc
cgccattggcgatcgcctgaaagtagtctttttgtccaactataacgttaaattagggcaacgggtttatcct
gccgctgatttatcagaacaaatttccactgcgggcaaagaggcttccggcaccggcaatatgaaattttcca
tgaacggagccttaaccattggtacgttggatggagccaatattgaaatccgagaggaggtgggggcagaaaa
ctttttcctctttggtctgaccacgccggaagtggagcaaaccttggctgagggttatcaaccctgggactac
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agctatttcgtcctttaatggattctttatttggccatgatccctatctcgtgtttgccgatttccaggccta
cgtggattgccaggttaaagtatcagcaacctataaagatcaggacaattgggcccgcatggccattctgaac
gtggcccgcatgggtaagttttcgtcggatcgtactattcgggaatacgccgaagacatctgggctatcaaac
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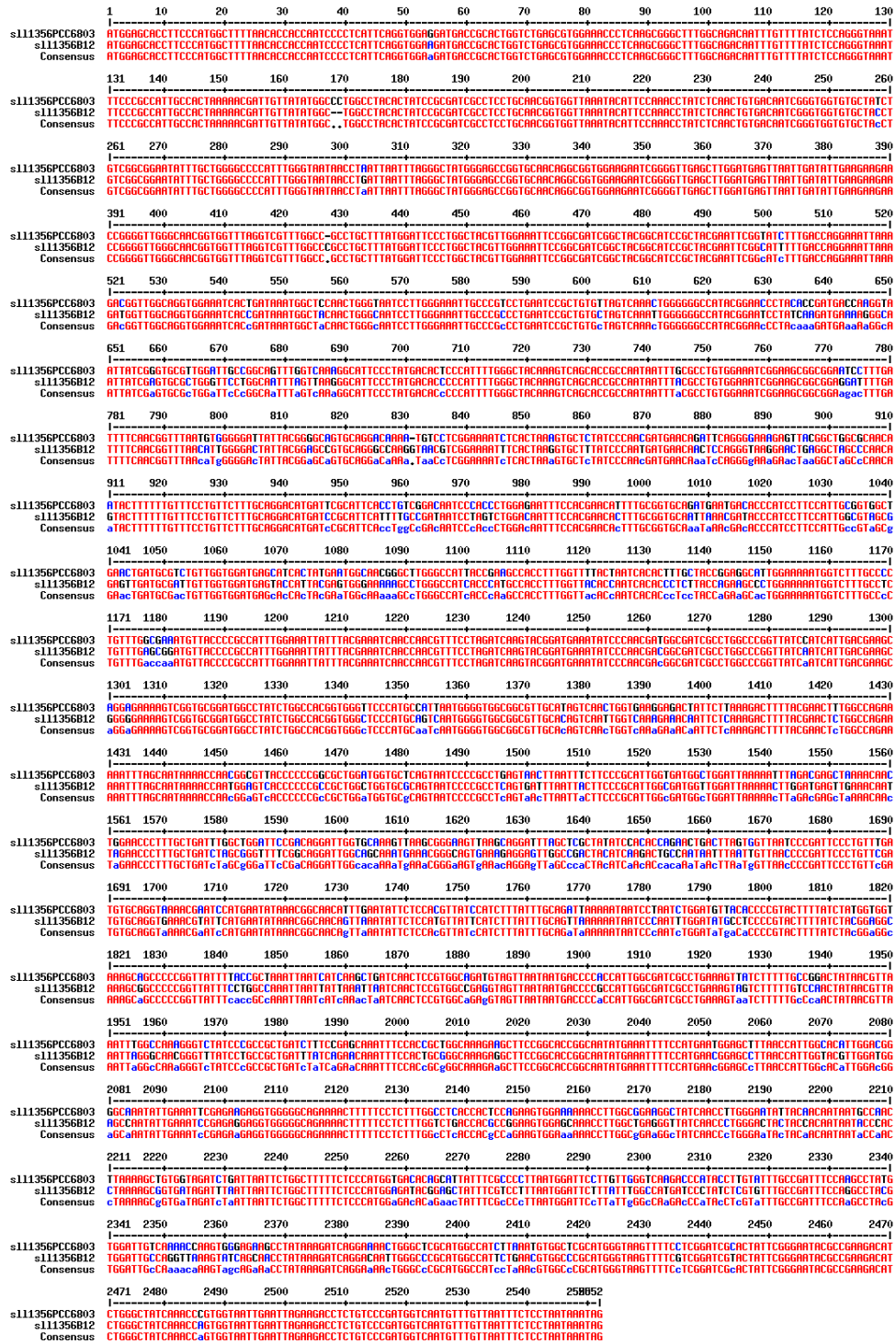

Fig 25. This figure shows alignment of sll1356 for PCC6803 and for B12 some differences are detected in single nucleotide or in triplets

Alignment ofGlgP1 was done, this gene works better in elevated temperature (Corpet, 1988).

The alignment of nucleotides sequences of *GlgP1* shows many differences, compared to reference sequence of PCC 6803. The percentage of similarity was 87,5% 3 deletions were detected and there were both single nucleotide

substitutions and grouped mutation Protein glycogen phosphorylase sll1356 of *Synechocystis* sp. PCC6803 was aligned with her corresponding of B12. Amino acid sequence of this protein was:

>slr1356 PCC6803

MEHLPMAFNTTNPLIQVEDDRTGLSVETLKRALADNLFYLQGKFPAIATKNDCYMALAYTIRDRLLQRWLNTF
QTYLNCDNRVVCYLSAEYLLGPHLGNNLINLGLWEPVQQAVEESGLSLDELIDIEEEPGLGNGGLGRLAACFM
DSLATLEIPAIGYGIRYEFGIFDQEIKDGWQVEITDKWLQLGNPWEIARPESAVLVKLGGHTEPYTDDQGNYR
VRWIAGSLVKGIPYDTPILGYKVSTANNLRLWKSEAAESFDFQRFNVGDYYGAVQDKMSSENLTKVLYPNDEQ
IQGKELRLAQQYFFVSCSLQDMIRIHLSDNPTLENFHEHFAVQMNDTHPSIAVAELMRLLVDEHHYEWQRAWA
ITEATFGFTNHTLLPEALEKWSLPLFGEMLPRHLEIIYEINQRFLDQVRMKYPNDGDRLARLSIIDEAGEKSV
RMAYLATVGSHAINGVAALHSQLVKETILKDFYELWPEKFSNKTNGVTPRRWMVLSNPRLSNLISSRIGDGWI
KNLDELKQLEPFADLAGFRQDWCKVKREVKQDLARYIHTRTDLVVNPDSLFDVQVKRIHEYKRQHLNILHVIH
LYLQIKNNPNLDVTPRTFIYGGKAAPGYFTAKLIIKLINSVADVVNNDPTIGDRLKVIFLPDYNVKFGQRVYP
AADLSEQISTAGKEASGTGNMKFSMNGALTIGTLDGANIEIREEVGAENFFLFGLTTPEVEKTLAEGYQPWEY
YNNNANLKAVVDLINSGFFSHGDTALFRPLMDSLLGQDPYLVFADFQAYVDCQNQVGEAYKDQENWARMAILN
VARMGKFSSDRTIREYAEDIWAIKPVVIELEDLCPDGQCLLISPNK

>slr1356 B12

MEHLPMAFNTTNPLIQVEDDRTGLSVETLKRALADNLFYLQGKFPAIATKNDCYMAGLHYPRSPPATVVKYIP
NLSQLQSGGVLPVGGIFAGAPFGPDFRAMGAGATGGGRIGVELGVNYRRTGVGQRWFRSFGPPALWIPWLRWK
FRRSATASATNSAFLTRKLKMVGRWKSPINGYNWAILGKLPALNPLCSNWGAIRNPIKMKRAIIECAGFLAIL
RAFPMTPPFWATKSAPPIIYACGNRKRRRILIFNGLTLGTITEPCRAKVTSENFTKVLYPNDEQLQGKELRLA
QQYFFVSCSLQDMIRIHFADNPSLDNFHEHFAVQLNDTHPSIGVAELMRLLVDEYHYEWEKAWAITHATFGYT
NHTLLPEALEKWSLPLFERMLPRHLEIIYEINQRFLDQVRMKYPNDGDRLARLSIIDEAGEKSVRMAYLATVG
SHAVNGVAALHSQLVKETILKDFYELWPEKFSNKTNGVTPRRWLVRSNPRLSDLITSRIGDGWIKNLDELKQL
EPFADLAGFRQDWQQMKRAVKEELADYIKTANNLIVNPDSLFDVQVKRIHEYKRQQLNILHVIHLYLQLKNNP
NLDMPPRTFIYGGKAAPGYFLAKLIIKLINSVAEVVNNDPAIGDRLKVVFLSNYNVKLGQRVYPAADLSEQIS
TAGKEASGTGNMKFSMNGALTIGTLDGANIEIREEVGAENFFLFGLTTPEVEQTLAEGYQPWDYYHNNTHLKA
VIDLINSGFFSHGDTELFRPLMDSLFGHDPYLVFADFQAYVDCQVKVSATYKDQDNWARMAILNVARMGKFSS
DRTIREYAEDIWAIKPVVIELEDLCPDGQCLLISPNK

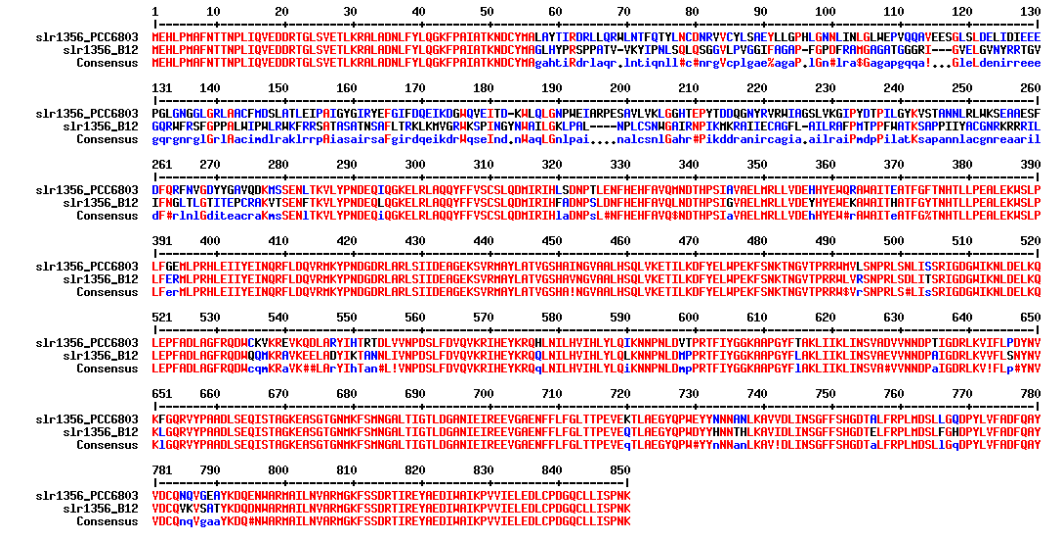

Fig 26. Alignment of amino acid sequences of GlgP1 the first part of alignment shows many gaps due to length of amino acid sequence, blue parts highlight differences while red letters mean correspondence. Length of sequence alignment was 854 aa, the first part of alignment shown a gap for sequence of B12. So, alignment between two sequences start further on

almost 240 aa. However, the two sequence show some substitutions of amino acids between two sequences moreover in the first part.

4.5 Genetic engineering

A key point to intervene on modulation of glycogen metabolism is to perform some molecular biology experiments that leads to preparation of constructs and then to the possibility of expression. The steps needed to prepare pAI plasmid were the insertion of promoter, the insertion of gene of interest in our case *GlgP2*, and at the end insertion of GFP a tag necessary to distinguish cells under microscope.

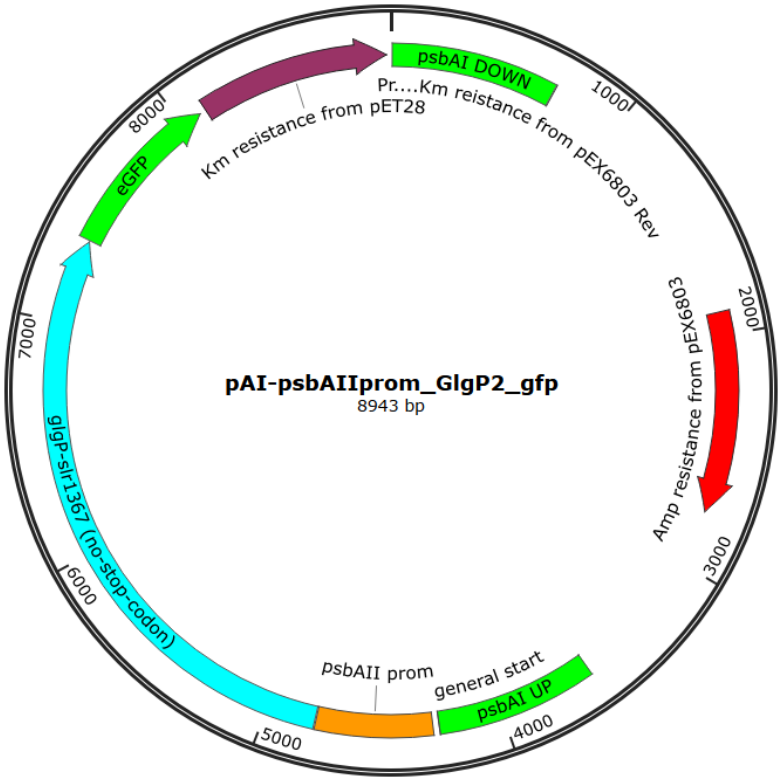

Fig 27. This map here reported shows: gene of resistance useful for selection with colour red, promoter in orange colour, in light blue gene GlgP2 is indicated and in green colour GFP gene is indicated.

So, two ways to achieve stable recombination concern natural transformation and electroporation, to reach this objective plasmid pAI have to be integrated.

This scheme shows pAI plasmid (expression plasmid) used to insert gene *GlgP2*. Orange colour indicates promoter, light blue colour indicates gene of interest, *GlgP2*. And GFP is indicated with green. Also in green colour sites psbAI up and down are indicated, these two sites are sites of recombination in genome, site psbAI is not active at transcriptional level, and it can give problems if it is used as recombination region (Formighieri

and Melis, 2014) Plasmid pAI was prepared at first inserting promoter, and then inserting gene GlgP2. GFP is a tag, and it is necessary to detect cells under microscopy. Map reported in Fig 30 indicate the plasmid that we want to generate.

The next step was to insert the GlgP2 gene into the pAI plasmid, which was already present in the laboratory (Allahverdiyeva et al., 2013). Results of amplification of PCR are shown in agarose gel, and they demonstrate high band at 2,5 Kbp, and PCR done with *rps* primers was implemented to verify quality of DNA. It evidences low band in correspondence of 250 bp. These results shows that gene and backbone pAI were correctly amplified.

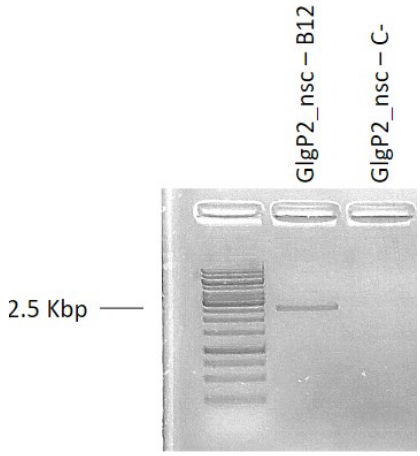

Fig 28. In this figure gene GlgP2 is indicated, band reports dimension of 2.5kb C- indicates water.

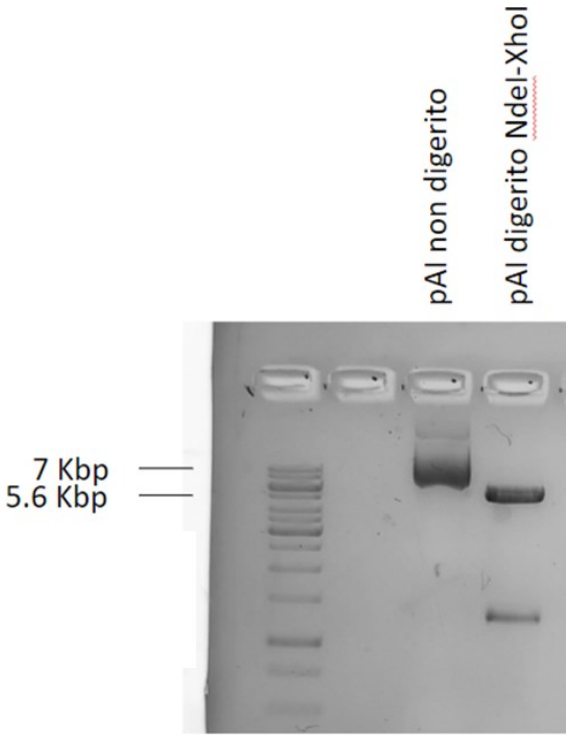

Fig 29. This figure reports amplification of backbone pAI, amplification of not digested plasmid is present but unclear so pAI plasmid was digested with NdeI and XhoI as restriction enzymes and band was better visible in correspondence of 5.6 Kbp.

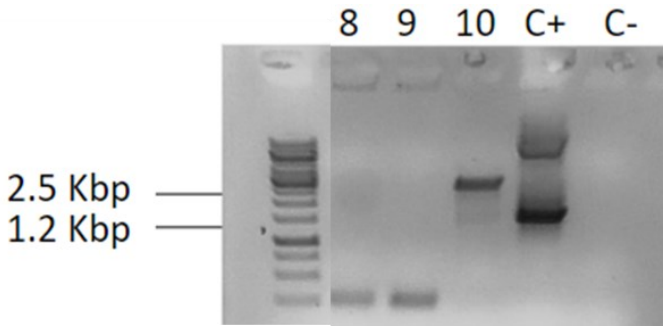

Fig 30. Colonies screening for gene GlgP2 if there is insertion band is 2.5 Kbp C+ pointed out positive control plasmid pAI and C- indicate negative control.

So, we have obtained some colonies we made colonies screening as already seen in paragraph 3.12, we had a positive colony n°10. Then miniprep was done as already described in paragraph 3.13.

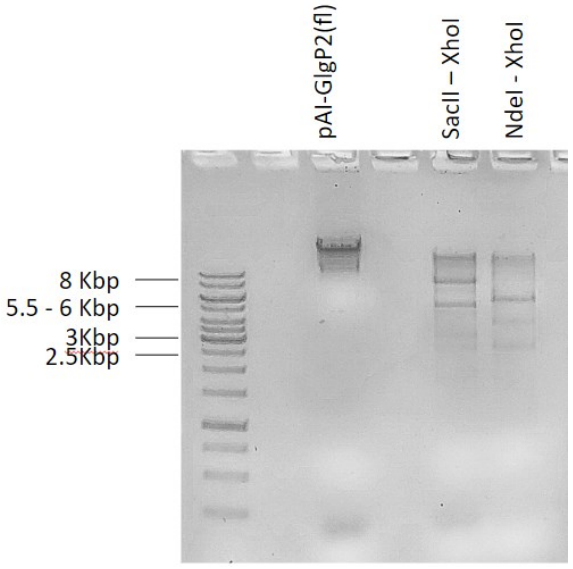

Fig 31. Agarose gel of digestion of miniprep of colony 10, plasmid pAI is digested and in this figure digestion with SacII and XhoI indicate promoter+gene instead digestion made with NdeI and XhoI point out only gene *GlgP2.*

The last step was to insert GFP into pAI plasmid (with the gene inserted). This agarose gel show that primers correctly amplify both pAI with gene GlgP2 inserted and GFP. in the following gel Fig 34. Both PCR products were well amplified backbone+promoter+gene and GFP

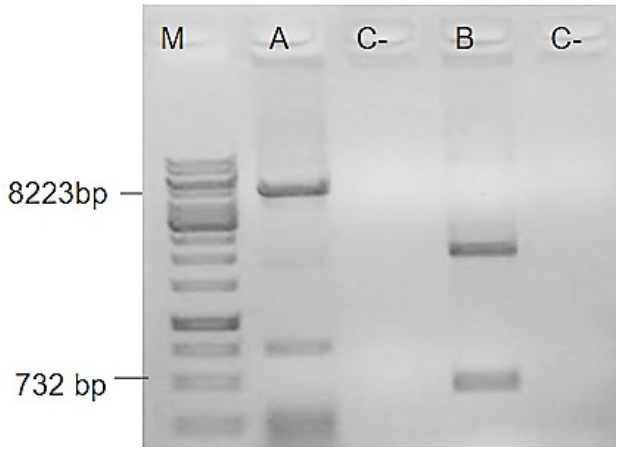

Fig 32. In this figure with letter A amplification of backbone pAI with promoter and gene denoted with letter A, it is enhanced a band with length of 8223 bp for backbone, and with letter B amplification of GFP is shown with a band at 732 bp.

Ligations gave colonies but they did not have GFP inserted so cloning are still going.

4.5.1 His-Tag

An alternative to GFP is His-tag, His-tag is a little tail of histidine, put at the end of gene sequence. (Romero-Fernández and Paradisi, 2020). His-tags are often used to make easier purification of protein, in our case GlgP2. While GFP is detectable at microscopy His-Tag cannot, but it needs to purify GlgP2 protein. It has a different purpose, and it can be an alternative in future. This map here reported (Fig 33) is an alternative vector to generate.

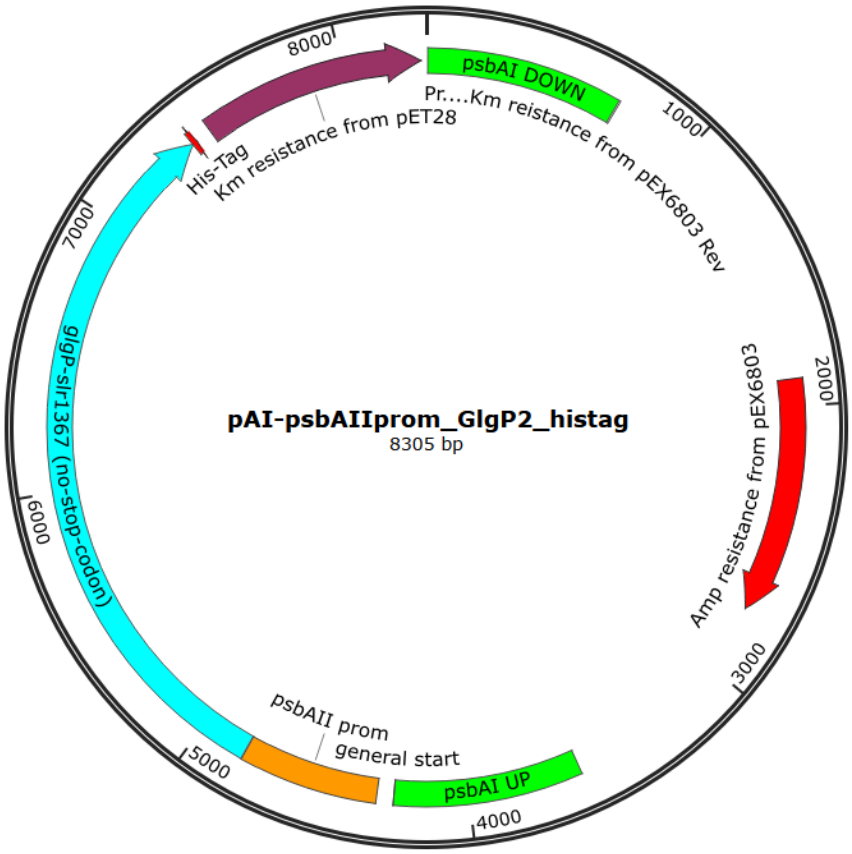

Fig 33. Map of pAI plasmid with His-Tag, promoter is indicated with orange colour, gene GlgP2 is pointed out with light blue colour, purple colour indicates Kanamycin resistance gene, while red colour indicates ampicillin resistance gene, His-Tag is enhanced in red colour after GlgP2 gene and before kanamycin resistance gene.

4.5.2 Cloning plasmid

Transient transformation is useful to modify cell avoiding integration of exogenous DNA in the genome of the cells, therefore avoiding destroying key genes.

For transient transformation plasmid pJET was used in a modified version as cloning vector to insert in order: promoter, gene *GlgP2*, and GFP. This plasmid was employed because the expression plasmid called pRL1383, needed for bacterial conjugation, was low-copy plasmid, so often difficult to perform cloning.

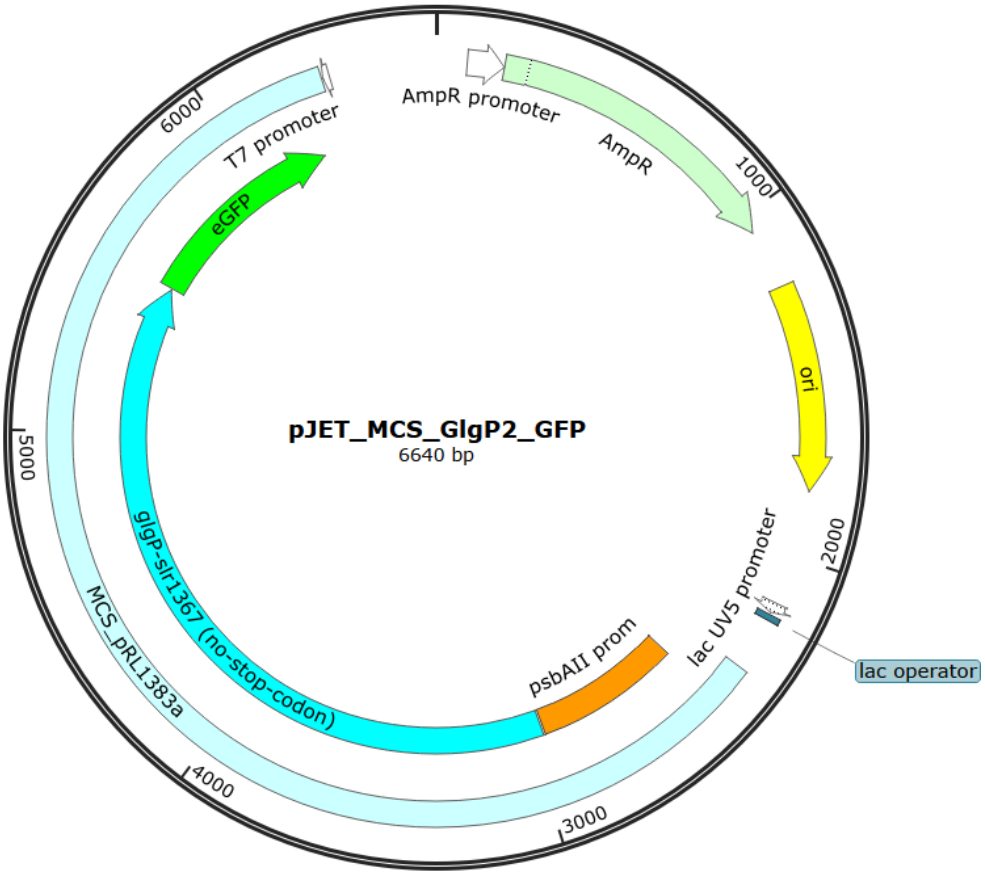

Fig 34. This map describes pJET modified and evidence: promoter in orange colour, gene of interest GlgP2 in light blue colour, GFP in green colour, gene of antibiotic resistance (ampicillin) in light green colour and origin of replication in yellow colour.

The key step was to insert promoter, PCR was made, and promoter was correctly amplified to do Gibson Assembly (paragraph 3.14)

Then next step was to insert promoter, results reports that promoter was successfully inserted into cloning plasmid pJET.

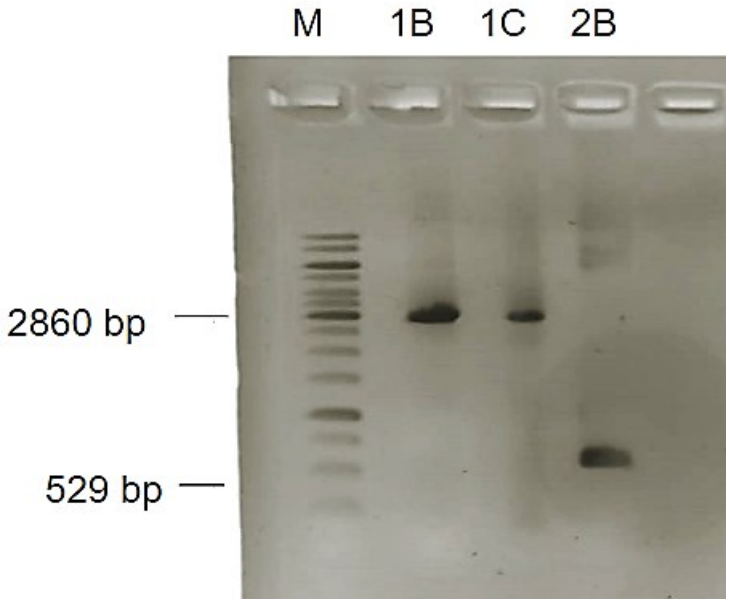

Fig 35. This figure reports agarose gel of Backbone+MCS, letters 1B and 1C indicate backbone pJET+ MCS length of fragment amplified was 2860 bp instead promoter is amplified, and it is indicated with letter 2B its length was 529 bp.

So, backbone+MCS called pJET MCS modified, and insert were amplified, and the next passage was to do GA (Gibson Assembly) and transform bacteria as seen in paragraph 3.11. Then screening of colonies was made.

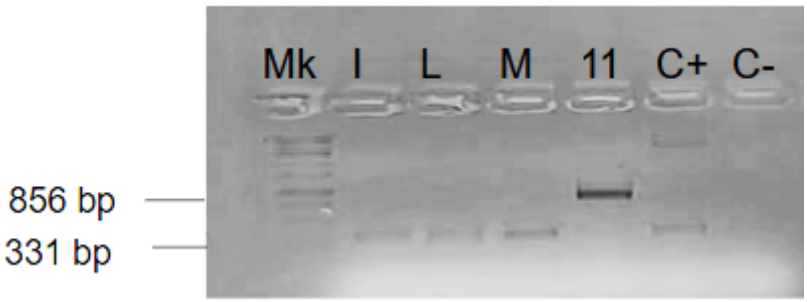

Fig 36. Here agarose gel of colonies screening is reported, with letters I, L, M some colonies are indicated, number 11 means positive colony. C+ is positive control, instead C- means negative control (water). Then backbone with inserted promoter and (1) and gene *GlgP2* (2) were re-amplified to confirm the possibility to do ligation. Length of fragments were 3385bp for backbone with promoter and 2565bp for gene *GlgP2.*

This agarose gel is a result of PCR made on *E. coli* colonies to verify if promoter was inserted. It highlighted a high band in correspondence of colony 11, this means that promoter was inserted into plasmid pJET.

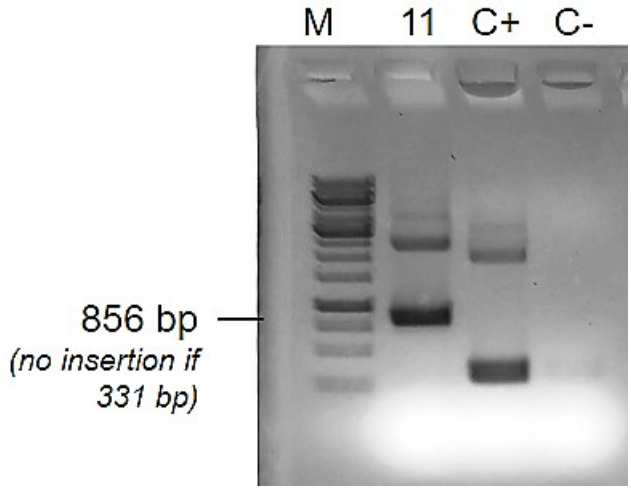

Fig 37. In this agarose gel control of miniprep is shown, number 11 indicates backbone with promoter, while C+ indicates amplification backbone without insert instead C- indicates water

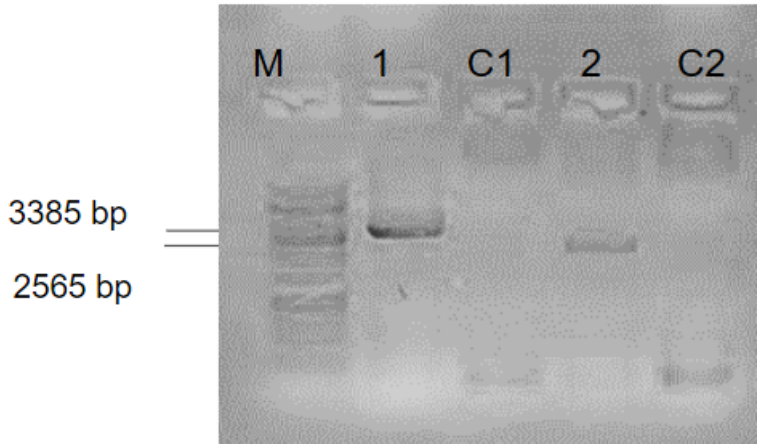

Fig 38. Amplification PCR of Backbone (mini-11) indicated with number 1 and gene GlgP2 with number 2, both bands are visible, so primers have correctly amplified.

But the insertion of gene GlgP2 into cloning plasmid pJET is still ongoing.

4.6 *Synechocystis* sp. Transformations

To reach manipulation of glycogen metabolism, some experiments on *Synechocystis* sp. were performed, for transformation both reference strain PCC 6803 and B12 were tested. Reference strain was used because many protocols are already tested, and transformations protocols have to be optimized.

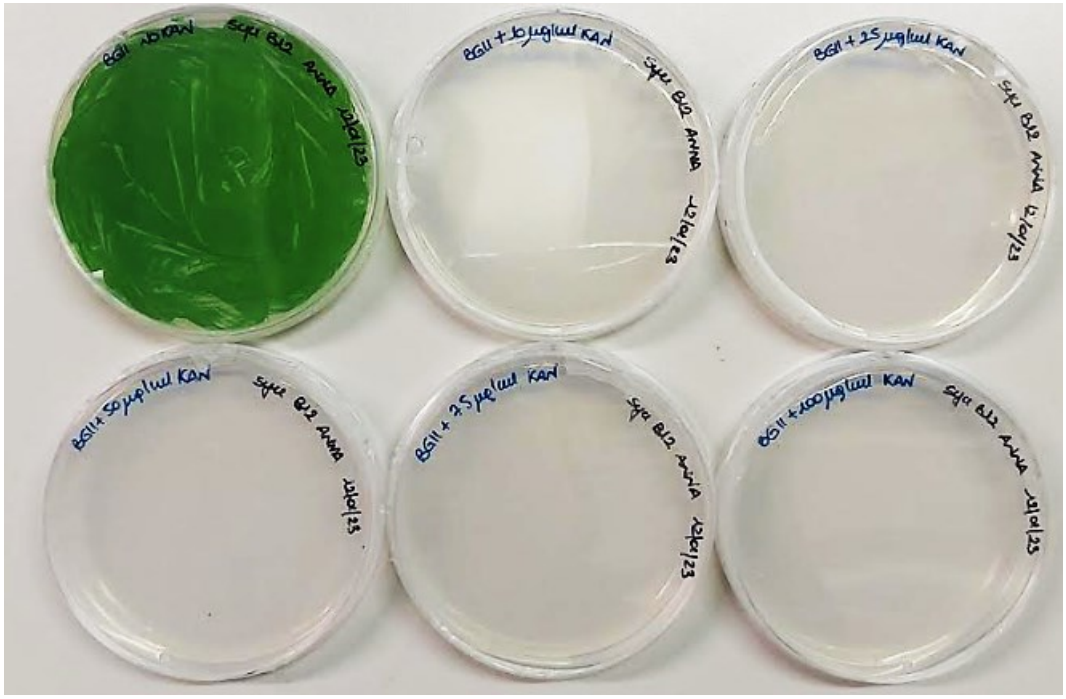

Fig 39. This figure shows test for kanamycin at different dosage negative control (without kanamycin) is the green plate, while dosage is: 10 µg/µl, 25 µg/µl, 50 µg/µl, 75 µg/µl and100 µg/µl.

As seen in fig 41 this is a test for *Synechocystis* sp. B12 for kanamycin, all concentrations of antibiotic are too dangerous for *Synechocystis* sp. B12 because there are no colonies in treated Petri dishes. But 10 µg/ml was chosen for next transformation because was the lowest.

Stable transformations performed with electroporation do not give colonies, but experiments have to be optimized to obtain results.

Techniques of stable recombination have to be optimized to reach results and to have mutant phenotypes both for PCC 6803, because it is an additional control and because many protocols are just tested, and for B12.

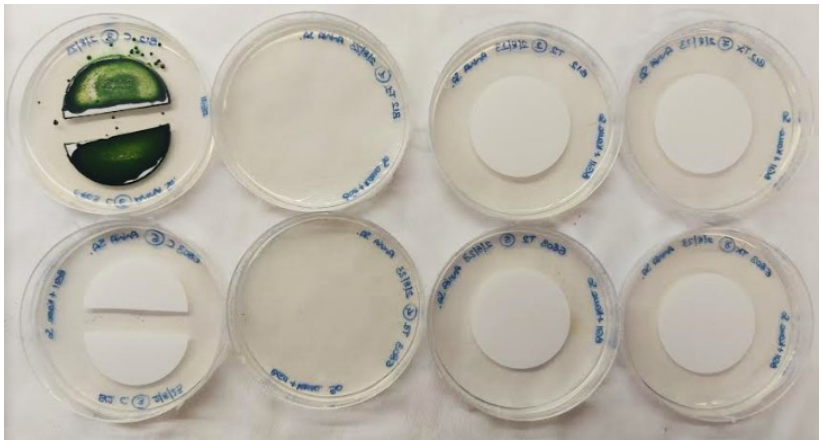

Fig 40. this image report Petri dishes with kanamycin seeded with cell treated with electroporation on left of image there are positive (green) and negative control.

Set up experiments for transformation through conjugation were performed, with the aim to have transient transformation, but they need to be optimized because there are still no colonies. In this image Petri plate with cells grown is positive control. PCC6803 was used because many protocols are already tested.

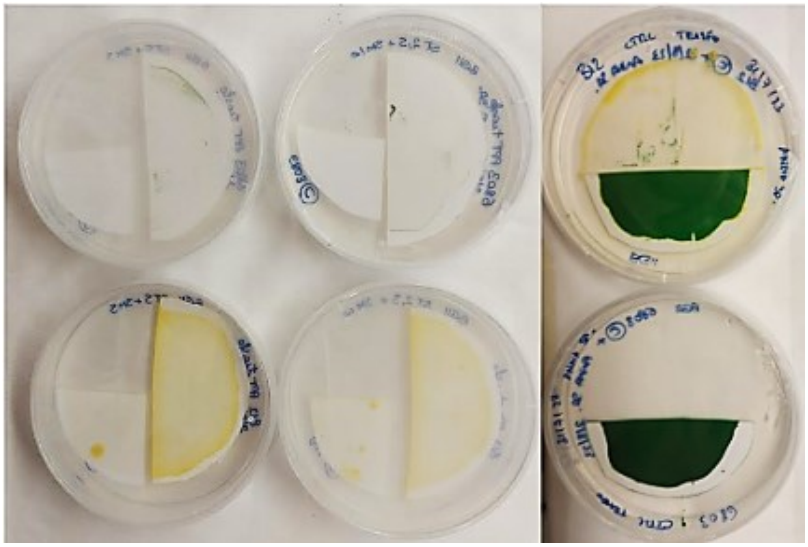

Fig 41. Petri dishes that show positive control enhanced in green colour on the right part of image, negative control in the left part of image and in centre Petri dishes with treatment. In yellow there are some bacteria of conjugation.

5 Discussion

To activate a large-scale plant of production of bioplastic by cyanobacteria, it is useful a careful evaluation, and an economic analysis about the feasibility, in economic terms (Rueda et al., 2023). In industrial perspective, it is vital to increase yields, lower the costs, enhance quality of compound. In a vision of sustainability, it is fundamental decrease environmental impact of process, products, and human activities. The possibility to use photosynthetic bacteria in industrial scale of useful metabolites production, it has great implications about sustainability and circular economy. Because cyanobacteria can use waste biomasses or wastewater, sun light, and CO_2 to produce high value products such as bioplastics. But there are many factors that have to be counted like: economic availability, engineering feasibility, process optimization. Briefly an industrial scale cultivation needs 3 things: LCA (life cycle assessment), TEA (techno-economic analysis), and MRV (monitoring reporting verification), (Rueda et al., 2023). However, it is needed to optimize the process to increase yields in PHB. Therefore, some effective ways to achieve these aims are to investigate environmental and growth conditions that lead to a boost in PHB production and to use some approaches of metabolic and genetic engineering. It is appropriate to investigate the optimal growth condition in cyanobacteria to find new useful and important information about improvement production of substances and scalability of process. Moreover biotechnology approaches can be implemented to provides new strands of cyanobacteria genetically modified to increase production of useful metabolites.(Robles-Bañuelos et al., 2022). The consideration of cyanobacteria for biobased product, can open several ways in biopolymers, and metabolites production, because biopolymers are often stored as reserve substances (Turetta et al., 2022)

Synechocystis sp. B12 is an organism extremely versatile to achieve aims of sustainability, and an effective way to quit conventional plastic derived from fossil fuels. It has peculiar characteristics that allow its, to manage efficiently stress factor like nitrogen and phosphorus absence in culture medium. This is a key point because ability of manage stress is vital to survive and assure a high production of bioproduct in industrial scale.

5.1 Cultivation in Nitrogen and Phosphorus starvation

Cyanobacterium *Synechocystis sp*. B12 grew in high light in agitation for 9 days and vital parameters were monitored with this cadence: day1, day 3, day7, and day9. It is important to underline that in small scale measurement can be obtained with an established schedule, instead in industrial scale measurement were recorded daily and sometimes twice a day. Growth conditions and stress factors applied can change in function

of what kind of metabolite it is desired (Hachicha et al., 2022). When results of PHB measured with Nile Red fluorescence of cells cultured in P starvation, were compared with Nile Red fluorescence of N starvation, fluorescence of P starvation shown higher values. Higher PHB content was evidenced also observing cells under confocal microscopy. Another consideration is that *Synechocystis sp.* B12 can manage efficiently P deprivation in culture medium, it is probably adapted to environments with low P content because it is able to survive and grow in the absence of phosphorus. The confirmation was that variation of OD_{750} attested a gradual increase during experiments. *Synechocystis sp.* B12 can resist both to high light and P starvation, it may be an indication that this strain had evolved strategies to easily face this stress factor. Yet this cyanobacterium has developed structural, metabolic, and genetic changes to manage the challenge of climate change. Because higher temperature, polluted environment, lacking key nutrient affects normal biochemical processes (Erratt et al., 2022).

5.2 Acclimation in Phosphorus starvation confirmed high accumulation of PHB.

In natural environment usually low content of P are in surface of water, however usually it is a nutrient that limit growth because it is essential in build-up of DNA and phospholipids (Rabouille et al., 2022). Cyanobacteria activate a cascade of genes like Pho regulon a group of genes as *sphS* and *sphR*, that allow *Synechocystis sp.* to acclimate themselves during P starvation (Rachedi et al., 2020). I found that this bacterium menage optimally the absence of P in medium, and that it accumulated high amount of PHB when this stress factor was applied, and it is confirmed with confocal microscopy. Experiment performed in acclimation of P starvation gave interesting results and helped to understand the accumulation of PHB in a mid-long period of time. Cells were acclimated for 5 days in P starvation before to start experiment. So, it is useful to investigate on feedstock substances and stress factor, in our case P starvation, to achieve the objective of promote cells growth and bioplastics accumulation. Time passed during acclimation in P starvation allow *Synechocystis sp.* B12 to adapt to environment and to be able to manage this stress factor. Results reported that acclimation led to an increase both in OD_{750} and PHB content. PHB content was verified with Nile Red fluorescence that reported values extremely high. This experiment permits to elucidate the trend in PHB accumulation inside cells.

5.3 Combined stress

Under many stress factors cyanobacteria try to respond, increasing synthesis of some metabolites or activating some metabolic pathways that

in normal situation are repressed (Rabouille et al., 2022). Experiment carried out in modified medium to combine two stress factors lacking N and P showed that this situation maybe is too stressful for *Synechocystis sp.* B12. Because OD_{750} do not shows a regular increase, this means that cells grow bad or do not grow due to maybe too stressful conditions. However, PHB accumulation seemed to show that for 3 days Nile Red fluorescence decrease, but then in day 7 and 9 it had a sensitive increase.

5.4 Sequences alignments

Nucleotides sequences alignment was performed, it was made on genes *GlgP1* and *GlgP2* both involved in glycogen breakdown pathway. Alignment of nucleotides sequences for reference strain PCC6 803 and for B12 have showed just few differences between two strains. There were more substitution and insertions or deletions in GlgP1 than GlgP2. Maybe these substitutions involved triplets that codify for key aminoacids, and this can concern many characteristics of final protein properties like: stability at high temperature, specificity to substrate, or capability to work at high variation of pH. Moreover, substitution concerning aspartic acid can modify the properties and the behaviour of the protein because aspartic acid is a polar amino acid characterized by negative charge, and by this way it can change hydrophobicity of protein, it has also a side chain with carboxylic group that can form H bond with other amino acid residues. Then amino acids sequence of slr1367 for reference strain PCC6803 was modelized using AlphaFold for 3D structure prediction. This method consents to understand batter the characteristics of subunits of protein like hydrophobicity, intermolecular bonds. It offers a new possibility to elucidate the role of slr 1367 in metabolic pathways, its dynamics in cytosol or in membranes, or maybe in some bacterial microcompartments (Greening and Lithgow, 2020). In alignment of amino acids sequence of GlgP1 there were the absence of a part of protein, so differences, are more evident there.

5.5 Genetic engineering

Experiments accomplished have shown possibility to generate the constructs to test potential of transformation. Some of results that have been achieved were insertion of promoter and gene GlgP2 into expression plasmid pAI, and test efficiency of primers to amplify GFP. It demonstrated that the way of genetic engineering is possible, and that there is concrete potentiality to overexpress gene GlgP2 to lead a manipulation of glycogen metabolism and consequently an increase in PHB accumulation. Then transformations are still in a development phase. Many studies demonstrate the possibility to transform reference strain PCC6803, so the possibility of transformation is confirmed, and it is possible to act at different metabolic and genetic levels to increase biomass or to enhance yields of some metabolites. Yet experiments are ongoing, it means that optimal conditions have still to be found. One factor that could have a role is that Synechocystis sp. B12 come from natural environment so, it has extracellular polysaccharides that trap other bacteria or impurities. These contamination despite cleaning procedures could have the effect to make transformations difficult to achieve, because exogenous bacteria often degrade extracellular DNA and reduce effectiveness of transformations. For conjugation, in plasmid pJET promoter is inserted and experiments to insert gene GlgP2 are still ongoing.

6 Conclusions

This thesis demonstrates the potential of employ *Synechocystis sp.* B12 in a context of carbon capture and circular economy. It has been reported as stress factor and genetic engineering can be the way to achieve the aims of bioplastics production. In this thesis was evaluated stress condition related to cells growth and PHB production. It is an indicator of how different stress impact in living conditions of cells. Synechocystis sp. B12 has shown the capability to manage expertly stress caused by P starvation and high light, it has confirmed a constant grow and a sensitive accumulation of PHB. Glycogen levels remained almost constant during P starvation but in N starvation after an extended period of time they start to decrease. Moreover, it was conducted genetic engineering experiment to achieve an increase in glycogen catabolism and potentially higher amount in PHB content, this is a starting point, because in future can be identified new target genes, individuated new key proteins, and considered alternative metabolic pathways to optimize yield of bioproducts. The capability to manage stress, and the availability to be genetically modified of *Synechocystis sp.* have confirmed its eligibility to be cultivated in industrial scale. Cyanobacteria, with their versatility, carry out perfectly challenges of global change and can easily enter in sustainable development not only for bioplastics but also for all useful and interesting metabolites, employable in several sector of industries.

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