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Use of struvite in the cultivation of the edible cyanobacterium *Limnospira indica* PCC8005 P1

Judith Mokuinema Wawina

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HAL Id: hal-04593700

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FACULTE DES SCIENCES ET TECHNOLOGIES
Master MICROBIOLOGIE 2eme ANNEE
Orientation MIND (microbiologie industrielle)
2022-2023

Use of struvite in the cultivation of the edible cyanobacterium *Limnospira indica* PCC8005 P1

Thesis presented by

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Internship from January 16th to June 30th

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Nuclear Medical Applications

Microbiology unit

sck: cen

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II. Abbreviations

BSA: Bovine serum Albumin

BCA: Bicinchoninic acid

EFSA: European Food Safety Agency

FDA: Food and Drug Administration

LC: Continuous Light

LD: Light/dark cycle

MELiSSA: Micro-Ecological Life Support System Alternative

MI: inspiration medium

PBS: Phosphate-buffered saline

PCC: Pasteur culture collection

ROS: Reactive Oxygen Species

SDS: Sodium dodecyl-sulfate

III. Introduction

A. History and classification

The appearance of cyanobacteria dates back 3 billion years, they are ubiquity, and these bacteria were responsible for oxygenating the atmosphere and oceans. On the other hand, the origin of first eukaryotic cells (including micro-algae) is estimated at 1.5 billion years ago with an estimated average number of species between 70,000 and 150,000 species, up to 1,000,000 for some publications. (sardet *et al.*2013)

Edible cyanobacteria were first discovered in Lake Chad in 1940, 10000 km from Lake Texcoco, another natural source of cyanobacteria. Their potential was already exploited by the Incas, Mayas and Aztecs over 3 thousand years ago and only about 10 species are now cultivated. The Kanembu tribe relies heavily on spirulina for protein, they harvest it from the Great Lake (Lake chad) and bake it into a type of bread called “dihe” (Castenholz *at al.*2001)

Cyanobacteria are classified into five different groups based on the physical distinctions between them: Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, Stigonematales (Lecointre and Le guyader ,2016) and according to pigmentary composition, there are 3 distinct taxonomic groups (phyla): chlorophytes, chromophytes and rhodophytes, which all have chlorophyll and are distinguished by additional pigments. E.g., phycocyanin, chlorophyll c and e, carotenoids, phycoerythrin. (Fleurence, 2021)

The taxonomic categorisation of cyanobacteria has undergone a recent revision, resulting in the establishment of a fresh genus named *Limnospira*, and a new species called *indica*. These have replaced the previous *Arthrospira* classification: *A.fusiformis*, *A.maxima*. (Nowicka-Krawczyk *et al.*2019).

The cyanobacterium *Limnospira indica* which is also included in commercial name Spirulina is a filamentous straight or spiral-shaped Oscillatoriales cyanobacterium. It is a gram-negative and obligate photoautotrophic, which means they require light for photosynthesis and cannot get energy from organic compounds.

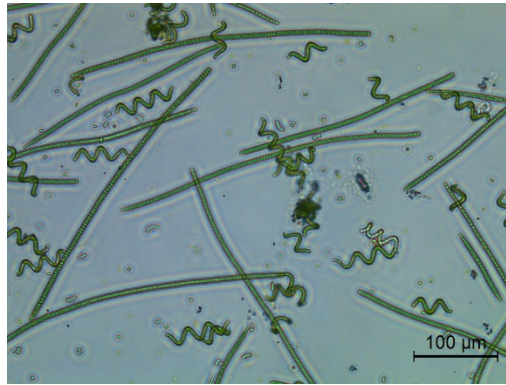


Figure1: The morphology of straight and spiral shaped *Limnospira indica* PCC8005 under optical microscopy.

B. Spirulina as food supplement

Spirulina is recognised as “a fantastic food source of the future” for Applied Microbiology since 1967. In 1970, Lake Texcoco was the site of the world’s first commercial production of spirulina. Between the years 2005 and 2013, the annual commercial output around the globe climbed from 3000 to 5500 tons.

Food regulatory agencies including the European Food Safety Agency (EFSA) and the Food and Drug Administration (FDA) have given their stamp of approval to dietary supplements containing spirulina. Its content in proteins is very important (between 60-70% of its dry weight) for food applications, mainly phycobiliproteins like phycocyanin and allophycocyanin.

Phycobiliproteins are gaining attention as a non-toxic and therapeutic alternative to synthetic food colorants (DK Saini *et al.*2018). Spirulina also contains natural pigment carotenoids as beta-carotenoid (a concentration of up to 4000 mg/kg) (Pietri,2011). (Appendix 1 & 2)

C. Spirulina and clinical application

Spirulina has been studied for its potential benefits for healthcare and to aid in improving medical conditions (metabolism, oncology, eye health).

Studies reported its hypolipemic, antihypertensive, antidiabetic, neuroprotective, antianemia, anticarcinogenic, hepatoprotective as well as anti-bacterial, anti-viral and immunomodulatory properties. (Trotta *et al.*2022)

Spirulina could help boost immunity to fight viral infections like CORONAVIRUS. Compounds such as ACE inhibitor peptides and calcium spirulan, found in Spirulina, have properties that can help the body resist infection and slow the progression of the disease. Additionally, probiotics, which are living microorganisms, have been found to reduce inflammation, lessen the severity and extent of tissue damage from infection, and possess antiviral protection. (Giacomo Fais *et al.*2022)

Spirulina would influence mood improvement by allowing the secretion of palmitoylethanolamide in the brain, which will induce the molecule of joy (pleasure, libido), improvement of concentration, mental energy (children's attention in this case), cognitive abilities and quality of life in general. (Anne-marie Pietri, 2011)

D. Spirulina and space exploration

The environment of space brings a lot of stress to the body and increases the amount of Reactive Oxygen Species (ROS). ROS are molecules that cells create from regular metabolism and from exposure to things like radiation, air pollution and other toxic chemicals. The ROS can damage cells, including their membranes, mitochondria, proteins and DNA. Microgravity, meaning low gravity, and radiation, which comes from space, can affect how the cells work, like the processes of repairing, replicating, transcribing and producing proteins.

For all these reasons, space agencies have encouraged eating spirulina as an additional source of nutrients and protection (for its antioxidants, immunomodulatory and anti-inflammatory activities) for astronauts in space missions. (Giacomo Fais *et al.*, 2022)

In order to make human spaceflight economically viable, MELISSA (Micro-Ecological Life Support System Alternative) project has designed a system to recycle waste (CO₂, organic waste) into food, water and oxygen by recreating a miniature terrestrial ecosystem (bacteria, algae, plants, chemical elements and natural processes). Spirulina has been chosen to be part of the system (recycling the waste of a space station crew), for its photosynthetic activity (Larissa Hendrickx *et al.*2006; Melissafoundation.org)

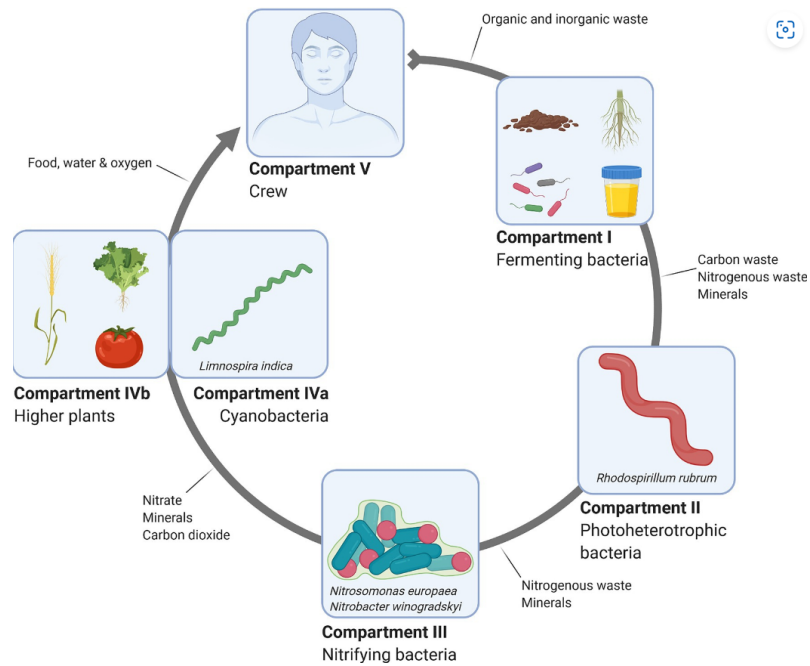


Figure 2: schematic overview of the MELiSSA loop (Tom vebeerlen *et al.*2021).

E. The INSPIRATION project

Along with Entrepreneurs for Entrepreneurs (Ovo), SCK CEN launched the INSPIRATION project. This aims to develop and support the cultivation of the cyanobacterium spirulina in the Democratic Republic (DR) of Congo.

In 2015, the INSPIRATION project began in the regions of Mooto and Mbandaka, where the first cultivation basins were installed. That needed some good preparation because spirulina acts like a sponge, absorbing all substances including potentially toxic ones from water. So before growing spirulina, SCK CEN chemists tested various water sources and identified the safest for cultivation. This research was done both on-site and remotely.

In July 2016, SCK CEN staff went back to DR Congo, this time with a spirulina inoculum and nutrient to ensure the culture started well. They also bought equipment, had sturdy, waterproof basin made, and provided a canopy to protect the spirulina from heavy rain and full sun, as that could dilute, disrupt or contaminate the spirulina culture.



Start of the INSPIRATION project in Mooto (RD Congo)

The Belgian NGO Congodorpen was a crucial partner in this enterprise and thanks to them, Denis Bwabwa was introduced to the project. Denis is an assistant professor at the Université Pédagogique Nationale (UPN), specialising in nutritional supplements for fish farming. He is our project leader in DR Congo. That means that he is in charge of the one-site investigation, awareness-raising and informing the population, and organising tasting sessions.

In the meantime, he has been captivated by the project that he is taking a doctorate at SCK CEN to develop spirulina cultivation in DR Congo and to find alternative, possibly local nutrients. That started with an internship at SCK CEN in 2019 before heading back to the UPN.



Denis Bwabwa internship at SCK CEN

F. Finding alternate source of nutrients

Limnospira indica grows naturally in lakes as mentioned above, to mimic these conditions in the laboratory, Chemical-based culture media containing a source of phosphorus, nitrogen, carbon and trace elements are used.

Chemical-based culture media are collections of chemicals that are used in various industries to cultivate cyanobacteria. Zarrouk medium is a type of chemical-based culture medium that has been conventionally used for many years and is known for its success in producing decent amounts of biomass (zarrouk, 1998). Other examples of chemical-based culture media include Central Food Technological Research Institute (CFTRI), Blue Green (BG-11), F/2, Conway, Schlosser medium, Kosaric, and Synthetic Human Urine (SHU). Sodium bicarbonate and sodium nitrate are two common chemicals typically present in these culture media. Sodium bicarbonate (NaHCO_3) helps to maintain a high pH in the culture medium, while sodium nitrate (NaNO_3) is an important nitrogen source that can affect the protein content and the amount of biomass produced (Hooi Ren Lim *et al.*2021)

Light, temperature, inoculum volume/concentration, agitation, aeration, dissolved particles, pH, water quality, and overall micronutrient richness are only a few of the environmental parameters influencing *Spirulina* production (Anil Kumar *et al.*2020)

These culture media are relatively expensive, and several studies have aimed to use alternative sources of these chemicals to mitigate this situation.

Rather than being an unusable waste, wastewater is now thought to be a source of valuable resources such as nitrogen, phosphorus, potassium, energy, and water. The global phosphorus cycle is already beyond the sustainable limit (Conjin *et al.*2020) and Wellmer, Scholz (2017) suggested that we need to have a new system of prioritizing resources, where phosphate rock is given higher priority than fossil fuels, as fossil fuels can be replaced with renewable energy, while phosphorus is essential and irreplaceable. The technology used to recover the phosphorus from municipal waste is at a high level, which means that it is reliable and effective. Studies have shown that recovering phosphorus in this way has a positive impact on the environment. However, it is only able to recover 5-25% of phosphorus from waste. (Bart Saerens *et al*, 2020)

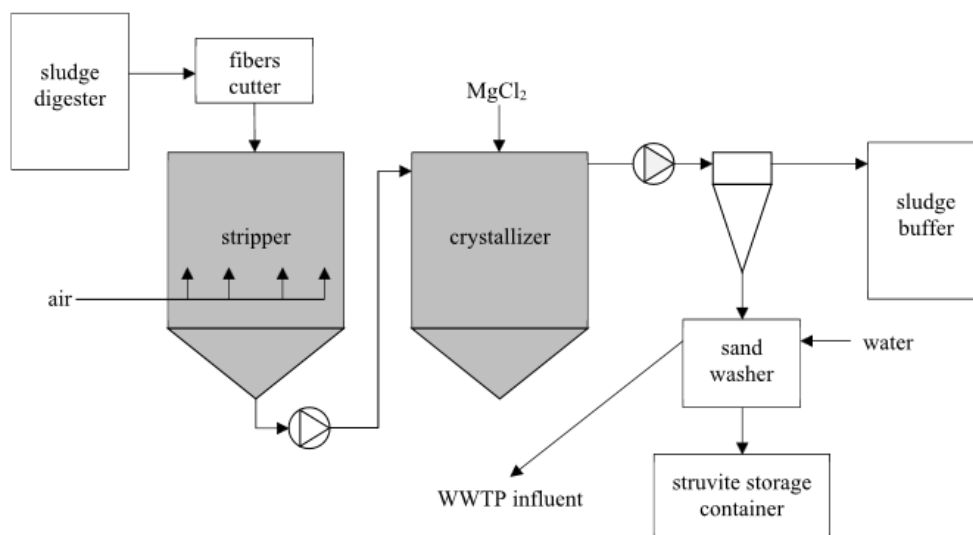


Figure3: Schematic overview of the struvite plant at the Leuven WWTP (Bart Saerens *et al.*2020)

Struvite is a chemical compound composed of nitrogen, magnesium, phosphorus, and oxygen ($\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$), which can be created by adding magnesium to wastewater (figure 3).

Contrary to the expense of chemicals (phosphorous, nitrogen, and magnesium), the use of struvite is much cheaper than the cost of chemical-based culture media.

According to Maarten Muys *et al.* (2020), most of the struvite is produced in the Netherlands (35-43%), Belgium (16-20%), and Germany (15%). This study showed that the struvite crystallisation process excludes pathogens, leaving them in the water rather than in the struvite and indicated that storage is a good strategy to reduce pathogens.

Two independents' studies were conducted to assess the impact of various concentrations of struvite in modified zarrouk or BBM medium on the growth rate of *Chlorella vulgaris* or *Arthrospira platensis*. In feasibility tests, struvite was used to replace the nitrogen, phosphorus and magnesium sources in these media, and they found that *Chlorella vulgaris* or *Arthrospira platensis* were able to survive for longer in the modified media with 700-800mg/l of struvite than the control (modified media without struvite). However, the challenge of contamination in waste products remains; they used sterilised and not sterilised struvite and found that sterilising the struvite limits contamination. (Niels *et al.*2015; Markou *et al.*2019)



Image of struvite crystallised.

Other sources of chemicals are also exploited such as

(i) the replacement of carbon source chemicals (NaHCO_3 and Na_2CO_3) in Zarrouk medium with organic medium such as cassava or taro or sweet potato flour, table sugar which achieve *Spirulina's* biomass dry weight. (Bandara and Arunakumara, 2020)

(ii) vermicompost of cow manure has higher levels of organic matter, nitrogen and phosphorus compared to fish sludge-based vermicompost, leading to higher biomass dry weight of *Spirulina*. (Hooi Ren Lim *et al.*2021)

(iii) Using corn steep liquor in Schlosser medium (mixotrophic conditions) to produce fibrinolytic enzyme enzymes by *Arthrospira platensis*. (Barros *et al.*2020)

(iv) According to Khatun *et al.* (2019), 50% of NaHCO_3 (sodium bicarbonate) and 75% of the micronutrients in Kosaric medium could be replaced with banana leaf ash extract without negatively affecting the maximum biomass dry weight. However, the result was not significant and viable in the long term because there was no difference in the Kosaric medium.

G. Objectives and strategy

In the frame of the doctoral research of Mr Denis Bwabwa we will investigating the use of struvite as an alternative source of nutrient for the cultivation of *Limnospira indica* PCC8005 P1 in various laboratory bench scale conditions.

The main objective of this work is to find out which components of the regular cultivation medium (i.e inspiration medium) can be efficiently replaced by using struvite. The final goal is to design a new optimized and cost-effective growth medium for spirulina production in the conditions of the Democratic Republic of Congo.

The growth of the cyanobacteria was followed by optical density measurement as well as other growth parameters including pH, light intensity and microscopy. The viability status of the culture was then analysed using a flow cytometry, and protein content was also monitored.

IV. Materials and Methods

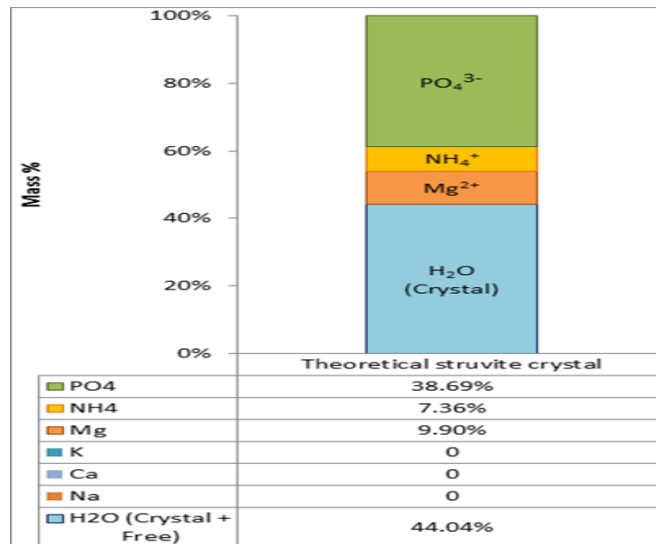
Strain and culture medium

The strain used is *Limnospira indica* PCC8005 P1, a gram-negative photosynthetic cyanobacterium spiral shape (P1), from the "Pasteur Culture Collection», which was cultivated in Zarrouk medium (Cogne *et al.*2003c) adjusted to 9.8 with carbonate buffer and maintained in axenic conditions (appendix 3). This is followed using 4 biological replicates in the Zarrouk and once at the stationary phase, the inoculums are collected and inoculated into the inspiration medium (Table1). The replicates in the inspiration medium were renewed every 3 weeks and used as stock for the experiments.

For each replicate, 50 mL of "Inspiration" medium was transferred aseptically into a 100 mL sterile glass Erlenmeyer flask with a cotton plug. Then, 5% (2.5 mL) of stock cultures of *Limnospira indica* PCC8005 P1 in the late exponential phase is transferred aseptically into the liquid "Inspiration" medium. The culture is then incubated at 30°C in a shaking light incubator (Edmund buhler GmbH) at 120 RPM and constant illumination of approximately 35 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ with fluorescent light. The culture is allowed to incubate for 15 to 30 days, with regular measurements of the optical density using a spectrophotometer Nanocolor Uv/Vis.

Preparation and treatment of struvite (solution stock)

Struvite from AQUAFIN (Belgium) is in unsterilised crystallised form. For this experiment, we will test both sterilised and not sterilised struvite. To optimise the struvite dissolution, 10g of crystallised struvite were dissolve in 1L of demineralised water (MilliQ), shaking for 2h and then autoclaved for 20min at 121°C (sterilised struvite).



Composition of struvite from AQUAFIN (bart saerens *et al.*2021)

Cultivation of Limnospira indica PCC8005 P1 in modified "inspiration" medium.

- Preparation of culture medium

For each condition, a volume of medium to be prepared was calculated in advance, knowing that we would need 500ml/condition. The tables representing all the deficiency conditions tested (Table1) and the halving concentration of certain components (Table2) are reported.

Table1: summary table for 500mL by condition (blue cells represent the missing components)

chemicals	Masse or volume	M1	M2	M3	M4	M5	M6	M7	M8	M9	M11
NaHCO ₃	8 g	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok
KNO ₃	1g		ok	ok	ok		ok	ok		ok	
NaCl	0.5g	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok
(NH ₄) ₃ PO ₄	0.05g or 5mL from 1% solution	ok		ok	ok			ok		ok	ok
FeSO ₄	5 mg or 500 µL from 1% solution	ok	ok	ok	ok	ok		ok	ok	ok	ok
MgSO ₄	0.05g or 5mL from 1% solution	ok	ok		ok				ok	ok	
K ₂ SO ₄	0.25g	ok	ok	ok					ok	ok	
CaCl ₂	0.05g or 5mL from 1% solution	ok	ok	ok	ok	ok	ok	ok	ok		ok
Green Tea	500 µL	ok	ok	ok	ok	ok	ok	ok	ok	ok	ok

Tab 2: summary table for 500mL modified inspiration medium by halving concentration (blue).

chemicals	½ M7	½ M8	M10
NaHCO ₃	8 g	8 g	8 g
KNO ₃	1g	0.5 g	0.5 g
NaCl	0.5g	0.5g	0.5g
(NH ₄) ₃ PO ₄	0.05g or 5mL from 1% solution	25 mg or 2.5mL from 1% solution	25 mg or 2.5mL from 1% solution
FeSO ₄	5 mg or 500 µL from 1% solution	5 mg or 500 µL from 1% solution	5 mg or 500 µL from 1% solution
MgSO ₄	25mg or 2.5mL from 1% solution	0.05g or 5mL from 1% solution	25mg or 2.5mL from 1% solution
K ₂ SO ₄	125mg	0.25g	125 mg
CaCl ₂	0.05g or 5mL from 1% solution	0.05g or 5mL from 1% solution	0.05g or 5mL from 1% solution
Green Tea	500 µL	500 µL	500 µL

- Culture conditions of the inoculum

The culture stock of *Limnospira indica* PCC8005-P1 (replicates 1 to 4) were centrifuged at 3000 rpm for 5min, washed with PBS 1% and then were resuspended in the interest medium with struvite or without struvite (as control).

To conduct the experiment, 17 mL of modified “Inspiration” medium and 2 mL (or 1 g/L) of struvite (sterilised and/or not sterilised), are aseptically transferred to a 50 mL sterile cell culture flask with a ventilated filter cap. Next, 5% (1mL) of stock cultures of *Limnospira indica* PCC8005 in the late exponential phase are transferred aseptically into the medium containing struvite. The culture is then incubated at 30°C in a shaking light incubator at 120 RPM and constant illumination of approximately 35 µmol/m²*s (continuous light) or exposed to light/dark cycles on a shaker bench lab at room temperature (Edmund Buhler GmbH). The culture was followed for 14 days, the optical density was measured daily using a spectrophotometer (Nanocolor Uv/Vis) at a wavelength of λ=750nm and pH using a pH-meter (Mettler Toledo). Each modified inspiration medium is used as blank, the sample volume used for analysis is 1mL.

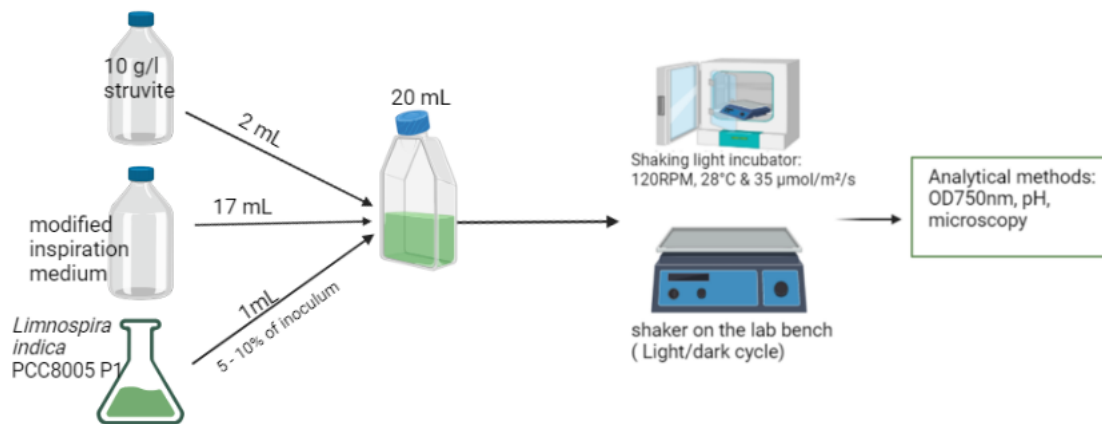


Figure 4: summary diagram for experiments with struvite (created by biorender).

To confirm the effect of struvite, experiments without struvite were conducted, i.e., 1 mL of *Limnospira indica* PCC8005 P1 were inoculated in 19 mL of each medium and then incubated under the same conditions as the test cultures.

- Alternatives Carbon source in inspiration medium

The carbonate source (NaHCO_3) in “inspiration” medium was replaced by either 5g of manioc flour (from Yoki) or 10,68g of sweet potato flour (from nu3 GmbH, Germany), then 1 mL of *Limnospira indica* PCC8005 was inoculated into 19 mL of each medium in a sterile 50 mL cell culture flask with vented filter cap and apply the same conditions as the previous experiments. (As a control, the "inspiration" medium without NaHCO_3 were used).

Microscopy

The observation of morphology of the cells of *L.indica* was done with a Leica optical microscope connected to a screen (figure 5). 10 μL of culture was placed on a slide and observation was made at different magnifications (using 5*, 10*, 20 *and 40* objectives).

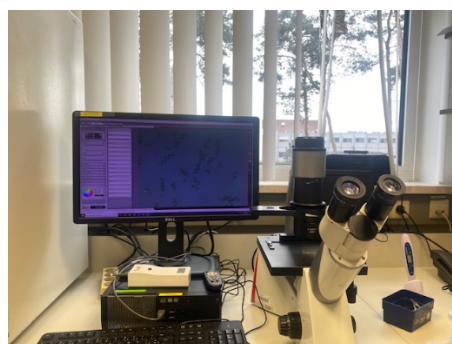


Figure 5: image of microscope connected to a screen.

Axenicity test

To check for the potential contamination of the medium, the axenicity test was done at the beginning and at the end of the experiment. To do this, 10mL of LB were transferred aseptically in a 50mL cell culture flask with vented cap and 9mL Zarrouk + 1mL LB in a other 50mL cell culture flask (these are two separated set-ups), and then 500 μ L of were *L.indica* was inoculated into these media. Culture was incubated and monitored up to 10 days.

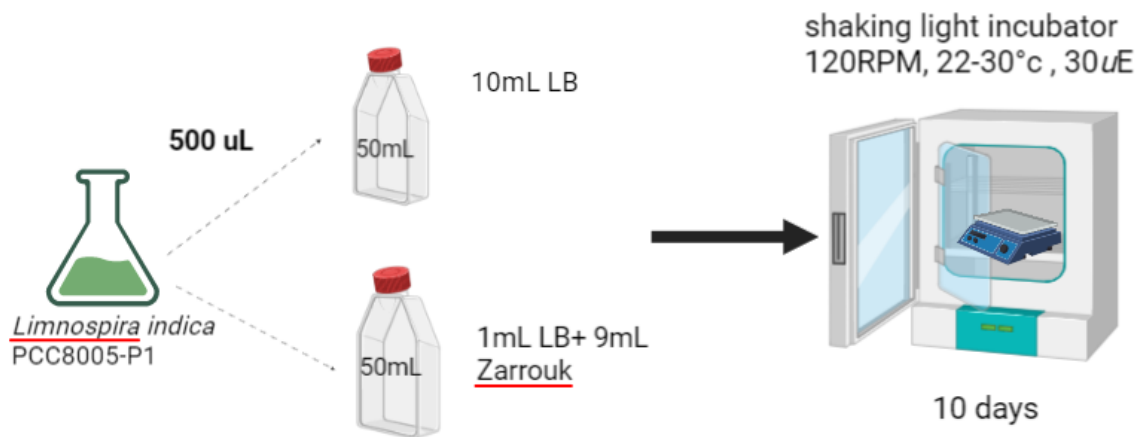


Figure 6: Summary scheme of axenicity test (created by biorender)

Flow cytometry

Flow cytometry measurement were performed using a BD ACCURI C6 flow cytometer (Beckton Dickinson, Erembodegem, Belgium) equipped with a blue (488nm) and a red (640nm) laser. 100 μ L of *Limnospira* culture were run in fast mode using the following thresholds: 10,000 in the forward scatter (FSC) and 800 in the fluorescent detector 4 (FL4), using the modified Zarrouk medium (Cogne et al., 2003) as blank background. FL3, associated to a 670 nm long pass filter and FL4, associated to a 675 \pm 12.5 nm filter were used to quantify pigments autofluorescence, respectively chlorophyll and phycocyanin. Previous work in our laboratory reported the importance of a population (P) in the total cell count cytogram, for the survival of a culture (Figure 7). More specifically healthy cultures were associated with a FL3 to FL4 ratio around 0.3, increasing to over 1 when the culture dies. In other words, cells containing approximately thrice the amount of phycocyanin compared to the amount of chlorophyll are more disposed to successful subculturing. In the rest of the paper, we will refer to these cells as "seed cells".

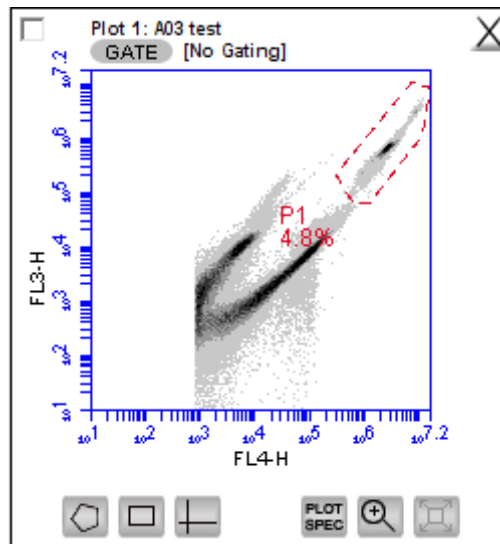


Figure 7: P1 colony highlighted in the BD Accuri flow cytometry data from samples of *L. indica*

Protein extraction and quantification

Extraction of spirulina was performed by taking 2mL of the stationary phase culture of *Limnospira indica* PCC8005 P1, first centrifuging to remove the medium and then resuspending the pellet with double the volume of pellet with 2% SDS lysis buffer (for 5ml: 0.125 ml 2M ammonium bicarbonate ,1 ml 10% SDS and 3.875 ml water). The resuspended pellet is then vortexed, incubated for 5 min at 95°C and then cooled on ice for 5 minutes. After a short spin, the solution is sonicated for 4 cycles of 10 secs at an amplitude of 40 cycli 1 per second while kept on ice. The final step involves centrifugation of the solution for 20 min at 14,000 rpm at 4°C, and the resulting supernatant is transferred to a new tube.

The BCA (Bicinchoninic acid) assay was utilised to quantify the protein content, employing a colorimetric method. The kit included various concentrations of BSA (bovine serum albumin) standard solutions (0.125; 0.250; 0.5; 0.75; 1.0; 1.5; 2.0 mg/ml). A mixture solution was then created by mixing Bicinchoninic acid solution (A) with copper sulfate solution (B). Next, two separate dilutions were made using the supernatant (which contained the protein), obtained from the extraction process.

For this purpose, the 96-well plate from CLARIOstar plus microplate reader (BMG Labtech) was used and filled first with 80ul of BCA mixture/well (of the total wells to be used), add

10ul/well of each standard BSA concentration in triplicate with three wells containing only water (as blank), add in duplicate 10ul of the samples (protein supernatant and the different dilutions). The plate was incubated at 37°C for 30min 500rpm in thermomixer (dark) and then the absorbance was measured at 562nm.

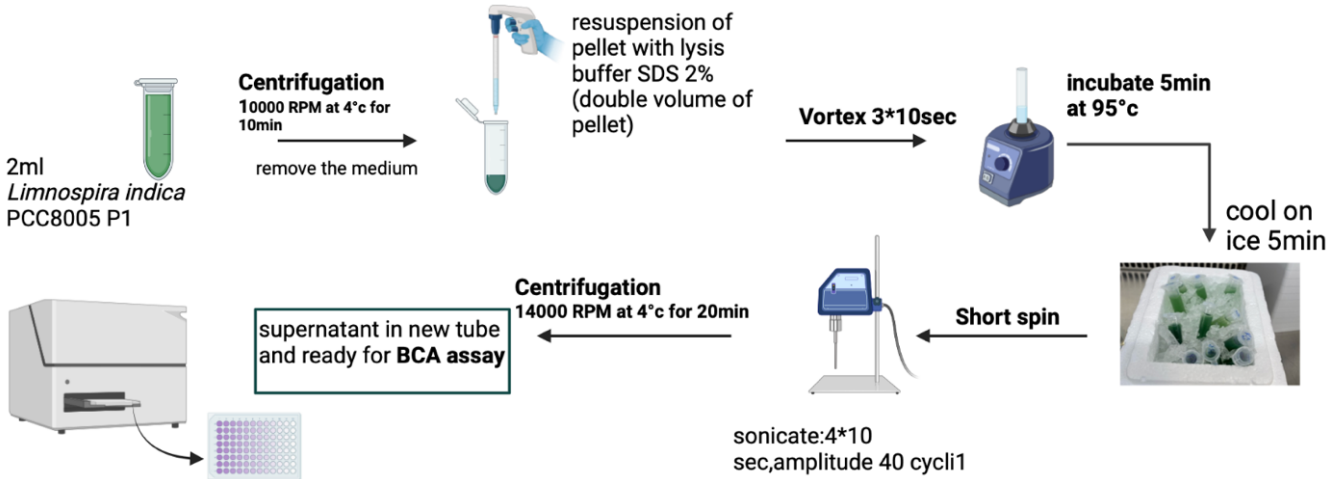


Figure 8: summary scheme for protein extraction and quantification (created by biorender).

Dry weight

For this experiment, 2 ml of each culture was taken in triplicate and filtered using filters, then these filters were dried at 80°C overnight and weighed with a precision balance.

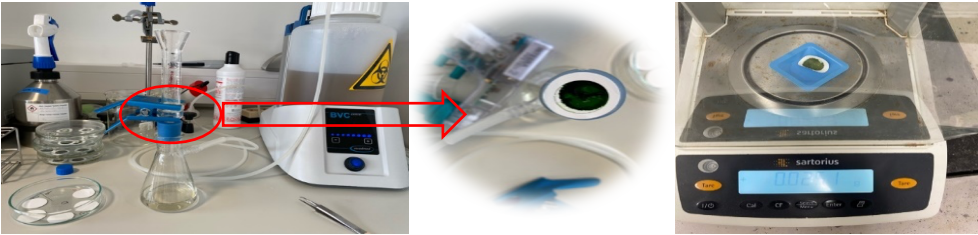


Figure 9: illustration of Spirulina filtration and weighing process.

V. Results

A. Growth performance under different conditions

1. OD evolution

The obtained results were generated through a combination of controlled laboratory conditions within an incubator, ensuring precise regulation of temperature (30°C) and light intensity (35 $\mu\text{mol}/\text{m}^2\cdot\text{s}$). Additionally, experiments outside the incubator were conducted under the influence of natural environmental conditions, encompassing variations in temperature and light intensity, as well as the light/dark cycle. The latter condition was used to mimic outdoor cultivation of *L.indica*.

We investigated the influence of the presence of struvite in inspiration medium on the growth of *Limnospira indica* PCC8005 P1 and we aimed to compare the grown performance of this strain in both the Zarrouk medium and inspiration medium.

The results showed that the growth of *Limnospira indica* PCC8005 P1 was significantly enhanced by continuous light. After 336 hours, OD reached 1.2 in Zarrouk medium and 1.4 in inspiration medium under continuous light. However, under light/dark conditions with a average of temperature and light intensity respectively 20 °C and 45 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, growth was delayed in both media. The pH of the medium was also monitored during the experiment. In Zarrouk medium, the initial pH was around 9.8 and reached a maximum of 11.5. In the inspiration medium, the values ranged from 8.5 to 10.5. (Figure 10)

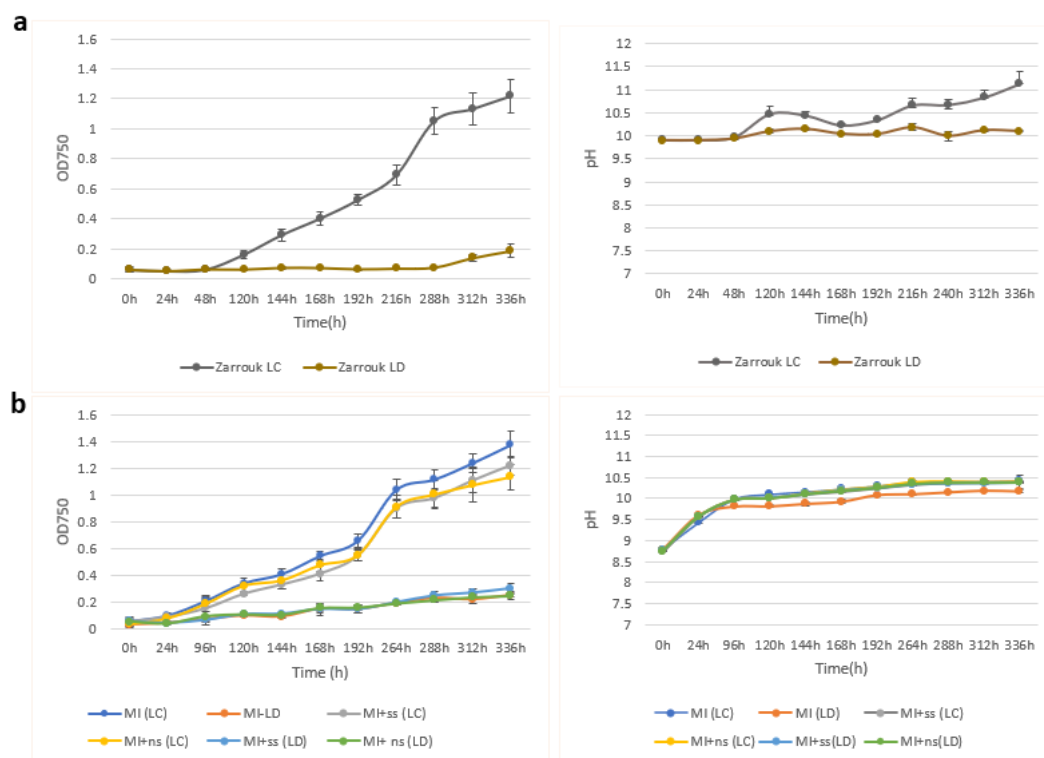


Figure 10: Evolution of optical density (OD) of *Limnospira indica* PCC8005 P1 **(a)** in Zarrouk medium and **(b)** in inspiration medium (MI) with sterilised struvite (ss), no sterilised struvite (ns) and without. The cultures were growing under light continuous (LC) and light/dark (LD) conditions. Each bar represents the average value \pm standard deviation (SD) of n = 4 replicates.

Removal of KNO_3 , $(\text{NH}_4)_3\text{PO}_4$, MgSO_4 or K_2SO_4

To further investigate the effect of selective removal of components from the inspiration medium (KNO_3 , $(\text{NH}_4)_3\text{PO}_4$, MgSO_4 , K_2SO_4) and the supplementation of struvite on the growth of *L.indica*, we conducted additional experiments.

The results revealed that the absence of $(\text{NH}_4)_3\text{PO}_4$ or K_2SO_4 led to a significant slowdown in the growth of *Limnospira indica* PCC8005 P1, under these conditions, growth was reduced compared with the complete inspiration medium, particularly under continuous light. In addition, the absence of KNO_3 or MgSO_4 resulted in poor growth, followed by cell death. In the case of light/dark cycle conditions, no growth was observed in the absence of these components (Figure 11a).

However, by adding struvite to each missing component, the growth of *Limnospira indica* PCC8005 P1 returned to levels like those of the complete inspiration medium for MgSO_4 and K_2SO_4 . In the case of KNO_3 and $(\text{NH}_4)_3\text{PO}_4$, growth was slightly lower, even with struvite supplementation. Furthermore, under light/dark cycle conditions, a late onset of growth was observed when the missing components were supplemented with struvite (Figure 11b).

We observed no significant difference in growth between the usage of sterilised and not sterilised struvite in our experiments even though the axenicity test showed potential contamination when the struvite was not sterilised than when it was sterilised. Additionally, the pH levels remained consistent in both the complete and incomplete inspiration medium. Although these specific results are not enumerated in the main text, they are available for reference in the appendix 4.

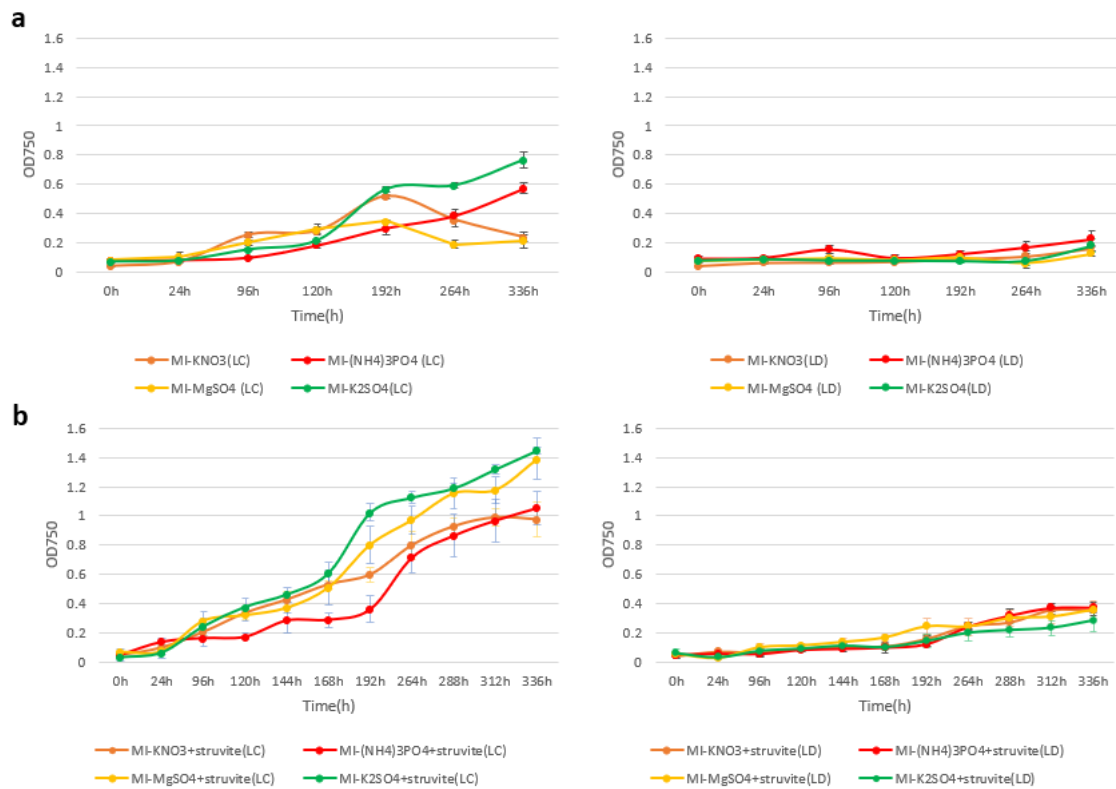


Figure 11: Evolution of optical density (OD) of *Limnospira indica* PCC8005 P1 under Light continuous (LC) and Light/dark cycle (LD). The cultures were grown in inspiration medium (MI) without (a) KNO_3 or $(\text{NH}_4)_3\text{PO}_4$ or MgSO_4 or K_2SO_4 while being supplemented by sterilised struvite (b). Each bar represents the average value \pm standard deviation (SD) of $n = 4$ replicates.

Removal of KNO_3 - $(\text{NH}_4)_3\text{PO}_4$ or MgSO_4 - K_2SO_4

Furthermore, we evaluated the effect of the combined removal of components (KNO_3 - $(\text{NH}_4)_3\text{PO}_4$ and MgSO_4 - K_2SO_4) and struvite supplementation on the growth of *L. indica*.

The results showed that the addition of struvite to medium without KNO_3 - $(\text{NH}_4)_3\text{PO}_4$ resulted in a slight change in the growth of *L. indica*. However, after 192 hours, growth stabilised or ceased completely, indicating growth limitation despite the presence of struvite (Figure 12a). In contrast, for the MgSO_4 - K_2SO_4 combination, initial growth was observed in the absence of struvite. However, after 144 hours, growth stopped and decreased thereafter, indicating growth limitation in the absence of these components. When struvite was added, the growth observed for 336 hours was lower than that of the complete inspiration medium, but

significantly higher than the growth of the previous pair in the presence of struvite (Figure 12b). Under the light/dark cycle condition, no growth was observed.

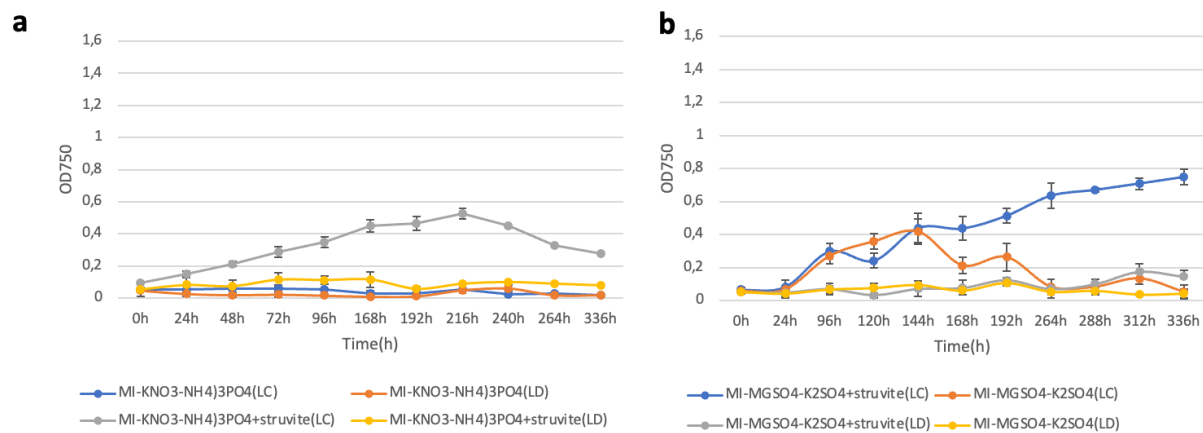


Figure 12: Evolution of optical density (OD) of *Limnospira indica* PCC8005 P1 in inspiration medium (MI) (a) without KNO₃ and (NH₄)₃PO₄ and (b) without MgSO₄ and K₂SO₄, both supplemented by struvite or not. The cultures were growing under continuous light (LC) and light/dark (LD) conditions. Each bar represents the average value ± standard deviation (SD) of n = 4 replicates.

Removal of KNO₃, (NH₄)₃PO₄, MgSO₄ and K₂SO₄

Subsequently, we carried out an experiment to evaluate the effect of the simultaneous absence of three components M11 (KNO₃-MgSO₄-K₂SO₄), both with and without the presence of struvite, on the growth of *Limnospira indica* PCC8005 P1. The results obtained revealed an initially weak growth, followed by a progressive decline when all three components were removed at the same time (figure 13a). The same observation was made in the experiment where we tried to remove all the 4 components (KNO₃-(NH₄)₃PO₄-MgSO₄-K₂SO₄) (figure 13b).

These results indicate that the simultaneous presence of KNO₃, (NH₄)₃PO₄, MgSO₄ and K₂SO₄ is essential to maintain adequate growth of *Limnospira indica* PCC8005 P1. In the absence of these components simultaneous, the growth of the strain was severely affected, resulting in a decrease in observed growth. The addition of struvite did not completely compensate for this absence, as growth remained low despite its presence.

These two experiments were repeated with an initial inoculum size of 10% due to the absence of any growth when starting with a 5% inoculum.

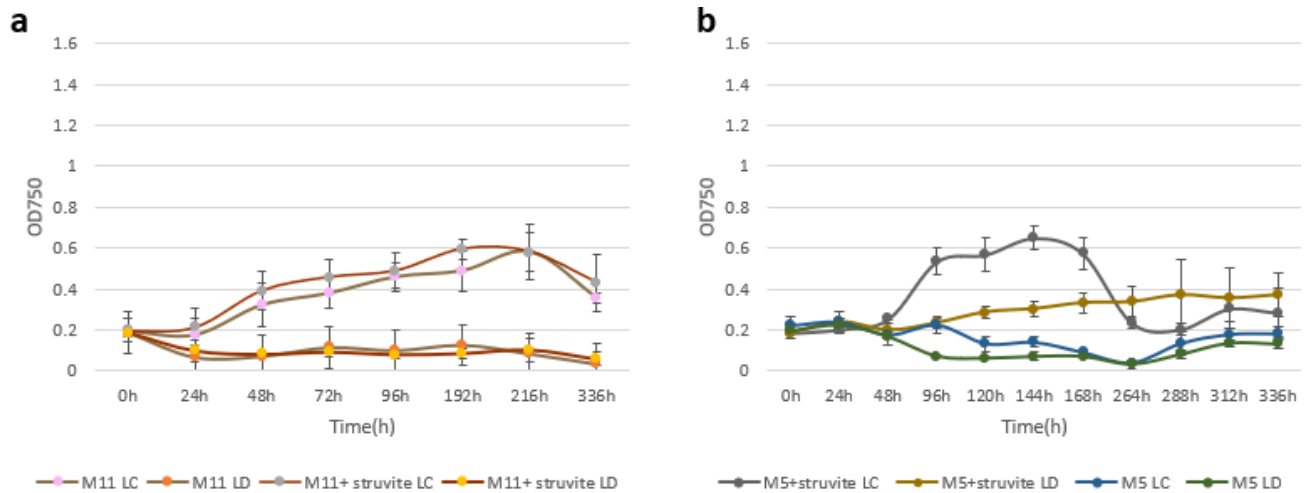


Figure13: Evolution of optical density (OD) of *Limnospira indica* PCC8005 P1 in inspiration medium (MI) **(a)** without KNO_3 , MgSO_4 and K_2SO_4 (**M11**) and **(b)** without KNO_3 , $(\text{NH}_4)_3\text{PO}_4$, MgSO_4 and K_2SO_4 (**M5**), both supplemented with struvite or not. The cultures were growing under light continuous (LC) and light/dark (LD) conditions. Each bar represents the average value \pm standard deviation (SD) of $n = 4$ replicates.

Removal of $(\text{NH}_4)_3\text{PO}_4$, MgSO_4 and K_2SO_4

When only KNO_3 was retained while supplementing the three other components with struvite, a significant growth comparable to that observed in the complete inspiration medium was observed. This finding suggests that nitrate (KNO_3) plays a crucial role as the key element for the growth of this microorganism. Furthermore, it indicates that struvite, at least partially, cannot fully substitute for the growth-promoting effects of nitrate (Figure 14).

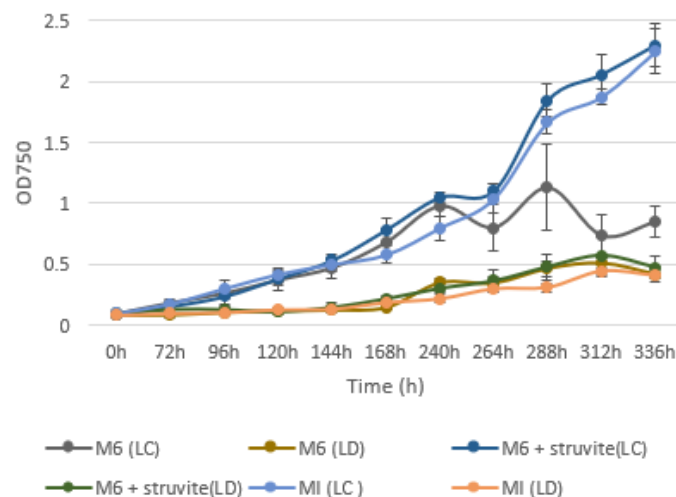


Figure14: Evolution of optical density (OD) of *Limnospira indica* PCC8005 P1 in inspiration medium (MI) and MI without $(\text{NH}_4)_3\text{PO}_4$, MgSO_4 and K_2SO_4 (**M6**) supplemented by struvite or not. The cultures were growing under continuous light (LC) and light/dark (LD) conditions. Each bar represents the average value \pm standard deviation (SD) of $n = 4$ replicates.

Partial Removal of KNO_3 , $(\text{NH}_4)_3\text{PO}_4$, MgSO_4 and K_2SO_4

Furthermore, we investigated the effect of halving the concentrations of the four components $\frac{1}{2}\text{M10}$ (KNO_3 - $(\text{NH}_4)_3\text{PO}_4$ - MgSO_4 - K_2SO_4) on the growth of *Limnospira indica* PCC8005 P1. Initially, we reduced the concentrations of each pair of components individually (KNO_3 - $(\text{NH}_4)_3\text{PO}_4$ and MgSO_4 - K_2SO_4), followed by reducing all four concentrations simultaneously. The results obtained revealed growth trends that were like the complete inspiration medium for the MgSO_4 - K_2SO_4 pair ($\frac{1}{2}\text{M7}$), and slightly lower for the KNO_3 - $(\text{NH}_4)_3\text{PO}_4$ pair ($\frac{1}{2}\text{M8}$), when cultures were exposed to continuous light. In addition, we also noticed growth in cultures subjected to the light/dark cycle. Interestingly, when all four compound concentrations were simultaneously reduced, we observed growth in cultures under continuous light, regardless of the presence or absence of struvite. However, no growth was observed for cultures exposed to the light/dark cycle. (Figure 15)

These results suggest that even when the concentrations are halved, growth is still possible but significantly attenuated compared to the complete medium. Notably, the presence or absence of struvite had no significant effect on growth under these conditions.

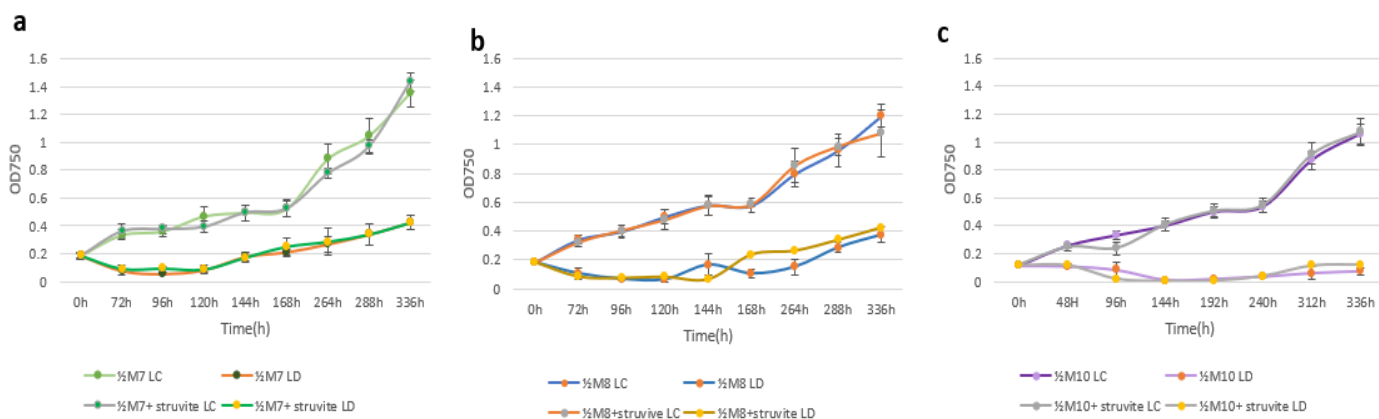


Figure15: Evolution of optical density (OD) of *Limnospira indica* PCC8005 P1 in inspiration medium (MI) without half concentration of **(a)** MgSO_4 and K_2SO_4 ($\frac{1}{2}\text{M7}$) **(b)** KNO_3 and $(\text{NH}_4)_3\text{PO}_4$ ($\frac{1}{2}\text{M8}$) and **(c)** KNO_3 , $(\text{NH}_4)_3\text{PO}_4$, MgSO_4 and K_2SO_4 ($\frac{1}{2}\text{M10}$) supplemented with

struvite or not. The cultures were growing under continuous light (LC) and light/dark (LD) conditions. Each bar represents the average value \pm standard deviation (SD) of n = 4 replicates.

Removal of carbon source

The experiments aimed at replacing the carbon source yielded contrasting outcomes. Remarkable growth was observed even in the absence of sodium bicarbonate (NaHCO_3), which was found to be significantly like the complete inspiration medium. Interestingly, this growth was observed not only under continuous light conditions but also in the light-dark cycle (Figure16). These findings suggest that sodium bicarbonate may not be a limiting factor for the growth of the studied organism, and alternative mechanisms may contribute to sustaining growth even in its absence. The substitution of sweet potatoes as a carbon source did not yield positive results, as no significant growth was observed. However, in the case of replacing the carbon source with cassava flour, a delayed initiation of growth was observed, accompanied by notably sluggish growth throughout the experiment (Table3).

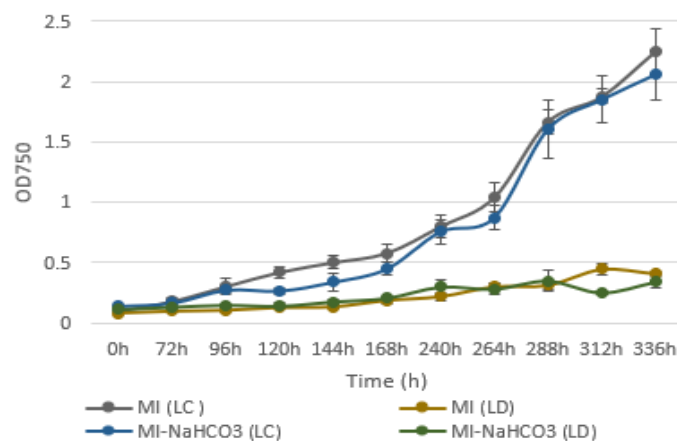


Figure16: Evolution of optical density (OD) of *Limnospira indica* PCC8005 P1 in inspiration medium (MI) and MI without NaHCO_3 . The cultures were growing under continuous light (LC) and light/dark (LD) conditions. Each bar represents the average value \pm standard deviation (SD) of n = 4 replicates

Table 3: OD and pH evolution of *Limnospira indica* PCC8005 P1 in inspiration medium with cassava flour as alternative carbon source under continuous Light (LC).

	average LC	SD	pH
0h	0.0995	0.024182	7.78
24h	0.12125	0.038147	9.06
168h	0.149	0.011769	9.4
336h	0.385	0.063887	9.86
672h	1.14	0.111131	10.01

Final composition

The results obtained from the previous composition tests have been utilised to formulate an optimal composition, which includes the components listed in Table 4.

Table 4: summary table for 500mL of optimised medium composition for Continuous Light and Light/dark.

chemicals	M12 for LC	M13 for LD
NaHCO ₃	4g	8g
KNO ₃	0,5g	1g
NaCl	0,5g	0,5g
FeSO ₄	5 mg or 500µL of 1% solution	5 mg or 500µL of 1% solution
CaCl ₂	25mg or 2,5mL of 1% solution	25mg or 2,5mL of 1% solution
Green Tea	500 µL	500 µL
Struvite	1g/l	1g/l

2. characteristics of cultures

Culture colour

One notable visual characteristic of the culture under investigation was its colour, wherein increased growth (biomass) corresponded to a darker green colour, indicating favourable growth conditions. Conversely, for instances where KNO₃ or NaHCO₃ were absent, a noticeable shift towards a green-yellow colour was observed during growth, regardless of the presence or absence of struvite. Additionally, flocs/aggregates formation was observed in cultures experiencing a deficiency of one or more components (Figure 17).

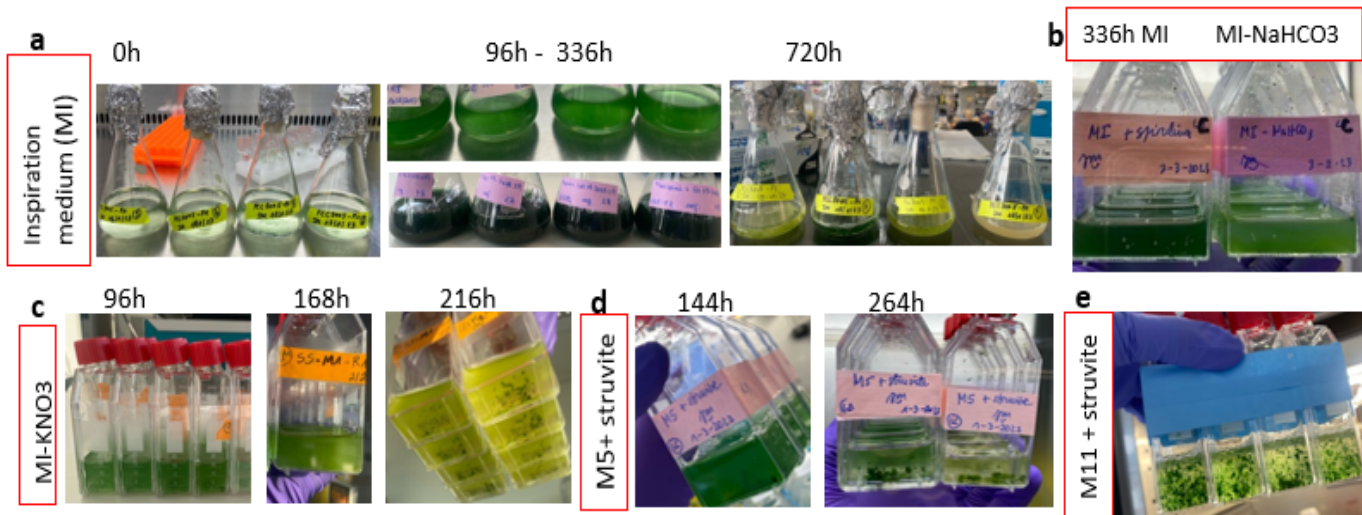


Figure 17: Colour evolution of *Limnospira indica* PCC8005 P1 in (a) complete inspiration medium (MI), without (b) NaHCO₃, (c) KNO₃ + struvite, (d) KNO₃; (NH₄)₃PO₄; MgSO₄ and K₂SO₄ + struvite (M5) and (e) KNO₃; MgSO₄ and K₂SO₄ + struvite (M11) and floccs formation in the supplemented medium.

Culture morphology

The cellular morphology of *Limnospira indica* P1 strain was investigated using light microscopy. The P1 strain is known for its spiral shape. Interestingly, when the cells were exposed to the light/dark cycle condition for multiple days, a distinct alteration in their shape was observed. Specifically, the cells exhibited a more contracted morphology compared to their typical spiral shape (Figure 18).

This finding suggests that the light/dark cycle condition may have an influence on the cellular structure and morphology of *Limnospira indica*, leading to observable changes in its shape.

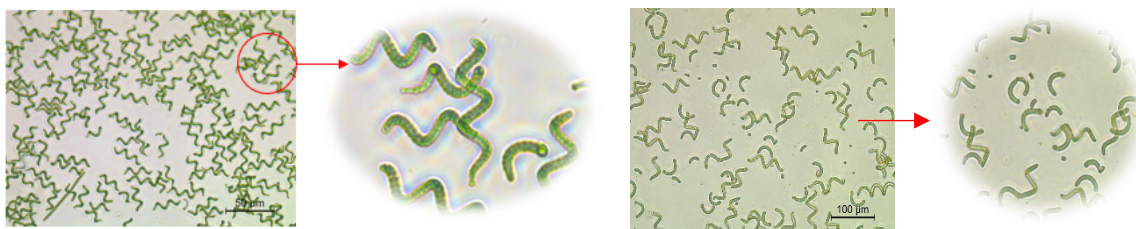


Figure 18: Morphology of *Limnospira indica* PCC8005 P1 at 336h under continuous Light (left) and light dark cycle (right) conditions as observed using optical microscopy.

B. Axenicity test

At the end of each experiment, we carried out this test to assess the purity of our cultures. A lot of contamination was observed in the culture with not sterilised struvite and cultures maintained under light/dark condition (outside of the incubator). the contamination was mainly observed in the LB medium, which changes colour in the presence of contamination (growth of other microorganisms) and remains intact when the culture is pure. (Figure 19)



Figure 19: image of contamination indicated by change in colour of LB medium (highlighted in red circle) in comparison to its typical colour evident in the Flacks remnants.

C. Viability of cells

Flow cytometry was employed as a method to evaluate cell viability in the study. For this purpose, a sample volume of 100 μ l was analysed. The focus was on the P population, representing the viable cell population. To determine viability, the median fluorescence intensity of chlorophyll (FL3-H) was compared to that of phycocyanin (FL4-H). In order to classify the cells as viable, the ratio of FL3-H to FL4-H should be around 0.3. This criterion was applied to distinguish viable cells from non-viable ones in the flow cytometry analysis.

In our study, the findings consistently demonstrated that all experiments conducted without the presence of KNO₃, regardless of the presence or absence of struvite, resulted in a ratio slightly exceeding 0.3. It is crucial to note that while this result is not fatal, since the ratio is not absolute, it does reinforce the importance of nitrate for the health and well-being of *L.indica* (Please refer to the appendix 5 for overview results).

D. Protein extraction and quantification

The BCA technique was employed to determine protein concentrations. The exact protein concentrations were calculated by subtracting the standard concentrations. A trendline curve was generated using the standards, and protein concentrations falling within the curve were used to calculate the protein quantity in mg/ml (x) using the equation of line ($y=ax+b$).

Additionally, dry weights were used to relate the protein concentration to a given mass quantity. (Please refer to the appendix 6 for a detailed calculation example).

For instance, we calculated the protein concentration for cultures with complete inspiration medium under continuous light and under the light/dark cycle (at 360 h) , which we related to their dry weight respectively.

Table 5. Protein quantification

Average of replicates	mgProtein concentration/ mgDry weight
Inspiration medium (LC)	2.63
Inspiration medium (LD)	2

VI. Discussion

The goal of this project was to identify the optimal composition of inspiration medium supplemented with struvite for promoting the growth of *Limnospira indica* PCC8005 P1 and achieving high spirulina production. This research aims to establish conditions that can be replicated on a larger scale for production in DR Congo.

To achieve our goal, we conducted experiments under controlled environmental conditions. The results of these experiments indicate that the inclusion of struvite in the culture medium has a positive impact on the growth of *Limnospira indica* PCC8005 P1. Moreover, struvite supplementation partially or completely compensates for the absence of individual key components such as KNO_3 , $(\text{NH}_4)_3\text{PO}_4$, MgSO_4 , and K_2SO_4 .

Nitrogen is an essential nutrient for the development and composition of spirulina, as it is required to produce proteins including photosynthetic pigments. The observed chlorosis, characterised by a yellow-green colour, in the absence of KNO_3 despite the presence of struvite, has been previously documented in other microalgae species such as *Chlorella vulgaris* (Tao Li *et al.*2016). Nitrogen limitation negatively affects the photosynthetic activity and leads to dynamic changes in the lipid, protein, and carbohydrate composition of *Spirulina platensis* (Xiaoting Li *et al.*2018). Studies on *Chlorella vulgaris* have shown that nitrogen

limitation can significantly affect the regulation of genes involved in carbon fixation and photosynthesis (Z. Ikaran *et al.* 2015).

The AQUAFIN struvite used in our experiments provides several important elements for *Spirulina* growth. Firstly, it supplies magnesium, a crucial element in cyanobacterial species, as it is a central component of the chlorophyll molecule essential for photosynthesis. Secondly, it contains phosphate, which is a key factor for energy metabolism, Lastly, it serves as a source of nitrogen.

While sterilisation of struvite reduces contamination, our results indicate that it does not significantly enhance growth compared to not-sterilised struvite in *Limnospira indica* PCC8005 P1, contrary to the findings reported by Markou *et al.* (2019).

Numerous studies have emphasised the significance of carbon sources in maintaining a basic pH for *Spirulina* growth. However, our experiment, as depicted in Figure 16, provided compelling evidence to suggest that spirulina can indeed grow without the presence of a carbon source directly in the medium, but it can dissolve the CO₂ from atmosphere in the culture medium as carbon source. In our final composition, we opted to include a carbon source (NaHCO₃), albeit at a reduced concentration, to ensure the overall quality of the results. This decision was made to provide optimal conditions for *Spirulina* growth and to minimize any potential limitations that may arise from carbon source deficiency.

In our experiments, we investigated the potential use of sweet potatoes and cassava flour as carbon sources for *Limnospira indica* PCC8005 P1 growth. However, we observed that neither sweet potatoes nor cassava flour were effective in promoting growth. It is possible that the lack of success can be attributed to the differences in the preparation and origin of these products. In the studies by Bandara and Arunakumara (2020), they utilised fresh flour prepared by themselves, which may have provided different nutritional characteristics compared to the commercially available products we used from the supermarket (appendix7). Similarly, Wei *et al.* (2009) and Agwa *et al.* (2014) employed cassava starch and waste as carbon sources, which could have yielded different results due to variations in composition and nutrient availability.

The light/dark condition had a profound impact on the growth of *Spirulina*, resulting in a significant delay or even the absence of substantial growth, even when the growth medium

was complete. In most instances, there was no observable growth under the light/dark cycle. However, in a few cases where growth was observed (Figure 14, 15 and appendix 8), it coincided with favourable environmental conditions, such as a temperature of 30 °C and an average light intensity of up to 168 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ (sunlight) compared to the average of normal temperature and light intensity respectively 20 °C and 45 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, observed in the laboratory from January to April.

The light/dark cycle also led to the formation of flocs in cultures where the inspiration medium was incomplete, even when supplemented with struvite. This posed challenges in obtaining accurate optical density measurements and the cultures progressively lost their characteristic spiral shape. These observations highlight the sensitivity of *Limnospira indica* PCC8005 to the light/dark condition and the significance of environmental factors in determining its growth behaviour. Further investigations into the underlying physiological and biochemical mechanisms involved in these growth responses could provide valuable insights for optimising Spirulina cultivation strategies and maximising its potential applications.

The choice to increase the inoculum size from 5 to 10% was crucial in ensuring successful cultivation and obtaining measurable results under different experimental conditions, namely under light/dark cycle and continuous light (as depicted in figure 13 and 15). These observations align with findings from previous studies conducted on *Spirulina platensis* (Nawal K.Z. Alfadhly *et al.* 2021), It is important to maintain an appropriate concentration /volume of inoculum to ensure the viability and acclimation of the culture.

Flow cytometry was exclusively conducted on cultures maintained under light continuous, as this method requires enough biomass for accurate analysis. We focused on evaluating the fluorescence signals of two pigments, phycocyanin and chlorophyll, within the viable population. The ratio chlorophyll/phycocyanin is anticipated to be lower to 0.3, due to the relatively higher abundance of phycocyanin in cyanobacteria. Cyanobacteria are known to have elevated phycocyanin levels compared to other pigments (DK Saini *et al.* 2018)

During our experimental investigations, we conducted an axenicity test to evaluate the presence of any unwanted contaminants prior to inoculation and at the conclusion of the culture, even if the inspiration medium wasn't sterilised there was no observable

contamination this would be partly because of the basicity of the medium limiting contamination.

Through our experimental endeavours, we successfully identified the specific components of the inspiration medium that could be replaced by struvite. This allowed us to formulate a culture medium suitable for controlled conditions, as well as one capable of withstanding environmental variations such as low temperature and low illumination. The controlled condition (incubator) tests served as a basis for potential scale-up, provided the outcomes were favourable. However, it is important to note that the light/dark condition emerged as a crucial variable impacting growth, potentially limiting our prospects.

Notably, the results obtained under light/dark (LD) conditions sometimes yielded contradictory findings. For instance, when testing the complete inspiration medium, different outcomes were observed depending on the specific environmental parameters during the experiments. Interestingly, when we reduced the concentrations of two (KNO_3 and $(\text{NH}_4)_3\text{PO}_4$ or MgSO_4 and K_2SO_4) or four components (KNO_3 , $(\text{NH}_4)_3\text{PO}_4$, MgSO_4 , and K_2SO_4) at the same time, using the same temperature and light intensity conditions within the same week, no growth was observed when reducing four components simultaneously. This observation led us to hypothesise that the growth of *Limnospira indica* PCC8005 P1 is influenced by multiple factors that must align simultaneously.

The utilisation of struvite in the cultivation of *Limnospira indica* PCC8005 P1 represents a significant advancement in terms of cost reduction for the growth medium knowing that its price varies around 80 euros per tonne. Moreover, it opens the possibility of cultivating this organism in open ponds, which holds promise for successful production under the environmental conditions of the Democratic Republic of the Congo.

For the continuation of this study, another crucial aspect to consider is the constant struvite concentration, even when supplementing four components simultaneously. It is plausible that by increasing the struvite concentration, it would be possible to supplement more than three components at once without impeding growth. However, in order to validate this hypothesis, further studies need to be carried out in this specific direction.

Therefore, future studies should focus on exploring the effects of varying struvite concentrations and its potential impact on the simultaneous supplementation of multiple components. This additional research will provide valuable information and confirm the viability of this theory.

VII. Conclusions

In conclusion, our work has produced convincing results indicating that *Limnospira indica* PCC8005 shows remarkable adaptability to struvite, using it as a vital source of nutrients for robust growth. This result marks a significant step towards the valorisation of waste products (struvite) and the large-scale, profitable production of spirulina adapted to the environmental and economic conditions of the Democratic Republic of Congo.

Throughout our research, we carried out continuous light and light/dark experiments, to assess the influence of light intensity and environmental parameters on the growth of *Limnospira indica* PCC8005 P1. The results of this research enabled us to formulate optimised growth media compositions: M12 for continuous light conditions and M13 for the light/dark cycle.

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IX. Appendix

1. Composition of Spirulina grown in controlled environment: amino acids, macronutrients, traces elements, vitamins, phytonutrients and minerals salts (Giacomo Fais et al, 2022).

Macronutrients	% dry weight	Carbohydrate	Mg/100g dry weight
Proteins	50-70	Glucose	54.4
Carbohydrate	15-20	Galactose	2.6
Lipids	7-16	Mannose	9.3
Vitamins	mg/100g dry weight	Rhamnose	22.3
Carotene	140	xylose	7
Biotin	0.005	Lipids	g/100g dry weight
Folic acid	0.01	Arachidic	0.048
Riboflavin	4	Gamma linolenic	1
Thiamine B1	3.5	Myristic	0.041
Vitamin E	100	Oleic	0.017
Vitamin B12	0.32	palmitic	2
Vitamin K	2.2	Amino acids	g/100g dry weight
niacin	14	Leucin	4.94
Minerals	mg/100g dry weight	Isoleucine	3.2
Calcium	700	Lysin	3.02
Copper	1.2	Methionine	1.15
Iron	100	Phenylalanine	2.78
Magnesium	400	Threonine	2.97
Manganese	5	Tryptophane	0.93
Phosphorus	800	valine	3.51
potassium	1400	Phytonutrients	g/100g dry weight
sodium	900	Cis beta-carotene	0.073
zinc	3	Chlorophyll a	1
Beta carotene trans	0.26	phycocyanin	12

2. Major Cyanobacterial pigments and their uses (D.K Saini et al, 2018)

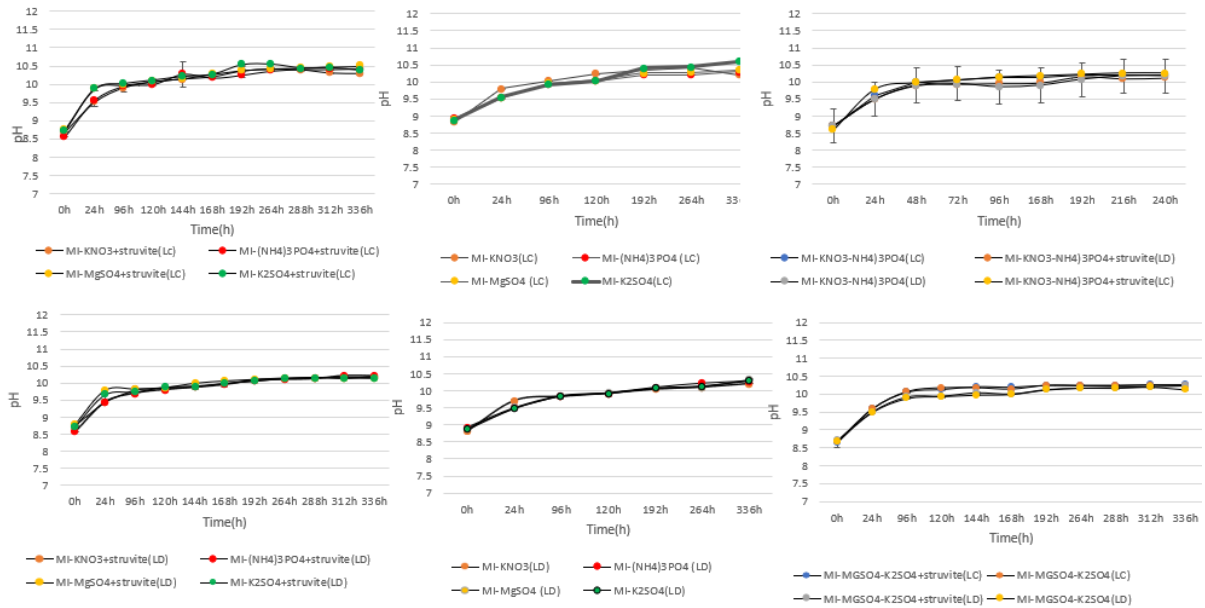
Table 1
Major Cyanobacterial pigments and their uses.

S.No.	Pigment	Absorption spectrum	Molecular weight (kda)	Color	Uses and activity	Reference
1.	Chlorophyll a	662, 430 nm	892	Green	Pharmaceutical and cosmetics (Deodorant)	da Silva Ferreira and Sant'Anna, 2017
Phycobiliproteins						
2.	Phycocerythrin	490-570 nm	240 000	Red	Immunofluorescence techniques, antibody labeling	Sonani et al., 2018
3.	Phycocerythrocyanin	560-600 nm	232 000	Orange		Chandra et al., 2017
4.	Phycocyanin	610-625 nm	232 000	Blue	Food colorant (ice Cream, sweets); cosmetics; Immunofluorescence techniques; Antibody labeling,	Tavanandi et al., 2018
5.	Allophycocyanin	650-660 nm	105 000	Bluish green		Rastogi et al., 2015
Carotenoids						
6.	β -Carotene	425, 450, 480 nm	536.87	Orange	Antioxidants, used as pro-vitamin A	Nagy et al., 2018
7.	Astaxanthin	477 nm	105 000	Red	Antioxidant, Anti cancer	Fonseca et al., 2015; Guerin et al., 2003
8.	Fucoaxanthin	420, 444, 467 nm	658.91	Olive green	Anti-obesity, anti-proliferative activity	Menichini et al., 2018
9.	Lutein	425, 448, 476 nm	568.87	Yellowish red		Zuluaga et al., 2017
10.	Zeaxanthin	428, 454, 481 nm	568.87	Yellow	Used as in age related eye diseases, anti-cancerous activity	Zuluaga et al., 2017
11.	Scytonemin	384 nm	544.56	Yellow brown	Anti-proliferative, anti-inflammatory.	Rastogi et al., 2016; Soule et al., 2007

3. Composition of medium Zarrouk (G. Cogne, 2002)

Component	g / L	Authorized supplier
K ₂ HPO ₄	0.5 g	VWR 26931.263
NaHCO ₃	10.5 g	SIGMA S6297
Na ₂ CO ₃	7.6 g	SIGMA S7795
NaNO ₃	2.5 g	SIGMA S5506
K ₂ SO ₄	1.0 g	MERCK / VWR 1.05153.0500
NaCl	1.0 g	MERCK / VWR 1.06404.1000
MgSO ₄ . 7H ₂ O	0.08 g or 8 mL from 1% solution	MERCK / VWR 1.05886.0500
CaCl ₂	0.03 g or 3 mL from 1% solution	SIGMA C1016-500
FeSO ₄ . H ₂ O	0.01 g or 1 mL from 1% solution	VWR 1.03965.0500
EDTA.Na ₂	0.08 g or 8 mL from 1% solution	SIGMA E5134-500 (.2H ₂ O)
MnCl ₂ . 4H ₂ O	0.23 g or 23 mL from 1% solution	MERCK / VWR
ZnSO ₄ . 7 H ₂ O	0.11 g or 11 mL from 1% solution	MERCK / VWR
CUSO ₄ . 5 H ₂ O	0.03 g or 3 mL from 1% solution	MERCK / VWR

4. pH evolution



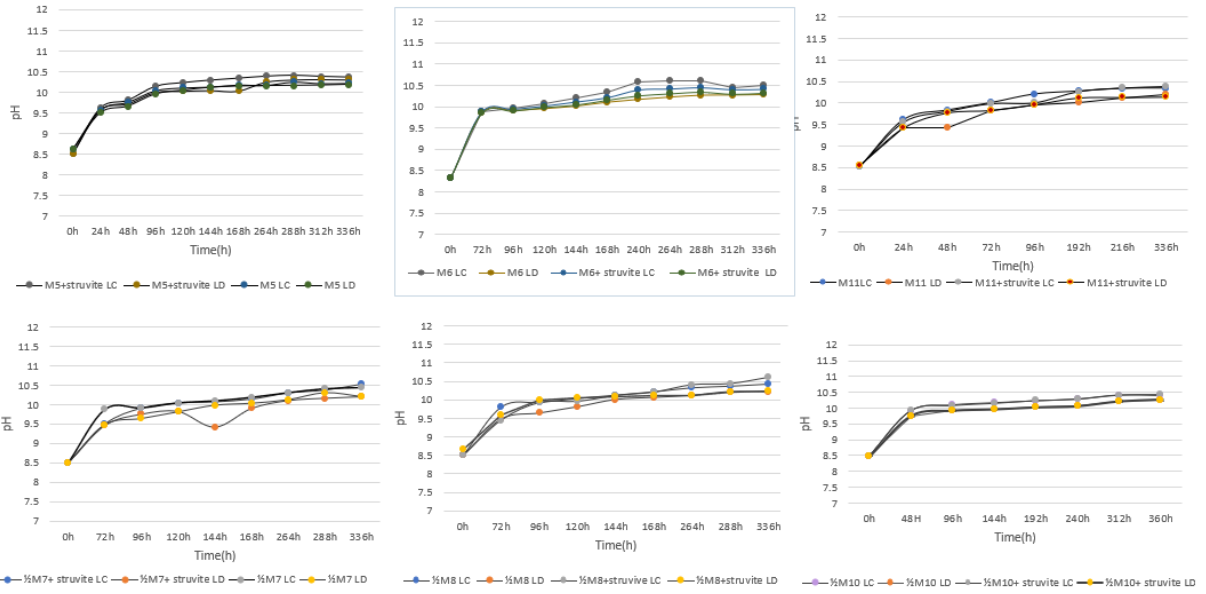


Figure: evolution of pH

5. Overview results

		MI				KNO3				(NH4)3PO4				MgSO4			
		LC		LD		LC		LD		LC		LD		LC		LD	
		struvite	NO	struvite	NO	struvite	no	struvite	no	struvite	no	struvite	no	struvite	no	struvite	no
Growth (OD)	>1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0,5 <= OD < 1																
	< 0,5																
Morphology	normal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	flocs																
	darkgreen																
colors	green	+	+	+	+					+	+	+	+	+	+	+	+
	yellow-G					+	+	+	+								
	yellow-G																
Health (flow cytometry)	<= 0,3																
	>= 0,3																
	growing	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AFTER 14 DAYS (336h)	died																

		K2SO4				CaCl2				MgSO4-K2SO4				KNO3-(NH4)3PO4			
		LC		LD		LC		LD		LC		LD		LC		LD	
		struvite	NO	struvite	NO	struvite	no	struvite	no	struvite	no	struvite	no	struvite	no	struvite	no
Growth (OD)	>1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0,5 <= OD < 1																
	< 0,5																
Morphology	normal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	flocs																
	darkgreen																
colors	green	+	+	+	+					+	+	+	+	+	+	+	+
	yellow-G					+	+	+	+								
	yellow-G																
Health (flow cytometry)	<= 0,3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	>= 0,3																
	growing	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AFTER 14 DAYS (336h)	died																

		KNO3-(NH4)3PO4-MgSO4-K2SO4				(NH4)3PO4-MgSO4-K2SO4				KNO3-MgSO4-K2SO4				half concentration MgSO4 & K2SO4			
		LC		LD		LC		LD		LC		LD		LC		LD	
		struvite	NO	struvite	NO	struvite	no	struvite	no	struvite	no	struvite	no	struvite	no	struvite	no
Growth (OD)	>1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0,5 <= OD < 1																
	< 0,5																
Morphology	normal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	flocs																
	darkgreen																
colors	green	+	+	+	+					+	+	+	+	+	+	+	+
	yellow-G					+	+	+	+								
	yellow-G																
Health (flow cytometry)	<= 0,3																
	>= 0,3																
	growing	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AFTER 14 DAYS (336h)	died																

6. Protein quantification calculations

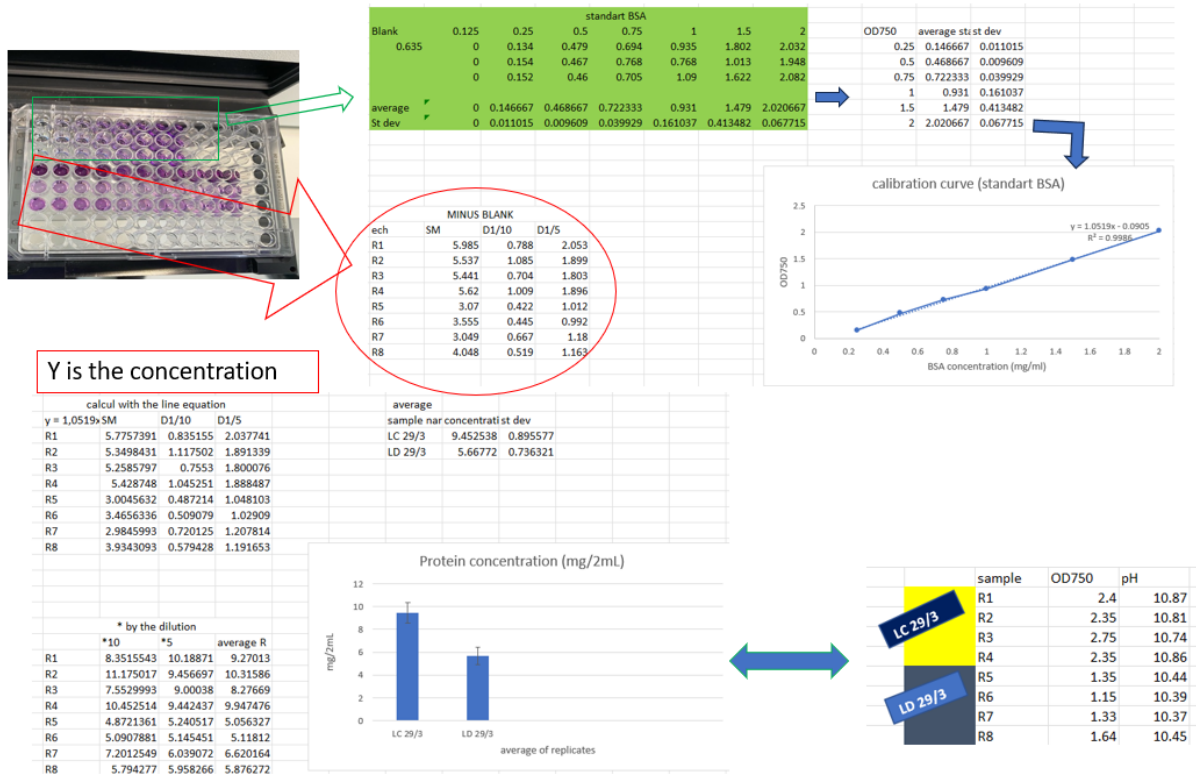
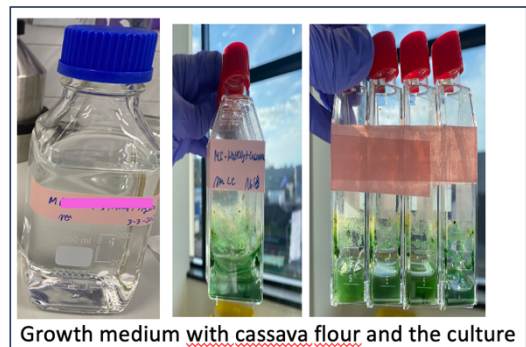
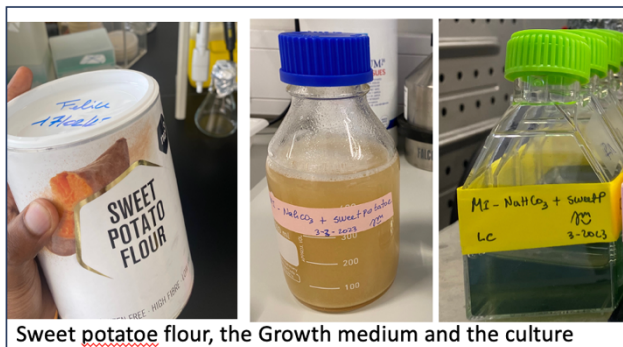
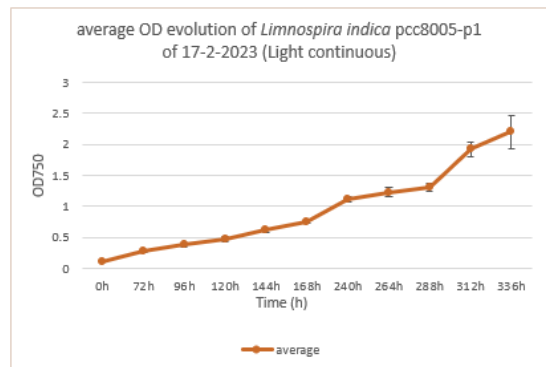
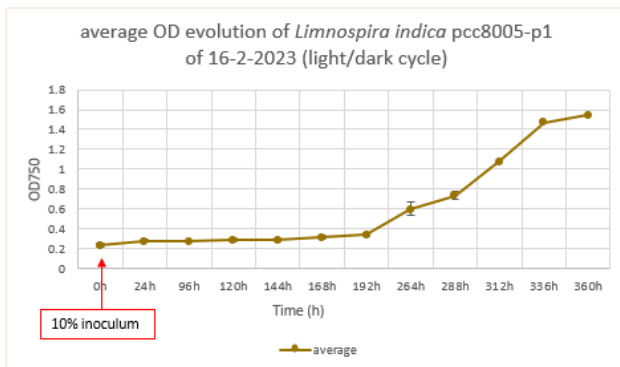


Figure: example of calculation for the stock culture in inspiration medium.

7. Images of cassava and sweet potatoes flours



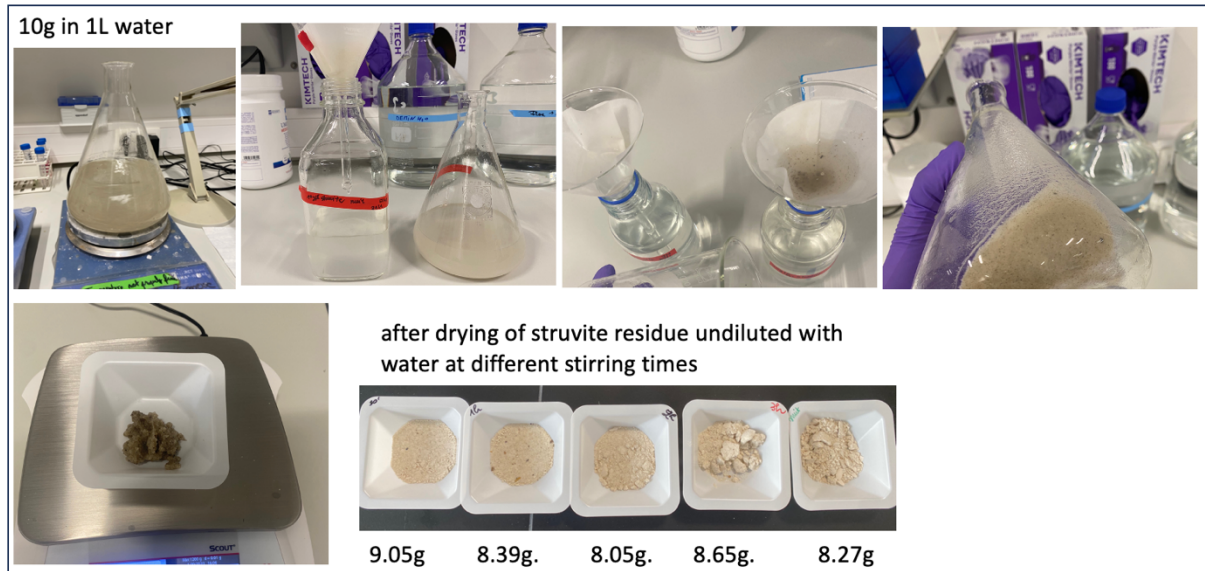
8. Graphic of OD evolution in inspiration medium



Appendix for additional information

a. Preparation of struvite

Furthermore, we observed a limited dissolution of struvite in the water, prompting us to make multiple attempts at agitation. Regrettably, our endeavours proved unsuccessful in achieving the desired outcome.



b. Molar Composition of key components of inspiration medium

For 1L of INSPIRATION medium:

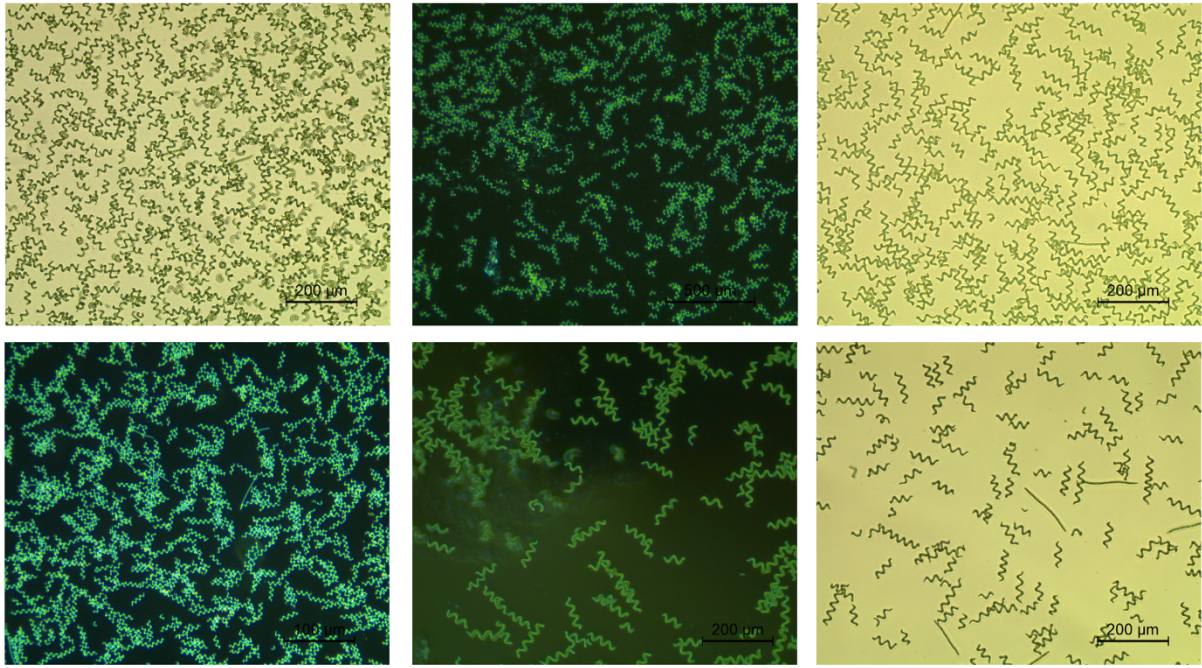
- 19.78 mM/L of nitrate from KNO_3^-
- 2 mM/L of ammonium from $(\text{NH}_4)_3\text{PO}_4$
- 0.6 mM of phosphate from $(\text{NH}_4)_3\text{PO}_4$
- 0.8 mM of magnesium from MgSO_4
- 5.7 mM of potassium from K_2SO_4

c. Images from microscopy

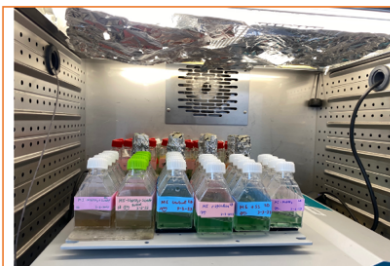
Objectives:

5x: 500 μm 20x :100 μm

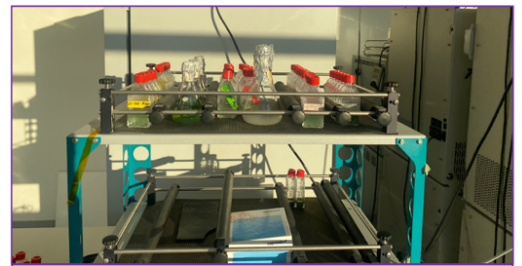
10x: 200 μm 40x:50 μm



d. Images of cultures



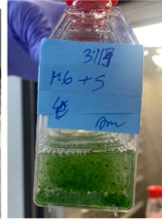
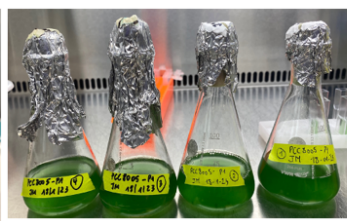
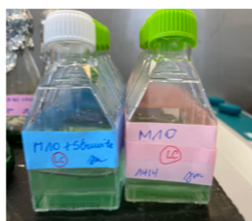
Cultures on shaking light continuous incubator



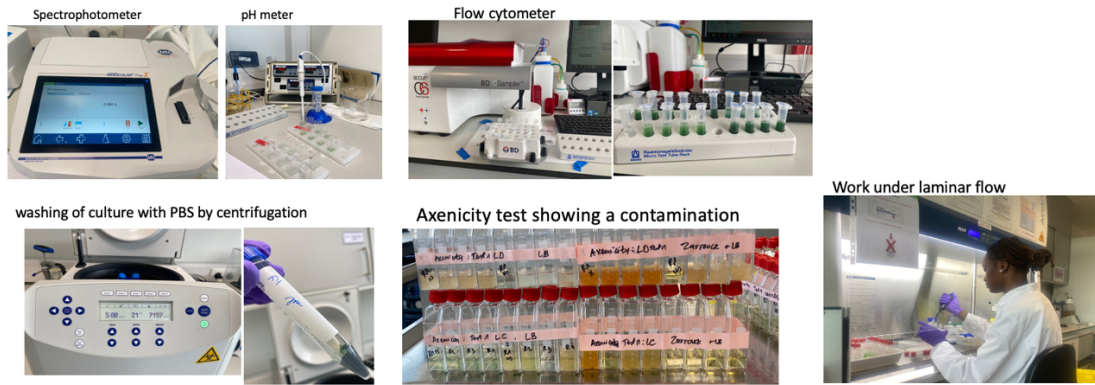
Cultures on shaker under light/dark cycle



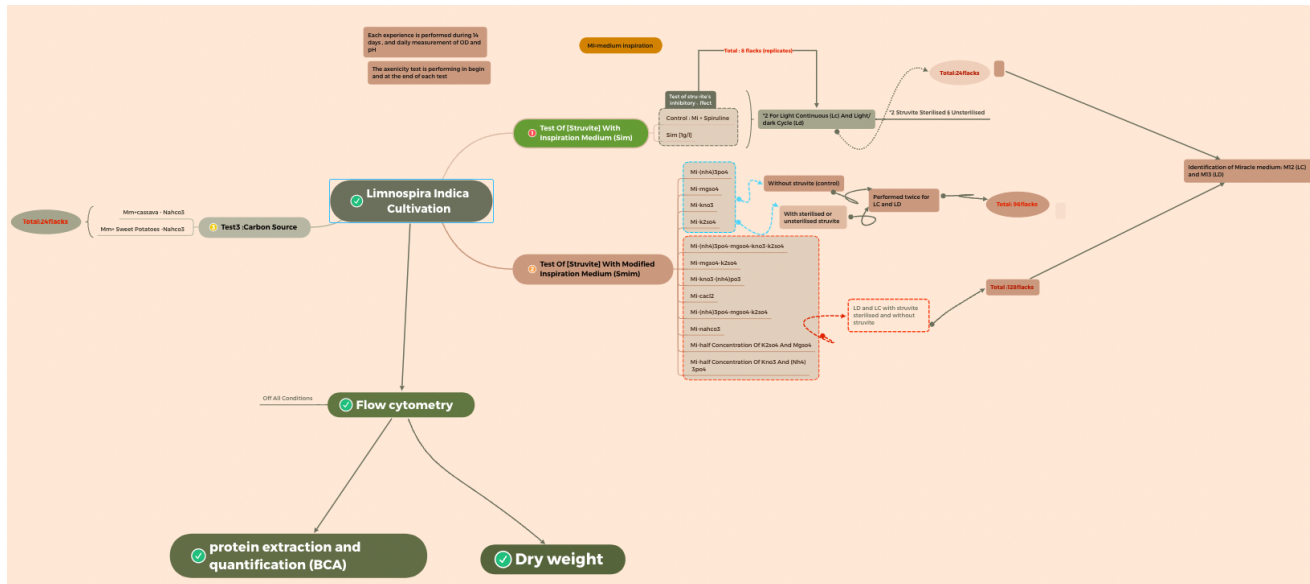
Cultures



e. Images during lab work



f. Mind map of all experiments carried out.



Resume (English)

Spirulina cultivation, renowned for its nutritional and therapeutic benefits, presents a challenge due to the high cost of chemical-based growth media. To address this, we have explored struvite, a nutrient-rich by-product of wastewater treatment, as a viable alternative. Our results demonstrated that struvite can effectively replace 3 components ($(\text{NH}_4)_3\text{PO}_4$, MgSO_4 et K_2SO_4) of the Inspiration medium. We also established that spirulina growth is influenced by both environmental factors (temperature and light) and the composition of the growth medium. As a result, an optimised struvite-based medium has been identified for large-scale production in the Democratic Republic of Congo.

Keywords : *Limnospira indica* cultivation, Struvite, Protein content, Nutrition

Résumé (Français)

Depuis des nombreuses années, la spiruline suscite un grand intérêt dans la recherche en raison de ses propriétés nutritives et thérapeutiques. Dans le cadre du projet INSPIRATION de la SCK CEN, l'objectif est d'utiliser la spiruline pour lutter contre la malnutrition en république démocratique du Congo. Pour atteindre cet objectif, il est nécessaire de mettre en place un milieu de culture moins coûteux favorisant la croissance de la spiruline et permettant une production à grande échelle.

Limnospira indica aussi connu commercialement sous le nom de spiruline, est une cyanobactérie qui se développe naturellement dans les lacs alcalins. Cependant, sa culture en laboratoire nécessite l'utilisation de milieux de croissance à base des produits chimiques contenant une source de phosphore, d'azote, de carbone et d'oligo-éléments, entraînant des coûts de production élevée à grande échelle. Cette problématique nous a conduit à l'étude de la struvite, un sous-produit du traitement des eaux usées riche en éléments chimiques essentiels à la croissance de la spiruline.

Dans le cadre de cette étude, nous avons réalisé des expériences à la fois dans des conditions environnementales contrôlées, à l'aide d'un incubateur, et dans des conditions non contrôlées, sur la paillasse du laboratoire. Les conditions contrôlées nous ont permis d'obtenir des résultats indiquant que l'ajout de struvite dans le milieu de culture avait un effet positif sur la croissance de *Limnospira indica*. Nous avons également démontré que la struvite pouvait efficacement remplacer trois composants spécifiques, à savoir le $(\text{NH}_4)_3\text{PO}_4$, MgSO_4 et K_2SO_4 , du milieu Inspiration. Nos résultats dans les conditions non contrôlées ont révélé que la croissance de la spiruline dépendait de plusieurs paramètres environnementaux, tels que la température et la lumière, en plus de la composition du milieu de culture. Cela souligne l'importance de prendre en compte ces paramètres pour optimiser la culture de la spiruline.

Cependant, nous avons réussi à formuler une composition de milieu optimisée avec la struvite, ce résultat ouvre la voie à une production économique à grande échelle de ce superaliment en utilisant la struvite comme alternative plus abordable aux composants chimiques traditionnels.

Mots clés : culture de *Limnospira indica*, Struvite, teneur en protéine, nutrition.