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Corso di Laurea in Biotecnologie Vegetali e Microbiche

Chloroplast Isolation from the fresh water green algae *Scenedesmus obliquus*: a biochemical and morphological characterization

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Summary

Microalgae are photosynthetic unicellular organisms. They belong to a large group of organism, which include also prokaryotic organisms like spirulina (*Arthrospira platensis*). Since it is necessary to reduce carbon dioxide emissions on a global scale, scientists started to investigate and select micro-algae due to their special characteristics in terms of growth speed and energy accumulation. Indeed, they grow faster than higher plants, accumulate biomolecules (proteins, starch and lipids) depending on their environmental conditions and do not require expensive nutrients for their growth. The fact that microalgae can be easily cultivated in photo-bioreactors under controlled conditions, makes them one of the most studied organisms in the field of biofuel production. However, the cost of the biomass is still too high compared to both fossil fuels and other biofuels obtained from land plants. Therefore, the possibility to produce biofuel (on an industrial scale) from microalgae is far away from the reality. In order to reduce the costs it is necessary to work on different aspects such as strain improvement, bio-refinery, economic related issues and the biological studies. The importance of studying the biology and the physiology of selected micro-algal cell stands on the creation of provisional models, which are important to maximize the production of a certain molecular component. In this thesis, we worked specifically on the isolation and molecular characterisation of the chloroplast, organelle in which photosynthesis occurs. We used *Scenedesmus obliquus* as model organism, a fresh water green algae known to be one of the most productive strain of triacylglycerol (TAGs) under stress conditions. In literature, there are not procedures to extract and isolate *S.obliquus* chloroplasts, so the aim of the thesis was to develop a new "tailor made" procedure for this specific organism. We cannot exclude that the same protocol could also work with similar micro-algae like *Chlorella spp*. Using a combination of treatments such as osmotic shock and ultra-sonication we achieved an efficiency of ≈70% in cell wall disruption. The obtained chloroplast were observed using scanning electron microscopy (SEM) for a morphological characterisation. As result, the whole cells not treated (control) showed an electron dense and smoot external layer (assumed to be the cell wall), while the treated samples showed the characteristic cup-shape of chloroplasts. The PCR purity assay underlined a mitochondrial contamination in the isolated chloroplasts, this result could be explained by the existence of a micro-tubular connections between the two organelles. The biochemical analysis, as it was expected, showed a constant presence of starch,

contained in both samples (control and treated), but a very different in proteins and total carbohydrates contents. Indeed, the starch is accumulated in the chloroplast and so both, the whole cell and the chloroplast, should contain almost the same amount of it. In contrast, since the chloroplast lacks of cell wall, which is rich in cellulose and proteins, it should contain much less than the whole cell. In conclusion, we developed a suitable chloroplast isolation protocol for metabolic studies either *in vivo* or *in vitro*. Furthermore, using scanning electron microscopy, we obtained more information about the external surface of chloroplasts. Moreover we observed how osmotic shocks and ultra-sonication treatments morphologically affect these organelles. Surely, there would be still some improvements to achieve, especially regarding the mitochondrial contamination and the cell-wall disruption efficiency but this thesis is a good starting point for researchers who want to study isolated chloroplast from *S.obliquus* or similar micro-algae cells.

"All exact science is dominated by the idea of approximation.
When a man tells you that he knows the exact truth about anything,
you are safe in infering that he is an inexact man."

"Le scienze estate sono dominate dall'idea dell'approssimazione.
Quando un uomo dice di sapere la verità assoluta a proposito di qualcosa,
saresti al sicuro nel dire che colui è un uomo inesatto."

Beltrand Russel

1. Introduction

To understand clearly this thesis, in the introduction, a set of basic information about microalgae will be provided. Mainly taking in account what they are and how they are classified. The most important features of the chloroplast will follow the general overview. Finally, a specific description of the fresh water green algae *S.obliquus*, since it is the organism used as a model.

1.1 Microalgae

1.1.1 Classification

Microalgae are unicellular photosynthetic organisms, therefore, they are able to convert carbon dioxide (CO_2) into biomass using the light radiation [1]. The variability within this large group is very high, for example, they can be eukaryotic or prokaryotic. Eukaryotic microalgae possess membrane bound organelles such as, nucleus, mitochondria, Golgi complex and chloroplast. Whereas prokaryotic microalgae consist of a single compartment with thylakoid membranes, structure where photosynthesis occurs. Microalgae can be classified in according to the morphology and pigments composition of their photosynthetic apparatus (**Figure 1**).

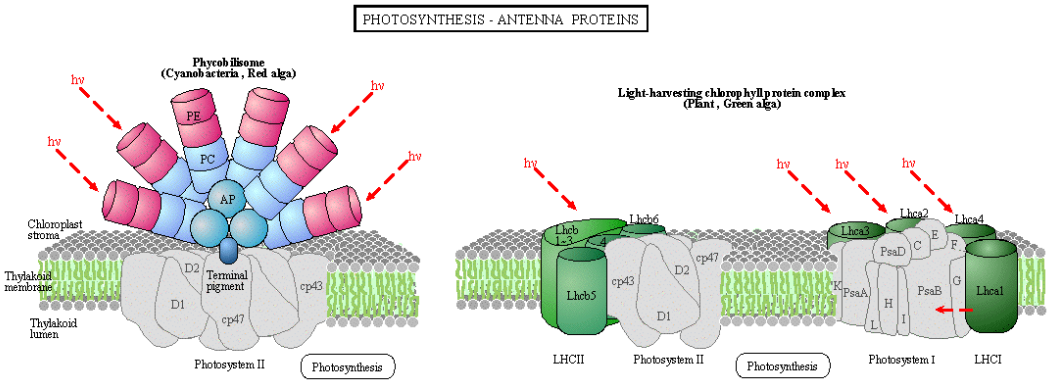

Figure 1: Schematic representation of antenna proteins in different algae. [Grossman A R, Schaefer M R, Chiang G G, and Collier J L. "The phycobilisome, a light-harvesting complex responsive to environmental conditions." Microbiol Rev (1993). 57(3):725-49]

1.1.2 Ecology

Microalgae have been isolated from a large variety of habitats within marine water, fresh water and soil. They are responsible for 50% of the oxigen (O_2) released in the atmosphere [2]. They take on a big importance for their primary production (conversion of inorganic substrate into biomass). Since they perform most of the primary production, it is clear their ecologic role. They provide the first source of biomass for greezling microrganisms which in turn can be predate by the plankton and so on up to the food chain. Microalgae can also cause water eutrophization, this phenomena is due to an excessive growth (algal bloom), resulting in a decrease in dissolved oxygen in water when an enrichment in nutrients (nitrogen and phosphorus) occurs. The huge biomass produced in an algal bloom will sediment on the water backdrop where heterotrophic bacteria can use it as a carbon sorce consuming oxygen (**Figure 2**).

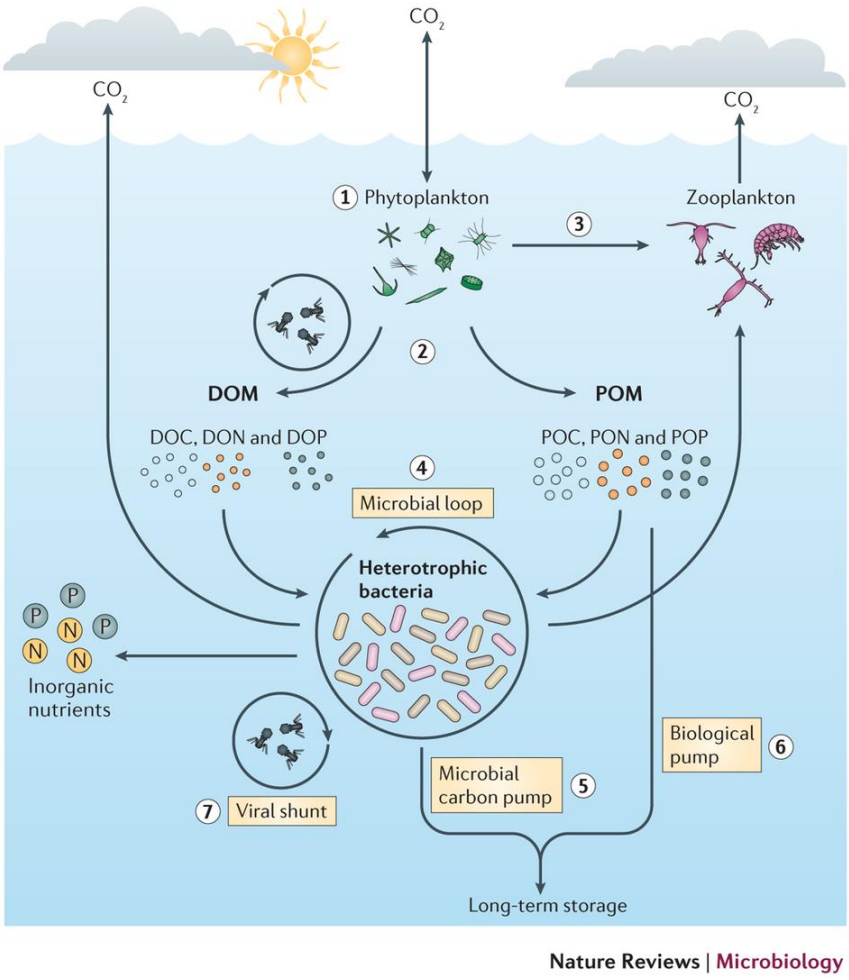

Figure 2 Bacterial transformation of phytoplankton-derived organic matter[3]. (Phytoplankton is mainly microalgae).

1.1.3 Uses

Microalgae are worldwide employed for several purposes such as food and feed applications, pharmaceutics, cosmetics, biopolimers and pigments production. However, since the world oil feedstock is limited, research is focusing on the potential application of microalgae for biofuels production. Microalgae seem to be one of the most interesting candidate to produce biomass and, subsequently, biofuels due to their high growth rate and peculiar physiologic characteristics described in the next sub-chapters.

1.2 *Scenedesmus obliquus*

1.2.1 Classification and ultrastructure

Scenedesmus obliquus is a fresh-water microalgae belonging to the family of the *Scenedesmaceae*, division of *Chlorophyte* (green algae). Generally, it forms non-motile colonies called *coenobium* but the composition of the growth medium influences the cell morphology, Francis and Trainor found that yeast extract (1.5% w/v) causes a switching from 4-8 cells colonies to single cells[4]. The cell has a characteristic lemon shape, elliptical and slightly pointed on the sides (**Figure 3**).

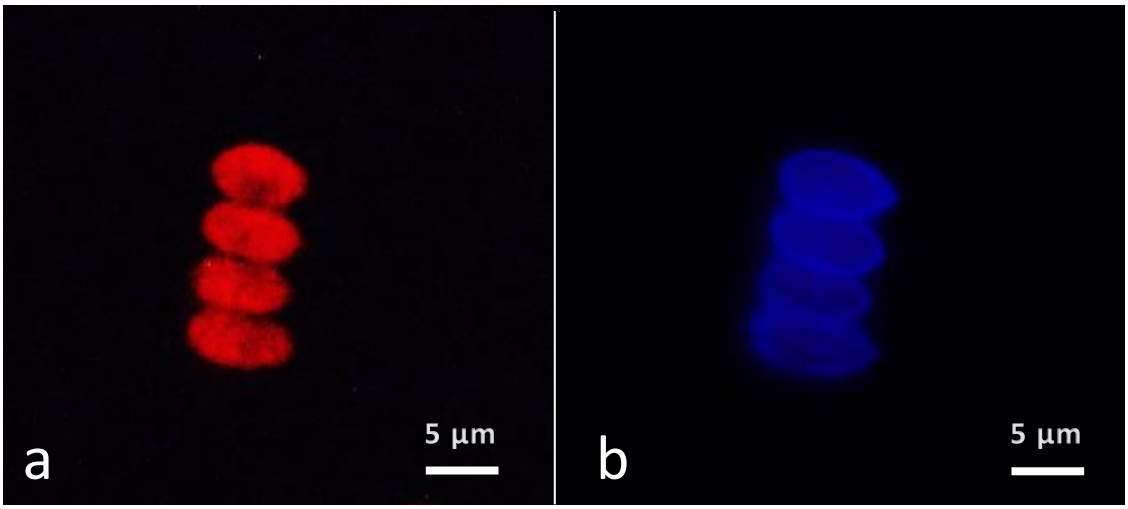

Figure. 3 Chloroplast view through chlorophyll red emission obtained by green excitation (a), cell wall stained by calcofluore white (b).

S.obliquus possess a trilaminar cell wall mainly made of cellulosic, hemicellulosic and pectic compounds. In addition, the cell wall contains glycoproteins and sporopollenin as well [5] (one of the most chemically inert biological polymers [6]). As an eukaryotic green algae, *S.obliquus* has a nucleus, mitochondria, Golgi complex and one big chloroplast

which takes most of the cell volume. In the chloroplast, there is the pyrenoid, a sub compartment which allows a localized CO_2 enrichment [7]. This carbon-concentrating mechanism enhance the carbon fixation made by, the photosynthetic enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO).

1.2.2 Physiologic characteristics

S.obliquus adapts very well to many different conditions and the optimum temperature optimum oscillates around 25 °C but can grow efficiently within a big range of temperature (between 14°C and 30°C). This characteristic is important since outdoor biomass production is required on the large scale. When it is grown under optimal conditions, *S.obliquus* accumulates starch as energy stock. However, previous studies indicated that this microalgae accumulates triacylglycerols (TAGs) under particular stress conditions, such as nitrogen starvation [8] (**Figure 4**).

Furthermore, in 1942 Gaffron and Rubin reported H_2 production by *S.obliquus*, under darkness and anaerobic conditions [9]. The H_2 production is carried out by the hydrogenase, enzyme that converts protons (H^+), produced by the H_2O photolysis, in hydrogen molecules (H_2) [10].

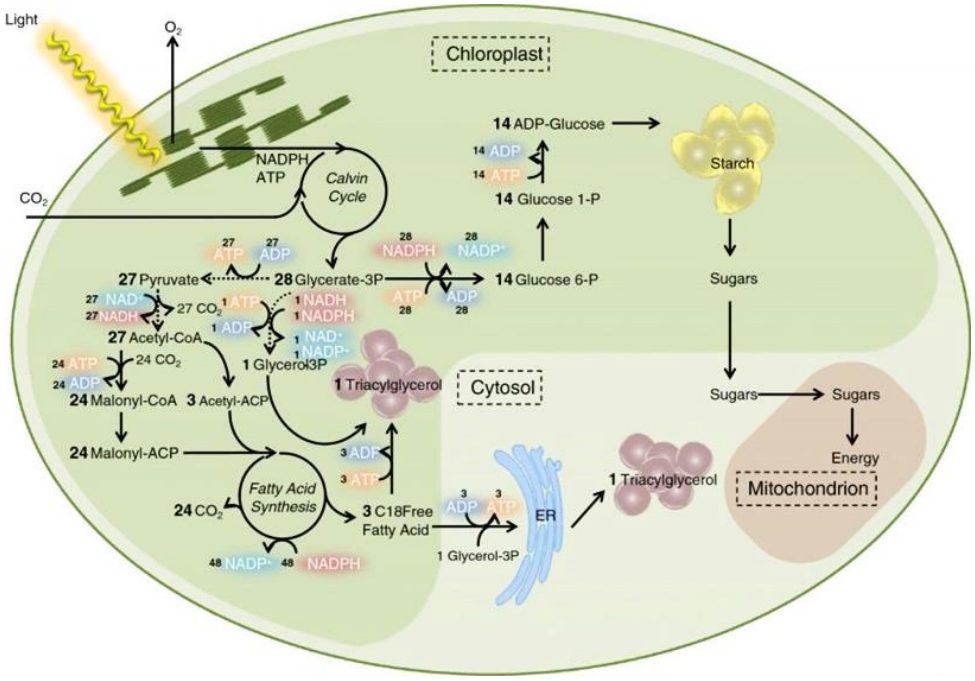

Figure 4 *S. obliquus* TAG synthesis pathway [8].

All the properties previously described make *S.obliquus* one of the most promising microalgae for biodiesel production. However, many problems have still to be solved: the difficulty in standardize the process, cells self-shading in high concentrated cultures, the high cost in lipid extraction and biomass harvesting. The research is focusing on solving these problems using different approaches such as strain improvement via metabolic engineering, bio-refinery based research, cell biology/physiology and modelling.

1.3 *S. obliquus* chloroplast

1.3.1 Photosynthesis

The chloroplast is an important cellular compartment where many essential biological processes occur, photosynthesis being one of them. The photosynthesis is the process that allows the cell to convert energy from light radiation into energy in form of biomass. This process is ideally split in two parts, which are the light dependent phase and the light independent phase (**Figure 5**). The light dependent phase refers to the light absorption on the thylakoids membranes by the photosystems, the reactions occurring in this phase are the H_2O photolysis, the ADP phosphorylation and the $NADP^+$ reduction:

$$2\ H_2O + 2\ NADP^+ + 3\ ADP + 3\ P_i + light \rightarrow 2\ NADPH + 2\ H^+ + 3\ ATP + O_2$$

In the light independent phase the carbohydrates synthesis by CO_2 fixation occurs:

$$3\ CO_2 + 9\ ATP + 6\ NADPH + 6\ H^+ \rightarrow C_3H_6O_3\text{-phosph.} + 9\ ADP + 8\ P_i + 6\ NADP^+ + 3\ H_2O$$

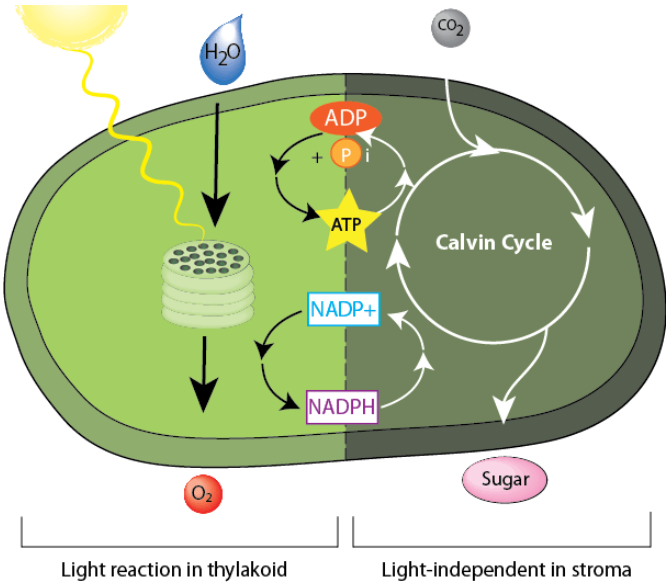

Figure 5 Schematization of light dependent and not dependent photosynthetic reactions.

S.obliquus, being a green algae, has a photosynthetic apparatus very close to the higher plants, therefore, two membrane-proteins/pigments complexes called *photosystem I* and *photosystem II* (PSI and PSII) placed on thylakoid membranes containing chlorophyll a and b as photosynthetic pigments. The PSII requires photons with a wavelength of 680 nm and it is involved in the H_2O photolysis while the PSI require photons with a higher wavelength (700nm) and it provides the electrons for the $NADP^+$ reduction (**Figure 6**).

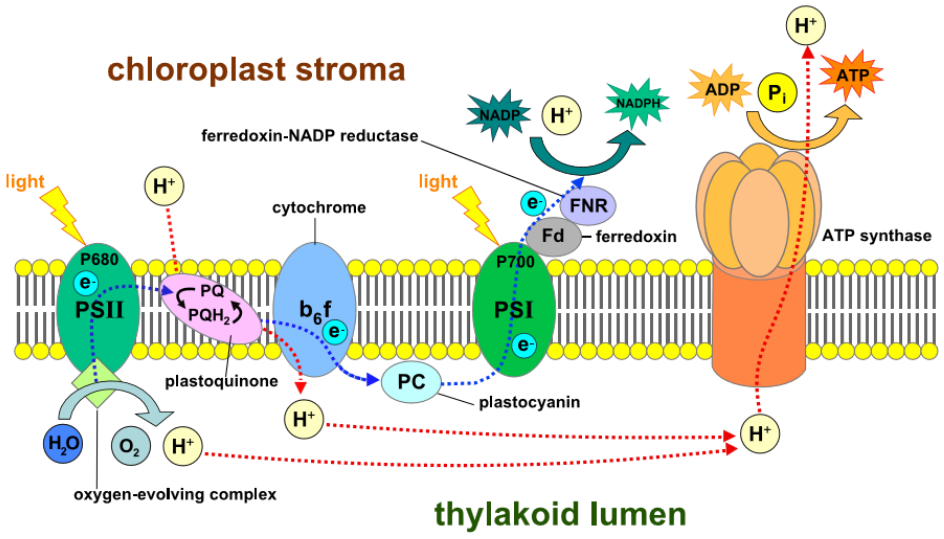

Figure 6 Scheme of a Photosynthetic apparatus. [Buchanan. Biochemistry and molecular biology of plants]

The operating principle of this complex molecular machinery is the electron excitation. Indeed, the photosystems are able to excite electrons (originated from the water photolysis). The electrons are, then, transported along the 'electron transport chain', during this process electrons lose energy and that energy is used to generate a proton (H^+) gradient between the stroma and the lumen. The H^+ gradient serves as fuel for ATP synthase, enzyme located in the thylakoid membrane as well, which phosphorylate ADP producing ATP.

1.3.2 Fatty acids synthesis

In green algae, fatty acids synthesis occurs in the chloroplast via malonyl-CoA beta-oxidation, the same molecular mechanism of higher plants. (**Figure 7**).

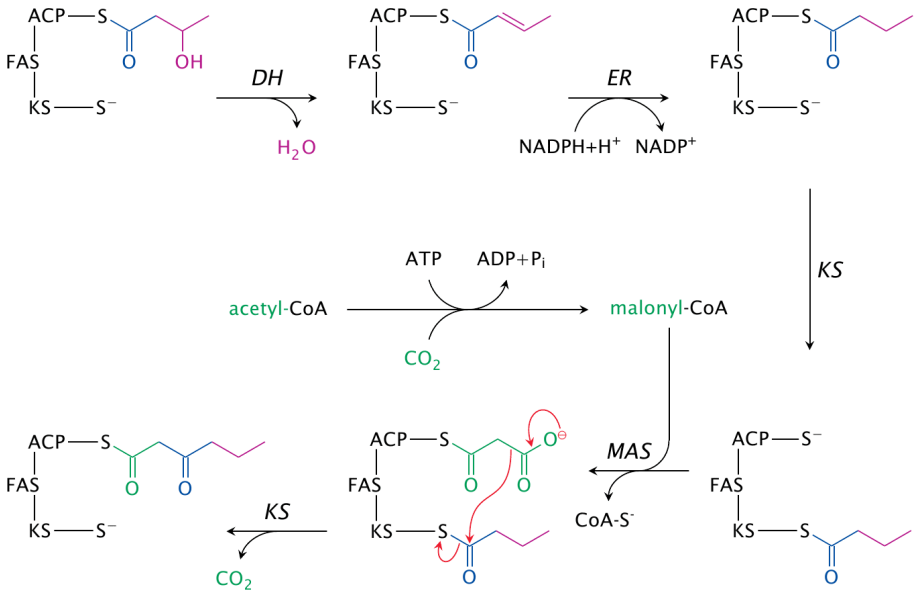

Figure 7 Fatty acids synthesis reactions.

Fatty acids have mainly two functions. The first one is structural, as they are the principal compound in membranes. The second one is energy storage, as part of triacylglycerols (TAGs). TAGs are an important source of energy. The energy yield of a gram of fatty acids (which are the building blocks of TAGs) is approximately 9 kcal (37 kJ), compared to the 4 kcal (17 kJ) of carbohydrates [11].

Since fatty acids are the building blocks of TAG, It is important to investigate the environmental conditions in which their synthesis is improved.

1.3.3 Photorespiration

Photorespiration is an important biological process occurring in algae and plant cells. Basically, this process is referred to the incorporation of molecular oxygen (O_2) instead of carbon dioxide (CO_2) by RuBisCO (light independent phase). RuBisCO is the most abundant protein/enzyme on Earth [12] and its function is to carboxylate (incorporate) CO_2 in a 5 carbons compound, ribulose bisphosphate, and split it in 2 molecules with 3 carbon atoms (3-Phosphoglycerate)[13] (**Figure 8**).

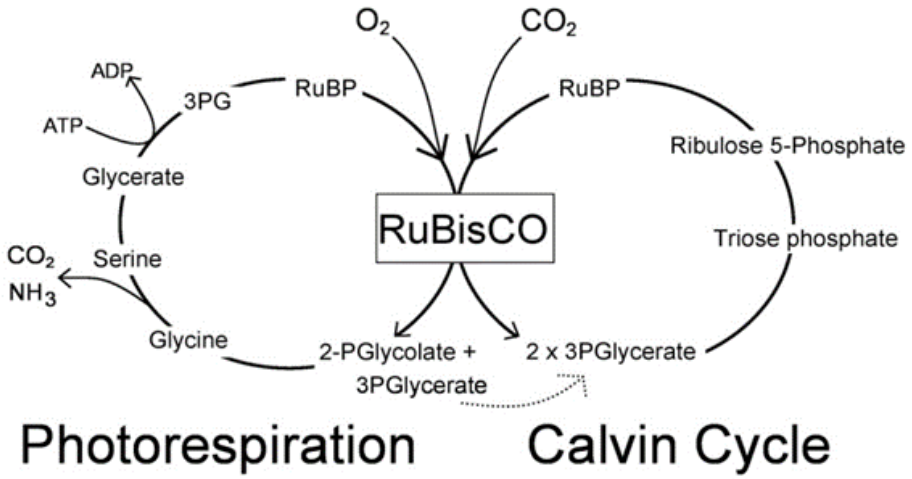

Figure 8 Simplified scheme of photorespiration and Calvin cycle pathways.

Unfortunately, this enzyme has a higher affinity for O_2 as substrate than CO_2. Therefore, when this two substrates are at the same concentration it incorporates O_2 rather than CO_2 resulting in the production of 1 molecule of 2-phosphoglycolate (2 carbon atoms) instead of 2-phosphoGlycerate (3 carbon atoms). 2-phosphoglycolate can be converted, then, in 2-phosphoGlycerate with an energy cost of 1 ATP and 1 NADPH. Hence, photorespiration requires more energy than normal carboxylation, so, in biomass production has to be avoided. Furthermore, the process reduces also the cell CO_2 intake resulting in less efficient carbon assimilation [14]. This waste of energy and potential in biomass accumulation during photorespiration, is relevant in large-scale production. Indeed, in closed photobioreactors the process is higher because oxygen (O_2) is accumulated in the medium during the growth.

1.4 Thesis aim and description

1.4.1 Aim

The aim of this thesis was to develop a suitable protocol to isolate chloroplasts from *S.obliquus* UTEX 393. Furthermore, we wanted to characterize the chloroplast morphology and macromolecular content, so, compare it with the data collected from the whole cell.

1.4.2 Description

The thesis project consisted of 4 phases:

1. Biomass production

The biomass was produced in a lab-scale photobioreactor (Algaemist®). In this phase, microalgae were grown under day/night cycles in continuous mode.

2. Cell disruption

Once the biomass is collected, a suitable cell disruption method had to be found. This method should have been strong enough to break the high resistant cell wall and, at the same time, mild enough to preserve the chloroplast intactness.

3. Chloroplasts isolation and testing

A chloroplast isolation procedure is necessary to isolate chloroplasts from the rest of the cell. After the isolation, the fraction containing chloroplasts was used to test the following characteristics: purity and integrity.

4. Biochemical morphological characterization

The last phase of this project is the biochemical and morphological characterization of the fraction obtained from the isolation procedure developed.

Figure 9 Thesis project flowchart.

2. Materials and methods

2.1 Organisms and growth conditions

The fresh water microalgae *Scenedesmus obliquus* wild type UTEX 393 was used. A single colony has been picked up from a Petri plate and inoculated in a 250mL shake flask containing GB medium (table1) to obtain a pre-culture.

Table 1 Medium composition (for stocks composition see appendix-1)

Component	Concentration
KNO_3	9.89 mM
Na_2SO_4	0.7 mM
Stock1 (micronutrients)	3 mL/L
Stock2 (micronutrients)	3 mL/L
Stock3 (vitamins)	1 mL/L
$MgSO_4$ x $7H_2O$	0.5924 mM
$CaCl_2$ x $2H_2O$	0.4898 mM
K_2HPO_4	2.4687 mM
HEPES (only for shaking flasks)	100 mM
$NaHCO_3$ (only for shaking flasks)	10 mM

The microalgae was grown for two weeks in an Infors HT multitron® incubator (2,5% CO_2; 180µmol/m^2·s; 23°C) under 18/6 day/night cycles. The pre-culture was used to inoculate 3 lab-scale flat panel photo-bioreactors (algaemist ®) with a working volume of 400mL, named R-43, R-44 and R-47 (for photobioreactors characteristics see Appendix-5). The reactors were inoculated to an optical density at 750nm of 0.2. Triplicated independent experiments were ran to ensure reproducibility. The reactors were run in batch mode until the light at the back of the reactor reached the desired set-point. Then, turbidostatic mode started. Under turbidostatic mode, the turbidity inside the reactors was kept constant. The same medium used for the shake flasks (GB medium) was used for feeding the cultures in reactors, but without HEPES and $NaHCO_3$. The growth parameters were based on the settings used previously by Breuer et al 2013 (Table 2).

Table 2 Algaemist settings/growth conditions

Parameters	Value
Light supply	167µmol photons/s*m^2
Day/night cycles	16h/8h
CO_2	On demand (pH regulation)
pH	7.00
Air supply	235 mL/min.
Temperature	27.5 °C

2.2 Cell wall disruption and chloroplast isolation

2.2.1 Cell wall disruption

In order to separate the chloroplasts from the whole cells a sonication treatment was performed. Reactor samples of 3mL cell suspension (OD750 ~ 2.32) were taken after 7.5 h the beginning of the day cycle and placed in a 15mL falcon tube. Then cells were pelleted at 3600xg for 10min and resuspended in 3mL of breaking buffer (see Appendix-2). The cells suspended in the breaking buffer were kept for few minutes in the darkness, sonicated with a probe sonicator (Sonics-vibracell) for 2 times 30 seconds each, at 100% of power (between 17 and 14 Watts) and then stored in ice (for whole procedure see appendix-4). Once the putative chloroplasts were obtained an isolation procedure has been performed (see "2.2.2 Chloroplast isolation").

2.2.2 Chloroplast isolation

Taking advantage of the higher density of the chloroplast (due to the presence of starch granules) than the debris and whole cells, the chloroplasts have been purified.

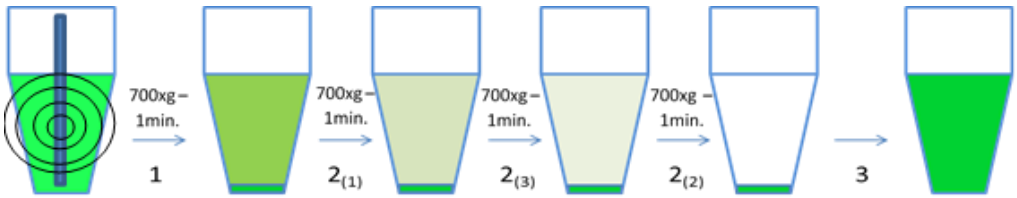

Figure 10 Chloroplast enrichment scheme. 1) The cell suspension were sonicated, pelleted at 700xg for 1 minute and resuspended in an isolation buffer. 2) The procedure has been repeated 3 times to enrich the pellet in chloroplast. 3) The chloroplasts were suspended, finally, in the isolation buffer and stored at 4°C.

The procedure consisted in three sequential mild centrifugations (700xg for 1min at 4°C) (**Figure 10**). After each centrifugation, the pellet obtained was gently resuspended in an isolation buffer [15] (see Appendix-2) using a small paintbrush avoiding the breakage of the fragile organelles.

2.3 Chloroplast purity

2.3.1 Calcofluore White staining and osmotic burst assay

To verify the presence of the cell wall, cells were stained with calcofluore white (CFW) provided by Sigma-Aldrich©, a specific dye for chitin and cellulose. For the staining procedure, 10µL of cell suspension (with an $OD_{(750nm)} \sim 2.3$) were placed on a microscope glass plate and dried for 10min in a flow cabinet. Then, a 5µL drop of CFW (not diluted) was placed on dried cells and incubated for 5min, after this process the samples were observed with a fluorescent microscope (OLYMPUS© IX71).

To quantify the amount of chloroplast extracted, *i.e.* the efficiency of the process, an osmotic shock assay was developed. Suspended cells were counted using a microscope-counting chamber (C-Chip in CYTO®) with or without the induction of an osmotic shock. The osmotic shock was induced by replacing the isolation buffer (containing Sorbitol 0.3M) with pure MilliQ water. The efficiency of the chloroplast release was calculated using the following formula:

$$\eta\ (\%) = \frac{Cc(f)}{Cc(i)} * 100$$

Where $\eta\ (\%)$ is the efficiency in percentage, $Cc(f)$ is the cell concentration after the osmotic shock and $Cc(i)$ is the cell concentration before the treatment.

2.3.2 Polymerase chain reaction (PCR) purity assay

A polymerase chain reaction (PCR) to test the purity of the chloroplasts was performed. Considering that the mitochondria are only present in the cytoplasm, we decided to determine the cytoplasmic contamination amplifying two marker sequences with selective primers (**table 3**): one specific of the chloroplast genome and another one for the mitochondrial genome [16].

Table 3 Specific information about the primers and the origin of the sequences amplified.

Code	Amplicon size	Marker gene	Sequence
1a Forw. Chloroplast	549 bp	Protein chloroplast envelope	CAACAGGAGCAGAAGCATCA
1a Rev. Chloroplast			CAGAGCTTCACCAGCAACTG
2a Forw. Mitochondria	276 bp	NAD+ dehydrogenase subunit-4	GGCAATGCTTCCATGTCTTT
2a Rev. Mitochondria			TGGTCCCAAATAAGCACTCC

The PCR was set for 35 cycles with a denaturation step of 95°C, an annealing step of 55°C, an elongation step of 72°C and a final storage phase at 4°C (parameters resumed in the **Table 4**).

Table 4 PCR setting.

Phase	Preliminary Denaturation	Denaturation	Annealing	Elongation		Post Elongation	Storage
Temperature	95°C	**95°C**	**55°C**	**72°C**	**X 35 cycles**	72°C	4°C
Time	3 min.	**30 sec.**	**30 sec.**	**1 min.**		5 min.	∞

Both the samples, after (putative chloroplasts) and before (control) the chloroplast isolation were analysed. To obtain the DNA a Chloroform:Phenol:Isopropylic acid (C:P:I) extraction was performed. The biomass for the extraction was obtained for both, chloroplast and whole cells, picking some pelleted cells. The whole procedure for the DNA extraction is described in the Appendix-3.

2.4 Scanning Electron Microscopy

For each sample, a drop of 10 µL was put on a poly-L- lysine coated glass slide and left on it for 1 h. Then it was gently rinsed in PBS and fixed with a drop of 10 µL of 2% glutaraldehyde in PBS for 1h. After that the sample was rinsed in PBS and stored at 4 °C in PBS. Before the analysis sample were fixed with OsO_4, dehydrated with ethanol solution at increasing amount (until 100 %), dried with a supercritical CO 2 dryer and finally coated with titanium. Analysis were carried out with Ultra high resolution field emission scanning electron microscope (FESEM) FEI Magellan 400.

2.5 Biochemical analysis

The biomass used for all the biochemical analysis was obtained by performing the chloroplast isolation (see paragraph 2.2.2). Then, the crude chloroplasts were freeze-dried with a Sublimator 2X3X3 (ZirBus technology). The biomass used as control was obtained freeze-drying the whole cells (**Figure 11**).

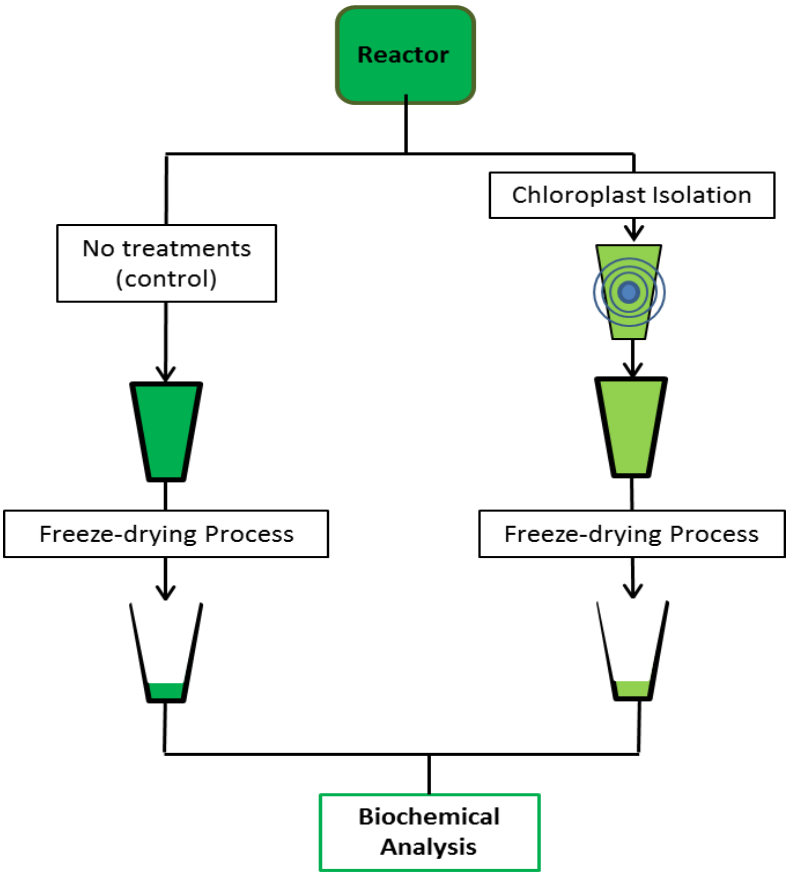

Figure 11 Biochemical characterisation experimental design.

2.5.1 Total carbohydrates

Once the freeze-drying procedure was completed, 10mg of sample were used to measure total carbohydrates based on the Colorimetric total carbohydrate assay, according to Dubois, for micro-algae [17].

2.5.2 Starch

For the starch analysis, 10mg of freeze-dried samples were weighted in bead beating tubes. Then, after an appropriate cell disruption (Precellys 24 - Bertin©) all the starch was hydrolysed to glucose using thermostable α-amylase and amyloglucosidase enzymes from the Total Starch assay (Megazyme International, Wicklow, Ireland), and the glucose was determined spectrophotometrically. An adapted protocol for microalgae was used [18].

2.5.3 Proteins

The total protein concentration was determined using a colorimetric assay (Bio-Rad DC protein assay) as described by P.R. Postma et al.[22] with the difference that 5mg of freeze dried biomass were used for the analysis. The protocol used, to quantify the protein content, is similar to the documented Lowry assay[19] but with the following improvements: The reaction reaches 90% of its maximum colour development within 15 minutes thereby saving valuable time, and the colour changes not more than 5% in 1 hour or 10% in 2 hours after the addition of reagent.

2.6 Protoplast regeneration

In order to test the presence and viability of the protoplasts we grew the cells obtained after several treatments. The treatments used were:

1. Sonication in the breaking buffer plus the chloroplast isolation by centrifugation.

2. Sonication in the breaking buffer, followed by the isolation and a syringe treatment (we passed through a needle [0.4x20mm] with a speed of 0.5mL/s).

3. Sonication in the media (without using the breaking buffer).

4. Normal cells taken from the reactor as control.

The cells were placed 250mL Erlenmeyer shake flasks with 10mL of fresh media and grown in an Infors HT multitron® incubator (2,5% CO2; 180µmol/m^2·s; 23°C) under 18/6 day/night cycles (**Figure 12**).

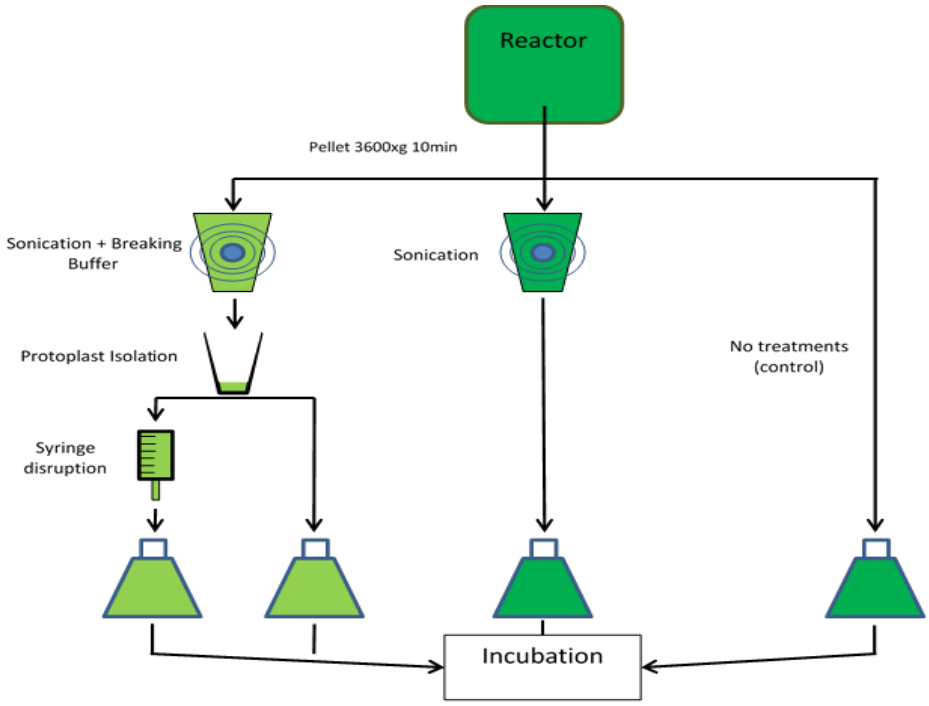

Figure 12 Experimental designs for the protoplast regeneration.

3. Results and discussion

3.1 Growth in photo-bioreactors

Three days after the inoculation the culture exhibited an intense green colour, indicating that the growth occurred, so the turbidostatic mode (1g/L) has been set. After 24 hours the concentration set was correctly reached (≃1g/L) (**Figure 13**). The dry weight during the sampling period showed the desired biomass concentration in all the three reactors was achieved. Furthermore the measurements of the overflow volume and the calculation of the dilution rate ($Eq.2$) confirmed that the steady state was reached (**fig. 13**).

As long as the cell concentration can influence the sonication process, these results ensured a good reproducibility of the 3 biological replicates for all the experiments. To

confirm the data obtained from the dry weight also the dilution rate was calculated using the following formula:

$$Eq.2 \qquad D = \frac{f(24h)}{V}$$

Where D is the dilution rate, $f(24h)$ is the overflow volume collected in 24h and V is the reactor volume (400mL). The data obtained confirmed that the steady state of the growth was reached.

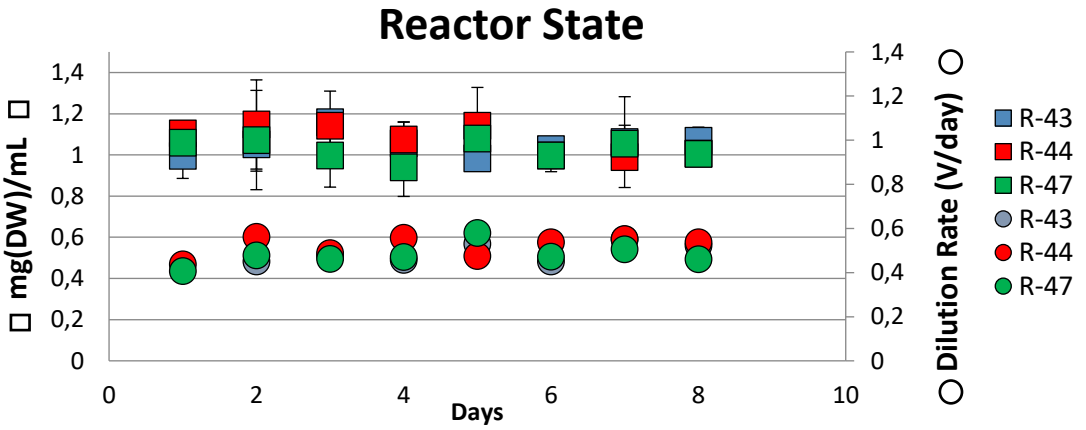

Figure 13 In the graph is shown the stability in the time of the dry-weight (diamonds) and the dilution rate (dots).

3.2 Cell wall disruption and chloroplast isolation

3.2.1 Cell wall disruption

The results of the disruption procedure, as described in the previous sub-chapter (2.2.1), showed an achievement in the removal of cell wall from cells. Both pictures, from fluorescent microscopy and scanning electron microscopy, were clear in showing a difference in the external surface of the cell (**Figure 14**). A change in the cell shape was observed, the cells appear to collapse onto the internal structure of the chloroplast, in the **Figure 15)** is appreciable a similarity of the morphology observed from optical microscopy and scanning electron microscopy. The untreated cells (whole cells) showed a characteristic *cenobia* arrangement (micro-colonies formed by 4 individuals), while the disrupted cells showed a single cell arrangement. Moreover, the whole cells present a clear electron dense external layer, which could be attributable to the cell wall.

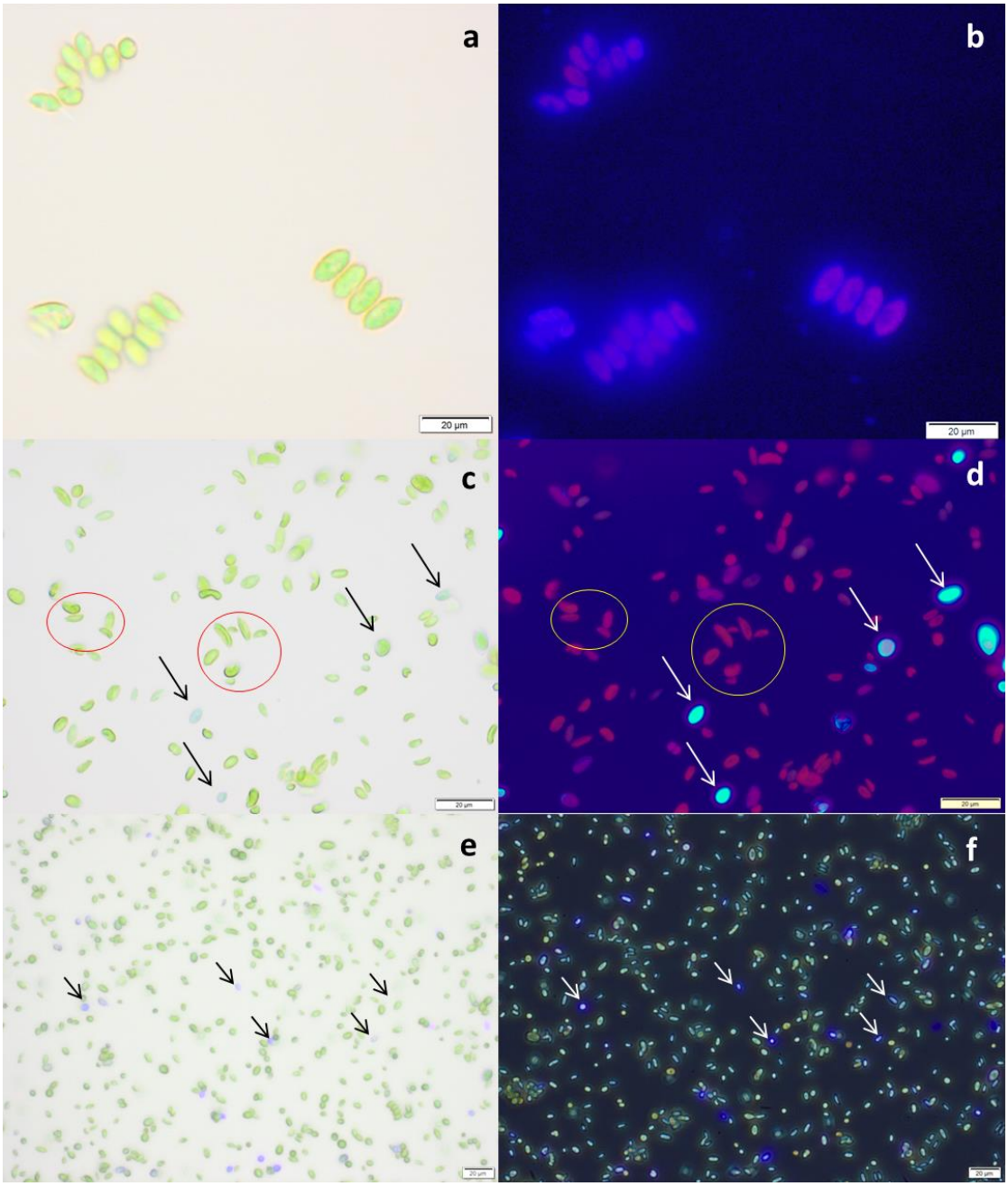

Figure 14 *S.obliquus* cells stained with calcofluore white observed in bright field (a) and the same sample observed under UV excitation (b) (330-350nm). In blue the cell wall on the edges of the cells (b), the chloroplast in red (chlorophyll fluorescence). In bright-field the typical cup-shape of chloroplasts is appreciable after the cell wall disruption (c), the cell wall in some whole cells under UV excitation as well (d). Arrows show whole cells and circles chloroplasts (c and d). Overview of stained cells less magnified in bright-field + UV (e) and in contrast-phase + UV (f), the arrows show whole cells.

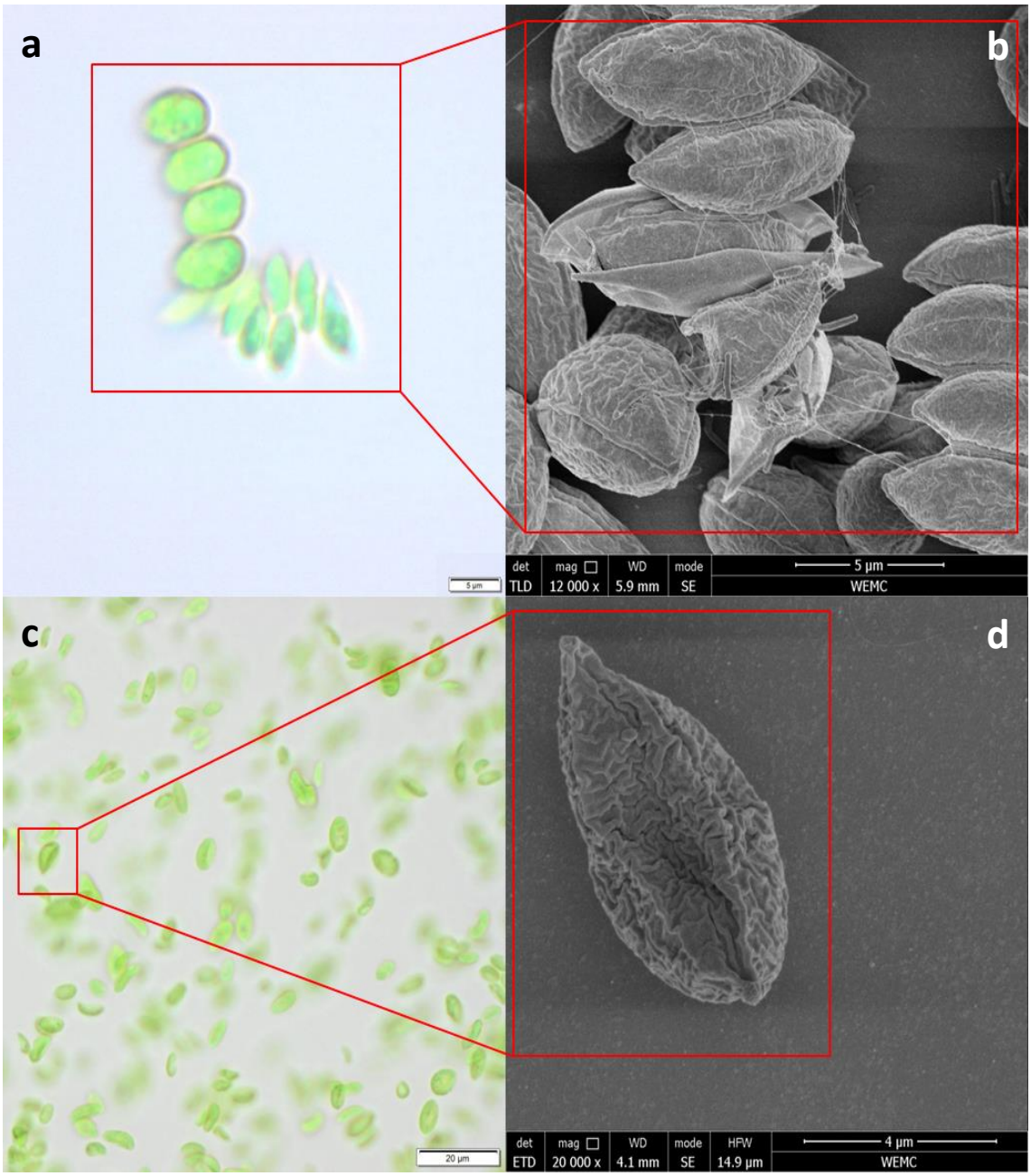

Figure 15 Above is appreciable the comparison between the *cenobia* arrangements photographed with optic microscope (a) and scanning electron microscope (b). Underneath, a comparison between the cells after the wall disruption photographed with optic (c) and scanning electron microscope (d).

The ornamentation of the surface result to be similar to the one of the control sample (whole cells), however, comparison with whole cells clearly shows that these have a transparent (white in SEM microphotography) layer covering them (**Figure 16**). In some of our SEM pictures, it was to still possible to observe the cell wall attached to the inner membranes. However, these membranes were not fully covered by it anymore. Another

indication of the lack of cell wall is the clear crumbled state of the membranes that in the control appear to be smoother. All over along the surface of the cells we observed the presence of hinge-like membranes (white rectangles, **Figure 16**) that could be related to the internal three-dimensional organization of the cytoskeleton.

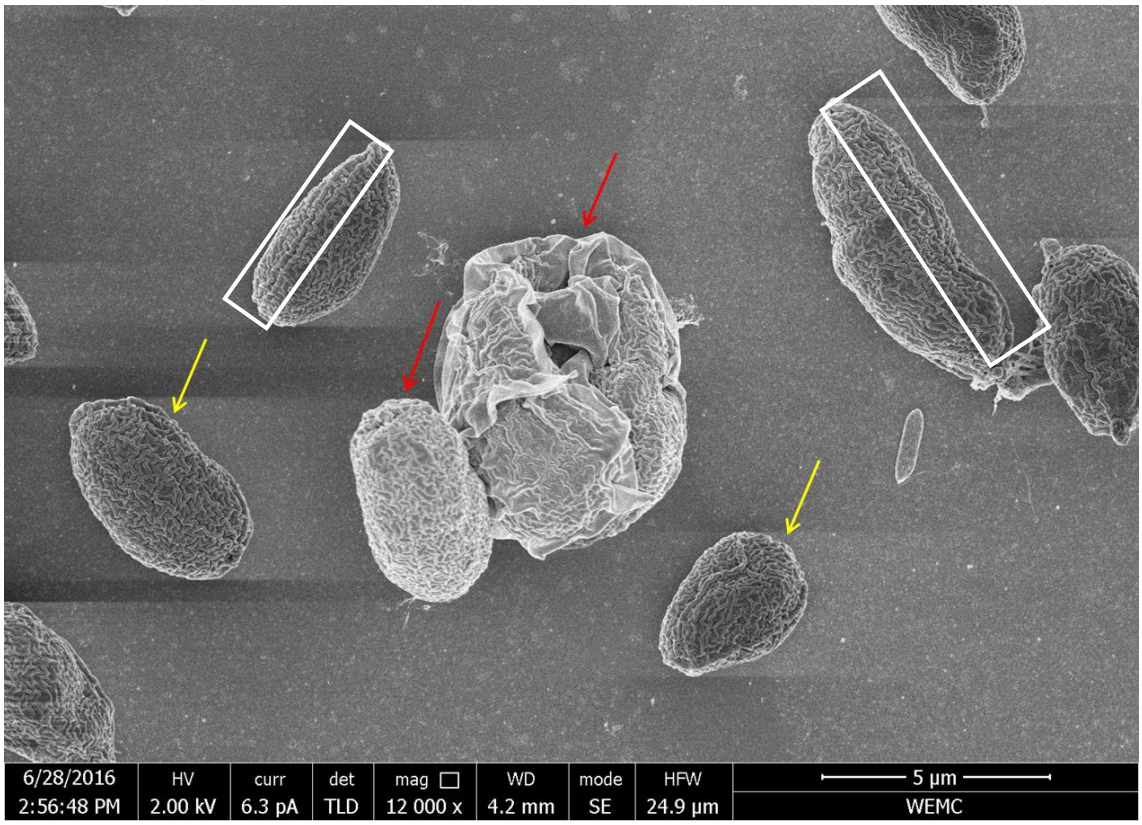

Figure 16 Overview of treated cells with still (red arrows) and without (yellow arrows) the cell wall.

3.2.2 Chloroplast isolation

After the sonication treatment we observed that parts of the cells were still covered by the cell wall due to the lightness of the treatment. On the other hand, to avoid the chloroplast disruption, we couldn`t use a harsher method. However, to obtain a high chloroplast concentration we separated most of the whole cells by mild centrifugation steps, talking advantage of the slightly higher density of the chloroplast.

The developed procedure allowed us to enrich the biomass in chloroplasts. Indeed, the bright-field microphotographs showed a morphologic difference (mostly in the size) between the cells removed with the supernatant and the cells pelleted on the bottom after the mild centrifugation (700xg, 1min) (**Figure17**).

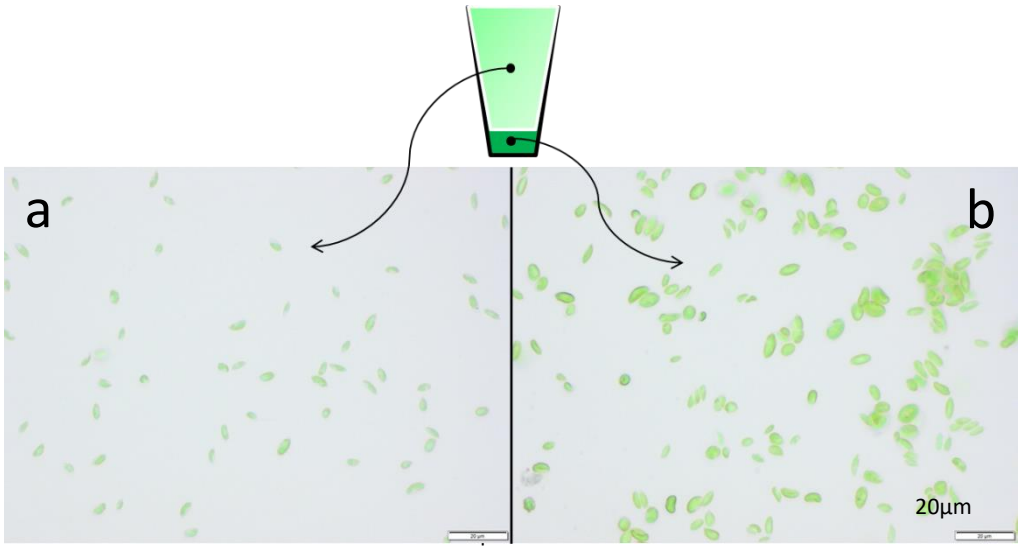

Figure 17 Whole cells photographed from a supernatant sample (a), enriched phase in chloroplasts photographed from a pellet suspension (b). Both samples were re-suspended in the isolation buffer (Sorbitol 0.3M).

3.3 Chloroplast purity

3.3.1 Cell wall disruption efficiency

The efficiency of the sonication process on the cell wall disrupting was estimated around 70% (**Figure.18**). This means that samples obtained after the isolation process contained still ≈30% of cells with still attached the cell wall. The percentages reported here refers to the number of the cell exploded after a hypotonic osmotic shock (see 2.3).

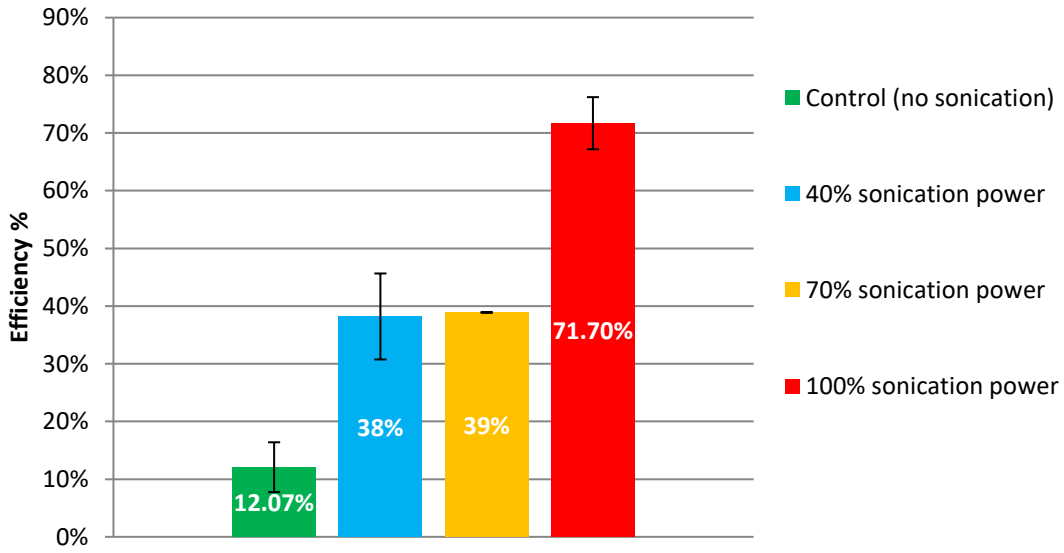

Figure 18 The graph shows the efficiency **(eq. 1)** of the sonication based cell wall disruption.

Even the control (no sonicated sample) showed a reduction in the number of cells after the osmotic shock. This phenomena can be explained by the fact that young auto-spores do not have any cell wall [20] right after the excretion from the mother cell (**Figure 19**). Therefore, also auto-spores are susceptible to the shock.

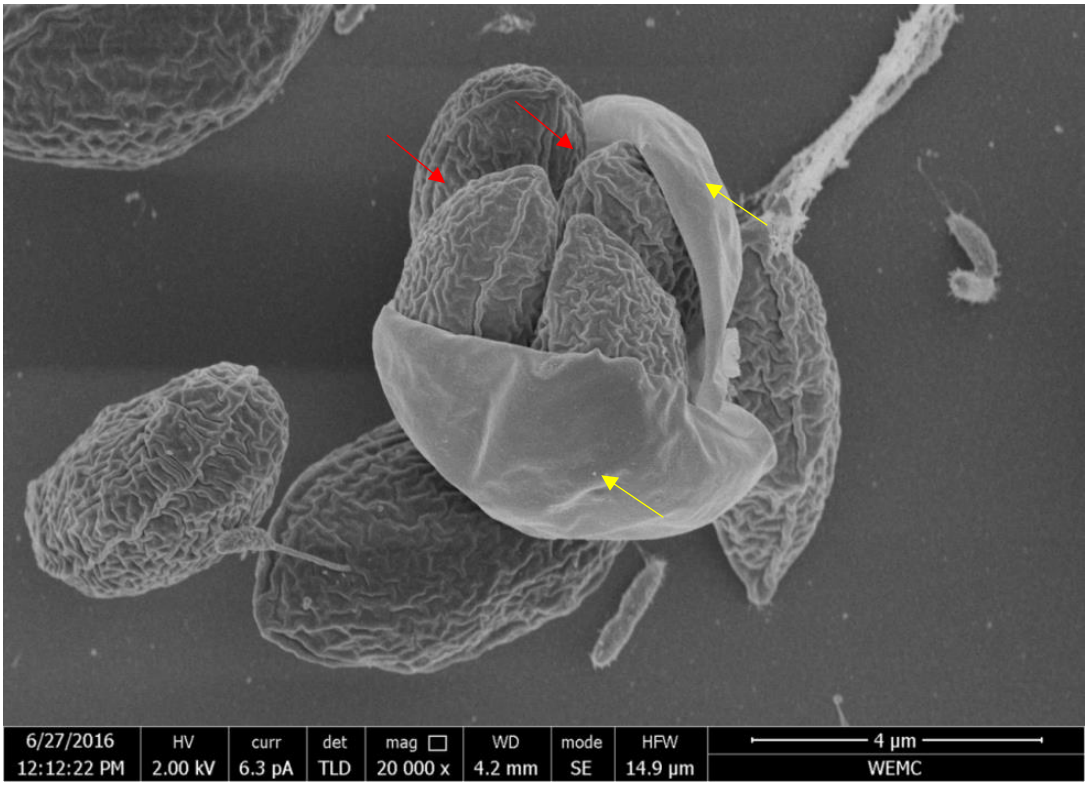

Figure 19 Auto-spores (red arrow) during the release process from the mother cell. The smooth cell wall is still appreciable all around the daughter cells (yellow arrow). NOTE: The picture was taken from no treated cell suspension.

The 30% of whole cells still remaining in the cell suspension could be more resistant to the procedure, also considering the normal heterogeneity that a culture has. The higher resistance of certain cells could be clarified by the fact that during *S.obliquus* cell cycle there is a phase in which the cell wall is thicker. However, this kind of behaviour is observed by Jeremy D. Pickett-Heaps and L. Andrew Staehelin (2008) in other microalgae species of *Scenedesmus* genera.

3.3.2 PCR purity assay

The PCR, performed to check the purity of the chloroplasts, showed an unexpected mitochondrial contamination. Indeed, the specific primers for the presumed

mitochondrial sequence amplified the target sequence in both in the treated and control samples (**Figure. 20**).

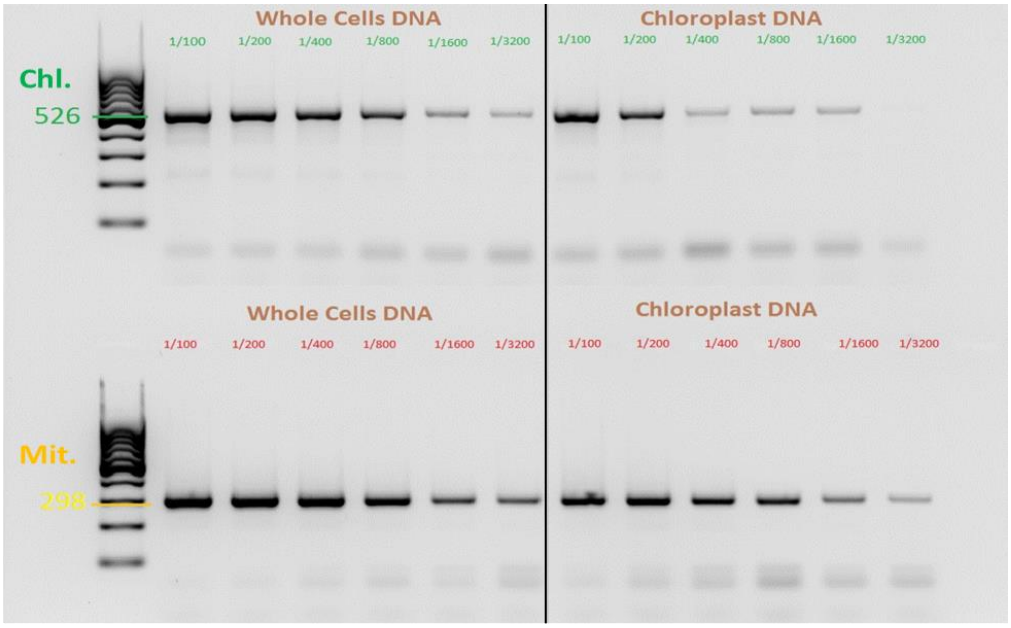

Figure 20 Image of the gel taken under UV excitation. Above, the amplified band of the chloroplast target sequence. Underneath, the mitochondrial target sequence band.

Although the result showed an amplification of the mitochondrial sequence, it is still not clear if this contamination can be considered more expanded (cytosolic content) or not. Indeed, the mitochondria could be still connected to the chloroplast surface, after the isolation procedure, due to its association to the cytoskeleton actin microtubules [21] (**Figure 21**).

Another interpretation of this result could be the implosion of the plasma membrane on the chloroplast. This interpretation can be speculated observing, carefully, the SEM pictures of magnified single isolated chloroplast (**fig. 22**). Indeed, the SEM microphotographs showed an excess of membranes (**Figure 22**; yellow arrows), in the area of the cytoplasmic invagination. On the pointed parts of the cell, crumbled membranes are appreciable as well.

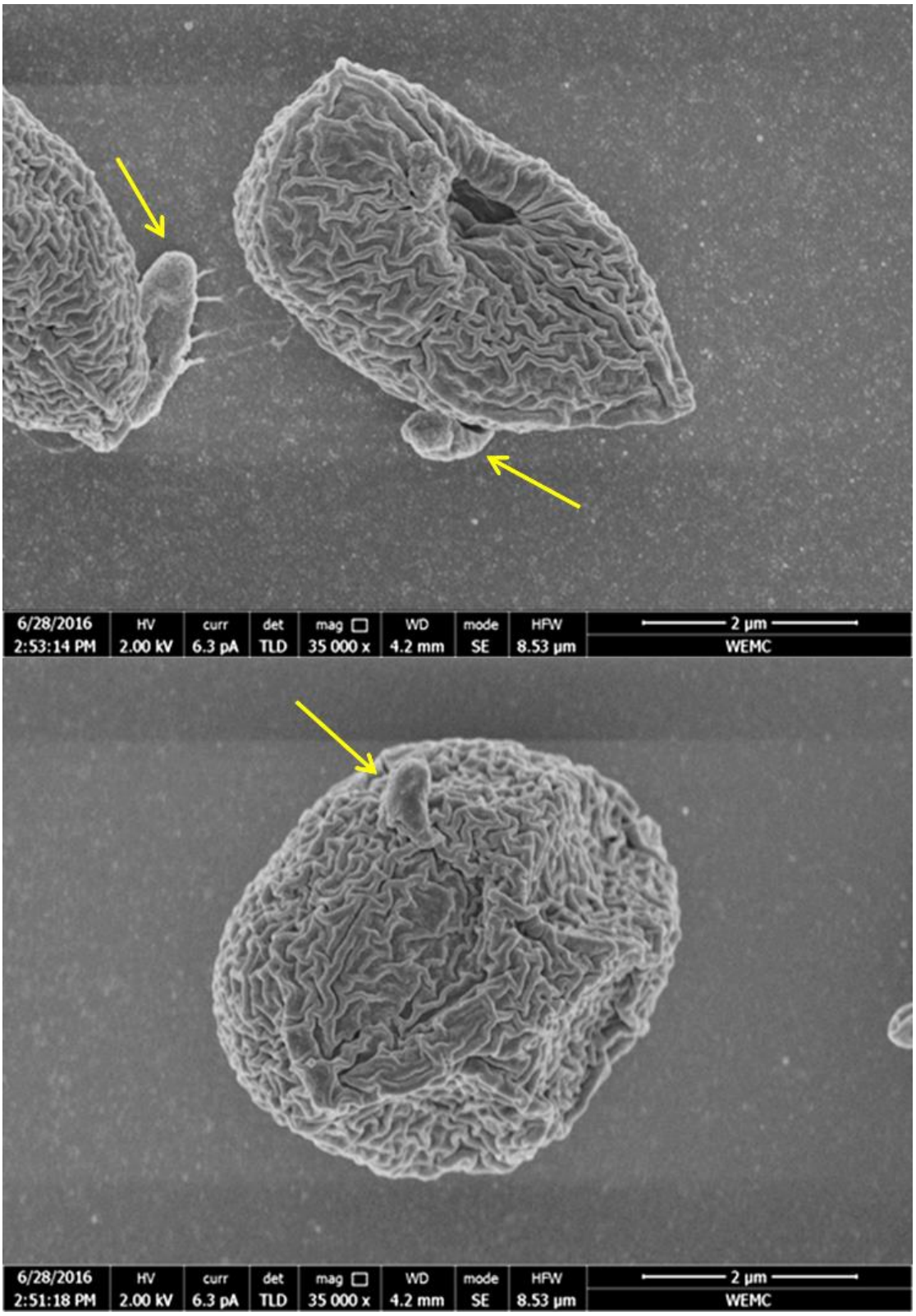

Figure 21 Single chloroplast views, putative mitochondria attached to the surface (yellow arrows).

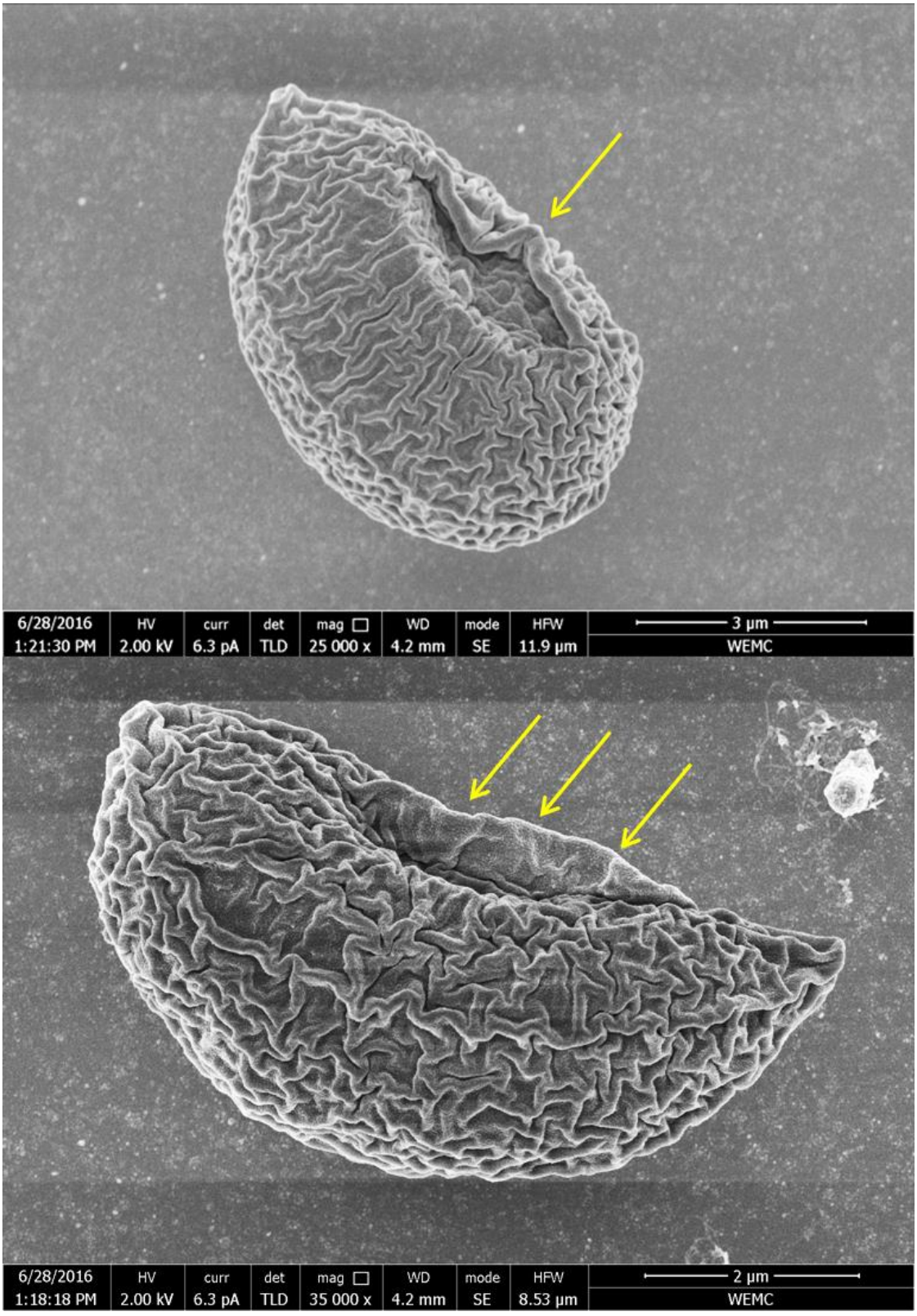

Figure 22 Crumpled membranes localized nearby the empty cytosolic invagination (yellow arrows).

3.4 Biochemical analysis

3.4.1 Starch

The starch content in the treated samples is, approximately, the same compared to the control (**Figure 23**). This result is in according with what we expected, the starch is only present in the chloroplast so the amount of starch and the whole cell should be similar.

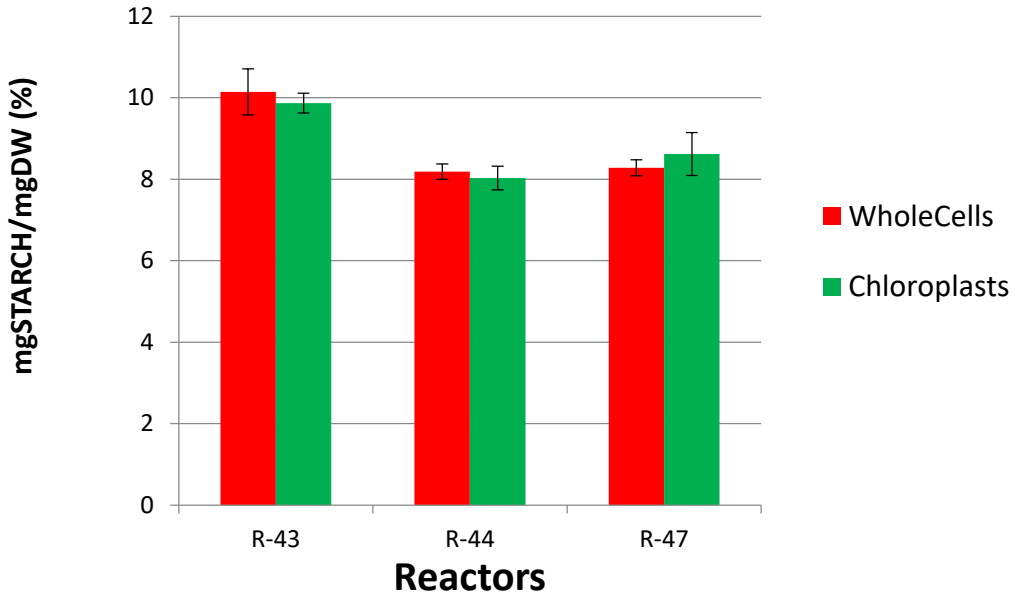

Figure 23 Graph showing the percentage of starch content on dry weight in three different biological replicates.

Although the result seems to be in according with our expectations, there is still something unclear in this result. Indeed, if we think that the chloroplast lacks of cell wall and cytoplasm, the weight (per single cell) should be less and consequently the starch should be more concentrated. This could be explained taking in account the harshness of sonication procedures and osmotic shocks induced to obtain chloroplasts. Some cells inevitably disrupt dissolving the starch in the buffer which is then lost during the isolation process. Therefore, the isolation protocol also disrupts some cells (also confirmed by the osmotic burst assay), so the isolation process we could also get rid of certain amount of starch, while in the whole cell (control) the starch is not lost. This could also explain why the difference is so high in in the results we obtained for the total carbohydrates and proteins (see next sub-chapter).

3.4.2 Total carbohydrates

Although the result seems to be in according with our expectations, there is still something unclear in this result. Indeed, if we think that the chloroplast lacks of cell wall and cytoplasm, the weight (per single cell) should be less and consequently the starch should be more concentrated. This could be explained taking in account the harshness of sonication procedures and osmotic shocks induced to obtain chloroplasts. Some cells inevitably disrupt dissolving the starch in the buffer which is then lost during the isolation process. Therefore, the isolation protocol also disrupts some cells (also confirmed by the osmotic burst assay), so the isolation process we could also get rid of certain amount of starch, while in the whole cell (control) the starch is not lost. This could also explain why the difference is so high in in the results we obtained for the total carbohydrates and proteins (see next sub chapter).

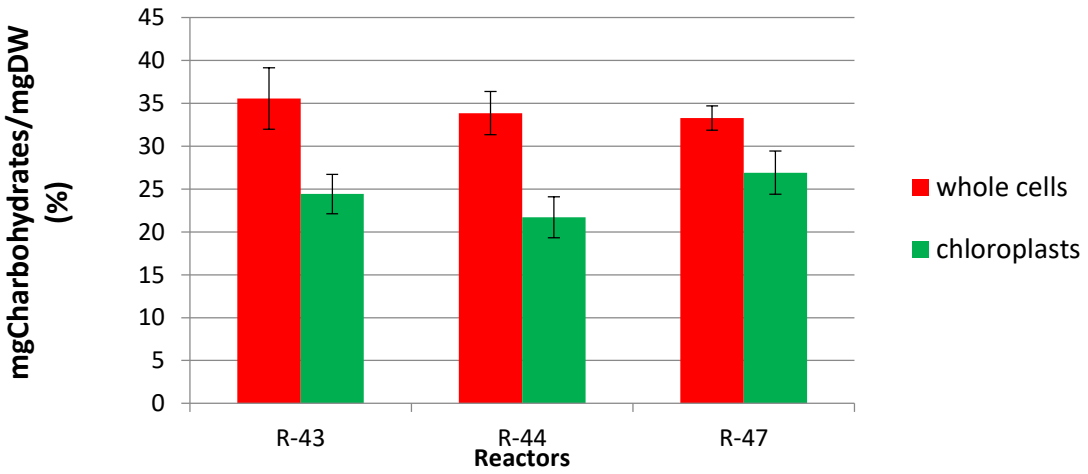

Figure 24 Graph showing the percentage of total carbohydrates on dry weight content in three different biological replicates.

3.4.3 Proteins

Even higher the difference in protein content, indeed, the variance is about the 20% in all the 3 replicates. This big variability could also be associated to an overestimation due to the presence of empty cell walls still present in the control samples. The protein content in green algae can vary between 30% and 60% (citation), therefore, the data obtained seems to be in according with previous experiments. However, previous experiments made do not take in account the presence of the empty cell walls.

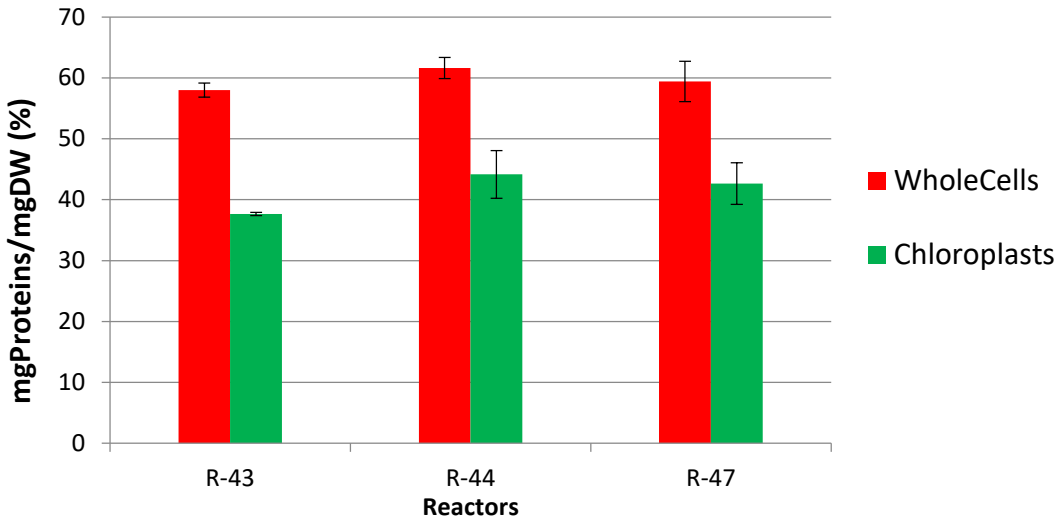

Figure 25 Graph showing the starch content in three different biological replicates.

3.5 Protoplast regeneration

After the chloroplast isolation, we could still observe a growth in the cell suspension. Since the chloroplast are not able to grow independently, we associated this phenomenon with a possible presence of protoplast. The presence of protoplasts could also be confirmed by the SEM microphotographs. This experiment, indeed, showed that the cells treated were still able to grow (**Figure 26**).

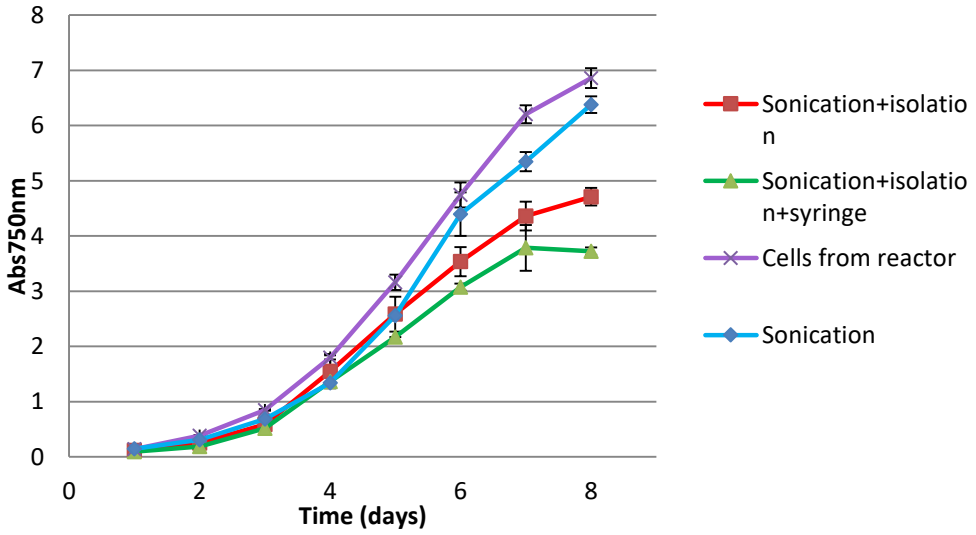

Figure 26 The graph show the growth curve of 4 samples differently treated and incubated for 8 days.

We observed that the samples only treated with the sonication, without adding the breaking buffer had the same grow rate compared to the cells taken from the reactor (any treatment). The cells also treated with the breaking buffer and the whole isolation process had instead a reduced growth. This data confirms the effectiveness of the breaking buffer. Furthermore, the samples treated with the syringe needle after the isolation process grew even less than the isolated protoplasts suggesting an effective disruption of the plasma membrane and the cytosol.

4. Recommendations

4.1 Cell disruption

In order to understand if the chloroplast is still covered by the plasma membrane, a further treatment after the sonication is suggested. With the knowledge maturated during this thesis, I would suggest a treatment with a mild lysing solution containing a detergent compound. In theory a mild detergent solution should be able to disrupt the external membrane (whether is present or not) and at the same time keep the chloroplast intact.

4.2 Chloroplast isolation

To increase the purity of chloroplast and to improve the exclusion of the cell debris (mainly composed of cell walls and cytosolic components), we suggest to use a more effective method. A differential density gradient centrifugation would be the ideal method to improve the isolation process due to the higher resolution in separating particles by density. For a sucrose differential isolation an ultracentrifuge is required.

4.3 Biochemical analysis

The biochemical analysis performed were not complete, lipids and pigments are still missing. Furthermore, the carbohydrates and the proteins could be overestimated because in the reactor culture many empty cell walls were still present in the samples. Therefore, to have a final big picture of the biochemical composition, lipids and pigments quantification are required. Moreover, to have a more precise biochemical characterization we suggest to use, also for the control samples, a preliminary washing step to get rid of the empty cell walls present in the cultures.

5. Acknowledgments (ringraziamenti)

Per la realizzazione di questa tesi, e più in generale per il raggiungimento di questo traguardo tanto sudato ed atteso, è stato necessaria la partecipazione (diretta ed indiretta) di un numero spropositato di persone. Sarebbe complicato e molto dispendioso in termini di tempo citarli tutti, quindi mi limiterò a considerare quelli che in un modo o nell'altro sono stati essenziali.

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Se dovessimo fare una classifica su "chi ho importunato di più negli ultimi due anni a Pisa", probabilmente, tra i primi posti ci piazzerei i miei colleghi di corso. L'ambiente creato, tra i banchi e i laboratori dell'Università (e non solo), è stato tra i più stimolanti e piacevoli che mio abbia mai vissuto. L'eterogeneità e la condivisione sono state le qualità che più mi hanno colpito del nostro gruppo ed è per questo che sono sicuro che in futuro avrò il piacere di collaborare con voi. Grazie colleghi!

You would notice something different in these lines, they are written in English. This is because they are dedicated to a special person who supported me, from the beginning to the end of this project. When I say "for this project", I do not mean only the technical support for the thesis (even if it was significant), but I also refer to the support in every-day life which made me a better person in a scientific environment and in the society. Thank you Mirka!

Last but not least, un ringraziamento particolare va a tutti i miei più cari amici, che direttamente mi hanno sostenuto in questa impresa e con cui mi sono divertito come un animale in questi due anni di "panico". Fabrizio, Zoe, Massimo, Piergiorgio, dall'Italia. Vincenzo, Fabian, Ernico, Claudio, Martim e Daniel dall'Olanda. Queste sono solo alcune delle fantastiche persone con cui ho condiviso parte della mia vita e con cui la condividerò ancora.

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Appendix-1

Stock solutions for freshwater media for *Scenedesmus obliquus*			
(Stock I)			
#	**Component**	**Amount (g/l)**	**Concentration in media (g/L)**
1	$Na_2EDTA.2H_2O$	9.92	0.02976
2	$MnCl_2.4H_2O$	1.27	0.00381
3	$ZnSO_4.7H_2O$	0.4	0.0012
4	$CoCl_2.6H_2O$	0.094	0.000282
5	$CuSO_4.5H_2O$	0.11	0.00033
6	$Na_2MoO_4.2H_2O$	0.0084	0.0000252
Stock II			
#	**Component**	**Amount (g/l)**	**Concentration in media (g/L)**
1	NaFeEDTA	3.4	0.0102
Vitamin (stock3)			
#	**Component**	**Amount (g/l)**	**Concentration in media (g/L)**
1	Biotin	0.025	0.000025
2	Vitamin B1	1.1	0.0011
3	Vitamin B12	0.135	0.000135

Appendix-2

Breaking Buffer – 100mL							
N.	**Compound**	**Final concentration (M/%)**	**Form**	**M/M**	**Amount to add (g/L or mL/L)**	**Dilution factor**	**Amount to add (g or mL)**
1	NaCl	0.5	solid	58.44	29.22	0.1	2.922
2	MgCl2*6H2O	0.05	solid	203.31	10.1655	0.1	1.01655
3	Na_ascorbate	0.005	Solid	198.11	0.99055	0.1	0.099
4	DTT	0.005	Liquid	154.25	0.77125	0.1	0.077
	3-4: To add fresh before the experiment						

	Isolation buffer - 500 mL	
N.	**Compound**	**Amount**
1	50 mM HEPES - KOH (pH= 7.5)	5.96 g
2	1% BSA	5 g
3	300 mM SORBITOL	27.4 g
4	1mM MgCl2*6H2O	0.1 g
5	2mM Na-EDTA (pH= 8)	2mL from 0.5M stock

Appendix-3

S.obliquus **DNA EXTRACTION PROTOCOL**

Procedure:

1. Add 300µL of 0.2% SDS to bead beating tubes
2. Pick pelleted biomass from 100µL of culture and dissolve in the SDS.
3. Add 150 µL of chloroform:phenol:isopropylic Acid (5:24:1) solution to the bead beating tubes.
4. Place tubes in the bead beater (Precellys 24 - Bertin©) and lyse the cells (4000 rpm; 2X60 seconds: 20 seconds in between).
5. Centrifuge (5 minutes; 15.000xg).
6. Transfer 200 µL from the supernatant into an eppendorf tube (carefully avoiding touching the beads).
7. Add Na-acetate buffer (pH 5.5) for 1/10 of the transferred volume (20µL for 200µL).
8. Add 400µL of cold ethanol (100%).
9. Place the tubes in the freezer for 1 hour (in the while cool the rotor of an eppendorf centrifuge at 4°C).
10. Centrifuge for 15 minutes at 15.000xg (4°C). NOTE: Pay attention the pellet is nearly invisible, in order to avoid the contact place the tubes in a fixed position and then pipet the supernatant from the opposite side of the pellet.
11. Remove the supernatant carefully.
12. Resuspend the pellet in 250µL of cold ethanol (70%)
13. Centrifuge for 5 minutes 15.000xg (4°C)
14. Remove all the supernatant and place the tube with the pellet in a heater (37°C) in order to dry all the ethanol.
15. Add 15µL of pure water (RNAse and DNAse free).
16. Dissolve the DNA by pipetting, measure the DNA concentration spectrophotometrically and store at -20°C.

Appendix-4

CHLOROPLAST ISOLATION PROTOCOL

1. Sample 3 mL of cell suspension from the reactor (7.5hours after light on) and place it in a 15 mL falcon tube.
2. Centrifuge the cells (3600 x g for 10 minutes with cold rotor, 4°C) and re-suspend the cells in 3mL of breaking buffer.
3. Place the tube in a Beaker filled with ice and sonicate for 1 minute (2 times for 30 seconds) at 100% of power using a probe sonicator in continuous mode (10/14 Watts) [make sure that the probe is not in contact with the plastic surface of the tube].
4. Place the tube in ice [After the sonication all the steps have to be performed below 4°C].
5. Centrifuge the suspension (700 x g for 1 minute at 4°C) and resuspend with the pipette in 1.8 mL of cold isolation buffer and transfer to 2mL Eppendorf tubes.
6. Rinse the chloroplast centrifuging (700 x g for 1 minute at 4°C) and replacing the isolation buffer 2 times.
7. After the last centrifugation suspend the chloroplast in 0.5mL of cold isolation buffer and transfer the suspension in another Eppendorf.
8. Washed once with Phosphate buffer (pH: 7.5) and pellet 1100xg for 2 minutes.
9. Store them in the freezer (-20°C).

Appendix-5

ALGAEMIST SCHEMATIZED VIEW