3

Materials and methods

3.1. Description of the experimental set-ups

Part of the experimental work presented in this thesis was carried out in a laboratory set-up while other part was developed in a bench scale pilot plant (Figure 3.1). The laboratory setting was composed of round shape 2 L glass flasks that were placed in a growth chamber (Sclab ER refrigerated chamber), aerated with air enriched with $2\,\%$ CO_2 at a flow rate of $0.027\,\mathrm{vvm}$ and shaken at $350\,\mathrm{rpm}$ with magnetic stirrers. The temperature was maintained at $22.0 \pm 0.2\,^{\circ}\mathrm{C}$ and the frontal light intensity provided by the lamps of the chamber was $145\,\mu\mathrm{E}\,\mathrm{m}^{-2}\,\mathrm{s}^{-1}$. Both aeration and light were supplied for $12\,\mathrm{h}$ per day.

The bench scale pilot plant consisted on two 70-L cylindrical photobioreactors, aerated with air enriched with $2\,\%$ CO_2 at a flow rate of $0.027\,\mathrm{vvm}$ by means of a fine bubble membrane diffuser (ABS KKI215) and illuminated for $12\,\mathrm{h}$ a day with a metal-halide lamp (Philips Master HPI Plus 400 W/767). Complete mixing in the reactor was reached by means of aeration and internal recirculation (WILO Star-RS 24/4) at a rate of five volume renewals per hour.

Figure 3.1: Laboratory and bench-scale set-ups.

The photobioreactors included a transparent truncated conical structure (Figure 3.2) that allowed the light to reach the deepest part of the cylindrical reactors. The specific criteria and procedure of the cones design is the subject of Chapter 4. In this case, the upper base has a diameter of $226\,\mathrm{mm}$ and the opposed base has a diameter

of $30\,\mathrm{mm}$. Cones height is $1120\,\mathrm{mm}$, but only $970\,\mathrm{mm}$ were submerged, where the cones diameter is $200\,\mathrm{mm}$. The material of the cones is PVC ($\mathrm{transmittance} \approx 88\,\%$), since methacrylate, although having a higher transmittance, could not be shaped as a cone with only 10° of aperture.

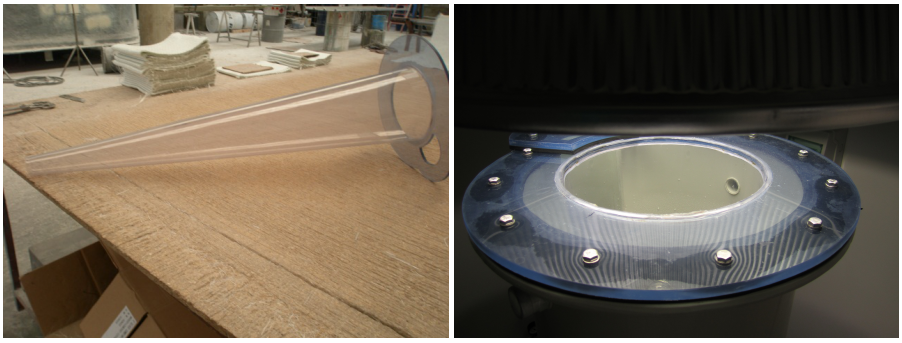

Figure 3.2: Plastic cone used in the bench scale set-up.

In the sections below, criteria followed to establish the culture conditions are detailed, as well as the applied analytical methods for growth, nutrients consumption and lipid production monitoring.

3.2. Culture conditions

3.2.1. Strain selection

Five selection criteria were taken into account to select the strain or strains to be used in the experiments:

Freshwater growth Urban, as well as industrial wastewater is usually produced inland. This kind of water can be treated by means of microalgae cultures before flowing into the sea or the rivers. Furthermore, using a freshwater microalgae strain makes easier the installation of culturing devices (photobioreactors) in a wider range of localizations than if a coastal area should be selected, including industrial areas where companies liable to profit from this technology are usually located. Finally, chloride presence in seawater favors equipment corrosion, making its life cycle shorter. Thus, selecting a freshwater strain diminishes maintenance and spare parts replacement.

High lipid content Since one of the main objectives of microalgae culturing is the oil for biodiesel production obtaining, one of the essential criteria to select the microalgae strains is their oil content. Microalgae able to accumulate high amounts of oils will be prioritized.

High CO_2 tolerance Another potential of sustainable microalgae culturing is its ability to capture CO_2 from flue gases, mitigating the contribution of fossil fuel emissions to global warming. As selecting criterion, not only its ability to fix high amounts of CO_2 is taken into account, but also its tolerance to flue gases with high concentrations of that compound.

Production of interesting compounds for the industry The possibility to obtain other interesting compounds, with high value in the industry, is an important characteristic to achieve the economic feasibility of microalgae culturing.

Low light requirements A strain with a low compensation point will allow a more effective use of light per unit surface. Furthermore, the geographical area where this work is carried out does not have high irradiance values. In the case of using artificial light in certain periods of the culture, the low light requirements will also favor the sustainability of the culture.

Keeping these criteria in mind, the first selected species was *Scenedesmus obliquus* since it is a freshwater microalgae and presents high oil contents, reaching until $55\,\%$ of its dry weight (Mata, Martins, and Caetano 2010). It is also a species with high tolerance to extra- CO_2, growing even at $50\,\%$ of CO_2 in air and CO_2 biofixation rates of $288\,\mathrm{mg\,L^{-1}d^{-1}}$ (Tang et al. 2011) or $390.2\,\mathrm{mg\,L^{-1}d^{-1}}$ (Ho et al. 2010). Among the compounds that can be obtained from *S. obliquus* cultures, different nutraceutic compounds can be found. The most abundant fatty acids are palmitic acid (16:0), estearic acid (18:0) and oleic acid (18:1n9). It is also possible to find, to a greater or lesser extent depending on culturing conditions, essential fatty acids as linoleic (18:2n6), linolenic (18:3n3), eicosapentaenoic (EPA, 20:5n3) and docohexaenoic (DHA, 22:6n3) (Ho et al. 2010; Hodaifa, Martínez, and Sánchez 2008) According to Sorokin and Krauss 1958 this species reaches its maximum growth rate at a relatively low light intensity, thus it complies with the established selection criteria. With regard to other factors that have lead to choose *S. obliquus* as one of the species to be used, one advantage is that it may be easier to harvest than other species due to its way of reproduction. This species is reproduced by multiple fission producing coenobia, which are colonies that contain a number of cells, and frequently more than two daughter cells remain connected (Figure 3.3). The most frequent colonies are formed

by four cells (Ho et al. 2010), producing big structures that make easier the harvesting techniques such as filtration, centrifugation and sedimentation.

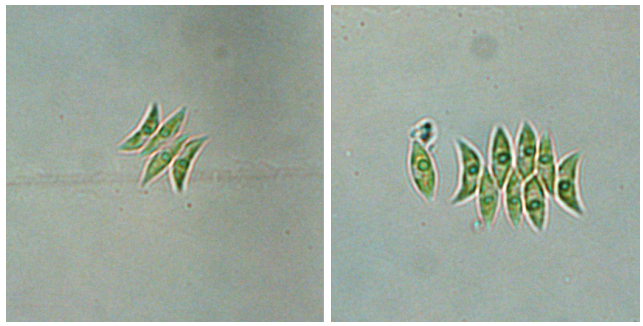

Figure 3.3: Coenobia formed by *S. obliquus*.

Species belonging to genus *Scenedesmus* have a thicker cell wall than others, making them more resistant to hydrodynamic stress (Hodaifa et al. 2010). It is worth saying that it is a species that has been used in wasterwater treatment (Godos et al. 2010) due to its robustness and its ability to use carbon, phosphorus and nitrogen in a versatile way (Hodaifa et al. 2010). The second working species selected was *Chlorella vulgaris* Beijerink which is also a freshwater microalgae. It is a spherical, unicellular green algae and has short time of generation and low nutritional requirements. Its reproduction takes place through the production of four or eight autospores by a mature cell (Iwamoto 2004). *C. vulgaris* is a nutritionally appreciated species and has been cultivated with commercial purposes for at least 40 years. The most appreciated substances that *C. vulgaris* produces are chlorophylls, beta-carotene, proteins, cellulose and lipids (Wijanarko et al. 2006). It has been usually reported as an easy to cultivate microalgae with not too high but significant lipid content, (Widjaja, Chien, and Ju 2009; Lv et al. 2010). A $50\,\%$ lipid content can be achieved under long N starvation (Illman, Scragg, and Shales 2000).

S. obliquus (CCAP 276/3A) and *C. vulgaris* (Beijerink CCAP 211/ 11B) were supplied by the Culture Collection of Algae and Protozoa in Scotland, UK.

3.2.2. Culture medium

An in depth bibliographic review was carried out to choose the most suitable culture medium for *S. obliquus* and *C. vulgaris* cultivation as shown in Table 3.1

BG11 medium was selected as culture medium due to:

Culture medium	Composition	References
BG11	$NaNO_3$, K_2HPO_4 $MgSO_4 \cdot 7\,H_2O$ $CaCl_2 \cdot 2\,H_2O$, Citric acid Ammonium Fe^{3+} citrate, $EDTANa_2$ Na_2CO_3 Trace minerals solution: H_3BO_3 $MnCl_2 \cdot 4\,H_2O$, $MnSO_4 \cdot 7\,H_2O$ $Na_2MoO_4 \cdot 2\,H_2O$ $CuSO_4 \cdot 5\,H_2O$ $Co(NO_3)_2 \cdot 6\,H_2O$	Tang et al. 2011; Rodolfi et al. 2009
DM	$Ca(NO_3)_2 \cdot 4\,H_2O$, KH_2PO_4, $MgSO_4 \cdot 7\,H_2O$, KCl, $FeSO_4 \cdot 7\,H_2O$, EDTA $\cdot$ 2 Na, H_3BO_3, $ZnCl_2$, $MnCl_2 \cdot 4\,H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$, $CuSO_4 \cdot 5\,H_2O$,	Ho et al. 2010; Cheng et al. 2011
MBM	KNO_3, $CaCl_2$, $MgSO_4$, K_2HPO_4, KH_2PO_4, NaCl, $FeSO_4 \cdot 7\,H_2O$, H_3B_3O, $MnCl_2$, $CuSO_4 \cdot 5\,H_2O$, 3 $(NH_4)_2 \cdot 7\,MoO_3 \cdot 4\,H_2O$	Watanabe 1960
JAWOROSKI	$Ca(NO_3)_2 \cdot 4\,H_2O$, KH_2PO_4, $MgSO_4 \cdot 7\,H_2O$, $NaHCO_3$, EDTAFeNa, $EDTANa_2$, H_3BO_3, $MnCl_2 \cdot 4\,H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$, Cyanocobalamin, Thiamine, HCl, Biotine, $NaNO_3$,Na2HPO4.12H2O	Hulatt and Thomas 2011

Table 3.1: Composition of culture media used by different authors.

- It does not contain vitamins, so it is more economically accessible.
- It has high nitrate content, allowing carrying out a wide range of tests with different concentrations of this compound, which is said to be a key factor in the microalgae oil accumulation ability (Illman, Scragg, and Shales 2000; Converti et al. 2009).
- It contains high concentrations of nitrogen and phosphorus, which allows simulating the effect of high strength wastewater over microalgae.

3.2.3. Temperature

Temperature directly affects to microalgae growth, thus the selection of its value was based on bibliographic data. Temperatures used by different authors are showed in Table 3.2.

It can be observed that all of the checked Works used temperatures of at least 25 °C, which is known to result in a higher growth rate, thus reducing experimentation time. However, in order to have more representative results, consuming less energy and taking into account the CCAP (Culture Collection of Algae and Protozoa) advice,

Temperature (°C)	Reference
28	Ho et al. 2010
30	Morais and Costa 2007
25	Mandal and Mallick 2009
25	Hodaifa et al. 2010
25	Kim et al. 2011
25	Yoo et al. 2010
25	Tang et al. 2011
25	Chinnasamy et al. 2010
30	Hodaifa, Martínez, and Sánchez 2008

Table 3.2: Temperatures used by different authors for microalgae culturing.

$25\,°C$ will be established in laboratory assays while between $20\,°C$ to $25\,°C$ will be maintained in bench scale assays.

3.2.4. Air flow rate and CO_2 concentration

Photoautotrophic organisms need CO_2 for growing, and this may be supplied to the culture by means or aeration, preferably with CO_2 enriched air. Concentrations used by different authors are shown in Table 3.3 CO_2 requirements can be calculated by means of the stoichiometric of carbon assimilation by the algae. $1.85\,g$ of CO_2 is needed to produce $1\,g$ of biomass (Posten 2009). Air flow rate and composition of the CO_2-air mixture must be established in order to guarantee a sufficient carbon supply but not exceeding inhibition concentrations. CO_2 enriched air was supplied during the light hours, thus in a 12:12 cycle. To determine the amount of CO_2 in the mixture, a review of works studying the effect of CO_2 concentration on microalgal biomass and lipids productivity was made. In accordance to their results a CO_2 concentration of $2\,\%$ v/v was selected for both, laboratory and bench scales.

CO_2 concentrations ($\%_{v/v}$)	References
5 / 10	Zhang, Kurano, and Miyachi 2002
0,04 / 6 / 12 / 18	Morais and Costa 2007
0,03 / 5 / 10 / 20 / 30 / 50	Tang et al. 2011
5 / 10 / 20 / 30 / 40 / 50 / 60 / 70	Ho et al. 2010

Table 3.3: CO_2 concentrations tested by different authors.

The CO_2 enriched air flow rate was calculated in accordance to expected biomass productivity and the $g\,(CO_2)\,g\,biomass^{-1}$ ratio. An aeration rate of $0.027\,vvm$

($0.04\,\mathrm{L\,air\,min^{-1}}$) was determined. Furthermore, assays to determine the CO_2 consumption, in accordance to standard UNE-EN ISO 9439 (ISO 2000), were carried so as to assure that CO_2 was not a limiting factor (data not shown). In order to maintain the same culture conditions in the bench scale experimentation, aeration rate and CO_2 concentrations were the same than in laboratory scale, resulting in a flow rate of $2\,\mathrm{L\,air\,min^{-1}}$.

3.2.5. Light: Type and intensity

The utilization of solar light is one of the main premises of the photobioreactor design. However, bench scale assays were carried out indoor to keep the culture conditions under control, so an artificial light source was needed. In order to obtain representative results, the illumination system should provide similar characteristics than solar light. The main criteria to have into account when selecting an artificial light source are (Carvalho et al. 2011):

- High energetic efficiency.
- Low heat loss.
- Highly reliable.
- Long lasting and durability.
- Relatively low size.
- Low cost.
- Emission in the desired spectrum..

Regarding to the light characteristics, the source selection was based in the following:

Color temperature: it is the parameter used to measure the warmth or the coldness of the light. It is based on the color emitted by and incandescent source, since it depends only on its temperature, thus it is possible to describe the color of a light source as its temperature. Direct solar light has a temperature of about $5.300\,\mathrm{K}$.

	Global irradiance ($\mathrm{W\,m^{-2}}$)	PAR irradiance ($\mathrm{W\,m^{-2}}$)	Ratio $\frac{\text{diffuserad.}}{\text{globalrad.}}$
January	285.00	128.25	0.58
February	362.50	163.13	0.55
March	482.22	217.00	0.48
April	498.89	224.50	0.52
May	502.00	225.90	0.51
June	476.36	214.36	0.49
July	484.55	218.05	0.48
August	511.00	229.95	0.47
September	528.89	238.00	0.44
October	401.11	180.50	0.49
November	307.50	138.38	0.56
December	252.50	113.63	0.60
Year	441.11	198.50	0.50

Table 3.4: Irradiance data in Santander. Source: *Photovoltaic Geographical Information System (PVGIS)*

Spectral distribution: this concept is related with the previous one, but it has into account the presence of peaks at certain wavelengths, or by the contrary, if the light has a continuous distribution. Solar spectral distribution within the PAR region is almost continuous.

Intensity: this is one of the key parameters in the photobioreactor design field. The light intensity distribution per unit surface (irradiance) it is not a scalable parameter, thus irradiance in the bench scale reactor should be the most similar as possible to outdoor irradiance.

Average annual irradiance in Santander, the city where this work was developed, is $441.11\,\mathrm{W\,m^{-2}}$, from which approximately $198.5\,\mathrm{W\,m^{-2}}$ are PAR. Average wavelength of PAR is $550\,\mathrm{nm}$, thus its intensity is about $132.333\,\mathrm{lux}$ or $910\,\mu\mathrm{E\,m^{-2}\,s^{-1}}$ (Table 3.4). Due to the different radiometric units used, the equivalence among units is shown in Table 3.5.

Having this data into account and according with the cones dimensions, cells in the external layer on the culture in the bench scale reactors are expected to receive about $65\,\mu\mathrm{E\,m^{-2}\,s^{-1}}$.

The frontal light intensity in the laboratory scale cultures, provided by the fluorescent tubes of the germination chamber, was $147\,\mu\mathrm{E\,m^{-2}\,s^{-1}}$, and could be diminished by placing more or less dense meshes between the light sources and the flasks.

Parameter	Unit	Abbreviation	Equivalence
Light intensity (SI)	candela	cd	$1\,\mathrm{lm\,sr^{-1}}$
Illuminance	lux	lx	$1\,\mathrm{lm\,m^{-2}}$
Luminous flux	lumen	lm	$1\,\mathrm{lx\,m^{2}}$
Radian flux	watt	W	$1/0.0015\,\mathrm{lm}$
Irradiance	$\mathrm{W\,m^{-2}}$		$1/0.0015\,\mathrm{lx}$
Photon flux density	$\mu\mathrm{E\,m^{-2}\,s^{-1}}$		$0.214\,\mathrm{W\,m^{-2}}$

Table 3.5: Equivalence of radiometric units.

Regarding the bench scale experimentation, after examining the light sources available in the market, it was found that those that better reproduced the solar spectrum were fluorescent tubes. However, this kind of lamps has a low power, since tubes with length of $59\,\mathrm{cm}$ and diameter of $2.56\,\mathrm{cm}$ have a consumption power of 18 W and emission power of 1.6 W. In addition, they present pronounced peaks, on the contrary to solar spectrum.

The most suitable lamps seemed to be metal-halide lamps, similar to Hg lamps but containing also other gases able to emit in other spectral regions. Lamps with color temperature similar to that of the Sun are available in the market. Heat losses are considered to be low in comparison to incandescent lamps and its energy efficiency is good (Carvalho et al. 2011). The lamp used in the bench scale pilot plant was a metal-halide lamp (Philips Master HPI Plus 400 W/767 lamp) with a luminous flux of 35000 lm and color temperature of 5500 K.

3.2.6. Light-dark cycles

According to numerous authors, two of the main factors related with the growth and lipid accumulation of microalgae are the light intensity and the alternation of light and dark conditions.

In Table 3.6, intensities and light-dark cycles used by different authors are detailed.

Since the experimentation is oriented to outdoor culture, in order to be near to the average L/D cycle in Spain, a 12/12 cycle was chosen.

3.3. Biomass growth measurement

The monitoring of the biomass growth can be made by means of direct methods or indirect methods. The direct methods are those in which the number of cells is

Light flux (μE m^{-2} s^{-1})	Light-dark Cycle (h (light)/ h (dark))	Reference
60	-	Ho et al. 2010
75	14/10	Mandal and Mallick 2009
298	continuous	Hodaifa, Martínez, and Sánchez 2008
150	continuous	Kim et al. 2011; Yoo et al. 2010
180	-	Tang et al. 2011
80	continuous	Qin, Liu, and Hu 2008
80	12/12	Chinnasamy et al. 2010
150	-	Gouveia and Oliveira 2009

Table 3.6: Intensities and light-dark cycles used by different authors.

counted in a certain culture volume, thus estimating the population size. Cell counting under the microscope is a direct method. The indirect methods use a parameter related with the number of cells, such as the dry weight or the absorbance. In general, it must be an easy to measure parameter in order to make the biomass growth monitoring faster and easier. In this work, direct and indirect methods were applied, since it was observed that the indirect methods, both dry weight and absorbance, could rise even when the culture had reached the stationary phase. Cell counting was the most suitable method to detect the phase of growth.

3.3.1. Cell counting

In order to quantify the cells in the cultures, a Thoma counting chamber was used. A Thoma chamber is divided in 16 squares, each one with 25 squares inside, 9 of them divided in half, in such a way that each group of 16 squares is surrounded by 16 half squares and 4 quarters of square. Each group of 16 squares have a volume of $4 \times 10^{-6}\,\mathrm{mL}$ ($0.1\,\mathrm{mm}$ depth and surface of $0.04\,\mathrm{mm}^2$). At least 10 of the 16 squares that composed the grid were counted. The number of cells per unit volume was calculated dividing the average number of cells in the grid by the volume of the grid. Two snapshots of the cells counting are showed in Figure 3.4 and Figure 3.5.

3.3.2. Dry weight

For dry weight measuring, a certain culture volume was filtered with $0.45\,\mu\mathrm{m}$ Whatman paper filter GF/C $47\,\mathrm{mm}$, which is a usual standard to collect suspended solids in potable and natural or industrial wastes. The filter was placed in a porcelain crucible previously weighted. It was dried at $103\,^{\circ}\mathrm{C}$ during $6\,\mathrm{h}$ or until constant weight. After that, it was cooled at room temperature in a desiccator. Then, the

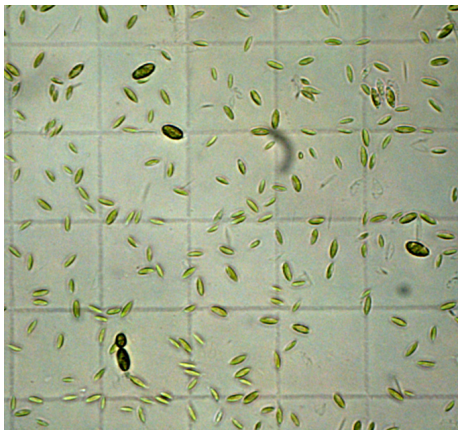

Figure 3.4: Thoma chamber grid before the cell counting under optical microscope.

Figure 3.5: Thoma chamber grid during the cell direct counting under optical microscope.

crucible and the filter were weighted together and the difference in weight with the previous datum was attributed to biomass.

3.3.3. Absorbance

The first step was to identify the most suitable wavelength to use in the growth monitoring of the selected species. A bibliographic review showed that in the case of *S. obliquus* the wavelength used was between $560\,\mathrm{nm}$ to $685\,\mathrm{nm}$. Then, a spectral scanning with a DR 3800 Hach Lange spectrophotometer was made.

Wavelength (nm)	Reference
685	Ho et al. 2010
670	Morais and Costa 2007
650	Tang et al. 2011
560	Sancho, Castillo, and Yousfi 1999

Table 3.7: Wavelengths used by different authors during *S. obliquus* growth monitoring.

The content in pigments of a given species determines which regions of the spectrum will be attenuated with higher or lower intensity. Knowing of this phenomena is important to calculate the amount of light useful for photosynthesis that goes through the culture, since photometers give the total PAR light intensity ($400\,\mathrm{nm}$ to $700\,\mathrm{nm}$), without differencing the different regions of the spectrum.

From an aliquot of a culture in exponential phase and another of a culture in stationary phase, progressive dilutions were made. In order to obtain a concentrated sample, aliquots were centrifuged at 3852g for 15 min, and the pellet was resuspended in $20\,\mathrm{mL}$ of fresh medium (the zero reading was made with fresh medium). The sample was diluted at $15\,\%$ and that was the A0 sample. Further dilutions were made as show in Table 3.8 and Table 3.9

	Dilutions (from A0)	PS (g/L)	Nr cel/mL	Abs. (685 nm)
A0		0.327	1.99×10^8	1.259
A1	9:10	0.294	1.79×10^8	1.232
A2	8:10	0.262	1.59×10^8	1.079
A3	7:10	0.229	1.39×10^8	0.951
A4	6:10	0.196	1.20×10^8	0.874
A5	5:10	0.164	9.96×10^7	0.746
A6	4:10	0.131	7.97×10^7	0.609
A7	3:10	0.098	5.98×10^7	0.471
A8	2:10	0.065	3.98×10^7	0.322
A9	1:10	0.033	1.99×10^7	0.164
A10	0.5:10	0.016	9.96×10^6	0.078

Table 3.8: Data from dilutions used in the absorbance characterization of *S. obliquus* in exponential phase.

A spectral scanning was made to each sample with a 1 inch glass cuvette.

	Dilutions (from A0)	PS (g/L)	Nrcel/mL	Abs. (685 nm)
A0		5.775	2.97×10^8	
A1	1:5	1.155	5.94×10^7	1.522
A2	1:7	0.826	4.24×10^7	1.522
A3	1:10	0.578	2.97×10^7	1.482
A4	1:15	0.385	1.98×10^7	1.100
A5	1:20	0.289	1.48×10^7	0.849
A6	1:25	0.231	1.19×10^7	0.694
A7	1:30	0.192	9.88×10^6	0.587
A8	1:40	0.144	7.42×10^6	0.443
A9	1:80	0.072	3.71×10^6	0.220

Table 3.9: Data from dilutions used in the absorbance characterization of *S. obliquus* in stationary phase.

In the graphs (Figure 3.6 and Figure 3.7) it can be seen the *S. obliquus* cultures presents two mean absorption peaks: one of them is well defined around $685\,\mathrm{nm}$, while the other one is less defined around $440\,\mathrm{nm}$. These two peaks belong to the blue and red regions of the spectrum, which is a characteristic of microalgae containing Chlorophylla. In spite of this, the two scans show also a substantial absorption in the intermediate areas of the spectrum, that is to say the green and yellow regions, although noticeable minor than in the red and blue regions. These results are coherent with that of Yun and Park 2001. In accordance with that obtained in this tests and the reviewed bibliography, $685\,\mathrm{nm}$ was selected as the wavelength for the growth monitoring of *S. obliquus*. The same procedure was carried out with *C. vulgaris*, and being also a green algae containing Chlorophylla, $685\,\mathrm{nm}$ resulted also the most suitable wavelength to monitor the growth by means of the absorbance.

3.4. Analytical methods for nutrients determination

Nitrogen and phosphorus are the main nutrients for microalgae feeding. The analytical methods employed for their determination are explained below. Before the analysis, samples were filtered by using $0.45\,\mu\mathrm{m}$ Whatman paper filter GF/C $47\,\mathrm{mm}$. Nutrients were photometrically determined by using a DR 3800 Hach Lange spectrophotometer. Dilution with distilled water was made if concentration was expected to be above the upper limit of the determination range.

3.4.1. Nitrogen as nitrate (NO_3-N)

As it is indicated in the composition of BG11 medium, nitrogen is supplied to microalgae mainly as nitrate, but also as ammonium. Nitrogen as nitrate was

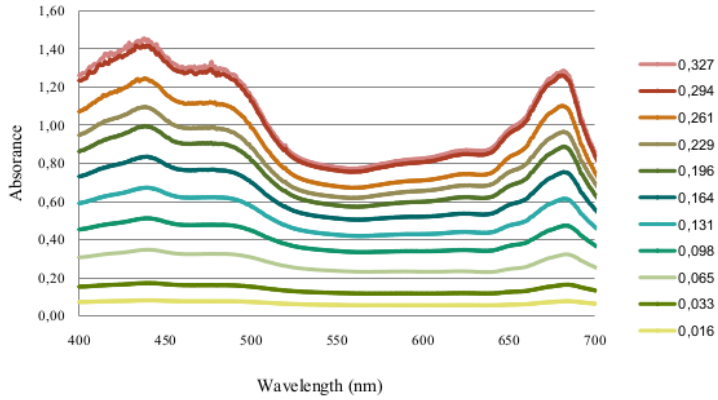

Figure 3.6: Spectral scan within the PAR region of a *S. obliquus* culture in exponential phase. The key expresses dry weight in g L^{-1}.

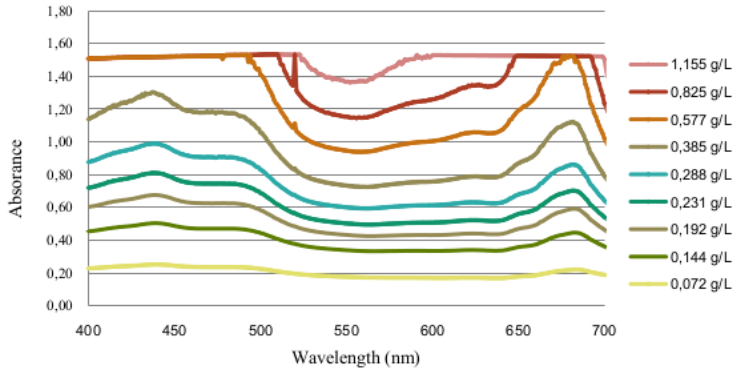

Figure 3.7: Spectral scan within the PAR region of a *S. obliquus* culture in stationary phase. The key expresses dry weight in g L^{-1}.

photometrically determined by means of Spectroquant Nitrate Test 1.14773.0001 (Merck Millipore). The method is based on the reaction of nitrate ions with a benzoic derivative in concentrated sulfuric, appearing a red compound that is quantified at

515 nm. 10 mm cuvettes were used, for which the measuring range corresponds to 0.5 mg (NO_3−N) L^{-1} to 20.0 mg (NO_3−N) L^{-1}. 1.5 mL of sample was needed for the determination.

3.4.2. Nitrogen as nitrite (NO_2−N)

Nitrogen as nitrite in the culture medium was photometrically determined by means of NitriVer® 3 test (Hach). The method is the USEPA Diazotization Method 354.1 (EPA 1983), and it is valid for determination of low range nitrite (up to 0.30 mg (NO_2−N) L^{-1}). A sample of 10 mL was needed. Nitrite in the sample reacts with sulfanilic acid to form an intermediate diazonium salt. This couples with chromotropic acid to produce a pink colored complex directly proportional to the amount of nitrite present. The measurement wavelength is 507 nm.

3.4.3. Nitrogen as ammonium (NH_4−N)

Nitrogen as ammonium was also determined by means of a photometric kit. In this case Spectroquant Ammonium Test 1.14752.0001 (Merck Millipore) was used. The method is analogous to EPA 350.1 (EPA 1983) or ISO 7150-1 (ISO 1984) and consists on the reaction of ammonia with a chlorinating agent to form monochloramine, which in turn reacts with thymol to form a blue indophenols derivative that is determined at 640 nm. 10 mm cuvettes were used, for which the measuring range corresponds to 0.05 mg to 3.00 mg (NH_4−N) L^{-1}. 5 mL of sample were needed for the determination.

3.4.4. Phosphorus as ortophosphate

Phosphorus as ortophosphate was determined by means of the photometric test Spectroquant 1.14848.0001 (Merck Millipore). The method is analogous to EPA 365.2+3 (EPA 1983) or ISO 6878 (ISO 2004). It consists on the reaction of orthophosphate ions with molybdate in sulfuric solution, originating molybdophosphoric acid. Ascorbic acid reduces this to phosphomolybdenum blue which is quantified at 880 nm. 10 mm cuvettes were used, for which the measuring range corresponds to 0.05 mg to 5.00 mg (PO_4−N) L^{-1}. 5 mL of sample were needed for the determination.

3.5. Lipid extraction

In order to extract the oily fraction from microalgae, a hydrolysis unit (B-411/E-416 Büchi, Switzerland) and a Soxhlet extraction system B-811 (Büchi, Switzerland) were used. Firstly microalgae samples were centrifuged at 3852g for 10 min and washed

three times with deionized water. After this, the biomass sample was freeze dried (VirTis BT2KE lyophilizer) and homogenized in a mortar. The obtained pellets were stored in the freezer at $-30\,^{\circ}\mathrm{C}$ until the extraction. Before the hydrolysis, the samples were dried at $60\,^{\circ}\mathrm{C}$ during $20\,\mathrm{h}$. $1\,\mathrm{g}$ of the sample was resuspended in deionized water and HCl was added so that the final suspension was $4\,\mathrm{M}$ HCl. Then, this suspension was hydrolyzed in the hydrolysis unit (Figure 3.8). The hydrolysis breaks the lipid bounds as lipoproteins, liposaccharides or sterol esters, so that the fat is accessible for extraction. The HCl with biomass suspension was boiled during 30 minutes to break down high molecular weight compounds into acid soluble constituents. After that, the sample was filtered over a glass tube filled with 50 g of quartz sand and 5 g of celite 545 (*Hydrolysis System B-411 Technical data sheet*).

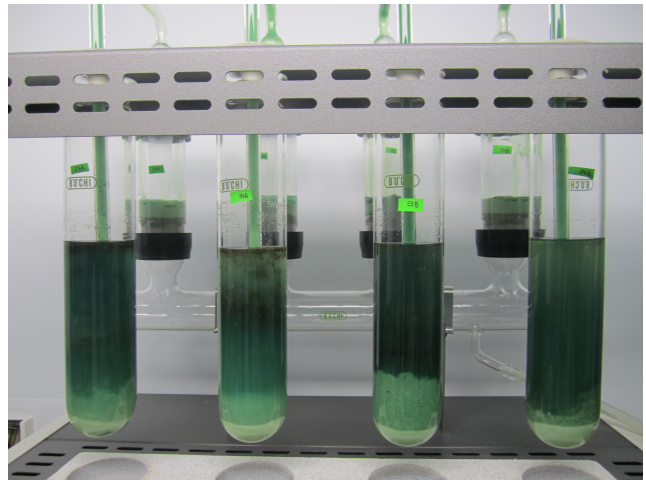

Figure 3.8: B-411 Büchi Hydrolysis unit during hydrolysis process.

Lipids were n-hexane extracted by warm Soxhlet method using the extraction system B-811 (Büchi, Switzerland) (Figure 3.9). N-hexane was used as solvent, due to its lower toxicity in comparison with other solvents and its selectivity towards neutral lipids, which are those that are desirable for biodiesel production. This method is based on that proposed by BUCHI experts for the determination of total fat in a dehydrated vegetable food flakes samples (*Extraction System B-811 Technical data sheet*). The extraction is followed by the rinsing: the sample tube is lifted and the rinsing removes all sample residues from the tube, pouring into the sample glass. Finally, during the drying, the solvent is collected by evaporation and then condensation, and the

concentrated extract remains in the sample glass for storing or further analysis (Figure 3.10).

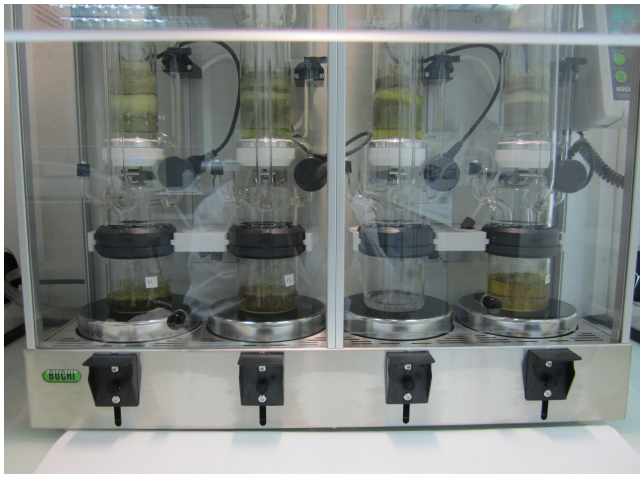

Figure 3.9: B-811 Büchi Extraction unit during extraction process.

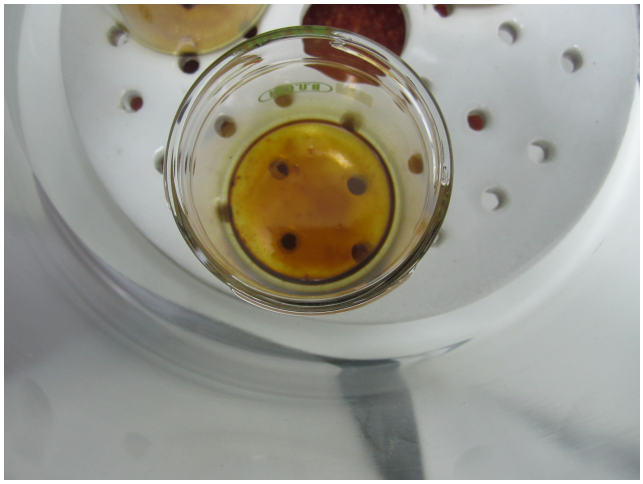

Figure 3.10: Sample glasses with the final oil sample.

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