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TECHNICAL NOTE 37.7

Photoheterotrophic compartment

Light limitation tests

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TECHNICAL NOTE 37.7:
Photoheterotrophic compartment. Light biomass control law

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1. Introduction

The development of compartment II, and particularly the heterotrophic subcompartment, has been performed at different levels. In order to characterise the growth of *Rhodospirillum rubrum* cells some batch cultures have been carried out using monodimensional illumination. At first, it was studied using different volatile fatty acids as carbon source at different light intensities (Lenguaza *et al.* 1997). Secondly, the growth rate was evaluated using two different kinds of light source, halogen and incandescent lamps (Cabello *et al.* 1999). Finally, several continuous cultures have been performed in a photobioreactor under light limitation conditions.

The tests reported in the present technical note have been performed in continuous experiments in a 2.4 L photobioreactor at different illumination conditions and at different dilution rates. The results obtained will be used to build the kinetic models that will be later applied to the control of this compartment.

Moreover, the biomass obtained in the steady states has been analysed from the macromolecular composition point of view. These results will be useful in order to develop a stoichometric model of compartment II.

2. Material and methods

The bacterial strain used was *Rhodospirillum rubrum* (ATCC 25903) and was obtained from the American Type Culture Collection. The strain was received freeze-dried and was revived using R8AH medium (ATCC medium 550). This medium was also used for routinely subculture of the stock strain.

The culture medium used during the tests was based on the basal salt mixture formulated by Segers & Verstraete and was modified in order to provide the required amount of nutrients to the compartments III and IV, since the runs reported here where also part of a connection test of compartments II, III and IV at laboratory scale. Acetic acid was used as a carbon and electron source and biotin as the only vitamin. The culture medium composition is described in Appendix 1.

The photobioreactor (Applikon ADI 1030 Bio Controller) used consists of a 2.4 L cylindrical glass vessel stirred mechanically by a Rushton propeller (figure 1). The external surface of the reactor was completely illuminated by 15 halogen lamps (Sylvania professional BAB 38º 12V 20W, improved version, cool beam, UV filtered, green box, code type 215). The temperature was controlled by means of a thermostatic bath, which impelled the water through the external glass jacket of the photobioreactor. The pH was maintained at 6.9 by means of the auxiliary control unit of the system, which added HCl (1.5 M) or NaOH (1.5 M) depending on the deviation from the set point value. Biomass concentration was determined measuring the dry weight and the carbon source profiles were followed during the tests by liquid chromatography analysis.

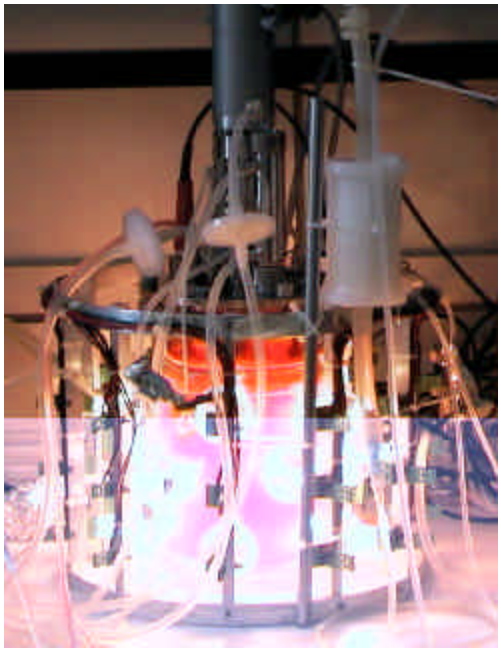


Figure 1: Applikon photobioreactor

3. Experimental results and discussion

The continuous runs have been carried out at three different dilution rates: 0.04, 0.08, and 0.12 h^{-1}. The incident light intensity and the carbon concentration have been modified in order to assure light limitation in several growth cultures. When every steady state was achieved, the produced biomass was harvested and freeze dried. These freeze dried samples were later used to perform the biomass analyses, which are presented in the second part of this chapter.

A. Kinetic data

The first continuous culture is represented in figure 2. It was started at the end of a batch culture(time equal to zero hours) and it was carried out at 136 W/m^2 and using 1 g C/L in the fresh medium of the carbon source, acetic acid. The first dilution rate was 0.02 h^{-1} and, once the steady state was achieved, at time equal to 195 h, it was increased to 0.04 h^{-1}.

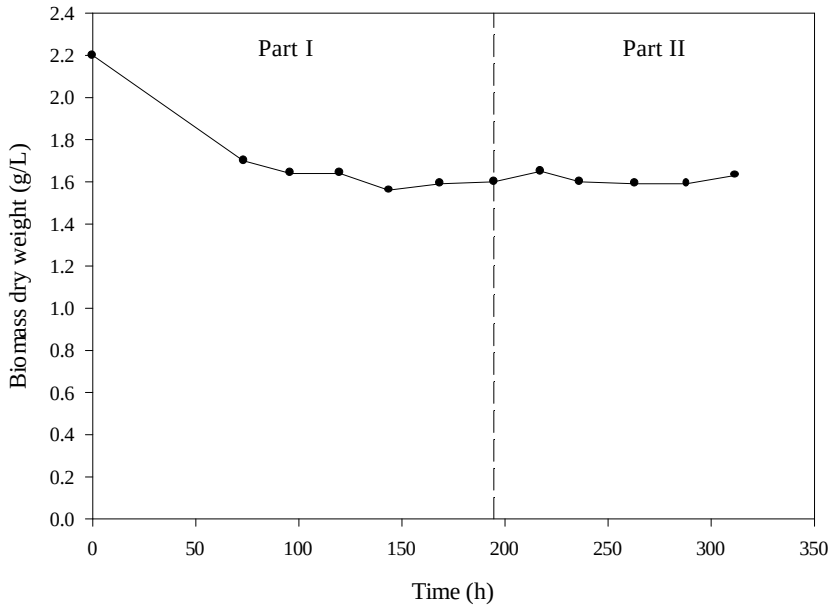


Figure 2: Continuous cultures of *R. rubrum* with acetic acid as carbon source. at F_R= 136 W/m^2 and 1 g C/L in the inlet. Part I: D=0.02 h^{-1}; Part II: D=0.04 h^{-1}.

In figure 2, it can be observed the fact that the dry weight does not vary considerably in the step up in the dilution rate and, in both steady states, it is around 1.61 g DW/L. This result can be explained because of the high affinity of the *R. rubrum* cells to this carbon source, acetic acid, making the change in the biomass concentration almost negligible.

In figure 3, the results of a different test at 0.04 h^{-1} are presented. Initially, the steady state at 136 W/m^2 was reproduced, in order to check the concordance between two different experiments (part I). It was verified that within measurement precision, all the carbon source was consumed and cell concentration reached 1.60 g/L of biomass dry weight, a value very similar to the previously obtained under the same conditions (part II of figure 2).

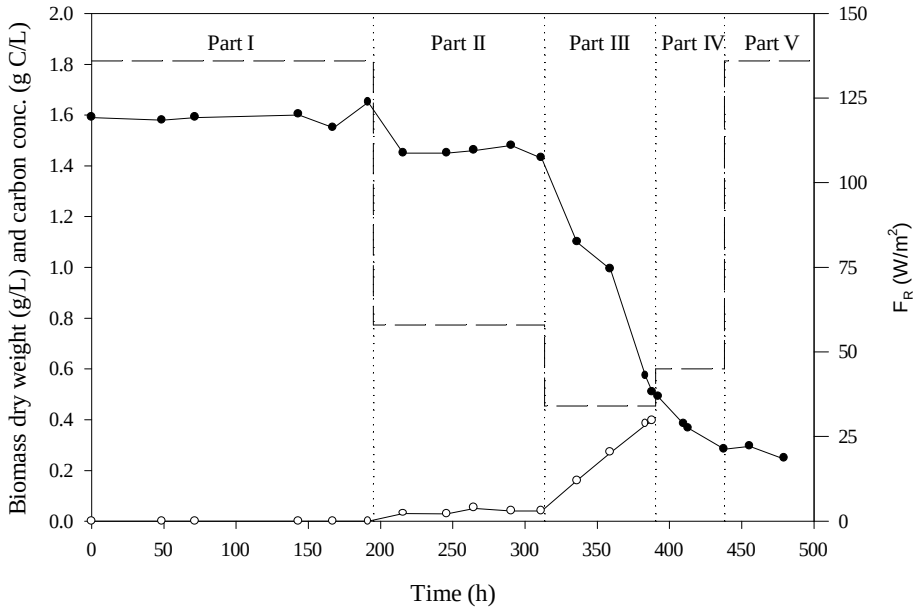


Figure 3: Continuous cultures of *R. rubrum* with acetic acid as carbon source at D=0.04 h^{-1} and using 1 g C/L in the inlet. Part I: F_R= 136 W/m^2; Part II: F_R=58 W/m^2; Part III: F_R=34 W/m^2; Part IV: F_R=45 W/m^2; Part V: F_R=136 W/m^2.

At time equal to 195 hours, a F_R step down was done, decreasing the light intensity from 136 W/m^2 to 58 W/m^2. The dry weight became stable at 1.46 g/L and it started to be detected a small amount of carbon source not consumed in the culture medium (0.038 g C/L). Then, the incident light intensity was decreased again from 58 W/m^2 to 34 W/m^2, observing several consequences in the culture. On one hand, the remaining carbon source started to increase significantly. On the other hand, at the same time, the *R. rubrum* cells started to form aggregates and to attach to the glass surface, decreasing considerably the cell concentration in suspension and increasing light shielding to the suspended culture. As it could be possible that 34 W/m^2 was a too low illumination level to maintain the cells growing at this dilution rate, the light intensity was increased to 45 W/m^2. However, the cells continued attaching to the reactor surface. It was clearly an unstable state, and as more cells were attached, less incident light intensity could be used by the cells in suspension. As the cells were washing-out of the reactor, it was decided to increase F_R back to its original value, 136 W/m^2, which had provided satisfactory illumination conditions at the beginning of the experiment (part I). Then, it was observed the fact that, once the cells are attached, they do not detach easily

when the culture conditions are brought back to a more advantageous situation, known not to trigger cell attachment. In figure 4, it can be observed the appearance of the photobioreactor when the *R. rubrum* cells were attached to the wall surface.

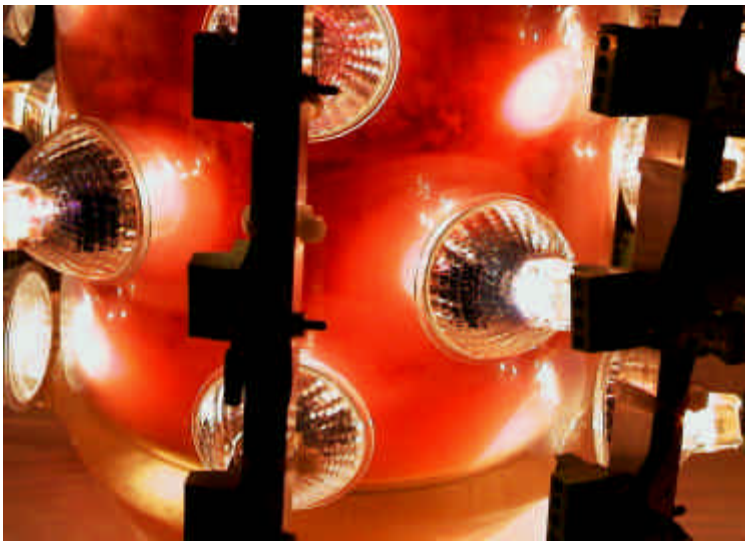


Figure 4: *R. rubrum* cells attached to the glass surface of the photobioreactor (darker areas).

In figures 5 and 6, microscopical observation of *R. rubrum* cells at 1000x are given. In can be observed that cells are filled with intracellular granules. Moreover, the cells are immobile and tend to form aggregates. Initially, when the aggregates start to be formed, they are in suspension and composed of a reduced number of cells. However, as the number of aggregates in suspension increase, they start to attach to the glass surface and there are many cells in that condition.

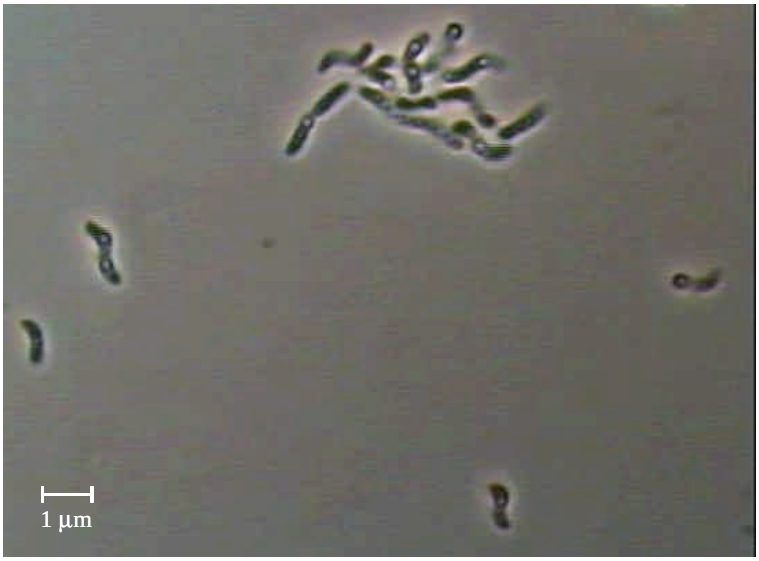


Figure 5: *R. rubrum* cells with intracellular granules. Microscopical observation at 1000x. This morphology is associated to a situation where cells will finally attach to the glass wall of the reactor.

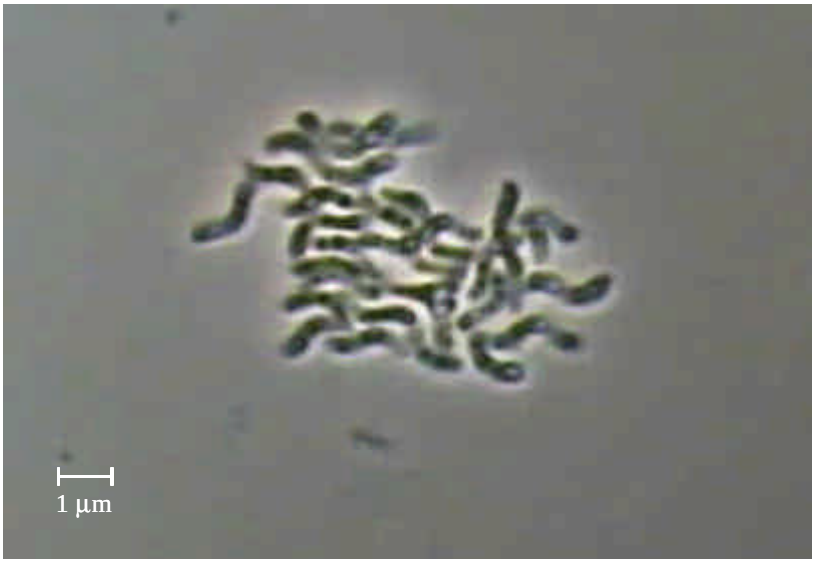


Figure 6: *R. rubrum* cells with intracellular granules. Microscopical observation at 1000x.

In figure 7 the results obtained in the first experiment carried at D=0.08 h^{-1}, using a F_R equal to 260 W/m^2 are shown. The cells were growing at 0.04 h^{-1} and, when they were close to the steady state (1.41 g/L), the dilution rate was increased to 0.08 h^{-1}. Once the step up in the dilution rate had been done, non-consumed acetic acid started to appear in the culture. Initially, the remaining carbon source concentration was very low, but measurable. In addition, at the same time, the *R. rubrum* cells began to aggregate and to attach to the reactor wall, attenuating the incident light. As the experiment progressed, the cells continued attaching and the culture suspension was washed-out.

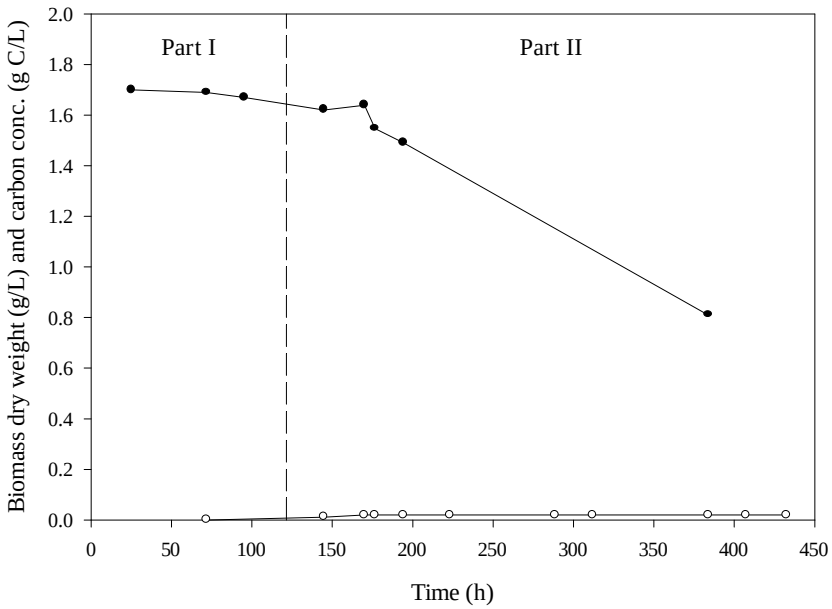


Figure 7: Continuous cultures of *R. rubrum* with acetic acid as carbon source using 1 g C/L in the inlet and F_R= 260 W/m^2. Part I: D=0.04 h^{-1}; Part II: D=0.08 h^{-1}.

As a first hypothesis, it would seem that *R. rubrum* cells are so sensitive to the carbon concentration in the culture that, when its accumulation begins, as a result of the decrease consumption due to low level, they change its morphology and stop growing.

Therefore, in order to maintain the culture growing successfully at 0.08 h^{-1}, the carbon source concentration in the fresh medium was reduced to the half. In figure 8, the results of the test at 0.08 h^{-1} and 0.5 g C/L of acetic acid are presented.

The first steady state achieved at 0.08 h^{-1} was obtained using an incident light intensity of 260 W/m^2 and 0.5 g/L of carbon in the fresh medium (part I). As the dry weight tended to be stable at 0.83 g/L and there was no carbon source in excess in the medium, a F_R step down was done, changing from 260W/m^2 to 192 W/m^2 (part II). In these conditions, the dry weight did not experiment any variation and the carbon source concentration in the culture continued being negligible. After that, another time the incident light intensity was decreased from 192 W/m^2 to 136 W/m^2 (part III). The consequences of that change were that the dry weight in the steady state decreased slightly to 0.79 g/L and still all the carbon source was consumed. Despite the fact that the dry weight measurements seem to decrease continuously in part III, the cells were not attached at all and, by microscopic analysis, it was verified that her physiological state (assessed by absence of granules, aggregation, motility) was satisfactory. These results seem to confirm that if acetic acid is not accumulated in excess, the culture operates properly at this dilution rate and light intensity.

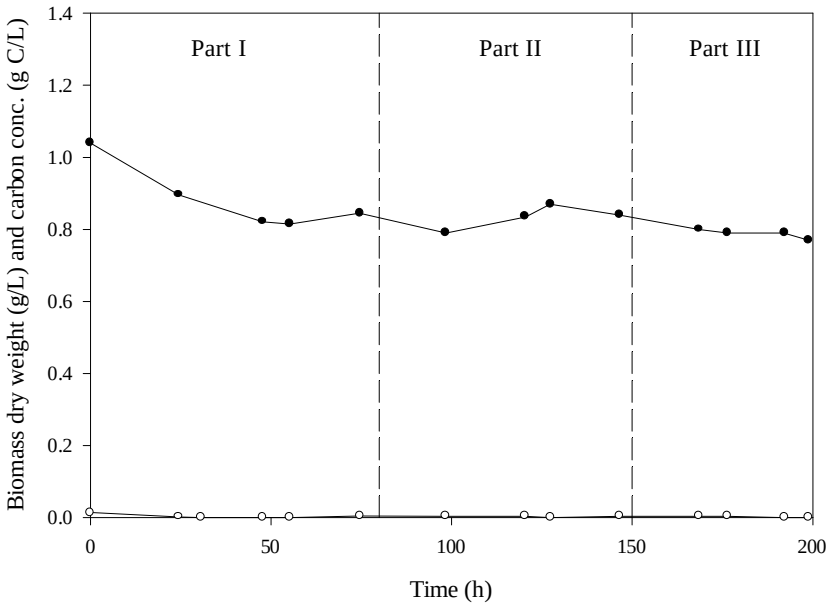


Figure 8: Continuous cultures of *R. rubrum* with acetic acid as carbon source using 0.5 g C/L in the inlet and D=0.08 h^{-1}. Part I: F_R= 260 W/m^2; Part II: F_R=192 W/m^2; Part III: F_R=136 W/m^2.

In figure 9, the results of cultures carried out at D=0.08 h^{-1} are presented. In part I, the F_R was 136 W/m^2, the light intensity tested in the previous culture, and the dry weight at the steady state was again 0.79 g/L. Subsequently, the light intensity was reduced to 92 W/m^2 (part II). Taking into account an average value to filter measurements oscillation, the dry weight did not vary significantly from the previous steady state.

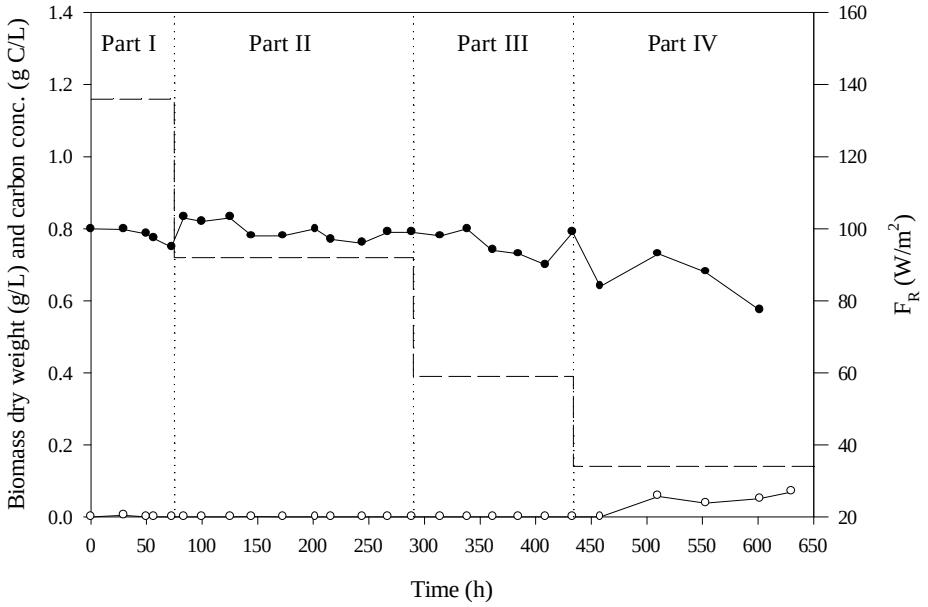


Figure 9: Continuous cultures of *R. rubrum* with acetic acid as carbon source using 0.5 g C/L in the inlet and D=0.08 h^{-1}. Part I: F_R= 136 W/m^2; Part II: F_R=92 W/m^2; Part III: F_R=59 W/m^2; Part IV: F_R=34 W/m^2.

Next, the F_R was decreased to 59 W/m^2 (part III), diminishing the dry weight to 0.76 g/L. The last change in the F_R value corresponded to a step down from 59 W/m^2 to 34 W/m^2. Although apparently dry weight started to stabilize at 0.66 g/L, free carbon source appeared in the culture and the *R. rubrum* cells started to form aggregates and to attach to the glass wall of the reactor. The experiment was stopped due to the wash-out of the culture.

In figure 10, the results obtained in an experiment at D=0.12 h^{-1} are provided. The test was started at the maximum possible light intensity, 260 W/m^2, and using 0.50 g/L of carbon in the fresh medium. The dry weight was stable at 0.75 g/L but there were some cells starting to attach to the reactor surface. Therefore, as the light intensity could not be increased in order to avoid a total attachment of the cells, the carbon source in the fresh medium was reduced to 0.40 g/L of carbon and biomass concentration stabilized around 0.71 g/L (part II). Then, the F_R was reduced to 224 W/m^2. As the cells appear to grow properly, the light intensity was decreased once more to 192 W/m^2. At these

conditions, the *R. rubrum* cells started to incorporate intracellular granules, although not in a significant amount. Therefore, the F_R was reduced to 136 W/m^2, starting to appear aggregates and making the performance of the reactor unstable. In the next step, the incident light intensity was reduced again, first to 92 W/m^2 and later to 59 W/m^2. The result was clear and conclusive: the cells formed aggregates and continued attaching more and more to the glass surface of the reactor.

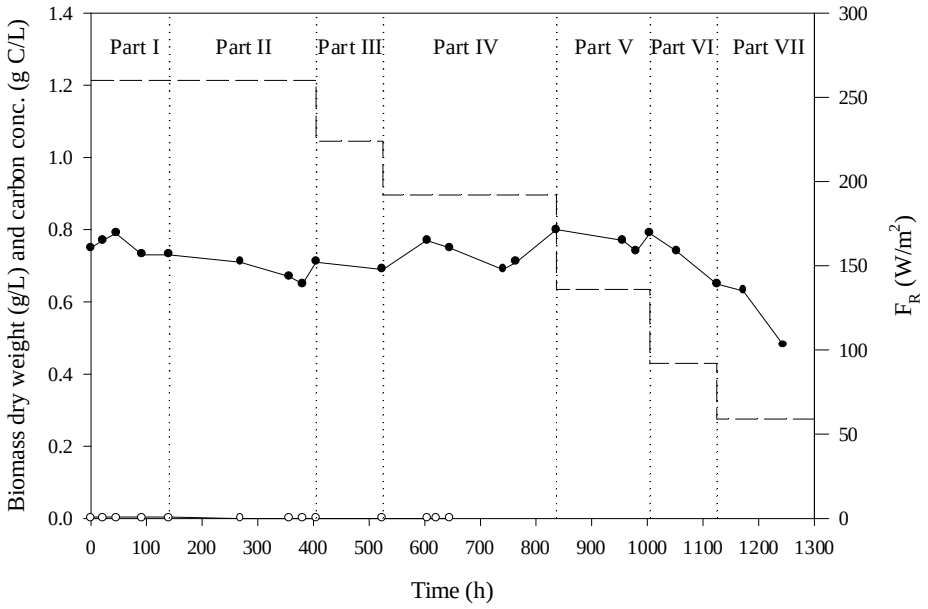


Figure 10: Continuous cultures of *R. rubrum* with acetic acid as carbon source at D=0.12 h^{-1}. Part I: F_R= 260 W/m^2, 0.5 g C/L in the inlet; Part II: F_R= 260 W/m^2, 0.4 g C/L in the inlet; Part III: F_R= 224 W/m^2; Part IV: F_R= 192 W/m^2; Part V: F_R= 136 W/m^2; Part VI: F_R= 92 W/m^2; Part VII: F_R= 59 W/m^2.

B. Biomass analyses

During the light limitation tests 17 different samples of *R. rubrum* cells have been freeze dried, particularly when a steady state was reached, in order to analyse the macromolecular composition of the cells. The analyses carried out consist on carbohydrates, proteins, lipids, DNA, RNA, PHB, glycogen, and elemental determination.

The samples have been obtained in different culture conditions, as is shown in table 1, corresponding to the steady states reported previously.

No.	F_R (W/m^2)	D (h^{-1})	Inlet C conc. (g C/L)	Dry weight (g/L)
1	136	0.04	1.00	1.41
2	136	0.04	1.00	1.40
3	58	0.04	1.00	1.26
4	260	0.04	1.00	1.49
5	260	0.08	1.00	Attached
6	260	0.08	1.00	Attached
7	260	0.08	0.50	0.83
8	136	0.08	0.50	0.79
9	136	0.08	0.50	0.79
10	92	0.08	0.50	0.79
11	59	0.08	0.50	0.76
12	34	0.08	0.50	Attached
13	18	0.08	0.50	Attached
14	260	0.12	0.50	0.75
15	260	0.12	0.40	0.71
16	260	0.12	0.40	0.71
17	224	0.12	0.40	0.71
18	192	0.12	0.40	0.75
19	138	0.12	0.40	0.75
20	92	0.12	0.40	Attached
21	58	0.12	0.40	Attached

Table 1: Summary of the operational conditions of the freeze dried analysed samples.

I. Carbohydrates determination

The carbohydrates determination has been carried out as explained in Appendix 3. Each sample has been analysed three times, and for each sample, the mean value of the three analyses as the dispersion of them is reported in table 2.

No.	Carbohydrates conc. (% of the DW)	Standard error
1	10.23	0.91
2	10.89	0.91
3	10.16	0.20
4	9.91	0.45
5	8.69	0.38
6	10.44	1.03
7	10.67	0.80
8	10.11	0.95
9	11.01	1.66
10	11.05	0.66
11	11.47	1.48
12	11.87	0.95
13	11.66	1.14
14	12.27	1.30
15	10.54	0.83
16	9.57	0.90
17	10.69	1.75
18	10.03	1.26
19	10.98	0.47
20	9.31	0.80
21	9.90	0.99

Table 2: Results of the carbohydrates determination.

II. Protein determination

The protein determination has been carried out as is explained in Appendix 4. Each sample has been analysed three times, and for each sample, the mean value of the three analyses and its standard error is reported in table 3.

No.	Protein conc. (% of the DW)	Standard error
1	64.23	3.24
2	60.35	3.60
3	60.74	3.09
4	62.04	5.76
5	60.70	5.70
6	59.83	4.22
7	66.59	9.84
8	63.67	7.14
9	63.65	3.63
10	63.18	2.74
11	55.32	2.95
12	59.19	5.29
13	48.14	5.97
14	64.17	1.72
15	63.49	5.03
16	64.22	3.67
17	57.90	3.89
18	58.54	7.66
19	63.89	6.09
20	54.69	5.62
21	51.72	2.52

Table 3: Results of the protein determination.

III. Lipids determination

The lipids determination has been carried out as is explained in Appendix 5. Palmitic acid, palmitoleic acid and vaccenic acid have been determined as they have been identified as the most representative lipids in the biomass analysed samples. The concentration of each of these lipids as well as the dispersion of the analyses are reported in table 4.

No.	Palmitic Acid (mg/g)	Standard error	Palmitoleic Acid (mg/g)	Standard error	Vaccenic Acid (mg/g)	Standard error	Total Lipid Conc. (mg/g)	% of the DW
1	11.5	0.23	15.3	0.64	38.7	0.23	65.5	6.55
2	9.4	0.81	13.8	1.04	37.3	1.21	60.5	6.05
3	16.2	0.58	21	0.17	37.6	0.12	74.8	7.48
4	10.5	0.87	12.8	0.92	36.4	0.87	59.7	5.97
5	13.8	1.04	14.5	0.46	34.9	0.69	63.2	6.32
6	8.8	2.48	11.6	1.79	30	2.60	50.4	5.04
7	10.6	1.10	12.8	1.33	30.5	1.15	53.9	5.39
8	10.8	0.35	14.8	0.29	31.5	0.40	57.1	5.71
9	9	2.25	12.9	2.48	27.6	2.42	49.5	4.95
10	9.6	0.17	14.5	0.00	30.6	0.06	54.7	5.47
11	10.4	0.64	15.5	0.64	30.4	0.98	56.3	5.63
12	13	0.98	16.9	0.87	36.5	1.21	66.4	6.64
13	13.1	2.48	16.3	2.37	35.5	2.60	64.9	6.49
14	10.2	0.92	11.6	0.29	30.5	0.40	52.3	5.23
15	10.1	0.75	12.7	0.92	27.7	0.98	50.5	5.05
16	9.4	0.69	13.4	0.92	26.1	0.92	48.9	4.89
17	10.1	0.40	14.5	0.06	29.5	0.06	54.1	5.41
18	8.5	2.42	12.4	2.48	25.6	2.83	46.5	4.65
19	10.4	0.92	13.7	0.98	33.6	0.92	57.7	5.77
20	13.6	2.14	16.9	1.73	39.2	2.08	69.7	6.97
21	10.7	0.46	13.8	0.52	31.3	0.58	55.8	5.58

Table 4: Results of the lipid determination.

IV. DNA determination

The DNA determination has been carried out as is explained in Appendix 6. Each sample has been analysed three times, and for each sample, the mean value of the three analyses as well as the dispersion obtained is reported in table 5.

No.	DNA Conc. (% of the DW)	Standard error
1	2.26	0.99
2	4.45	1.36
3	3.19	0.07
4	3.92	0.92
5	1.65	0.08
6	2.02	0.69
7	4.04	0.46
8	3.07	0.04
9	3.73	0.80
10	5.31	0.36
11	3.99	0.63
12	1.72	0.62
13	2.90	0.19
14	2.29	0.60
15	3.15	0.19
16	1.48	0.97
17	2.48	0.08
18	2.33	0.36
19	1.17	0.04
20	0.66	0.05
21	2.26	0.99

Table 5: Results of the DNA determination.

V. RNA determination

The RNA determination has been carried out as is explained in Appendix 7. Each sample has been analysed three times, and for each sample, the mean value of the three analyses as well as the calculated deviation is reported in table 6.

No.	RNA Conc. (% of the DW)	Standard error
1	5.37	0.69
2	2.95	0.32
3	3.96	1.15
4	3.34	0.13
5	5.95	2.16
6	4.14	2.26
7	7.34	7.12
8	9.45	6.11
9	10.56	14.09
11	2.09	10.47
12	1.86	0.75
13	1.55	0.07
14	2.06	0.09
15	1.09	0.08
16	4.10	0.08
17	3.20	1.30
18	5.75	0.43
19	2.97	0.17
21	6.25	0.29

Table 6: Results of the DNA determination.

VI. PHB determination

The PHB determination has been carried out as is explained in Appendix 8. Each sample has been analysed three times, and for each sample, the mean value of the three analyses as well as the calculated deviation is reported in table 7.

No.	PHB Conc. (% of the DW)	Standard error
1	8.61	4.09
2	1.98	1.54
3	9.87	3.41
4	7.81	3.75
5	9.06	6.95
6	7.45	2.49
7	5.46	6.15
8	6.62	4.84
9	6.47	6.81
10	6.53	7.91
11	19.91	16.57
12	12.36	12.55
13	32.45	5.59
14	7.55	5.57
15	12.81	15.63
16	12.00	9.94
17	20.90	9.44
19	13.93	8.93

Table 7: Results of the PHB determination.

VII. Glycogen determination

The glycogen determination has been carried out as is explained in Appendix 9. Each sample has been analysed three times, and for each sample, the mean value of the three analyses as well as the calculated deviation is reported in table 8.

No.	Glycogen Conc. (% of the DW)	Standard error
1	2.03	0.02
2	2.84	0.10
3	2.54	0.17
4	2.44	0.37
5	1.84	0.13
6	2.60	0.11
7	1.86	0.14
8	2.24	0.34
9	2.59	0.15
10	2.89	0.19
11	2.48	0.49
12	2.90	0.36
13	2.19	1.10
14	2.60	0.11
15	1.29	0.18
16	1.98	0.22
17	2.15	0.17
18	2.51	0.24
19	2.18	0.39
20	2.56	0.17
21	2.61	0.36

Table 8: Results of the glycogen determination.

VIII. Elemental analysis

The elemental composition analysis has been carried out as is explained in Appendix 10. The mean value for C, N, H and S as the dispersion of them are reported in table 9.

No.	C	Std. Dev.	N	Std. Dev.	H	Std. Dev.	S	Std. Dev.
1	47.89	0.1498	10.54	0.0451	7.59	0.2303	0.40	0.0200
2	49.11	0.0707	10.87	0.0636	7.29	0.2121	0.35	0.0141
3	50.23	0.0000	10.45	0.0354	7.62	0.0566	0.36	0.0071
4	48.17	0.0141	10.78	0.0707	7.43	0.1344	0.36	0.0283
5	49.89	0.1061	11.27	0.0919	7.54	0.0071	0.38	0.0000
6	47.45	0.0212	11.26	0.0919	7.54	0.0495	0.38	0.0071
7	46.85	0.2835	11.29	0.1050	6.58	0.5064	0.34	0.0666
8	48.05	0.0212	11.19	0.0283	7.19	0.0919	0.41	0.0141
9	47.46	0.1131	11.20	0.0283	7.28	0.1273	0.41	0.0000
10	47.47	0.0919	11.05	0.0990	7.16	0.1556	0.39	0.0071
11	48.62	0.0495	10.35	0.1909	7.27	0.0707	0.43	0.0424
12	50.73	0.1202	10.86	0.0212	7.73	0.1202	0.37	0.0141
13	50.00	0.0071	9.42	0.0141	7.12	0.0283	0.32	0.0141
14	47.50	0.0354	11.43	0.0707	6.95	0.1556	0.41	0.0424
15	47.01	0.0566	11.90	0.0566	7.04	0.0141	0.40	0.0071
16	47.20	0.2828	11.20	0.0424	6.91	0.1344	0.36	0.0283
17	48.00	0.2248	11.05	0.1328	7.04	0.1992	0.41	0.0153
18	47.61	0.0141	10.64	0.0071	7.00	0.0566	0.38	0.0071
19	49.38	0.1202	10.92	0.0212	7.23	0.1131	0.38	0.0071
20	49.53	0.1980	10.16	0.0566	7.33	0.0849	0.34	0.0212
21	49.13	0.1556	9.32	0.0283	7.09	0.0919	0.31	0.0283

Table 9: Results of the elemental composition analysis. Percentages do not add to 100% due to the lack of determination of oxygen. phosphorous and ashes.

In order to stand out possible tendencies, the data obtained in all of the biomass analyses done are summarized in table 10 as well as the operational conditions.

No.	F_R (W/m^2)	D (h^{-1})	Inlet C Conc. (g C/L)	Dry weight (g/L)	Carbohydrates (% of the DW)	Proteins (% of the DW)	Lipids (% of the DW)	DNA (% of the DW)	RNA (% of the DW)	PHB (% of the DW)	Glycogen (% of the DW)	Total (% of the DW)
1	136	0.04	1.00	1.41	10.23	64.23	6.55	2.26	5.37	8.61	2.03	97.25
2	136	0.04	1.00	1.40	10.89	60.35	6.05	4.45	2.95	1.98	2.84	86.67
3	58	0.04	1.00	1.26	10.16	60.74	7.48	3.19	3.96	9.87	2.54	95.40
4	260	0.04	1.00	1.49	9.91	62.04	5.97	3.92	3.34	7.81	2.44	92.99
5	260	0.08	1.00	Attached	8.69	60.70	6.32	1.65	5.95	9.06	1.84	92.37
6	260	0.08	1.00	Attached	10.44	59.83	5.04	2.02	4.14	7.45	2.60	88.92
7	260	0.08	0.50	0.83	10.67	66.59	5.39	4.04	7.34	5.46	1.86	99.49
8	136	0.08	0.50	0.79	10.11	63.67	5.71	3.07	9.45	6.62	2.24	98.63
9	136	0.08	0.50	0.79	11.01	63.65	4.95	3.73	10.56	6.47	2.59	100.37
10	92	0.08	0.50	0.79	11.05	63.18	5.47	5.31	***4.49***	6.53	2.89	96.03
11	59	0.08	0.50	0.76	11.47	55.32	5.63	3.99	2.09	19.91	2.48	98.41
12	34	0.08	0.50	Attached	11.87	59.19	6.64	1.72	1.86	12.36	2.90	93.64
13	18	0.08	0.50	Attached	11.66	48.14	6.49	2.90	1.55	32.45	2.19	103.19
14	260	0.12	0.50	0.75	12.27	64.17	5.23	2.29	2.06	7.55	2.60	93.57
15	260	0.12	0.40	0.71	10.54	63.49	5.05	3.15	1.09	12.81	1.29	96.13
16	260	0.12	0.40	0.71	9.57	64.22	4.89	1.48	4.10	12.00	1.98	96.26
17	224	0.12	0.40	0.71	10.69	57.90	5.41	2.48	3.20	20.90	2.15	100.58
18	192	0.12	0.40	0.75	10.03	58.54	4.65	2.33	5.75	***14.73***	2.51	96.03
19	138	0.12	0.40	0.75	10.98	63.89	5.77	1.17	2.97	13.93	2.18	98.71
20	92	0.12	0.40	Attached	9.31	54.69	6.97	***3.00***	***4.00***	***18.06***	2.56	96.03
21	58	0.12	0.40	Attached	9.90	51.72	5.58	***3.00***	6.25	***19.58***	2.61	96.03

Table 10: Summary of all data obtained in the biomass analyses. Glycogen results has not been added up to the total weight, since it is considered to be included in the total carbohydrates measure.

In table 10, all of the results (except glycogen) for each biomass sample have been added up in order to check if they constitute the hundred per cent of the cell composition. As it can be observed in this table, the addition of all of the macromolecular contents is near to hundred per cent of the cell mass. The difference between these results and the theoretical hundred per cent is due to the lack of the mineral elements not measured by the biomass analyses. Moreover, the dispersion of the analyses, usually RNA and PHB determinations, make difficult to reach the theoretical 100%. In few cases, an experimental result was outside its possible range and has been calculated theoretically taking into account the percentage of the other compounds (RNA result in sample number 10) and the total weight measured in the other samples. In samples number 20 and 21, the experimentally obtained PHB content was not probable as well as the DNA and RNA results. In this case, DNA and RNA have been assumed using common values and the PHB value has also been calculated theoretically.

Analysing the data reported in table 10, some tendencies have been observed by comparison of the macromolecular contents versus the growing conditions, especially the incident light intensity. The inlet carbon concentration of the fresh medium has not a significant influence on the cell composition. It has been modified in order to provide light or carbon limitation depending on the case. The tendencies reported below are the same in every dilution rate experiment series, but in the 0.08 h^{-1} set of experiments, the conclusions can be observed more clearly. In figures 11 and 12 the content of each macrocomponent analysed versus the incident light intensity are represented for the experiments carried out at 0.08 h^{-1}.

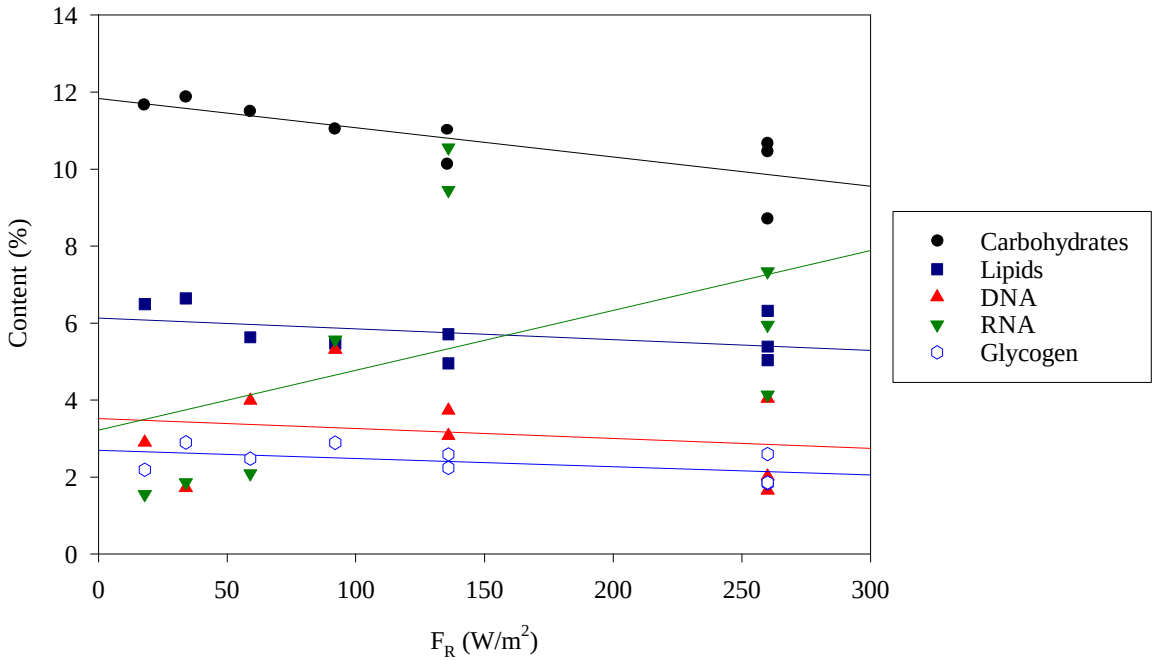


Figure 11: Carbohydrates, lipids, nucleic acids and glycogen content versus the incident light intensity for the 0.08 h^{-1} set of experiments.

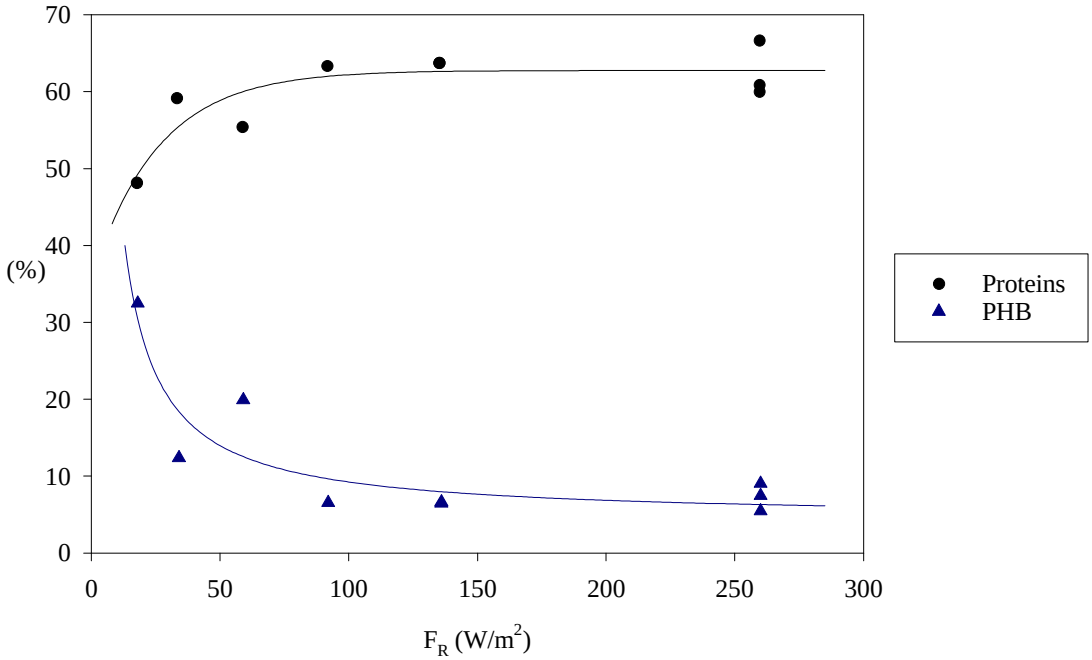


Figure 12: Proteins and PHB content versus the incident light intensity for the 0.08 h^{-1} set of experiments.

On one hand, in figures 11 and 12, can be observed that the content of carbohydrates, lipids, DNA, glycogen and PHB decrease slightly when the incident light intensity is increased. On the other hand, the content of proteins and RNA are higher at light increased levels.

This behaviour can be explained considering the fact that when the overall available light is higher, the overall available light-carbon ratio is upper too, and possibly the cell readapt its composition (increasing its enzymatic capacity) to be prepared for growing at a higher specific growth rate. Consequently, if the cell is foreseeing to increase its specific protein production rate, the RNA content is also increased.

When the overall available light is reduced and it is low enough to limit the growth rate, the carbon source starts to be stoichometrically in excess and intracellular polymers are accumulated, mainly PHB, as a reserve of carbon and energy. In the environmental conditions studied, PHB is accumulated in greater amount than glycogen, probably due to the lack of enough light and maybe CO_2 too. Therefore, the PHB production depends on de ratio between the available light intensity and the free carbon concentration in the culture. As a preliminary study, since we cannot determine the available light distribution inside the culture and the carbon concentration has not been

determined in all of the experiments, in figure 13, it has been represented de percentage of intracellular PHB measured versus de ratio F_R/C_0 (incident light intensity – carbon concentration in the inlet medium ratio). Every dilution rate tested (0.04, 0.08, and 0.12h^{-1}) has been represented separately.

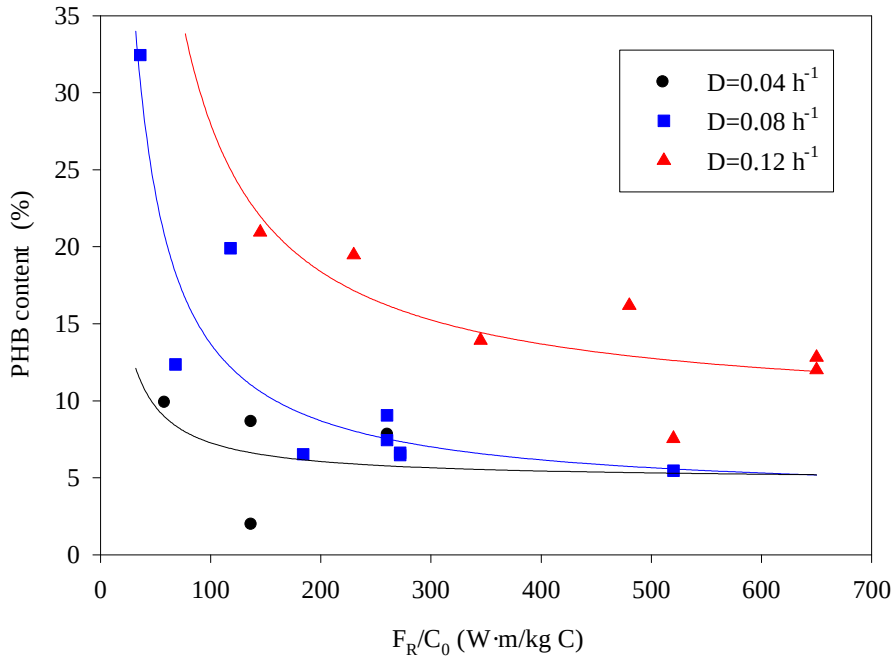


Figure 13: PHB content versus the incident light intensity-inlet carbon concentration of the fresh medium ratio for the three different dilution rates tested.

In figure 13, it can be observed the fact that the PHB content accumulated as intracellular polymer increases considerably in proportion to the decrease of the F_R/C_0 ratio. Moreover, analysing this figure it can be stand out the fact that at higher dilution rates, the *R. rubrum* cells accumulate a greater amount of PHB.

This is because when the dilution rate is increased, the amount of carbon that is introduced in the culture is also augmented whereas the light intensity inside the culture is increased slightly, because of the decrease in the biomass concentration. Therefore, when the dilution rate is increased, the available light intensity-free carbon in the culture ratio is decreased.

4. Conclusions

Taking into account the results obtained in these experiments several points can be concluded. On one hand, it seems that *R. rubrum* cells are so sensitive to the carbon source remaining in the culture that, when the acetic acid concentration is higher than 0.1 g/L of carbon, the cells form aggregates and attach to the reactor surface. Actually, during the continuous culture, if the cells are not able to consume the entire carbon source, they tend to accumulate it as intracellular granule, changing its morphology and stop growing.

On the other hand, once the cells are changing their morphology it is important to re-establish the initial conditions as soon as possible. Otherwise, the attachment problem can only be reversed by stopping the continuous culture and maintaining a batch culture until the cells consume the excess of carbon source and detach again.

These results appear to indicate that there is a combination of light intensity and acetic acid concentration that allow the proper operation of the reactor. The decrease in light intensity decreases acetic acid consumption in the appropriate way and triggers a mechanism of carbon source accumulation. The lack of cell mobility can also be a consequence of the limited energy supply, which could result in low levels of ATP availability.

Therefore, at this point the combined factors of low energy availability and high acetic acid levels can be proposed as the triggering factors for the cell attachment and carbon compounds accumulation mechanism. A more detailed explanation will be pursued in future experiments.

The cell composition analyses have verified that the intracellular granules observed at the microscopy are formed generally by β-polihydroxibutirate, although glycogen has been also accumulated. It is probably that the accumulation of intracellular granules of PHB is driven by the value of the ratio between the available light intensity and the free carbon concentration in the culture. When this ratio is low, there is not enough light availability to consume the entire carbon source and the free carbon is accumulated as PHB.

Moreover, the several steady states achieved may be obtained under carbon limitation instead of light limitation, because when the growth is limited by the light, the cells change its morphology and do not grow properly. Therefore, the present experiments allow to approximate the light intensity values necessary to obtain a certain dilution rate. However, the mathematical model used to explain the data has to include a term explaining the attachment problem.

In next experiments, it would be studied whether this behaviour only happens when the acetic acid is used or also occurs when others carbon sources are being consumed.

5. References

Albiol J. (1994) Study of the MELISSA photoheterotrophic compartment. Kinetics and effects of C limitation. ESA/YCL/2148.JAS.ESA-EWP-1808.

6. Appendixes

Appendix 1: Culture medium composition

Component	g/L medium
CH_3COOH	2.500
EDTA-Na · 2 H_2O	0.100
$MnCl_2$ · 2 H_2O	0.008
$FeSO_4$ · 7 H_2O	0.033
KH_2PO_4	0.400
$NaHCO_3$	0.250
$MgSO_4$· 7 H_2O	1.200
$CaCl_2$· 2 H_2O	0.091
$(NH_4)_2SO_4$	2.728
$CuSO_4$· 5 H_2O	$4.0 \cdot 10^{-6}$
Na_2HPO_4	0.489
$ZnSO_4$· 7 H_2O	$4.3 \cdot 10^{-6}$
$(NH_4)_6Mo_7O_{27}$ · 4 H_2O	0.177
K_2SO_4	0.550
Trace elements solution	1.00 mL/L medium
Biotin solution	1.00 mL/L medium
A5 solution	1.00 mL/L medium
B6 solution	1.00 mL/L medium

Dissolutions	
A5 (g/L solution)	
H_3BO_3	2.860
$MnCl_2$ · 4 H_2O	1.810
$ZnSO_4$· 7 H_2O	0.222
$CuSO_4$· 5 H_2O	0.079
MoO_3	0.015
B6 (g/L solution)	
NH_4VO_3	0.023
$KCr(SO_4)_2$ · 12 H_2O	0.096
$NiSO_4$ · 7 H_2O	0.048
$(NO_3)_2Co$ · 6 H_2O	0.049
Na_2WO_4 · 2 H_2O	0.018
$Ti(SO_4)_2$ + $TiOSO_4$	0.048
Trace elements (g/L solution)	
$NiSO_4$· 6 H_2O	0.500
$MnCl_2$ · 4 H_2O	0.500
$FeSO_4$· 7 H_2O	0.500
$ZnSO_4$· 7 H_2O	0.100
$CoCl_2$ · 2 H_2O	0.050
$CuSO_4$· 5 H_2O	0.005
H_3BO_3	0.100
Na_2MoO_4· 2 H_2O	0.050
Biotin solution (g/L solution)	
Biotin	0.015

Appendix 2: Acetic acid determination

The range of concentrations that can be determined is 0.001-1.000 g/L. For the analysis, 50 μL of sample are used. Samples have to be analyzed three times.

Instrumentation

- Liquid chromatograph Waters LC Module I Plus
- Ultraviolet detector at 210 nm
- Integration software Millenium 2.0

Mobile phase

Sulphuric acid (0.015 M) prepared with Milli-Q water (resistance 18.2 Ω), adjusted at pH=3.00 with diluted acetic acid and filtered through a 0.45 μm membrane.

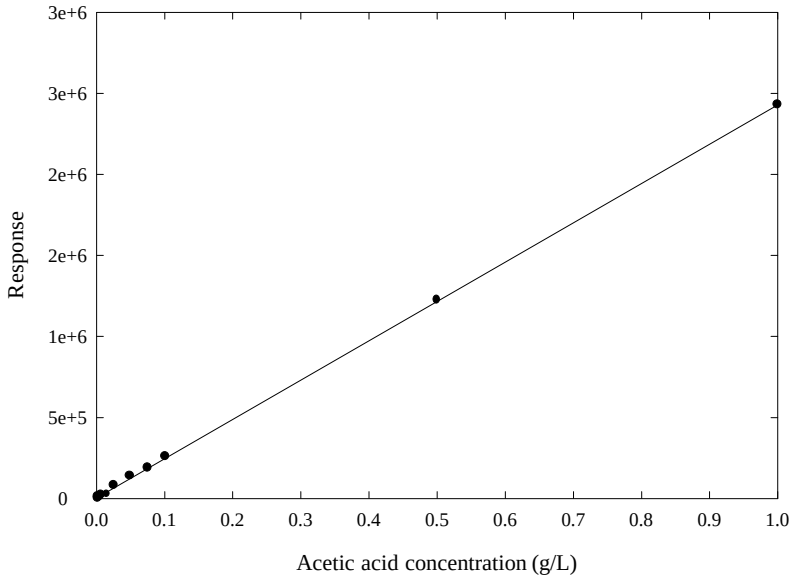
Analysis physical conditions

- Column: ionic exchange Aminex HPX-87H from Bio Rad
- Flow rate: 0.6 mL/min, isocratic
- Injection volume: 20 μL
- Analysis time: approximately 30 minutes
- Temperature: 25 ºC

Sample preparation:

- Centrifuge (12000 g, 10 min, 8 ºC)
- Filter (0.22 μm membrane)

Calibration



Appendix 3: Total carbohydrates determination

1.- Reactants:

1.- Concentrated sulphuric acid (96-97%).

2. -Phenol 5% (w/v)

3.- Glucose

2.- Sample treatment:

- Centrifuge 10 mL of culture suspension. Discard supernatant.
- Wash with DW (distilled water).
- Centrifuge in the same conditions. Discard the supernatant.
- Dissolve in DW.

(Alternatively dissolve a sample of freeze dried biomass in DW).

3.- Analysis

-Add to 1 mL sample

1 mL Phenol and mix carefully

5 mL of sulphuric acid. Mix carefully.

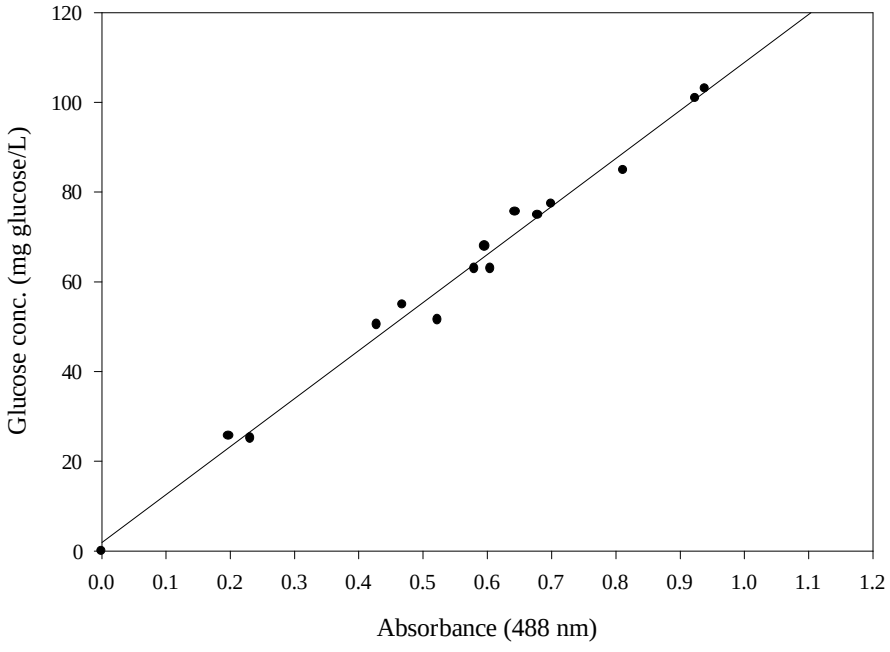
- Wait 10 min.
- Cool the tubes (15 min. in water 25 °C).
- Read absorbance of the sample and the Blanc at 488 nm, against DW.

4.-Results

-Prepare a calibration curve using glucose samples (0-100 mg/L).

Straight line fitted: Glucose conc. (mg/L) = -3.30 + 103.57 · Abs (488 nm) r^2=0.99

- Use the standard curve to calculate the concentration of the 1 mL sample by interpolation of the absolute absorbance.



Appendix 4: Protein determination (Lowry modified method)

1.- Reactants:

1.- Reagent A. 20 g of Na_2CO_3 dissolved in 1000 mL of distilled water (DW).

2.- Reagent B. 0.5 g $CuSO_4 \cdot 5H_2O$ and 1 g of Na-K tartrate dissolved in 100 mL of DW.

3.- Reagent C. 50 mL of reagent A + 1 mL of reagent B. This reagent cannot be conserved.

4.- Reagent D. Foling-Ciocalteus reagent, diluted 1:2 (v/v) in DW.

5.- Albumin stock solutions in the range 400-40 µg prot./mL

6.- Freeze dried biomass solutions. 0.0025 g biomass/5 mL DW.

2.- Prodecure:

1.- Prepare the following stock solutions of albumin:

400 µg/mL	0.04 g in 100 mL of DW
320 µg/mL	20 mL solution 400 µg/mL in 25 mL
200 µg/mL	10 mL solution 400 µg/mL in 20 mL
160 µg/mL	10 mL solution 400 µg/mL in 25 mL
100 µg/mL	5 mL solution 400 µg/mL in 20 mL
80 µg/mL	5 mL solution 400 µg/mL in 25 mL
40 µg/mL	2 mL solution 400 µg/mL in 20 mL

2.- Prepare the biomass solutions.

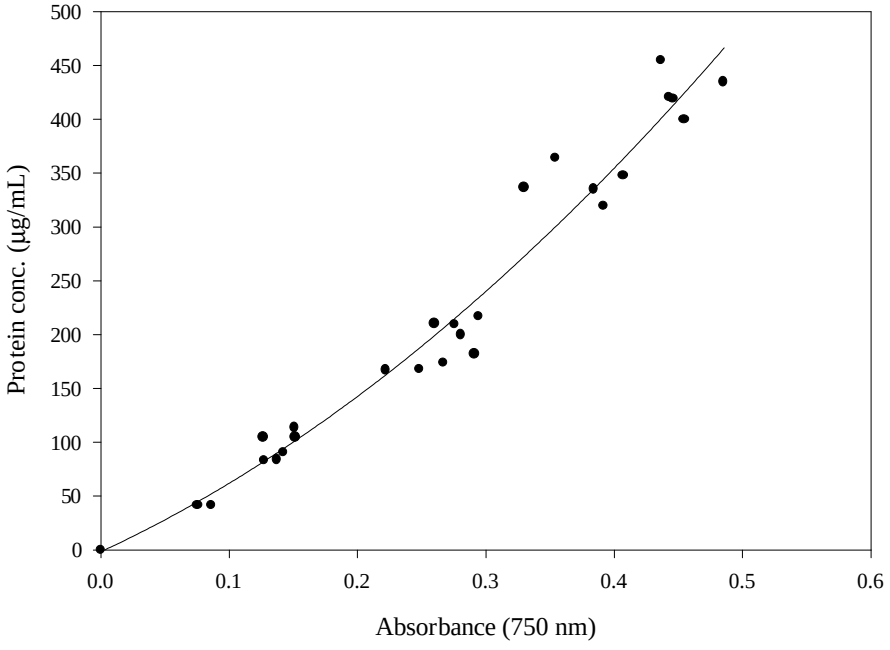
3.- Take (three times) 0.5 mL of each stock solution, 0.5 mL of each biomass solution and 0.5 mL of DW.

4.- Add up 0.5 mL of NaOH 1 M to every sample.

5.- Boil at 100 ºC for 10 minutes each sample. Cool in a water bath.

6.- Add 5 mL of reagent C to each sample. Shake and wait 10 minutes.

7.- Add 0.5 mL of reagent D to each sample. Mix. Repose in the dark for 30 minutes. Measure the absorbance at 750 nm.



Appendix 5: Lipids determination

The lipid determination has been carried out by gas chromatography with a FID and a mass spectrometer as a detector.

Appendix 6: DNA determination

1.- Reactants:

1.- Reagent A. 15 g diphenylamine + 15 mL conc. H_2SO_4 + 1 L CH_3COOH

2.- Reagent B. 2 L $HCLO_4$ 0.2 N

3.- Reagent C. 1 L $HCLO_4$ 0.5 N

4.- Reagent D. 1 L chloroform + 500 mL methanol

5.- Reagent E. NaOH 5 mM

2.- Prodecure:

1.- Add to 25 g of biomass sample 10 mL of reagent B. Keep 15 min. at 4 ºC. Centrifuge 5 min. at 6000 rpm. Discard de supernatant.

2.- Repeat step num. 1.

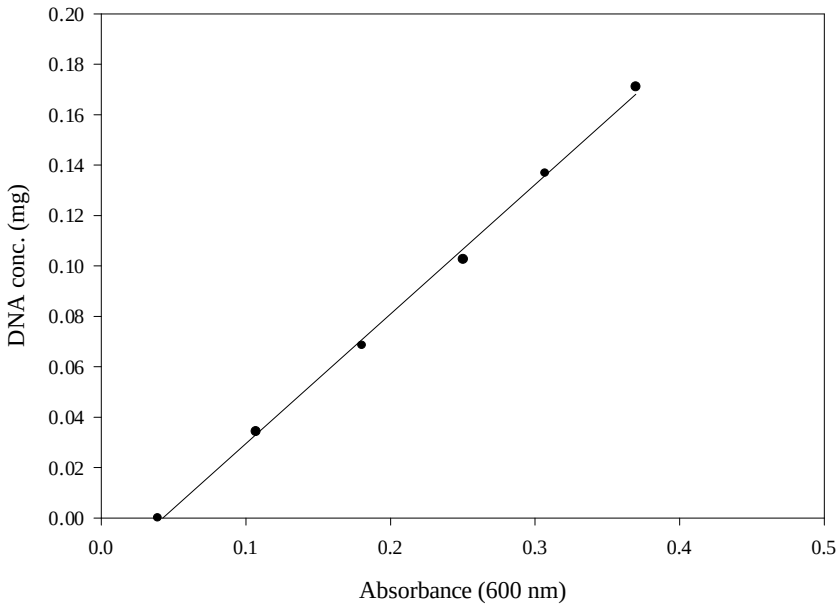
3.- Add to the pellet 10 mL of reagent D. Wait for 5 min. and centrifuge 5 min. at 6000 rpm. Discard de supernatant.

4.- Repeat step num. 3.

5.- Add to the pellet 5 mL of reagent C. Keep at 70 ºC during 45 min. Centrifuge.

6.- Take three samples of the supernatant (0.5 mL, 1 mL, and 1.5 mL) and prepare 5 samples of the stock solution of DNA Na salt (0.1 g, 0.2 g, 0.3 g, 0.4 g, and 0.5 g). Add to all of the samples reagent C until 2 mL. Add 4 mL of reagent D and keep at 30 ºC during 16-24 h.

7.- Measure the absorbance at 600 nm.



Appendix 7: RNA determination

1.- Reactants:

1.- Reagent A. $HCLO_4$ 0.2 N

2.- Reagent B. Dissolve 2.5 g orcinol in 5 mL ethanol 95%

3.- Reagent C. Standard RNA. Concentration: 100 mg/mL (store at 4 ºC)

4.- Reagent D. 0.3 g of $CuCl_2 \cdot 2H_2O$/200 mL of conc. HCl (prepared the same day of the analysis)

5.- Reagent E. 2 mL reagent A and 100 mL of reagent C

6.- Reagent F. KOH 0.3 N

7.- Reagent G. 1 L chloroform + 500 mL methanol

2.- Prodecure:

1.- Add to 25 g of biomass sample 10 mL of reagent A. Keep 15 min. at 4 ºC. Centrifuge 5 min. at 6000 rpm. Discard de supernatant.

2.- Repeat step num. 1.

3.- Add to the pellet 10 mL of reagent G. Wait for 5 min. and centrifuge 5 min. at 6000 rpm. Discard de supernatant.

4.- Repeat step num. 3.

5.- Add to the pellet 2 mL of reagent F. Keep at 30 ºC during 18-24 h.

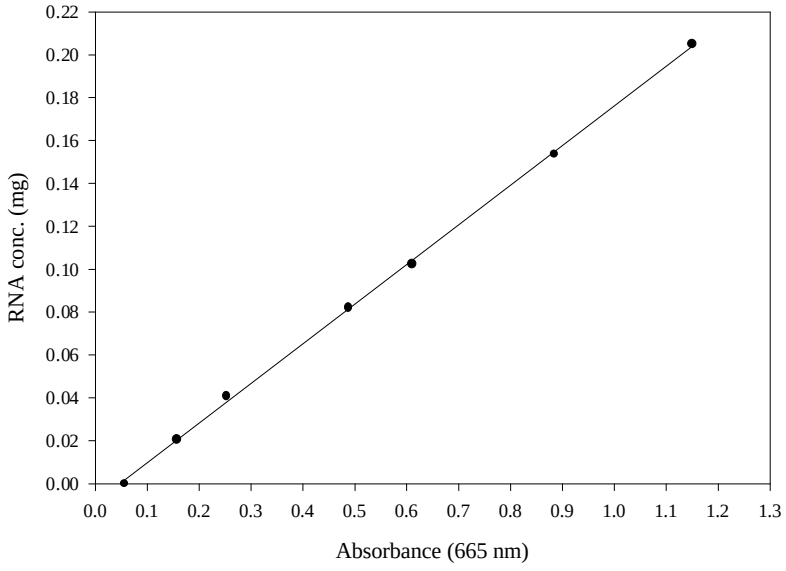
6.- Cool down and add conc. $HClO_4$ to reach pH=1

7.- Centrifuge at 6000 rpm for 6 min. Do not discard the supernatant.

8.- Wash the pellet with 1 mL of reagent A. Centrifuge at 6000 rpm for 6 min. Do not discard the supernatant.

9.- Mix both supernatants and take three samples (0.1 mL, 0.2 mL, and 0.3 mL). Take six samples of standard RNA solution (2 mg standard RNA in 20 ml chloroform) - 0.2mL, 0.4 mL, 0.8 mL, 1 mL, 1.5 mL, and 2 mL -. Add H_2O until 2 mL and 2 mL reagent G.

10 Keep during 35 min. at 100 ºC. Cool down with water and measure the absorbance at 665 nm.



Appendix 8: PHB determination

Prodecure:

1.- Add to 10-15 mg of biomass sample 1 mL NaClO (10-14 % Cl)

2.- Keep at 37 ºC for 1 hour.

3.- Add 4 mL of Milli Q Water. Mix up and centrifuge for 10 min at 12000 rpm.

4.- Discard de supernatant and wash the pellet with 5 mL of acetone.

5.- Mix up and centrifuge for 10 min at 12000 rpm.

4.- Discard de supernatant and wash the pellet with 5 mL of absolute ethanol.

6.- Mix up and centrifuge for 10 min at 12000 rpm.

7.- Discard de supernatant and add to the pellet 3 mL of chloroform.

8.- Keep in a boiling water bath for 1-2 min. Cool down.

9.- Centrifuge for 10 min at 12000 rpm.

10.- Put the supernatant away and repeat the extraction with chloroform twice.

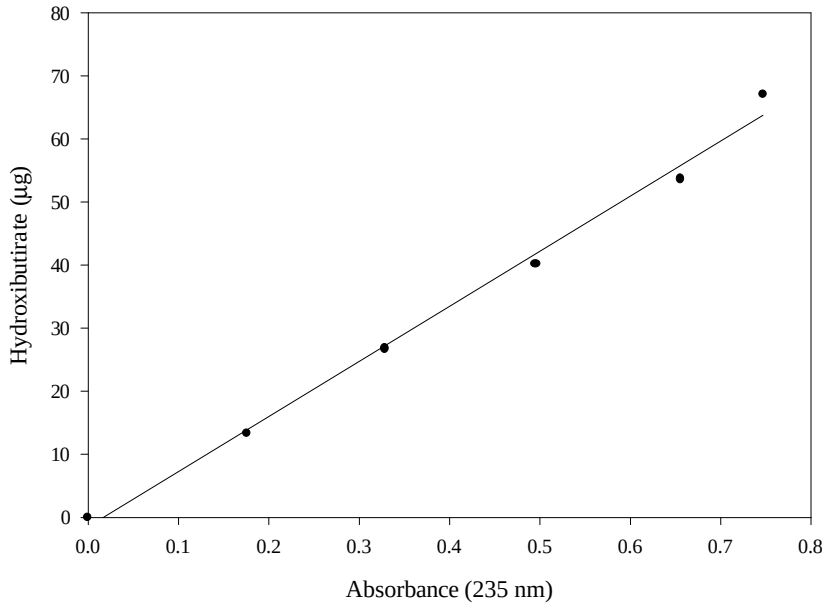
11.- Add chloroform to the supernatant to obtain a total volume equal to 10 mL.

12.- Take 1 mL, 3 mL, and 5 mL of biomass samples with chloroform.

13.- Evaporate the chloroform in a boiling water bath.

14.- Add 10 mL of conc. H_2SO_4 and incubate covered for 10 min. in a boiling water bath.

15.- Cool down and measure the absorbance at 235 nm.



Appendix 9: Glycogen determination

1.- Take 20 mg of biomass sample

2.- Add 10 mL of HCl 0.6 M

3.- Keep at a boiling water bath for 1 hour. Cool down.

4.- Filtrate through a 0.22 μm filter.

5.- Measure the glucose concentration with a glucose analyser (enzymatic analysis).