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MELISSA

Memorandum of Understanding

ECT/FG/MMM/97.012

Contract Number: ESTEC/CONTRACT11549/95/NL/FG

TECHNICAL NOTE 52.1

Study of the Na, K, Ca, and Mg influence on the composition of the Compartment II biomass

Version: 1

Issue: 0

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November 2002

Document Change Log

Version	Issue	Date	Observations
0	0	22/07/02	Draft Version
1	0	25/11/02	Final Version

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TECHNICAL NOTE 52.1:
Na, K, Ca and Mg uptake rate tests in Compartment II

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1. Introduction

In previous Technical Notes and reports the composition of the *R. rubrum* cells have been studied, particularly from the macrocomponents point of view, since it is very interesting to have the cell composition well characterised. Moreover, some tests studying the uptake of the main oligoelements such as Mn, Fe, Ni, Zn, Cu, etc. have been done as well.

In the present Technical Note, the mineral requirements of the *R. rubrum* cells are studied and this work continues with the task of the previous studies. The consumption of metallic elements such as sodium, potassium, calcium and magnesium by the *R. rubrum* cells has been analysed. All of them are essential for the cells, as then will be explained, but they are needed in small amounts.

In order to obtain some preliminary results about how the absence of some oligoelements such as Na, K, Ca, and Mg could affect the growth of the *R. rubrum* cells, it was carried out a first round of batch experiments. Afterwards, a run of continuous cultures in an 8-litre photobioreactor was done to determine approximately the amount of each element assimilated by the cells in several growing conditions.

These studies are of great importance as they determine the minimum amount of the nutrients, which compose the medium, necessary for the growth of the cells. Thus, the culture medium will be better adapted to the needs of the cells. Additionally, it is very important to know the uptake of all of the nutrients needed by the cells to build stoichometric models and mass balances of this compartment of the MELISSA loop.

2. Materials and methods

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

Batch experiments

The batch tests were carried out in 12 cylindrical glass tubes, of approximately 5 mL of working volume and 1 cm of external diameter. The first advantage of these tubes was the possibility to insert them into the spectrophotometer chamber to measure the optical density, without the need to take out a sample of culture.

Illumination was set up in monodimensional conditions inside a dark chamber with internal black surface (figure 1). The lamps were of the halogen type (Sylvania professional BAB 38º 12V 20W, improved version, cool beam, UV filtered, green box, code type 215).

Photosynthetically active radiation (PAR) was measured using a quantum sensor (Licor Li-190SA) attached to a LI-189 portable meter. The sensor gives the photosyntethic photon flux density (PPFD) in $\mu mols \cdot s^{-1} \cdot m^{-2}$. Conversion of quantum units to radiometric units (W/m^2) has been done using a constant factor obtained by integration of the lamp spectral data (Cabello *et al.*, 1999). The factor used were 0.368 for the 350-950 nm range.

The tubes were located at a different distances from the lamps in order to receive the same light intensity, as it is shown in figure 2. The light intensity was around 30 ± 1 $\mu mols \cdot s^{-1} \cdot m^{-2}$ or, doing the conversion to radiometric units, 11.04 ± 0.37 W/m^2.

The temperature was the laboratory one (approximately 28 ºC) and a fan inside the dark chamber avoided the accumulation of warm air around the tubes.

Biomass dry weight was calculated from the optical density at 700 nm, measured directly from the tubes, and its value interpolated on a calibration curve made using previously determined values of dry weight (average from all of the tubes) and optical density (Appendix 1).

The carbon source used in this test was acetic acid and the composition of the fresh medium is detailed in Appendix 2. There were six different compositions of the fresh medium, as each variety is deficient in one nutrient depending on the case: Ca^{2+} (tubes 1 and 2), Na^{+} (tubes 3 and 4), Mg^{2+} (tubes 5 and 6) or K^{+} (tubes 7 and 8). Moreover, there was a medium with all of the nutrients (tubes 9 and 10) whereas one medium was lacking in the four ions at the same time (tubes 11 and 12).

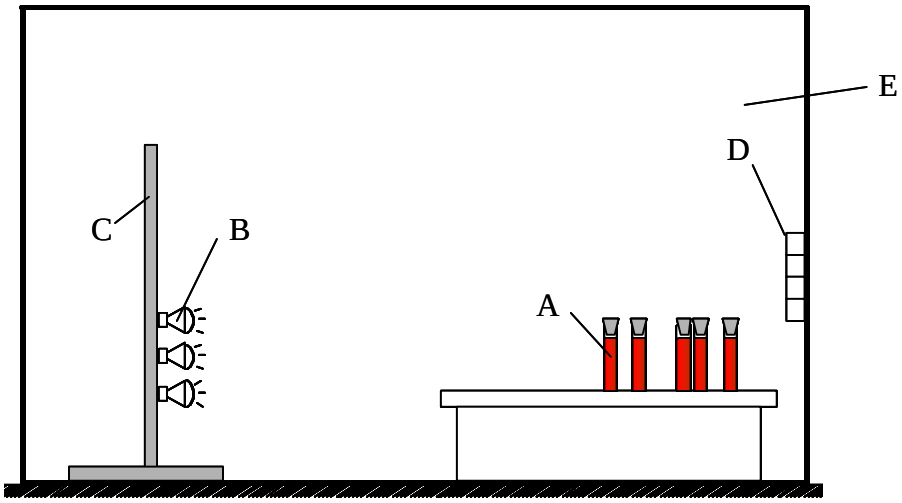


Figure 1: Experimental set-up: A: culture tubes; B: halogen lamps; C: lamps support; D: fan; E: dark chamber.

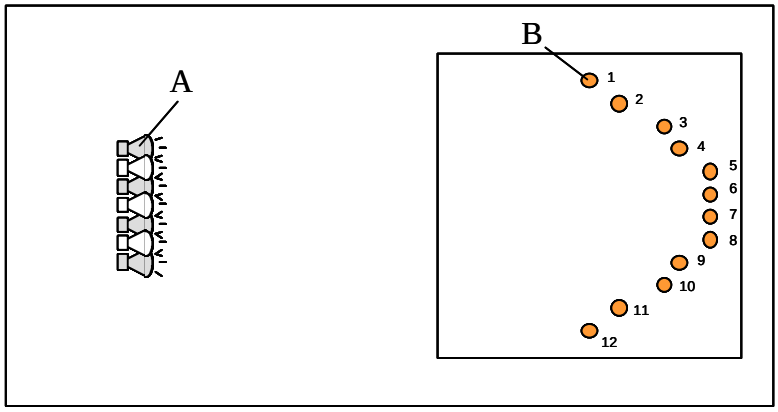


Figure 2: Experimental culture tubes distribution. All of the tubes receive the same light intensity. A: halogen lamps; B: culture tubes.

Continuous cultures

The continuous culture tests were carried out in an 8-litre *Bioengineering* photobioreactor (figure 3). The carbon source was acetic acid and the fresh medium is detailed in appendix 2. The carbon source concentration in the culture medium was analysed by the gas chromatography technique. The biomass concentration was determined measuring the dry weight. 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 NH_4OH (1.5 M) depending on the deviation from the set point value.

The concentration of the four nutrients studied, Na, K, Ca, and Mg, was analysed in the fresh medium and in the culture medium. These concentrations were measured by the ICP-OES technique.

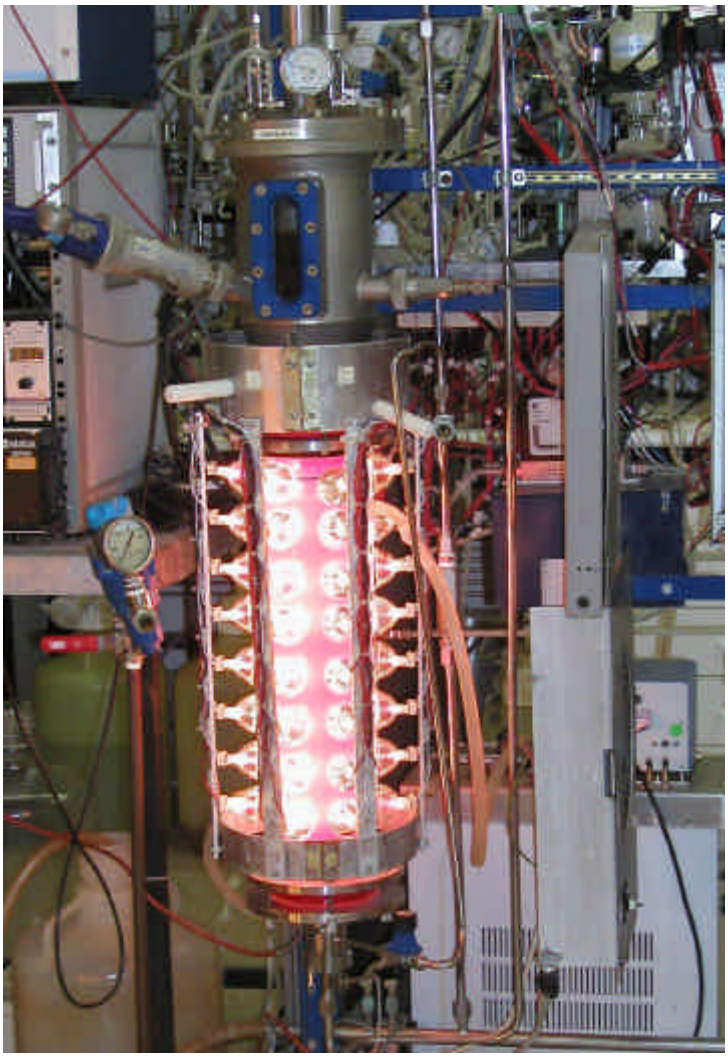


Figure 3: 8-litre *Bioengineering* photobioreactor.

3. Experimental results and discussion

Batch experiments

The influence of the Na^+, K^+, Ca^{2+}, and Mg^{2+} ions on the growth rate of the *R. rubrum* cells has been studied in batch cultures. The test consisted of subculturing the cells several times in a fresh medium deficient, at least, in one of the studied ions. There were 12 glass tubes using six different fresh media, as is detailed in table 1.

All of the tubes were inoculated the first time using the same inoculum, which nearly had not Na^+, K^+, Ca^{2+}, and Mg^{2+} ions. In the next batch cultures, the inoculum used was the previous cells grown in each tube.

Tube No.		
1, 2	Medium I:	deficient in Ca^{2+} ions.
3, 4	Medium II:	deficient in Na^+ ions
5, 6	Medium III:	deficient in Mg^{2+} ions
7, 8	Medium IV:	deficient in K^+ ions
9, 10	Medium V:	deficient in Ca^{2+}, Na^+, Mg^{2+}, and K^+
11,12	Medium VI:	with Ca^{2+}, Na^+, Mg^{2+}, and K^+

Table 1: the fresh media used in the tests. In Appendix 2, the composition of all of the six media is detailed.

Batch tests using Medium I (without Ca^{2+} ions)

In figure 4, it can be observed that the growth rate is decreasing with increasing time. In the first batch culture, the specific growth rate of the exponential phase was 0.0211 ± 0.0007 h^{-1} while, in the second batch culture, the specific growth rate decreased to 0.0069 ± 0.0002 h^{-1} and in the third batch culture the cells did not grow. After finishing the batch culture, the cells were subcultivated in the same fresh medium.

The specific growth rates have been calculated by linear regression in figures 5 and 6.

These results seem to indicate that Ca^{2+} ions are needed in the metabolism of the *R. rubrum* cells, as their lack progressively decreases the growth rate of the cells.

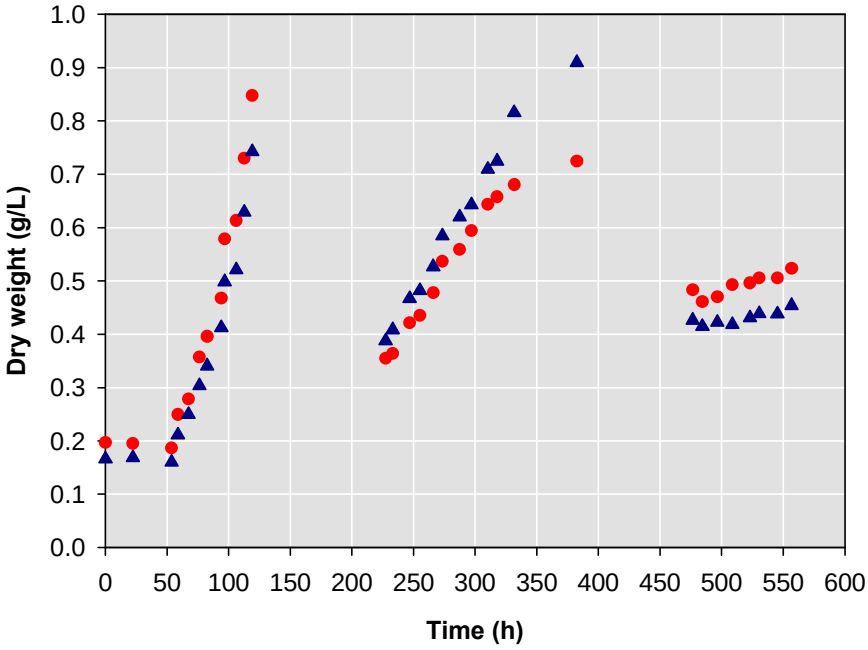


Figure 4: experimental data, dry weight *vs*. time,obtained in three batch tests using medium I, deficient in Ca^{2+} ions. The two different kinds of symbols in the graph correspond to the two glass tubes used in the test.

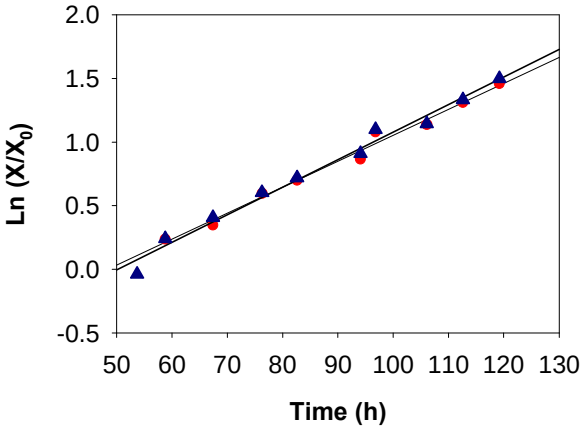


Figure 5: Linear regression from the data obtained in the first batch culture represented in figure 4. The slope of the lines corresponds to the specific growth rates and the values obtained are: 0.0204 h^{-1} and 0.0217 h^{-1}.

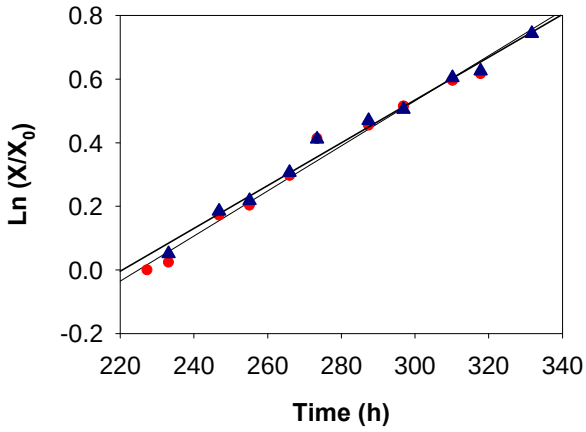


Figure 6: Linear regression from the data obtained in the second batch culture represented in figure 4. The slope of the lines corresponds to the specific growth rates and the values obtained are: 0.0071 h^{-1} and 0.0067 h^{-1}.

Batch tests using Medium II (without Na^+ ions)

In figure 7, the results obtained in the batch tests carried out using a fresh medium deficient in Na^+ ions (medium II have been depicted). During the three batch cultures the cells have grown without apparent problems.

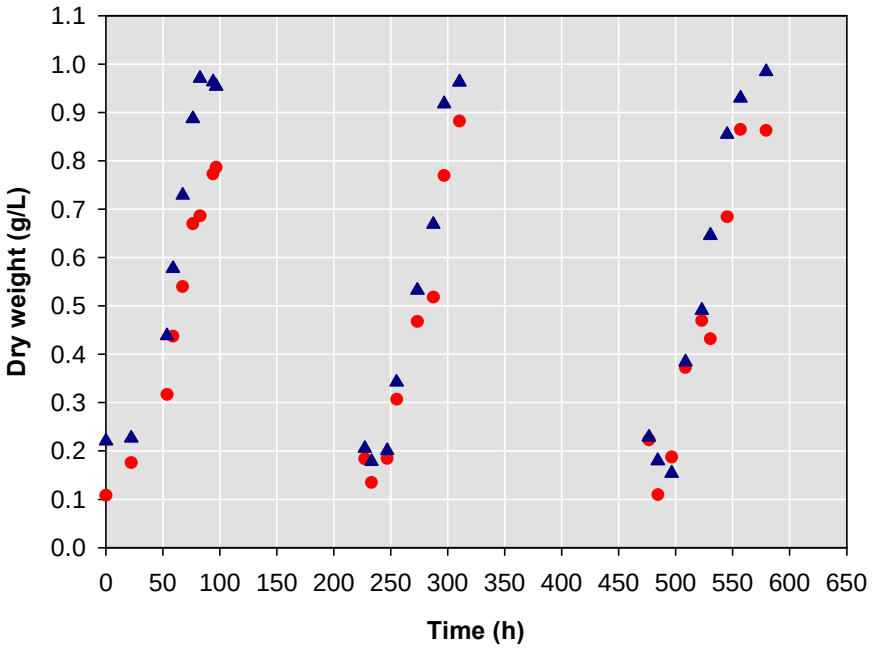


Figure 7: experimental data, dry weight *vs*. time, obtained in three batch tests using medium II, deficient in Na^+ ions. The two different kinds of symbols in the graph correspond to the two glass tubes used in the test.

In figure 8, 9 and 10 the specific growth rates have been determined, observing the fact that they do not decrease significantly due to the lack of Na^+ ions.

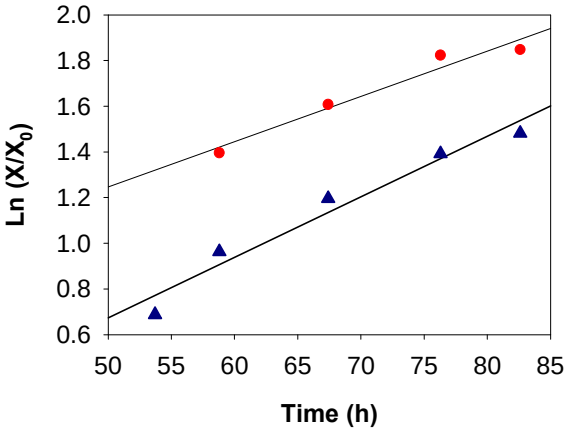


Figure 8: Linear regression of the first batch culture. The specific growth rates are: 0.0199 h^{-1} and 0.0265 h^{-1}.

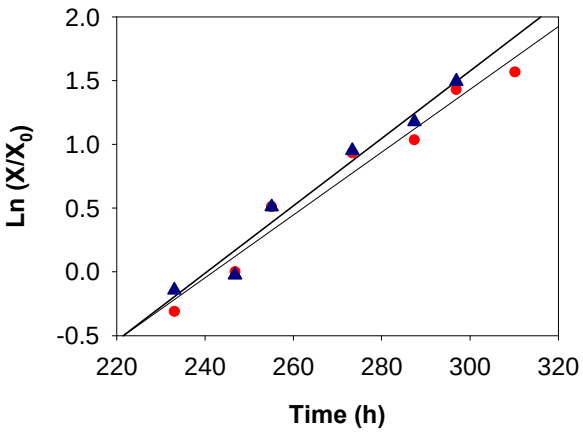


Figure 9: Linear regression of the second batch culture. The specific growth rates are: 0.0246 h^{-1} and 0.0264 h^{-1}.

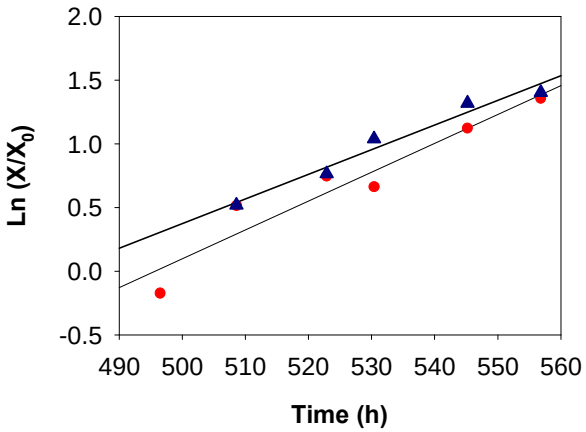


Figure 10: Linear regression of the third batch culture. The specific growth rates are: 0.0227 h^{-1} and 0.0194 h^{-1}.

These results seem to indicate that the lack of Na^+ ions does not affect the growth of *R. rubrum* cells drastically, although these tests don't allow to state that Na^+ ions are not essential for the cells.

Batch tests using Medium III (without Mg^{2+} ions)

In figure 11, the results obtained in the batch tests using the medium deficient in Mg^{2+} ions have been represented.

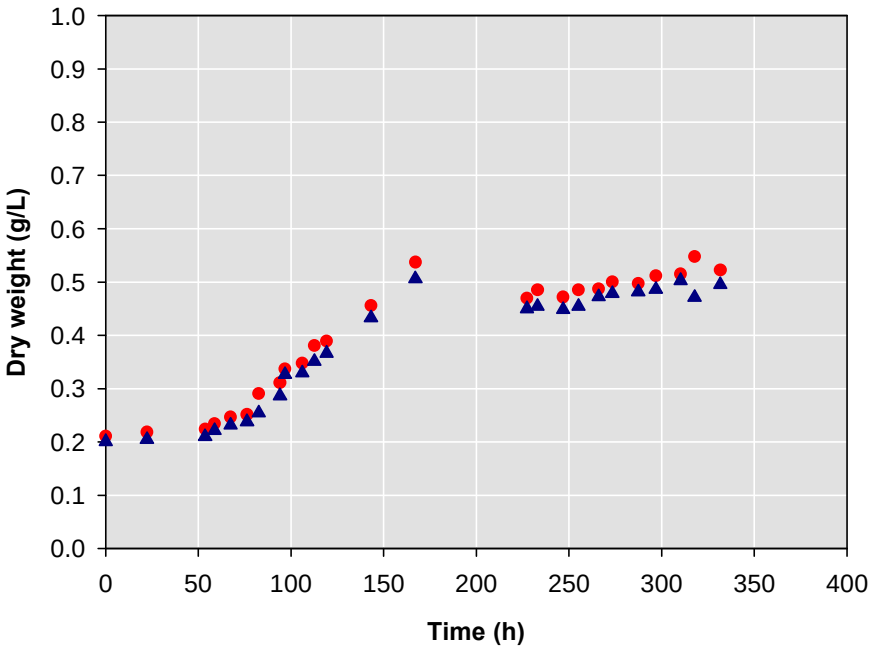


Figure 11: experimental data obtained in two batch tests using medium III, deficient in Mg^{2+} ions. The two different kinds of symbols in the graph correspond to the two glass tubes used in the test.

As can be observed in figure 11, the cells did not grow satisfactory in this medium, as the specific growth rate in the first batch culture was very small compared with the values obtained in the other cultures.

In the first batch culture, the specific growth rate in the two tubes was 0.0081 h^{-1} and 0.0080 h^{-1}, data determined in figure 12.

As the cells didn't grow properly, the two cultures were subcultivated in medium III again. The cells nearly did not grow.

This behaviour was predictable as the Mg^{2+} ions play an important role in several processes such as in the molecules of chlorophyll. Thus, without Mg^{2+} ions, the cells cannot grow properly.

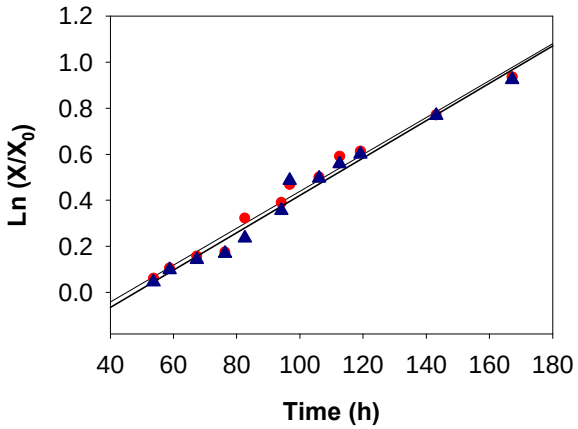


Figure 12: Linear regression of the first batch culture. The specific growth rates are: 0.0081 h^{-1} and 0.0080 h^{-1}.

These results indicate that Mg^{2+} ions are essential for *R. rubrum* cells, since they cannot grow without them.

Batch tests using Medium IV (without K^+ ions)

In figure 13 are represented the experimental data obtained in batch cultures using medium deficient in K^+ ions.

As in the case of the medium II tests, without Na^+ ions, the cells grow satisfactory. In figures 14, 15, and 16 are determined the specific growth rates obtained in these results. The results do not allow to observe any influence in the growth of the cells and more tests would be necessary to determine whether the K^+ ions are essential for the cells.

The fact that the cells are able to grow without Na^+ ions or without K^+ ions may indicate the possibility that the cells could use them indistinctly. Thus, when one type of ions is lacking, the cells use the other for the same purposes.

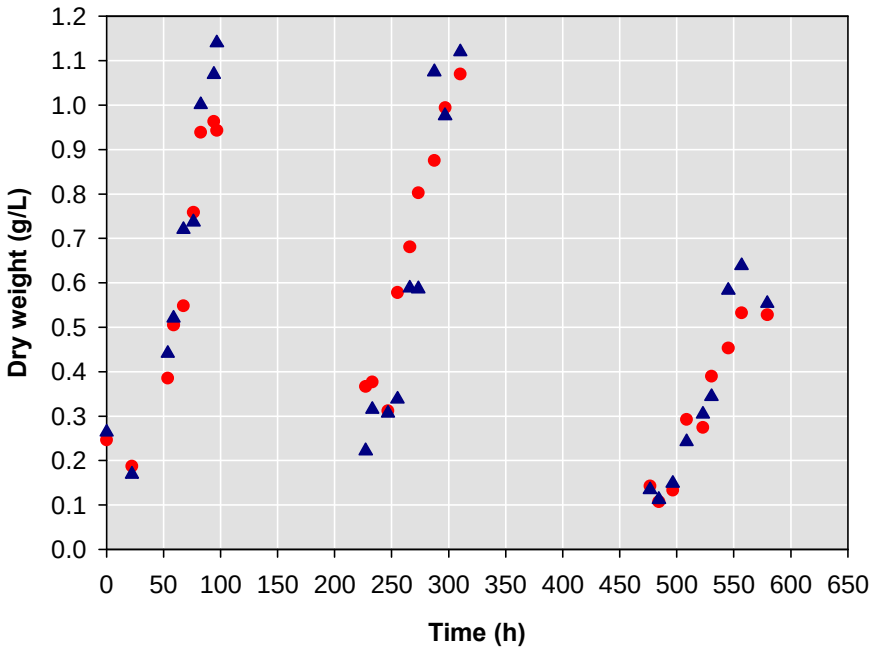


Figure 13: experimental data obtained in three batch tests using medium IV, deficient in K^+ ions. The two different kinds of symbols in the graph correspond to the two glass tubes used in the test.

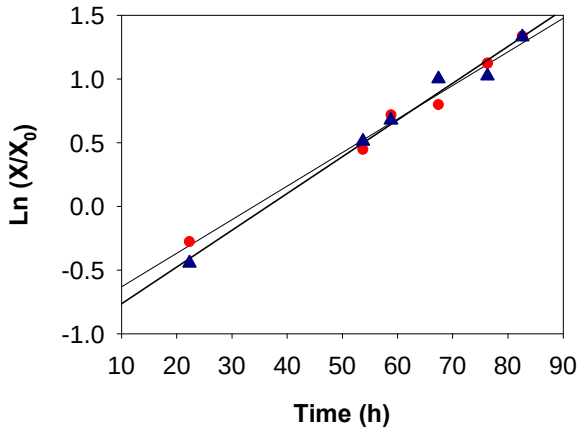


Figure 14: Linear regression of the first batch culture. The specific growth rates are: 0.0263 h^{-1} and 0.0289 h^{-1}.

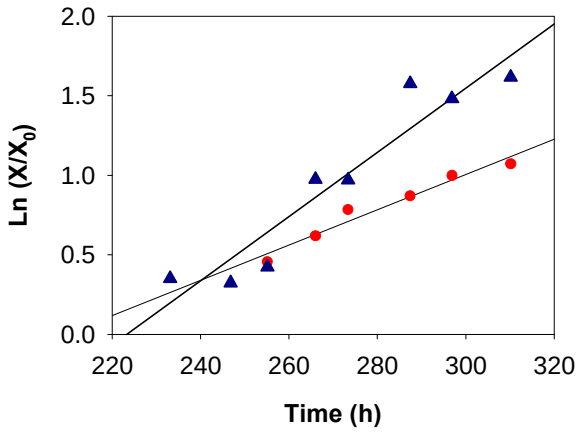


Figure 15: Linear regression of the second batch culture. The specific growth rates are: 0.0101 h^{-1} and 0.0202 h^{-1}.

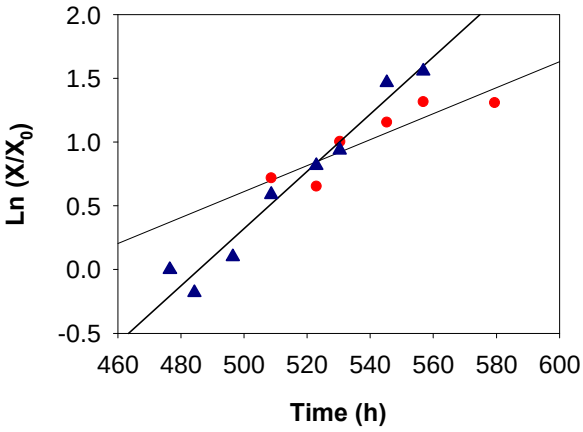


Figure 16: Linear regression of the third batch culture. The specific growth rates are: 0.0102 h^{-1} and 0.0224 h^{-1}.

Batch tests using Medium V (without Ca^{2+}, Na^{+}, Mg^{2+}, and K^{+} ions)

As can be observed in figure 17, the cells cannot grow in this medium without Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} ions. However, the cell concentration is maintained nearly constant.

This medium was used to prepare the inoculum of all of the tubes. In the culture used as inoculum, the cells did not grow satisfactory too and the initial cell concentration subsultivated as inoculum in this medium kept approximately constant.

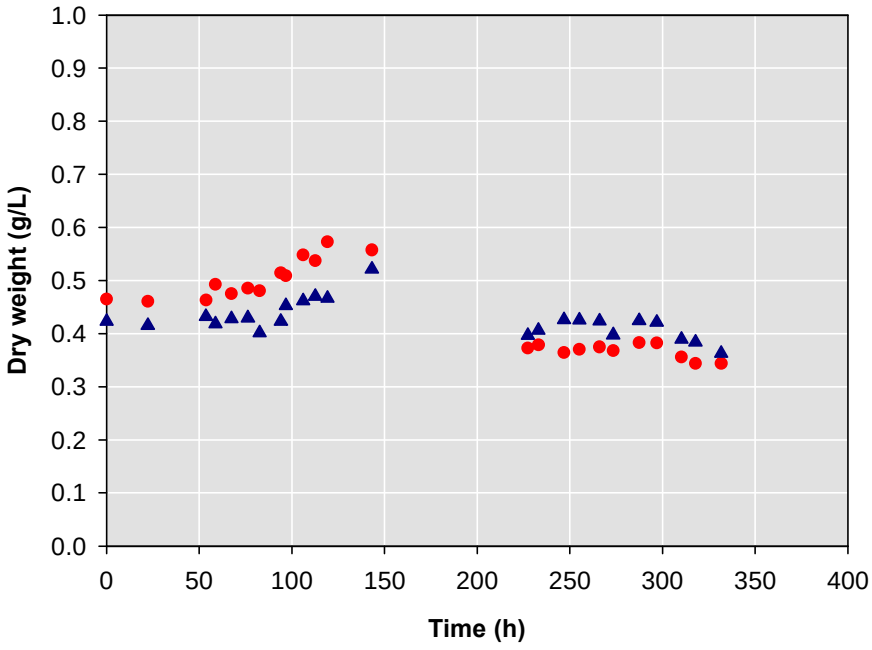


Figure 17: experimental data obtained in two batch tests using medium V, deficient in all of the studied ions. The two different kinds of symbols in the graph correspond to the two glass tubes used in the test.

Batch tests using Medium VI (with Ca^{2+}, Na^{+}, Mg^{2+}, and K^{+} ions) *(Control tubes)*

In figure 18 are represented the results obtained using a medium with all of the studied nutrients. In this medium the cells grew satisfactory.

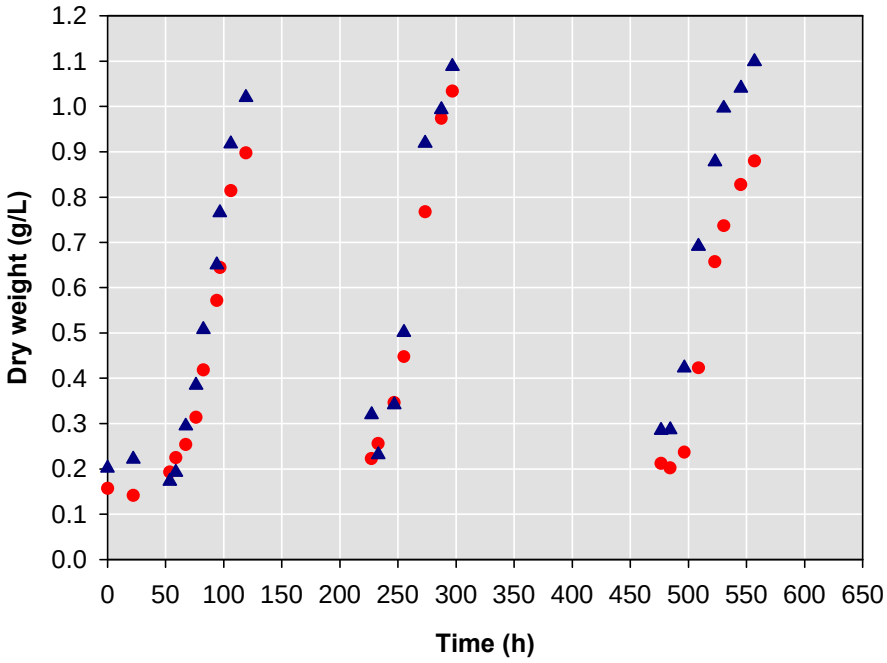


Figure 18: experimental data obtained in three batch tests using medium VI, with all of the studied ions. The two different kinds of symbols in the graph correspond to the two glass tubes used in the test.

In figures 19, 20, and 21 the specific growth rates have been determined. The values obtained, 0.0315 h^{-1}, 0.0240 h^{-1} and 0.0273 h^{-1}, are superior than the ones obtained in the previous tests using media without, at least, one kind of ions.

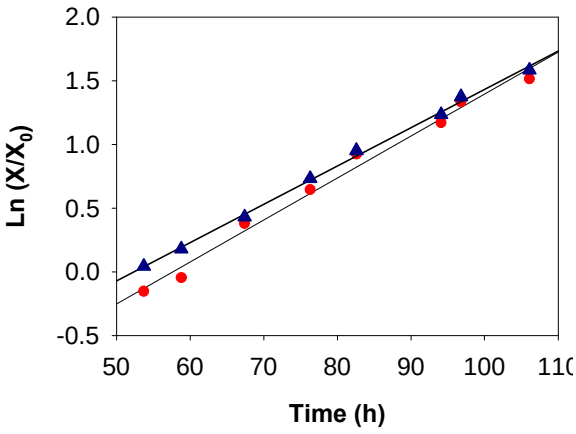


Figure 19: Linear regression of the first batch culture. The specific growth rates are: 0.0329 h^{-1} and 0.0301 h^{-1}.

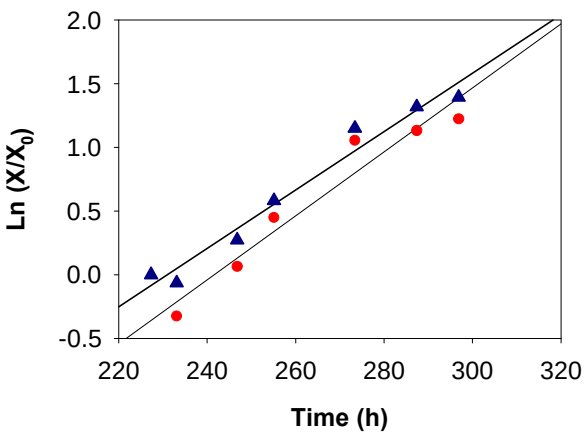


Figure 20: Linear regression of the second batch culture. The specific growth rates are: 0.0251 h^{-1} and 0.0229 h^{-1}.

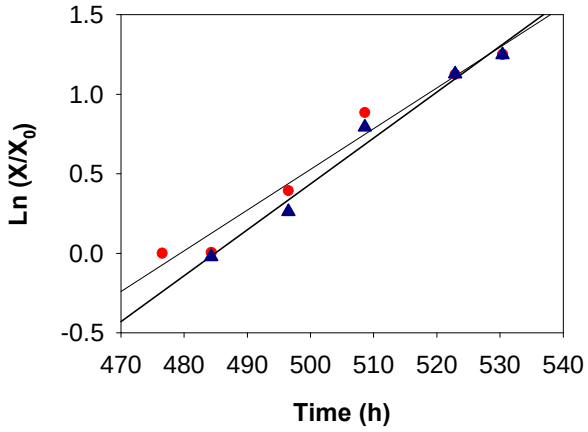


Figure 19: Linear regression of the third batch culture. The specific growth rates are: 0.0256 h^{-1} and 0.0289 h^{-1}.

Thus, in all of the previous tests, even in Na^+ and K^+ tests, there was a limiting factor of the growth different to the light intensity, as this parameter has been maintained constant and equal in all of the tubes.

Continuous cultures

In order to determine the amount of oligoelements required by the cells, a run of continuous cultures have been carried out. These tests consisted on determine the concentration of the studied ions in the fresh medium and in the culture. Thus, the difference between this both concentrations corresponds to the amount of oligoelements assimilated by the cells. The obtained results are presented in table 2.

	Na^+	K^+	Ca^{2+}	Mg^{2+}
Inlet medium	109 ± 1	102 ± 3	19.2 ± 0.8	22.5 ± 0.3
Outlet medium	93 ± 3	98 ± 2	14.1 ± 0.5	14.3 ± 0.1
Difference	16 ± 4	4 ± 5	5.1 ± 1.3	8.2 ± 0.4
$Y_{Ol/X}$ (mg olig/g cell)	20.0 ± 5	5.0 ± 6	6.4 ± 1.6	10.3 ± 0.5

Table 2: Mass balance of the studied ions between the inlet and the outlet of the photobioreactor. The error of the result is expressed as standard deviation.

In figure 20 can be observed the run of continuous cultures carried out in the 8 L photobioreactor. Three steady states were achieved, but only the samples corresponding to the third steady state could be analysed by the ICP-OES technique.

In all of the steady states achieved, a sample of broth of culture was taken in order to analyse the concentration of the studied metals. All the samples were centrifuged to separate the biomass from the liquid and then were analysed by the ICP-OES technique. The results were not satisfactory as the concentration of the ions was approximately the same in the fresh medium than in the culture. Maybe, the cells were broken while they were centrifuged. Another sample of culture was taken from the bioreactor (corresponding to the third steady state, at 215 W/m^2 and 0.035 h^{-1}) and in this case, the biomass was separated from the liquid by filtration. In this case the results were reasonable. That is the reason for having experimental results of only one steady state.

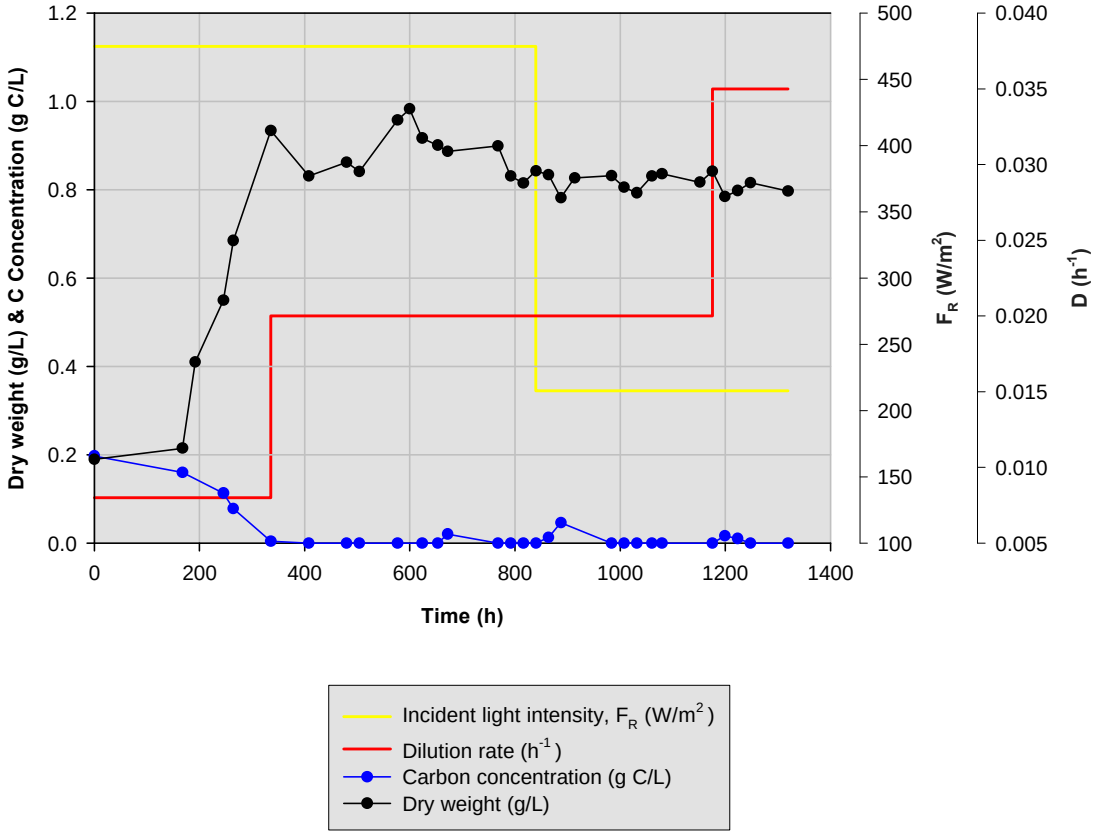


Figure 20: Continuous cultures carried out in the 8 L photobioreactor. The data used in the figure are reported in table 3.

The results reported in table 2 indicate that Na^+ ions are necessary for the growth of the cells, as it is consumed in a considerable amount. On the opposite, K^+ ions are not consumed or, at least, consumed in a small dose, since the standard deviation associated with the result is higher than the amount consumed.

Time (h)	Dry weight (g/L)	C conc. (g/L)	F_R (W/m^2)	D (h^{-1})
0.00	0.19	0.1972	475	0.008
167.83	0.215	0.16	475	0.008
191.75	0.41		475	0.008
245.75	0.55	0.1132	475	0.008
264.25	0.685	0.078	475	0.008
335.75	0.934	0.0044	475	0.008
408.00	0.831	0	475	0.02
479.83	0.862	0	475	0.02
504.00	0.841	0	475	0.02
576.92	0.958	0	475	0.02
599.75	0.983		475	0.02
623.83	0.917	0	475	0.02
652.92	0.901	0	475	0.02
672.00	0.887	0.0204	475	0.02
767.75	0.899	0	475	0.02
791.83	0.831	0	475	0.02
816.00	0.815	0	475	0.02
840.00	0.843	0	475	0.02
863.75	0.834	0.0132	215	0.02
887.50	0.782	0.0464	215	0.02
913.75	0.827		215	0.02
983.75	0.832	0	215	0.02
1007.75	0.806	0	215	0.02
1031.75	0.793	0	215	0.02
1060.67	0.831	0	215	0.02
1079.83	0.836	0	215	0.02
1151.75	0.817		215	0.02
1175.75	0.842	0	215	0.02
1199.50	0.785	0.0164	215	0.035
1223.75	0.798	0.0104	215	0.035
1248.00	0.816	0	215	0.035
1319.50	0.797	0	215	0.035

Table 3: Data obtained in the continuous cultures, which are represented in figure 20.

4. Conclusions

Taking into account the results reported in table 2, several facts can be concluded. The results obtained in the bioreactor indicate that the *R. rubrum* cells consume approximately 20.0 ± 5 mg Na^+ per g cell. On the other hand, the results about the consumption of K^+ ions seem to indicate that this ion is consumed in a very small amount or even is not consumed. Interestingly, in the batch cultures where one ion is lacking, the cells grow properly.

These results suggest that in case Na^+ ions or K^+ ions are lacking, the cells can use both for the same purposes. Thus, the lack of one of these ions does not affect drastically the growth of the cells. But, when the two ions are present in the culture, it seems that the cells prefer Na^+ ions than K^+ ions, as the first are consume in a superior amount.

Mg^{2+} ions are consumed in a higher amounts according the continuous test results. These results are coherent with the batch test results (figure 11), as the cells could not grow significantly without Mg^{2+} ions.

Concerning the consumption of Ca^{2+} ions, batch and continue tests indicate that these ions are consumed in a small amount. The cells can grow in the batch test without these ions, but they grow more and more slowly until its available Ca^{2+} is depleted.

These tests provide a preliminary results about the consumption of the studied ions and in the future further tests will be done.

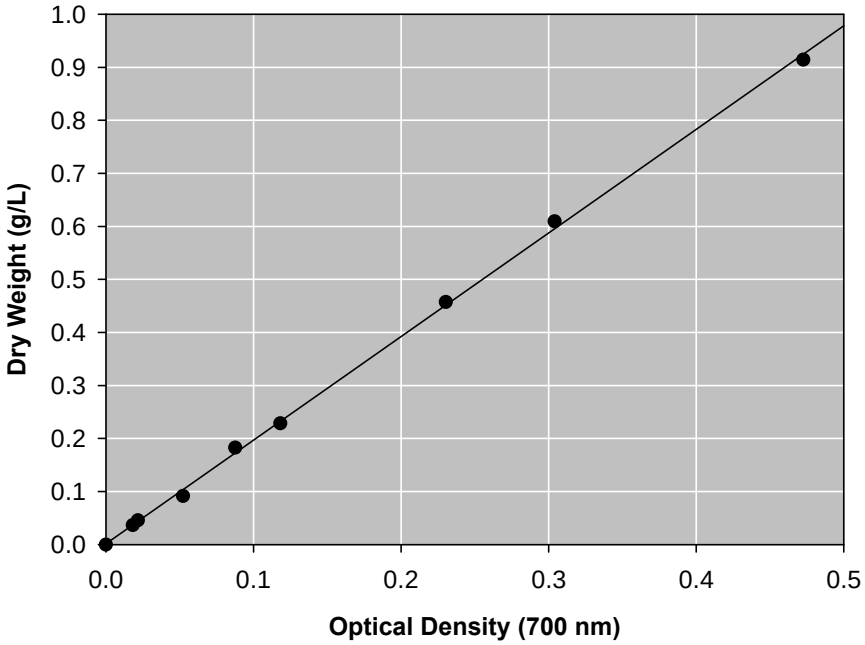
5. References

– TN 43.610: CABELLO, F.; ALBIOL, J.; GODIA, F. Scientific tests for *Rhodospirillum rubrum* 98. Test bench evaluation of IR enriched lamps. H_2 consumption test proposal. September, 1999. MELISSA. Contract 11549/95/NL/FG.

6. Appendixes

Appendix 1

With the purpose of knowing the dry weight from the optical density measurement a calibration between absorbance and dry weight have been done. Dry weight was done using Millipore 0.22 μm filters.



Appendix 2

Reactant (g/L)	I	II	III	IV	V	VI	Bioreactor
CH_3COOH	1.875	1.875	1.875	1.875	1.875	1.875	1.25
$(NH_4)_2SO_4$	0.925	0.925	0.925	0.925	0.925	0.925	0.925
EDTA-Na· $2\ H_2O$	0.02	0	0.02	0.02	0	0.02	0.02
$MnCl_2$· $4\ H_2O$	0.01	0.01	0.01	0.01	0.01	0.01	0.01
$FeSO_4$ · $7\ H_2O$	0.02	0.02	0.02	0.02	0.02	0.02	0.02
KH_2PO_4	0.03	0.03	0.03	0	0	0.03	0.03
K_2HPO_4	0.034	0.034	0.034	0	0	0.034	0.034
$NaHCO_3$	0.07	0	0.07	0.07	0	0.07	0.07
$MgSO_4$· $7\ H_2O$	0.2	0.2	0	0.2	0	0.2	0.2
$CaCl_2$· $2H_2O$	0	0.05	0.05	0.05	0	0.05	0.05
Trace elements *	1 mL of solution per L medium						
Biotin *	1 mL of solution per L medium						

***** *Trace elements solution*

Reactant	**Quantity**
Fe citrate	0.3 g
$MnSO_4$ · H_2O	0.002 g
H_3BO_3	0.001 g
$CuSO_4$· $5H_2O$	0.001 g
$(NH_4)_6Mo_7O_{27}$ · $4H_2O$	0.002 g
$ZnSO_4$	0.001 g
Distilled water	1 L

***** *Biotin solution*

Reactant	**Quantity**
Biotin	0.015 g
Distilled water	1 L