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ANALYSIS OF CARBON LIMITATION IN THE *SPIRULINA* COMPARTMENT

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Analysis of Carbon Limitation in the *Spirulina* Compartment

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Content

Page

Introduction and Objectives 4

1- Material and Methods 6

1.1- General Considerations 6

1.2- Experiments and Analysis 8

1.2.1- Determination of Molar Volumetric Oxygen Evolution Rate 9

1.2.2- Determination of Molar Volumetric CO_2 Consumption Rate 10

1.2.3- Calculation of the Volumetric Mass Transfer Coefficient K_La 10

1.2.4- Determination of the Volumetric Rates for Total Biomass Growth 10

1.2.5- Determination of the Volumetric Rates for the Main Compounds of the PRC and of the Photosynthetic Quotient 11

2- Results 11

2.1- Volumetric Mass Transfer Coefficient 11

2.2- Rates and Mass Fractions 12

3- Discussion 14

4- Modelling 15

4.1- General Case 15

4.2- Physical CO_2 Transfer Rate Approximation 16

Conclusion 17

Notations 18

References 19

Introduction and Objectives

At high pH values ($8 < pH < 11$), the total dissolved carbon ($C_T = CO_2 + HCO_3^- + CO_3^{2-}$) is essentially composed of bicarbonate HCO_3^- and carbonate CO_3^{2-} ions. For example, at pH 9.5, the concentration of carbonate and bicarbonate ions is about 2000 times higher than the CO_2 concentration. This is an important specificity because this creates a large buffer reserve of total dissolved carbon in the culture media. This implies that for gas transfer analysis inside a photobioreactor, the assumption of non-accumulation of dissolved gases in the medium used in the classical approach is no longer valid. The mass balance equations can not be written at steady state and must account for an accumulation term in the liquid medium. This entails solving non-stationary differential equations.

Cyanobacteria are known to concentrate intracellular bicarbonate if the extracellular concentration is low (Miller and Colman, 1980; Coleman and Colman, 1981; Badger and Andrews, 1982; Kaplan *et al.*, 1982). This concentration proceeds against the HCO_3^- concentration gradient but maintains the activity of the Rubisco at a high level. However, this active transport consumes a part of the produced ATP and the energy yield of photosynthesis thus decreases, resulting in lower growth rates.

The study of the limitation by the carbon source and its modeling are therefore complex problems since information has to be obtained at three levels:

- the study of the CO_2 gas-liquid transfer and of any limitation by the CO_2 transfer rate;
- the study of chemical equilibria in complex media with high ionic strength to calculate the CO_2-HCO_3^--CO_3^{2-} concentrations in the culture medium as a function of pH;
- the study of HCO_3^- limiting concentrations on the physiology and metabolism of *S. platensis* in order to postulate a kinetic law.

Moreover, the general modeling of these coupled phenomena requires mass balances on O_2 and CO_2 in the gas phase to be performed to supplement the above information.
Two principal cases may be examined:

<u>1- Limiting concentration of bicarbonate:</u>

In this case, the Rubisco controls the bioconversion rates (growth and exopolysaccharide synthesis rates) which depend on the bicarbonate concentration. This

bicarbonate concentration is related to the gas-liquid mass balance and to the dissociation equilibria. Either at steady state or in transient state, the bicarbonate concentration is obtained by solving the mass balance equations (Cornet *et al.*, 1995, TN 19.4) enabling to link the bicarbonate concentration to process parameters i.e. gas-liquid volumetric transfer coefficient K_La, gas flow rate, input molar fraction of CO_2, and total pressure.

2- Non-limiting concentration of bicarbonate:

The bioconversion rates depend on other parameters (light energy transfer, mineral limitations). However, the CO_2 mass balance remains to be solved because the total dissolved carbon concentration can either slowly increase or decrease, depending on the process parameters and on the physiological activity of the micro-organisms.

In a previous study (Cornet *et al.*, 1995, TN. 19.4), the problem of dynamic gas liquid CO_2 mass transfer was investigated and solved when the volumetric mass transfer coefficient K_La and the volumetric biomass growth rate $<r_X>$ were known. The aim of this Technical Note is to provide experimental results for the biological limitation of *S. platensis* by the carbone source, in order to be able to predict the volumetric biomass growth rate $<r_X>$ from the knowledge of the bicarbonate concentration in the liquid phase of the reactor. The problem of the activity coefficients calculation in electrolytic media in order to obtain the respective concentrations of bicarbonates and carbonates will be turn off by working with the pseudo-species total dissolved carbon concentration C_T. This note is a summary of the thesis of Marty (1997) which then appears as a reference document if more details are needed.

1- Material and Methods

1.1- General Considerations

All the experiments described in this TN were batch cultivations.

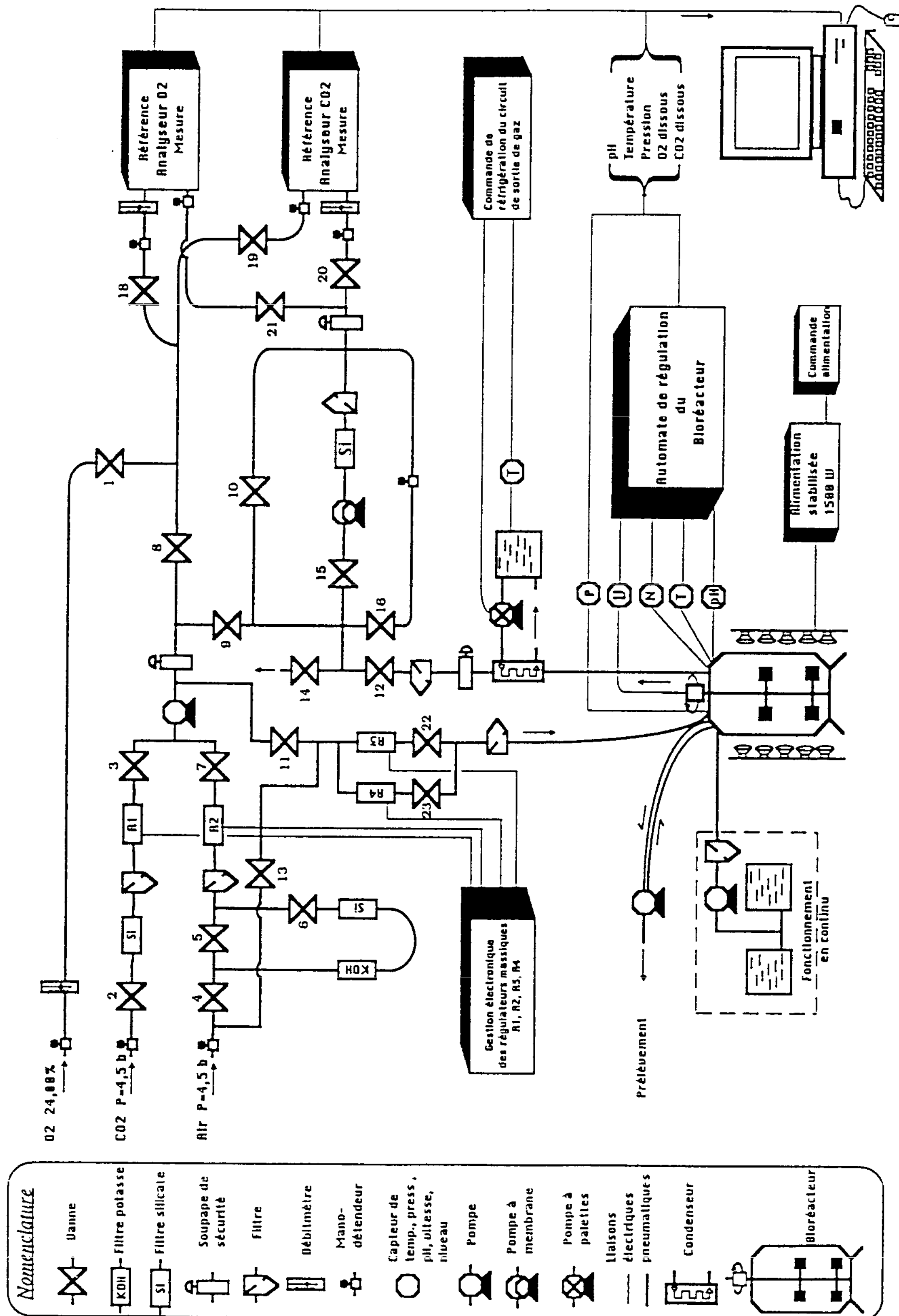
The pilot plant used in this study was already described in previous works (Cornet *et al.*, 1995, TN. 19.4). The scheme of the plant with the gas and liquid circuits and differential analysers is given in Figure 1. The photoreactor was an agitated vessel with two rushton turbines of 5 liters of working volume, radially illuminated by 55 halogen lamps Claude BAB 38, 20 W.

- *Gas phase*: the O_2 an CO_2 mass balances on the gas phase were performed with two differential analysers; a paramagnetic analyser for oxygen and an infra-red analyser for CO_2.

- *Liquid phase*: samples were regularly taken on the liquid phase in order to analyse off-line the concentrations for the main components, i.e. total dissolved carbon, total biomass, total sugars (including exopolysaccharide EPS), proteins, pigments, nucleic acids. The polyhydroxybutyrate (PHB) content of the cells was also determined in order to have a complete analysis of the biomass produced in conditions of carbon limitation (only the lipid content of the cells was not investigated because of the inaccuracy of the method). The methods used were as follows:

- total dissolved carbon: Gas Phase Chromatography method (Marty *et al.*, 1995);
- total biomass: dry mass and turbidimetry (750 nm);
- total sugars (including EPS): phenol method (Herbert *et al.*, 1971);
- proteins: method of Lowry modified by Peterson (1983);
- pigments: spectrophotometric method (Cornet, 1992);
- ADN: method of Burton (1956);
- ARN: method of Schneider (1957);
- PHB: method of Law and Slepecky (1960).

Figure 1: General scheme of the pilot plant used for the study.



All the controlled and measured variables on the process are summarized in Table 1. The strain used was *S. platensis* PCC 8005 cultivated on Zarrouk medium except for the carbonate-bicarbonate buffer. The pH was 9.5 and the temperature 36°C. The gas flow rate was 30 sl/h (0.1 vvm), and the rotation speed 300 rpm.

Controlled Parameters	**Measured Parameters**	
	On-line	***Off-line***
pH	O_2 partial pressure	Total dissolved carbon
Temperature	O_2 differential molar fraction	Biomass concentration
Rotation speed	CO_2 differential molar fraction	Total sugars (including EPS)
Incident light flux		Proteins
Gas flow rate	pH	Phycocyanins
Input CO_2 and O_2 molar fraction	Temperature	Chlorophylls
	Pressure	Nucleic acids
		PHB

Table 1: controlled and measured variables during batch cultures of *S. platensis* in the cylindrical and radially illuminated photobioreactor.

1.2- Experiments and Analysis

Two series of batch cultures experiments were performed for two incident radiant light fluxes illuminations. These values of mean radial incident flux were respectively 48 and 210 $W.m^{-2}$ in the range 350-750 nm. For each series, the input CO_2 molar fraction in the gas phase was decreased in the following order: 3000, 2400, 1800, 1200, 700 and 350 ppm, and for each fraction, the experiment was run until a pseudo-steady state regime was reached for the volumetric rate of total carbon consumption. **This regime was determined when the total dissolved carbon concentration in the liquid phase of the reactor became constant, which corresponds to a linear volumetric growth rate for biomass $<r_X>$. Then, only in these pseudo-steady state cases, the mean biomass growth rate was related to a constant and corresponding limiting total dissolved carbon concentration.**

This protocol then led to 12 stationnary phases (6 experiments for each of the couple of incident fluxes tested), which are summarized and labelled in Table 2.

Incident Flux F_0 ($W.m^{-2}$)	Input CO_2 molar fraction $y_{CO2}{}^E$ (ppm)	Label experiment
48	3000	A1
	2400	A2
	1800	A3
	1200	A4
	700	A5
	350	A6
210	3000	B1
	2400	B2
	1800	B3
	1200	B4
	700	B5
	350	B6

Table 2: different culture conditions for each pseudo-steady state reference phase.

1.2.1- Determination of Molar Volumetric Oxygen Evolution Rate

The molar volumetric oxygen evolution rate was determined with the hypothesis of no oxygen accumulation in the gas phase (pseudo-steady state). From the oxygen and inert mass balances in the gas phase, this rate was then given by:

$$< r'_{O_2} > = \frac{G^E}{V_L}\left[y_{O_2}^S \frac{(1-y_{O_2}^E)}{(1-y_{O_2}^S)} - y_{O_2}^E \right] \quad (1)$$

1.2.2- Determination of Molar Volumetric CO_2 Consumption Rate

This rate was determined from a mass balance equation on CO_2, using the same hypothesis as for oxygen:

$$< r'_{CO_2} >= \frac{G^E}{V_L}\left[y^S_{CO_2}\frac{(1-y^E_{CO_2})}{(1-y^S_{CO_2})} - y^E_{CO_2}\right] \quad (2)$$

1.2.3- Calculation of the Volumetric Mass Transfer Coefficient K_La

Because the O_2 and CO_2 mass transfer coefficients are very close (the difference is likely in the accuracy of the measurements), the same value for the two gases was assumed and determined from the mass balance in the liquid phase of the reactor, considering no accumulation term, even for the CO_2 because of the pseudo-steady state hypothesis in the corresponding phases. Taking a plug flow assumption for the gas phase, K_La is then given by:

$$K_La =< r'_{O_2} > \frac{H_{O_2}}{P}\frac{\ln\left[\dfrac{y^E_{O_2}\dfrac{P}{H_{O_2}} - C_{O_2}}{y^S_{O_2}\dfrac{P}{H_{O_2}} - C_{O_2}}\right]}{(y^E_{O_2} - y^S_{O_2})} = < r'_{CO_2} > \frac{H_{CO_2}}{P}\frac{\ln\left[\dfrac{y^E_{CO_2}\dfrac{P}{H_{CO_2}} - \dfrac{C_T}{K}}{y^S_{CO_2}\dfrac{P}{H_{CO_2}} - \dfrac{C_T}{K}}\right]}{(y^E_{CO_2} - y^S_{CO_2})} \quad (3)$$

The experimental determined values were compared to the values given by the correlation of Calderbank and Moo-Young (1961) for air-water coalescent dispersions:

$$K_La = 0.025\left(\frac{P_w}{V_L}\right)^{0.4} v_s^{0.5} \quad (4)$$

1.2.4- Determination of the Volumetric Rates for Total Biomass Growth

The mean mass volumetric rate $<r_X>$ for total biomass growth was experimentally obtained from the slope of the biomass concentration time course during stationnary phases

for the total dissolved carbon. The mean molar volumetric rate $<r'_X>$ was determined by dividing $<r_X>$ by the C-molar mass of the produced biomass.

1.2.5- Determination of the Volumetric Rates for the Main Compounds of the PRC and of the Photosynthetic Quotient

The mean mass fraction x_i for compound i in biomass was determined for each experiment by taking the ratio of the mean linear mass volumeric rate for i on the biomass volumetric growth rate, that is:

$$x_i = \frac{< r_i >}{< r_X >} \quad (5)$$

In the same way, the percentage of recovery for the carbon (PRC) in the biomass was determined according to:

$$PRC = \frac{< r'_X >}{< r'_{CO_2} >} \times 100 \quad (6)$$

and the photosynthetic quotient Q_P by:

$$Q_P = \frac{< r'_{O_2} >}{< r'_{CO_2} >} \quad (7)$$

2- Results

2.1- Volumetric Mass Transfer Coefficient

From all the experiments, the K_La value was determined. It was equal to 12 ± 1 h^{-1}, which is in very good agreement with the Calderbank and Moo-Young correlation (1961) for the actual conditions (0.1 vvm, 300 rpm). This determination enables to calculate the molar volumetric CO_2 consumption rate $<r'_{CO2}>$ from available data of the liquid phase (equation 3), in comparison of data of the gas phase only (equation 2). In the following analysis an average value for $<r'_{CO2}>$ from equation (2) and (3) has been used, especially in PRC determination.

2.2- Rates and Mass Fractions

The different volumetric biomass growth rates corresponding to each limiting constant dissolved carbon concentration C_T obtained for the 12 experiments are given in Table 3.

F_0 ($W.m^{-2}$)	N° Exp.	y_{CO2}^E (ppm)	y_{CO2}^S (ppm)	Total Dissolved Carbon Concentration C_T ($mol.l^{-1}*10^3$)	Mass Biomass Volumetric Rate $<r_X>$ ($g.l^{-1}.h^{-1}*10^3$)	Molar Biomass Volumetric Rate $<r'_X>$ ($mol.l^{-1}.h^{-1}*10^4$)
48	A1	3000	1480	25.0 ± 0.9	10 ± 1	4.10 ± 0.01
	A2	2400	1120	16.0 ± 0.6	7.2 ± 0.5	2.30 ± 0.01
	A3	1800	710	14.0 ± 0.5	5.7 ± 0.5	2.20 ± 0.03
	A4	1200	650	8.4 ± 0.3	4.3 ± 0.5	1.7 ± 0.3
	A5	700	330	1.10 ± 0.04	3.2 ± 0.5	1.40 ± 0.03
	A6	350	140	0.87 ± 0.03	-	-
210	B1	3000	1000	0.70 ± 0.03	12.5 ± 1	5.0 ± 0.3
	B2	2400	960	0.40 ± 0.02	10.3 ± 1	4.1 ± 0.1
	B3	1800	670	0.26 ± 0.01	8.4 ± 0.5	3.3 ± 0.1
	B4	1200	470	0.17 ± 0.01	5.7 ± 0.5	2.5 ± 0.1
	B5	700	280	0.13 ± 0.01	3.1 ± 0.5	1.2 ± 0.1
	B6	350	100	0.10 ± 0.01	-	-

Table 3: total dissolved inorganic carbon limiting concentrations C_T for stationnary phases and the corresponding volumetric biomass growth rates.

The growth rates for experiments A6 and B6 were not determined because they were too low and several months would be necessary in order to have significant differences in biomass concentration.

From these results and the mass balances for the gas phase, the percentage of recovery for carbon was determined. The results are given in Table 4.

N° Experiment	Molar Biomass Volumetric Rate $<r'_X>$ ($mol.l^{-1}.h^{-1}*10^4$)	Molar Rate of CO_2 Consumption $<r'_{CO2}>$ ($mol.l^{-1}.h^{-1}*10^4$)	Percentage of Carbon Recovery *PRC* (%)
A1	4.1	4.3	95
A2	2.3	3.7	62
A3	2.2	2.5	88
A4	1.7	1.8	94
A5	1.4	1.3	107
A6	-	0.67	-
B1	5.0	5.4	93
B2	4.1	4.5	91
B3	3.3	3.6	92
B4	2.5	2.3	108
B5	1.2	1.4	86
B6	-	0.64	-

Table 4: percentage of carbon recovery in the biomass (PRC) from CO_2 gas mass balances during stationnary phases.

N° Exp.	Percentage of Macromolecules (%)						
	Proteins	Phycocya-nins	Chloro-phylls	Total Sugars	ADN + ARN	PHB	Total (without lipids)
A1	54	18	1.1	26	10	1	92
A2	40	13	0.7	11	10	1	63
A3	56	12	0.7	12	10	1	80
A4	56	11	0.6	12	10	1	80
A5	50	11	0.5	15	10	1	77
A6	49	5	0.8	12	10	1	73
B1	59	11	0.5	14	10	1	85
B2	50	10	0.5	12	10	1	70
B3	50	13	0.6	14	10	1	76
B4	49	8	0.6	12	10	1	61
B5	38	4	0.5	13	10	1	63
B6	38	-	0.1	13	10	1	63

Table 5: mean percentages for the main macromolecules involved in the biomass composition (except lipids) during stationnary phases.

For each phase, the ratio of mass volumetric rates was established (equation 5), giving the mean percentages for the main macromolecules involved in the biomass composition, and allowing to study the metabolic deviations occuring under bicarbonate limitation. These results are available in Table 5.

3- Discussion

From the results given in Table 4, it appears clearly that the PRC are good (that is within the range of 10% accuracy) for all the experiments except for experiment A2. For this latter experiment, this probably results in a problem on the determination of the volumetric growth rate in biomass for high biomass concentrations. Because the PRC is generally close to 100%, it seems possible to use the molar volumetric rate of CO_2 consumption $\langle r'_{CO2} \rangle$ instead of the molar volumetric growth rate $\langle r'_X \rangle$ when this last data is not available. This is particularly true for the experiments A6 and B6.

The main feature of the limitation of *S. platensis* growth by the carbon source appears in Table 3. The results clearly show the coupling between light and carbon limitation as previously stated in the literature (Miller and Colman, 1980; Coleman and Colman, 1981). For example, experiments A1 and B2 have growth rates very close for a total carbon concentration in the liquid phase C_T varying in a factor 60. At the opposite, experiments A6 and B1 present almost the same C_T, but a factor 8 on the growth rates. The fact that no direct proportionality was found between C_T and rates shows the coupling between the mechanism of intracellular carbon concentration and the photophosphorylation, directly dependent of the available radiant light energy in the medium.
Moreover, Table 3 shows that, at the opposite of O_2 limitation for heterotrophic microorganisms, the purely physical limitation by CO_2 transfer does not exist (at least at pH = 9.5), because, even in the more limiting condition of experiment B6, the CO_2 concentration in the liquid phase is very low ($5\ 10^{-8}$ mol.l^{-1}), but C_T is equal to 10^{-4} mol.l^{-1}. From the literature data, this value of C_T involves a higher intracellular total carbon concentration.

Table 5 displays the metabolic deviations occuring in bicarbonate limitation. The main features are the inhibition of the exopolysaccharide synthesis (the total sugars mass fraction remains equal to 12-13%, which corresponds only to the cell wall sugars), except in experiment A1 in which the limitation appears for the first time; and a progressive decrease of the pigments mass fraction (phycocyanins and chlorophylls), corresponding to a decrease of total proteins. As the mechanism of concentration for the bicarbonate proceeds against the

concentration gradient and uses ATP for active transport, and because the rate of ATP synthesis is imposed by the light transfer limitation (i.e. the ratio P/2e$^-$), the part of ATP consumed for bicarbonate transport leads to a new P/2e$^-$ ratio, and then to metabolic deviations.

The last conclusion concerns results summarised in the last column of Table 5. These results show the decrease in the total of the species mass fractions (without lipids). This total should have to be close to 90% because the lipid content in *S. platensis* is about 10%. This is the case only for experiments A1 and B1, but for other experiments, this total decreases with increases in carbon limitation. The « lacking matter » is an unknown compound, probably a lipid. This hypothesis lies on the fact that all the known components for *Spirulina* have been measured except lipids, and that the elemental analysis performed on the biomass of experiment B6 have led to a very reduced global formula, only possible with a high lipid content. It then seems possible to think that the decrease in pigments fraction is balanced by an incease in the lipid fraction corresponding to a lower P/2e$^-$ ratio available in the cells, and allowing that an important amount of ATP remains available for active transport of bicarbonate by the cells.

4- Modelling

4.1- General Case

Some different models of increasing complexity have been established and compared to experimental results. We first investigated simple models with the extracellular total carbon concentration as the main variable, then we introduced a new term of "maintenance" in order to take into account the ATP energy dissipated for bicarbonate active transport. Because it was impossible to modelize experimental results with such kinds of models, we tried to choose the intracellular total carbon concentration as the main variable. The arising problem was that this concentration was not measured in our experiments and we have no possibility to verify the assumptions on the laws for the concentration phenomena. Moreover, this kind of models involves at least 4 new and coupled parameters which remain to be identified. This coupling could be reduced by finding new independent equations.

At present time, a model able to take into account all the 12 experiments is still under investigation in the lab and we report here only the first conclusions from our previous analysis.

The model must have a term of inhibition of biomass growth rate by the intracellular total carbon concentration in the cells. As a first trial, the half saturation constant for this phenomena may be taken as the literature constant of the Rubisco. This approach imposes to have an independent equation for the bicarbonate concentration phenomena, giving the ratio of intracellular to extracellular carbon concentrations. The model must also have a term of "maintenance" to take into account the decrease of biomass growth rate due to ATP consumption for active transport. This term is linked to the radiant light energy transfer in the medium for two reasons:

- it exists only in the working illuminated volume of the reactor;
- we have theoretically established that the maintenance coefficient of the model was linked to the specific growth rate versus local available radiant light energy.

Finally, the model must be able to take into account metabolic deviations such as the inhibition of the EPS synthesis, and the decrease of the pigment synthesis rate corresponding probably to an increase of the lipid synthesis rate.

4.2- Physical CO_2 Transfer Rate Approximation

Even if it is not really the case, it is possible to express the molar volumetric biomass growth rate from the hypothesis of a physical limitation by the CO_2 transfer rate. In this case, with the assumption of plug flow for the gas phase in the reactor, we have the following relation, considering the total carbon concentration in the liquid phase as close to zero:

$$< r'_X > = < r'_{CO_2} > \cong K_L a \frac{P}{H_{CO_2}} \frac{(y^E_{CO_2} - y^S_{CO_2})}{\ln\left(\frac{y^E_{CO_2}}{y^S_{CO_2}}\right)} \quad (8)$$

It is then possible to predict the biomass growth rate from equation (8) if $K_L a$ and y^S are known. It can be shown that use of equation (8) gives an accuracy better than 10% if the total dissolved carbon concentration C_T is lower than 10^{-3} mol.l^{-1} (corresponding to a CO_2 concentration of 5 10^{-7} mol.l^{-1}), that is for experiments A5 to B6.

Conclusion

The kinetic and physiological results obtained in this study appear as basic informations for modelling the coupled light and carbon source limitations for *S. platensis* cultivated in photobioreactors. Nevertheless, this model appears very difficult to establish because of the number of phenomena involved and their own coupling. So, it is still under investigation at present time.

New additionnal informations would be of great interest for this purpose, such as the exact determination of the unknown compound, of its amount in the cells, and the accurate determination of the intracellular total carbon concentration for each stationnary phase. This requires new experimental methods to be developed to have an accurate assessment of the lipid and bicarbonate contents in cells.

Finally, these results, obtained in batch cultures, have to be verified in continuous cutures, allowing to keep low biomass concentrations in the reactor, then leading to very accurate determination of mass volumetric growth rates in biomass.

Notations

C_i	Concentration of compound i in the liquid phase $(mol.l^{-1})$
G	Molar gas flow rate $(mol.h^{-1})$
H_i	Henry's constant for the species i $(l.atm.mol^{-1})$
K	Factor for CO_2 to total carbon concentration conversion (-)
K_La	Volumetric mass transfer coefficient (h^{-1})
P	Total pressure (atm)
P_w	Mechanical power dissipated in an agitated vessel (W)
Q_P	Photosynthetic quotient (-)
r_i	Mass volumetric production or consumption rate for species i $(g.l^{-1}.h^{-1})$
r'_i	Molar volumetric rate for the species i $(mol.l^{-1}.h^{-1})$
v_S	Superficial velocity for the gas in a vessel $(m.s^{-1})$
V	Volume (l)
x_i	Mass fraction for the compound i in the liquid phase (-)
y_i	Molar fraction for the compound i in the gas phase (-)

Exponents

E	relative to an input in the reactor
S	relative to an output of the reactor

Indices

L	relative to the liquid phase
T	relative to total dissolved carbon

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