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Stoichiometric analysis of *Rs.rubrum* growth on different carbon substrates

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Technical Note 45.4

Stoichiometric analysis of *Rs.rubrum* growth on different carbon substrates

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Content

1. INTRODUCTION AND OBJECTIVES

This note deals with the second compartment (anoxigenic phototrophs) of the MELiSSA loop.

The purpose of the second compartment is to consume the organic acids incoming from the first compartment, under anaerobic conditions, in the light.

Several strains were proposed in TN1 to be used in this compartment. Among those, *Rs. rubrum* was considered to be most suitable to be used for the photoheterotrophic subcompartment (Albiol, 1994), *Rb. capsulatus* and *Thiocapsa roseopersicina* were selected for being used in the photoautotrophic subcompartment and the sulfur subcompartment respectively.

The carbon sources most likely to be found at the exit of compartment I of MELiSSA and therefore the carbon sources that will be used in compartment II have been identified as the following volatile fatty acids (VFA) : acetic, propionic, butyric, isobutyric, valeric, caproic, isovaleric and isocaproic.

In the photoheterotrophic subcompartment, the fatty acids become the carbon source and the electron donor.

This paper is an up-dated of the study realized by Poughon (1995). We interested only in the carbon metabolism, i.e. the carbon substrates considered by Poughon (1995) with substrates of minor importance in concentration : caproate, valerate, isocaproate and isovalerate.

In this work, the mass balance technique has been applied to *Rs.rubrum* growth under anaerobic photoheterotrophic conditions.

The main aim of this work is to establish the stoichiometric equations for *Rs. rubrum* growth, from the different carbon substrates found at the exit of the liquefying compartment, taking into account metabolic information from the literature and the experimental results obtained by the MELiSSA partners. The metabolic network proposed for writing these stoichiometric equations has been validated for different carbon substates by using a flux calculation method. **The proposed stoichiometric model is only valid in the working illuminated volume of the photobioreacteur** (Cornet et al., 1999) but this approach is a prequisite step in modeling metabolic pathway and stoichiometries during the short residence time of cells in the dark efficient zone of the photobioreactor.

The flux values are obtained by applying the pseudo-steady state assumption to the metabolic pools and using analytical information on proper cell composition including macromolecules and intracelular metabolites, substrate consumption, biomass and major byproduct synthesis.

This T.N. includes three parts :

- the first part is a brief review of the carbon metabolism taking into account the photometabolism of organic compounds used in the photoheterotrophic subcompartment. Closing this part, the biomass chemical and biochemical compositions are presented.

- in the second part, the methodology in building stoichiometries is presented. The main metabolic assumptions used to build the stoichiometries are described and discussed.

- in the third part, we present the stoichiometric model of *Rs. rubrum* on different carbon substrates, the flux calculation, the determination of the mass yields and the quantum yields for each carbon substrate used in this study.

2. DIFFERENTS METABOLIC ASPECTS OF *RS. rubrum*

2.1 The purple non-sulfur bacteria (PNSB) of the anoxygenic phototroph subcompartment

In order to establish the stoichiometries for *Rs. rubrum*, it is necessary to determine the characteristics of their metabolism i.e. their metabolic pathways for the anabolic reactions, the catabolic reactions and the energy generation. The general and specific metabolic characteristics of *Rs. rubrum* have been largely described in T.N. 23.3 (Poughon, 1995). In this study, we interested more especially in the carbon metabolism.

Rs. rubrum which has been chosen to colonize the anoxygenic phototrophs compartment is a genus of the *Rhodospirillaceae* (or PNSB). The *Rhodospirillaceae* are phototroph gram negative bacteria. The present strain studied is the one used at ESTEC and at UAB, obtained from the American Type Culture Collection : *Rs. rubrum*, ATCC 25903 (Albiol, 1994). Different metabolisms are to be considered with this strain but the most preferably is the growth as **photoheterotrophs** with various organic substrates under **anaerobic conditions** in the **light**.

It is known that the purple non sulphur photosynthetic bacteria are probably the most metabolically versatile organisms found in the nature (Madigan,1988). Indeed, the strain can grow also under the same conditions as photoautotrophs with molecular hydrogen (H_2) as electron donor and CO_2 as sole carbon source (Imhoff et al. 1989). Growth is also possible under microaerobic to aerobic conditions in the dark. Fermentative metabolism with pyruvate under anaerobic dark condition and oxydant dependant anaerobic dark metabolism are also possible (Imhoff and Trupper, 1971).

2.2. Photometabolism of organic coumpounds

Purple bacteria can grow when illuminated under anaerobic conditions in synthetic medium containing NH_4^+ as N-source and an organic coumpound as C-source. It has been observed that CO_2 yield (produced or assimilated) depends on the organic compound for PNSB (Ormerod and Gest, 1962). In the presence of the light as energy source and substrates more reduced than CO_2, it may be assumed that the major function of various metabolic cycles in photosynthetic bacteria is the synthesis of new cellular material rather than the production of energy (Doelle, 1975).

Willison (1988) studying the pyruvate metabolism in *Rb. capsulatus* described the central pathway of carbon metabolism in this organism (figure 1). This scheme of the central pathway of carbon metabolism is very useful because it shows the entry points in the central carbon pathway (TCA and Embden Meyerhoff pathways) of the main carbon compounds supposed to be present in compartment II (VFA, CO_2).

The functioning (regulation, stoichiometries) of the central pathway of carbon metabolism (TCA, Entner-Doudoroff and Embden-Meyerhoff pathways) are well-known and the stoichiometric equations that describe these pathways are reported in Annexe.

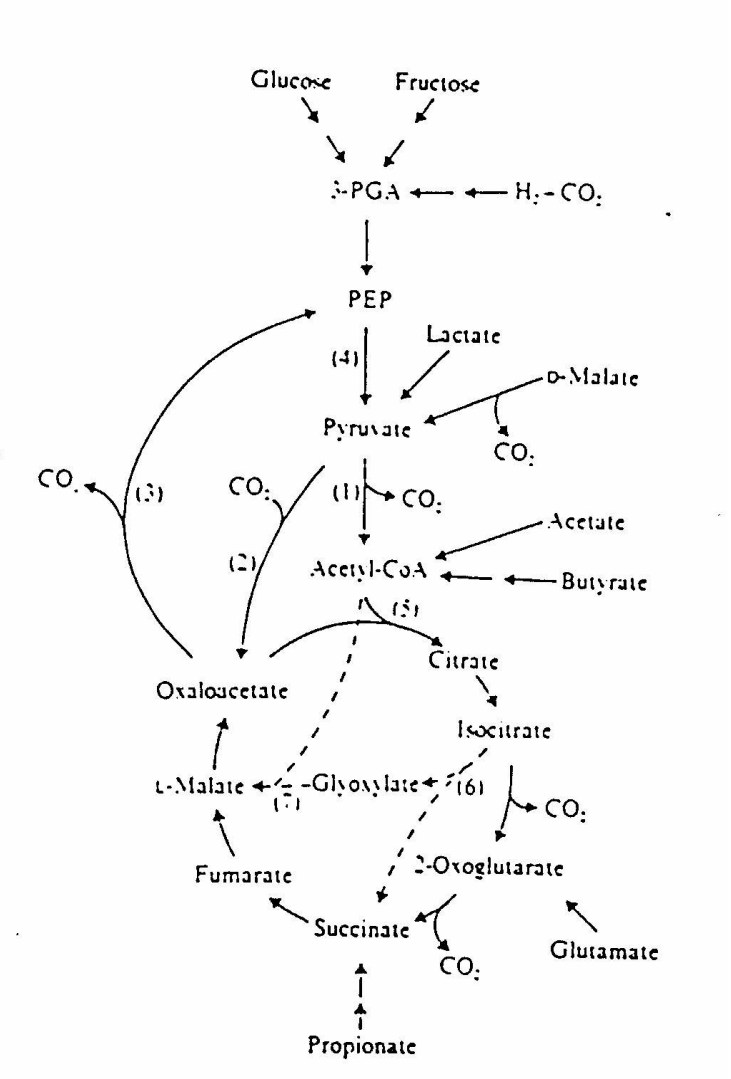

Figure 1 : Central pathway of carbon metabolism in *Rb. capsulatus*. (1) pyruvate dehydrogenase ; (2) pyruvate carboxylase ; (3) Phosphoenolpyruvate carboxykinase ; (4) pyruvate kinase ; (5) citrate syntase ; (6) isocitate liase; (7) malate syntase. Taken from Willison (1988).

The stoichiometric description of the reactions from the organic compounds (VFA) to the central carbon pathway is the purpose of the following lines. The fatty acids are usually degraded by β-oxydation.The fatty acid is first converted to the corresponding CoA ester by acyl-CoA synthetase. The CoA ester is then oxidized in the β-position and subsequentely cleaved to yield acetyl-CoA and the CoA ester of the fatty acid shortened by two carbon atoms. Even-numbered fatty acids yield only acetyl-CoA. With odd-numbered fatty acids the final β-oxydation cycle yields acetyl-CoA and propionyl-CoA.

Acetic acid ($C_2H_4O_2$)

Many strains of the *Rhodospiriliaceae* family are able to utilize acetate as the sole carbon and reducing power source for photosynthetic anaerobic growth (Sojka, 1978). Acetate is typically catabolized via the tricarboxylic acid cycle (TCA cycle).

The key enzymes for acetate metabolism in bacteria are isocitrate lyase and malate synthase which together with other enzymes of the tricarboxylic acid cycle constitute the glyoxylate shunt and allow bacterial cells to grow on acetate. However *Rs. rubrum* can grow on acetate as the sole organic substrate in spite of its lack of isocitrate lyase (another enzyme of glyoxylate shunt, malate synthase, is present). Malate synthase appears to be constitutive (Blasco et al., 1989). Thus, this microorganism lack the glyoxylate cycle.

In our metabolic network we have taken into account this consideration.

Rs.rubrum is able to assimilate acetic acid directly in absence of CO_2. Acetic acid is converted to acetyl-CoA by an acetic activated enzyme (acetate kinase and phosphotransacetilase). This one is the classical pathway.

Acetic acid + ATP +CoA-SH → Acetyl-CoA + AMP + PPi

Without CO_2, the main storage compound product formed is poly-β-hydroxybutyric acid (PHB). Because this synthesis is reductive, some acetic acid must be oxydized to provide the necessary reducing power via the TCA cycle (Doelle, 1975).

The formation of poly-β-hydroxybutyric acid depends on the amount of dissolved CO_2, because CO_2 shifts the synthesis toward polysaccharide (glycogen) formation.

Rs. rubrum has a further pathway of acetate transformation, with the end product being citramalate. At the same time there are data in the literature suggesting that the citramalate is essential for the acetate metabolism and may be involved in the oxidation of acetate to gyoxylate (Ivanovskii, 1997). Ivanovskii et al. (1997), present evidence for the existence in *Rs. rubrum* cells of a new anaplerotic cycle of acetate oxydation to glyoxylate via citramalate as an intermediate. The overall reaction proposed by them from citramalate cycle is acetate → glyoxylate + 4 [H]. The main function of this cycle is the oxidation of acetate to glyoxylate. However, the mechanism of its new pathway and its really implication in the acetate photometabolism has not been completely resolved. The same authors proposed a general scheme of the interaction of the citramalate cycle with another major pathway of carbon metabolism, the Calvin cycle.

For the stoichiometric model we have used the classical pathway of acetate metabolism.

Butyric acid ($C_4H_8O_2$)

As acetic acid, it can be assumed that butyric acid is assimilated to poly-β-hydroxybutyric acid (Doelle, 1975). It is supposed that butyric acid is converted to acetyl-CoA, as acetic acid via the β-oxydation of fatty acid pathway (T.N. 9.3). Poughon (1995) proposed the following global stoichiometric description:

$$\text{Butyric acid} + 2\ \text{CoA-SH} + NAD^+ + ATP + FAD^+ + H_2O$$
$$\downarrow$$
$$2\ \text{Acetyl-CoA} + FADH,H^+ + NADH,H^+ + PPi + AMP$$

Propionic acid ($C_3H_6O_2$)

The classical pathway for the assimilation and the catabolism of propionic acid is connected to a carboxylation to succinate (Olsen and Merrick, 1968). The metabolism of propionate is initiated by its activation to propionyl-CoA. The stoichiometric descriptions of this pathway is (Michael, 1999) :

$$\text{Propinic acid} + ATP + \text{CoA-SH} \rightarrow \text{Propionyl- CoA} + AMP + PPi$$

$$\text{Propionyl - CoA} + OAA \rightarrow \text{Pyruvate} + \text{Succinate} + \text{CoA-SH}$$

It must be noted that propionyl-CoA is an intermediate of alanine synthesis.

Greenberg (1960) presented evidence of another metabolic pathway for the metabolism of propionate. This time, the substrate of carboxylation reaction is propionyl-coenzyme A and the product of carboxylation reaction is methyl-malonyl- coenzyme A. This latter compound is then converted by the action of the separate methyl-malonyl isomerase into succinyl - CoA.

Global stoichiometric description is the following:

$$\text{Propionyl-CoA} + CO_2 + \text{ATP} \rightarrow \text{Succinyl-CoA} + \text{ADP} + \text{Pi}$$

For the stoichiometric model we are taking into account the first proposed reaction because, with this one we obtained more coherent flux values.

Caproic acid ($C_6H_{12}O_2$)

Caproate leads via the β-oxidation of fatty acid pathway to acetyl-CoA. The global stoichiometric descriptions of this pathway is (Gottschalk, 1986):

$$\text{Caproic acid} + \text{ATP} + 3\ \text{CoA-SH} + 2FAD^+ + 2NAD^+ + 2H_2O$$
$$\downarrow$$
$$3\ \text{Acetyl-CoA} + \text{AMP} + \text{PPi} + 2\ FADH,H^+ + 2NADH,H^+$$

Valeric acid ($C_5H_{10}O_2$)

Caproate leads via the β-oxidation of fatty acid pathway to acetyl-CoA and propionyl-CoA. The latter product itself is transformed via the reaction presented in the propionate pathway. The global stoichiometric descriptions of this pathway is (Gottschalk, 1986):

$$\text{Valeric acid} + \text{ATP} + 2\ \text{CoA-SH} + FAD^+ + NAD^+ + H_2O$$
$$\downarrow$$
$$\text{Propionyl-CoA} + \text{Acetyl-CoA} + \text{AMP} + \text{PPi} + FADH,H^+ + NADH,H^+$$

$$\text{Propionyl-CoA} + \text{OAA} \rightarrow \text{Pyruvate} + \text{Succinate} + \text{CoA-SH}$$

Branched VFA

The catabolism of branched fatty acids (i.e. isobutyric acid, isovalerate, isocoproate which have been considered in the studies of compartment II), is different from the non-branched ones.

In the case of isobutyric acid, two pathways can occur:

- the classical path leading to succinate:

$$\text{Isobutyric acid} + \text{ATP} + 2FAD^+ + 2NAD^+ + 2H_2O$$
$$\downarrow$$
$$\text{Succinic acid} + \text{AMP} + \text{PPi} + 2\ FADH,H^+ + 2NADH,H^+$$

- the second one leads to propionyl-CoA :

$$\text{Isobutyric acid} + \text{ATP} + FAD^+ + 2NAD^+ + 2H_2O + \text{CoA-SH}$$
$$\downarrow$$
$$\text{Propionyl-CoA} + CO_2 + \text{AMP} + \text{PPi} + FADH,H^+ + 2NADH,H^+$$

We used the last reaction in our stoichiometric modelization because with this one we obtained more coherent flux values.

In the case of isovaleric acid ($C_5H_{10}O_2$), the mechanism of β-oxydation is the one proposed by Green and Gibson (1960). It leads to acetoacetate and acetyl-CoA. The former is then transformed in two acetyl-CoA in the presence of succinyl-CoA.

The global stoichiometric descriptions of this pathway is:

$$\text{Isovaleric acid} + 2\text{ATP} + \text{CoA-SH} + \text{FAD}^+ + \text{CO}_2 + 2\text{H}_2\text{O}$$
$$\downarrow$$
$$\text{Acetoacetate} + \text{Acetyl-CoA} + \text{AMP} + \text{PPi} + \text{FADH,H}^+ + \text{ADP} + \text{Pi}$$

$$\text{Acetoacetate} + \text{Succinyl-CoA} + \text{CoA-SH} \rightarrow 2\ \text{Acetyl-CoA} + \text{Succinate}$$

In the case of isocaproic acid ($C_6H_{12}O_2$), the mechanism is similar but the final products are acetoacetate and propionyl-CoA.

The global stoichiometric descriptions of this pathway is:

$$\text{Isocaproic acid} + 2\text{ATP} + \text{CoA-SH} + \text{FAD}^+ + \text{CO}_2 + 2\text{H}_2\text{O}$$
$$\downarrow$$
$$\text{Acetoacetate} + \text{Propionyl-CoA} + \text{AMP} + \text{PPi} + \text{FADH,H}^+ + \text{ADP} + \text{Pi}$$

$$\text{Propionyl - CoA} + \text{OAA} \rightarrow \text{Pyruvate} + \text{Succinate} + \text{CoA-SH}$$

$$\text{Acetoacetate} + \text{Succinyl-CoA} + \text{CoA-SH} \rightarrow 2\ \text{Acetyl-CoA} + \text{Succinate}$$

2.3. Biomass elemental and biochemical compositions

The biomass composition can be expressed in two forms :

- an elemental composition (C, H, O, N composition) ;
- a general components composition (proteins, fats, DNA, ...).

The biochemical composition of the biomass, as well as the elemental composition changes with the growth conditions.

As a first assumption used in stoichiometric model it can be supposed that for the same growth conditions (C/N, light, dilution rate, ...) on different carbon sources , the biomass composition remains constant.

The biomass composition as a fonction of C/N and biomass concentration have been measured from experiments (Albiol, 1994).

From these experimental results, Albiol (1994) observed that the change in the biomass composition can be linked to the change of C/N ratio of the medium and of the light availability. Perhaps the most marked effect is in the biomass C/N ratio of the biomass. This is due to the fact that two important elements in the biomass composition are the proteins and

the carbon storage compounds. This two families of compounds differ, usually in their content of nitrogen.

Suhaimi (1987) found that the C/N ratio giving complete assimilation and recovery of ammonium in the biomass was 5, therefore this was the ratio selected for further studies and experiments in the MELiSSA project .

The phototrophic , purple, non-sulphur bacterium, *Rs. Rubrum* is known to produce intracellular energy and carbon storage products which can be generally described as being poly-β-hydroxybutyrate, PHB. Generally, environmental conditions and physiological abilities of *Rs. Rubrum* control the quantitative formation of this one. The PHB is accumulated in the cell when carbon and energy sources are in excess. Its accumulation represents an alternative for expending reducing power.

We are also assuming for the modeling network using different carbon substrates a biomass composition without PHB. We are considering the PHB as an exchangeable compound. Poughon (1995) considered in his modeling network that the PHB is fixed in the biochemical biomass composition. This approch allows to consider an active biomass and an intracelullar storage compound, PHB. The production of the last one can be a function of operating conditions. This approch is similar to that used by Cornet (1993) for *Spirulina platensis*.

We are assuming in this study an active biomass composition with C/N of 5 without PHB (table1).

	% mass	mol/Cmol	mass mol	mol/100gbio
Proteins	51.47	0.1153	108.0236	0.4765
Fats	14.24	0.0050	685.9914	0.0208
Carbohyddrates	12.77	0.0034	903.0000	0.0141
RNA	8.84	0.0077	277.8300	0.0318
DNA	3.93	0.0033	289.2192	0.0136

Table 1 :The biochemical composition of the active biomass.

Other aspects concerning the elemental composition, the biochemical composition and the biomass macrocomponents composition are presented by Poughon (1995) and Albiol (1994).

The elemental biomass composition is then deduced from the composition of macrocomponents of biomass and from the biochemical description of *Rs. Rubrum* (table 2).

C	H	O	N	S	P	Ash
1	1.6004	0.3620	0.2218	0.0036	0.0161	7.37%mass
45.9%	6.12%	22.17%	11.88%	0.44%	1.91%	7.37%

Table 2 : The elemental biomass composition.

2.4. Specific considerations on the *Rs. rubrum* metabolism

2.4.1 Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO)

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), is the key enzyme of the Calvin reductive pentose phosphate pathway. RubisCO catalyzes CO_2 fixation in most photosynthetic and chemolitoautotrophic organisms and is usually the predominant CO_2 fixation enzyme. Tabita et al. (1974) in their study found RubisCO in *Rs.rubrum* cells.

Rs.rubrum contains high levels of RubisCO in addition to the reductive TCA cycle enzymes, suggesting that the reductive TCA cycle might serve an auxiliary role in CO_2 fixation in this organism (Tabita, 1988).

Tabita et al. (1991) suggested that RubisCO synthesized in photoheterotrophycally grown cells may be employed to maintain a proper intracellular redox balance. They are also suggested that the RubisCO is functional in *Rs.rubrum*.

According to these informations we have considered the Calvin cycle is functional in photoheterotrophic growth. In previous studies (Poughon, 1995), it was assumed that the Calvin cycle is absent in heterotrophic conditions.

2.4.2 The nicotinamide nucleotide transhydrogenase

Generally the microorganisms have another possibility at their disposal to produce NADPH, via a transhydrogenase. The transhydrogenase is membrane-bound and transhydrogenation is protonmotive force driven ; it thus can only proceed at an energized membrane. The enzyme catalyses the following reversible reaction :

$NADH + NADP^+ + x\ H^+extra \leftrightarrow NAD^+ + NADPH + x\ H^+intra$ (Cunningham, 1992 and Bizouarn, 1993).

x is the number of protons translocated across the membrane for each e^- transferred between NAD(H) and NADP(H). It was concluded for *Rs. rubrum* that *x* was 1 (Bizouarn et al., 1993).

The membrane-bound tranhydrogenase is present in *Rs.rubrum*. Therefore the transhydrogenase reaction used in the metabolic network is the following :

$NADH,H + NADP^+ + 2\ H^+extra \leftrightarrow NAD^+ + NADPH,H + 2\ H^+intra$

2.4.3 The anaplerotic pathways used in the metabolic network

It is already known that the TCA cycle is fed by pyruvate dehydrogenase or by anaplerotic pathways. The most of anaplerotic pathways are feeding the Krebs cycle at the oxaloacetate level. Moreover, oxaloacetate serves as starting material for all the carbohydrates required for polymer synthesis.

The main anaplerotic pathways used in our metabolic network are presented below.

Pyruvate carboxylase is clearly the major enzyme involved in the generation of the tricarboxylic acid cycle intermediates from pyruvate. This enzyme catalyses the convertion of pyruvate into oxaloacetate.

(1) : Pyruvate + CO_2 + ATP + H_2O $\xrightarrow{pyruvatecarboxylase}$ OAA + ADP +P_i

Another anaplerotic reaction is catalysed by phosphoenolpyruvate carboxykinase (PEP carboxykinase).

(2) : OAA +ATP $\xleftarrow{PEP\ carboxykinase}$ PEP + ADP + CO_2

Willison (1988) in his study shown that PEP carboxykinase is present in *Rb. Capsulatus*.

We also considered in our metabolic network the anaplerotic reaction catalyzed by PEP carboxylase, which is involved in the transformation of PEP into oxaloacetate.

(3) : PEP + H_2O + CO_2 $\xrightarrow{PEP\ carboxylase}$ OAA + P_i

Poughon (1995) for the modeling of the metabolic network used only the last anaplerotic reaction.

From the biomass composition and from the metabolic pathways we have build and computed the flux values.

3. STOICHIOMETRIES : BUILDING AND RESOLUTION OF THE METABOLIC MATRIX

3.1. Basic Principles and the Importance of Metabolic Modeling

In this work, the mass balance technique has been applied to *Rs. rubrum*. This technique does not require information about enzyme kinetics.

Accurate analysis of the overall bacterial metabolism (anabolism, catabolism, and energy conversion steps) together with cell biomass composition is a prerequisite for establishing the set of mass conservation balances for each intermediate metabolite and for other components involved in the metabolism. The fluxes of each individual reactions are the unknowns. Generally, only a relatively small amount of metabolic fluxes need to be measured to create computer model of the organism's metabolism and estimate unknown fluxes.

All of the calculated fluxes are required to completely understand any given physiological state of a micro-organism.

3.2. Using Modeling to build the Metabolic matrix and to Determine Metabolic Fluxes

We used the mass balance technique in order to calculate the metabolic fluxes. This method takes into account the reaction stoichiometry obtained by an analysis of the internal behavior of the micro-organism.

The first research done on modeling metabolic networks used only about 10 reactions. Some of these original 10 equations were later broken down into more details, and 40 equations were used to specify the metabolic network of *Corynebacterium glutamicum* (Vallino and Stephanopoulos, 1992). Currently, about 100 equations are being used with

Corynebacterium melassecola (Pons et al., 1996).

The metabolic reaction can be depicted in term of matrices and vectors algebra. The bioreaction network equation (Vallino and Stephanopoulos, 1990) represents a metabolite balance and it is constructed by determining the time rate of the production and consumption of each metabolite in the network ($\mathbf{r_{metabolite}}$) as a function of all the unknown flows ($\mathbf{J_i}$ for the i th reaction in the network). The accumulation rate of a metabolite in a metabolic network is given by the summation of all reactions producing that metabolite minus the reactions consuming that metabolite.

$$r_j(t) = \sum_{m} a_{jm} J_m(t) - \sum_{k} a_{jk} J_k(t) \qquad (1)$$

where a is the stoichiometric coefficient, $J_m(t)$ is the flux through reaction m and the $r_j(t)$ is the accumulation rate of metabolite j.

The set of equations formed from such balances for each metabolite in the network is represented in matrix notation by :

$$\mathbf{M\ J(t) = r(t)} \qquad (2)$$

M is a ($c \times r$) matrix of the stoichiometric coefficients (c metabolites and r metabolic reactions) of all the reactions involved in the metabolism. Each column in the matrix represents a metabolic reaction.

The c metabolic constituents are divided into two categories :

- the exchangeable compounds, which are exchanged with the growth medium. On a macroscopic point of view these compounds are the products and the substrates involved during the cellular growth phase. Their accumulation rates are linked to the stoichiometric yields of the growth.

- the nonexchangeable compounds, which are only involved inside the cell. For these one the accumulation rate is equal to zero.

J(t) is the r dimensional flux vector and **r(t)** the c-dimensional vector of the accumulation rates of the metabolite.

By using the complete matrix **M** we must obtain a square matrix in order to solve equation (2).

In order to solve the stoichiometry building, the metabolic matrix **M** can be represented by two sub-matrices $\mathbf{M_{AC}}$ and $\mathbf{M_{NC}}$:

$$\mathbf{M} = \begin{bmatrix} \mathbf{M_{AC}} \\ \hline \mathbf{M_{NC}} \end{bmatrix} \qquad (3)$$

Where $\mathbf{M_{AC}}$ is the stoichiometric matrix associated to the nonexchangeable compounds for which the accumulation rate is known and set to zero ; $\mathbf{M_{NC}}$ is the stoichiometric matrix associated to :

- the compounds which are implicitly dependent to some other compounds through a chemical or biochemical balance (as NAD^+ and $NADPH^+$) ;

- the compounds for which the accumulation rate, $\mathbf{r_i(t)}$, is not known.

The principals steps which are followed for building an inversible matrix $\mathbf{M_{AC}}$ are :

❑ At first, the data available for the resolution of the metabolic network are determined. These matrix, $\mathbf{M_{AC}}$ and $\mathbf{M_{NC}}$ can be built from the knowledge of matrix **M**. The $\mathbf{M_{AC}}$ deduced from **M** and $\mathbf{M_{NC}}$ is generally not a square matrix.

❑ In a second step it is necessary to determine the number of degree of freedom of the system. Analysis of the maximal rank of the matrix involves the determination of the colinearities among rows and columns. The colinearities between the rows are due to the non independent reactions present in the matrix and the second one to the non independent balances.

❑ The third step consist in the determination and the elimination of the colinearities between the intermediates and between the columns. These colinearities create a number of cycles in the matrix. Each cycle can contain from two to ten or more reactions.

This is an essential step in the system resolution.

❑ In a fourth step, it was necessary to made assumptions and to give supplementary information in order to reduce the degrees of freedom to zero and to obtain an inversible matrix, $\mathbf{M'_{AC}}$.

This allows to calculate the metabolic flux vector **J(t)**. The flux vector **J(t)** can be determined only if $\mathbf{M'_{AC}}$ is an inversible matrix and if $\mathbf{r_{AC}(t)}$ is a known accumulation rate vector :

$$\mathbf{J(t) = M'_{AC}{}^{-1}\, r_{AC}(t)} \qquad (4)$$

In order to establish a stoichiometry, the accumulation rate vector for the exchangeable compounds, $\mathbf{r_{NC}(t)}$, must be calculated. In this aim, the following equation must be solved :

$$\mathbf{r_{NC}(t) = M_{NC}\, J(t)} \qquad (5)$$

Therefore the building of the stoichiometry for the growth and the calculation of the metabolic flux from a set of metabolic equations can then be reduced to the determination of an inversible matrix $\mathbf{M'_{AC}}$ and of its associated accumulation rate vector $\mathbf{r_{AC}(t)}$

Finally, the equation (5) can be solved and a stoichiometry can be established.

4. THE METABOLIC MATRIX AND THE STOICHIOMETRIES FOR *Rs. rubrum*

In this work the mass balance technique was applied to the metabolic network of *Rs. rubrum* under **photoheterotrophic anaerobic conditions in the light**.

The biochemical composition and the macrocomponents biochemical composition are supposed to be known.

A brief review of the main metabolic pathways which are taken into account to build the metabolic matrix are presented in the second part of this study. The *Rs. rubrum* metabolism has been detailed in the T.N. 23.3 by Poughon (1995).

4.1. Building the metabolic matrix

The first step in the resolution of the problem is to build the metabolic matrix.
The metabolic reactions involved in the description of the growth of *Rs.rubrum* can be classified in several items :

General carbon metabolism

TCA cycle
Embden-Meyerhof pathway
Pentose phosphate pathway
Calvin Benson cycle

Anabolism

Amino acids biosynthesis pathways
Lipids biosynthesis pathway
Carbohydrates biosynthesis pathway
DNA and RNA biosynthesis
Biomass synthesis

Reserve metabolism

Reserve material synthesis as glycogen and poly-β-hydroxybutyric acid (PHB).

Energetic metabolism

The cyclic and the non cyclic electron transport
ATP synthesis

Maintenance can be represented as an ATP hydrolysis.

Carbon substrates

In the metabolic matrix are also included the reactions concerning the entrance of the carbon organic compounds used in this study.

A complete reaction list for the metabolic matrix discussed below is included in the Annexe.
The matrix M built as described above from these reactions is a 125 x 117 matrix.
The total number of metabolic reactions in the network of *Rs.rubrum* is equal to 117. An analysis of the reaction list for this metabolic network indicates that there are a total of 125 metabolites (108 intermediates and 17 exchangeables).

For this study we have considered 17 exchangeables compounds :

- CO_2 as substrate or product
- NH_3 as N-source
- H_2SO_4 as S-source

- H_3PO_4 as P-source
- Biomass as the result of the growth
- H_2O
- acetate, propionate, butyrate, valerate, caproate, isobutyrate, isovalerate, isocaproate as carbon substrates
- PHB ....

This separation (exchangeable, nonexchangeable) is important for calculating the number degrees of freedom.

The analysis of the colinearities among the columns of the matrix shows that 6 cycles are detected.

It assumes that it is only possible to measure some fluxes for the exchangeables.

4.2. Resolution of the metabolic matrix

In our case the system presents17 degrees of freedom.

This indicates that 17 supplementary informations must be furnished to the system in order to completely specify it and to permit resolution. The number of degree of freedom must be reduced to zero before the matrix system can be solved. It will be done in the following way.

Suppressing the **6** detected cycles with 6 supplementary conditions

Each of the cycles can be suppressed by carefully choosing a reaction within the cycle and setting its rate to zero. The choice is made based on the conditions the cell is operating in. The anaerobic, photoheterotrophic conditions were used as the basis for the assumptions as seen below.

Fixing **11** exchangeables - taking into account the operating conditions (photoheterotrophic metabolism under anaerobiotic conditions in the light).

a) Suppressing the cycles

Preliminary tests have been done. For each simulation test we analyzed the fluxes values in order to not violated the thermodynamic constraints (i.e., no negative flows in irreversible reactions). This almost trivial confirmation is important for unacceptable flux distributions, indicate errors or inconsistencies in the metabolic network.

The metabolic fluxes maps obtained directed our choice of the following six conditions. The first five assumptions which we have considered are common for all the carbon substrates.

The maintenance assumption $\rightarrow J_m = 0$

The PEP carboxylase which catalyses the transformation of PEP into oxaloacetate is supposed to be inactive. $\rightarrow J_{21} = 0$

The pyruvate carboxylase which catalyses the transformation of pyruvate into oxaloacetate is supposed to be inactive. $\rightarrow J_{21'} = 0$

The shunt of glyoxylate is supposed to be inactive.$\rightarrow J_{25} = 0$

Phosphogluconate deshydrogenase $\rightarrow J_{17} = 0$

The last one depends of the level of entrance of VFA in Krebs cycle. In our metabolic network we have two different levels of entrance depending on the carbon substrate in the metabolism:

- acetyl-CoA level (for carbon substrates as acetate, butyrate, caproate, isovalerate)
- acetyl-CoA and propyonyl-CoA levels (for carbon substrates as propionate, isobutyrate, valerate, isocaproate).

We have condidered that $J_9 = 0$ for the carbon substrates which lead the Krebs cycle to acetyl-CoA level. For the other ones, which lead to acetyl-CoA and propionyl-CoA levels we supposed that $J_8 = 0$.

The six detected cycles are removed by using these 6 supplementary conditions.

b) Fixing the exchangeables

We have fixed 11 exchangeables by taking into account the operating conditions. The following exchangeables (oxygen, hydrogen, acetate, propionate, butyrate, caproate, valerate, isobutyrate, isovalerate, isocaproate and the PHB) are fixed.

The oxygen consumption reactions can be set to zero if the cell is operating anaerobically. The hydrogen and the PHB are also set to 0.

The organic carbon substrate uptake rate is the only known accumulation rate; it is set to -1 (consumption) as the reference for the calculation of the other rates. The accumulation rate vector, $\mathbf{r_{AC}}$ is composed of zero values excepted the value of the accumulation of the organic carbon source, set to -1.

5. MAIN RESULTS

5.1. The stoichiometries for the different carbon substrates

The metabolic matrix for the photoheterotrophic growth of *Rs. rubrum* can be resolved considering the hypotheses presented above. We obtained therefore by computation the metabolic fluxes for the carbon substrates considered and the accumulation rates vectors of the non fixed exchangeable compounds (biomasse, CO_2, NH_3, H_2O, P_i, H_2SO_4..).

Several other metabolic information can also be calculated such as :

- the in vivo ratio ATP/ reducing power : quantity of produced ATP/ quantity of consumed cofactors (NADH, NAD, NADP)
- the necessary quanta : J_{nrj4} x2/ $J_{biomasse}$
- the number of the propagation cycle : J_{nrj4}/J_{14}

The following stoichiometries are then obtained for the different carbon substrates using a biomass with a C/N of 5.

Acetic acid

$$C_2H_4O_2 + 0.4115\ NH_3 + 0.0299\ H_3PO_4 + 0.0066\ H_2SO_4$$
$$\downarrow$$
$$1.8550\ CH_{1.6004}O_{0.3621}N_{0.2218}S_{0.0036}P_{0.0161} + 0.1450\ CO_2 + 1.1843\ H_2O$$

The metabolic fluxes indicated that :
- a sum of 5.1 quanta/C-mol biomasse are involved in the reaction
- a sum of 9.47 quanta are involved in the reaction
- the propagation cycle number is 6.34
- the ratio $\frac{ATP}{reduced\ power}$ calculated for this biosynthesis is equal to 1.78

Propionic acid

$$C_3H_6O_2 + 0.7201\ NH_3 + 0.0523\ H_3PO_4 + 0.0116\ H_2SO_4 + 0.2462\ CO_2$$
$$\downarrow$$
$$3.2462\ CH_{1.6004}O_{0.3621}N_{0.2218}S_{0.0036}P_{0.0161} + 1.5726\ H_2O$$

The metabolic fluxes indicated that :
- a sum of 4.67 quanta/C-mol biomasse are involved in the reaction
- a sum of 15.15 quanta are involved in the reaction
- the propagation cycle number is 5.58

- the ratio $\frac{ATP}{reduced\ power}$ calculated for this biosynthesis is equal to 1.40

Butyric acid

$$C_4H_8O_2 + 1.0287\ NH_3 + 0.0748\ H_3PO_4 + 0.0165\ H_2SO_4 + 0.6374\ CO_2 \downarrow 4.6374\ CH_{1.6004}O_{0.3621}N_{0.2218}S_{0.0036}P_{0.0161} + 1.9609\ H_2O$$

The metabolic fluxes indicated that :
- a sum of 4.62 quanta/C-mol biomasse are involved in the reaction
- a sum of 21.43 quanta are involved in the reaction
- the propagation cycle number is 7.66
- the ratio $\frac{ATP}{reduced\ power}$ calculated for this biosynthesis is equal to 1.41

Caproic acid

$$C_6H_{12}O_2 + 1.6459\ NH_3 + 0.1196\ H_3PO_4 + 0.0264\ H_2SO_4 + 1.4198\ CO_2 \downarrow 7.4198\ CH_{1.6004}O_{0.3621}N_{0.2218}S_{0.0036}P_{0.0161} + 2.7374\ H_2O$$

The metabolic fluxes indicated that :
- a sum of 4.5 quanta/C-mol biomasse are involved in the reaction
- a sum of 33.39 quanta are involved in the reaction
- the propagation cycle number is 8.2
- the ratio $\frac{ATP}{reduced\ power}$ calculated for this biosynthesis is equal to 1.31

Valeric acid

$$C_5H_{10}O_2 + 1.3373\ NH_3 + 0.0972\ H_3PO_4 + 0.0215\ H_2SO_4 + 1.0286\ CO_2 \downarrow 6.0286\ CH_{1.6004}O_{0.3621}N_{0.2218}S_{0.0036}P_{0.0161} + 2.3491\ H_2O$$

The metabolic fluxes indicated that :
- a sum of 4.28 quanta/C-mol biomasse are involved in the reaction
- a sum of 25.78 quanta are involved in the reaction
- the propagation cycle number is 7.14
- the ratio $\frac{ATP}{reduced\ power}$ calculated for this biosynthesis is equal to 1.25

Isobutyric acid

$$C_4H_8O_2 + 1.0287\ NH_3 + 0.0748\ H_3PO_4 + 0.0165\ H_2SO_4 + 0.6374\ CO_2 \downarrow 4.6374\ CH_{1.6004}O_{0.3621}N_{0.2218}S_{0.0036}P_{0.0161} + 1.9609\ H_2O$$

The metabolic fluxes indicated that :
- a sum of 4.68 quanta/C-mol biomasse are involved in the reaction
-a sum of 21.71 quanta are involved in the reaction
- the propagation cycle number is 10.04
- the ratio $\frac{ATP}{reduced\ power}$ calculated for this biosynthesis is equal to 1.24

Isovaleric acid

$$C_5H_{10}O_2 + 1.3373\ NH_3 + 0.0972\ H_3PO_4 + 0.0215\ H_2SO_4 + 1.0286\ CO_2 \downarrow 6.0286\ CH_{1.6004}O_{0.3621}N_{0.2218}S_{0.0036}P_{0.0161} + 2.3491\ H_2O$$

The metabolic fluxes indicated that :
- a sum of 4.84 quanta/C-mol biomasse are involved in the reaction
- a sum of 29.16 quanta are involved in the reaction
- the propagation cycle number is 6.58
- the ratio $\frac{ATP}{reduced\ power}$ calculated for this biosynthesis is equal to 1.46

Isocaproic acid

$$C_6H_{12}O_2 + 1.6459\ NH_3 + 0.1196\ H_3PO_4 + 0.0264\ H_2SO_4 + 1.4198\ CO_2 \downarrow 7.4198\ CH_{1.6004}O_{0.3621}N_{0.2218}S_{0.0036}P_{0.0161} + 2.7374\ H_2O$$

The metabolic fluxes indicated that :
- a sum of 4.43 quanta/C-mol biomasse are involved in the reaction
-a sum of 32.84 quanta are involved in the reaction
- the propagation cycle number is 6.49
- the ratio $\frac{ATP}{reduced\ power}$ calculated for this biosynthesis is equal to 1.33

5.2. Global yields

We also calculated the mass yields of biomass synthesis for all of the carbon substrates ($Y_{X/S}$).

The calculated values are reported in table 3. We compared them to the experimental ones of Albiol et al.(1998) at 420 [350-950 nm] W/m^2 incident light on different VFA (acetate, propionate, butyrate, isobutyrate and isovalerate).

VFA	$Y_{X/S}$ (calculated)	$Y_{X/S}$ (experimental)
acetic acid	0.71	0.77 - 0.8
propionic acid	1.03	1.1
butyric acid	1.22	1.5
valeric acid	1.36	-
caproic acid	1.48	-
isobutyric acid	1.22	2.7 - 0.7
isovaleric acid	1.36	2.1
isocaproic acid	1.48	-

Table 3 : The mass yields of biomass synthesis (gX/gS).

6. DISCUSSION AND CONCLUSIONS

In this work we updated the literature review about the main metabolic aspects of *Rs.rubrum* under anaerobic photoheterotrophic conditions. It allowed us to modify a few hypothesis used by Poughon (1995) in order to solve the matrix system, to obtain coherent intracellular fluxes and then to have a better stoichiometric model.

In his work, Poughon (1995) fixed to three the number of propagation cycles and for the maintenance he considered the following relation : $J_m=3.25\ J_{ATP}$. We preferred to set the J_m value to zero in order to determine the minimun number of propagation cycles. According to Poughon (1995) and Gottschalk (1986) we considered that the glyoxylate shunt is inactive in *Rs. rubrum.*

Since we have taken into account different other carbon substrates we obtained a 125×117 metabolic matrix. To solve it, we needed to make more hypotheses (the PEP carboxylase, the pyruvate carboxylase and phosphogluconate deshydrogenase assumptions) than Poughon (1995).

Calculating the metabolic fluxes is useful to identify the direction of each reaction involved in the bacterial network. We checked very strictly that the directions correspond with the bacteria metabolism.

One main advantage of the metabolic network is that it ensures mass balance conservation , both through the establishment of the stoichiometric reactions describing cell anabolism and catabolism steps. The calculated fluxes make possible to compare calculated yield values with the experimental ones.

As presented in table 3, we can notice that for acetate and propionate the calculated values of the mass yields at 420 W/m^2 incident light are very close to the experimental ones of Albiol et al. (1998) (confidence interval 10%). Experimental and calculated values are quite different for isobutyrate and isovalerate for which photoinhibition seems to appear. In order to validate the proposed stoichiometric model, it is necessary to realize repeated tests for the different carbon substrates which is the purpose of a further T.N.

The proposed metabolic network and the metabolic fluxes maps of the preliminary tests in *Rs.rubrum* under photoheterotrophic anaerobic conditions indicates that there is carbon dioxide fixation via Calvin Benson cycle. This condition is a necessary one in order to not violate the thermodynamic constraints of calculated fluxes. From our point of view the concomitant functionnement of the Calvin-Benson cycle with acetate assimilation is an important result ; it is in accordance with Tabita et al. (1991) who detects the RubisCO synthesis in heterotrophic conditions. This consideration is different from the hypothesis made by Poughon (1995) in his previous work, who assumed that the Calvin cycle was inoperative in *Rs. rubrum* in heterotrophic conditions.

The proposed stoichiometries are only valid in the working illuminated volume of the photobioreactor. This study is also a prerequisite step in modeling metabolic pathways and stoichiometries during the short residence time of the cells in the dark efficient zone of the photobioreactor (Cornet et al., 1999).

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