A stoichiometric reaction scheme for *Saccharothrix algeriensis* growth and thiolutin production

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Keywords:
Factorial analysis
Stoichiometry
Reaction scheme
Secondary metabolism
Actinomycetes
Dithiolopyrrolone antibiotics

A B S T R A C T

A new bacterial species, *Saccharothrix algeriensis* NRRL B-24137, was isolated in 1992 in the Sahara desert. This filamentous bacterium is able to produce dithiolopyrrolones, molecules presenting antibacterial, antifungal, and anticancer properties. In this study, a "reaction engineering" approach was adopted to gain more knowledge on the growth of *Sa. algeriensis* and its dithiolopyrrolone production on a semi-synthetic liquid medium. The objective is to establish a reaction scheme of the bacterium metabolism from extracellular experimental information, relatively easy to obtain. The approach enabled us to show that *Sa. algeriensis* could grow using several substrates that were sequentially consumed and that substrate limitation may induce a secondary metabolism in antibiotic production. From these qualitative data, a general reaction scheme was extracted consisting of four reactions: growth via amino acids, glucose consumption for maintenance, growth using glucose, and thiolutin production. The stoichiometric coefficients and the reaction extends were identified using a factorial analysis based on the bilinear structure of the component mass balances in a batch reactor. The analysis of the reaction stoichiometry enabled us to draw some conclusions concerning the substrate consumption pathway.

1. Introduction

Almost a quarter of human deaths in the world result from infectious diseases. They are increasingly caused by bacteria that have developed a (or several) resistance(s) to antibiotics [1]. This phenomenon is concerning for humanity and demonstrates the importance of constant renewal of a pool of bioactive molecules. Thus, the production of new bioactive molecules from currently available strains that are pathogenic and antibiotic resistant is the subject of broader interdisciplinary research projects. In this context, a new bacterial species, *Saccharothrix algeriensis* NRRL B-24137 [2], was isolated in 1992 from the Sahara desert (Algeria). This filamentous bacterium belongs to the actinomycete family. It produces molecules belonging to the dithiolopyrrolones family, which possess antibacterial, antifungal, and interesting anticancer properties [3–5].

A previous work [6,7] highlighted the influence of the culture medium composition on the production of dithiolopyrrolones and of new molecules never before described in the scientific literature [8]. *Sa. algeriensis* should be able to produce different antibiotics by precursor directed biosynthesis. Hence, the findings from the *Sa. algeriensis* study present an undeniable fundamental interest, in addition to an obvious useful interest for pharmaceutical industry. As this bacterium was recently discovered and characterized, no comprehensive knowledge on its metabolism is available. Several studies have been undertaken in parallel to better understand the dithiolopyrrolones production pathway by *Sa. algeriensis*. In the study presented herein, a macroscopic approach was chosen to investigate the bacterium metabolism. "Omics" data were not used; instead the "reaction engineering" approach was used. This enabled us to analyze the metabolism from a qualitative and quantitative point of view, which allowed us to gain more knowledge about the mechanism of dithiolopyrrolones production.

Two broad approaches are available to model microbial bioproduction:

- Black box models, which do not require any knowledge of the microorganism. Only inputs (substrates) and outputs (products) of the system are taken into account. These models cannot be used to draw conclusions on the metabolic pathways and are purely descriptive. It remains purely descriptive.

  However efforts have been made in this context to systematically derive and compare macroscopic reaction schemes on the basis of extracellular component concentration measurements [9,10].

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doi:10.1016/j.procbio.2010.07.010

ıctured models, like the "Metabolic Flow Analysis" model, ch favor a more systemic approach to studying the microorism. They are generally complex and require a good wledge of the metabolic network of the bacteria. The ntification of hundreds of flux from a limited number of ısurements compels the assumption that all intermediate ıpounds are in a quasi-stationary state. This drawback may be ided if intracellular compounds can be measured. Note that knowledge of such metabolic pathways allows also, thanks ppropriate model reduction, to derive and legitimate macroɔic reaction schemes [11,12].

In this work, we adopted an intermediate approach based on an analysis of experimental data. Thus, a global reaction scheme of the bacterium metabolism can be established. The advantage of this type of model is that, while remaining at the extracellular level and with experimental information relatively easy to obtain, we can generate information on the cell's metabolism. The proposed model, called a stoichiokinetic model, consists of the representation of bacterial activity giving some reactions with invariant stoichiometry over time. This "reaction engineering" approach is widely used in chemical engineering to determine the occurring reactions in a complex reaction scheme. It is based on the bilinear structure of the component mass balances in a batch reactor. The first adaptation of the factorial analysis to the treatment of batch reactor data was performed by Hamer [13]. This method has undergone several adaptations since then [14–18]. This paper presents the results of this approach applied to the growth and production of thiolutin (the main dithiolopyrrolone produced) by *Sa. algeriensis* on a semi-synthetic medium. The consistency of the experimental data is first verified to further perform the qualitative and quantitative analysis using reliable data. This approach is presented hereafter for a culture performed under reference control conditions. Finally, the influence of the medium composition is analyzed.

2. Materials and methods

2.1. Microorganisms and conditions for the reference culture

Sa. algeriensis NRRL B-24137 was used in this study. Microbial spores were obtained from solid cultures on Petri dishes filled with conservation medium. They were maintained in 25% glycerol at −20 °C. A 100 ml volume of semi-synthetic medium was inoculated using 3.5 ml of this suspension and by incubating on a rotary shaker (New Brunswick Scientific, Edison, NJ, USA) at 250 rotations per minute (rpm) at 30 °C for 52 h. Five milliliters of the resulting preculture was used to inoculate each culture. Before inoculation, the pH was adjusted to 7 by the addition of 1 mol L^{-1} NaOH. Cultures were grown in NBS reactors containing 2 L of medium. The cultures lasted for at least 1 week. We maintained the pH at 7 ± 0.035 by the automatic addition of 1 mol L^{-1} NaOH or 1 mol L^{-1} HCl solutions. An aeration rate of one vessel volume per minute (0.5 vvm) was employed. The agitation rate was controlled to keep the dissolved oxygen level above 30% saturation with a starting rate of 150 rpm. The pH and dissolved oxygen levels were monitored using Ingold specific electrodes. The temperature was regulated at 30 °C. The percentages of O_2 and CO_2 exhaust gas were determined using a gas analyzer (Servomex 4100, paramagnetic transductor for O_2 and infrared transductor for CO_2).

2.2. Media composition for the reference culture

The conservation media had the following composition (per liter of distilled water): 10 g malt extract, 4 g yeast extract, 4 g glucose and 18 g agarose. Semi-synthetic medium used as the growth and production medium for the reference culture, containing the following (per liter of distilled water): 15 g glucose, 2 g yeast extract, 2 g NaCl, 2 g $(NH_4)_2SO_4{\cdot}2H_2O$, 0.5 g KH_2PO_4, 1 g K_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 1 g $CaCl_2{\cdot}2H_2O$, 2 g MOPS and 20 mmol uracil. The concentrations of chemical elements and free amino acids in the yeast extract used during this study (288620 Bacto™ Yeast Extract, Technical) are presented in Table 1. This reference culture was assayed in triplicate to verify reproducibility. To better control the medium composition, attempts were made to substitute yeast extract without success [19].

2.3. Analytical procedures

For the estimation of dry cell weight (DCW), 3 ml samples of homogenized culture broth were centrifuged at 16,000 *g* for 10 min in preweighed Eppendorf tubes.

Table 1
Concentrations of free amino acids and other elements of the yeast extract (288620 Bacto™ Yeast Extract, Technical).

Amino acids	[Amino acids] (μ mol) per g of YE	Atomic element	[Atomic element] (μg)
Per g of YE			
ASP	142	P	9751
THR	144	S	36877
SER	200	W	222
GLU	119	Zn	286
GLN	4	Co	5
PRO	42	Pb	4
CYS	30	B	41
GLY	204	Mn	3
ALA	415	Fe	117
VAL	236	Mg	802
MET	51	Al	276
ISO	164	V	66
LEU	311	Be	2
TYR	32	Ca	935
PHE	140	Zr	1
LYS	156	Ba	1
HIS	47	Na	1526
TRP	15	K	73,542
		NH_4^+	20,000
ARG	109		
ASN	108		

The pellet was washed first with 0.25 mol L^{-1} NaOH solution, then with 0.35 mol L^{-1} HCl solution and finally with distilled water. The supernatant was kept for other analyses. Eppendorf tubes containing the pellet were dried at 105 °C for 48 h and were then cooled for 30 min in a dessicator and weighed [20]. The relative error of the measurement is 5%.

The analysis of dithiolopyrrolone antibiotics was carried out by nonpolar chromatography (HPLC, Bio-Tek Instruments, column C_{18} ODB, Zorbac SB, Uptisphere, 5 μm, 150 mm × 4.6 mm). The supernatant from the DCW separation was filtered at 0.2 μm and used to measure metabolites. Additional information on this method is available in Bouras [21].

Glucose was quantified using a biochemical analyzer with glucose oxidase enzymes fixed on a membrane (YSI2700 select). Glucose was determined using an amperometric quantification following enzymatic oxidation. The amperometer data are linear for glucose concentrations ranging from 0 to 25 g L^{-1}. The relative error of the measurement is 3%.

Ammonium ions and α-amino nitrogen were quantified using specific enzymatic methods (Diagnostics Ammonia kit from Boehringer–Mannheim, using glutamate dehydrogenase and Microdon Kit using glutamate oxidase) and an automatic multiparametric analyzer (Mascott Lisabio). The signal is linear for concentrations in α-amino nitrogen and ammonium ions ranging from 0 to 500 mg L^{-1}. The relative error of the measurement is 5%.

Amino acids were quantified via an AminoQuant HP1900 (nonpolar C_{18} column) and the associated protocol [22]. Prior to analysis, proteins contained in the supernatant were precipitated overnight at 4 °C using 75% (v/v) methanol. Amino acids were automatically determined with orthophthal-aldehyde-9-fluorenylmethyloroformate (OPA-FMOC).

The elemental composition of the biomass was measured using the CHNS/O AE1110 and Flash-EA1112 analyzer (Thermofinnigan). The principle was to analyze a solid biomass sample via chromatography of the gases produced by the combustion with oxygen at 1800 °C. The analyzer provides the composition of C, H, O, N and S.

2.4. Data reconciliation and validation

In general, experimental data acquired during batch fermentations can be distorted by several error sources: sampling, dilutions, and methods of analysis, among others. Further, the use of raw experimental measures without testing their quality can be a source of error for modeling. To avoid this problem, methods of data reconciliation were developed, largely in the 1990s [16]. The principle of reconciliation requires that we adjust the experimental data to fit an exact mathematical model. It can be then written as a mathematical optimization problem:

$$\begin{cases} \underset{\hat{X}}{\text{Min}} \left[\frac{1}{2} (\hat{X} - X_m)^T V^{-1} (\hat{X} - X_m) \right] \\ M\hat{X} = 0 \end{cases} \quad (1)$$

The solution to this problem lies in researching reconciled data $\hat{X}$ close to X_m and satisfying the constraints of the exact model ($M\hat{X} = 0$). This approach assumes that the errors are random and that they follow a centered, normal distribution law with variance V known.

The exact model chosen here, which is valid regardless of the reaction mechanism involved, is the conservation of atomic elements. For batch fermentation, it is possible to calculate the vector D_j for the cumulative molar amount of a component i in the sample j and to establish the matrix E for the atomic composition of the system components. In a batch process, the balance is dynamic, and D_j changes in time while E remains constant. The conservation of atomic elements between 2 samples is written as follows:

$E\Delta D_j = 0$ with $\Delta D_j = D_{j+1} - D_j$ or on a matrix form: $MD = 0$ with:

$$M = \begin{bmatrix} -E & E & 0 & 0 \\ 0 & -E & \ddots & \\ \vdots & 0 & \ddots & E \\ 0 & \dots & 0 & -E \end{bmatrix} \quad (2)$$

This method is effective if all of the components involved in the atomic balance are measured. For the measurements performed in our experiments, we assumed that all of the constituents containing C and N elements were measured. In addition, we could achieve H and O balances because water was not measured. Practically, to obtain cumulative molar amounts that verify C and N balances from experimental data, computer programs developed in our laboratory using classic linear optimization methods for the problem of minimization were used. We used these methods in our study for data reconciliation.

It is also possible to detect samples where variation from the exact mathematical model is greater than the measurement error. This data consistency procedure was performed by cross-validation using 3 statistical tests: Generalized Likelihood Ratio (GLR) [23], Iterative Measurement Test (ITM) [24] and Residual Criterion (RC) [25]. A highly erroneous experimental sample detected by one of the three tests is generally removed from the database. It is sometimes corrected with the estimation bias proposed by the GLR test.

As a result of this process, we have a reliable experimental database written in a matrix form of reconciled and validated cumulative molar amounts (D_{rec}) that we obtained fully evaluating C and N balances.

2.5. Stoichiometric coefficients and reaction extends determination

It is possible to determine a reaction scheme and corresponding reaction extends from the reconciled cumulative molar amounts by exploiting the bilinear structure of the mass balances component. In the case of a batch reactor in which n_r reactions are performed and n_c components are measured for n_{obs} samples, the data matrix D_{rec}, consisting of n_{obs} rows containing the cumulative molar amount of the n_c measured species, can be written as the product of two matrixes R and S, respectively, the matrix of the reaction extend (n_{obs},n_r) and the matrix of the stoichiometric coefficients of the n_r reactions (n_r,n_c). When a qualitative analysis of the raw data enables to assume the active reactions, a direct determination of R and S by solving the constrained optimization problem based on reconciled data can be performed [17]:

$$\min_{R,S} \sum_i \frac{\sqrt{\sum_j (D_{i,j\,\mathrm{exp}} - D_{i,j\,\mathrm{rec}})^2}}{\mathrm{Max}(\left|D_{i,j\,\mathrm{exp}}\right|)} \quad (3)$$

With constraints: $R_{i,j} > 0$ et $R_{i+1,J} > 0$ for $i = 1$, n_{obs} and $j = 1$, n_r; S varifies the element balance:

The two constraints verify the physical coherence of the found solution with positive and growing reaction extends and with stoichiometric coefficients that follow the elemental balance.

To better understand this method, we propose a simple example. Consider a batch reactor system where four constituents A, B, C, D were measured at five different sampling times. We can then calculate the cumulative molar amount D_{rec}, i.e., quantities are positive for constituents produced and negative for constituents consumed.

$$D_{rec} = \begin{array}{c} \begin{matrix} A & B & C & D \end{matrix} \\ \begin{bmatrix} -1 & -1 & 0 & 3 \\ -3 & -2 & 1 & 7 \\ -5 & -3 & 2 & 11 \\ -7 & -4 & 3 & 15 \\ -8 & -5 & 3 & 18 \end{bmatrix} \end{array} \begin{matrix} t_0 \\ t_1 \\ t_2 \\ t_3 \\ t_4 \end{matrix}$$

From the theoretical knowledge of the system and the analysis of experimental data, an outline of the reaction scheme emerges lacking the stoichiometric coefficients and number of reactions. For example, the two following reaction equations were proposed:

$A \rightarrow \nu_1^C C + \nu_1^D D$ (RI)

$B + \nu_2^C C \rightarrow \nu_2^D D$ (RII)

The matrix S of stoichiometric values and R of individual reaction extends were written, and the cumulative molar amount can be calculated as follows:

$$S = \begin{array}{c} \begin{matrix} A & B & C & D \end{matrix} \\ \begin{bmatrix} -1 & 0 & \nu_1^C & \nu_1^D \\ 0 & -1 & \nu_2^C & \nu_2^D \end{bmatrix} \end{array} \qquad R = \begin{array}{c} \\ \begin{bmatrix} \xi_0^{RI} & \xi_0^{RII} \\ \xi_1^{RI} & \xi_1^{RII} \\ \xi_2^{RI} & \xi_2^{RII} \\ \xi_3^{RI} & \xi_3^{RII} \\ \xi_4^{RI} & \xi_4^{RII} \end{bmatrix} \end{array} \begin{matrix} t_0 \\ t_1 \\ t_2 \\ t_3 \\ t_4 \end{matrix} \qquad D_{calc} = S.R \quad (4)$$

Using the atomic balances, it is sometimes possible to determine some of the stoichiometric coefficients for reactions I and II. The minimization problem then consists of identifying the matrix of the reaction advancements and unknown (if there are any) stoichiometric coefficients, which can minimize the difference between matrix D_{rec} and $D_{calc} = S.R$. For the proposed example, the resolution of the minimization problem gives:

$$S = \begin{array}{c} \begin{matrix} A & B & C & D \end{matrix} \\ \begin{bmatrix} -1 & 0 & 1 & 1 \\ 0 & -1 & -1 & 2 \end{bmatrix} \end{array} \qquad R = \begin{bmatrix} 1 & 1 \\ 3 & 2 \\ 5 & 3 \\ 7 & 4 \\ 8 & 5 \end{bmatrix} \qquad D_{calc} = \begin{bmatrix} -1 & -1 & 0 & 3 \\ -3 & -2 & 1 & 7 \\ -5 & -3 & 2 & 11 \\ -7 & -4 & 3 & 15 \\ -8 & -5 & 3 & 18 \end{bmatrix} \quad (5)$$

If the gap between D_{rec} and D_{calc} is low, the proposed reaction scheme is compatible with the experimental data obtained. If, however, the gap is high, the proposed reaction scheme is not valid and must be reviewed. For the presented academic example, there is no gap between D_{rec} and D_{calc}.

3. Results

3.1. Data reconciliation

From the experimental values of glucose, carbon dioxide, ammonium and amino acid concentrations, elemental balances for carbon and nitrogen and data reconciliation were performed.

Biomass composition was determined four times during the culture. With less than 2% variation between these measures, the biomass composition was considered invariant over time. Hence, the biomass was considered as a chemical compound with the formula $C_{3.82}H_{6.75}O_{1.89}N_{0.8}$ and assumed to be constant during the experiments.

Individual amino acid concentrations were not sufficiently high in the elemental balance for us to perform data reconciliation on them. To account for amino acids, the 20 amino acids in the yeast extract were considered as one “pseudo amino acid” having the composition $C_{4.73}H_{9.69}O_{2.38}N_{1.26}$. This composition was calculated from the weight of the different amino acids in the yeast extract. This can be done, as the main amino acids were consumed at a similar rate during the first phase of the experiment, as can be seen in Fig. 1. In the first 20 h of the total 200 h of culture, 17 out of 20 amino acids in the yeast extract were completely consumed. Only lysine was slowly consumed by *Sa. algeriensis*. Its consumption began after the first 20 h, and it was 75% consumed after 40 h. Similarly, leucine was not fully consumed (75% consumption). Proline was not consumed (not shown on the graph). This amino acid is known to be consumed rarely by microorganisms. From these results, we can reasonably draw the conclusion that the main amino acids are consumed at the same kinetic rate and consider them as one component with an average molecular weight.

For each experiment, data reconciliation highlighted an increase in carbon at the very beginning of the fermentation. Carbon measured after 20 h was approximately 10% higher than the initial carbon introduced in the media. The quantity of carbon in the medium then stabilized to its highest value. Yet, no significant deviation was found in the nitrogen balance (Fig. 2). Therefore, we can suppose that an undetected carbohydrate from the yeast extract is

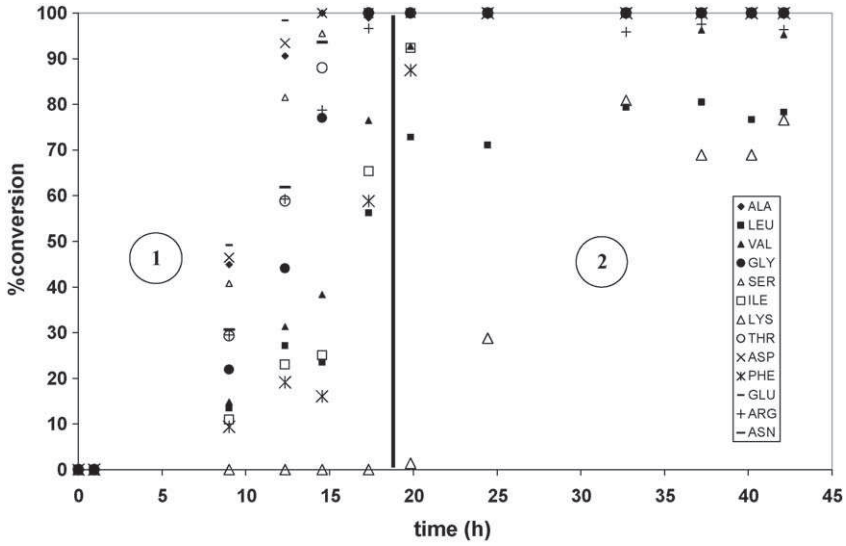

Fig. 1. Amino acid conversion during *Sa. algeriensis* growth on a semi-synthetic medium (glucose 15 g L^{-1}, YE 2 g L^{-1}, uracil 20 mM) at 30 °C, pH 7, 0.5 vvm and 250 rpm.

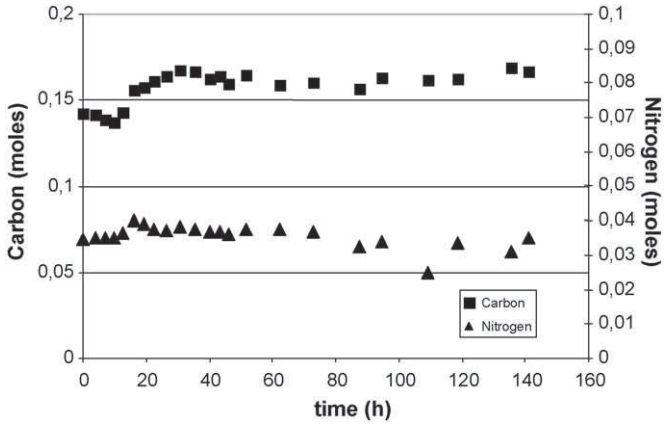

Fig. 2. Carbon and nitrogen balances during *Sa. algeriensis* growth on a semi-synthetic medium (glucose 15 g L^{-1}, YE 2 g L^{-1}, uracil 20 mM) at 30 °C, pH 7, 0.5 vvm and 250 rpm.

consumed during the first 20 h by *Sa. algeriensis* as a carbon source. We can further suppose that the carbon balance is constant when this carbon source is exhausted. Using the data reconciliation procedure, experimental values were corrected to satisfy the elemental C and N balances. In Fig. 3, an example of corrected data compared to raw experimental data is shown. The unknown carbohydrate compound was considered in the reconciliation procedure as glucose to satisfy the carbon balance. Therefore, for glucose, at the beginning of the culture, corrected values were superior to experimental values. For amino acids, the two sets of data were very similar because there was no significant deviation for the nitrogen balance. Further quantitative analysis was done with the values provided by data reconciliation.

3.2. Qualitative analysis of the growth of Sa. algeriensis in liquid medium

In Fig. 4, carbon dioxide production is presented for a batch culture of *Sa. algeriensis* on semi-synthetic medium under the conditions defined for the reference culture. This continuous measurement shows that this growth can be divided into four phases. The corresponding evolutions of the biomass, the glucose, the α-amino nitrogen, the ammonium and the thiolutin are presented in Fig. 5(a) and (b).

In the first phase, about 1 g L^{-1} of biomass is produced and glucose remains constant, whereas amino acids are consumed and ammonium ion values increase. Hence, amino acids are consumed

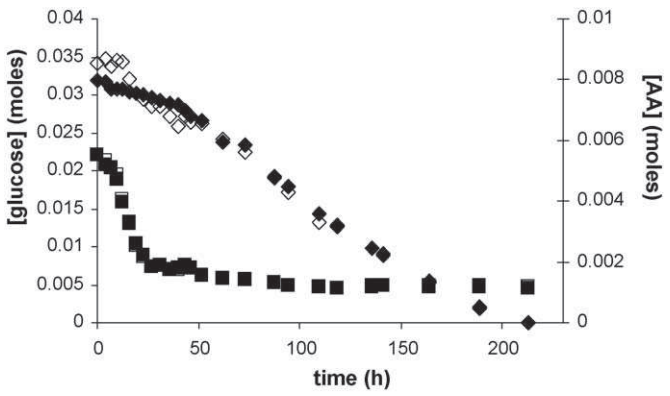

Fig. 3. Glucose (raw data (♦), reconciliated data (◊)) and amino acids (raw data (■), reconciliated data (□)) during *Sa. algeriensis* growth on a semi-synthetic medium (glucose 15 g L^{-1}, YE 2 g L^{-1}, uracil 20 mM) at 30 °C, pH 7, 0.5 vvm and 250 rpm.

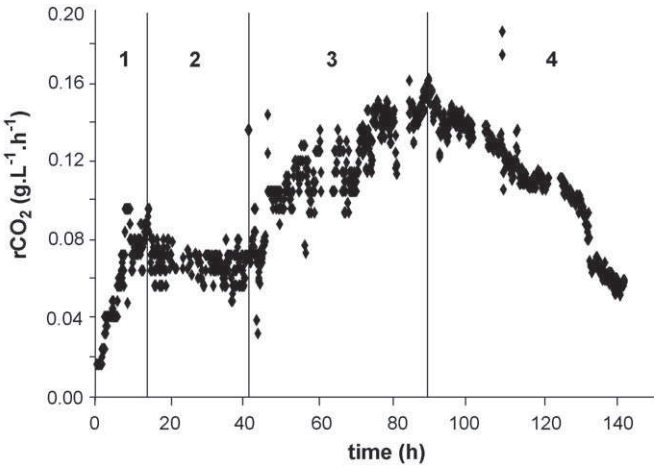

Fig. 4. Carbon dioxide production rate (g L^{-1} h^{-1}) versus time (h) during *Sa. algeriensis* growth on a semi-synthetic medium (YE 2 g L^{-1}, uracil 20 mM) at 30 °C, pH 7, 0.5 vvm and 150 rpm.

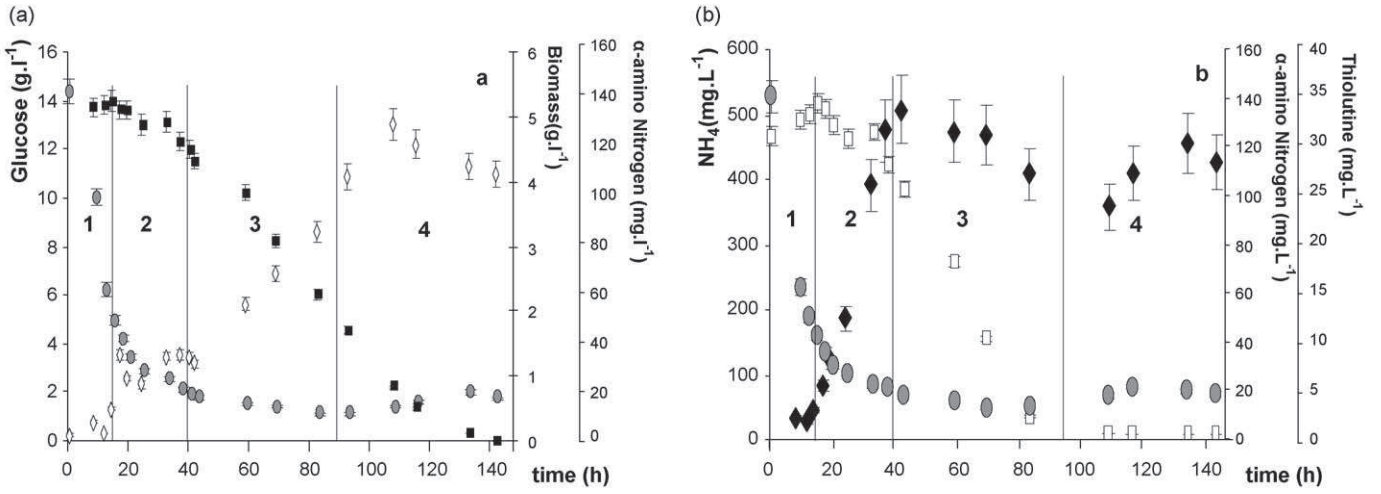

Fig. 5. Batch culture of *Sa. algeriensis* on a semi-synthetic medium (glucose 15 g L^{-1}, YE 2 g L^{-1}, uracil 20 mM) at 30 °C, pH 7, 0.5 vvm and 250 rpm. (a) Biomass (◊), α-amino N ( ) and glucose (■) (b) thiolutin (♦), α-amino N ( ) and ammonium (□).

as both a carbon and as a nitrogen source. This is not surprising. Indeed, different studies on microbial production in reactors were performed using media containing one or more amino acids as sole carbon and nitrogen sources or together with glucose [26,27]. In these studies, the authors obtain significant biomass production from amino acids used as carbon and nitrogen sources. The fact that ammonium ions are released in the broth could indicate that the amino acids are deaminated, with ammonium ion excretion, and integrated into the Krebs cycle, as explained by Voelker and Altaba [26]. The end of the first phase, as determined by the carbon dioxide concentration (Fig. 4), corresponds to the exhaustion of the main amino acids (Fig. 5(a)). The secondary metabolism of the bacterium is then activated to produce thiolutin. No growth is observed during this second phase, but glucose is believed to be consumed as an energy source for maintenance [28]. Then, in the third phase, the primary metabolism is activated again to achieve growth using glucose and ammonium as carbon and nitrogen sources, respectively. During this phase, thiolutin degradation is observed. The fourth phase begins as ammonium is exhausted. Again, thiolutin production is observed. Glucose is still consumed but no growth is observed. As already mentioned, bacteria use glucose as an energy source for maintenance.

The qualitative analysis of the experimental results indicates a sequential consumption of the carbon source substrates with a probable diauxic growth via an initial preferential consumption of amino acids followed by a glucose consumption. Thiolutin production is partially decoupled from the growth and is initiated by substrate deprivation.

3.3. Quantitative analysis of the growth of Sa. algeriensis in liquid medium

From the qualitative analysis, we suggest that *Sa. algeriensis* growth and thiolutin production occurs through a set of four reactions: growth via amino acids (R1), maintenance using glucose (R2), growth using glucose (R3) and thiolutin production (R4). These reactions occur during the different growth phases identified earlier as follows:

- Phase 1: growth via amino acids
- Phase 2: maintenance using glucose and thiolutin production
- Phase 3: growth using glucose
- Phase 4: maintenance using glucose and thiolutin production

Two more reactions could have been written to express the thiolutin degradation and the biomass decrease, but as the compounds resulting from these degradations were neither determined nor measured, these reactions were omitted. To identify the reaction scheme stoichiometry, the thiolutin data corrected to show no degradation and biomass was considered as constant during the last phase.

For each reaction, a stoichiometric relationship was established:

R1 Growth via amino acids:

$$C_{4.73}H_{9.69}O_{2.38}N_{1.26} + \mathbf{o_1}O_2 \Rightarrow \mathbf{x_1}C_{3.82}H_{6.75}O_{1.89}N_{0.8} + \mathbf{c_1}CO_2 + \mathbf{n_1}NH_4^+ + \mathbf{h_1}H_2O$$

R2 Maintenance using glucose:

$$C_6H_{12}O_6 + \mathbf{o_2}O_2 \Rightarrow \mathbf{c_2}CO_2 + \mathbf{h_2}H_2O$$

R3 Growth using glucose:

$$C_6H_{12}O_6 + \mathbf{o_3}O_2 + \mathbf{n_3}NH_4^+ \Rightarrow \mathbf{x_3}C_{3.82}H_{6.75}O_{1.89}N_{0.8} + \mathbf{c_3}CO_2 + \mathbf{h_3}H_2O$$

R4 Thiolutin production:

$$C_6H_{12}O_6 + \mathbf{o_4}O_2 + \mathbf{n_4}NH_4^+ + \mathbf{s_4}SO_4^{2-} \Rightarrow \mathbf{t_4}C_8H_8N_2O_2S_2 + \mathbf{c_4}CO_2 + \mathbf{h_4}H_2O$$

Nineteen stoichiometric coefficients must be determined. Yet, for each reaction, an elemental balance must be written. In R1 and R3, 4 out of 5 coefficients can be calculated using C, H, O, N balances. For R4, 5 out of 6 coefficients can be determined by adding the sulfur elemental balance. In R2, all three coefficients are determined using the three elemental balances (C, H, O). In the end, only 3 coefficients (x_1, x_3 and t_4) are determined using factorial analysis of the reconciled data from the reference culture.

These coefficients together with the reaction extends were evaluated. Equations were written in Cmol to compare reaction stoichiometries:

R1 Growth via amino acids:

$$CH_{2.05}O_{0.50}N_{0.26} + 0.046O_2 \rightarrow 0.969CH_{1.767}O_{0.49}N_{0.21} + 0.0317CO_2 + 0.055H_2O + 0.07NH_4^+$$

R2 Maintenance using glucose:

$$CH_2O + O_2 \rightarrow CO_2 + H_2O$$

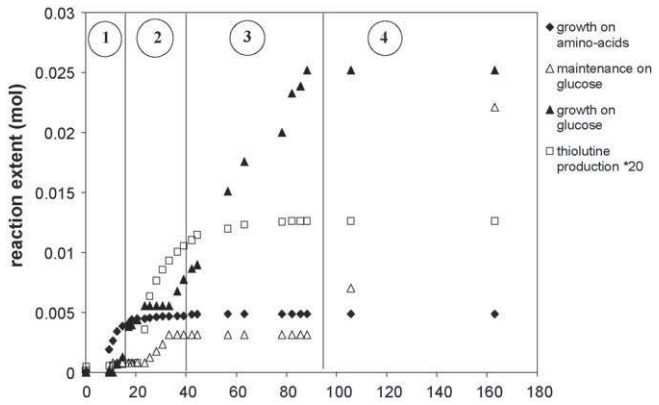

Fig. 6. Reaction extends for a batch culture of *Sa. algeriensis* on a semi-synthetic medium (glucose 15 g L^{-1}, YE 2 g L^{-1}, uracil 20 mM) at 30 °C, pH 7, 0.5 vvm and 250 rpm.

R3 Growth using glucose:

$$CH_2O + 0.648O_2 + 0.07NH_4^+ \rightarrow 0.337CH_{1.767}O_{0.49}N_{0.21} + 0.66CO_2 + 0.8H_2O$$

R4 Thiolutin production:

$$CH_2O + 0.695O_2 + 0.053NH_4^+ + 0.053SO_4^{2-} \rightarrow 0.213CHN_{0.25}O_{0.25}S_{0.25} + 0.79CO_2 + 0.97H_2O$$

Reaction extends obtained from factorial analysis are presented in Fig. 6. A good agreement between calculated and experimental molar content values was obtained for the different compounds, as shown in Fig. 7.

The analysis of stoichiometric coefficients revealed that growth via amino acids (R1) is carried out with a weak respiratory activity (Q_R = 0.689), compared to those obtained for the growth using glucose (R3, Q_R = 1). This phenomenon could be explained by the direct integration of amino acids at the level of protein synthesis without the participation of the central metabolism of the microorganism, and therefore, with less respiratory activity. The Q_R values inferior or equal to 1 indicate an oxidative metabolism for this bacterium, which is consistent with a secondary metabolism.

The identified reaction extends (Fig. 6) confirm the assumptions made for the occurrence of different reactions in the qualitative analysis. The four phases could be identified. The growth is carried out initially using the free amino acids and the unknown substrate from the yeast extract is considered as glucose. The reaction extends of R1 and R3 increase. The reaction extend of R1 was stopped when free amino acids were exhausted. The production of thiolutin (R4), uncoupled from the growth, begins and is accompanied by the maintenance phase that uses glucose (R2). The glucose (R3) is then used for growth until the exhaustion of ammonium, the nitrogen source (phase 3). In the fourth phase, only maintenance using glucose occurs. Therefore, the sequence of the reactions can be summarized:

Phase 1: R1 and R3
Phase 2: R2 and R4
Phase 3: R3
Phase 4: R2

Thus, the quantitative analysis confirms the results of the qualitative analysis, with two differences. In phase 1, the occurrence of R3 was not identified in the qualitative analysis because glucose was not consumed. In the quantitative analysis, this reaction occurs because the unknown substrate from yeast extract formed glucose. The other difference occurs in phase 4, where the production of thiolutin was not identified by quantitative analysis. This can be explained by the weak quantity of thiolutin produced in this phase, which balances the thiolutin degradation in phase 3 and is not detailed in the reaction scheme.

3.4. Influence of glucose and yeast extract initial concentrations on stoichiometric coefficients

A stoichiometric reaction scheme was built based on a reference medium containing 15 g L^{-1} glucose and 2 g L^{-1} yeast extract. To evaluate the influence of the initial composition of the medium on the scheme, 7 experiments were carried out by varying the initial glucose and yeast extract concentrations. In an 8th experiment, an amino acid cocktail was added, corresponding qualitatively and quantitatively to the 2 g L^{-1} amino acids in the yeast extract. The conditions of these 8 experiments are summarized in Table 2.

The qualitative analysis of these experiments provided similar results to the qualitative analysis of the reference culture: four phases could be distinguished from the continuous analysis of carbon dioxide production. The data reconciliation procedure was applied in each case. For experiments with initial yeast extract concentrations of 4 and 6 g L^{-1}, the increase in carbon after 20 h of culture reached approximately 15–20%, whereas for experiments with initial concentrations in yeast extract of 0.5 g L^{-1}, the increase was not significant. This observation confirms that the yeast extract adds an unknown carbohydrate substrate that is consumed by *Sa. algeriensis* during the first 20 h of culture.

To test the validity of the reaction scheme on these new experiments, it would be possible to apply the stoichiometry identified using the reference culture and to determine the reaction extends by solving the minimization problem under constraints of elemental balances and the increase of reaction extends. The comparison between experimental and modeled values could validate the stoichiometry.

Here, another approach was performed. The complete factorial analysis was carried out for each experiment, allowing us to determine the three unknown coefficients x_1, x_3 and t_4 and the reaction extends of the 4 reactions, as it was performed with the reference culture. The sensitivities of the stoichiometric coefficients are presented in Table 2.

For each experiment, experimental values were near the modeled values (refer to the appendix section) using the identified stoichiometric coefficients. Hence, the reaction scheme with the four phases was confirmed. The analysis of the stoichiometric coefficients revealed that they do not appear to be sensitive to the concentration of glucose. For experiments in which the initial glucose concentration was varied and the yeast extract was fixed at 2 g L^{-1}, the stoichiometric coefficients obtained did not vary by more than 2% for x_1 and 15% for x_3.

Coefficient t_4 is always invariant but it has no significance. The couple t_4 and the R4 reaction extend cannot be discriminated by factorial analysis; thiolutin production occurs only in this reaction and the weight of this product in the carbon and nitrogen balances is negligible. A variation of the t_4 coefficient could be compensated by the R4 reaction extend to satisfy thiolutin production without affecting the carbon and nitrogen balance. A limitation of the method is highlighted here; it is not adapted for the minority products that have little weight in elemental balances.

Stoichiometric coefficients are not sensitive to glucose concentrations; however, they are sensitive to the yeast extract initial concentrations. Coefficients x_1 and x_3 take different values, varying approximately 40–50% compared to the reference culture. Coefficient t_4 remains unchanged because of the limitation of this method. Therefore, the initial concentration of the yeast extract influences the stoichiometry of the proposed reaction scheme. This

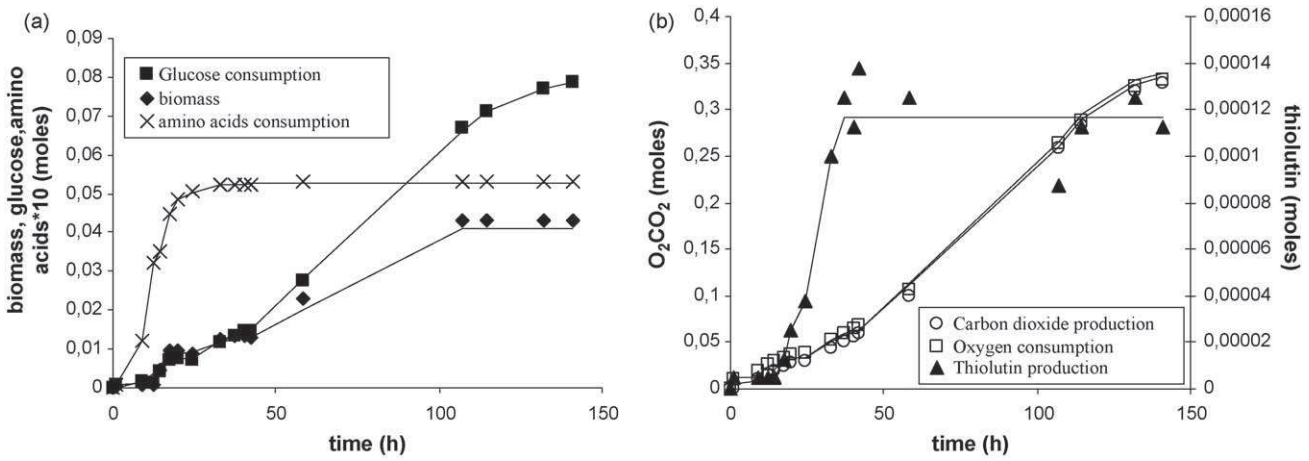

Fig. 7. Calculated values (lines) versus experimental values (points) for biomass, glucose and amino acids (a) and thiolutin, oxygen and carbon dioxide (b) for a batch culture of *Sa. algeriensis* on a semi-synthetic medium (glucose 15 g L^{-1}, YE 2 g L^{-1}, uracil 20 mM) at 30 °C, pH 7, 0.5 vvm and 250 rpm.

Table 2
Variability of the identified stoichiometric coefficients for different glucose and yeast extract initial concentrations.

$[Glucose]_0$ (g L^{-1})	$[Yeast\ extract]_0$ (g L^{-1})	Other compound	x_1	x_3	t_4
15	2	–	0.969	0.337	0.213
3	2	–	0.969	0.285	0.213
5	2	–	0.969	0.285	0.213
8	2	–	0.969	0.318	0.213
15	2	Amino acids mix at the same composition as in YE at 2 g L^{-1}	0.95	0.337	0.213
15	0.5	–	0.975	0.324	0.213
15	4	–	0.759	0.165	0.213
15	6	–	0.597	0.203	0.213

point is confirmed by the experiment using 2 g L^{-1} yeast extract and an amino acid concentration equivalent to 2 g L^{-1} of yeast extract. The stoichiometric coefficients identified for this experiment are the same as the ones obtained for the reference culture. This finding proves that the amino acid consumption is not responsible for the differences but is due to other components from the yeast extract. The number of important components in the yeast extract (minerals and oligo-elements, among others) able to interfere with bacteria metabolism makes it difficult to propose an explanation for the yeast extract influence on the stoichiometry. It was not developed further in this study, but it appears that the proposed reaction scheme cannot be applied for yeast extract concentrations other than 2 g L^{-1}.

The next steps of this study are to establish a kinetic model for each reaction using the identified reaction extends, to express the reaction rate as a function of the substrate concentrations, and to simulate the evolution of the compounds.

4. Conclusions

This study shows that it is possible to elucidate *Sa. algeriensis* metabolic reactions from extracellular compound measurements. This "reaction engineering" approach enables us to perform qualitative and quantitative analyses of reliable data with respect to elemental mass balances. A scheme with four reactions was proposed as a macroscopic view of the growth of *Sa. algeriensis* and the associated production of thiolutin. It was shown that *Sa. algeriensis* was able to grow using both free amino acids and glucose as a carbon source. The amino acids were preferentially consumed. They seemed to be directly integrated into protein synthesis and to enrich the medium with ammonia. This ammonia was then consumed during growth using glucose. It was further established that the production of thiolutin was the result of a secondary metabolism, decoupled from growth. Under our experimental conditions, thiolutin production seemed to be initiated as soon as nitrogen source depletion occurs.

The proposed reaction scheme was tested under different medium compositions. It was validated for all experiments carried out with a yeast extract concentration of 2 g L^{-1}. For other concentrations, our results showed that the same reaction scheme could be applied using different stoichiometric coefficients. An unknown component of the yeast extract interfered with the reaction scheme. This method of reaction elucidation has limitations. Furthermore, it cannot be applied to molecules that have only a small role in mass balances.

These results demonstrate an interesting method for determining the behavior of poorly known bacterium. On an industrial scale, the quantity of produced biomass is difficult to estimate, and this difficulty increases in the case of a filamentous microorganism. CO_2 online monitoring is a direct and continuous means to follow the evolution of a filamentous microbial culture. Hence, it provides insights for the production of thiolutin on an industrial scale. Moreover, our stoichiometric reaction scheme will help to target the critical points of antibiotic production (nutritional deprivations) for the study of the key enzymes (or enzymatic complexes) responsible for the final step of pyrrothine nucleus acylation in dithiolopyrrolones biosynthesis. Moreover, the establishment of the kinetic model will be combined with potential intracellular data concerning the expression of key enzymes. Hence, the production of thiolutin could be simulated.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2010.07.010.

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