REACTION ENGINEERING APPROACH APPLIED TO THE PRODUCTION OF ANTIBIOTICS BY *Saccharothrix algeriensis*

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Abstract. The relatively short product life cycle in very competitive industrial fields like pharmaceutics and food industries never let time to realize all experiments which would give enough information to get a good knowledge of the kinetic description of reactions performed in a batch reactor. Now, a good mastery of reaction kinetics is necessary to optimize and control those reactors. In this article, we develop a method to establish reliable kinetic models from raw data through data reconciliation and factor analysis. The objective is to reduce a complex reaction system from observable data. The method is based on the bilinear structure of the molar holdup regarding stoichiometric coefficient and reaction yields. By reducing the noise intensity using data reconciliation based on elemental balances, errors become negligible compared to reaction extends and in that case, the discrimination of reactions is possible and reliable. This approach was successfully applied to the analysis of the growth of *Saccharothrix algeriensis*, a bacterium belonging to the actinomycete family and able to synthesize antibiotics. The suggested approach enables to establish a general reaction scheme consisting in four reactions (growth on glucose, growth on amino-acids, production of antibiotics and glucose degradation for maintenance) and to validate the assumption of a diauxic growth.

1 Introduction

Almost a quarter of the deaths in the world results of infectious diseases. They are more and more caused by bacteria having developed a (or several) resistance to antibiotics [13]. This phenomenon is worrying for humanity and stresses the importance of constantly renewing the pool of bio-active molecules. Thus, the production of new bio-active molecules on the pathogenic currently available antibiotic resistant strains is the subject of more and more interdisciplinary research projects. In this context, a new bacterial specie *Saccharothrix algeriensis* NRRL B-24137 [20] has been isolated in 1992 from the Sahara desert (Algeria). This filamentous bacterium belongs to the actinomycetes family. It produces molecules belonging to the dithiolopyrrolones family which present antibacterial, antifungal, and interesting anticancer activities [19, 15, 11]. A previous work [2, 3] highlighted the influence of the culture medium composition on the dithiolopyrrolones production, including on the production of new molecules never described in the scientific literature [6]. This capacity to produce antibiotics upon request by precursor-directed biosynthesis facts that the study of *Saccharothrix algeriensis* presents a fundamental undeniable interest, in addition to an obvious useful interest. As this bacterium has been recently discovered and characterised, no deep knowledge on its metabolism is available. Several studies have been undertaken in parallel to better understand the dithiolopyrrolones production pathway by *Saccharothrix algeriensis*. For the study presented here, a macroscopic approach has been chosen to investigate the bacterium metabolism. No data coming from "omics" are used but the "reaction engineering" approach adopted to analyse the metabolism from a qualitative and quantitative point of view enables to get more knowledge about the mechanism of dithiolopyrrolones production. Two broad approaches exist to model microbial bio-productions:

- The black box models which do not require any knowledge on the microorganism. Only the inputs (substrates) and the outputs (products) of the system are taken into account. These models do not enable to draw assumptions on the metabolic pathways. It remains purely descriptive.
- The structured models, like the "Metabolic Flow Analysis" model, which favour a more systemic approach of the microorganism. They are generally complex and require a good knowledge of the metabolism network of the bacteria. The quantification of hundreds of fluxes from a limited measurements number compels to assume that all intermediate compounds are in a quasi-stationary state. This drawback may be avoided if intracellular compounds can be measured.

In this work, we adopted an intermediate approach based on an analysis of experimental data. It allows establishing a global reaction scheme of the bacterium metabolism. The advantage of this type of model is that, while remaining at the extra cellular scale, with experimental information relatively easy to obtain, it allows getting information on the metabolism of the cell. Stoichio-kinetic models consist of the decomposition of bacterial growth in some reactions of invariant stoichiometry with 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

batch reactor data is due to Hamer [9]. This method underwent several adaptations [1, 10, 8, 12, 7]. This paper presents the results of this approach applied to the growth and production of thiolutine (the main dithiolopyrrolone produced) by *Saccharothrix algeriensis*, on a semi-synthetic medium. The consistency of the experimental data is first checked in order to further perform the qualitative and quantitative analysis on reliable data. These three parts are presented successively hereafter.

2 Material and Methods

2.1 Microoganism and culture conditions

Saccharothrix algeriensis NRRL B-24137 was used in this study. Microbial spores were obtained from solid culture 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 by 3.5 ml of this suspension and incubated on a rotary shaker (New Brunswick Scientific, Edison, NJ, USA) at 250 rotation per minute (rpm) at 30˚C for 52 h. Five ml of the resulting preculture was used to inoculate each culture. Before inoculation, the pH was adjusted to 7 by addition of 1 mol.L^{-1} NaOH solution. Cultures were performed in NBS reactors containing 2 L of medium. Culture lasted for at least one week. pH was maintained at 7±0.035 by the automatic addition of 1 mol.L^{-1} NaOH and 1 mol.L^{-1} HCl solutions. 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 level were monitoring using Ingold specific electrodes. Temperature was regulated at 30˚C. Percentages of O_2, CO_2 exhaust gas were determined by a gas analyzer (Servomex 4100, paramagnetic transductor for O_2 and infrared transductor for CO_2).

2.2 Media composition

The conservation media had the following composition (per litter of distilled water): 10 g malt extract, yeast extract 4 g, glucose 4 g and agarose 18 g. Semi-synthetic medium used as growth and production medium contained (per litter of distilled water): glucose 15 g, yeast extract 2 g, NaCl 2 g, $(NH4)_2SO_4$ 2 g, KH_2PO_4 0.5 g, K_2HPO_4 1g, $MgSO_4.7H_2O$ 0.2g, $CaCl_2.2H_2O$ 1 g and MOPS 2 g, Uracil 20 mmol. Attempts were made to substitute yeast extract to better control the medium composition without success [17]. The concentrations in chemical elements and free amino acids of the yeast extract used during this study (288620 BactoTM Yeast Extract, Technical) are presented in Table 1. Its global elementary composition was evaluated to $C_{3.32}H_{6.54}O_{2.17}N_{0.79}$.

Amino-acids	μmol per g of YE	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	73542
ARG	109		
ASN	108		

Table 1: Concentrations in free amino-acids and other elements of the yeast extract (288620 BactoTM Yeast Extract, Technical)

2.3 Analytical procedures

For estimation of dry cell weight (DCW), 3 ml samples of homogenised culture broth were centrifuged at 16000 g for 10 min in preweighed Eppendorf tubes. Pellet was washed first with 0.25 mol.L^{-1} NaOH solution, then with 0.35 mol.L^{-1} HCl solution and then with distilled water. Eppendorf tubes containing pellet were dried at 105˚C for 48 h, and weight after cooled for 30 min in a dessicator [5]. The measurement relative error is 5%. Supernatant coming from DCW determination was filtered at 0.2 μm and used to measure metabolites. The analysis of

dithiolopyrrolone antibiotics was carried out by non polar chromatography (HPLC, Bio-Tek Instruments) with the method described by Bouras et al. [3]. Glucose was quantified with a biochemical analyzer with fixed glucose oxydase enzymes on membrane (YSI2700 select). Glucose was determined with amperometric quantification after its enzymatic oxidation. The amperometer answer is linear for a concentration in glucose between 0 and 25 $g.L^{-1}$. The measurement relative error is 3%. Ammonium ions and α-amino nitrogen were quantified using specific enzymatically methods (Diagnostics Ammonia kit from Boehringer-Mannheim, using glutamate deshydrogenase and Microdon Kit using glutamate oxydase) and an automatic multiparametric analyser (Mascott Lisabio). The signal answer is linear for concentrations in α-amino nitrogen and ammonium ion between 0 and 500 $mg.L^{-1}$. The measurement relative error is 5%. Amino acids quantification was done with AminoQuant HP1900 (non polar column C18) and associated protocol. Before analysis, proteins contained in the supernatant were precipated with methanol 75% v/v one night at 4˚C. Amino acids were automatically derivated with orthophthal-aldehyde-9-fluorenylmethyloroformate (OPA-FMOC).

2.4 Data reconciliation

Experimental values were reconciliated according to the chemical elemental (C, N, O, H, ⋯) mass balances. The objective is double: first, it enables to check the consistency of experimental data and second, it can slightly correct them so that the mass balances on chemical elements are completely satisfied. Let $d_{i,j}$ be the molar content of component j in sample i. For *nc* components and *ns* samples, we get the matrix of the molar contents: $D(ns,nc) = [d_{i,j}]$. The E atomic element matrix of the reactive system is $E(nc,ne) = [e_{i,k}]$, where $e_{i,k}$ is the mole number of element k in the component i and *ne* the number of atomic element involved in the reaction system. A_j, the jth column of the matrix $A(ne,ns)$ corresponding to the molar amount of each element k in each sample j can be calculated by:

$$A_j = E^T D_i \tag{1}$$

where and D_i the ith row of D. For a batch mode, the mass balance is dynamic, the values of D change with time but the values of E remain constant as long as the biomass composition remains constant during experiments. So, elemental balance constraint between two successive samples, can be written as:

$$E^T (D_{i+1} - D_i) = 0 \tag{2}$$

Let $X_j = D_{i+1} - D_i$ the jth column of a $X(nc,ns-1)$ matrix. The mass balance constraint can then be written as:

$$E^T X = 0 \tag{3}$$

Data reconciliation consists in solving the optimization problem [8]:

$$\begin{cases} \underset{\hat{\mathbf{X}}}{Min}\left[\left(\hat{\mathbf{X}} - \mathbf{X_m}\right)^T V^{-1}\left(\hat{\mathbf{X}} - \mathbf{X_m}\right)\right] \\ E^T \hat{X} = 0 \end{cases} \tag{4}$$

Where X_m is the (nc, ns-1) matrix of the measured values of the molar variation between two successive samples, $\hat{X}$ is the (nc, ns-1) matrix of the reconciliated values and V the measurement variance-covariance (nc,nc) matrix calculated from the measurement relative errors. As the constraint is linear, the minimisation problem has an analytic solution. To check the data consistency, three statistical tests are performed: the Generalized Likelihood Ratio [14], the Iterative Measurement Test [16] and the Residual Criterion [18].

2.5 Stoichiometric coefficients and reaction extends determination

It is possible to extract a reaction scheme and the corresponding reactions extends from the cumulative molar amount by exploiting the bilinear structure of the component mass balances. In the case of a batch reactor in which *nr* reactions are performed and *nc* components are measured on *ns* samples, the data matrix D, consisting of *ns* rows containing the cumulative molar amount of the *nc* measured species can be written as the product of two matrixes $R(ns,nr)$ and $S(nr,nc)$, the reaction extends matrix and the stoichiometric coefficients matrix of the *nr* reactions respectively. 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 reconciliated data can be done [12]:

$$\underset{R,S}{Min} \sum_i \frac{\sqrt{\sum_j (D_{exp}(i,j) - D_{calc}(i,j))^2}}{Max(|D_{exp}(i,j)|)} \text{ and } \begin{cases} D_{calc} = RS \\ \text{R is coherent with the elemental balances} \\ s_{i,j} > 0 \text{ for i = 1, ns and j = 1, nr} \\ s_{i+1,j} - s_{i,j} > 0 \text{ for i = 1, ns-1 and j = 1, nr} \end{cases} \tag{5}$$

3 Results

3.1 Data reconciliation

For the amino-acids to have a sufficient weight in the elemental balance to perform data reconciliation, the 20 amino-acids from the yeast extract have been assimilated to one "pseudo amino-acid" with the composition $C_{4.73}H_{9.69}O_{2.38}N_{1.26}$ calculated from the weight of the different amino-acids in the yeast extract. This can be done as the main amino acids are consumed nearly at a same rate during the first phase of the experiment as shown on Figure 1.

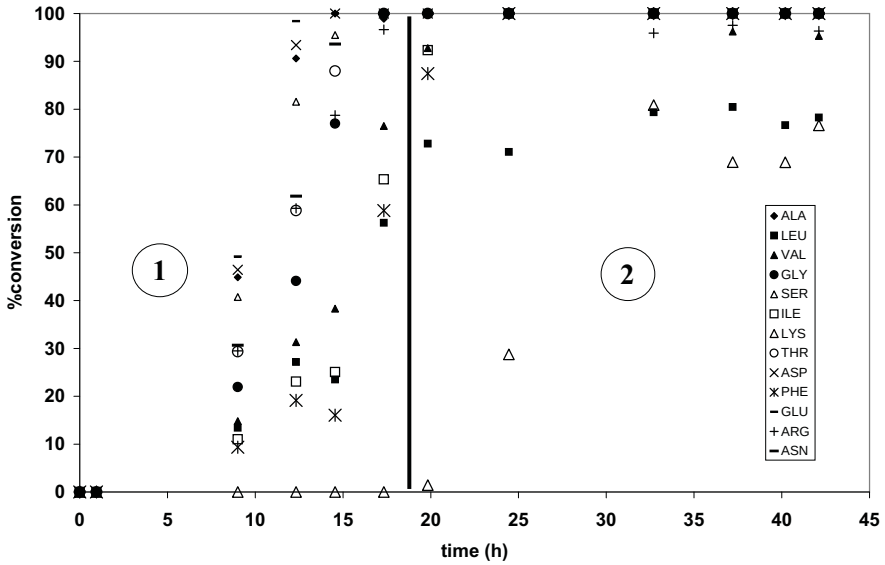

Figure 1: amino-acid conversion during *Saccharothrix algeriensis* growth on a semi-synthetic medium (glucose: 5 $g.L^{-1}$, YE: 2 $g.L^{-1}$), pH 7, 30 ˚C, 250 rpm.

The biomass composition has been evaluated at different time during growth and the results did not show any significant evolution. So, biomass was assimilated to a chemical compound with the formula $C_{3.82}H_{6.75}O_{1.89}N_{0.8}$ assumed to be constant during the whole experiments.

Data reconciliation performed on all measurements enlightens a lack of carbon at the very beginning of the fermentation. This indicates that a non measured carbohydrate coming from the yeast exact is consumed by *Saccharothrix algeriensis* preferably to glucose as carbon source. This compound was assimilated to glucose for the quantitative analysis with the quantity given by the data reconciliation (Figure 2). No deviation was found on nitrogen balance.

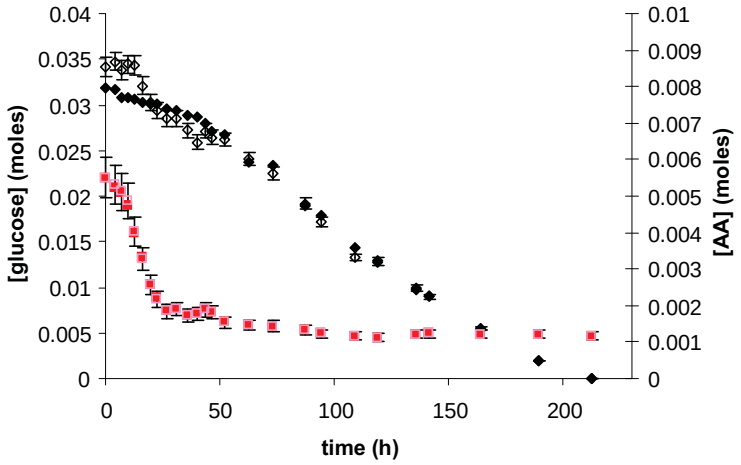

Figure 2: glucose (raw data (♦), reconciliated data (◇)) and amino-acids (raw data (▪), reconciliated data (□)) during *Saccharothrix algeriensis* growth on a semi-synthetic medium (YE: 2$g.L^{-1}$, Uracil: 20 mM) at 30˚C, pH 7, 0.5 vvm and 250 rpm.

3.2 Qualitative analysis of the growth of *Saccharothrix algeriensis* in liquid medium

The continuous measurement of the carbon dioxide production by Sa. algeriensis shows that its growth on the semi-synthetic medium can be divided into four phases (Figure 3).

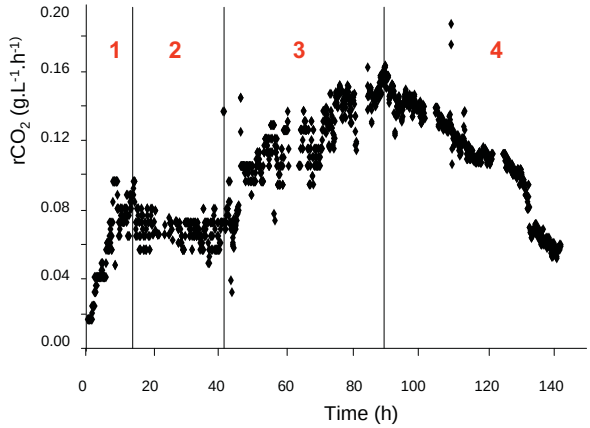

Figure 3: carbon dioxide production rate ($g.L^{-1}h^{-1}$) versus time (h) during *Saccharothrix algeriensis* growth on a semi-synthetic medium (glucose: 5 $g.L^{-1}$, YE: 2$g.L^{-1}$, Uracil: 20 mM) at 30˚C, pH 7, 0.5 vvm and 150 rpm).

The corresponding evolution of the biomass, the glucose, the α-amino nitrogen, the ammonium and the thiolutine are represented on Figure 4.

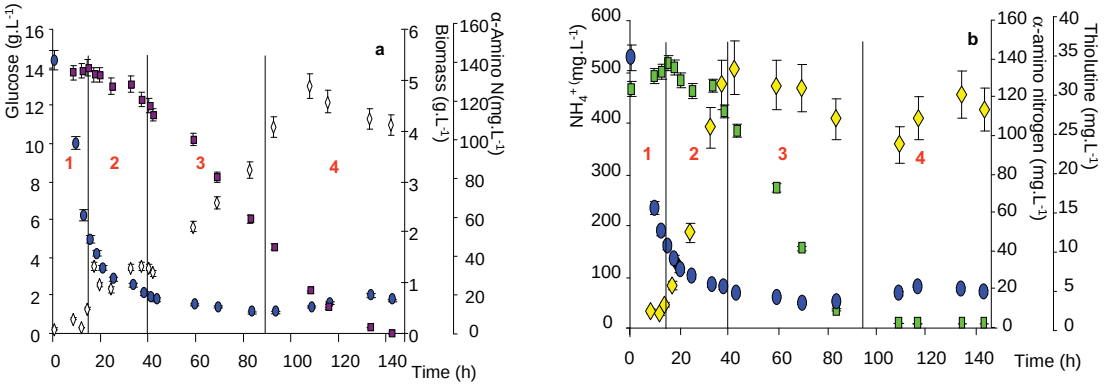

Figure 4: Batch culture of *Saccharothrix algeriensis* on a semi-synthetic medium (YE: 2 $g.L^{-1}$, Uracil: 20 mM) at 30˚C, pH 7, 0.5 vvm and 150 rpm. (left) Biomass (◇), α-amino N (●), glucose (■) - (right) Thiolutine (◇), α-amino N (●), ammonium (■).

For all experiments, the four phases have been observed. First, the amino-acids are consumed as a carbon and nitrogen source. When this substrate is exhausted, a secondary metabolism of the bacterium is activated to produce thiolutine. No growth is observed during this second phase but some carbohydrates are consumed certainly as energy source for maintenance. Then the primary metabolism is activated again to achieve the growth on glucose and ammonium as carbon and nitrogen source respectively. During this phase, a thiolutine degradation is observed. The fourth phase begins as the ammonium is exhausted and again a thiolutine production is observed. The qualitative analysis of the experimental results indicates a sequential consumption of the carbon source substrates, a probable diauxic growth, a preferential consumption of small molar weight amino-acids and a thioluthine production partially decoupled from the growth and initiated when a substrate deprive occurs.

3.3 Quantitative analysis of the growth of *Saccharothrix algeriensis* in liquid medium

From the qualitative analysis, we suggest to represent *Saccharothrix algeriensis* growth and thiolutine production through a set of four reactions: growth on amino-acids (R1), maintenance on glucose (R2), growth on glucose (R3) and thiolutine production (R4). These reactions occur during the different growth phases formerly identified as follows:

- Phase 1: growth on amino-acids (R1) and maintenance on glucose (R2)
- Phase 2: maintenance on glucose (R2) and thiolutine production (R4)
- Phase 3: growth on glucose (R3)
- Phase 4: maintenance on glucose (R2) and thiolutine production (R4)

[glucose]0 (gL^{-1})	[Yeast Extract]0 (gL^{-1})	Other compound	x1	x3	t4
15	2	Uracil 20 mM	1.2	0.56	0.16
15	2	benzoic acid 5mM Uracil 20 mM	1.2	0.61	0.16
15	2	Uracil 20 mM	1.2	0.54	0.16
15	2	-	1.2	0.52	0.16
3	2	-	1.2	0.44	0.16
5	2	-	1.2	0.44	0.16
8	2	-	1.2	0.5	0.16
15	6	Uracil 20 mM	0.94	0.32	0.16
15	4	Uracil 20 mM	0.74	0.26	0.16
15	2	Uracil 20 mM Amino-acids mix at the same composition as in YE at $2gL^{-1}$	0.95	0.53	0.16
15	2	Uracil 40 mM	1.2	0.54	0.16

Table 2: variability of the stoichiometric coefficients with the glucose and the yeast extract initial concentrations

Two more reactions could have been written to express the thiolutine degradation and the biomass decrease but as the compounds resulting from these degradations were not determined nor measured, these reactions were omitted. To identify the reaction scheme stoichiometric, thiolutine data were corrected to consider no degradation and biomass was considered to remain constant during the last phase. For each reaction a stoichiometric relationship was established:

R1 Growth on amino acids
$C_{4.73}H_{9.69}O_{2.38}N_{1.26} + o_1\ O_2 \rightarrow x_1\ C_{3.82}H_{6.75}O_{1.89}N_{0.8} + c_1\ CO_2 + n_1\ NH_4^+ + h_1\ H_2O$
R2 Maintenance on glucose
$C_6H_{12}O_6 + o_2\ O_2 \rightarrow c_2\ CO_2 + h_2\ H_2O$
R3 Growth on glucose
$C_6H_{12}O_6 + o_3\ O_2 + n_3\ NH_4^+ \rightarrow x_3\ C_{3.82}H_{6.75}O_{1.89}N_{0.8} + c_3\ CO_2 + h_3\ H_2O$
R4 Thiolutine production
$C_6H_{12}O_6 + o_4\ O_2 + n_4\ NH_4^+ + s_4\ SO_4^{2-} \rightarrow t_4\ C_8H_8N_2O_2S_2 + c_4\ CO_2 + h_4\ H_2O$

18 stoichiometric coefficients have to be determined. For R1 to R3, 4 coefficients can be calculated by elemental balance (C, H, O, N) and 5 for R4 (C, H, O, N, S). So, only 3 coefficients (x1, x3 and t4) are to be determined by factorial analysis. These coefficients together with the reaction extends have been evaluated for experiments at different glucose and yeast extract initial concentrations (Table 2) with a good agreement between calculated and experimental molar content values, as shown on Figure 5 for biomass and thiolutine.

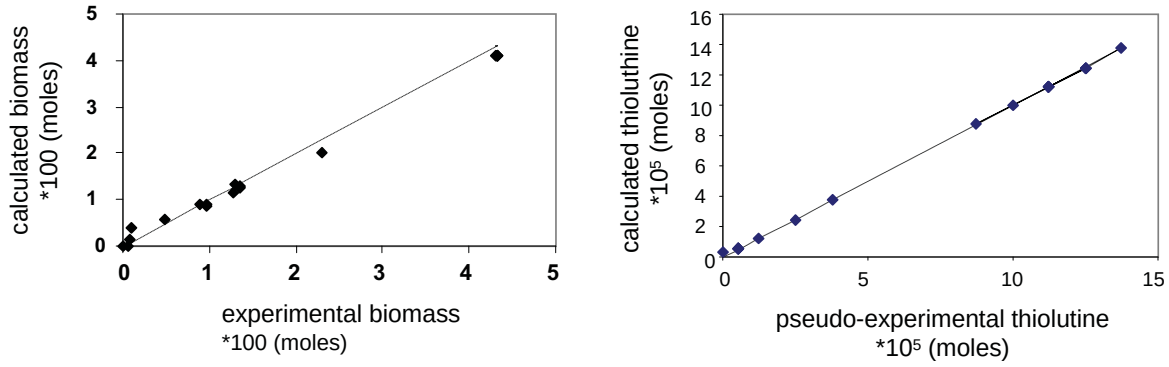

Figure 5: calculated values versus experimental values for biomass (left) and thiolutine (right) for a batch culture of *Saccharothrix algeriensis* on semi synthetic media (glucose: 5 $g.L^{-1}$), YE: 2 $g.L^{-1}$), pH 7, 30˚C, 250 rpm.

Stoichiometric coefficients do not appear to be sensitive to the glucose concentration but different values where obtained for different yeast extract initial concentrations, indicating a probable different metabolism pathway. This aspect was not analysed further in this study. Finally, for a yeast extract initial concentration of 2 gL^{-1} , the fol-

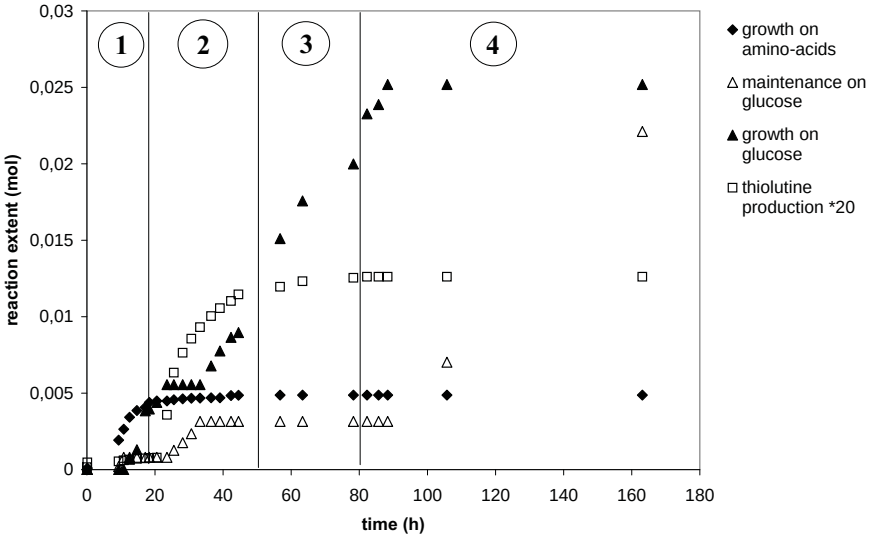

Figure 6: reaction extends for a batch culture of *Saccharothrix algeriensis* on a semi-synthetic medium (glucose: 5 $g.L^{-1}$, YE: 2 $g.L^{-1}$), pH 7, 30 ˚C, 250 rpm.

lowing reaction scheme can represent the growth of *Saccharothrix algeriensis* and the thiolutine production. It has been written in Cmol to compare reactions stoichiometries.

R1 Growth on amino acids
$CH_{2.05}O_{0.50}N_{0.26} + 0.046\ O_2 \rightarrow 0.969\ CH_{1.767}O_{0.49}N_{0.21} + 0.0317\ CO_2 + 0.055\ H_2O + 0.07\ NH_4^+$
R2 Maintenance on glucose
$CH_2O + O_2 \rightarrow CO_2 + H_2O$
R3 Growth on glucose
$CH_2O + 0.648\ O_2 + 0.07\ NH_4^+ \rightarrow 0.337\ CH1.767O0.49N0.21 + 0.66\ CO_2 + 0.8\ H_2O$
R4 Thiolutine production
$CH_2O + 0.695\ O_2 + 0.053\ NH_4^+ + 0.053\ SO_4^{2-} \rightarrow 0.213\ CHN_{0.25}O_{0.25}S_{0.25} + 0.79\ CO_2 + 0.97\ H_2O$

The growth on amino acids (R1) is carried out with a weak respiratory activity (Q_R = 0.689) compared to those obtained for the growth on glucose (R3), Q_R = 1. The assimilated amino acids would be integrated directly into the level of the proteinic synthesis without participating to the central metabolism of the micro-organism. The reaction extends presented on Figure 6 confirms the assumptions made on the occurence of the different reactions: the growth is carried out initially on the free amino acids and the unknown substrate brought by the yeast extract assimilated to glucose (R1). When free amino acids are exhausted, the growth takes on glucose (R3). The production of thiolutine (R4) is uncoupled from the growth. It is carried out during the phase of maintenance on glucose (R2). A kinetic model for each reaction will be established from these reaction extends to express the reaction rate as a function of the substrate concentrations.

4 Conclusions

This study shows how it has been possible to acquire some knowledge on *Saccharothrix algeriensis* metabolism from extra cellular compound measurements. The "reaction engineering" approach enables to perform a qualitative and a quantitative analysis on reliable data with respect to elemental mass balances. So, a four reaction scheme has been suggested as a macroscopic view of its growth and the associated production of thiolutine. It was shown that this bacterium was able to grow on both free amino acids and glucose as carbon source. The amino acids of weak molar weight are consumed preferentially to glucose. They seem to be directly integrated to the proteinic synthesis and enriched the medium with ammonia. This ammonia is then assimilated during the growth on glucose. It has been established that the thiolutine production was the result of a secondary metabolism, decoupled from growth. In our experimental conditions it seems to be initiated as soon as a nitrogen source depletion occurs. These results open an interesting way to deepen the knowledge on the behaviour of this bacterium of a rare kind. They give tracks for an exploration of the metabolism to an intracellular level and pose the bases for the production of dithiolopyrrolone at an industrial scale.

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