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

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ABSTRACT

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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- Structured models, like the "Metabolic Flow Analysis" model, which favor a more systemic approach to studying the microorganism. They are generally complex and require a good knowledge of the metabolic network of the bacteria. The quantification of hundreds of flux from a limited number of measurements compels the assumption that all intermediate compounds are in a quasi-stationary state. This drawback may be avoided if intracellular compounds can be measured. Note that the knowledge of such metabolic pathways allows also, thanks to appropriate model reduction, to derive and legitimate macroscopic 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.