**CHEMICAL ENGINEERING TRANSACTIONS**, Volume 14, 2008
Edited by G. Marino, E. Bardone, A. Viglia
 ISBN 978-88-95608-02-0


# *Saccharothrix algeriensis*, a new antibiotic producer: investigations on its secondary metabolism

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The constant growth of the antibiotic world market leads to study rare actinomycete. Therefore, the bacterium *Saccharothrix algeriensis* which products new antibiotics of dithiolopyrrolone class is studied in liquid culture.
The aim of this work is to better apprehend the mechanisms which govern the bacterial growth as well as the dithiolopyrrolones synthesis and to capitalize this knowledge in a general reactional scheme of the metabolism.
The growth of *Saccharothrix algeriensis* was performed in a batch reactor, on a semi-synthetic medium. The growth, the thiolutine (the main dithiolopyrrolone) and the carbon dioxide production and the substrates consumption were monitored. The influence of the substrate concentration on growth and on the production of dithiolopyrrolones was evaluated. It seems that diauxic growth occurs on amino acids then on glucose. The thiolutine synthesis would be induced by carbon substrates exhaustion and by ammonium ions deficiency. Glucose seems to exert a catabolite repression on the secondary metabolic activities of the cell.

## 1. Introduction

The production of new bio active molecules is now a major concern to answer to the fact of the proliferation of microbial pathogen strains resistant to the molecules currently available (Butler et al. 1996; Critchley et al. 2007; Linares et al. 2007). Thus researches tend to exploit the biodiversity in order to find new bio active molecules with microbial origin (Boudjella et al, 2005). In this context, the bacterium *Saccharothrix algeriensis*, belonging to actinomycetes family and producing new antibiotics of dithiolopyrrolone class, has been isolated in Saharan soils in Algeria (Zitouni et al. 2004). Dithiolopyrrolone antibiotics have others interesting properties: antifungi, insecticide and anticancer activities (Webster and Chen, 2000; Minamiguchi et al. 2001, Webster et al. 2002, Xu 1998).
*Sa algeriensis* is able to produce at least five dithiolopyrrolone antibiotics: thiolutine, senecyol-pyrrothine (SEP), tigloyl-pyrrothine (TIP), isobutyryl-pyrrothine (ISP) and butanoyl-pyrrothine (BUP) as shown in figure 1. *Sa algeriensis* is the only known microorganism able to produce TIP and SEP (Lamari et al. 2002; Bouras et al. 2006). Recent works showed that the nature of the radical ® depends on the culture medium composition. Each association "organic acid-heterocycles" leads to different molecules with different properties, which makes it an interesting model to study (Bouras et al. 2006; Bouras et al. 2007). But few data exist on the macroscopic behaviour of the bacterium in a semi-synthetic medium.

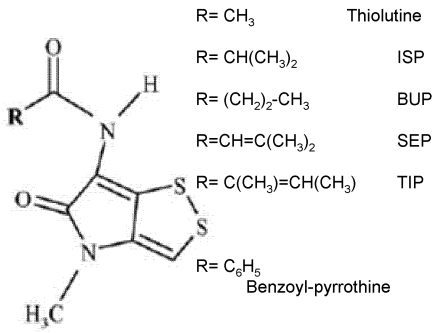


**Fig.1** *Dithiolopyrrolonne antibiotic structures*

The aim of this work is to study the development of *Sa algeriensis* in different conditions to capitalize knowledge on its metabolism and at term to control qualitatively and quantitatively dithiolopyrrolone production. The development of *Sa algeriensis* was followed on semi-synthetic medium where majority of the substrates was identified. The cultures have been carried out in batch reactor with probes for monitoring and controlling physical and chemical environment of the bacterium. In parallel substrates and products concentrations have been followed by offline measurements.

## 2. Materials and Methods

### 2.1 Flasks and reactors cultures conditions

*Sa algeriensis* NRRL B-24137 was used in this study. Spores were maintained in 25% glycerol at -20°C. Spores were obtained from solid culture of *Sa algeriensis* on conservation medium.

For the precultures, a volume of 100 mL of semi synthetic medium was inoculated by 3.5 mL of spores suspension and incubated on a rotary shaker (New Brunswick Scientific Co., NJ, USA) at 250 rotation per minute (rpm) and 30°C for 52 h.

Reactors were inoculated with 100 mL of preculture. Cultures were performed in a NBS fermentor containing 2 L of medium. Culture lasted for at least one week. pH was maintained at 7± 0.035 by automatic addition of NaOH solution (1 $mol.L^{-1}$) and HCl solution (1 $mol.L^{-1}$). Aeration rate of one vessel volume per minute (vvm) was employed. The agitation rate was controlled to keep the dissolved oxygen level above 30% of the saturation with a starting rate of 150 rpm. The pH and dissolved oxygen level were monitored using Ingold specific electrodes. Temperature was regulated at 30°C. $O_2$ and $CO_2$ content in the exhaust gas were determined by a gas analysor (Servomex 4100, paramagnetic transductor for $O_2$ and infrared transductor for $CO_2$).

### 2.2 Culture media compositions

*Conservation media: International Streptomyces project 2 (ISP2)*

The ISP2 medium had the following composition (per liter of distilled water): 10 g malt extract, yeast extract 4 g, glucose 4 g and agarose 18 g.

*Growth and production medium*

The 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, $(NH_4)_2SO_4$ 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.

**2.3 Analytical procedures**

For the estimation of dry cell weight (DCW), 3 mL samples of homogenized culture broth were centrifuged at 16000 g for 10 min in preweighed Eppendorf tubes. Pellet was washed twice with distilled water. Supernatant was kept for other analysis. Eppendorf tubes containing pellets were dried at 105°C for 48 h, and weighted after being cooled for 30 min in a dessicator (Bouras et al., 2006). The measurement relative error is 5 %.

The analysis of dithiolopyrrolone antibiotics was carried out by non polar chromatography (HPLC, Bio-Tek Instruments (Bouras et al., 2007)) The measurement relative error is 10 %. Supernatant coming from DCW determination was filtered at 0.2 µm and used to measure metabolites.

A biochemical analyzer with enzymes fixed on membrane has been used (YSI2700 select) to quantify glucose. Amperometric quantification after the enzymatic oxidation allows determining glucose concentration in the culture broth. 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 have been quantified using specific enzymatically methods (Diagnostics Ammonia kit from Boehringer-Mannheim, using glutamate deshydrogenase and Microdom kit using glutamate oxydase respectively) 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 %.

## 3. Results

**3.1 Growth on semi-synthetic medium**

Figures 2 and 3 present the evolution of glucose, α-amino nitrogen, biomass, ammonium ion and thiolutine during a batch culture of *Sa algeriensis* on semi-synthetic medium. Four periods can be distinguished. The first period (1) is a growth phase of the microorganism. It is accompanied by a diminution of α-amino nitrogen concentration (figure 2); it is to say amino acids consumption, and an increase in the ammonium ions concentration. It could be explained by the deamination of the consumed amino acids from yeast extract (figure 3). There is no apparent glucose consumption, by against the amino acids are quickly consumed by the bacterium. The second period (2) is characterized by a brake in the growth. During this period, the rest of the amino acids and the glucose are consumed. The ammonium ions concentration is stable. The third period is a growth phase on glucose and ammonium ions longer than the first one on amino acids (3). The ammonium ions could be used as nitrogen source. The last period (4) is characterized by a decrease of biomass concentration that seems to coincide with ammonium ions exhaustion. It is probably caused by cell lysis.

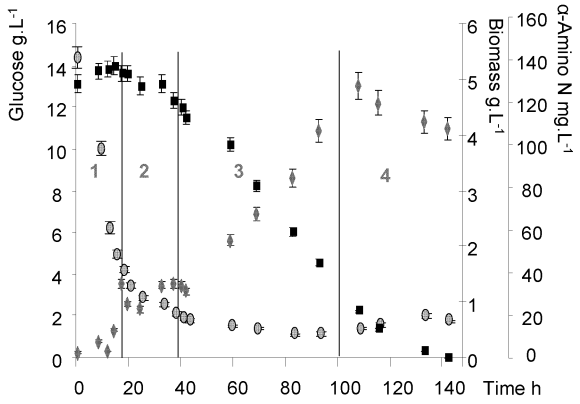


**Fig.2** *Sa. algeriensis batch culture on semi-synthetic medium in batch reactor, 30°C, pH 7.00 ± 0.035 upH, 0.5 vvm and 250 rpm.* ○ *α-amino N (mg.L$^{-1}$),* ■ *glucose (g.L$^{-1}$) consumption and* ◈ *biomass production (g.L$^{-1}$).*

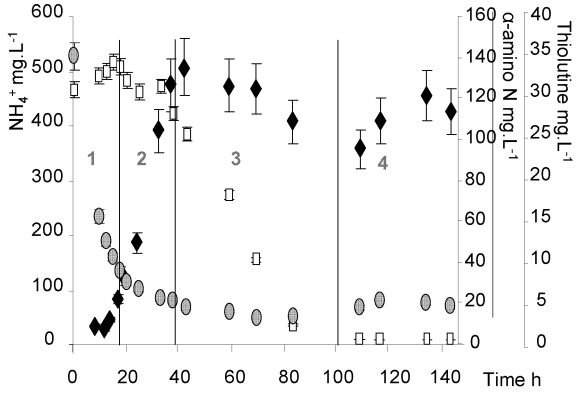


**Fig.3** *Thiolutine production* ♦ *(mg.L$^{-1}$),* α*-amino N* ○ *(mg.L$^{-1}$), and ammonium ions* □ *(mg.L$^{-1}$) in Sa algeriensis batch culture on semi-synthetic medium, 30°C, pH 7.00 ± 0.035 upH, 0.5 vvm and 250 rpm.*

*Sa algerinsis* growth seems to be diauxic (Narang and Pilyugin 2006) on semi-synthetic medium. Indeed, in the first time, the amino acids are used for biomass production (period 1), and then when they are almost exhausted, after an adaptation period (period 2), the bacterium uses glucose and ammonium ions for its development (period 3). The fact that other actinomycetes have a diauxic growth confirms our comments (Novotna et al. 2003).

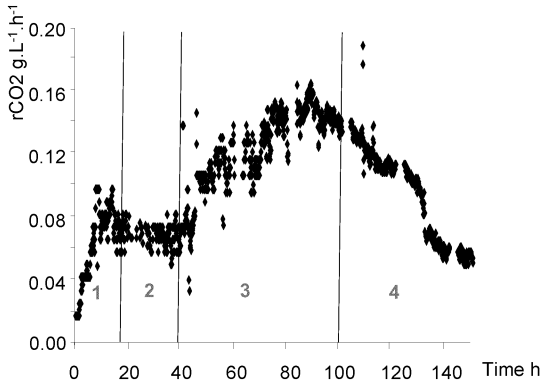


**Fig.4**: *Sa. algeriensis batch culture on semi-synthetic medium in batch reactor, 30°C, pH 7.00 ± 0.035 upH, 0.5 vvm and 250 rpm. ♦ Carbon dioxide consumption speed ($r_{CO2}$ in $g.L^{-1}.h^{-1}$).*

Figure 4 shows the $CO_2$ production speed evolution during the same culture.
It appears that the evolution phases of $CO_2$ production speed is perfectly calked on the phases of the biomass development (Figure 2). The period 1 is accompanied by an increase of the $CO_2$ production speed. It does not change during the period 2 because of this adaptation phase. The next period (3) is characterized by an increase of the $CO_2$ production speed. The last period of cells lysis (4) is accompanied by a decline in this production speed. From a practical point of view, the on-line study of the carbon dioxide production by gas analysis allows observing the important metabolic events, like biomass development or changes of consumed substrate. As a result, the on-line $CO_2$ measures can be used as a mean of monitoring the culture in absence of off-line measures.

**3.2 Secondary metabolism induction**

The thiolutine production in parallel to the α-amino nitrogen and ammonium ions consumptions is presented in figure 3. The thiolutine concentration starts to increase at the end of the period 1 when most of the amino acids are exhausted from the culture broth. In the period 3, the thiolutine concentration decreases. The thiolutine would be degraded or transformed into other dithiolopyrrolones derivatives by the microorganism. There is a second phase of thiolutine production following ammonium ion depletion during the period 4. Carbon and nitrogen limitations induce a slowdown in growth or metabolic activity of the microorganism which would cause the thiolutine synthesis in the culture broth (Wilson and Bushell 1995; Melzoch et al. 1997). However, the fact that a microorganism produces sequentially its secondary metabolites is rarely described in literature (Bushell and Fryday 1983).

**3.3 Glucose catabolite repression**

Figure 5a presents the thiolutine production as a function of the time at three glucose concentrations (3, 5.5 and 8 $g.L^{-1}$). Figure 5b presents biomass production at three different times of culture for these same glucose concentrations. The three concentrations allow the thiolutine production. On the other side, it is important to notice that they have no impact on biomass production. However, production depends on the initial glucose concentration. Culture containing 8 $g.L^{-1}$ glucose reaches the lower production (14.8 $mg.L^{-1}$ in 30 h) while the one containing the least of glucose allows the best production of thiolutine (3.28$mg.L^{-1}$ in 30 h). The more initial glucose concentration in the culture medium the less production of thiolutine in the first 30 h of culture is.

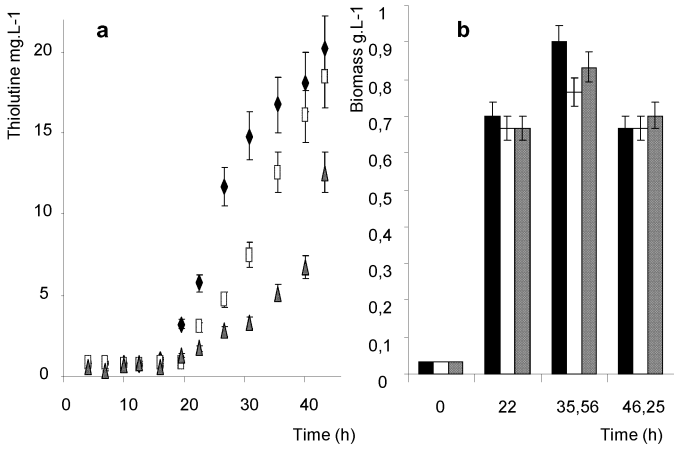


**Fig.5** *a) Thiolutine production ($mg.L^{-1}$) versus different glucose concentrations. ♦: 3 $g.L^{-1}$ ; □ :5.5 $g.L^{-1}$ and ▲ :8 $g.L^{-1}$. b) Biomass production ($g.L^{-1}$) versus different glucose concentrations black: 3 $g.L^{-1}$ ; white :5.5 $g.L^{-1}$ and grey :8 $g.L^{-1}$. Sa. algeriensis batch culture on semi-synthetic medium in batch reactor, 30°C, pH 7.00* ± 0.035 upH, 0.5 vvm and 250 rpm

Moreover, the observation of figure 5b shows that whatever the time of culture, biomass produced is nearly the same in the three conditions. It seems that the glucose has a repressive effect on the secondary metabolism. Then it does not affect the growth of the bacterium. This regulation exists in other actinomycetes (Demain 1998; Hodgson 2000) but not on theirs biosynthetic activities.

## 4. Conclusion

*Sa algeriensis* is able to grow from several substrates sequentially consumed. On the other hand, from a practical point of view, the $CO_2$ consumption speed measure allows an easily monitoring of the various culture phases. The secondary metabolism of this actinomycete seems to be inducted by exhaustion of different carbon and nitrogen substrates which are in the culture broth. Thiolutine production occurs when the substrate which is currently metabolised became limiting. As the several changes of substrate during the batch culture, the production phenomenon can occur several times during a batch culture.

The establishment of a general reaction scheme should allow validating the hypotheses the diauxic growth of the microorganism. It will be tested on other experimental conditions. A mathematical model representing the growth of the bacterium can be developed. At term, it will allow the control of the produced derivate, and certainly the production of dithiolopyrrolone class new members

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