

## Simultaneous production and decomposition of different rifamycins during *Amycolatopsis mediterranei* growth in shake flask and in stirred tank bioreactor

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The present work deals with the kinetics of rifamycins production and degradation by *Amycolatopsis mediterranei* in submerged culture using industrial medium. Cultivations were conducted in shake flasks and stirred tank bioreactor to evaluate the influence of transfer of process to higher scale on overall process kinetics and to obtain better understanding for the kinetics of rifamycins production and degradation. Both rifamycins B and SV increased with time in both shake flasks and bioreactor cultures and reaching their maximal value after about 200 h. The maximal production of rifamycins B and SV was about 4832 mg l<sup>-1</sup> and 3967 mg l<sup>-1</sup>, respectively, in bioreactor cultures. These values were about 75% higher than those obtained in the corresponding batch culture in shake flasks under the same cultivation conditions. On the other hand, rifamycin O is produced as an intermediate product reaching its maximal value after about 100 h and decomposed again with time and totally disappeared at the end of cultivation time.

**Keywords:** *Amycolatopsis mediterranei*; Rifamycins; Batch cultivation

### 1. Introduction

The rifamycins are a family of ansamycins characterized by an aliphatic bridge spanning a naphthalene nucleus. Among different types of rifamycins, rifamycin B, O, S and SV are the most important members because of their relative higher stability in fermentation broth. The rifamycins and semisynthetic drugs derived from them exert their antibiotic activity by specific inhibition of bacterial DNA-dependent RNA polymerase of different microbial species [31-33]. At higher concentrations, these antibiotics also inhibit the RNA-dependent DNA polymerase of retroviruses as mono-therapy or in combination with other drugs [20,23,30]. Therefore, antibiotics of the rifamycin class such as rifampicin, rifabutin and rifapentine have been employed on a global basis in a number of well established combination regimes for the clinical treatment of tuberculosis, leprosy, AIDS-related mycobacterial infections and many other enteric infections [14,15,18,21,27,29]. Moreover, rifamycins enhance anticancer drugs accumulation. Thus, it is applied in multi-drug resistance and used in combination with chemotherapy for cancer treatment [9]. Since the first industrial production of rifamycins in the mid 1960s by the Italian drug company Lepetit SpA, the production is carried out by *Amycolatopsis mediterranei* (formerly known as *Streptomyces mediterranei* and *Nocardia mediterranei*). The production of rifamycins is carried out mainly in submerged culture using either free cells in batch/fed-batch cultures [2,6,17,19,30] or immobilized cells [1,7,9,10].

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Nowadays, the industrial production of rifamycins is usually carried out using complex media with complex nitrogen source and high glucose concentration [3,4,16,17]. In the biosynthesis of rifamycins, only one general pathway is operating in which the later biosynthesis step splits off into several branches leading to different rifamycins types. The key intermediate in the biosynthesis of a considerable number of rifamycins is rifamycin S, which can be reduced in presence of NADPH to rifamycin SV, a precursor of rifamycin B. On the other hand, rifamycin B can be transformed by oxidative degradation to rifamycin O which is readily hydrolysed to rifamycin S. Thus, the inter-conversion between different types of rifamycins under different conditions is possible. However, it is also worthy to note that the biosynthesis ratio of different types of rifamycins in culture is not only medium composition dependent but also strain dependent [5,19].

In the present work, the production of rifamycins was studied in both shake flask and bioreactor level using industrial media. The cultivations were undertaken in complex media containing glucose, defatted soybean meal and defatted peanut flour. The analysis of the different growth and production patterns obtained could be partially explained by the influence of the scale of production process and its relation to growth and production kinetics.

## 2. Materials and Methods

### 2.1 Microorganism

The rifamycins producer strain *Amycolatopsis mediterranei* (ATCC 21789) was obtained from the American Type Culture Collection (Chicago, IL, USA).

### 2.2 Propagation and selection

Bennett's agar medium was used for propagation and colony selection. This medium was consisted of ( $\text{g l}^{-1}$ ): meat extract, 1.0; enzymatic hydrolysate of casein, 2.0; yeast extract, 1.0; glucose monohydrate, 12.0 and agar, 20.0. The pH was adjusted to 7.2 before sterilization. The inoculated plates were incubated at 28 °C for 13 days [24]. After incubation, typical high rifamycins producer colonies were selected and used to inoculate the Q2 medium according to the method of Farid *et al.* [12].

### 2.3 Medium for maintenance

The Q2 agar medium was used for maintenance of cells according to the method of El-Tayeb *et al.* [8]. This medium consists of ( $\text{g l}^{-1}$ ): yeast extract, 4.0; malt, 10.0; glucose monohydrate, 4.0; oat flakes, 20.0 and agar, 20.0. The pH was adjusted to 7.6 before sterilization and the slants were incubated at 28 °C for 8 days. After incubation, the surface growth of typical slant cultures was used to inoculate the vegetative medium.

### 2.4 Inoculum preparation

The inoculum for rifamycin B production was prepared in a vegetative medium containing ( $\text{g l}^{-1}$ ): glucose monohydrate, 20.0; defatted soybean meal, 20.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.4;  $\text{CaCO}_3$ , 2.5;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05 and  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.003. The pH was adjusted to 6.9 and 250 ml Erlenmeyer flasks with 50 ml vegetative medium were incubated at 28 °C for 48 h in a rotatory incubator shaker at 250 rpm (Innova 4330, New Brunswick Scientific Co., NJ, USA). The grown cells were used to inoculate both 250 ml baffled flask containing 25 ml fermentation medium and 3 L stirred tank bioreactor in concentration of 5% ( $\text{v v}^{-1}$ ).

## 2.5 Rifamycins production medium

The fermentation medium was composed of ( $\text{g l}^{-1}$ ): defatted peanut flour, 25.0; defatted soybean meal, 10.0; glucose monohydrate, 140.0; propylene glycol, 10.0; sodium diethyl barbiturate, 1.7;  $(\text{NH}_4)_2\text{SO}_4$ , 9.6;  $\text{CaCO}_3$ , 11.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0;  $\text{KH}_2\text{PO}_4$ , 1.0;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.012 and  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.003. The pH of medium was adjusted to 8.1 before sterilization and the inoculated flasks were incubated at 28 °C for 8 days at 250 rpm. on a rotary shaker. In case of bioreactor experiments, cultivation was carried out in a 3 L stirred tank bioreactor Bioflow III (New Brunswick Scientific Co., New Brunswick, NJ, USA) with a working volume of 2.0 L. Agitation was performed using a two 4-bladed Rushton turbine impellers ( $d_{i(\text{impeller diameter})} = 65 \text{ mm}$ ;  $d_{t(\text{tank diameter})} = 135 \text{ mm}$ ,  $d_i d_t^{-1} = 0.48$ ). Agitation was started at 300 rpm and increased gradually during the process up to 800 rpm to keep the DO value above 20% saturation. Aeration was performed by filtered sterile air [ $1 \text{ v v}^{-1} \text{ min}^{-1}$ ] at the beginning of cultivation and increased gradually with time up to  $2 \text{ v v}^{-1} \text{ min}^{-1}$  cascaded with agitation speed to keep the DO value above 20% saturation. Dissolved oxygen concentrations were analyzed by polarographic electrode (Ingold, Germany). Foam was suppressed, when necessary, by the addition of silicon antifoam reagent (Fluka, Switzerland).

## 2.6 Sample preparation and determination of total dry weight

Samples, in the form of 2 flasks of 25 ml broth for each, or 15 ml broth (in case of bioreactor cultures) were taken at different times during cell cultivation in preweighed centrifugation tube (falcon, USA), centrifuged at 3000 rpm for 20 min. A small fraction of the supernatant was frozen at -20°C and left for glucose and carbohydrates analysis. The centrifuged cells were washed twice by distilled water followed by centrifugation. Centrifugal tubes were then dried to constant weight at 80°C to determine the total dry weight (TDW).

## 2.7 Rifamycins determinations

Rifamycin B in the fermentation broth was determined spectrophotometrically according to the method of Pasqualucci *et al.* [28] as follows: Samples were prepared for assay by taking two 1-ml aliquots of fermentative broths. One aliquot was diluted 1:6 with buffer A (acetate buffer of pH 4.63 prepared by mixing of 100 ml 1N NaOH and 200 ml 1N glacial acetic acid, and completing the final volume to 1 L with dist. water) and the other was diluted similarly with buffer B (buffer A containing 0.1% w/v sodium nitrite) as blank. After shaking for 5 min, both samples were filtered using Whatman filter paper no 1. Buffer A treated samples were measured using spectrophotometer (Smart Spec 3000, BioRad, USA) against their blank at 425 nm to determine the rifamycin B concentration. Rifamycin SV was determined similarly at 447 nm. For rifamycin O determination, samples diluted with buffer C (buffer A containing 0.1%w/v ascorbic acid) were measured at 425 nm against samples diluted with buffer A as blank.

## 2.8 Glucose determination

Glucose was determined enzymatically using glucose oxidase-glucose peroxidase kit (Diamond Diagnostics, Cairo, Egypt). The intensity of developed color was determined colorimetrically at 550 nm using spectrophotometer (Smart Spec 3000, BioRad, USA).

# 3. Results and Discussion

The kinetics of cell growth and rifamycins production by *A. Mediterranei* in shake flasks and bioreactor cultures were studied under uncontrolled pH conditions. The cultivation temperature, type and age of inoculum were the same in both cultures. Thus, data were taken from two parallel experiments and the only difference was the production scale.

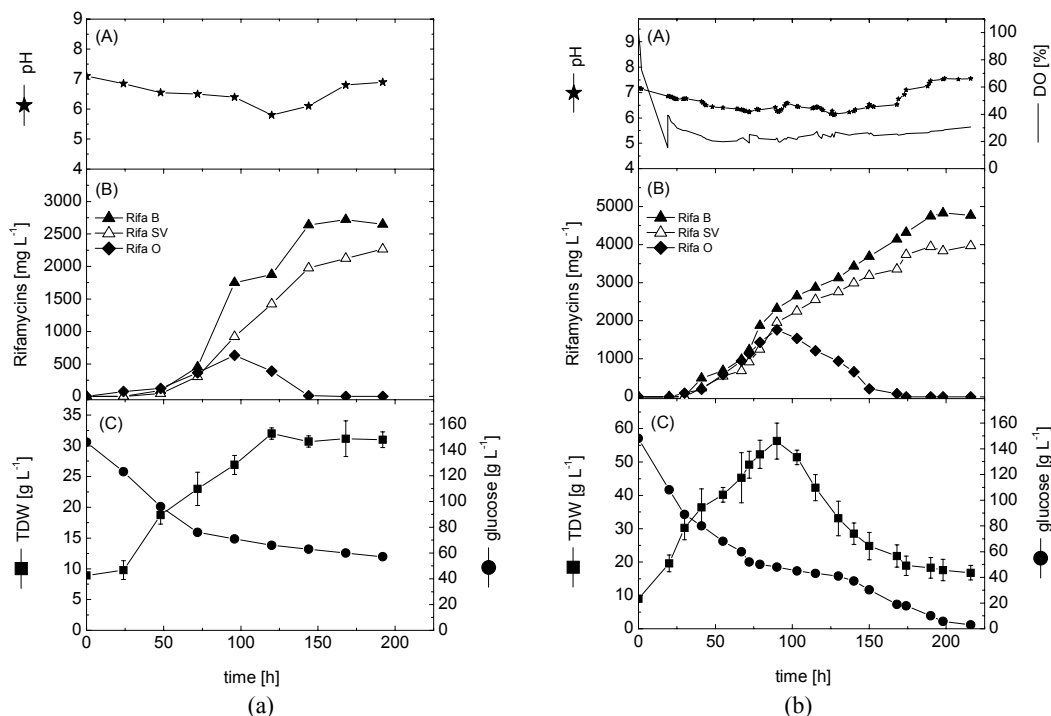
### 3.1 Production of rifamycins in shake flask cultures

The changes in cell growth, glucose concentration, rifamycins production and pH during cell cultivation are depicted in fig. 1a. As shown, a fairly short lag phase was observed followed by exponential growth of cells. The total dry weight reached its maximal value of about  $32 \text{ g l}^{-1}$  after 120 h and kept more or less constant for the rest of cultivation time. The initial increase in cell mass was important to support high biomass to maximize the yield of rifamycins production [22]. Concomitantly, glucose concentration in the fermentation medium decreased rapidly during the exponential growth phase with rate of  $0.97 \text{ g l}^{-1} \text{ h}^{-1}$ . On the other hand, the pH of culture slightly decreased and reached 5.6 at the end of growth phase. The initial drop in pH was most probably due to accumulation of intermediate organic acids from carbohydrate metabolism whereas the subsequent increase in pH was due to assimilation of complex nitrogen sources in culture and release of ammonium ion. Rifamycins production began early during the growth phase with very low rate. The rifamycin O production rate was about  $6.58 \text{ mg l}^{-1} \text{ h}^{-1}$  during the cell growth phase reaching its maximal value of  $632 \text{ mg l}^{-1}$  after 96 h. The rifamycin O concentration decreased thereafter with rate of  $12.96 \text{ mg l}^{-1} \text{ h}^{-1}$  and was completely absent from the culture medium after 144 h. Similarly, rifamycin B and SV production started during the early growth phase. The maximal rates of antibiotics production were observed from 72 h to 144 h of cultivation. The rifamycin B concentration kept more or less constant thereafter, whereas rifamycin SV concentration increased gradually until the end of cultivation time. The maximal production of rifamycin B and SV reached  $2720 \text{ mg l}^{-1}$  after 144 h, and  $2265 \text{ mg l}^{-1}$  after 192 h, respectively. Thus, we can conclude that, after termination of cell growth, the glucose consumed was mainly directed to rifamycins B and SV production.

### 3.2 Production of rifamycins in bioreactor cultures

Figure 1b shows different changes in culture during the batch production of rifamycins after the process scaling up to bioreactor level. Compared to shake flask culture, shorter growth phases were observed in this culture. The total dry weight reached its maximal value of about  $56.3 \text{ g l}^{-1}$  after 90 h and decreased thereafter until the end of cultivation time. The decrease in biomass may be due to the high shear stress in bioreactor compared to gentle shaking in shake flasks. Concomitantly, glucose concentration in the cultivation medium decreased gradually during the cultivation process. The glucose consumption rate in bioreactor culture was about  $1.45 \text{ g l}^{-1} \text{ h}^{-1}$ . This value was higher by about 50% than those obtained in shake flask cultures. The pH profile of culture was similar to shake flask culture. During the growth phase, the pH decreased gradually and reached its minimal value of 6.05 after 140h. The pH of culture increased thereafter until the end of cultivation time. The drop in pH during growth phase and the subsequent increase of pH could be also attributed to the successive consumption of carbohydrates and acid production during the early growth phase, followed by the consumption of protein concomitant with the release of ammonia. The production of rifamycins began after about 20 h. Rifamycin O was firstly produced and reached a maximal value of  $1760 \text{ mg l}^{-1}$  after 90 h. During that time, the rate of rifamycin O production was about  $25.14 \text{ mg l}^{-1} \text{ h}^{-1}$ . The concentration of rifamycin O in culture reduced thereafter with rate of  $20.95 \text{ mg l}^{-1} \text{ h}^{-1}$ . When comparing these data with shake flask culture, it was clear that the production of rifamycin O and its degradation took place at higher rates in bioreactor culture. Similarly, rifamycin B and SV production started after a short lag phase of about 30 h and 40 h, respectively and increased gradually till the end of cultivation time. The production of rifamycin B and SV reached maximal of  $4832 \text{ mg l}^{-1}$  after 198 h, and  $3967 \text{ mg l}^{-1}$  after 216 h, respectively. These values were about 1.7 folds higher than those value obtained in shake flask cultures. The production rates of rifamycin B and SV were  $29.94 \text{ mg g}^{-1} \text{ h}^{-1}$  and  $25.93 \text{ mg g}^{-1} \text{ h}^{-1}$ , respectively. It is also worthy to note that the difference between the rate of rifamycin B and SV production in bioreactor and in shake flasks could be attributed to the higher aeration conditions which favour production of the more oxidized form rifamycin SV. It is also noticed that after 90 h cultivation, glucose consumption was mainly directed toward rifamycin B and SV production. These results agree in principal with our data published before [6]. From

the results of this work, it seems clear that with proper control of aeration conditions, the yield and rates of different types of rifamycins could be improved. For better understanding of the effect of production scale on cell growth and antibiotic production kinetics, different kinetic values were calculated and summarized in table 1.



**Fig. 1** Kinetics of cell growth and rifamycins production during *A. mediterranei* cultivation in shake flask (a) and stirred tank bioreactor in batch culture (b).

**Table 1** Kinetic parameters of cell growth and rifamycins production by *A. mediterranei* during shake flask and bioreactor cultivations in batch cultures.

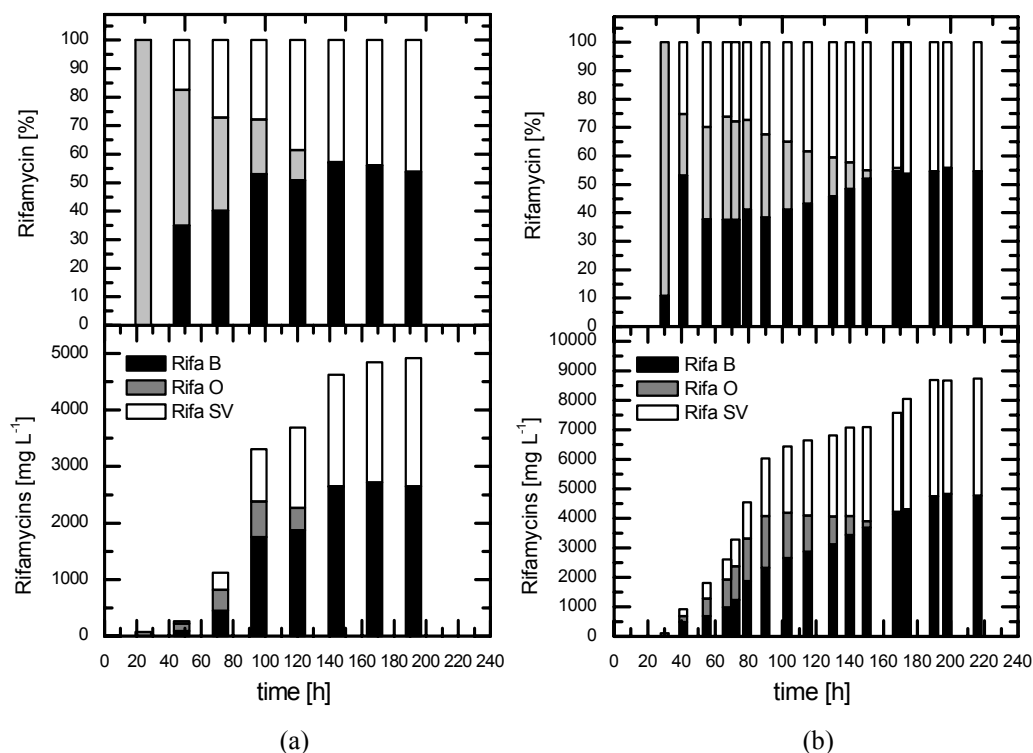
1) Parameter	Artikel II. Type of cultivation vessel	
	Artikel III. Shake flask	(i) Bioreactor
$TDM_{max}$ [g l <sup>-1</sup> ]	32.0	56.3
$P_{rifam B}$ [mg l <sup>-1</sup> ]	2720	4832
$P_{rifam SV}$ [mg l <sup>-1</sup> ]	2265	3967
$P_{rifam O}$ [mg l <sup>-1</sup> ]	632	1760
$-Q_S$ [g l <sup>-1</sup> h <sup>-1</sup> ]	0.97	1.45
$Q_{rifam B}$ [mg l <sup>-1</sup> h <sup>-1</sup> ]	22.0	29.94
$Q_{rifam SV}$ [mg l <sup>-1</sup> h <sup>-1</sup> ]	16.92	25.93
$Q_{rifam O}$ [mg l <sup>-1</sup> h <sup>-1</sup> ]	6.58	25.14
$-Q_{rifam O}$ [mg l <sup>-1</sup> h <sup>-1</sup> ]	12.96	20.95

Abbreviations:  $TDM_{max}$ : maximal total dry mass;  $P_{rifam B}$ : maximal volumetric rifamycin B production;  $P_{rifam SV}$ : maximal volumetric rifamycin SV production;  $P_{rifam O}$ : maximal volumetric rifamycin O production;  $-Q_S$ : glucose consumption rate;  $Q_{rifam B}$ : volumetric rifamycin B production rate;  $Q_{rifam SV}$ : volumetric rifamycin SV production rate;  $Q_{rifam O}$ : volumetric rifamycin O production rate;  $-Q_{rifam O}$ : volumetric rifamycin O degradation rate.

### 3.3 Production ratios between rifamycins B, O and SV at different production scales

Figure 2 shows the ratios between different types of rifamycins produced in both shake flasks and bioreactor cultures. As shown, the rifamycin O production started from the early time of cultivation, superior to rifamycins B and SV, with high production rate in both shake flask and bioreactor culture. After about 140 h, the rifamycin O was almost disappeared from the culture media. This was due to its conversion to either rifamycin B and/or SV. The transformation of rifamycin O to other types of rifamycins is usually mediated by its conversion to the intermediate form, rifamycin S, in the first transformation step [13]. Both rifamycins B and SV were produced in the production media after 40 h in both cultures. The concentration of both rifamycins B and SV increased with time until the end of cultivation.

The production of rifamycin B in high concentration was mostly due to addition of barbital in the cultivation media. The addition of barbital (5,5-diethylbarbituric acid) to the fermentation medium shifts the metabolic pathway to more rifamycin B biosynthesis [25,26]. On the other hand, the high rifamycin SV production in our study was due to the strain used. However, the biosynthesis ratio between different types of rifamycins is not only nutrient dependent but also strain specific [5,19]. However, as shown in fig. 2, the ratio between different types of rifamycins produced was almost the same in both shake flask and bioreactor cultures. Thus, we can conclude that the differences in cultivation conditions during process transfer from shake flask to bioreactor, which are mainly aeration/agitation effects, are not critical factors for the inter conversion between different types of rifamycins.



**Fig. 2** Production of different types of rifamycins in fermentation medium during *A. Mediterranei* growth in shake flask (a) and bioreactor (b) cultures.

## 4. Conclusion

Rifamycins production was higher in bioreactor cultures compared to shake flask. Most probably this was due to better mixing and aeration conditions in bioreactor, which lead to better mixing between gas and liquid phases. These resulted in better oxygen transfer and more oxygen availability for the cells. These conditions favour firstly the growth of aerobic *A. mediterranei* and thus we can conclude also that oxygen is one of the main limiting factors in rifamycins production.

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