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Research Article

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Enhanced production of Rifamycins B and SV by medium supplementation with Vermiculite

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ABSTRACT

Rifamycins are broad spectrum and important antibiotics used in the treatment of pathogenic diseases. They are generally produced by the fermentative cultivation of Amycolatopsis mediterranei, using submerged cultivation system. The medium composition greatly influences the antibiotic production. Trace elements are one of the major factors need to be optimized to enhance the production of rifamycins. In the present work, we investigated the possible improvement of rifamycins B and SV production through the addition of vermiculite (dehydrated magnesium-aluminum-ion silicate) as a cheap and affordable source of trace elements. The obtained results showed that the addition of 20 g/L vermiculite gave the highest volumetric production of rifamycins B and SV (767.5 and 1265 mg/L for Rif B and SV, respectively). Moreover, addition of vermiculite at the beginning of the cultivation resulted in the highest volumetric productivities in comparison to control cultivation (770 and 1305 mg/mL of rifamycin B and SV, respectively). Comparative growth curve kinetics between vermiculite-supplemented medium and vermiculite free medium demonstrated that vermiculite significantly enhanced cell growth, cell growth rate, glucose consumption rate, as well as the production rates of both rifamycins B and SV compared to cultivation without vermiculite. In addition, vermiculite supplementation increased both maximal volumetric productivity and production rates increased by about 32 and 52% for rifamycin B and SV, respectively, as compared to vermiculite-free cultivation.

Keywords: *Amycolatopsis mediterranei*, Rifamycins, vermiculite, submerged fermentation.

INTRODUCTION

Rifamycins are a group of antibiotics belong to ansamycin family and include different members such as (Rifs B, O, S, and SV). This antibiotic family is characterized by their unique structure, which comprises an aromatic moiety bridged by an aliphatic chain. The importance of rifamycins is based on their wide medical application as broad spectrum antibiotics against large group of microbes belong to G+ve and G-ve bacteria. Moreover, rifamycin is one of the best medicine of choice in the treatment of tuberculosis, leprosy, AIDS-related mycobacterial infection, peptic ulcer, and toxoplasma infection [1-3]. In addition, recent research reported on the anti-inflammatory and immunomodulatory activities of rifamycin SV [4]. Nowadays, rifamycins and rifamycins-derivatives are present in

the pharmaceutical market under different trade names such as: rifampicin, rimactane, rifampin, rifabutin, rifapentine, rifalazil, rifadin, rifaximin[5]. Rifamycins are produced by the actinomycetes *Amycolatopsis mediterranei* in either submerged culture or in solid state fermentation[6,7]. However, the industrial production of this antibiotic is carried out in submerged culture system either in batch or in fed-batch culture [8,9]. Moreover, many studies were also carried out for the production of rifamycins using immobilized cell systems to reduce the production time and increase the antibiotic yield [10-13]. However, *A. mediterranei* produces different types of rifamycins and is characterized by high strain intra-population variation. In our previous study[14], we characterized the highly rifamycin producing colony which synthesize mainly rifa B and rifa SV (the most important members of the rifamycin family and highly considered as starting compounds for the production of new generation semi-synthetic antibiotics). The production of rifamycins by *A. mediterranei* is highly affected by the medium composition and cultivation conditions [8, 9, 15]. As reported by many researchers, the production of secondary metabolites such as antibiotics by microorganisms is highly affected by inorganic trace elements [16-19]. They act as important enzyme catalysts in many metabolic pathways. Vermiculite (dehydrated magnesium-aluminum-ion silicate) is a common material with many industrial and agricultural applications. It is widely used in industries as insulation material, packing material, additive to fireproof, soilless growing media, seed germination, soil conditioner, and egg incubation. More recently, based on its mineral content, it was also used as medium supplement during bacterial and fungal inoculant preparation in submerged solid state and submerged cultures [20-22]. In addition, vermiculite supplementation to the fermentation medium showed positive impacts on citric acid production by *Aspergillus niger*[23].

Our previous studies demonstrated the potential application of vermiculite to support oxytetracycline production by *Streptomyces rimosus*[24]. This research was focused on the evaluation of vermiculite suitability to support rifamycins B and SV production as cheap source for balanced inorganic nutrients required for antibiotic production.

EXPERIMENTAL SECTION

Microorganism, propagation and selection

Amycolatopsis mediterranei ATCC 21789 was used in this study for Rifamycin B and SV production. This strain was initially obtained from American Type Culture Collection (University Boulverad Manassas, VA, USA). The strain was first propagated in ISP-2 medium composed of (g/L): malt extract, 10.0; yeast extract, 4.0; and glucose, 4.0.

Propagation and selection of highly producer colony

The grown vegetative cells were sub-cultured on Bennett's agar medium for colony selection [14].This medium have the following composition in (g/L): yeast extract, 1.0; enzymatic hydrolysate of casein, 2.0; yeast extract, 1.0; glucose, 12.0; and agar 20.0. The pH was adjusted to 7.2 before sterilization. The inoculated plates were incubated at 28°C for 14 days. Of different colonies arisen on the agar plate, small dark red colony was selected and further propagated on Q2 maintenance medium composed of (g/L): yeast extract, 4.0; malt extract, 10.0; glucose, 4.0; oat flakes, 20.0 and agar 20.0. After 8 days cultivation, the arisen colonies were used to inoculate vegetative medium to support high cell growth. The vegetative medium contained (g/L):glucose, 20.0; yeast extract, 5.0; $MgSO_4.7H_2O$, 0.016, KH_2PO_4, 3.0; zinc acetate, 0.001, and the pH of the medium was adjusted to 7.0 pH before sterilization. Cultivation was carried out in 250 mL Erlenmeyer flask of 50 mL broth. The inoculated flasks were incubated at 28°C for 48 h on a rotary shaker at 200 rpm. The grown cells were used to inoculate fermentation medium in a final concentration of 5% (v/v).

Rifamycin production medium

The initial fermentation medium for rifamycins production was composed of (g/L): glucose, 40.0; yeast extract, 5.0; KH_2PO_4, 3.0; K_2HPO_4, 1.5; $MgSO_4.7H_2O$, 0.016, zinc acetate, 0.001, the pH was adjusted to 7.0 before sterilization [8]. Glucose was sterilized separately and added to the cultivation medium before inoculation. The inoculated flasks were incubated at 28°C for 7 days at 200 rpm on rotary shaker. In vermiculite supplemented cultures, vermiculite was added to the fermentation medium in different concentrations ranged between (0 to 40 g/L) before sterilization.

Sample preparation and determination of total dry weight

Samples in the form of three flasks (50 mL each) were withdrawn at different time intervals during the cultivation for analysis. Samples were filtered using dry and pre-weighed filter paper (Whatman filter paper No. 1). The supernatants were taken for determination of glucose and antibiotic activity. The filtered biomass was washed twice

by distilled water and subsequently dried in an oven at 100°C for a constant weight. In case of vermiculite medium which contains insoluble fractions, the differences between the weight of the inoculated and uninoculated medium was taken as the cell dry weight.

Rifamycins determination

Rifamycin B and SV were determined in the filtrate using spectrophotometric method as described by Passqualucci et al. [25]. Samples were prepared for the assay by taking two 1 mL aliquots of broth. One aliquot was firstly diluted 1:6 with buffer A (acetate buffer of pH 4.63) and the other was similarly diluted with buffer B (which composed of buffer A with addition of 0.1 $NaNO_3$, w/v) as the blank. After shaking for 5 min, both samples were filtered using Whatman filter paper No. 1. Buffer A treated sample was measured using spectrophotometer (Smart Spec 3000, BioRad, USA) against their blank at 425 nm to determine rifamycin B concentration. Rifamycin SV was also determined similarly at 447 nm.

Glucose determination

Glucose concentration in the fermentation broth was measured by enzymatic methods using automated glucose analyzer (Biochemical analyzer 2700, Yellow Springs Instruments, OH, USA).

RESULTS AND DISCUSSION

Effect of different vermiculite concentration on rifamycins production

This experiment was designed to investigate the effect of the addition of different concentrations of vermiculite on the production of rifamycins B and SV. From the obtained results presented in Figure 1, it can be seen that the addition of different vermiculite concentrations had no noticeable effect on cell growth, where the addition of varying concentrations (10-50 g/L) only increased cell growth from the control (no addition) by about 6.6% in all applied concentrations. On the other hand, vermiculite addition significantly affected the production of rifamycins B and SV. It can be observed that increasing the vermiculite concentrations up to 20 g/L increased the volumetric production of both rifamycin types, where the maximal production was obtained (767.5 and 1265 mg/L for Rif B and SV, respectively). This increase corresponds to about 29 47% increase from the values obtained in control culture (595 and 860 mg/L for Rif B and SV, respectively). Concerning the data of the yield coefficients ($Y_{P/X}$), it can also be seen that the maximal values of the rifamycin yields in terms of cell growth are obtained at 20 g/L of vermiculite (96.5 and 159.1 mg Rif B and SV/g cells, respectively). It previous research, vermiculite addition to culture medium showed positive effect on citric acid and oxytetracycline production [23,24]. In this study, the significant increase in rifamycins volumetric and specific antibiotic production is due that vermiculite include some important trace elements which are necessary for rifamycins production such as iron and magnesium. Ferrous ion level in fermentation medium showed specific regulatory role in many secondary metabolites production by actinomycetes such as in case of Collisymycin A biosynthesis by *Streptomyces* sp. and iturin A production by *Bacillus amyloliquefaciens*[26,27]. However, addition of iron to the cultivation medium should not exceed certain level otherwise it became inhibitory to different metabolites biosynthesis [24,26].

Effect of vermiculite addition time on rifamycins production

This experiment was conducted to investigate the effect of time of addition of vermiculite to the cultivation medium on the production of rifamycinx B and SV. The results obtaine din Table 1 shows clearly that the addition of vermiculite significantly affected the production of both rifamycins. However, the maximal production of both rifamycin B and SV was obtained when the vermiculite was added at the beginning of the cultivation. The maximal volumetric production of 770 and 1305 mg/mL of rifamycin B and SV was obtained when the vermiculite was added at 0 h. The addition of vermiculite after cell growth progression resulted in a gradual decrease in the volumetric productivity reaching the minimal values (605 and 900 mg/L for Rif B and SV, respectively) were obtained when the vermiculite was added after 96 h of cultivation. Similarly, the maximal specific productivities were also recorded (97.5 and 165.2 mg/g cells, for Rif B and SV, respectively) when the vermiculite was added at the beginning of the cultivation. Afterwards, the specific productivity decreased gradually reaching their minimal values when vermiculite was added after 96 h of cultivation. This is attributed to the parallel decrease in the volumetric productivity of both rifamycins.

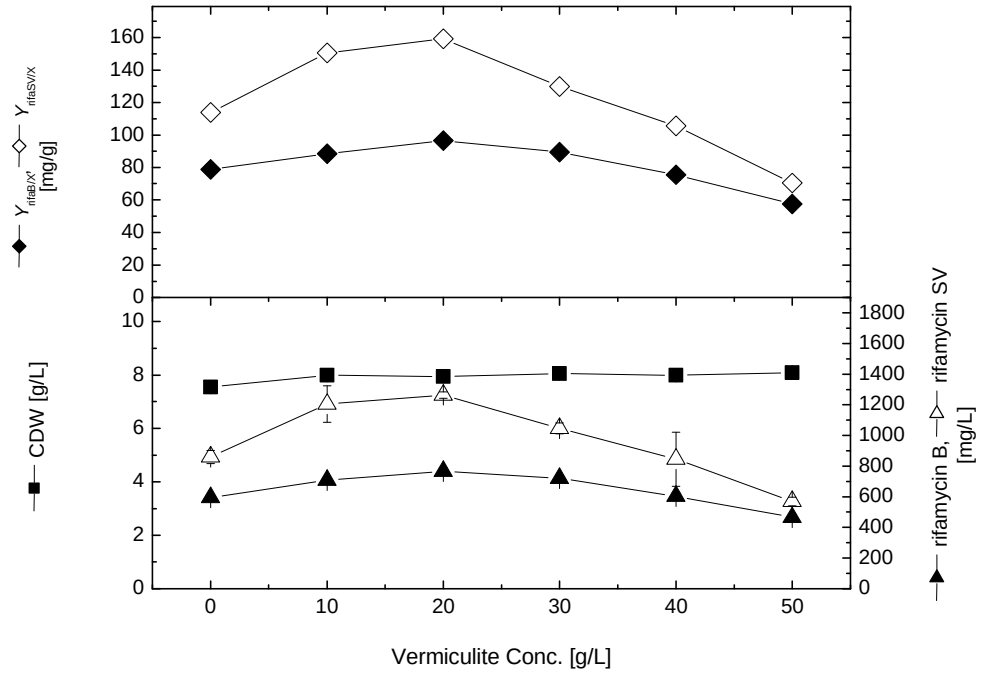

Figure 1: Effect of different vermiculite concentrations on the cell growth and both of volumetric and specific rifamycins production by *A.mediterranei* after 144 hours cultivation in submerged culture

Table 1: Effect of vermiculite addition time on the cell growth and rifamycins volumetric and specific production by *A.mediterranei*

Addition time [h]	Final pH	CDW [g/L]	Rifamycin B [mg/L]	Rifamycin SV [mg/L]	$Y_{rifaB/X}$ [mg/g]	$Y_{rifaSV/X}$ [mg/g]
0	7.1	7.9	770	1305	97.5	165.2
24	7.0	7.8	735	1220	94.2	156.4
48	6.9	7.4	630	910	85.1	123.0
72	7.0	7.5	608	902	81.1	120.3
96	6.9	7.5	605	900	80.1	120.0
Control (without)	6.7	7.6	598	890	78.7	117.1

Kinetics of cell growth and production of rifamycin B and SV by *A. mediterranei* with and without vermiculite supplementation

The present experiment described the kinetics of cell growth, glucose consumption and production of rifamycin B and SV by *A. mediterranei* cultivated in medium containing vermiculite in comparison to cultivation performed in vermiculite-free medium. From Figure 2, it can be clearly seen that cells grew exponentially with time in both cultivations without entering a lag phase, and entered the stationary growth phase after 96 h. At that time, the maximal cell growth reached 7.1 and 7.9 g/L for cultivations without and with vermiculite, respectively. By the end of the cultivation time, the maximal cell growth obtained in cultivation with vermiculite (8.03 g/L) was about 8.5% from that obtained in cultivation without vermiculite (7.4 g/L). It is noteworthy to mention that the cell growth rate in vermiculite cultivation (0.082 g/L/h) was about 11% higher than the growth rate obtained in cultivation without vermiculite (0.074 g/L/h). Concomitantly, the growing cells in cultivation with vermiculite consumed glucose rapidly and with a glucose consumption rate of 0.364 g/L/h, which is about 9% higher than the glucose consumption rate obtained in cultivation without vermiculite (0.334 g/L/h). Concerning rifamycin productivity, it can be clearly observed that the production of both Rif B and SV increased with time in both vermiculite-supplemented and free cultivations, until reaching the maximum at 120 h, then decreased by the end of the cultivation. However, it can be seen that the maximal volumetric productivities of Rif B and SV (790 and 1320 mg/L, respectively) in vermiculite cultivation were higher than those obtained in vermiculite-free cultivation (598 and 870 mg/L). This corresponds to about 32 and 51.7% increase in vermiculite-supplemented cultivations for rifamycin B and SV, respectively. Looking to the data of the production rate of rifamycins B and SV in both cultivations, it can be observed that the

Rif B production rate increased from 4.98 to 6.58 mg/L/h in vermiculite-free and supplemented cultivation, respectively, corresponding to an increase of 32% in the production rate. Similarly, the Rif SV production rate increased by about 52% in vermiculite-supplemented culture compared to vermiculite-free culture.

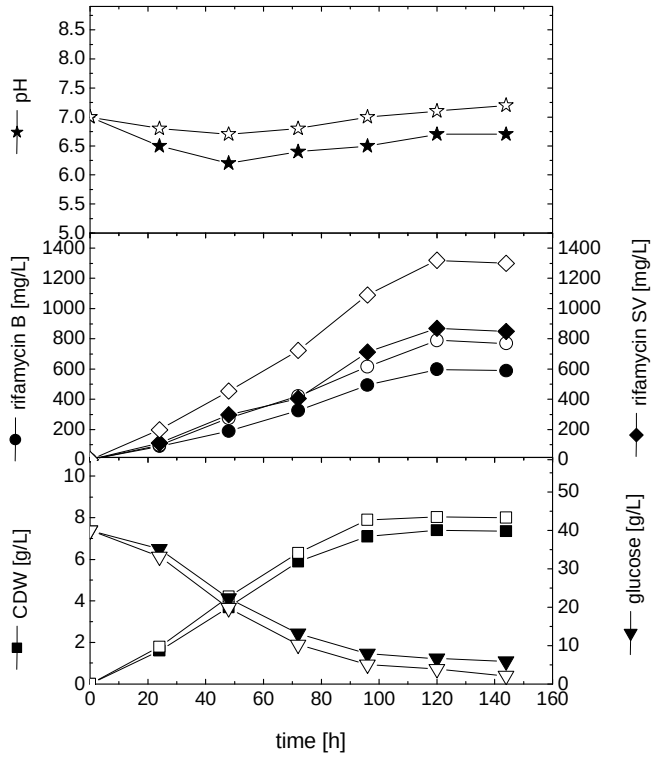

Figure 2: Time profile for the changes of cell growth, glucose consumption, rifamycins production, pH during cultivation of *A. mediterranei* in submerged. (Openedand closed symbols represent the results of cultivation in medium with and without vermiculite addition, respectively)

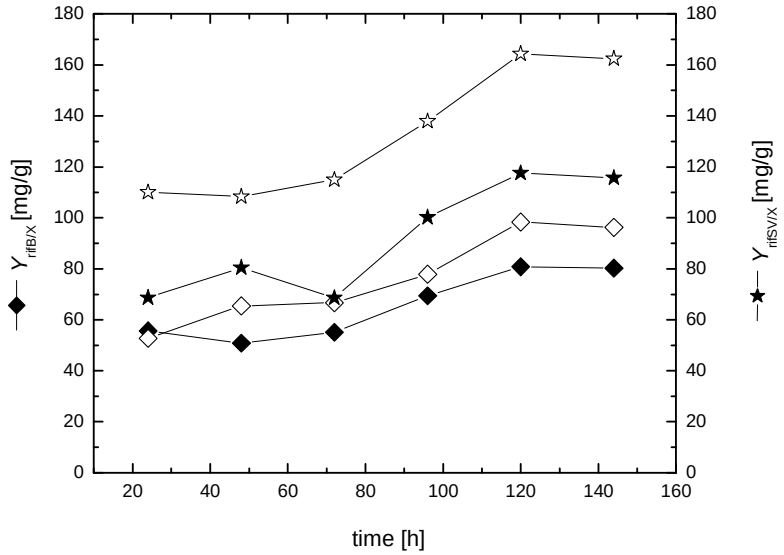

Figure 3: The specific production for rifamycins B and SV during cultivation time (Opened and closed symbols represent the results of medium with and without vermiculite addition, respectively)

The results shown in Figure 3 represent the specific productivities of both rifamycin B and SV during cultivation times in vermiculite-supplemented and vermiculite-free medium. It can be clearly seen that during the first 72 h of cultivation the specific productivities remained more or less constant. However, from 72 h to 96 h, there was a marked increase in the values obtained for the specific productivities of both rifamycin B and SV in both cultures. On the other hand, the obtained results showed that the rifamycin B specific productivity generally increased in vermiculite cultivation by a value ranging from 12.1 to 28.9% over the cultivation period. For rifamycin SV, the specific productivity showed a more significant increase, which ranged from 34.5 to 67.4% during the cultivation process.

CONCLUSION

The results of this study clearly demonstrate that vermiculite addition to the cultivation medium at the beginning of cultivation enhances antibiotic production without significant effect on biomass production. Therefore, we can conclude that the increase in antibiotic production is mainly attributed to the increase in metabolic activity toward antibiotic production through the addition of some necessary trace elements such as iron and magnesium which enhance rifamycins biosynthetic pathway. Vermiculite should be considered as cheap trace element source in microbial cell fermentation for secondary metabolites production.

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