

Full Length Research Paper

Optimization of industrial production of rifamycin B by *Amycolatopsis mediterranei*. I. The role of colony morphology and nitrogen sources in productivity

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A systematic approach to process optimization for production of rifamycin B was applied to a strain of *Amycolatopsis mediterranei*. Examination of the growth revealed 6 different morphologically distinct colonies on Bennett's agar medium. Rifamycin B production in shake flasks by the six different colony types ranged between 0.5 and 1.2 g/l. There was a clear correlation between the colony morphology and rifamycin B productivity. The highest yield of rifamycin B (1.03-1.2 g/l) was obtained by using the orange-red colored colonies, rosette shaped, devoid of hollow center and 2-3 mm in diameter. Variability in colony morphology, however, remained and the appropriate colonies had to be picked up for preparing the inoculum of each experiment. Addition of yeast extract to the fermentation medium at different times increased rifamycin B production. The highest antibiotic production was obtained upon the addition of 0.1% yeast extract after 2 days of incubation, where the yield increased from 1.15 to 1.95 g/l (70%). The use of 1.8% KNO₃ in the fermentation medium, instead of 0.96% (NH₄)₂SO₄, markedly increased rifamycin B production from 1.15 to 2.92 g/l (154%). It was also observed, upon microscopical examination, that KNO₃ decreased branching and fragmentation of the mycelia in the fermentation medium.

Key words: Rifamycin B, fermentation, biotechnology, *Amycolatopsis mediterranei*, strain selection.

INTRODUCTION

Among infectious diseases, tuberculosis remains a leading cause of death worldwide. It is estimated that over 8 million people contract tuberculosis each year, and approximately 2 to 3 million people die of this disease. In addition, it is thought that as many as 2 billion people have been exposed to the tuberculosis bacillus and are therefore at risk of developing active disease. This problem is further compounded by a dramatic increase in multidrug-resistant strains of *Mycobacterium tuberculosis*. An additional factor is human immunodeficiency virus, which has significantly increased the incidence of tuberculosis in sub-Saharan Africa and elsewhere (De

Cock and Chaisson, 1999; Dye et al., 1999; Keshavjee and Bacerra, 2000; WHO, 2002). Rifampicin is considered one of the most effective antibiotics for the treatment of tuberculosis. Apart from its application against pathogens of tuberculosis and leprosy, it has also been found to be effective against several pathogens including *Mycobacterium avium* and penicillin-resistant pneumococci (Anne et al., 2000; Anon, 1999; Reynaldo-Dietze et al., 2001; Russell, 1998).

Rifamycins are produced mainly by the Gram-positive bacterium *Amycolatopsis mediterranei*, previously named *Streptomyces mediterranei* and later *Nocardia mediterranei*, which was first isolated from the soil at a French pine forest (Pape and Rehm, 1985). Rifamycins were first isolated from a fermentation culture at the Lepetit Laboratory in Milan, Italy, by Sensi and coworkers. Different strains of this organism produce

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different types of rifamycins (A, B, C, D, E, S, SV etc.) according to the conditions of production. These rifamycins are usually interconvertible by chemical means and vary considerably in their antimicrobial spectrum and extent of activity. Being more stable, easier to purify, soluble at physiological pH and good starting material for the synthesis of active derivatives such as rifampicin, rifamycin B was chosen for development (Krishna et al., 2000; Pape and Rehm, 1985; Sensi and Thiemann, 1967).

It has been previously reported that members of the genus streptomyces show genetic instability and, in several cases, variants with altered abilities to elaborate antibiotics (Fishman and Gershberger, 1983; Ono et al., 1982; Schremppf, 1982). Inorganic and organic nitrogen compounds have been reported to play a key role in promoting rifamycin biosynthesis by serving either as a precursor or stimulant (Gresham and Inamine, 1986; Lee and Rho, 1994).

This study aimed at selection of the most active antibiotic producing colony from *A. mediterranei*-RCP 1001 (N1) strain, which suffered generally from low and inconsistent productivity of around 500 mg/l of rifamycin B. In addition, the effect of KNO₃ and yeast extract on rifamycin B production was studied.

MATERIALS AND METHODS

Bacterial strain

A. mediterranei-RCP 1001 (N1) strain was obtained from El-Nasr Company for Pharmaceutical Chemicals, Egypt. The organism was maintained on Q/2 agar slants and propagated on Bennett's agar. Selected colonies were subcultured onto Q/2 agar slants and stored at 4°C to be used within 27 days. For long term storage, the surface growth on Q/2 agar slants was harvested in 10% skim milk and lyophilized.

Chemicals

Chemicals used throughout this work were of laboratory reagent grade unless otherwise indicated. Glucose, KNO₃, NaNO₂ and propylene glycol were the products of ADWIC, Egypt. Sodium diethyl barbiturate (SDB) was the product of Grindstedvaerket A/S, Denmark. Potassium sodium tartarate tetrahydrate, 3,5-dinitrosalicylic acid, CaCO₃, MgSO₄.7H₂O, FeSO₄.7H₂O, KH₂PO₄, ZnSO₄.7H₂O, CoCl₂.6H₂O, (NH₄)₂SO₄ and NaOH were the products of E. Merck, Darmstadt, Germany. Glacial acetic acid was the product of Aldrich Ltd, England.

Media

Yeast extract, malt extract, beef extract, tryptone, skim milk and bacto agar were the products of Difco Laboratories, Detroit, U.S.A. Soybean meal, oat flakes and peanut meal were obtained from local commercial suppliers.

Bennett's agar medium was used for propagation and selection and consisted of: beef extract, 1.0 g; tryptone, 2.0; yeast extract 1.0g; dextrose 12.0; bacto agar 20.0g and distilled water to 1000 ml. The pH was adjusted to 7.2 (Margalith and Beretta, 1960). Q/2 agar medium was used for maintenance and consisted of: yeast

extract, 4.0 g; malt extract, 10.0 g; dextrose, 4.0 g; oat flakes, 20.0 g; bacto agar, 20.0 g and distilled water to 1000ml. The pH was adjusted to 6.9. Vegetative medium (V1) was used for preparation of inoculum and consisted of: dextrose, 20.0 g; soybean meal, 20.0 g; MgSO₄.7H₂O, 0.4 g; CaCO₃, 2.5 g; FeSO₄.7H₂O, 0.01 g; ZnSO₄.7H₂O, 0.05 g; CoCl₂.6H₂O, 0.003 g and distilled water to 1000 ml. The pH was adjusted to 6.7. Fermentation medium (F1) was used for the antibiotic production and consisted of: peanut meal, 25.0 g; soybean meal, 10.0 g; dextrose, 140.0 g; propylene glycol, 10.0 ml; sodium diethyl barbiturate, 1.7g; (NH₄)₂SO₄, 9.6g; CaCO₃, 11.0g; MgSO₄.7H₂O, 1.0g; FeSO₄.7H₂O, 0.005g; ZnSO₄.7H₂O, 0.012 g; KH₂PO₄, 1.0 g; CoCl₂.6H₂O, 0.003 g and distilled water to 1000ml. The pH was adjusted to 8.1.

Strain propagation and selection

One loopful from the culture on Q/2 agar slant was transferred into a sterile tube containing 6 ml of normal saline and seven glass beads, then homogenized by vortex for 5 min and ten-fold serial dilutions were prepared in normal saline. Aliquots of 0.1 ml from dilutions 10⁻⁵, 10⁻⁶, 10⁻⁷, and 10⁻⁸ were transferred onto the surface of Bennett's agar plates. The plates were incubated at 28°C for one day then inverted, and the incubation was continued for 13 days. Plates with 15–30 well distinguished colonies were used for selection. Developed colonies with different morphological characteristics were selected and characterized. Colonies showing secondary surface development of mycelia, as observed under the stereomicroscope, were rejected.

Each of the selected colonies was suspended in one ml saline and homogenized by vortex. Aliquots of 0.1 ml of this mycelial suspension were used to inoculate each of 3 slants of Q/2 medium and incubated at 28°C for 8 days. Slants showing homogenous growth, tough surface and dark red color were selected and stored at 4°C to be used within 27 days.

Preparation of inoculum

Surface growth of the selected slant was harvested in flasks containing 100 ml of vegetative medium (V1). The flasks were incubated at 28°C in a rotary incubator shaker (Controlled Environment Incubator Shaker, New Brunswick Scientific Co., NJ, USA) at 100 rpm for 56 to 72 h. Flasks showing the following parameters were selected for inoculation of the fermentation medium: pH, 7.6-7.9; percentage packed mycelial volume (Rho et al., 1991), 9 – 12%; color, intense orange to red; time for methylene blue decolorization, 50 – 70 s.

Determination of percentage packed mycelial volume (PMV)

The percentage packed mycelial volume was determined as described by Rho et al. (1991). Twenty ml of the previously prepared vegetative culture were centrifuged at 4,000 rpm for 5 min. After decantation, the mycelia were washed with saline by centrifugation three times, then resuspended in saline to the original volume. The resuspended mycelia were allowed to settle down for 20 min in 25 ml measuring cylinder, then the packed mycelial volume was measured and the percentage was determined.

Decolorization of methylene blue

One ml of 1% methylene blue solution was added to 10 ml of the vegetative culture, mixed well and the decolorization time was determined.

Table 1. The morphological characteristics of the different colonies of *A. mediterranei* on Bennett's agar medium and their corresponding rifamycin B production.

Colony type	Diameter (mm)	Morphological characteristics	Range* of Rifamycin B (g/l)
1	2-3	Orange-red, rosette, irregular edge.	1.03-1.2
2	3-5	Orange, mucoid, flattened, irregular edge.	0.95-1.0
3	7-10	Orange-red, folding toward the center irregular edge.	0.76-0.82
4	3-5	Orange, mucoid, hollow center, irregular edge.	0.50-0.60
5	5-7	Reddish orange, mucoid, rounded, irregular edge.	0.70-0.80
6	1-2	Reddish brown, mucoid, irregular edge.	0.62-0.70

*Range of 5 different experiments.

Production of rifamycin B in shake flasks

The fermentation was carried out in six flasks each containing 50 ml of the fermentation medium (F1) and inoculated with 2.5 ml (5% v/v) of the inoculum. The flasks were incubated at 28°C in a rotary incubator shaker at 250 rpm for 8 days. Rifamycin B concentration, remaining glucose concentration and pH were determined in each Colony type 1 Colony type 2 flask on days 2, 4, 6, 7, and 8. The results were the average of the 6 flasks.

Determination of remaining glucose concentration

Remaining glucose concentration in the fermentation broth was determined by the dinitro salicylic acid method as described by Bernfeld (1955).

Assay of rifamycin B

The concentration of rifamycin B in the fermentation broth was determined spectrophotometrically as described by Pasqualucci et al. (1970) using a Shimadzu UV-160 A spectrophotometer, Japan.

RESULTS

The morphological characteristics of the growing colonies on Bennett's agar plates showed considerable morphological variation as indicated in Table 1 and illustrated in Figure 1. In addition, a considerable variation in rifamycin B production by the different types of colonies was observed (Table 1). The highest yield of rifamycin B was obtained by using colony type 1, which has orange-red color, rosette shape, devoid of a hollow center and 2-3 mm in diameter.

Microscopical examination of the culture in the vegetative medium (V1) of the six colony types showed Gram-positive short thin filaments arranged radially around hollow centers and no spores could be detected (Figure 2). Microscopical examination of the culture in the fermentation medium (F1) varied with time where the mycelia showed frequent branching at the early exponential phase (day 2-4), followed by increased branching and subsequent gradual fragmentation into short rods (day 4-6).

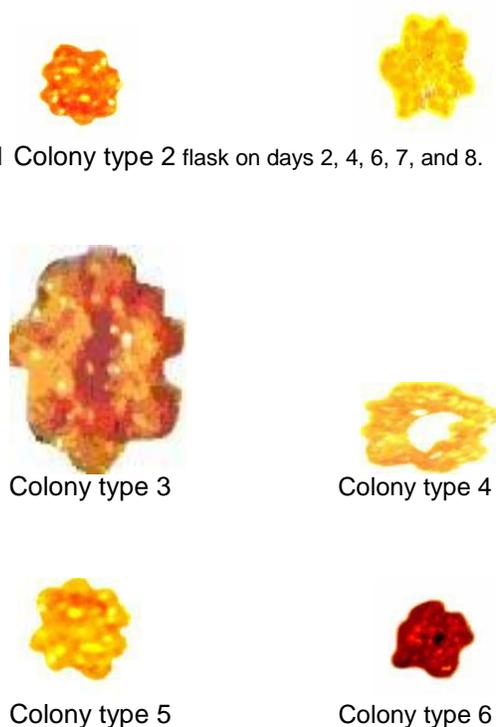


Figure 1. Different colony types of *A. mediterranei* grown on Bennett's agar plates (18 x).

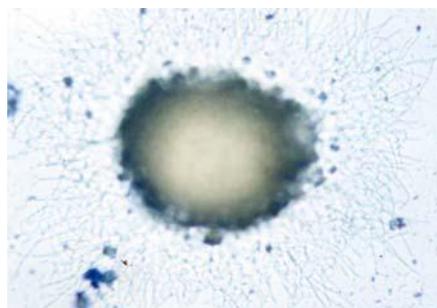


Figure 2. *A. mediterranei* grown in early stage in fermentation medium (F1) as seen under the microscope stained with methylene blue (1000 x).

The replacement of 4.5% of the glucose content in F1 medium with 4.5% glycerol decreased rifamycin B production (Figure 3). Elimination of or increasing KH_2PO_4 concentration above 0.1% decreased the production of rifamycin B (Figure 3). The addition of 0.05, 0.1 and 0.2% of yeast extract at different times of the fermentation process increased rifamycin B production (Figures 3 and 4). The highest increase was obtained when 0.1% yeast extract was added after 2 days of incubation, where the yield increased from 1.15 g/l to 1.95 g/l (70%). The addition of 0.9 and 1.8% KNO_3 , instead of 0.96% $(\text{NH}_4)_2\text{SO}_4$, to the F1 medium increased rifamycin B production from 1.15 g/l to 2.74 g/l and to 2.92 g/l, respectively (Figure 5). Furthermore, the microscopical examination of the mycelia in the fermentation medium containing KNO_3 revealed a decrease in branching and fragmentation.

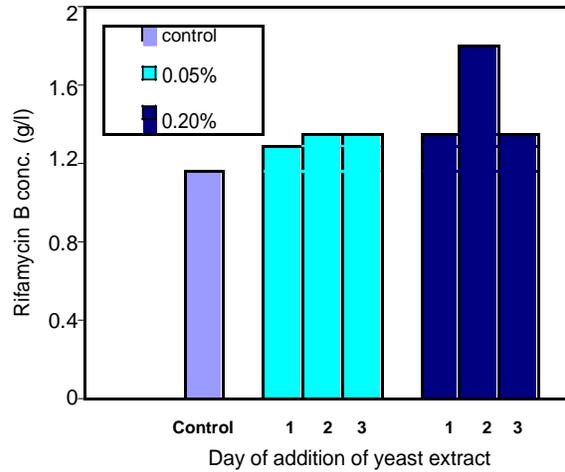


Figure 4. Effect of addition of 0.05 and 0.2% yeast extract to F1 medium on rifamycin B production by strain N1.

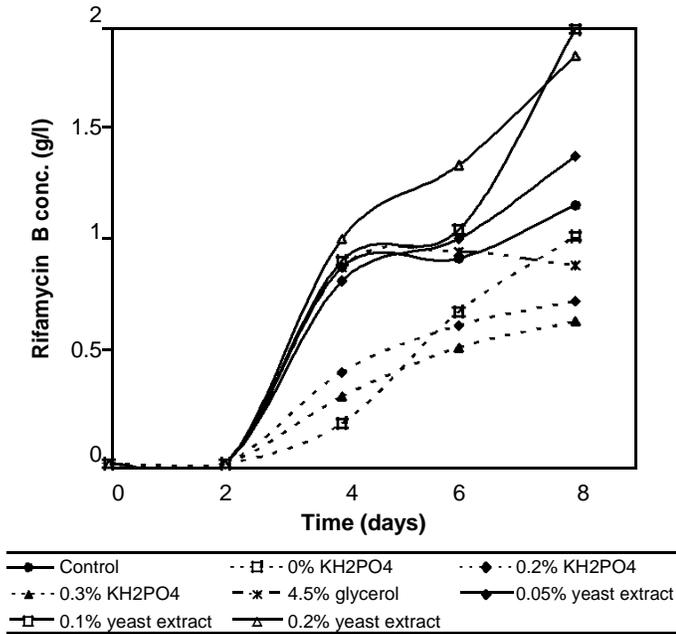


Figure 3. Effect of addition of yeast extract after 2 days, replacement of 4.5% glucose with 4.5% glycerol and the use of different concentrations of KH_2PO_4 , in F1 medium, on rifamycin B production by strain N1.

DISCUSSION

Commercial production of an antibiotic requires the use of a fairly stable strain with consistent high productivity. Such consistency could be a function of both the strain and of the process. In the present work we emphasized the selection and identification of distinguishing characteristics of the most productive variant for a strain with potential commercial application for production of

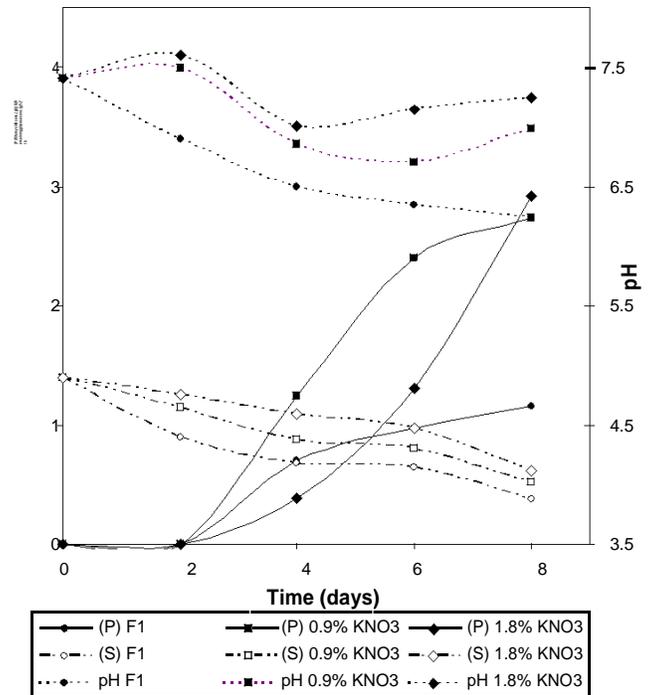


Figure 5. Effect of replacement of $(\text{NH}_4)_2\text{SO}_4$ with KNO_3 in F1 medium on rifamycin B production (P), remaining glucose concentration (S) and pH using strain N1.

rifamycin B and aimed at defining some fundamental conditions necessary to maintain such variant at high levels of antibiotic productivity.

The morphological characteristics of the growing colonies on Bennett's agar medium showed considerable variation, where 6 different colony types were recognized. Subculture of such colony types on Q/2 agar slants also

showed variation in the color and the homogeneity of the surface growth. The highest yield of rifamycin B was obtained by using type 1 colonies (Figure 1). Such intra-strain morphological variability and correlation between colony morphology and antibiotic productivity is not uncommon within species of the genus *Streptomyces* (Fishman and Gershberger, 1983; Ono et al., 1982; Schrenpf, 1982). In a study using a different strain of *A. mediterranei* (CBS 42575), Farid et al. (1996) demonstrated that the highest yield of rifamycin B (0.78 g/l) was obtained by using the red colored colonies, 2–3 mm in diameter and rosette shape, a somewhat similar description of our colony type 1. Unfortunately, we could not examine a colored picture of those author's colonies, which we can use for comparison. It can be concluded that using our strain of *A. mediterranei*, the least productive colonies are buff, orange or brown in color, <2 mm or >3 mm in size, hollow-centered or folding toward the center, mucoid, regular edge while the most productive colonies are orange-red in color, rosette shape with irregular edge and 2-3 mm in size.

Microscopical examination of the organism showed Gram-positive short thin filaments and no spores could be detected. It has previously been reported that sporulation is best noticeable in chemically defined media (Margalith and Beretta, 1960). In the fermentation medium (F1), the mycelia showed frequent branching followed by subsequent gradual fragmentation into short rods. Branching and fragmentation of the mycelia was also reported by Lee and Rho (1994). Several authors reported the tendency of the mycelia to fragment into short rods. It should be noted that such fragmentation as well as the presence of meso-diaminopimelic acid and of arabinose and the absence of glycine are characteristics of the genus *Nocardia* (Pape and Rehm, 1985; Thiemann et al., 1969). However, *Nocardia mediterranei* was moved from the genus *Nocardia* to a new genus *Amycolatopsis*, on the basis of lacking mycolic acids in the cell wall and being not susceptible to typical nocardia phages (Lancini and Cavalleri, 1997; Le Chevalier et al., 1986).

The maximum rifamycin B production by the selected typical colony type 1 ranged between 1.03 g/l and 1.2 g/l. Although this yield is considerably higher than that reported by Farid et al. (1996), it is still too low for industrial application. This low yield and even this limited fluctuation in production are considered major problems in industry. Attempts to optimize the fermentation process were carried out by studying the effect of inorganic phosphate and of organic and inorganic nitrogen sources.

Phosphate has been known to act as a regulatory substance in the synthesis of primary and secondary metabolites in microorganisms (Mertz and Doolin, 1973). In our study, increasing the concentration of KH_2PO_4 above 0.1% caused a marked decrease in rifamycin B production (36- 45%), while total elimination of KH_2PO_4 caused only 12% decrease (Figure 3). This rather unexpected result may be a reflection of the proposal that

production of one rifamycin is influenced by synthesis of another (Lal et al., 1995). For example, Lancini and Sensi (1967) reported that in production of rifamycin B, any shift in metabolism which stimulates simultaneous production of other rifamycins may suppress synthesis of rifamycin B. More specifically, using a strain of *Nocardia mediterranei* which produced rifamycin B and to a lesser extent rifamycin Y, they noted that although several fermentation variables did not suppress production of rifamycin Y, decreasing the level of KH_2PO_4 drastically did - and hence increased production of rifamycin B. Our results may reflect a similar case, whereby an increase in KH_2PO_4 favors synthesis of a rifamycin which suppresses synthesis of rifamycin B rather than result in a total lower yield of rifamycins.

Yeast extract was reported by Kawaguchi et al. (1984) to play a regulatory role in the production of rifamycin B by *Nocardia*. They further reported that yeast extract contains 3'-(1-butyl phosphoryl) adenosine (the B-factor) that regulates the production of the rifamycin precursor 3-amino -5-hydroxybenzoic acid which is the starter unit (chain initiator) for the assembly of a polyketide in rifamycin biosynthesis. In our study, the addition of 0.05, 0.1 and 0.2% yeast extract at different times of the fermentation process (1, 2 and 3 days) increased rifamycin B production (Figures 3 and 4). The highest yield was obtained upon using 0.1% yeast extract after 2 days of incubation with 70% higher yield than the control (8 days, total fermentation time). It was observed that, at all tested concentrations, the highest stimulation of rifamycin B production was when yeast extract was added after 2 days of incubation. It is further noted that the lowest concentration of yeast extract (0.05%) showed only moderate influence on antibiotic production, while the highest concentration (0.2%) showed its influence from the 4th day onward. Interestingly, a concentration of 0.1% yeast extract, while showing only a moderate influence up to the 6th day, gave a sharp increase in antibiotic productivity afterwards. Kawaguchi et al. (1988) reported that the addition of B-factor, isolated from yeast extract, after 2 days of incubation caused maximum rifamycin B production and that earlier addition at day 0 and day 1 gave slightly lower production. They suggested that this was probably due to degradation of the B-factor before initiation of the antibiotic biosynthesis.

In our study, the fermentation medium F1 contained 0.96% $(\text{NH}_4)_2\text{SO}_4$ as inorganic nitrogen source, which has also been used by Lysko and Gorskaia (1986). They studied the influence of various forms of inorganic nitrogen on biosynthesis of rifamycin B and found that only $(\text{NH}_4)_2\text{SO}_4$ provided the pH levels of the fermentation broth suitable for the antibiotic biosynthesis and the highest levels of antibiotic production. However, Lee and Rho (1994) reported that inorganic nitrogen sources play an important role in determining the production profile of rifamycin B with KNO_3 showing a positive influence on antibiotic production. Accordingly, the effect of 0.9 and

1.8% KNO₃ (representing half and same nitrogen equivalent to 0.96% (NH₄)₂SO₄, respectively) was tested. When 0.9% KNO₃ was used, production by the 8th day was 138% higher while with 1.8% KNO₃ antibiotic production was more sluggish but showed a very steep positive slope starting on the 6th day with 154% increase by the 8th day. From our results, it was concluded that KNO₃ was superior to (NH₄)₂SO₄ as an inorganic nitrogen source for the production of rifamycin B. Since the optimum pH during the trophophase was reported to be 6.5 (Lee et al., 1983), and since the pH profiles for the production with 0.9 and 1.8% KNO₃ did not seem to be significantly different (Figure 5), a pH effect may not explain the different antibiotic productivities under the 2 conditions.

An interesting observation was that while a slightly higher rifamycin B production occurred with 1.8% than with 0.9% KNO₃ by day 8, productivity trends were much higher with 0.9% than 1.8% KNO₃ at all other time samples. In both cases however, production almost always significantly exceeded the control but the fact remained that with 1.8% KNO₃ the slower start was compensated for by a consistent positive trend in apparent rate of production, when compared to 0.9% KNO₃. Unfortunately, we were not able to determine biomass and hence specific production rates. A possible significant observation is that in all cases glucose consumption was rather slow and incomplete but occurred at somewhat higher apparent rates with (NH₄)₂SO₄, 0.9% KNO₃ and 1.8% KNO₃, respectively in decreasing order (Figure 5) especially during the trophophase. The faster utilization of glucose with (NH₄)₂SO₄ than with KNO₃ was also observed by Lee and Rho (1994) and might be the cause of the lower yield of the antibiotic with (NH₄)₂SO₄ in our study as explained by Pape and Rehm (1985).

The microscopical characteristics of the mycelia grown with (NH₄)₂SO₄ differed from that with KNO₃. In addition, the culture medium was much more viscous and the mycelia were more profoundly branched in the medium containing (NH₄)₂SO₄ than that containing KNO₃. It is possible that this excessive branching caused an increase in viscosity of the culture medium and subsequently interfered with mixing and aeration. It may be concluded that the microscopical characteristics of *A. mediterranei* were significantly affected by the constituents of the culture media which possibly also influenced availability of oxygen, and that rifamycin B productivity was also related to morphological changes as previously reported by Rho et al. (1991) and Lee and Rho (1994).

In conclusion, selection of the most productive colony type increased the yield of rifamycin B from 0.5 g/l to a range of 1.03 to 1.2 g/l and the use of 0.1% yeast extract or 1.8% KNO₃ led to a further increase in the yield to 1.95 g/l (70%) and 2.92 g/l (154%), respectively. This yield is still much lower than the level for the economic

production of rifamycin B. Thus, several studies to increase the yield need to be performed.

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