

Full Length Research Paper

# Optimization of industrial production of rifamycin B by *Amycolatopsis mediterranei*. III. Production in fed-batch mode in shake flasks

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Optimization of the fermentation process using the gene amplified variant of *Amycolatopsis mediterranei*, NCH, in the fermentation medium F2 was carried out by the application of fed-batch regime. The addition of 12% glucose alone at day 4 or simultaneously with 0.1% yeast extract at day 2 led to an increase in the yield of rifamycin B by 46% and 57%, respectively. The application of fed-batch regime together with replacing  $(\text{NH}_4)_2\text{SO}_4$  with the better yielding inorganic nitrogen sources  $\text{NH}_4\text{NO}_3$  or  $\text{KNO}_3$  (F2m1 and F2m2 media, respectively) increased the production of rifamycin B. The use of F2m1 medium alone or with an additional 12% glucose added at day 4 increased the yield by 53% and 120%, respectively. However, further addition of 0.1% yeast extract led to an increase in the yield by only 63%. The addition of 3% soytone or 0.05%  $\text{NH}_4\text{NO}_3$  to F2m1 at day 3 increased the yield by 72% and 61%, respectively, compared to productivity in F2 medium. The use of F2m2 medium increased the yield by 50%. The addition of 12% glucose at day 4 or of 0.1% yeast extract at day 2 to F2m2 medium led to an increase in the yield by 119 and 55%, respectively, compared to F2 medium. However, when both 12% glucose and 0.1% yeast extract were added at similar scheduled times only 64% increase in the yield occurred. By applying the three most effective optimization regimes determined using variant NCH with F2m2 medium on a standard rifamycin B producing strain, *Nocardia mediterranei* ATCC 21789, a similar pattern of increase in the antibiotic yield was observed. Thus, the use of F2m2 instead of F2 medium either alone or with an additional 12% glucose added at day 4 increased the yield by 36 and 75%, respectively, whereas the addition of 0.1% yeast extract to F2 medium increased the yield by 15%. In conclusion, the application of fed-batch technique with the optimum modifications of the medium constituents increased rifamycin B production by variant NCH to a maximum of 17.17 g/l compared to a yield of 5.3 g/l by the tested standard strain under the same conditions. The increase in rifamycin B production using the standard strain confirms the usefulness of the tested medium modifications in the improvement of rifamycin B production and its possible application in fermentations using other rifamycin B producer strains and also shows the superiority of variant NCH as a producer, when compared to the standard strain.

**Key words:** Rifamycin B, fermentation, biotechnology, *Amycolatopsis mediterranei*, optimization, fed-batch and physiological factors.

## INTRODUCTION

One of the purposes of fed-batching is to make the substrate(s) available to the microorganism at an optimum concentration for a longer period of time or at specified times of the process. In the fed-batch mode, gradual addition of concentrated additives, e.g. nutrients, takes place. Fed-batch cultures can be used for

fermentation processes in which the products are either growth associated or non-growth associated (Trevan et al., 1988). In the course of the development of the fermentation process for production of rifamycin B, fed-batch fermentations have been developed in order to extend the production phase, where sources of sugar, organic and inorganic nitrogen were fed batchwise or continuously (Ghisalba et al., 1984; Lancini and Cavalleri, 1997).

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Our previous attempts to improve rifamycin B productivity by development of an amplified variant NCH of *A. mediterranei* by application of gene amplification technique and by modification of the fermentation medium resulted in a 10.4-fold increase in productivity from 1.15 to 11.99 g/l (El-Tayeb et al., 2004a, b). In the present work, we tried to optimize the fermentation process by replacing  $(\text{NH}_4)_2\text{SO}_4$  with  $\text{NH}_4\text{NO}_3$  or  $\text{KNO}_3$  and fed-batching different concentrations of carbon, organic and inorganic nitrogen sources and finally integration of the optimum addition regimes. To test the usefulness of the medium modifications in the improvement of rifamycin B production and its possible application in fermentations using other rifamycin B producer strains, we also applied the most effective optimization regimes determined using variant NCH on a standard rifamycin B producing strain.

## MATERIALS AND METHODS

### Bacterial strains

The lyophilized standard strain *Nocardia mediterranei* ATCC 21789 was propagated on International Streptomyces Project (ISP) or Bennett's agar media. The amplified variant NCH of *A. mediterranei*-RCP 1001, previously obtained as described by El-Tayeb et al. (2004a), and the standard strain were maintained on 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,  $\text{KNO}_3$ ,  $\text{NH}_4\text{NO}_3$  and  $\text{NaNO}_2$  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,  $\text{CaCO}_3$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{KH}_2\text{PO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NaOH}$  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, soytone, skim milk, bacto agar and ISP medium were the products of Difco Laboratories, Detroit, U.S.A. Oat flakes was obtained from commercial suppliers.

Media used for propagation, selection and maintenance as well as the vegetative medium V2 and the fermentation medium F2 were those previously reported by El-Tayeb et al. (2004a,b). The ISP medium was reconstituted and sterilized according to the indicated manufacturer instructions.

The modified fermentation medium F2m1 is composed of: glucose, 120.0 g; soytone, 30.0 g;  $\text{NH}_4\text{NO}_3$ , 1.0 g;  $\text{KH}_2\text{PO}_4$ , 1.0 g;  $\text{CaCO}_3$ , 8.5 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.8 g; sodium diethyl barbiturate, 1.0 g and distilled water to 1000.0 ml. The pH was adjusted to 7.1. The modified fermentation medium F2m2 is composed of: glucose, 120 g; soytone, 35 g;  $\text{KNO}_3$ , 12 g;  $\text{KH}_2\text{PO}_4$ , 1.0 g;  $\text{CaCO}_3$ , 8.5g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.8 g; sodium diethyl barbiturate, 1.0 g and distilled water to 1000.0 ml. The pH was adjusted to 7.1.

## METHODS

The methods used for maintenance, propagation, selection, preparation of inoculum and production of rifamycin B in shake flasks as well as determination of remaining glucose concentration, biomass and assay of rifamycin B were those previously reported by El-Tayeb et al. (2004a,b). For fed-batch experiments, aliquots of 5 ml of glucose and of 0.5 ml of soytone, yeast extract or  $\text{NH}_4\text{NO}_3$  were taken from sterile stock solutions containing suitable concentrations, and added to the fermentation culture at the indicated times to give the required final concentrations.

## RESULTS

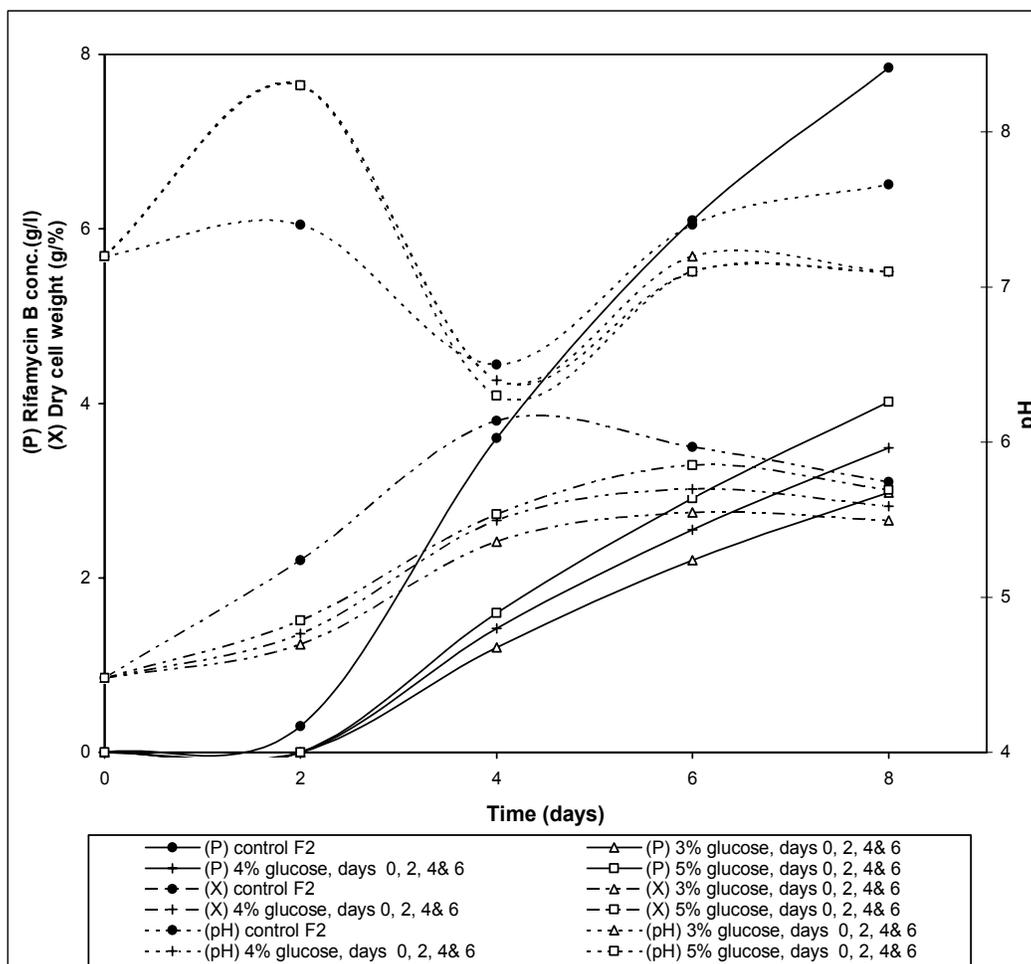
### Application of fed-batch technique

Successive additions of 3%, 4% or 5% glucose to an initially glucose-free F2 medium at days 0, 2, 4 and 6 caused a marked reduction in rifamycin B production (Figure 1). On the other hand, addition of 12% glucose to the F2 medium (initially containing another 12% glucose) at day 4 (Figure 2) increased rifamycin B production from 7.85 to 11.45 g/l (46%). Augmenting this addition with addition of 0.1% yeast extract at day 2 (Figure 2) increased rifamycin B production from 7.85 to 12.36 g/l (57%).

### Application of fed-batch technique to modified F2 medium

We have previously determined that both  $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$  are superior to  $(\text{NH}_4)_2\text{SO}_4$  as sources of inorganic nitrogen in F2 medium and led to an increase in the yield from 7.85 to 11.99 (53%) and 11.76 g/l (50%), respectively (El-Tayeb et al., 2004a,b). Thus, F2m1 and F2m2 were formulated by replacing 0.6%  $(\text{NH}_4)_2\text{SO}_4$  in F2 medium with 0.1%  $\text{NH}_4\text{NO}_3$  and 1.2%  $\text{KNO}_3$ , respectively. The use of F2m1 medium, instead of F2, either alone or with an additional 12% glucose added at day 4 increased the yield by 53% and 120%, respectively. However, further addition of 0.1% yeast extract led to an increase in the yield by only 63% (Figure 3). The batch-wise addition of 0.025, 0.05 and 0.075%  $\text{NH}_4\text{NO}_3$  to F2m1 medium at day 3 increased the yield by 52, 61 and 57%, respectively (Figure 3), as compared to the yield in F2 medium, with 0.05%  $\text{NH}_4\text{NO}_3$  producing the highest yield, 12.62 g/l. The batch-wise addition of 1, 2 and 3% soytone to F2m1 medium at day 3 increased the yield by 58, 65 and 72%, respectively (Figure 3), as compared to the yield in F2 medium, with 3% soytone producing the highest yield, 13.53 g/l.

The batch-wise addition of 0.1% yeast extract at day 2 or of 12% glucose at day 4 to F2m2 medium (Figure 4) increased rifamycin B production to 12.17 (55%) and 17.17 g/l (119%), respectively, compared to productivity in F2 medium, whereas their combined addition resulted in only 64% increase in the yield.



**Figure 1.** Effect of replacing 12% glucose in F2 medium with successive additions of 3%, 4% and 5% of glucose at days 0, 2, 4 & 6 on rifamycin B production (P), biomass (X) and pH by variant NCH.

### Production of rifamycin B by the standard strain (*N. mediterranei* ATCC 21789)

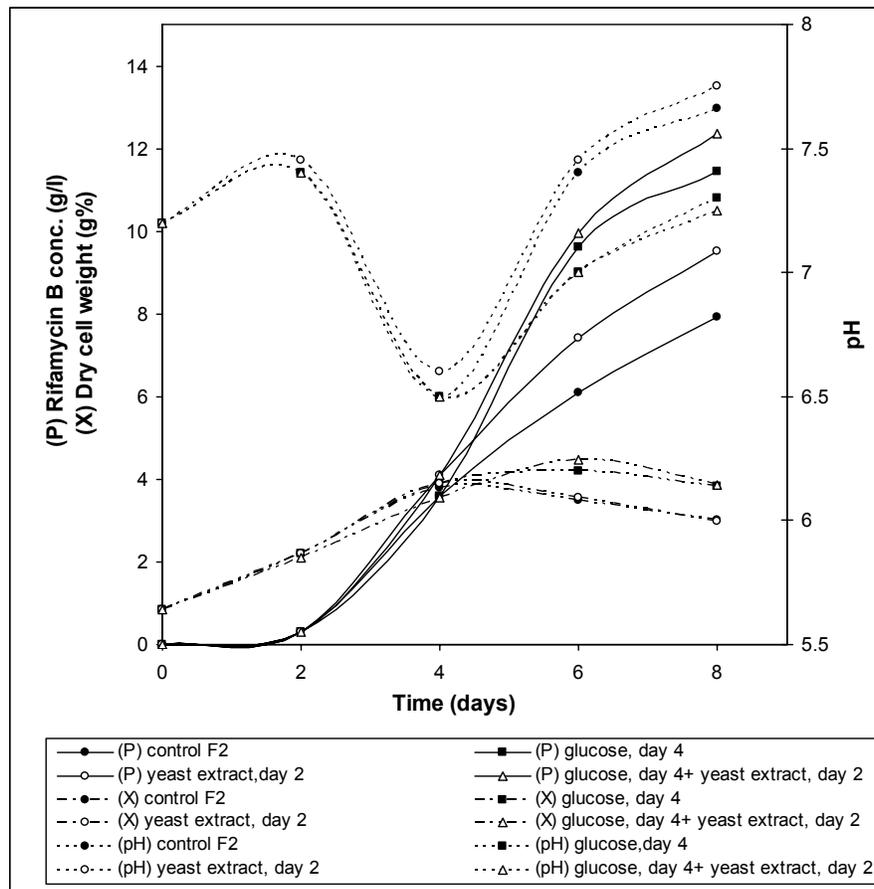
The colonies of *N. mediterranei* ATCC 21789 on Bennett's agar plates varied from 1–5 mm in size; from yellow to orange in color and were covered with white aerial mycelia. Colonies showing nearly the same morphological characteristics (orange, rosette, irregular edge and 2-3 mm in diameter), as the previously described colony type 1 of the N1 or NCH strain (El-Tayeb et al., 2004a,b), were selected for rifamycin B production.

Microscopical examination of the culture in the vegetative medium (V2) showed Gram-positive short thin filaments arranged radially around hollow centers and no spores could be detected. Microscopical examination of the culture in the fermentation medium (F2) showed characteristic fragmentation of the filaments into short rods similar to those of strain N1 and variant NCH (El-Tayeb et al., 2004 a,b).

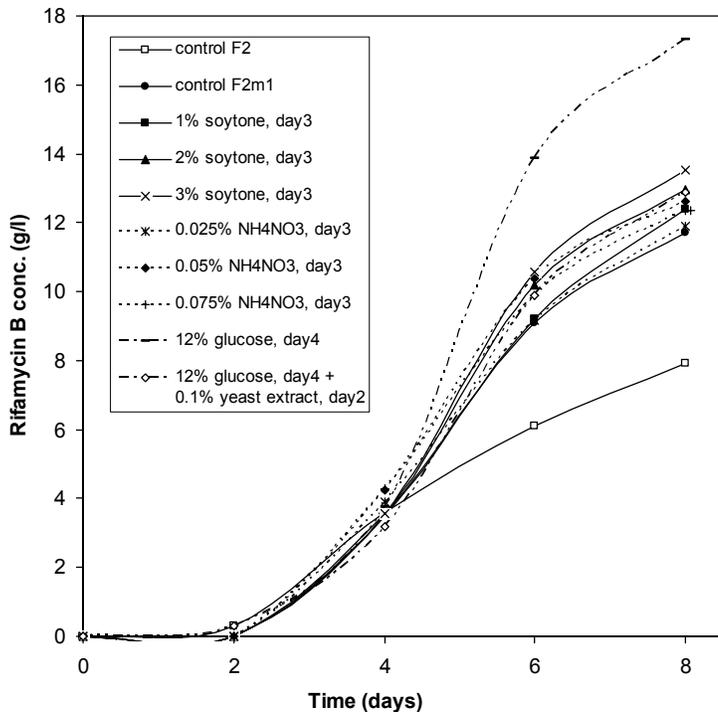
The standard strain produced a yield of 3.02 g/ml in F2 medium (Figure 5). The addition of 0.1% yeast extract at day 2 increased rifamycin B production from 3.02 to 3.49 g/l (16%). The use of F2m2 increased rifamycin B production from 3.02 to 4.12 g/l (36%), while addition of 12 % glucose at day 4 to F2m2 markedly increased rifamycin B production from 3.02 to 5.3 g/l (75%).

### DISCUSSION

Our previous attempts to increase rifamycin B production using the amplified variant of *A. mediterranei* (NCH) by modification of the fermentation medium resulted in a yield of 11.99 g/l (El-Tayeb et al., 2004a,b). In the present work, another approach was carried out by fed-batch addition of carbon, inorganic or organic nitrogen sources. Our observation and the previous report of Ghisalba et al. (1984) showed that a consistent lag time occurred before the beginning of the logarithmic growth and of detectable

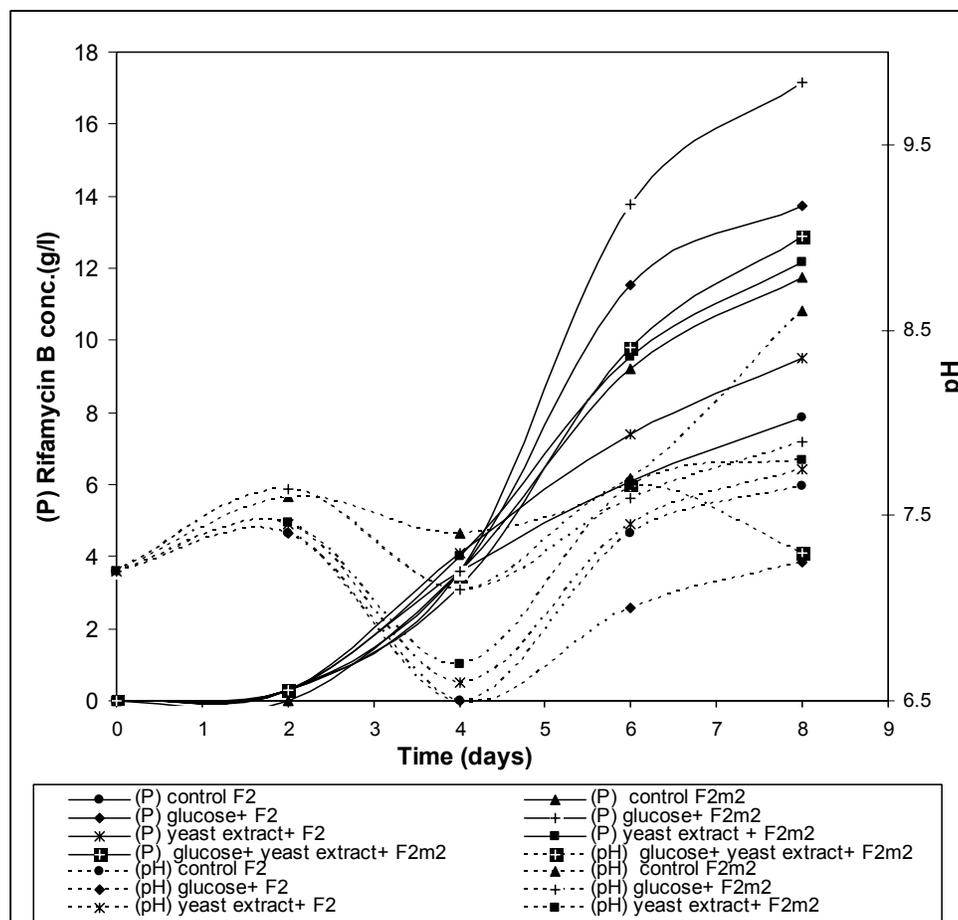


**Figure 2.** Effect of addition of 12% glucose at day 4 alone or in combination with 0.1% yeast extract at day 2 to F2 medium on rifamycin B production (P), biomass (X) and pH by variant NCH.



antibiotic production which may be attributed to the osmotic pressure caused by the high concentration of glucose and other soluble nutrients present in the fermentation medium as reported by Pape and Rehm (1985). We therefore, tried to reduce the initial glucose concentration, by the successive additions of 3, 4 and 5% glucose at days 0, 2, 4 and 6 to glucose-free F2 medium. These additions gave a total glucose concentrations equal to or above the control (12, 16 and 20%). However, the results showed that the lag phase persisted (Figure 1) and rifamycin B production was lower than that produced in F2 medium. In addition, a high pH was observed during the trophophase, which markedly affected the growth.

**Figure 3.** Effect of addition of 12% glucose at day 4 alone or in combination with 0.1% yeast extract at day 2 and of different concentrations of  $\text{NH}_4\text{NO}_3$  and of soytone to F2m1 medium on rifamycin B production by variant NCH.

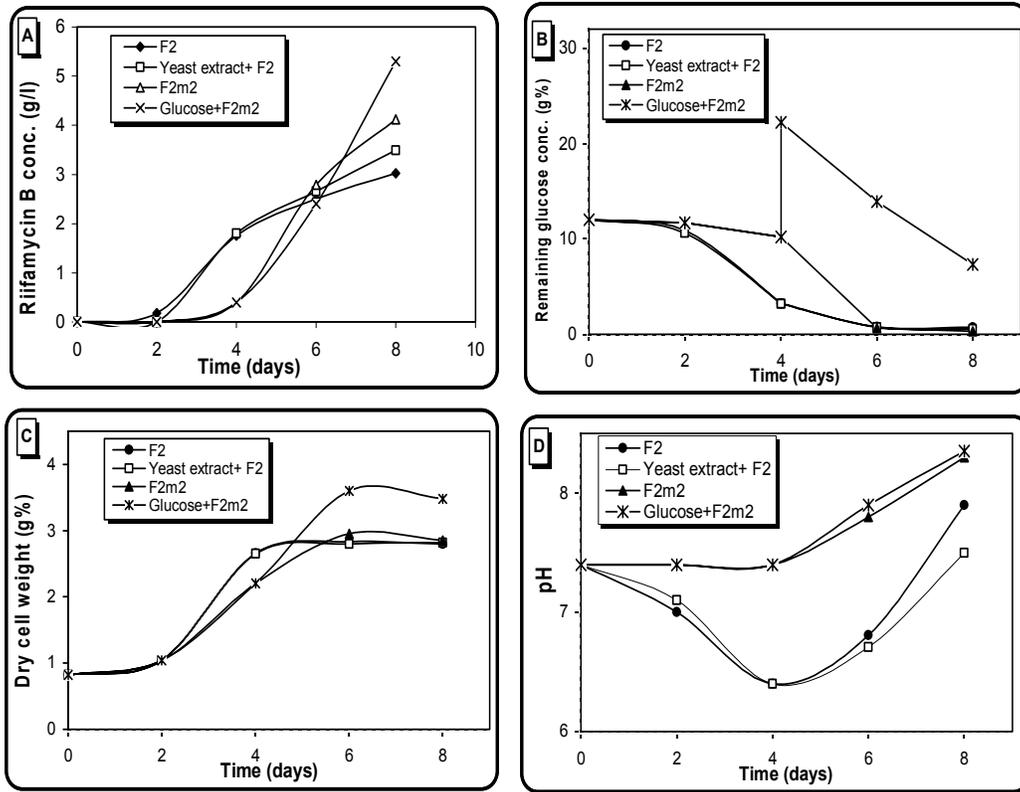


**Figure 4.** Effect of single or combined additions of 0.1% yeast extract at day 2 and of 12% glucose at day 4 to F2m2 medium on rifamycin B production (P) and pH by variant NCH.

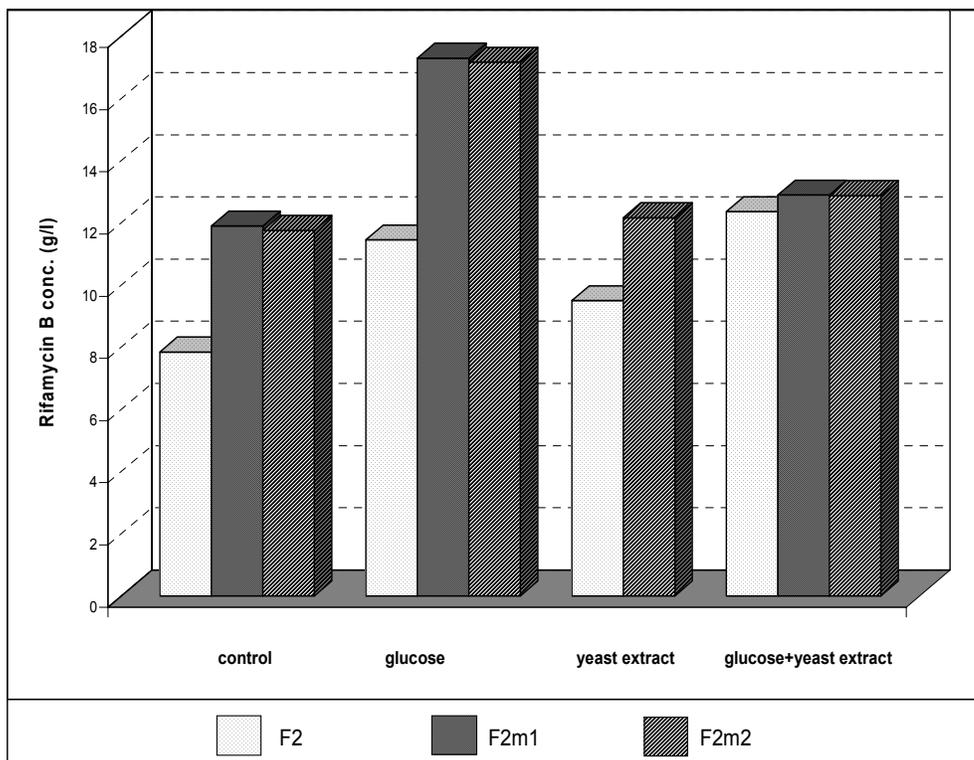
This increase in pH may be due to the utilization of intermediate organic acids by the microorganism after the consumption of the relatively small amounts of glucose initially present as well as the appearance of nitrogenous materials metabolized from proteins (Lee et al., 1983). This observation could suggest that high initial concentration of glucose in the fermentation medium is required for both growth and production of the antibiotic. Based on the reported need for glucose for the biosynthesis of the antibiotic during the idiophase (Lee and Rho, 1994) and on our observation that the apparent rate of glucose consumption increased between day 2 and 4, we tried the batch-wise addition of 12% glucose at day 4 to F2 medium. This led to an increase in the yield from 7.85 to 11.45 g/l (46%) along with a sharp increase in sugar utilization and a slight decrease in pH (Figure 2).

Yeast extract contains the B factor that regulates the production of the rifamycin B precursor 3-amino-5-hydroxybenzoic acid which is the starter unit (chain initiator) for the assembly of a polyketide in rifamycin biosynthesis as reported by Kawaguchi et al. (1984, 1988). In a previous study, we have shown that the

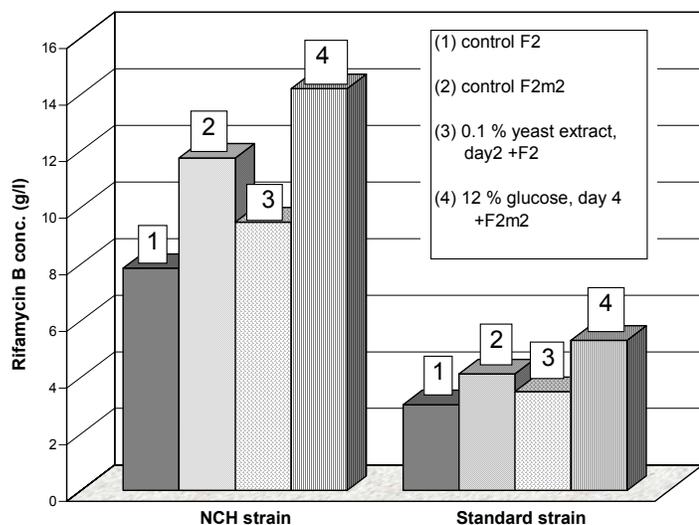
addition of 0.1% yeast extract at day 2 to F2 medium increased rifamycin B production by 21% (El-Tayeb et al., 2004 a). Thus we tried to further increase the yield, by augmenting the positive effect of fed-batching 12% glucose at day 4 (46%) with addition of 0.1% yeast extract at day 2. The yield increased, however, by only 57% (Figures 2 and 6). Relatively large amounts of nitrogen are necessary to stimulate rifamycin B production (Ghisalba et al., 1984). In addition, nitrate stimulates rifamycin production by its regulatory effect on lipid and rifamycin biosynthetic pathways (Rui-Shen et al., 1979). We have previously determined that both  $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$  are superior to  $(\text{NH}_4)_2\text{SO}_4$  as sources of inorganic nitrogen in F2 medium and led to an increase in the yield from 7.85 to 11.99 and 11.76 g/l, respectively (El-Tayeb et al., 2004a,b). Thus, F2m1 and F2m2 media were formulated by replacing 0.6%  $(\text{NH}_4)_2\text{SO}_4$  in F2 medium with 0.1%  $\text{NH}_4\text{NO}_3$  and 1.2%  $\text{KNO}_3$ , respectively. Therefore, we tried to further increase the yield of rifamycin B by the batch-wise addition of 0.025, 0.05 and 0.075%  $\text{NH}_4\text{NO}_3$  to F2m1 medium (Figure 3) at day 3, where the yield increased by 52, 61



**Figure 5.** Effect of addition of 0.1% yeast extract at day 2 to F2 medium and of 12% glucose at day 4 to F2m2 medium on rifamycin B production (A), remaining glucose concentration (B), biomass (C) and pH (D) by the standard strain ATCC 21789.



**Figure 6.** Comparison between rifamycin B production on day 8 by variant NCH in F2, F2m1 and F2m2 media with the addition of 12% glucose at day 4 alone or in combination with 0.1% yeast extract at day 2.



**Figure 7.** Comparison between rifamycin B production on day 8 by variant NCH and the standard strain ATCC 21789 in F2m2 medium.

and 57%, respectively, as compared to the yield in F2 medium, and by 2, 8, and 5% as compared to that of F2m1 medium. These increases are considered only minor and thus, the batch-wise addition of inorganic nitrogen sources to the modified fermentation media was not worthy of further investigation at this stage.

The addition of organic nitrogen compounds is generally recognized to be essential for high yields of rifamycin B production by serving either as a precursor or stimulant for antibiotic biosynthesis (Gresham and Inamine, 1986). The batch-wise addition of 1, 2 and 3% soytone to F2m1 medium at day 3 increased the yield by 58, 65 and 72%, respectively (Figure 3), as compared to the yield in F2 medium.

Furthermore, the batchwise addition of 12% glucose to F2m1 medium increased the yield of rifamycin B to 17.3 g/l (120% compared to F2 medium). Based on the lower cost of  $\text{NH}_4\text{NO}_3$  and the comparable high yield of  $\text{NH}_4\text{NO}_3$  (F2m1) to that of  $\text{KNO}_3$  (F2m2), F2m1 medium was chosen for further fed-batch mode fermentations using the fermentor.

By the batch-wise addition of 12% glucose or 0.1% yeast extract to F2m2 medium, it was possible to increase the yield of rifamycin B to 17.17 (119%) and 12.17 g/l (55%), respectively, and the addition of both only increased the yield to 12.88 g/l (64%), compared to the yield in F2 (Figures 4 and 6). However, the biomass was comparable in all these experiments. It was also noted that the pH was lower and the remaining glucose was higher (day 6-8) when both yeast extract and glucose were added to either F2m1 or F2m2 media than those observed upon using these media alone or with addition of either yeast extract or glucose (data not shown). These results suggest that the effect of the combination of yeast extract and glucose added to F2m1 or to F2m2 media is

on the biosynthesis of rifamycin B and is not related to growth of the microorganism. Thus, the stimulation of rifamycin B production obtained by fed-batch addition of 12% glucose to these media could be due to the development of either a more active system for rifamycin B production or a less active system for rifamycin B inhibition (Krishna et al., 2000) and the better availability of glucose during the idiophase (Lee and Rho, 1994).

This standard strain (*N. mediterranei* ATCC 21789) showed microscopical characteristics on both V2 and F2 media similar to those observed with strain N1 and variant NCH, whereas the morphology of the colonies was only slightly different. On Bennett's agar medium, the selected colonies had nearly the same morphological characteristics as colony type 1 of strain N1 or variant NCH (El-Tayeb et al., 2004a,b). One may suspect that all these strains belong to the same morphological group. As regards production of rifamycin B, the selected colonies produced only 3.02 g/l in F2 medium compared to 7.85 g/l produced by variant NCH (Figures 5 and 7).

The use of the most effective medium constituents in their optimum concentrations determined from the previous optimization experiments using strain N1 and variant NCH was tried using this standard strain (Figure 5). The use of F2m2 medium increased rifamycin B production to 4.12 g/l (36%) as compared to 3.02 g/l in F2 medium. It was observed that the effect of  $\text{KNO}_3$  on biomass, glucose utilization and pH showed the same pattern during the time course of fermentation as that observed with strain N1 and variant NCH (El-Tayeb et al., 2004a,b). The addition of 0.1% yeast extract at day 2 to F2 medium increased rifamycin B production from 3.02 to 3.49 g/l (16%). By further addition of 12% glucose at day 4 to F2m2 medium, the yield of rifamycin B increased from 3.02 to 5.3 g/l (75%).

In conclusion, among all the tested regimes, the batch-wise addition of glucose together with modification of the fermentation medium, by using F2m2 medium, increased rifamycin B production by NCH strain to a maximum of 17.17 g/l compared to a yield of 5.3 g/l by the standard strain under the same conditions (Figure 7). The increase in rifamycin B production using the standard strain confirms the usefulness of the tested medium modification regimes in the improvement of antibiotic production and its possible application in fermentations using other rifamycin B producer strains and also shows the superiority of variant NCH as a producer, when compared to the standard strain.

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