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Full Length Research Paper

Optimization of industrial production of rifamycin B by *Amycolatopsis mediterranei*. IV. Production in the fermentor

El-Tayeb, O.M.[1]*, Hussein, M. M. M.[2], Salama, A.A.[2] and El-Sedawy, H.F.[1]

[1]Microbial Biotechnology Center, Faculty of Pharmacy, Cairo University, Cairo, Egypt.
[2]Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

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Optimization of the physical and physiological parameters of the fermentation process using the gene amplified variant of *Amycolatopsis mediterranei* (NCH) was carried out. Optimization of the physical parameters by controlling the pH at 6.5 for 3 days then at 7 thereafter and by adjustment of aeration at 1 vvm for 3 days then controlling the dissolved oxygen (DO) at 30% saturation increased the yield from 9.77 to 11.96 (22%) and 13.39 g/l (37%), respectively. Replacing 12% glucose in the fermentation medium (F2m1) with 5% glucose syrup (F2m3 medium) resulted in a drop of the yield from 9.77 to 7.5 g/l, while further addition of another 5% glucose syrup at day 4 increased the yield from 7.5 to 13.81 g/l (84%); with a further increase in the yield to 14.25 g/l (90%) upon controlling DO. Whereas, the combined addition of 0.1% yeast extract at day 2 to F2m3 medium along with the addition 5% glucose syrup at day 4 increased the yield from 7.5 to 15.35 g/l (105%); with a further increase in the yield to 16.3 g/l (117%) upon controlling DO. The fed-batch addition of both 3% soytone at day 3 and 5% of glucose syrup at day 4 to F2m3 medium increased the yield from 7.5 to 16.2 g/l (116%) and by extending the fermentation period to 10 days the yield reached 17.9 g/l (139%). Upon applying all optimum physical and physiological conditions in the fermentor the yield increased from 7.5 to 17.43 g/l in 8 days (132%) and by extending the fermentation period to 10 days the yield reached 19.4 g/l (159%). Further process optimization by examination and analysis of the kinetics of the process would most certainly further increase the yield and quantitatively define the process to a level that could be tested on a pilot scale.

Key words: Rifamycin B, fermentor, biotechnology, *Amycolatopsis mediterranei,* optimization, fed-batch and process development.

INTRODUCTION

In recent years, rifamycins have acquired an added significance all over the world. Although rifamycins are primarily used against *Mycobacterium tuberculosis* and *M. leprae*, they are also effective against *M. avium* associated with acquired immunodeficiency diseases and penicillin-resistant pneumococci (Anon, 1999; Anne et al., 2000; Reynaldo-Dietze et al., 2001 and Russell, 1998).

*Correspondingauthor. E-Mail: omtayeb@link.net.

In view of this importance, *Amycolatopsis mediterranei* has been the focus of research of many laboratories. Yields of rifamycins vary from a few milligrams per liter to as high as 15 g/l. Fermentation processes and fermentation media have been continuously updated to obtain better yields of rifamycin (Lancini and Hengeller, 1971; Ghisalba et al., 1984; Lysko and Gorskaia, 1986; Schupp and Divers 1986; Chiao et al., 1988; Lal et al., 1995; Lancini and Cavalleri, 1997 and Venkateswarlu et al., 2000). Parameters such as pH, temperature, aeration

and agitation may have a substantial effect on the antibiotic production. Optimization of antibiotic production necessitates studying of the different physical and physiological factors affecting its production; taking into concern that the optimal fermentation conditions are related to the strain used (Pape and Rehm, 1985 and Venkateswarlu et al., 2000). For fermentation, *A. mediterranei* is first grown in seed medium, and then cultures are transferred to production medium. The use of reasonably cheaper ingredients in the production medium considerably lowers the production cost of rifamycin. Thus, several manipulations of the media, such as replacement of the costly carbon source, glucose, by cheaper ingredients such as maize flour, peanut meal and glycerol have been attempted (Ghisalba et al., 1984; Lal et al., 1995).

This study aimed at improvement of the fermentation process parameters to achieve better yield of rifamycin B and to lower the cost of production. Thus, we applied the optimized process previously developed in shake flasks (El Tayeb et al., 2004 bc) on fermentation experiments carried out in laboratory fermentors. The significant parameters that govern the fermentation process, such as aeration, dissolved oxygen, pH and the composition of the fermentation medium as well as the different regimes of fed-batch of some fermentation medium components were studied.

MATERIALS AND METHODS

Bacterial strain

The amplified variant NCH of *Amycolatopsis mediterranei*–RCP 1001, previously obtained as described by El-Tayeb et al. (2004a) was 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, NH_4NO_3 and $NaNO_2$ 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, $CaCO_3$, $MgSO_4.7H_2O$, KH_2PO_4, HCl 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, soytone, tryptone, skim milk and bacto agar were the products of Difco Laboratories, Detroit, U.S.A. Sunflower oil and oat flakes were obtained from commercial suppliers. Glucose syrup was obtained from the Egyptian Co. for Production of Starch and Glucose, Cairo.

Media used for propagation, selection and maintenance as well as the vegetative medium V2 and the fermentation medium F2m1 were those previously reported by El-Tayeb et al. (2004a, b). The modified fermentation medium F2m3 is composed of: glucose syrup 50.0 g, 120.0 g; soytone, 30.0 g; NH_4NO_3, 1.0 g; KH_2PO_4, 1.0 g; $CaCO_3$, 8.5g; $MgSO_4.7H_2O$, 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 and preparation of inoculum as well as determination of remaining glucose or reducing sugars concentration, biomass and assay of rifamycin B were those previously reported by El-Tayeb et al. (2004a,c).

Production of rifamycin B in the fermentor

The production of rifamycin B was carried out in a 6.6 litre laboratory glass fermentor (Bioflo 3000 Benchtop fermentor, New Brunswick Scientific Co., NJ, USA). The inoculum in a concentration of 5% was added to 2.5 litres of F2m1 medium, unless otherwise indicated. Agitation was carried out using 2 six blade Rushton impeller separated by 9.5 cm with the lower impeller 2.5 cm above the agitator tip. Aeration using a multiorifice ring sparger was controlled by a pressure regulator and monitored by a flowmeter. The percent dissolved oxygen (DO) was monitored and/or controlled through the fermentor's control unit using polarographic DO probe. DO control was achieved by cascading the airflow and the agitation speed simultaneously. The pH was monitored by a pH electrode; calcium carbonate was omitted from the medium when the pH was automatically controlled by the fermentor's control unit, which applied 1N HCl, and 1N NaOH actuated by peristaltic pumps. The temperature was adjusted at 28°C, the agitation rate at 500 rpm and the aeration rate at 1 vvm, unless otherwise indicated. Foam was suppressed by sterile sunflower oil using a foam controller pump and a foam probe. Samples were taken every 24 h and rifamycin B concentration, remaining glucose or reducing sugars concentrations, dry cell weight and, where indicated, viscosity of the broth mixture was determined. For fed-batch experiments, aliquots of 150 ml of glucose, 150 ml of glucose syrup, 150 ml of soytone, 25 ml of yeast extract 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.

Determination of viscosity

Ten ml of the fermentation broth culture were placed in the small adaptor of the rotational viscometer (Cole-Parmer 98936, USA). The spindle and speed combination was carefully selected to measure the correspondent viscosity in centipoise (cp) according to the table present in the instruction and operation manual supplied by the manufacturer.

RESULTS

By applying the process previously developed in shake flasks (El-Tayeb et al. 2004a, b) using F2m1 medium on fermentation experiments carried out in the fermentor (operated at 1 vvm, 500 rpm and 28°C) with NCH strain, the yield decreased from 11.76 to 9.77 g/l (Figure 1). Different manipulations of the fermentation process were carried out in batch-mode by changing the aeration rate, dissolved oxygen and pH or the application of fed-batch

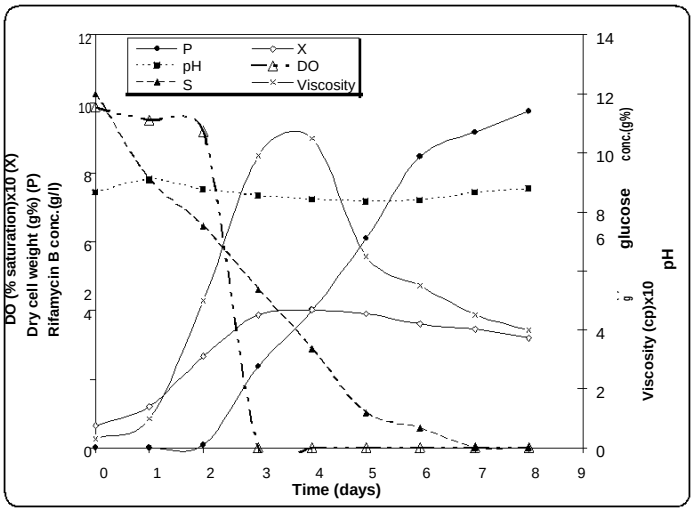

Figure 1. Time course of rifamycin B production in F2m1 medium by variant NCH in the fermentor. Conditions: 1 vvm, 500 rpm and 28°C.

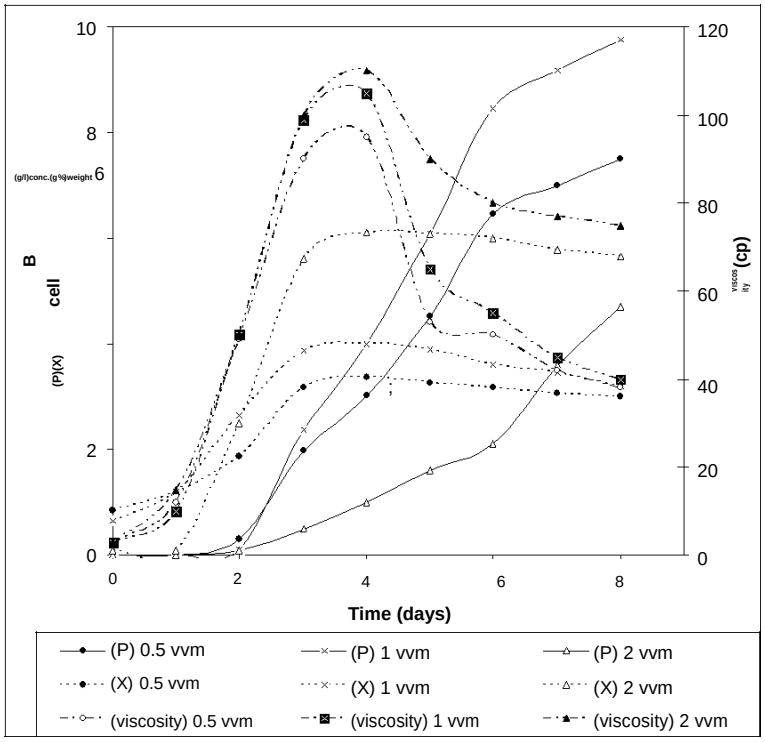

Figure 2. Effect of aeration rate on rifamycin B production (P), biomass (X) and viscosity in F2m1 medium by variant NCH. Conditions: 500 rpm and 28°C.

mode by the addition of glucose syrup, yeast extract and soytone, as described below.

The effect of the use of aeration rates of 0.5, 1 and 2 vvm on rifamycin B production, dry cell weight and viscosity is illustrated in Figures 1 and 2. Maximum rifamycin B production was obtained upon using 1 vvm 9.77 g/l) followed by 0.5 vvm (7.5 g/l) then 2 vvm (4.7 g/l), while the highest viscosity was obtained upon using aeration rate of 2 vvm followed by 1 vvm then 0.5 vvm. Different regimes for controlling DO were tested (Figure 3). The best regime was the use of 1 vvm for 3 days, then controlling DO at 30% saturation to the end of

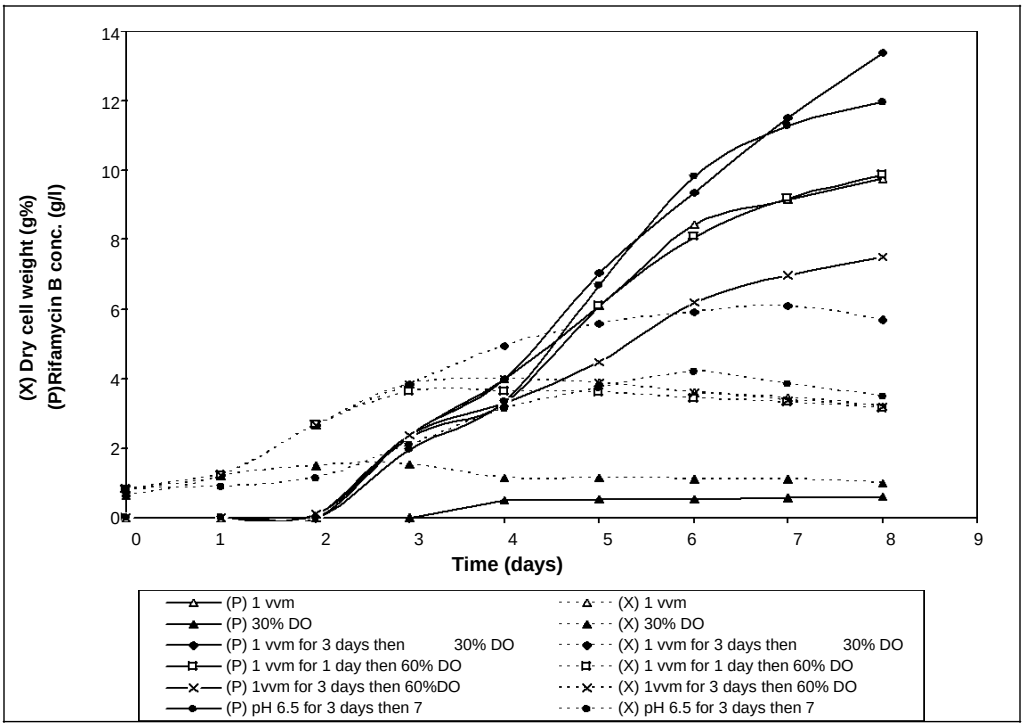

Figure 3. Effect of controlling DO and pH on rifamycin B production (P), and biomass (X) by variant NCH in F2m1 medium. Conditions: 500 rpm and 28°C.

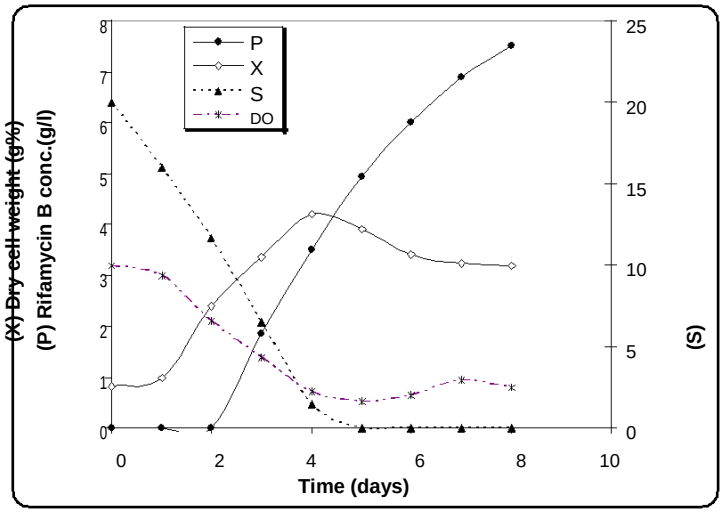

Figure 4. Effect of replacing 12% glucose in F2m1 medium by 5% glucose syrup (F2m3) on process parameters by variant NCH. Conditions: 1 vvm, 500 rpm, pH 6.5 for 3 days then 7 and 28°C.

fermentation, where the yield increased from 9.77 to 13.39 g/l (37%). Controlling the pH at 6.5 for 3 days then at 7 to the end of the fermentation process (Figure 3) increased rifamycin B production from 9.77 to 11.96 g/l (22%).The effect of the replacement of glucose in F2m1 medium with 5% glucose syrup (F2m3) on process parameters is illustrated in Figure 4. The use of F2m3 decreased rifamycin B production from 9.77 to 7.5 g/l. Different regimes of fed-batch addition of glucose syrup to F2m3 were tested (Figure 5). All the tested regimes increased rifamycin B production from 7.5 g/l to a yield ranging from 12.94 g/l to 13.81 g/l (73 to 84%). The best

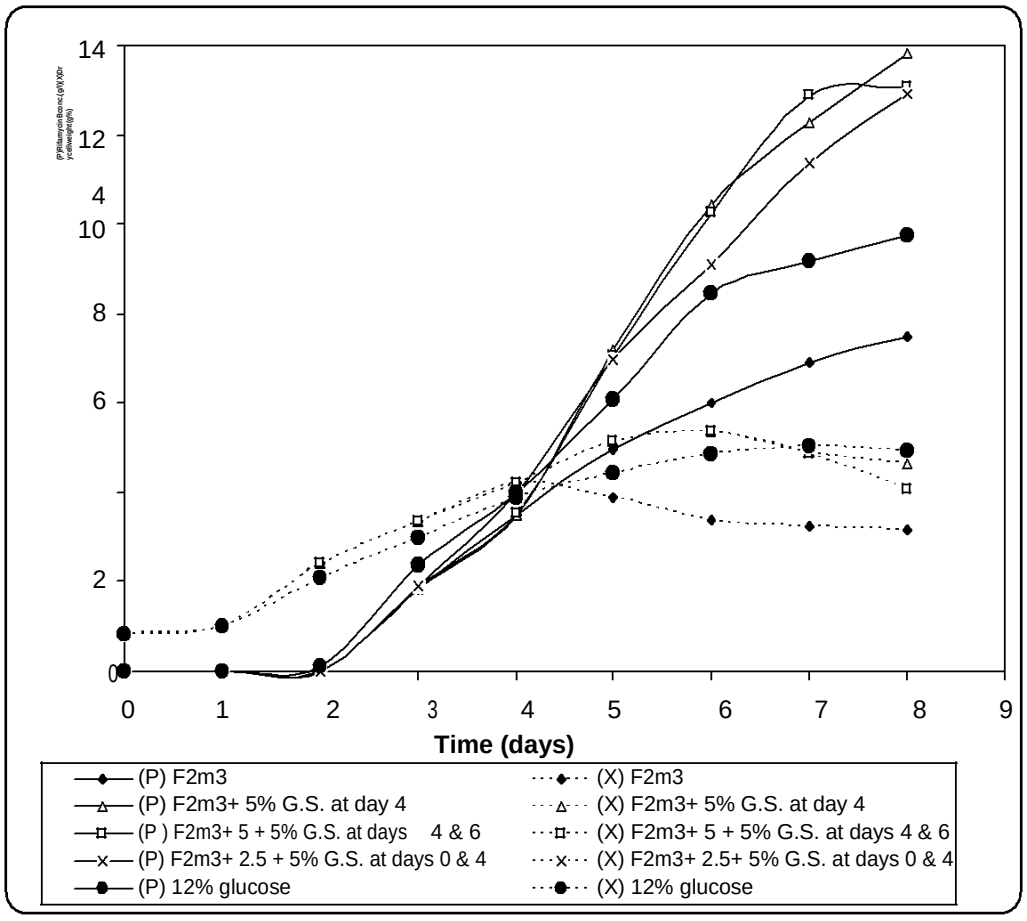

Figure 5. Effect of different regimes for addition of glucose syrup (G.S.) to F2m3 medium on rifamycin B production (P) and biomass (X) by variant NCH. Conditions: 1 vvm, 500 rpm, pH 6.5 for 3 days then 7 and 28°C.

regime was the addition of 5% glucose syrup at day 4 where the yield increased from 7.5 to 13.81 g/l (84%) with a further increase to 14.25 g/l (90%) upon applying the optimum regime for controlling DO (Figure 5). Whereas, the combined addition of 0.1% yeast extract at day 2 along with the addition of 5% glucose syrup at day 4 (Figure 6) increased the yield from 7.5 to 15.35 g/l (105%); with a further increase to 16.3 g/l upon using the best regime for controlling DO (117%). While, the combined addition of 3% soytone at day 3 along with the addition of 5% glucose syrup at day 4 (Figure 6) increased the yield from 7.5 to 16.2 g/l (116%) and by extending the fermentation period to 10 days, the yield reached 17.9 g/l (139%).

Integration of the optimum physical and physiological conditions was carried out in the fermentor (Figure 7). By applying the fed-batch addition of 0.1% yeast extract at day 2, 3% soytone at day 3 and 5% glucose syrup at day 4 to F2m3 medium under the best regime of controlling pH and DO, the yield increased from 7.5 to 17.43 g/l (132%) and by extending the fermentation period to 10 days, the yield reached 19.4 g/l (159%).

DISCUSSION

The results of production of aerobic fermentation on products in shake flasks usually could not be extrapolated to indicate possible performance in the fermentor (Dewitt et al., 1989). Both physical and biological factors are quite different in fermentors and in shake flasks. Moreover, controls on the reaction in the shake flasks are extremely limited while in the fermentor such controls are almost limitless. Accordingly, results obtained in the shake flasks should be taken only as preliminary indicators for the conditions necessary for successful industrial production and must be verified in studies carried out in the fermentor. For large-scale fermentation, the use of reasonably cheaper ingredients in the production medium considerably lowers the cost of

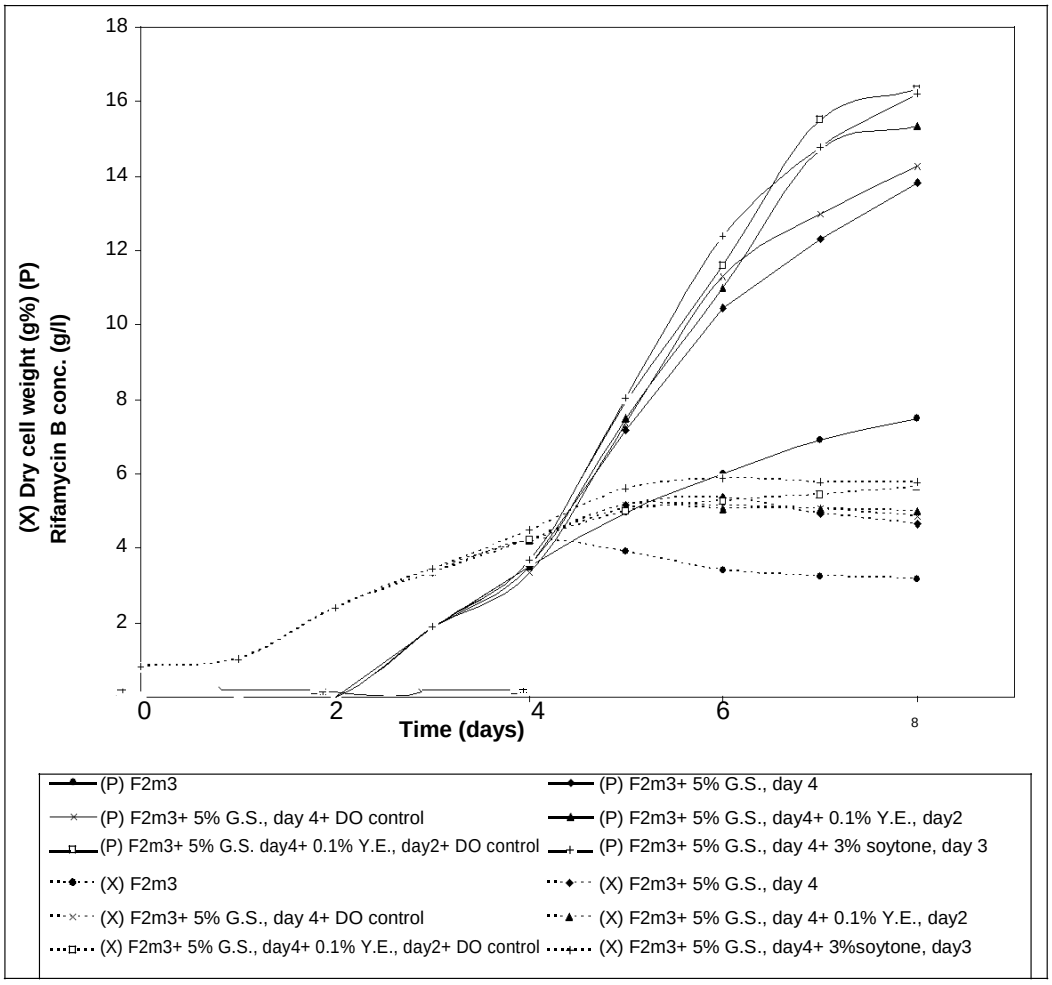

Figure 6. Effect of fed-batching 5% glucose syrup (G.S.) at day 4 alone or combined with either 0.1% yeast extract (Y.E.) at day 2 or 3% soytone at day 3, to F2m3 and of controlling DO (1vvm for 3 days then 30% DO) on rifamycin B production (P) and biomass (X) by variant NCH. Conditions: 1 vvm , 500 rpm, pH 6.5 for 3 days then 7 and 28°C.

production. Thus, several manipulations of the F2 medium by the use of a lower concentration of soytone (3% instead of 3.5%) and the replacement of $(NH_4)_2SO_4$ by the more productive and cheaper NH_4NO_3 (F2m1) or along with the replacement of the costly carbon source, glucose, by the cheaper glucose syrup (F2m3) as well as studying the influence of different physical and physiological parameters have been attempted in a fermentor.

By applying the process previously developed in shake flasks (El-Tayeb et al., 2004a, b) using F2m1 medium on fermentation experiments carried out in the fermentor (operated at 1 vvm, 500 rpm and 28°C) with NCH strain, the yield decreased from 11.76 to 9.77 g/l (Figure1). This lower yield observed upon using the fermentor was in accordance with the findings of Alvarez et al. (1990), who observed that there were both qualitative and quantitative differences in the production of rifamycin under shake flask and pilot-scale fermentations. In shake flasks, only rifamycin B was produced while in fermentors both rifamycin B and W were produced, with the production rate of rifamycin B decreasing with the accumulation of rifamycin W. They did not explain this difference in behavior of the strain in shake flasks and fermentors.

Aeration has an important effect on rifamycin B production. A characteristic of A. mediterranei fermentation is the very high oxygen requirement in a definite period of the growth-production cycle. The time at which this occurs varies with the strain and the medium composition, but it always coincides with the phase in which glucose is rapidly metabolized. If this oxygen demand is not met by an adequate oxygen supply through aeration, a very low final yield of rifamycin B is obtained (Virgilio et al., 1964 and Lancini and Cavalleri, 1997). In several reports, airflow of 1 vvm is carried out at pilot scale (Lee et al., 1983; Lee and Rho, 1994 and Lancini and Cavalleri, 1997). In our study, the maximum rifamycin B production was obtained (Figures 1 and 2) upon using 1 vvm (9.77 g/l) followed by the use of 0.5 vvm (7.5 g/l) then by the use of 2 vvm (4.7 g/l). Therefore,

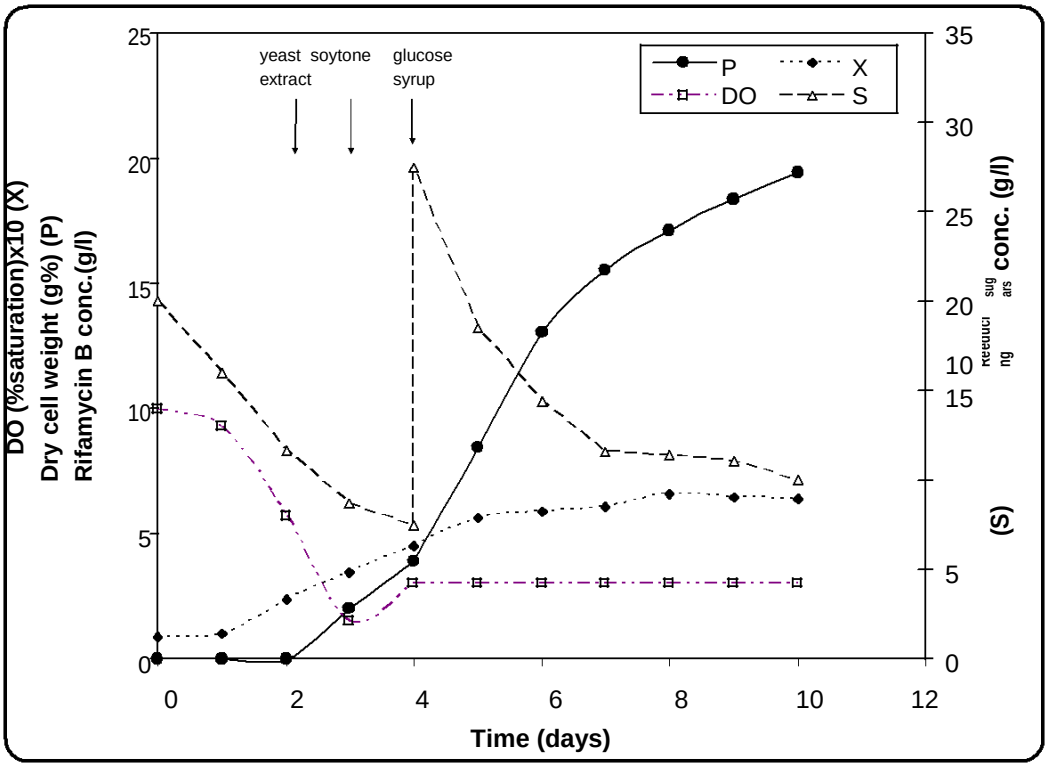

Figure 7. Effect of fed-batching 0.1% yeast extract at day 2, 3% soytone at day 3 and 5% glucose syrup at day 4 to F2m3 with controlling pH and DO on process parameters by variant NCH. Conditions: 1 vvm for 3 days then 30% DO, pH 6.5 for 3 days then 7, 500 rpm, and 28°C.

1 vvm was selected for further fermentation studies performed in the fermentor. The lowest yield of rifamycin B obtained upon using 2vvm was associated with the highest biomass and consequently the highest viscosity (Figure 2). This cell mass exceeded the level that can be supported by the optimum oxygen transfer rate (OTR) required for maximum antibiotic production in the fermentor as reported by Margaritis and Zajic (1978).

It was also observed that, in experiments using 1 vvm or 0.5 vvm, the recorded dissolved oxygen decreased to zero on the 3rd (Figure 1) or 4th day (data not shown) of fermentation process and remained so till the end of fermentation period. Based on this result and taking in consideration the reported 30% saturation as a minimum oxygen demand for growth and production phases (Lee and Rho, 1994), we tried different regimes of controlled dissolved oxygen (Figure 3) to maintain optimal DO level throughout the fermentation. A marked increase in the yield from 9.77 to 13.39 g/l (37%) was achieved by using 1 vvm for 3 days then 30% controlled DO to the end of the fermentation period (Figure 3).

Controlling the pH during the fermentation has been reported to contribute to improving rifamycin B production. Thus, Lee et al. (1983) reported a characteristic pH behavior pattern of A. mediterranei and recommended a pH strategy of 6.5 at the trophophase (day 1-3) and 7.0 at the idiophase (day 4-8). This pH profile was applied and this increased the yield of rifamycin B (Figure 3) from 9.77 g/l to 11.86 g/l (22%).

To lower the production cost of rifamycin B, the cheaper glucose syrup was used instead of the costly carbon source, glucose, in the F2m3 medium. From the results using shake flasks (El-Tayeb et al., 2004a), we have already found that 5% glucose syrup was the lowest optimum concentration for both growth and production of rifamycin B. In the fermentor, the yield of rifamycin B upon using 5% glucose syrup under controlled pH was 7.5 g/l (Figure 4) compared to 11.96 g/l when using 12% glucose (Figure 5). This reduction may be attributed to the low glucose content in glucose syrup. The glucose syrup used is a product of an acid hydrolysis process and contains less than 25% of its carbohydrates in the form of glucose, the remaining carbohydrates being a mixture of disaccharides, trisaccharides and dextrins; the response of the organism to which is not known. This fact is further supported by the observation that with 5% glucose syrup in the fermentor there was clear carbon starvation as evidenced by the exhaustion of the used carbon source to less than 0.15% on day 4 (Figure 4). Since the utilization of glucose at the idiophase influences rifamycin B production (Lee et al., 1983) and taking into consideration the suggestion of Sokol et al. (1982) to add

carbon sources to the fermentation when the glucose level decreased below 5% of the initial concentration, different regimes for batch-wise addition of glucose syrup were carried out (Figure 5). All the tested regimes increased rifamycin B from 7.5 g/l to a yield ranging from 12.94 g/l to 13.81 g/l (72% to 84%), and the highest antibiotic production was achieved by addition of 5% glucose syrup to F2m3 medium at day 4 with a further increase to 14.25 g/l (90%) upon controlling DO (Figure 6). The use of this regime thus, attained two goals: lowered cost and higher yield. Therefore, in all subsequent experiments this regime was used. The use of the two regimes: initial 7.5% then 5% glucose syrup added at day 4, and initial 5% then two increments of 5% glucose syrup added at days 4 and 6, led to a lower yield compared to that obtained upon using initial 5% then one increment of 5% glucose syrup at day 4. It seems that glucose syrup in concentrations above 10%, used in two or three increments, might contain some undesirable ingredients such as hydroxyl-methyl furfural, which may lead to inhibition of rifamycin B biosynthesis and/or biomass formation. An interesting finding is that the addition of the third increment of 5% glucose syrup, although increasing the apparent rate of production from day 6 to 7, caused abrupt cessation of production thereafter along with reduction of the biomass (Figure 5).

By the addition of the optimum concentration of yeast extract (0.1%) previously determined by El-Tayeb et al. (2004a) to F2m3, rifamycin B production increased from 7.5 to 15.35 g/l (105%) with a further increase to 16.3 g/l (117%) upon controlling DO (Figure 6). The batch-wise addition of 3% soytone to F2m3 medium (Figure 6) increased the yield from 7.5 to 16.2 g/l (116%), by extending the fermentation period to 10 days the yield reached 17.9 g/l (139%). An integrated fermentation process was carried out combining all of the following optimized conditions: controlled DO (1 vvm for 3 days then 30% of saturation to the end of fermentation), controlled pH (6.5 for 3 days then 7.0 to the end of fermentation), the addition of 0.1% yeast extract at day 2, of 3% soytone at day 3 and of 5% glucose syrup at day 4 to F2m3 medium. These conditions afforded a yield of 17.43 and 19.4 g/l in days 8 and 10, respectively (Figure 7).

In conclusion, through improvement of the fermentation process, this work achieved its target where the yield of rifamycin B increased from 7.5 g/l to 17.43 g/l (132%) and by extending the fermentation period to 10 days the yield reached 19.4 g/l (159%). Although higher yields are achieved by extending the fermentation period to more than 8 days, sufficient economic studies on the cost of labor, power, etc. must be carried out to determine whether the extension of the fermentation period is beneficial or not. A comparable yield (19.11 g/l) was reported by Jin et al. (2002) in fed-batch fermentation in 60,000 L fermentor with a mutant industrial strain of *A. mediterranei.*

Further process optimization by examination and analysis of the kinetics of the process through mathematical modeling and/or genetic analysis and modification of the strain would most certainly further increase the yield and quantitatively define the process to a level which could be tested on a pilot scale and hence suites the design of an industrial process.

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