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Optimization of industrial production of rifamycin B by *Amycolatopsis mediterranei*. II. The role of gene amplification and physiological factors in productivity in shake flasks

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Amplification of gene expression of the most productive colony type of *Amycolatopsis mediterranei* strain N1 under stress of chloramphenicol, resulted in isolation of a variant NCH with productivity of 2.56 g/l compared to 1.15 g/l by the parent strain N1 (2.2 fold increase) . This amplified variant has a further advantage of reduced variation in colony morphology with predominance of the most productive colony type. Using variant NCH, modification of the fermentation medium F1 by the addition of 0.1% yeast extract or the use of 1.8% KNO₃ resulted in 3.8 and 5.8-fold increase in productivity, respectively, compared to strain N1. When the F1 medium was replaced by a new medium F2 containing soytone, instead of the particulate constituents (peanut meal and soybean meal) the yield by variant NCH reached 7.85 g/l (6.8-fold increase). Modification of the F2 medium by addition of glycerol or the replacement of glucose by glucose syrup decreased rifamycin B production. Changing the concentration of soytone increased the yield only slightly while replacing it with peptone or tryptone or the addition of 1 % corn steep liquor failed to increase the yield. On the other hand, the addition of 0.1 % yeast extract, or the replacement of 0.6% (NH₄)₂SO₄ by 1.2% KNO₃ or 0.4% NH₄NO₃, to F2 medium led to 8.2, 10.2 and 10.4-fold increase in productivity, respectively, compared to productivity of strain N1 in F1 medium. The change in the concentrations of either MgSO₄ or CaCO₃, the use of different types of antifoams and the use of higher concentrations of sodium diethyl barbiturate did not significantly influence the yield. These collective optimization attempts thus resulted in a 10.4-fold increase in productivity, from 1.15 to 11.99 g/l.

Key words: Rifamycin B, fermentation, biotechnology, *Amycolatopsis mediterranei*, optimization, gene amplification, physiological factors.

INTRODUCTION

Attempts have been made to improve fermentation and downstream processing parameters for better yields of rifamycins. Continuous efforts since 1960 have led to the development of several industrial strains of *Amycolatopsis mediterranei* either with the ability to produce higher amounts of rifamycin B or mutant strains

that could directly produce active rifamycins and their derivatives (Chiao et al., 1988; Lal et al., 1995; Lancini and Hengeller, 1971; Lysko and Gorskaia, 1986; Ghisalba et al., 1982; Schupp and Divers 1986). Gene amplification is of widespread occurrence in prokaryotic and eukaryotic organisms (Anderson and Roth, 1977; Schimke et al., 1982) where selection for increased gene dosage can be applied to generate strains that carry multiple copies of a gene and consequently high gene expression products (Young, 1984).

Biotechnology production processes are the result of time-consuming, expensive research. For each producing

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strain, the medium and the other process parameters must be adjusted to allow the maximal expression of the producing capacity. It is obvious that such informations are industrial properties, and for rifamycin, the composition of the media actually used for industrial production are not published. However, some information can be extrapolated from published laboratory data and patent literature that give a fairly good idea of the most suitable ingredients and their concentrations (Lancini and Cavalleri, 1997; Pape and Rehm, 1985).

Our previous attempts to improve rifamycin B productivity of the industrial strain N1 by selection of the best producing colony type and by modifying the fermentation medium F1 resulted in an increase in the yield from 0.5 to 2.92 g/l (El-Tayeb et al., 2004). This is much lower than the economically viable yield. Therefore, in this study, we tried two approaches: the improvement of the producer strain by gene amplification and studying different physiological parameters for the process including media constituents.

MATERIALS AND METHODS

Bacterial strains

A. mediterranei – RCP 1001 mutant strain N1 was obtained from El-Nasr Company for Pharmaceutical Chemicals, Egypt.

Chemicals

Chemicals used throughout this work were of laboratory reagent grade unless otherwise indicated. Glucose, KNO₃, NH₄NO₃, 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. Chloramphenicol was obtained from Chemical Industries Development Co. (CID), Egypt. Sunflower oil was obtained from local commercial suppliers.

Media

Tryptone, peptone, yeast extract, malt extract, beef extract, skim milk, soytone, and bacto agar were the products of Difco Laboratories, Detroit, U.S.A. Corn steep liquor and glucose syrup were obtained from the Egyptian Co. for Manufacture of Starch and Glucose, Egypt. Oat flakes, soybean meal and peanut meal were obtained from local commercial suppliers.

Media used for propagation, selection and maintenance as well as the vegetative medium V1 and the fermentation medium F1 were those previously reported by El-Tayeb et al. (2004). The vegetative medium V2 (Lee and Rho, 1994) is composed of: glucose, 20.0 g; yeast extract, 5.0 g; tryptone, 2.5 g; malt extract, 5.0 g and distilled water to 1000.0 ml. The pH of was adjusted to 7.0. The fermentation medium F2 (Lee and Rho, 1994) is composed of: dextrose, 120.0 g; soytone, 35.0 g; (NH₄)₂SO₄, 6.0 g; KH₂PO₄, 1.0 g; CaCO₃, 8.5 g; MgSO₄.7H₂O, 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 and assay of rifamycin B were those previously reported by El-Tayeb et al. (2004). Yields of rifamycin B indicated are those obtained on day 8, unless otherwise indicated. The biomass was determined by dry cell weight method as described by Virgilio et al. (1964).

Gene amplification

Gene amplification was carried out as described by Kallio et al. (1987). A 5% v/v inoculum of strain N1 was added to a flask containing 100 ml vegetative medium (V1) containing 15 µg/ml chloramphenicol. The shake flask was incubated for 3 days at 28°C. One ml of this culture was used to inoculate another flask containing 30 µg/ml of the antibiotic, then the shake flask was incubated for 3 days at 28°C and the procedure was repeated using 60 µg/ml chloramphenicol. Several aliquots of 1 ml of the last culture were transferred onto the surface of Bennett's agar plates containing 120 µg/ml chloramphenicol. These plates were incubated for 12–15 days at 28°C. Typical colonies (El-Tayeb et al., 2004) were selected and cultured onto the surface of Q/2 agar slants, incubated for 8 days at 28°C and used for propagation and selection. The variant obtained, NCH, was maintained as lyophilized material in skim milk.

RESULTS

The variant NCH was compared to strain N1 for productivity in F1 medium (Figure 1) whereby it produced 2.56 g/l compared to 1.15 g/l by strain N1. The pattern of productivity by both cultures was somewhat similar, with variant NCH showing higher slope of increase between days 6 and 8. Both sugar consumption and pH were similar with both strains. The variant NCH showed the same colonial morphology and microscopical characteristics as the parent strain N1 on Bennett's agar and F1 medium. However, it showed less variation in colony morphology with the predominance of the best productive colony type.

Modification of the F1 medium was carried out by the addition of yeast extract and by changing the concentrations of each of glucose and KH₂PO₄ as well as replacing (NH₄)₂SO₄ by KNO₃ (Figure 2). Addition of 0.1% yeast extract after 2 days of incubation increased rifamycin B production from 2.56 to 4.32 g/l (68%), while the use of 0.9 and 1.8% KNO₃ markedly increased rifamycin B production from 2.56 to 6.33 g/l (2.5-fold) and to 6.72 g/l (2.6-fold), respectively. Upon microscopical examination, It was observed that KNO₃ decreased branching and fragmentation of the mycelia in the fermentation medium. On the other hand, the use of glucose in concentrations above or below 14% (control) reduced the yield by 15-17% (Figure 2). In addition, the use of KH₂PO₄ in concentrations above 0.1% (control) caused a marked decrease (37-45%) in the yield (Figure 2). Thus, these optimization attempts of the fermentation

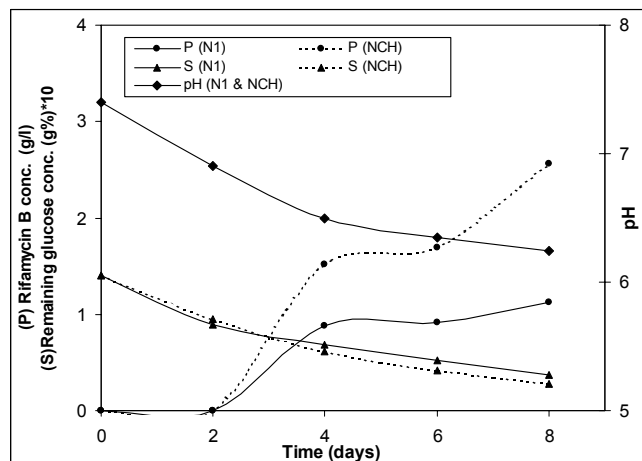


Figure 1. Comparison between strain N1 and variant NCH with respect to rifamycin B production (P), remaining glucose concentration (S) and pH in F1 medium.

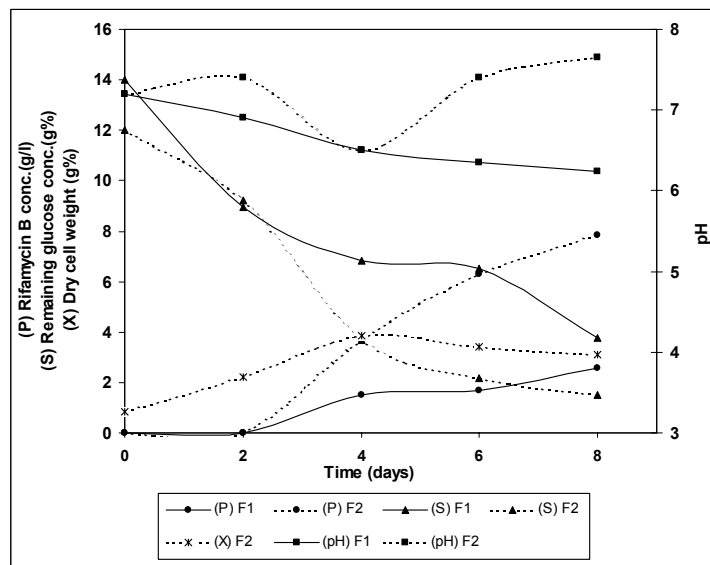


Figure 3. Comparison between F1 and F2 media with respect to rifamycin B production (P), remaining glucose concentration (S), biomass (X) and pH by variant NCH.

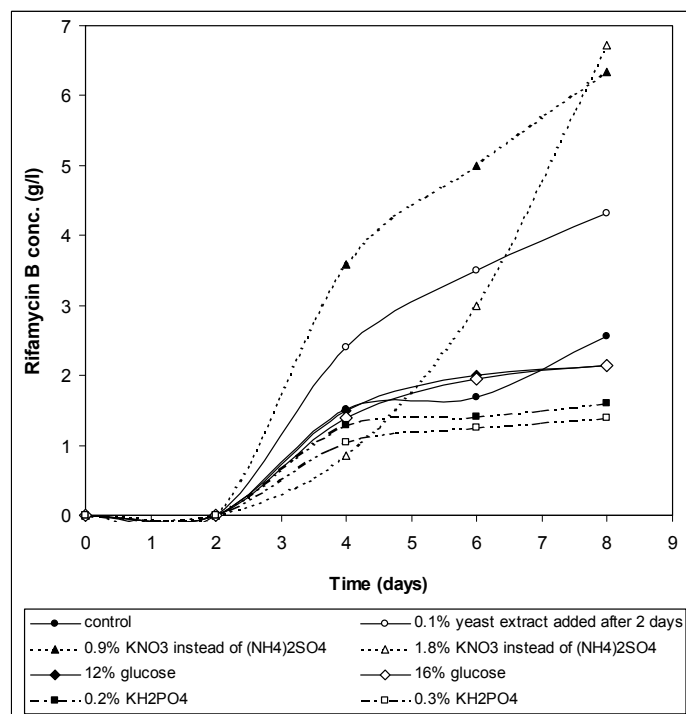


Figure 2. Effect of different modifications in F1 medium on rifamycin B production by variant NCH.

process using variant NCH resulted in a significant increase in the yield from 2.56 g/l to a maximum of 6.72 g/l (2.6-fold).

To further increase the yield of rifamycin B using variant NCH, we resorted to radical modification of both the vegetative and the fermentation media. In addition to

failing to reach the desired yield, F1 medium was a particulate medium in which following biomass production was not possible through either dry cell weight or turbidity. We thus shifted to a soluble medium, F2. It was thought appropriate then to also shift the vegetative medium to a particle free one, V2. This approach resulted in an increase of rifamycin B production from 2.56 to 7.85 g/l (3.1-fold) when compared to production by variant NCH on unmodified F1 medium (Figure 3). Using F2 medium we noted that while biomass formation started immediately and reached its peak after 4 days, production started only on day 2 and reached its maximum on day 8.

Further optimization of rifamycin B production by the variant NCH in F2 medium included changes of most of its ingredients, their concentrations and the time of their addition and the results are presented in Table 1. In all cases, rifamycin B production, remaining glucose concentration, biomass and pH were followed in a time course. The carbon sources glucose syrup and glycerol, the nitrogen sources peptone, tryptone, corn steep liquor, soytone at a concentration of less than 3% and concentrations of $(NH_4)_2SO_4$ as well as different concentrations of $MgSO_4$ and $CaCO_3$ failed to increase the yield (Table 1 and Figures 4 and 5). Different concentrations of SDB and different types of antifoam gave comparable yields (Table 1). On the other hand, the addition of 0.1 % yeast extract after 2 days of incubation (Table 1) increased the yield from 7.85 to 9.49 g/l (21%) and the replacement of $(NH_4)_2SO_4$ with 0.6 to 1.2% KNO_3 or with 0.05 to 0.8% NH_4NO_3 , increased rifamycin B production and the highest yield was achieved with 1.2%

Table 1. Effect of different modifications in F2 medium* on rifamycin B production by variant NCH on day 8.

Variable factors	Conc. (g%)	Rifamycin B conc. (g/l)	Variable factors	Conc. (g%)	Rifamycin B conc. (g/l)
Carbon Source Glycerol ⁽¹⁾	6.0 ⁽¹⁾	6.56	Glucose syrup ⁽³⁾	5.0	6.11
	6.0 ⁽²⁾	7.68		10.0	6.22
				15.0	6.34
				20.0	3.52
				25.0	1.84
Inorganic Nitrogen (NH ₄) ₂ SO ₄ KNO ₃ ⁽⁴⁾ NH ₄ NO ₃ ⁽⁴⁾	0.4	7.65	Organic Nitrogen Soytone Tryptone ⁽⁵⁾ Peptone ⁽⁵⁾ Yeast extract ⁽⁶⁾ Corn steep liquor ⁽⁷⁾	2.0	5.86
	(0.6)	7.85		2.5	6.95
	0.8	6.93		3.0	7.76
	0.96	6.79		(3.5)	7.85
	0.6	8.76		4.0	7.71
	0.9	8.85		4.5	7.69
	1.2	11.76		3.5	6.5
	1.8	4.59		3.5	6.28
	0.05	11.56		0.1	9.49
	0.1	11.73		1.0	6.69
	0.2	11.88			
	0.4	11.99			
	0.6	10.32			
0.8	9.07				
MgSO₄	0.04	5.80	CaCO₃	0.6	7.55
	(0.08)	7.85		(0.85)	7.85
	0.12	7.90		1.1	7.70
	0.16	6.27			
SDB ⁽⁸⁾	(0.1)	7.85	Antifoam Low density silicone oil High density silicone oil Sunflower oil	0.2	7.62
	0.15	7.97			
	0.17	8.13			
	0.2	7.91			
			0.2	7.63	
			0.2	7.57	

*The concentrations of the F2 medium (control) are between brackets.

(1) Glycerol added after 1 day of incubation.

(2) Glycerol added after 3 days of incubation.

(3) Instead of glucose.

(4) Instead of (NH₄)₂SO₄.

(5) Instead of soytone.

(6) Yeast extract added after 2 days of incubation.

(7) Corn steep liquor added after 1 day of incubation.

(8) Sodium diethyl barbiturate.

KNO₃ or 0.4% NH₄NO₃ which increased the yield from 7.85 to 11.76 (50%) and 11.99 g/l (53%), respectively (Table 1 and Figure 6).

DISCUSSION

Although our previous optimization attempts to improve the fermentation process using strain N1 in F1 medium increased rifamycin B production, the yield was too low to

be used for an economically viable technology and some variation in colony types continued to be observed (El-Tayeb et al., 2004). To obtain higher yields and to overcome the problem of variation in colony types, another approach to improve the strain had to be tried. Gene amplification as a method of strain improvement had been successfully used with *Bacillus subtilis* producing α -amylase (El-Tayeb et al., 2000; Kallio et al., 1987). By application of gene amplification to N1 strain (by exposing the parent strain N1 to increasing

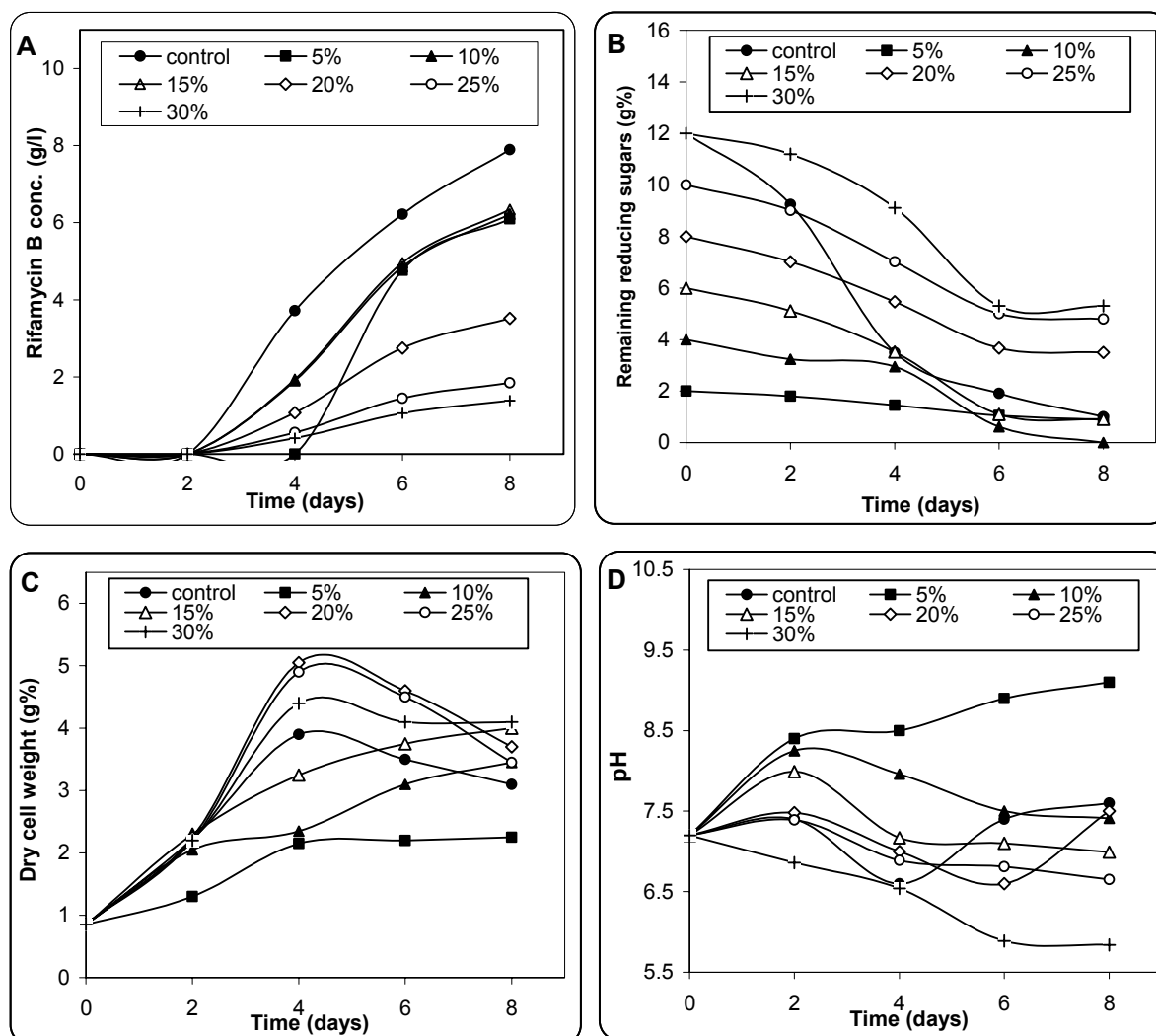


Figure 4. Effect of replacing glucose with different concentrations of glucose syrup in F2 medium on rifamycin B production (A), remaining reducing sugars (B), biomass (C) and pH (D) by variant NCH.

concentrations of chloramphenicol), we achieved 2.2-fold higher yield (Figure 1) with less colony variation and predominance of the most productive colony type.

The increase in the production of rifamycin B after treatment of the parent strain with chloramphenicol could be explained by the finding of Lenski et al. (1994) that repeated subculture of a plasmid-containing strain under selective conditions eventually gave rise to a variant of the plasmid that was much more stable in the absence of selection than the original form of the plasmid. Salyers and Amabile-Cuevas (1997) suggested that exposure of a bacterium with a newly acquired plasmid or conjugative transposon to antibiotic concentrations high enough to be slightly selective but low enough to allow bacteria to replicate could foster adaptive mutations that have the effect of fixing the element in its new host. They further

suggested that any gene carried on the plasmid would have the chance during this period of selective pressure to increase their expression levels or to adopt to a better fit with their new host. On the basis of the above explanations, one may suspect the possible presence of plasmids in colony type 1 which are involved in the biosynthesis of rifamycin. Although Ghisalba et al. (1984) reported that no plasmids have been isolated from *Nocardia mediterranei* ATCC 13685 and its mutants and indicated that plasmids do not play a significant role in rifamycin biosynthesis, a genetically manipulated industrial strain such as strain N1 may show different genomic constitution. However, it should also be noted that, Kallio et al. (1987) studied α -amylase production by *Bacillus subtilis* in two different gene expression systems where α -amylase gene was incorporated either into a

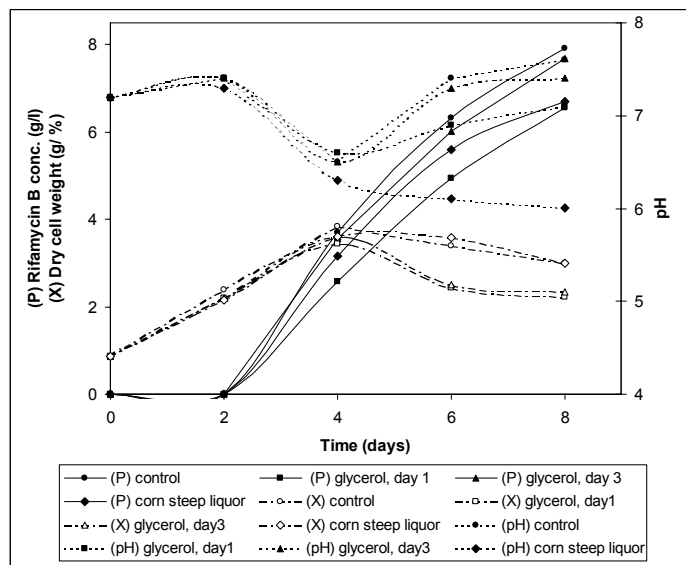


Figure 5. Effect of addition of 6% glycerol after 1 or 3 days and of 1% corn steep layer after 1 day to F2 medium on rifamycin B production (P) and pH by variant NCH.

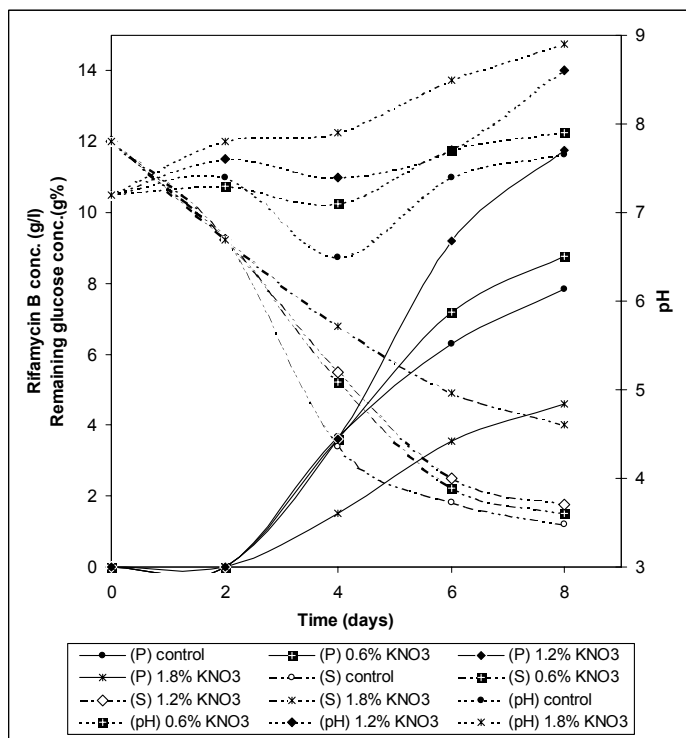


Figure 6. Effect of replacing $(\text{NH}_4)_2\text{SO}_4$ with KNO_3 in F2 medium on rifamycin B production (P), remaining glucose concentration (S) and pH by variant NCH.

plasmid or in the chromosome. They found that gene amplification by chloramphenicol resulted in higher amplification of the chromosomally encoded gene copies

for α -amylase than in plasmid containing or the parental strains. Further genetic studies on variant NCH are needed in order to determine whether the amplification of the gene for rifamycin B biosynthesis is plasmid or/and chromosomally encoded.

Optimization of the process using variant NCH in F1 medium by the addition of yeast extract, changing concentrations of either glucose or KH_2PO_4 or replacing $(\text{NH}_4)_2\text{SO}_4$ by KNO_3 gave results which were similar to those previously obtained with strain N1 ((El-Tayeb et al., 2004). When glucose concentrations above or below the control were used the yield decreased by 15–17% (Figure 2). In conclusion, optimization of rifamycin B production by variant NCH using a modified F1 medium increased the yield from 2.56 g/l to a maximum of 6.72 g/l (2.6-fold).

To further increase the yield of rifamycin B we shifted to soluble media, V2 and F2, recommended by Lee and Rho (1994). This approach resulted in a 3.1-fold increase of rifamycin B yield by variant NCH from 2.56 to 7.85 g/l as compared to production in the unmodified F1 medium. This increase in the yield was associated with a marked increase in the apparent rate of glucose consumption till day 4 along with a decrease in pH from day 2 to day 4 (Figure 3). After day 4, the apparent rate of glucose consumption decreased, with F2 medium, along with a rise in pH from 6.5 to 7.7 while higher remaining glucose concentration was still available with F1 medium. The rise in pH after day 4 is possibly due to the disappearance of carbohydrates and the metabolism of the intermediate organic acids accumulated, as well as the slow release and metabolism of nitrogenous materials from proteins. Lee et al. (1983) concluded that the utilization of glucose at the idiophase is an influencing factor for rifamycin B production, since almost all the carbon units in the rifamycin B molecule are derived from glucose. They added that the optimal pH condition in the idiophase was found to be in the range of pH 7.0 to 7.5.

Further attempts to optimize rifamycin B production by variant NCH in F2 medium were carried out by changing most of its ingredients, their concentrations and the time of their additions. The fermentation process lasts for 8 days and a consistent lag time of about 48 h was observed before the beginning of antibiotic production. Ghisalba et al. (1984) mentioned that the long lag phase observed during the fermentation of *A. mediterranei* is one of the problems encountered in rifamycin B production. However, they did not give any suggestion as to how this problem may be overcome.

Replacing glucose with the less expensive glucose syrup (5–30%) decreased the yield. The use of 5, 10 and 15% glucose syrup decreased biomass till day 5 and consequently antibiotic production by 19 to 22% along with high pH values (above 7.5) in the trophophase (Figure 4). Taking into consideration that the glucose syrup used was prepared by acid hydrolysis of starch and hence contained mainly dextrans, maltose and low

content of glucose, this rise in pH may point to a possible establishment of a critical balance in utilization of glucose, dextrans, and amino acids released from soluble proteins of the medium as energy sources with dextrans being least favored. With such possibility, a slight rise of pH could be expected as a result of release of ammonia from amino acids along with continuous consumption of intermediate organic acids produced during metabolic activity. However, the higher concentrations of glucose syrup (20-30%) markedly decreased the yield by 55 to 82%. It is possible that these high concentrations of glucose syrup might contain some undesirable ingredients such as hydroxy-methyl furfural, which led to inhibition of rifamycin B biosynthesis even though the results do not suggest inhibition of biomass production (Figure 4). It is interesting that with all the tested concentrations of glucose syrup, the pattern of remaining reducing sugars was somewhat similar and differed from the control, showing a slower rate of carbohydrate utilization. It seems that the organism continuously replenishes the reducing sugars by hydrolysing more higher saccharides.

Since *A. mediterranei* can utilize either glucose or glycerate as a precursor for the biosynthesis of 3-amino-5-hydroxybenzoic acid which is the chain initiator molecule in the biosynthesis of rifamycins (White et al., 1974), we used a supplement of 6% glycerol to the F2 medium containing 12% glucose after 1 or 3 days of incubation (Figure 5). The addition of glycerol after 1 day was associated with reduced biomass during the idiophase with somewhat lower rifamycin B production, while its addition after 3 days led to a similar reduction in biomass but the yield was comparable to the control.

As for the content of organic nitrogen sources in F2 medium, the soytone concentrations of 3% and higher gave comparable rifamycin B production while lower concentrations resulted in some reduction of the yield (Table 1). Replacement of soytone by other enzymatically hydrolyzed proteins such as tryptone and peptone (Table 1) slightly decreased the yield. The addition of 0.1% yeast extract after 2 days increased the antibiotic yield by 21% (Table 1). In contrast, the use of 1% corn steep liquor after 1 day decreased the yield along with a marked decrease in pH during the idiophase, while the biomass was comparable to the control (Figure 5). Yeast extract here is regarded more as a source of cofactors than of nitrogen nutrients. In one sense corn steep liquor is a comparable substrate. However, since it did not produce the same effect one may assume that corn steep liquor acts as a nitrogen source but not as a source of stimulatory cofactors such as the B-factor present in yeast extract (Kawaguchi et al., 1984, 1988). This conclusion should be taken in conjunction with the observed lower pH which corn steep liquor produced after day 4 which is not favorable for antibiotic production (Lee et al., 1983). In this respect, Krishna et al. (2000) reported that some organic nitrogen compounds do not

stimulate rifamycin SV production because of feed back effects which are strain specific. As for inorganic nitrogen sources, 0.4% $(\text{NH}_4)_2\text{SO}_4$ led to a rifamycin B yield comparable to the control (0.6%), while higher concentrations (0.8–1%) resulted in a slight reduction in yield (Table 1). Lee and Rho (1994) suggested that the use of high concentrations of ammonium ion repressed rifamycin B production.

Since nitrate stimulates rifamycin production by its regulatory effect on lipid and rifamycin biosynthetic pathways (Rui-Shen et al., 1979), we replaced $(\text{NH}_4)_2\text{SO}_4$ in F2 medium with different concentrations of KNO_3 and NH_4NO_3 . The use of 0.6, 0.9 and 1.2% KNO_3 in F2 medium, led to an increase in rifamycin B production by 12, 13 and 50%, respectively (Table 1 and Figure 6). It is to be noted that 1.2% KNO_3 contains an equivalent nitrogen as 0.6% $(\text{NH}_4)_2\text{SO}_4$ present in F2 medium (control). A similar increase has been previously observed when using KNO_3 in F1 medium with variant NCH (Figure 2) and strain N1 (El-Tayeb et al., 2004). In contrast, the use of 1.8% KNO_3 led to 42% decrease in rifamycin B production, along with an increase in pH observed all over the time course of the fermentation, which also affected growth.

When $(\text{NH}_4)_2\text{SO}_4$ was replaced by 0.05-0.8% NH_4NO_3 (Table 1) rifamycin B production increased from 7.85 g/l to a maximum yield of 11.99 g/l (53%). All the tested concentrations also showed almost the same pattern of glucose consumption and of biomass production but slight differences in pH patterns. This is in contrast with the findings of Lysko and Gorskaia (1986) who tested different inorganic nitrogen sources, and found that $(\text{NH}_4)_2\text{SO}_4$ but not NH_4NO_3 , NH_4Cl and NaNO_3 provided optimum pH levels for antibiotic production. This disagreement may be due to differences in strains, since Rui-Shen et al. (1979) reported that some strains did not utilize nitrate as a nitrogen source and that the effect of nitrate is strain specific.

Although SDB was reported to act as an activator or an inhibitor of certain enzymes associated with rifamycin B production, causing a shift toward the production of rifamycin B (Lal et al., 1995; Mejia et al., 1998), concentrations of SDB above that present in F2 medium (0.1%) slightly increased the yield of rifamycin B (Table 1). Similarly, changes in concentrations of MgSO_4 and CaCO_3 did not cause a major shift in antibiotic yield (Table 1). Since antifoams, which are necessary when the process is conducted in a stirred tank fermentor, may affect growth and consequently antibiotic production, different types of antifoams namely, low and high density silicone oils and sunflower oil were tested. They all resulted in comparable antibiotic yields (Table 1).

In conclusion, the application of gene amplification technique increased rifamycin B production from 1.15 to 2.56 g/l (2.2-fold) and reduced morphological colony variation. Modification of the fermentation medium F1 increased the yield from 2.56 to 6.72 g/l (2.6-fold). When

the V1 and the F1 media were radically replaced by V2 and F2 media, the yield increased from 2.56 to 7.85 g/l (3.1-fold). Modification of the F2 medium by the addition of 0.1% yeast extract after 2 days of incubation or the use of either 1.2% KNO₃ or 0.4% NH₄NO₃, instead of (NH₄)₂SO₄, increased the yield from 7.85 to 9.49, 11.76 and 11.99 g/l, respectively. These yields are promising for further optimization for industrial production.

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