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EFFECT OF SOME PARAMETERS ON THE PRODUCTION OF RIFAMYCINS B AND SV BY *AMYCOLATOPSIS MEDITERRANEI*

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The effect of different parameters on the production of rifamycins B and SV were studied. The optimum production was obtained after four days. The concentration ratio of starch to glucose at 20 : 10 g/L was favourable for the antibiotic production. The effect of nitrogen compounds indicated that the phenylenediamine, potassium phthalimide and uracil have a stimulating effect on the production of rifamycin B by *Amycolatopsis mediterranei* similar to the barbital; specially uracil at 2 g/L.

Key words: *Nocardia mediterranei* CBS 42575, Rifamycins B and SV. $NaNO_2$, Uracil, Phenylenediamine.

Introduction

Rifamycins were first isolated from fermented broth of an *Actinomycetes* strain which classified as *Streptomyces mediterranei* [1,2]. Afterwards, this microorganism was transferred from *Streptomyces* to *Nocardia* on the basis of the presence of meso-diaminopimilic acid (DAP) in its cell-wall [3]. Later, on the taxonomic position of this organism was altered into *Amycolatopsis* genus on the basis of its cell-wall chemical composition [4]. Subsequently, other strains produce members of the rifamycin family were isolated such as *Streptomyces tolipophorus,* and *Micromonospora lacustris* [5,6].

Rifamycins are a complex of atleast six different antibiotics B, G, O, S, SV and Y. Rifamycins were produced by submerged fermentation procedure in the laboratory and pilot plant scale using organic or semi-synthetic media [1-8]. Rifamycins G and Y are inactive. Whereas, rifamycin B shows good stability, also biogenetically derived from rifamycins S [9] and it appears to be moderately active against gram positive bacteria only because it is transformed spontaneously into the active rifamycin S [10]. Rifamycin S considers a key intermediate for a variety of transformation reactions leading to the rifamycins by chemical method or excreted from *Nocardia mediterranei* broth. It was reduced to rifamycin SV which was isolated from a mutant strains of *Nocardia mediterranei* [11,12], and it is clinically used in the therapy against gram-positive and tubercular infections.

The biosynthesis of rifamycins were studied genetically using the mutants of *Nocardia mediterranei* [13,14] or chemically with the effect of some organic chemicals such as barbital and pH on the production of rifamycin B [15-17].

The present research represents the effect of some parameters rather than the barbital on the fermentation production of rifamycins B and SV by *Amycolatopsis mediterranei* CBS 42575.

Experimental

Microorganism. Rifamycin producing organism, *Amycolatopsis mediterranei* CBS 42575 was used and maintained on a medium containing (g/l): malt extract 10; glucose, 4; yeast extract 4; agar-agar, 20; distilled water 1 liter. The pH was adjusted to 6.8 with glass electrode pH meter.

Fermentation medium. The basal fermentation medium [12] was used. It is consists of (g/L): glucose, 20; KH_2PO_4,3; K_2HPO_4, 1.5; MgSO4.7H_2O, 1.0; $FeSO_4$, 0.016; Zn (acetate), 0.001; yeast extract, 5.0; distilled water, 1 liter, and the pH was adjusted to 6.8.

Determination of rifamycins. Rifamycins B and SV were determined spectrophotometrically [18]. The rifamycin B and SV concentrations in the fermentation broth were obtained by the formula:

$$\text{Rifamycin B (mcg. ml)} = \frac{A\ (425).\ 50.000}{21.5}$$

$$\text{Rifamycin SV (mcg.ml)} = \frac{A\ 447.\ 50.000}{15.6}$$

Extraction and detection of rifamycins. The culture broth of *Amycolatopsis mediterranei* at the proper times was filtrated through Watman No. 1 filter paper at pH 8.0. The filtrate was adjusted to pH 2.0 with 0.1N sulfuric acid and extracted three times by ethyl acetate. Extracts were collected and evaporated under vacuum till dryness. Dried extract was dissolved in methanol-chloroform (1:1 v/v) and spotted on thin layer chromatography (TLC) aluminium sheets silica gel 60 F254 (Merck). The spots were developed with the solvent

system of benzene - chloroform - ethyl acetate (1:2:1 v/v/v), and the rifamycins were detected on TLC according to their colors and R_f values (19) as indicated in Table 1.

Determination of protein and reducing sugars. Protein in the culture filtrate was measured by the Folin reagent [20] using bovine albumine as a standard. The reducing sugar in the culture filtrate was measured chlorimetrically [21,22] using glucose as a standard.

All the results were recorded in an average of the doublicate flasks.

Rifamycins B and SV. Authentic samples of rifamycins B and SV were kindly donated by Dr. L. Cellai, Institute d'strutturistica Chimica. G. Giacomello. Consiglio Nazionale Delle Ricerche. Area Della Ricerca Roma, Italy. and Dr. M.H. Itan, Biotechnology Research Department Vrorea Advanced Institute of Science and Technology, Seoul.

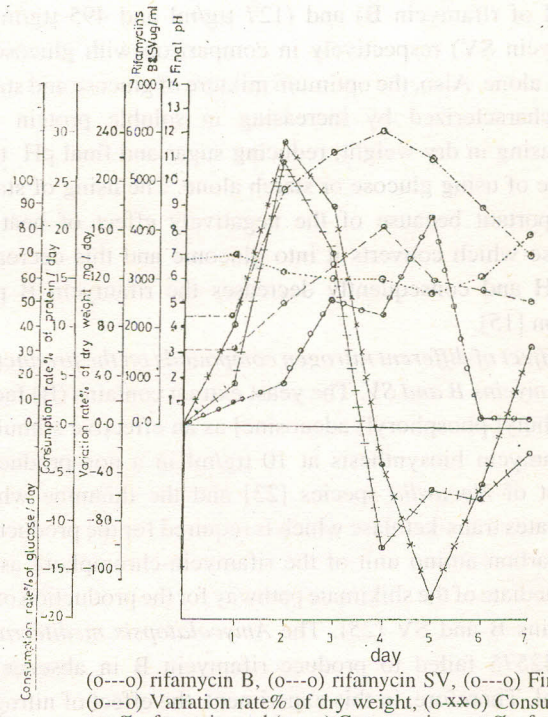
Results and Discussion

Biochemical changes on the production of rifamycins B and SV at different incubation periods. In this experiment, spore suspension of one slant of 10 days old was used to inoculate 250 ml conical flasks each containing 50 ml of fermentation medium (containing 1.0 g/L barbital), that were then incubated aerobically on rotary shaker (200 r.p.m.) at 28-30°C for different times. At the end of each incubation time, the filtrate of each flask adjusted to 50 ml with distilled water, pH; dry weight of the biomass; soluble protein in the filtrate; reducing sugar and rifamycins were measured and detected. The results obtained in (Table 1, Fig.1) showed that the optimum fermentation time for the production of rifamycins B and SV was 4 days, which was characterized by decreasing in consumption rate % of glucose, decreasing in variation rate % of dry weight and stability of final pH of fermentation around 6.0. At the same time, increasing soluble protein in the fermented broth and consumption rate % of protein was decreased consequently. These results indicated that the production of rifamycin B and SV was carried out through a cell-growth phase inside the cell and the antibiotic were diffusible to the fermented broth through different fermentation period.

TABLE 1. R_f VALUES AND COLOR SPOTS OF DIFFERENT RIFAMYCINS ON TLC.

rifamycin	R_{f*}	colour spot
rifamycin O	0.8	pale yellow
rifamycin S	0.65	purple
rifamycin SV	0.32	deep yellow
rifamycin B	0.17-0.20	reddish yellow

*The solvent front allow to ascend 10 cm above the line application



(o---o) rifamycin B, (o---o) rifamycin SV, (o---o) Final pH, (o---o) Variation rate% of dry weight, (o-xx-o) Consumption rate% of protein, and (o---o) Consumption rate% of glucose.

Fig. 1. Illustration of biochemical changes of rifamycins B & SV at different fermentation periods.

TABLE 2. BIOCHEMICAL CHANGES OF THE PRODUCTION OF RIFAMYCINS B AND SV AT DIFFERENT TIMES.

Incubation times (days)	Final pH	Dry weight mg/50ml	Variation rate %of dry weight /day	Soluble protein µg/ml	Consumption rate % of protein/ day	Reducing sugars µg/ml	Consumption rate % of glucose/ day	Rifamycins B µg/ml	Rifamycins SV µg/ml
0.0	6.80	5.8	00.0	66.00	00.0	24600	00.0	1543	2240
1	7.00	42.0	36.2	5928	10.18	22500	8.53	1553	2246
2	6.30	258.0	216.0	4200	29.15	18675	17.00	2490	4845
3	6.00	399.0	141.0	3240	22.85	9075	51.40	3278	5647
4	6.00	306.0	-93.0	3384	-4.44	4912	45.87	4052	6009
5	5.42	271.0	-35.0	4008	-18.44	727	85.20	2955	5567
6	6.10	210.0	-61.0	4272	-6.50	703	3.30	2755	4486
7	7.90	186.0	-24.0	3888	8.98	6.78	3.55	2562	3519

Effect of starch on the production of rifamycins B and SV. In this experiment, the fermentation medium without glucose was used as a basal medium and 1 g/L of barbital was added to stimulate rifamycin B production. Glucose was replaced with equal amount of starch. Two ml of the inoculum which was maintained in 20% peptone were used to inoculate 50 ml of the fermentation medium in 250 ml conical flasks that was incubated aerobically on a rotary shaker at 28-30°C and 200 rpm for 4 days. At the end of the incubation time, the filtrate of each flask adjusted to 50 ml with distilled water, the biochemical changes and rifamycins B and SV were measured.

The results in Table 3 showed that the mixture of 10 g glucose to 20 g starch was favourable for production of rifamycins B and SV with increasing of (150 µg/ml and 444 µg/ml of rifamycin B) and (127 µg/ml and 495 µg/ml of rifamycin SV) respectively in comparison with glucose or starch alone. Also, the optimum mixture of glucose and starch was characterized by increasing in soluble protein and decreasing in dry weight; reducing sugar and final pH than in case of using glucose or starch alone. The using of starch is important because of the negatively effect of heat on glucose which converts it into gluconic and this decreases the pH and consequently decreases the rifamycin B production [15].

Effect of different nitrogen compounds on the production of rifamycins B and SV. The yeast extract contains (B) factor [3-(1-butyl phosphoryl) adenosine] as an effective stimulant of rifamycin biosynthesis at 10 µg/ml in a non-producing mutant of *Nocardia* species [23] and the thiamine which stimulates trans-ketolase which is required for the production of 7-carbon amino unit of the rifamycin-chromphore as an intermediate of the shikimate pathway for the production of rifamycins B and SV [25]. The *Amycolatopsis mediterranei* CBS-42575 failed to produce rifamycin B in absence of barbital. Therefore, in this experiment, the effect of nitrogen compounds and some aromatic amino acids on the production of rifamycins B and SV was studied in comparison with barbital. They were used with concentration of 0.7 g/L of each compound and were added to the fermentation medium. At the end of the incubation time the biochemical changes and rifamycins were measured. The rifamycins were extracted and detected on TLC silica gel using the solvent systems mentioned in the materials and methods. The results recorded in Table 4 showed that the phenylenediamine, pot. phthalimide and uracil have the stimulation effect similar to barbital on the *Amycolatopsis* strain for the production of rifamycin B. On the other hand, p-amino-benzoic acid, 4-methyl-amino phenol, nicotinic acid, pyridoxine, L-treptophan and DL-treptophan give a slightly inhibit of rifamycins B and SV production in comparison with barbital and control. Whereas, the amino acids (tyrosine and methionine) inhibit the production of rifamycin B and stimulate the production of rifamycin SV. These results show that some nitrogen compounds (such as tyrosine) do not afect on the rifamycin B production but this is not a proof for using it as a precursor of seven-carbon amino unit of other rifamycins [26].

Effect of different concentrations of uracil on the production of rifamycins B and SV. In the following experiment, different concentrations of uracil were added to the basal fermentation medium. Uracil concentrations were used from 1.0 to 5.0 g/L. At the end of incubation period the biochemical changes and rifamycins B and SV production were measured. The results obtained in Table 5 showed that the concentration of 2 g/L uracil was favourable for the production of rifamycins B and SV with final pH 6.78. On the other hand, increased uracil concentrations reduced the rifamycins production by the producer strain under study.

Table 3. Effect of Starch and Glucose on the Production of Rifamycins B and SV.

Concentration g/L glucose:starch		Final pH	Dry weight mg/ 5ml Broth	Protein µg/ml	Reducing sugars µg/ml	Rifamycins B µg/ml	Rifamycins SV µg/ml
30	0.0	6.80	66.23	1077.6	4068.7	1890	2773
25	5	6.72	69.10	1430.4	1638.7	1904	2830
20	10	6.63	45.6	950.4	1968.8	1960	2858
15	15	6.32	44.15	955.2	1511.3	1980	2858
10	20	6.50	42.76	967.2	1601.3	2040	2900
5	25	7.02	53.13	1272.0	1605.0	1638	2490
0.0	30	7.04	73.68	916.8	1743.8	1596	2405

Table 4. Effect of Different Nitrogen Compounds on the Fermentative Production of Rifamycins B and SV After 4 Days.

Nitrogen Compound	Final pH	Dry weight mg/flask	Soluble protein µg/ml	Reducing sugars µg/ml	Rifamycins B. µg/ml	Rifamycins SV µg/ml
Barbital	6.82	380.2	1829.0	3398.0	730	1666
P.aminobenzoic acid	6.53	239.6	2256.0	9375.0	-	385
Phenylenediemine	6.55	454.0	1725.0	1350.0	732	1683
Pot.phthalimide	6.57	296.8	1564.8	3810.0	639	1506
4-methyl amino phenol	6.91	231.6	3892.8	7792.5	-	513
nicotenic acid	6.24	318.5	1636.8	9150.0	-	721
pyridoxine	6.50	317.7	2635.2	7312.5	-	433
uracil	6.63	361.0	1699.2	24.94.5	813	1891
tyrosine	6.58	401.8	2376.0	1590.0	-	2035
L-tryptophane	6.42	340.2	2760.0	2310.0	-	1154
DL-tryptophane	6.51	306.6	3009.6	3900.0	-	1089
methionine	6.75	205.6	2572.8	1113.0	-	1763
Control without any additive	6.58	325.2	1915.2	2895.0	-	1731

Where (-): rifamycin B was not detected on TLC.

TABLE 5. EFFECT OF DIFFERENT CONCENTRATION OF URACIL ON THE PRODUCTION OF RIFAMYCINS B AND SV.

Concentration g/L	Final pH	Dry weight µg/ml	Protein mg/50ml (flask)	Reducing sugars µg/ml	Rifamycins B µg/ml	Rifamycins SV µg/ml
1	6.64	328.3	2203.2	1470.0	1442	2884
2	6.68	323.8	2160.0	1335.0	1907	3910
3	6.65	273.2	2294.4	1334.3	1023	2115
4	6.65	367.6	1944.0	1230.0	1000	2019
5	6.60	268.7	2016.0	1402.5	1402	2916
Control without uracil or barbital	6.65	336.2	2580.5	1322.5	0.0	3109

All these results showed that the production of rifamycins specially rifamycin B can be stimulated with some aromatic amino derivatives (uracil, phenylene-diamine and pot. phthalimide) instead of barbital. *Amycolatopsis mediterranei* CBS-42575 is unable to produce rifamycin B on all tested media specially without addition of barbital. However, the strain successfully used the uracil to stimulate the production of rifamycins B and SV with increasing of 83 µg/ml and 225 µg/ml of rifamycins B and SV respectively in comparison with barbital. The effect of uracil on the fermentation production of rifamycin B may be similar to the effect of barbital which stimulate biosynthetic pathways of rifamycin B by the production of D-sedoheptulose-7-phosphate or by activation of transketolase [11]. So, addition of uracil or barbital at other fermentation stages into the fermentation medium,with exception of zero time, are failed to produce rifamycin B.

References

1. P. Sensi, P. Margalith and M. T. Timbal, Faormaco. Ed. Sci., **14,** 146, (1959).
2. P. Margalith and G. Beretta, Rifamycin Xl, Taxonomic Study on *Streptomyces Mediterranei nov.* sp. Mycopathol et Mycol., Appl., **8** (4), 321 (1960).
3. J. E. Thiemann, G. Zucco and G. Pelizza, A proposal for the Transfer of *Streptomyces mediterranei.*, Margalith and Beretta 1960 to the genus *Nocardia* as *Nocardia mediterranei* (Margalith and Beretta), Comb. Nov. Arch. Microbiol., **67,** 147 (1969).
4. M. P. Lechevalier, H. Prauser, D. P. Lubeola and J. S. Ruan, Internat. J. Systematic Bacteriol., **29** (1983).
5. T. Kishi, H. Yamana, M. Muroi, S. Harada, T. H. Arai and K. Mizuno, J. Antibiot., **25,** 11 (1972).
6. W. D. Celner, W. P. Cullen, A. R. English, M. T. Jefferson, J.R. Oscarson, J.B. Routien and F.C. Sciavilino, 3-Methyl-thiorifamycin SV and CP-33, 650 2 novel Sulphur Containing Ansamycins from a New Species of *Micromonospora* In: 15th Intersci. Conference Antimicrob Agents Chempther (1975), pp. 260.
7. J. Birner, P.R. Hongson, W.R. Lane and E.H. Baxter, J. Antibiot., **6** 356 (1972).
8. P. Margalith and H. Pagani, Appl. Microbiol., **9,** 325 (1961b).
9. G. C. Lancini, G.G. Gallo, G. Sartori and P. Sensi, J. Antibiot., **22,** 369 (1969).
10. S. Furesz and M. T. Timbal, Chemotherapia, **7,** 200 (1963).
11. G. C. Lancini and C. Hangeller, J. Antibiot., **22,** 637 (1969).
12. J. Birner, P. R. Hodgson, W. R. Lane and E. H. Baxter, J. Antibiot., **25** (b), 356 (1972).
13. O. Ghisalba and J. Nuesch, J. Antibiot., **31,** 202 (1978a).
14. O. Ghisalba and J. Nuesch, J. Antibiot., **31,** 215 (1978b).
15. P. Morgalith and H. Pagani, Mycopathol. Amycol. Appl., **9,** 320 (1961).
16. D. Kluepfel, G. C. Lancini and G. Sartori, Appl. Microbiol., **13** 600 (1965).
17. J. G. Lee, C. Y. Choi, B. L. Seong and M. H. Han, J. Ferment. Technol., **61** 49 (1983).
18. C.R. Pasqualucie, A. Vigevani, P. Radaelli and C.G. Gallo, J. Pharm. Sci., **59** 685 (1970).
19. B. L. Seong, H. J. Son, T. I. Mheen, Y. H. Park and M. H. Han, J. Ferment. Technol., **63** (6), 515 (1985).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. L. Randall, J. Bio. Chem., **193,** 265 (1951).
21. M. Somogi, J. Biol. Chem., **195,** 19 (1953).
22. N. Nelson, J. Biol. Chem., **153,** 375 (1944).
23. T. Kawaguchi, T. Asahi, T. Satoh, T. Liozuni and T. Beppu, J. Antibiot., **37,** 1587 (1984).
24. M. Shimamura, J. Yoshitake and T. Imai, Agric. Biol. Chem., **37,** 2245 (1973).
25. O. Ghisalba and J. Nuesch, J. Antibiot., **34,** 64 (1981).
26. A. Karlsson, G. Sartori and R. J. White, Fur. J . Biochem., **47,** 251 (1974).