Journal of Industrial Microbiology, 5 (1990) 283–288
Elsevier

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The effect of neutral resins on the fermentation production of rubradirin

V.P. Marshall, S.J. McWethy, J.M. Sirotti and J.I. Cialdella

Research Laboratories, The Upjohn Company, Kalamazoo, MI, U.S.A.

Received 27 March 1989
Revised 18 July 1989
Accepted 24 July 1989

Key words: Neutral resins; Rubradirin; Fermentation production

SUMMARY

Rubradirin is an antibiotic of complex chemical structure which is active *vs.* methicillin resistant staphylococci. Its development has been limited due to inadequate production yields. The incorporation of neutral resins into fermentations of *Streptomyces achromogenes* v. *rubradiris*, UC® 8051 resulted in the enhanced production of rubradirin. Resins HP-20, HP-21, XAD-2, XAD-7 and XAD-16 were employed in flask and tank fermentations. The incorporation of these resins promoted 2- to 4-fold enhancements of the rubradirin activity produced in flask fermentations, and the incorporation of XAD-16 and HP-21 into tank fermentations promoted production titer increases >5 fold.

INTRODUCTION

Rubradirin is an antibiotic of complex chemical structure (Fig. 1) produced by *Streptomyces achromogenes* v. *rubradiris* [3]. Rubradirin consists of quinone, dihydropicolinic acid, coumarin and nitrosugar moieties. It is primarily active *vs.* Gram-positive bacteria including methicillin resistant staphylococci [3,4]. Rubradirin inhibits ribosomal

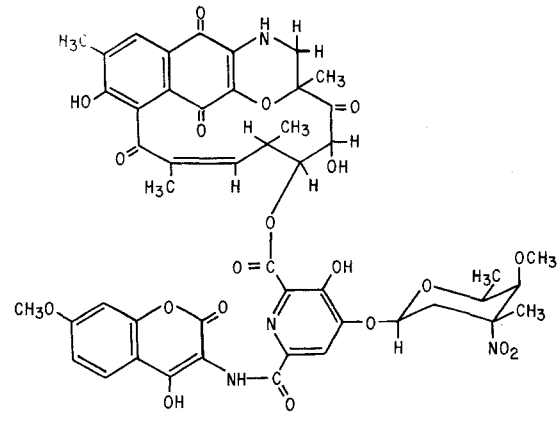

Fig. 1. The structure of rubradirin.

Correspondence: V.P. Marshall, Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001, U.S.A.

functions by selectively inhibiting protein chain initiation [3].

XAD-2 resin has been employed previously to enhance the fermentation production of paulomycin (volonomycin) by *Streptomyces paulus*. Growth of *S. paulus* was sensitive to paulomycin's incorporation into agar media at concentrations $\geqq 10$ μg/ml [1]. The role of XAD-2 in the submerged paulomycin fermentation process appeared to have been related partially to the sequestering of paulomycin away from its sensitive producer. Another means through which XAD-2 functioned to promote increased paulomycin production was by its stabilization to prevent conversion to paulomenol [2]. In the current study, the neutral resins were employed to enhance the fermentation production of rubradirin.

MATERIALS AND METHODS

Fermentation methods

Flask fermentations. *S. achromogenes* v. *rubradiris*, UC 8051 was stored and maintained on sterile soils in the culture collection of The Upjohn Company. The cultures were inoculated into a seed medium (GS-7) which contained Cerelose (C.P.C. International) and Pharmamedia (Procter and Gamble) each added at 25 g per liter of tap water. The medium was adjusted to pH 7.2 with NH_4OH and was autoclaved for 30 min. The inoculated 100 ml volumes of GS-7 were shaken in wide-mouth 500 ml fermentation flasks at 250 rpm for 48–72 h at 28°C. The mature seed cultures were used as the source of inoculum (5% seed rate) for the fermentation medium. The fermentation medium contained distiller's solubles, 20 g; corn steep liquor, 15 g; corn starch, 10 g; and $NaNO_3$, 2 g per liter of tap water. The medium was adjusted to pH 7.2 with KOH, and was autoclaved for 30 min. The inoculated fermentation medium was employed in the manner described for GS-7. After 24 h of growth, the washed resins were added aseptically as sterilized aqueous slurries at the concentrations indicated. The resin slurries (XAD-2, XAD-7, XAD-16, HP-20 and HP-21) were sterilized by autoclaving for 30 min. Amberlite XAD-2, XAD-7 and XAD-16 resins are products of Rohm and Haas, and Diaion HP-20 and HP-21 resins are products of Mitsubishi Chemicals, Ltd.

Tank fermentations. Pilot fermentations incorporated two seed stages. Primary seed cultures (GS-7 in shaken flasks) were inoculated using soil stock culture and were incubated for 72 h on a rotary shaker (250 rpm) at 28°C. Secondary seed vessels contained 250 l of GS-7 medium and were inoculated with 300 ml of the primary seed. Operating conditions were: temperature, 28°C; agitation rate, 280 rpm; aeration rate, 200 standard liters per min (slm); back pressure, 7 psi; and incubation time, 48 h.

The fermentation medium contained distiller's solubles, 10 g; corn steep liquor, 20 g; corn starch, 20 g; $NaNO_3$, 3 g; $CaCO_3$, 5 g; and polypropylene glycol, 0.2 ml per liter of tap water. Additions of soybean oil and non-ionic resins are detailed in the Results section. Tank fermentation (250 l) were inoculated with 12.5 l of secondary seed and incubated for approximately 90 h at 28°C. Other conditions were: agitation rate, 280 rpm; aeration rate, 200 slm; back pressure, 7 psi; and pH control at 7.5 using sulfuric acid.

Biounit assay methods

The antibacterial potency of the *S. achromogenes* v. *rubradiris* beers was determined by a biounit assay *vs. Micrococcus luteus*, UC® 130 performed in the presence of standards composed of authentic rubradirin. One biounit of anti-*M. luteus* activity was defined as the amount of antibiotic that when applied to a 12.7 mm paper disc (Schleicher and Schuell No. 740-E) produced a zone of growth inhibition of 20 mm when applied onto a seeded agar culture. Microgram values were assessed through comparison of the experimental biounit activities to those produced by authentic rubradirin.

Aliquots (2 ml) of whole fermentation beers were taken at 1, 2, 3 and 4 days and were mixed with an equal volume of acetone. The mixtures were allowed to stand for 15 min and then were agitated for 1 min before centrifugation at 5000 $\times$ *g* for 5 min. The supernatant fluids were then assayed for anti-*M. luteus* activity following serial dilution (2-

fold increments) in 50% aqueous acetone buffered with 50 mM potassium phosphate, pH 7.0. Such dilutions were routinely assayed between 4- and 2096-fold. The buffered acetone solutions had no antibacterial activity in this assay. The assay trays were developed by incubation at 32°C.

Stock solutions of authentic rubradirin (1 mg/ml) were prepared as acetone solutions. For individual assays, the stocks were diluted 10-fold in acetone and were then further diluted in acetone (2-fold increments) another 32-fold. Following this primary dilution in acetone, further dilutions (2-fold increments) were made in deionized water. Each beer assay was performed in the presence of the first 6 aqueous dilutions of the standard.

Bioautographic methods

The composition of the rubradirin complex produced in the flask fermentations was monitored by bioautography *vs. M. luteus*. Chromatography was performed on Analtech silica gel GF uniplates developed in ethyl acetate–acetone–H_2O (8:5:1). Ten to 20 μl of the acetone-treated beer supernatants (see Biounit assay methods) were applied to the plates. One μg of authentic rubradirin was chromatographed simultaneously. The plates were then developed and the antibiotic activities were absorbed onto seeded agar trays of *M. luteus* which were then incubated overnight at 32°C.

MIC methods

The minimum inhibitory concentration (MIC) of rubradirin *vs. S. achromogenes* v. *rubradiris* was determined by growth inhibition in broth. The organism was inoculated into a 100 ml volume of trypticase soy broth (TSB) contained in a 500 ml wide-mouth flask and was shaken at 250 rpm at 28°C for 72 h. Aliquots (0.05 ml) of this culture were inoculated into 5 ml volumes of TSB contained in 25 ml flasks. Rubradirin was dissolved in dimethylformamide (DMF), and was added aseptically to the flasks at concentrations ranging from 1–1000 μg/ml. Control cultures lacking rubradirin were also grown with and without DMF. Growth inhibition was determined after incubation of the cultures at 28°C for 72 h as described above.

RESULTS AND DISCUSSION

Data presented in Table 1 show the titer enhancing effect of the incorporation of XAD-2, XAD-7, HP-20 and HP-21 into flask fermentations at concentrations of 40 and 70 g of resin per liter. In general, these resins promoted rubradirin bioactivity increases of 2- to 4-fold on 3 and 4 days of fermentation. Fig. 2 shows a similar effect produced through the addition of XAD-16 to 3 day flask fermentations of *S. achromogenes* v. *rubradiris*. Data

Table 1

The effects of resins on the production of rubradirin by *S. achromogenes* v. *rubradiris* UC® 8051 in shaken flask fermentations

Resin	Level of resin addition (g/l)	Rubradirin bioactivity (μg/ml)		
		Day 2	Day 3	Day 4
None	–	44	50	25
XAD-2	40	44	142	100
XAD-2	70	44	142	100
XAD-7	40	70	100	70
XAD-7	70	100	100	40
HP-20	40	65	200	140
HP-20	70	131	142	70
HP-21	40	35	142	70
HP-21	70	35	200	100

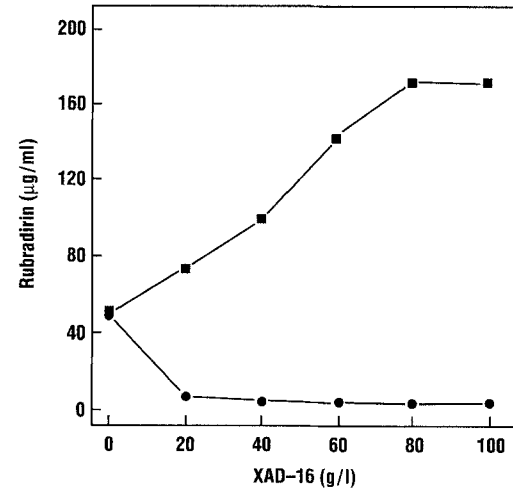

Fig. 2. The effect of XAD-16 on the production of rubradirin at 3 days of fermentation: (■), acetone elution; (●), no elution.

presented in this figure show that rubradirin removed from XAD-16 by acetone treatment resulted in production titers *ca.* 4-fold greater than those observed in fermentations lacking resin. Note that without elution, the quantity of rubradirin in the fermentation supernatants decreased as a function of resin addition.

Fig. 3 is a bioautogram *vs. M. luteus* which shows the composition of the bioactivity produced in flask fermentations containing XAD-2. Positions 1 and 8 show the bioactivity of authentic rubradirin. Positions 2–7 show the bioactivity produced in fermentations containing XAD-2 at concentrations of 0, 10, 20, 30, 40, and 50 g/l, respectively. An obvious increase of rubradirin bioactivity is produced as a function of resin addition up to 30 or 40 g of XAD-2 added per liter.

In an initial attempt to scale up the rubradirin resin process from shaken flasks to pilot tanks, titers were disappointingly low (Table 2, Run A). This fermentation included 60 g/l of Diaion HP-21 resin sterilized with the medium using pH control at 8.5. Addition of HP-21 (60 g/l) as a separate sterile injection at approximately 20 h of fermentation (Table 2, Run C) conferred 5–13-fold more bioactivity than the control lacking resin (Table 2, Run B). A subsequent comparison of HP-21 (Table 2, Run D) and XAD-16 (Table 2, Run E) added as separate sterile injections indicated that XAD-16 functioned somewhat better than HP-21. In addition, the rubradirin activity produced was enhanced several fold by the incorporation of soybean oil at 10 ml/l (Table 2, Run D) as compared to rubradirin activity produced in the absence of soybean oil (Table 2, Run C).

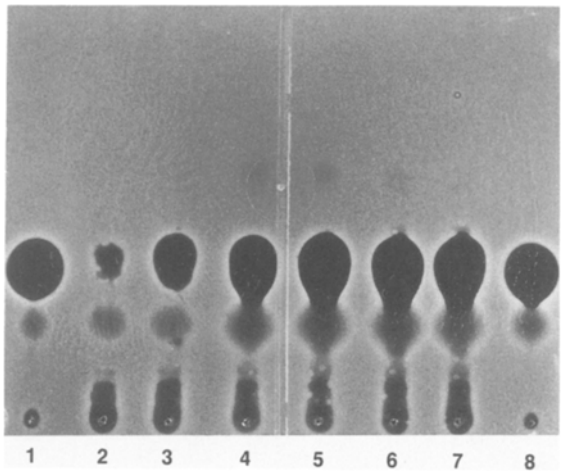

Fig. 3. The effect of XAD-2 on the fermentation production of rubradirin. Positions 1 and 8 represent the bioactivity of 1 μg of authentic rubradirin (R_f = 0.38). Positions 2–7 represent the bioactivity of 20 μl of eluate from fermentations containing XAD-2 resin at concentrations of 0, 10, 20, 30, 40 and 50 g/l, respectively.

Table 2

Rubradirin bioactivity in 250-l pilot fermentors

Rubradirin (μg/ml)					
Hours	Run A	Run B	Run C	Run D	Run E
44	8	11	51	41	46
70	30	6	41	154	246
90	22	4	52	316	346

1. Resins (60 g/l) were sterilized separately and added at 24 h of fermentation (except Run A where resin was sterilized with media components and Run B performed in the absence of resin). 2. Resins employed: HP-21 (Runs A, C, D); XAD-16 (Run E); none (Run B). 3. Soybean oil addition (10 ml/l): Runs D, E. 4. pH Control: pH 7.5 (except Run A = pH 8.5).

The resins also performed more favorably in flask fermentations when added as presterilized aqueous slurries 24 h after inoculation. It appears that autoclaving in the presence of media components may alter the resins making them less able to bind rubradirin, or may promote the binding of essential metabolites required by the producer.

Rubradirin was tested as an antibiotic *vs. S. achromogenes* v. *rubradiris* and was found to be active at *ca.* 250 μg/ml (MIC at 72 h). However, at 24 h rubradirin inhibited growth at concentrations as low as 1 μg/ml. As *S. achromogenes* v. *rubradiris* is sensitive to rubradirin to some degree, it would appear that resin may function by limiting the concentration of free antibiotic.

Another means through which resin may function is by removing rubradirin from solution and thus preventing its degradation. In the case of the

paulomycin production process, resin prevented the conversion of paulomycin to its degradation product termed paulomenol [2]. Although this explanation appears to be plausable, we have no direct evidence that resin functioned through the stabilization of rubradirin. The possible role of rubradirin as an end product inhibitor was not investigated. Nonetheless, the incorporation of these resins into the rubradirin fermentation process promoted significantly improved production titers.

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