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Improvement of the kirromycin fermentation by resin addition

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The kirromycin yield of *Actinoplanes* sp A8924 was improved from 30–50 mg L^{-1} to 350 mg L^{-1} by mutant selection and medium optimization. The incorporation of polystyrenic resins into the fermentations promoted a further four-fold enhancement of kirromycin productivity to 1500 mg L^{-1}. The positive effect of resin addition appears to be due to removal of kirromycin from the fermentation broth because kirromycin's minimal inhibitory concentration against the producing strain remained at *ca* 350 mg L^{-1}.

Keywords: aurodox; kirromycin; antibiotic; production yield; resin; end-product toxicity

Introduction

Aurodox-type compounds belong to a class of antibiotics which selectively inhibit bacterial protein synthesis by acting on elongation factor Tu (EF-Tu) [13,14]. These antibiotics show a characteristic antibacterial spectrum: they inhibit anaerobes, neisseriae and streptococci, but are ineffective against *Staphylococcus aureus* [3,4,6]. Most of the aurodox-type antibiotics are produced by microorganisms belonging to the genus *Streptomyces*. Recently a new producer of kirromycin was isolated and identified as *Actinoplanes* sp A8924 [1]. Ongoing chemotaxonomic and molecular studies show that it is very similar to *Actinoplanes liguriae* DSM43865 (F Marinelli, unpublished data). *Actinoplanes* sp A8924 is the first species of *Actinoplanes* known to produce an aurodox-type antibiotic. An important feature of this strain is that it does not co-produce toxic humidin-type macrolides, which often complicate purification of aurodox-type antibiotics from *Streptomyces* spp [1]. The productivity of the original soil isolate was low, 30–50 mg L^{-1}. In this paper, we describe approaches to improve kirromycin production.

Materials and methods

Cultivation conditions

Actinoplanes sp A8924 and its mutant A/8924/F were maintained on slants of ISP No. 1 medium [16]. Stock cultures of washed mycelium frozen at −80°C were used as inocula (2%) for 100-ml volumes of preculture medium AF/MS (glucose 20, yeast extract 2, soybean meal 8, $CaCO_3$ 1, NaCl 4 g L^{-1} in distilled water, pH adjusted with 10 N NaOH to 7.3 before sterilization). The medium was sterilized at 121°C for 20 min. After 48 h of growth, 5% of preculture was used to inoculate production media. Fermentations were carried out in 500-ml Erlenmeyer flasks containing 100-ml volumes of medium at 28°C agitated at 200 rpm or in 4-L bioreactors (Chemap-Braun CMF200, Melsungen, Germany) containing 3 L of medium at 28°C stirred at 400 ÷ 1100 rpm in a cascade with dissolved oxygen concentration kept at the minimum saturation of 20%.

Production media

Glucose, maltose, dextrin, glycerol, starch and sucrose were tested as carbon sources in a basal medium (BM) composed of soybean meal 10, peptone 4, meat extract 4, yeast extract 10, NaCl 2.5 and $CaCO_3$ 5 g L^{-1} in distilled water. The optimized medium (AUR/M) consisted of maltose 20, dextrin 10, soybean meal 15, peptone 4, meat extract 4, yeast extract 2 and $CaCO_3$ 2 g L^{-1} in distilled water. Media were sterilized at 121°C for 30 min. Medium pH was adjusted to 7.2 with 10 N NaOH before sterilization.

Mutagenic treatment and overlay assay

After 48 h of growth in AF/MS medium, the mycelium was filtered, repeatedly washed and sonicated for 30–60 s at 25°C in a Rapidis Ultrasonic Disintegrator 180 (Ultrasonic Ltd, PBI, Italy) at 30 W power. Sonicated suspensions were treated with 0.5 or 1.0 mg L^{-1} *N*-methyl-*N'*-nitro nitrosoguanidine in 0.02 M sodium borate buffer pH 9.2 for 15, 30, 45, 60 min. Surviving cells were plated on ISP No. 2 agar [16]. After incubation for 3–4 days at 28°C, the resulting colonies were overlayed with Todd Hewitt agar (Difco, Detroit, MI, USA) seeded with 10^6 cells ml^{-1} of *Bacillus megaterium* NRRL 10778. After overnight incubation at 37°C, colonies capable of producing wide inhibition zones were selected and tested in liquid medium as described above.

Kirromycin minimal inhibitory concentration (MIC)

Samples of cultures of *Actinoplanes* sp A8924/F growing in liquid medium were collected at different fermentation times and streaked onto solid medium (glucose 10, casitone 2, meat extract 1, yeast extract 1, agar 15 g L^{-1} in distilled water, pH 6.8) containing increasing amounts of kirromycin (from 10 to 1000 mg L^{-1} final concentration). Surviving colonies were scored after 1 week of incubation at 28°C.

Resins

Resin S-112 is a polystyrene resin supplied by Dow Chemical (Fombio, Italy) and previously used for the isolation of kirromycin in the downstream process (E Restelli, unpub-

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lished results). Resin SP205 is a brominated polystyrene resin manufactured by Mitsubishi Kasei Corporation (Kaseico, Japan). A unique feature of this resin is its high specific gravity which can simplify its separation from exhausted media. The resins were conditioned with methanol, extensively washed with distilled water and added to AUR/M before sterilization or sterilized separately and then added to the fermentation medium at selected times.

Kirromycin analysis

Culture samples containing mycelium and resin were collected, their pH adjusted to 9.0 with diluted NaOH and mixed with an equal volume of acetone. Samples were stirred at room temperature for 20 min and centrifuged at 15 000 × *g* in an Eppendorf centrifuge (Eppendorf, Hamburg, Germany) for 5 min. A sample from the supernatant phase was injected for HPLC analysis on a Bakerbond (JT Baker Inc, Phillipsburg, NJ, USA) 5-μm column (4.6 × 250 mm) eluted by a linear gradient from 30% phase B (40 mM HCO_2NH_4: CH_3CN: tetrahydrofuran 20 : 40 : 40) in phase A (40 mM HCO_2NH_4: CH_3CN: tetrahydrofuran 80 : 10 :10) to 40% B in 15 min. The flow rate used was 1.5 ml min^{-1} and the injection volume was 30 μl. The column effluent was monitored at 254 nm. Pure samples of kirromycin were used as external reference standards.

Results

Strain selection and medium optimization

A first productivity increase was achieved by using maltose or dextrin carbon sources (25 g L^{-1}) in the BM production medium. The kirromycin yield was improved three-fold (150–170 mg L^{-1}). A spontaneous mutant, *Actinoplanes* A8924/F, which produced 300 mg L^{-1}, was then selected by overlay with *B. megaterium* NRRL 10778. Cultivation of *Actinoplanes* A8924/F in optimized AUR/M increased productivity up to 350 mg L^{-1}. Attempts at further improvement were unsuccessful. Over 50 000 colonies mutagenized with *N*-methyl-*N'*-nitro nitrosoguanidine were screened, but no higher-producing mutant was selected.

Addition of polystyrenic resins

Two polystyrenic resins (S-112 and SP-205) were added to *Actinoplanes* sp A8924/F flask fermentations at different cultivation times and at different concentrations. The addition of 5% resin S-112 or SP-205 in the first 24 h of microbial growth gave more than a three-fold improvement in kirromycin production: *ca* 1000 and 1200 mg L^{-1}, respectively (Table 1). Under these conditions, kirromycin was completely adsorbed on the resin. As shown in Figure 1, the antibiotic concentration in the medium decreased as a function of the amount of resin added.

The first attempt to scale-up the kirromycin resin process showed that the mixing conditions in our tanks represented the crucial factor, causing most of the resins to float on the surface or to stick to the reactor walls. Figure 2 shows the time course of *Actinoplanes* sp A8924/F fermentation in a 4-L bioreactor. Kirromycin production started at 24 h and reached maximum (350–400 mg L^{-1}) after 72 h. Under the best conditions so far achieved (mechanical stirring at 450 ÷ 1000 rpm in a cascade with the dissolved oxygen concentration), an improved yield of *ca* 1500 mg L^{-1} was obtained at 96 h by adding resin S-112 at the time of inoculation. It is worth noting that under the same conditions, the addition of resin SP-205, which has a higher specific gravity and was the most effective in shake flasks, gave a yield of only 700 mg L^{-1}. Kirromycin was not completely adsorbed on resin SP-205 but 400 mg L^{-1} (*ca* 57% of the total amount) was found in the fermentation medium after 72 h of fermentation.

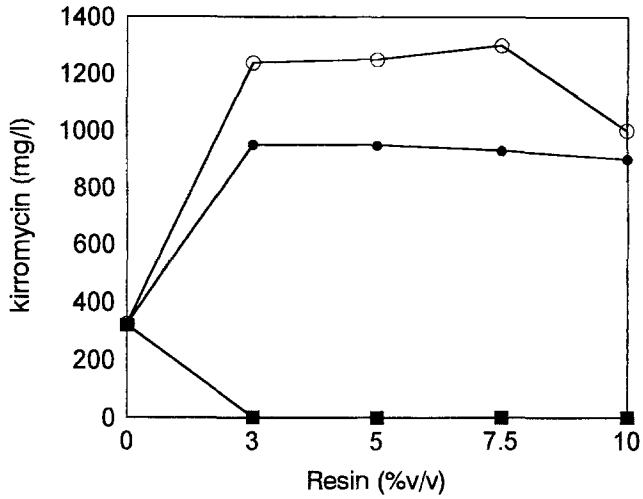

Figure 1 Effect of concentrations of resin S-112 or SP-205 added at inoculation on kirromycin production measured after 96 h of growth. Kirromycin bound to resin S-112 (●) or to resin SP-205 (○); kirromycin in the fermentation medium (■).

Table 1 Effect of addition of resin S-112 (A) or SP-205 (B) to kirromycin production in shake flasks

(A)

S-112 (% v/v)	Time of addition (h)	Kirromycin (mg L^{-1})[a]
none	–	356
5	0[b]	941
5	24	956
5	48	642
5	72	300

(B)

SP-205 (% v/v)	Time of addition (h)	Kirromycin (mg L^{-1})[a]
none	–	356
5	0[b]	1254
5	24	1115
5	48	987
5	72	574

[a]Kirromycin measured after 96 h of fermentation.
[b]No difference in the effectiveness of resins sterilized with AUR/M *vs* those sterilized separately was observed.

Kirromycin effect

In order to explain the improvement caused by resin addition, the effect of kirromycin on both growth and pro-

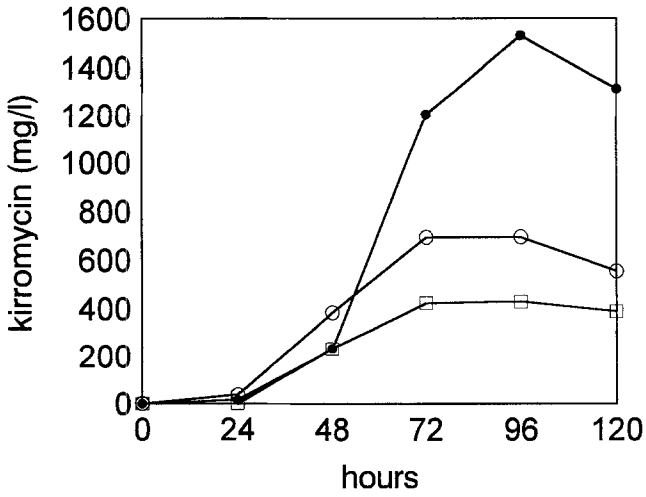

Figure 2 Production of kirromycin by *Actinoplanes* sp A8924/F in a control 4-L fermentation (□) or after the addition of resin S-112 (●) or SP-205 (○) at inoculation.

ductivity of *Actinoplanes* sp A8924/F was investigated. The minimal inhibitory concentration (MIC) of kirromycin at the beginning of growth in the production medium was 250–300 mg L^{-1}. This value increased to *ca* 350 mg L^{-1} after 4–5 days of cultivation, when production of kirromycin reached its maximum (350 mg L^{-1}). A typical time course for kirromycin production in shake flasks is shown in Figure 3. Addition of increasing amounts of kirromycin to the liquid culture when it was inoculated with *Actinoplanes* sp A8924/F, reduced its production by a corresponding amount, so that the total concentration of kirromycin never exceeded 350 mg L^{-1}. The growth of *Actinoplanes* sp A8924/F and consequently antibiotic production was delayed by the addition of increasing concentrations of kirromycin and almost completely inhibited at 250–300 mg L^{-1}.

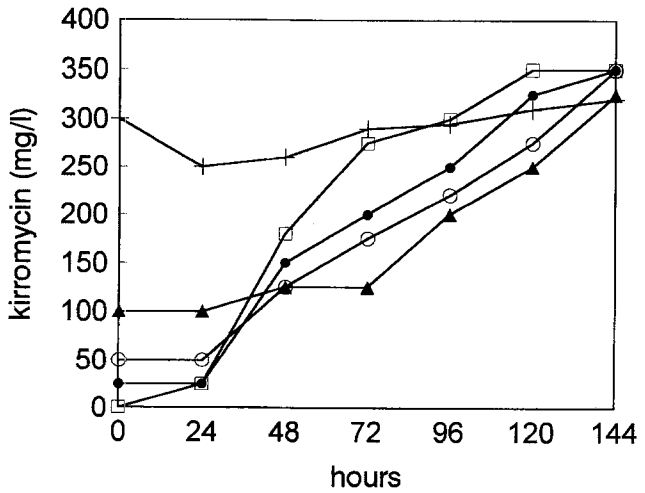

Figure 3 Effect of different concentrations of kirromycin in mg L^{-1} added at inoculation on antibiotic production: 0 (□), 25 (●), 50 (○), 100 (▲), 300 (+).

Discussion

Kirromycin productivity was increased from 30–50 mg L^{-1} to 350 mg L^{-1} using traditional approaches. A further significant improvement (to 1500 mg L^{-1}) was obtained by kirromycin adsorption onto polystyrenic resins during *Actinoplanes* sp A8924/F cultivation. The addition of resins to shake flask or bioreactor processes as a methodology to enhance antibiotic yields and simplify isolation procedures has been described for rubradirin [12], paulomycin (volonomycin) [11], myxalamids [5], angiolam A [9], myxovirescins [17] and trichothecenes [8]. Cultivation in the presence of resins was also used for preparation of radiolabeled antibiotics [7] and more recently for discovering new exfoliamycins [15]. In the case of paulomycin and rubradirin, two mechanisms of action have been proposed to explain the resin effect. Since these two antibiotics inhibit the growth of their producing microorganisms at relatively low concentrations, the resin may function by sequestering the toxic end-product. Alternatively, removal of the antibiotic from the solution may prevent its degradation [12].

In this paper, we report that the maximum amount of kirromycin produced by *Actinoplanes* sp A8924/F (350 mg L^{-1}) corresponds to the MIC of the antibiotic *vs* its producing strain. We conclude that the increase of kirromycin productivity by resin addition was due to the sequestering of the toxic end-product from the cultivation medium. Kirromycin was stable during the period of its accumulation and degradation products have never been isolated (E Restelli, personal comunication).

It is worth noting that aurodox (X-5108), a methylated homolog of kirromycin, produced by *Streptomyces goldiniensis* ATCC 21386, also limits its own production at 300–400 mg L^{-1} [2,10,18]. Further improvement in kirromycin productivity could be achieved by selection of mutants resistant to increasing concentrations of kirromycin.

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