Journal of Microbiology and Biotechnology
Vol. 5. No. 5, 297-301, 1995

Improved Production, and Purification of Aclacinomycin A from *Streptomyces lavendofoliae* DKRS

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An anthracycline antibiotic, aclacinomycin A (aclarubicin), was produced from a mutant strain of *Streptomyces lavendofoliae*. The mutant strain which showed a 4-fold higher productivity of aclacinomycin A compared with the parent strain was also found to produce a significantly higher amount of aclacinomycin A than the reported production strain, *Streptomyces galilaeus*. The aclacinomycin A was produced up to 125 mg/l using potato starch and soybean meal as carbon and nitrogen sources, respectively, on a 3 liter scale fermentation in a 5 liter jar fermentor. The mutant strain also produced significant amount of aclacinomycins B and Y. Aclacinomycin A was isolated from the culture broth by solvent extractions and further purified by silica gel column chromatography. The yield of aclacinomycin A with over 99% purity was found to be over 60% starting from 3 liters of culture broth.

Aclacinomycin A, an antitumor antibiotic, is a member of the anthracycline antibiotic group consisting of doxorubicin, daunomycin, carminomycin and so on (5, 6). The anthracycline represents a group of glycosidic compounds which have basic structures of 7, 8, 9, 10 - tetrahydro-5, 12-naphthacene quinone (7). Although doxorubicin and daunomycin have been used in the treatment of various tumor types, their therapeutic uses have been hampered by adverse side-effects, notably by cardiotoxicity and bone marrow suppression (2). Since aclacinomycin A as an antitumor antibiotic shows low cardiotoxicity, this antitumor antibiotic is expected to have wider uses for the treatment of acute leukemia and malignant lymphoma (2, 8, 9).

Aclacinomycin A was originally isolated from *Streptomyces galilaeus* (*S. galilaeus*) by Oki *et al.* (6). Fermentation of this strain gave rise to the production of aclacinomycin A with a yield of 46 mg/l together with some 20 aclacinomycin analogues including a red pigment, cinerubin (5). This pigment was reported to complicate the further purification in the downstream procedures (5). Other studies also described the production of aclacinomycin A using different strains of *Streptomyces* (1, 7, 10). Depending on the strains, the production of aclacinomycin A is accompanied by concomitant production of its major analogue compounds such as aclainomycin B and/or Y. The present study describes a development of a mutant strain of *Steptomyces lavendofoliae* (*S. lavendofoliae* DKRS) which produces a 4-fold higher amount of aclacinomycin A than the parent strain. Compared with *S. galilaeus* which is currently in use for aclacinomycin A production (5), *S. lavendofoliae* DKRS produced a higher amount of aclacinomycin A and the isolation procedures could also be less complicated due to the much lower occurrence of red pigment, cinerubin.

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Key words: aclacinomycin A, *Streptomyces lavendofoliae* DKRS, Fermentation

MATERIALS AND METHODS

Mutagenesis

S. lavendofoliae DKRS was obtained by mutagenizing *S. lavendofoliae* ATCC 15872. *S. lavendofoliae* ATCC 15872 was incubated at 30°C for 6 days on a solid plate containing glucose 1.0%, casamino acid 0.2%, meat extract 0.1%, yeast extract 0.1%, agar 1.5%, pH 7.0-7.2. Spores of the strain were collected by glass wool filtration of the culture suspended with sterile water. These spores were washed with 0.05 M Tris-malate buffer 3 times and resuspended in the buffer to make 10^6-10^8/ml concentration. *N*-Methyl-*N'*-nitro-N-nitroso-guanidine (NTG) was mixed to 500 μg/ml and incubated at 30°C for 20 minutes. After NTG treatment, spores

were collected by glass wool filtration, washed with sterile saline 3 times, and plated on a solid plate. The incubation was carried out at 30°C for 7 days and each mutant colony was cultivated as below and analyzed by HPLC to check the level of aclacinomycin A production.

Fermentation

A slant culture of *S. lavendofoliae* DKRS was inoculated into a 500 ml Erlenmeyer flask containing 50 ml of seed medium composed of glucose 1.0%, potato starch 1.0%, soybean meal 0.5%, NaCl 0.1%, $CaCO_3$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.05% with pH adjusted to 7.0. The flask was shaken at 200 rpm on a rotary shaker at 28°C for 24 hours. In order to find an optimal carbon source, 5 ml of the seed culture was transferred to 500 ml Erlenmeyer flasks containing 50 ml of a basic production medium I consisting of soybean meal 1.5%, NaCl 0.1%, $CaCO_3$ 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CuSO_4$ 0.001%, and K_2HPO_4 0.1%. This basic production medium I was supplemented with 4% each of glucose, lactose, galactose, maltose, potato starch or sucrose. For the determination of an optimal nitrogen source, 5 ml of the seed culture was transferred to 500 ml Erlenmeyer flasks containing 50 ml of a basic production medium II consisting of potato starch 4%, NaCl 0.1%, $CaCO_3$ 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CuSO_4$ 0.001%, and K_2HPO_4 0.1%. This medium was supplemented to make 1.5% with each of soybean meal, peptone, yeast extract or ammonium sulfate. The flasks were incubated at 200 rpm on a rotary shaker at 28°C for 144 hours. The final production medium containing potato starch 4%, soybean meal 1.5%, K_2HPO_4 0.1%, NaCl 0.1%, $CaCO_3$ 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001% and $CuSO_4$ 0.001% was used for the production of aclacinomycin A in a 5 liter jar fermentor. Fermentation continued at 28°C for 144 hours with an aeration rate of 3 liters per minute. 20 ml of the fermentation broth was sampled for pH, packed cell volume, and the productivity analyses.

Purification of Aclacinomycin A

The procedure for the isolation and purification of aclacinomycin A is presented in Fig. 1. 3 l of culture broth was filtered and the mycelium cake was extracted with 1.5 l of methanol with intensive shaking for 1 hour. The extract was filtered and the filtrate was re-extracted with 500 ml of ethyl acetate. The ethyl acetate layer was washed with purified water several times and then concentrated in a vacuum evaporator. The concentrate was put on a silica column (2.5×50 cm) (silica gel 70-230 Mesh, Merck, Germany), washed with methylene chloride

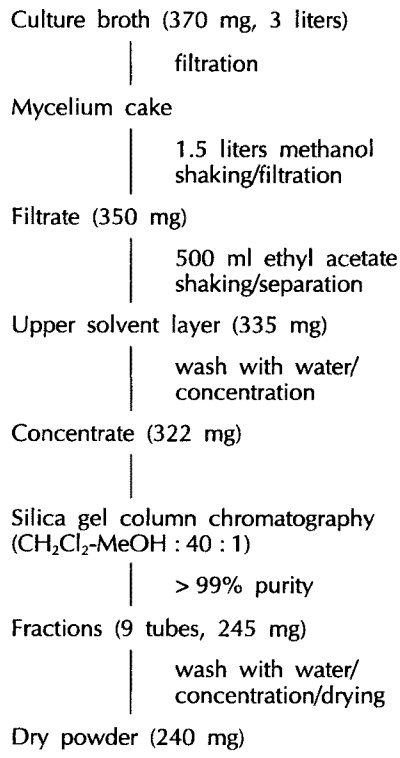

Fig. 1. Process for purification of aclacinomycin A from culture broth.

and eluted with methylene chloride-methanol (40 : 1) at a flow rate of 1 ml/min. Each fraction was analyzed by HPLC. Fractions showing more than 99% purity (9 fractions, 12 ml each) were collected and washed with water several times. The washed organic solvent layer was concentrated and dried in vacuum to yield pure yellow powder. For HPLC analyses, 10 ml of culture broth was centrifuged at 5,000 g for 10 minutes. The mycelium cake was extracted with 5 ml of methanol with intensive shaking and then centrifuged again. The supernatant was mixed with 2 ml of ethyl acetate. The upper layer was analyzed by HPLC. Authentic samples of aclacinomycin A were purchased from Sigma and aclacinomycin Y, which was not commercially available, was prepared in-house and characterized to confirm its identity. HPLC was performed on a silica column (Toyosoda, 5 μm, 4.6×250 mm, Japan) using chloroform-methanol-acetic acid-water-triethylamine (68 : 20 : 10 : 2 : 0.01) as an eluting solvent. This solvent system is a modification of the published method (4), which resulted in an improved resolution of aclacinomycin A and Y (Detailed information is described in an accompanying paper). Elution was carried out at 1.0 ml/min and the eluant was monitored at 432 nm.

RESULTS AND DISCUSSION

Selection of Aclacinomycin A-production Strain

S. lavendofoliae ATCC 15872 was subjected to NTG mutagenesis. A number of colonies obtained through NTG treatment of the parent strain were preliminarily screened for the larger size and the darker colors and these colonies were tested for the increased level of aclacinomycin A production. One such a mutant strain named as *S. lavendofoliae* DKRS was found to produce a 4-fold higher amount of aclacinomycin A compared with the parent strain. This strain was selected as the production strain. The selected mutant strain also produced considerable amounts of aclacinomycins B and Y (See Table 3 in Fermentation Section.). It is noteworthy that aclacinomycin Y was not found in the parent strain. This difference in the spectrum of aclacinomycin production indicates that some changes were introduced by mutagenesis to metabolic pathways in aclacinomycin biosynthesis.

Media Optimization for the Fermentation of Aclacinomycin A

Optimization of fermentation medium was performed with respect to carbon and nitrogen sources. Optimum concentrations of these carbon and nitrogen sources were determined in preliminary experiments (data not shown) and the maximum amounts of aclacinomycin A obtained during the 6 days of fermentation are presented in the following tables. First, various carbon sources at fixed concentration of 4% were supplemented to basic nitrogen medium (basic production medium I) containing soybean meal (Table 1). Potato starch gave the best result, although the highest amount of aclacinomycin A was obtained only at the later stage of cultivation. Glucose and sucrose showed rapid appearances of aclacinomycin A, but with lesser final amounts. Second, various nitrogen sources were supplemented, at a fixed concentration of 1.5%, to basic carbon medium (basic production medium II) containing potato starch (Table 2). Soybean meal was found to give the highest amount of aclacinomycin A. Yeast extract also produced considerable amount of aclacinomycin A. Therefore, potato starch and soybean meal were selected as proper carbon and nitrogen sources, respectively, for the production of aclacinomycin A.

Table 1. Effect of different carbon sources on aclacinomycin A production.

Carbon Source*	Maximum Aclacinomycin A Concentration (mg/l)
Glucose	6.7
Lactose	0.3
Galactose	5.6
Maltose	7.5
Potato starch	9.4
Sucrose	7.9

*Each carbon source was added to basic nitrogen medium (basic production medium I) to give a final concentration of 4%. The cultures were shaken at 200 rpm on a rotary shaker at 28°C for 144 hours and analyzed by HPLC.

Aclacinomycin A Fermentation

Fermentation was carried out in a 5 liter jar fermentor using 3 liters of the optimized production medium. *S. lavendofoliae* DKRS in the optimized fermentation medium showed a rather long period of lag phase as shown in Fig. 2. Cell growth was very slow during 72 hours of cultivation, followed by a rapid increase. After 96 hours of cultivation, packed cell volume remained constant at about 30%. Change in pH values during fermentation was negligible. During the 6 days of fermentation, a sharp increase in the amount of aclacinomycin A was observed on the 5th day followed by a rapid decrease on the 6th day. Up to 125 mg/l of aclacinomycin A was produced. The maximum amounts of aclaci-

Table 2. Effect of different nitrogen sources on aclacinomycin A production.

Nitrogen Source*	Maximum Aclacinomycin A Concentration (mg/l)
Soybean meal	17.7
Peptone	9.8
Yeast extract	16.4
Ammonium sulfate	7.1

*Each nitrogen source was added to basic carbon medium (basic production medium II) to give a final concentration of 1.5%. The cultures were shaken at 200 rpm on a rotary shaker at 28°C for 144 hours and analyzed by HPLC.

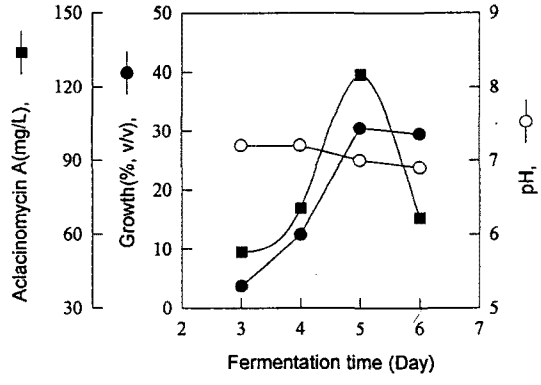

Fig. 2. Time course of aclacinomycin A production, pH, and packed cell volume.

Streptomyces lavendofoliae DKRS was cultured in 3 liters of production medium in a 5 liter jar fermentor. Fermentation was continued at 28°C for 144 hours with an aeration rate of 1 VVM. 20 ml of fermentation broth was sampled and analyzed for the content of aclacinomycin, pH, and packed cell volume.

nomycins A, B and Y obtained from fermentations with the parent strain and the mutant strain are summarized in Table 3. Thus, the amount of aclacinomycin A obtained in this study using the strain *S. lavendofoliae* DKRS is roughly 4 times higher than that of the parent strain and is also considerably higher than the amounts found in previous literature. It was reported that *S. galilaeus* produced 46 mg/l of aclacinomycin A in a fermentation study using a similar medium (5). The mutant strain also produced considerable amounts of aclacinomycins Y as well as B, although the parent strain did not produce aclacinomycin Y. It was also reported that *S. galilaeus* did not produce appreciable amount of aclacinomycin Y.

Purification of Aclacinomycin A

It was first determined whether the produced aclacinomycin A occurred in the medium or inside the cell. Over 95% of the total amount of aclacinomycin A produced was found in the mycelium cake. The purification of aclacinomycin A was performed as outlined in Fig. 1 (See Materials and Methods). 3 liters of culture broth contained about 370 mg of aclacinomycin A after fermentation. The mycelium cake obtained by filtration of the culture broth was extracted successively with methanol and ethylacetate. This solvent extract was concentrated and further purified using silica gel column chromatography. Starting from 370 mg of aclacinomycin A contained in the culture broth, 270 mg of aclacinomycin A of 99% purity was obtained as a yellowish dry powder. The overall yield of the entire purification procedure, therefore, was about 65%. The yield of each step is shown in Table 4. The purification procedure developed in this study could lead to an improved yield compared with the previously reported process (5), where 25% of the overall purification yield was obtained. This improvement in purification yield seemed to be partly due to much lower occurrence of a red pigment, cinerubin. It was reported that the separation of aclacinomycin A from the red pigment was very difficult owing to their similar chromatographic behaviours. For the removal of the red pigment, metal ions were used to selectively precipitate the red pigment over yellow aclacinomycins prior to column chromatography (5).

From the results obtained in this study, it is concluded that aclacinomycin A could be efficiently produced from *S. lavendofoliae* DKRS with higher productivity. The purification processes newly developed in this study would be of value in the development of process for the production of aclacinomycin A on a large scale. Aclacinomycin Y which was found to be produced in high amount by the strain used in this study has not been substantially characterized for its potential use as an antitumor antibiotic. Careful studies with animal would be required to determine its efficacy as an antitumor antibiotic compared with similar compounds including aclacinomycin A.

Table 3. Production of aclacinomycins A, B and Y during fermentation with the parent strain (*S. lavendofoliae* ATCC 15872) and the mutant strain (*S. lavendofoliae* DKRS).

Strains	Aclacinomycins (mg/l)*		
	A	B	Y
Parent strain (*S. lavendofoliae* ATCC 15872)	33	29	-
Mutant strain (*S. lavendofoliae* DKRS)	125	78	69

*Each strain was cultivated in a 5 liter fermentor using 3 liters of production medium for 6 days and the maximum amounts of different aclacinomycins during the fermentation were pressented in this table.

Table 4. Yields of each step for aclacinomycin A purifiaction.

Purification Step	Weight (mg)	Yield (%)
Culture broth	370	100
Methanol extract	350	95
Ethyl acetate extract	335	91
Concentrate	322	87
Column fractions	245	66
Final powder	240	65

Acknowledgment

This work has been funded by the Korean Ministry of Science and Technology (Project No. G71010). Authors deeply appreciate the financial support.

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(Received February 9, 1995)