ENHANCED SINEFUNGIN PRODUCTION BY MEDIUM IMPROVEMENT, MUTAGENESIS, AND PROTOPLAST REGENERATION OF *STREPTOMYCES INCARNATUS* NRRL 8089

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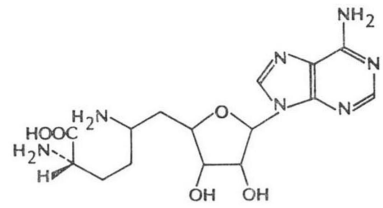
(Received for publication November 5, 1984)

Increased production of sinefungin, a very potent antifungal and antiparasitic nucleoside antibiotic was achieved by medium and strain improvement. When soybean-meal, dextrin and yeast extract were added as carbon and nitrogen sources to the fermentation medium, instead of corn steep liquor, soya-oil and glucose; the antibiotic yield increased from 40 μg/ml to 126 μg/ml with low biomass production. Strain improvement was attempted by two methods. The mean antibiotic yield of the variants after multistep mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and ethyleneimine was 466 μg/ml. Protoplasts of the parental strain were prepared by lysozyme digestion from mycelia grown in a medium containing 0.7% glycine. The mean activity of the regenerated protoplasts was 664 μg/ml. Thus, the overall sinefungin production could be increased 16-fold.

Two strains are known to produce sinefungin also designated 32232 RP[1]. These are: *Streptomyces griseolus*, isolated at Eli Lilly Research Laboratories[2], and *S. incarnatus*, isolated at Rhône-Poulenc Research Laboratories in France[1].

Sinefungin is a nucleoside, in which a molecule of ornithine is linked to the 5'-end of adenosine through a C-C bond. It has outstanding antifungal[3] and antiparasitic[4~7] properties *in vitro* and *in vivo* but provokes nephrotoxicity and bone marrow depression in dogs.

As sinefungin can be considered as a model compound against fungal and parasitic infections, several laboratories undertook its chemical synthesis, with the goal of preparing structural analogues[8~11]. The total synthesis, however, needs many steps, and the overall yield is very low. Thus, an improvement in the yield of the microbial production of sinefungin would be very useful and should allow chemical and enzymatic modification of the nucleoside.



Sinefungin

The various fermentation conditions described in this paper allowed a 3- to 12-fold increase of antibiotic production over the published yield. In some media however, the increased yield was accompanied by increased biomass and pigment production. The best results were obtained with regenerated protoplasts of the parental strain. Stable variants were isolated which produced an average of 664 μg/ml sinefungin in the improved medium, allowing a 16-fold increase over the 40 μg/ml originally achieved.

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Materials and Methods

Strain

Streptomyces incarnatus NRRL 8089 was provided by Dr. J. LEBOUL, Rhône-Poulenc Research Laboratories, Vitry, France.

Seed Medium

Consisted of glucose 1%, Bacto-peptone 1%, yeast extract 0.5%, NaCl 0.5% and $CoCl_2 \cdot 6H_2O$ 0.003%, pH 7.0 (medium A).

Fermentation medium

The original fermentation medium described in the Rhône-Poulenc patent consisted of glucose 1%, soya-oil 2%, corn steep liquor 2%, $CaCO_3$ 0.5% and $CoCl_2 \cdot 6H_2O$ 0.0025% (medium B).

Culture Conditions

A 2% volume of 24-hour culture from medium A was transferred to the fermentation medium and incubated in Erlenmeyer flasks at 28°C on a rotatory shaker.

Fermentation Studies

Once a day the cultures were centrifuged, the wet biomass weighed, and the antibiotic activity determined against *Candida albicans*. The highest antibiotic production was observed on day-6.

Analysis of the Culture Supernatant

This was performed by thin-layer chromatography on cellulose plates with the following solvents: A; butanol - acetic acid - water, 40: 15: 5, B; isoamyl alcohol - formic acid - water, 3: 2: 1, C; methanol - chloroform - 28% NH_4OH, 3: 1: 1. The Rf value of sinefungin in the three solvent system was respectively 0, 0.34 and 0.25. The compounds were visualized by UV light, by ninhydrin and by autobiography.

The antibiotic concentration was estimated by the agar diffusion disc plate method using *C. albicans* as the test organism on a medium consisting of yeast extract 0.5%, asparagine 0.5%, glucose 1% and agar 2%. A standard inhibition curve was established by using authentic sinefungin provided by Eli Lilly, U.S.A.

Preparation of Mutants

Spores of the variant LB-12 derived from the original *S. incarnatus* NRRL 8089 were treated with 500 μg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) for 1 hour. The spores of the best variant N-4 were further treated with 7% of ethyleneimine. Both mutational treatments gave 1~2% survivals following colony formation on N_8-agar medium (see Table 1). After each step 30~40 colonies were tested for their morphology, pigment and antibiotic production. The developing colonies were isolated, and their antibiotic activity was estimated after 6 days culture in N_1 liquid medium.

Preparation of Protoplasts

Medium S_1[12] was inoculated with a spore suspension of *S. incarnatus* from N_8 medium. After 48 hours of incubation 2% of the culture was transferred into medium S_2[12] containing glucose 2%, yeast extract 0.4%, malt extract 1% and various concentration of glycine (Merck).

Ten ml of mycelial suspension was then harvested by centrifugation and washed with a 0.5 M sucrose. The washed mycelia were centrifuged, resuspended in medium P[13]. The suspension was centrifuged again and resuspended in the same medium containing 1 mg lysozyme/ml, and incubated at 32°C for various times.

The lysozyme treated cell suspension was centrifuged at 700 rpm for 5 minutes, and filtered through glass wool to remove intact cells. The protoplast suspension (7×10^5/ml) was diluted gently in medium PWP[14] and plated on the regeneration medium R_4[12] or R_3[14].

The characterization of the mutagen-treated cells and the regenerated protoplasts was performed according to SHIRLING and GOTTLIEB[15].

Table 1. Antibiotic activity and biomass production in the various fermentation media tested.

	N_1	N_2	N_3	N_4	N_5	N_6	N_8	B
Component (g/liter)								
Dextrin	10	10	10	—	—	—	10	—
Glucose	—	—	10	—	10	—		10
Maltose	—	—	—	10	10	10	5	—
Yeast extract	8	—	—	—	—	2	2	—
Soya-oil	—	—	—	—	—	—	—	20
Soybean-meal	10	20	—	—	20	10	—	—
Corn steep liquor	—	—	—	—	—	—	—	20
Bacto-peptone	—	—	10	10	—	—	—	—
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Na_2HPO_4	2	2	2	2	2	2	2	—
$CaCO_3$	1	1	1	—	—	—	1	5
Mean activity (μg/ml)	126±50	316±50	63.5±25	5±3	501±100	218±50	50±25	40±20
*Mean biomass (g/ml)	0.20	0.70	0.70	0.37	0.85	0.40	0.20	0.2
Sinefungin (μg) / Wet mycelium (g)	630	451	90.7	13.5	589	545	250	200
Melanine on solid media	+	−	++	++	+	+	+	+

* Biomass is defined as the wet weight mycelium.
Each experiment was performed by running ten identically prepared cultures in parallel.

Results

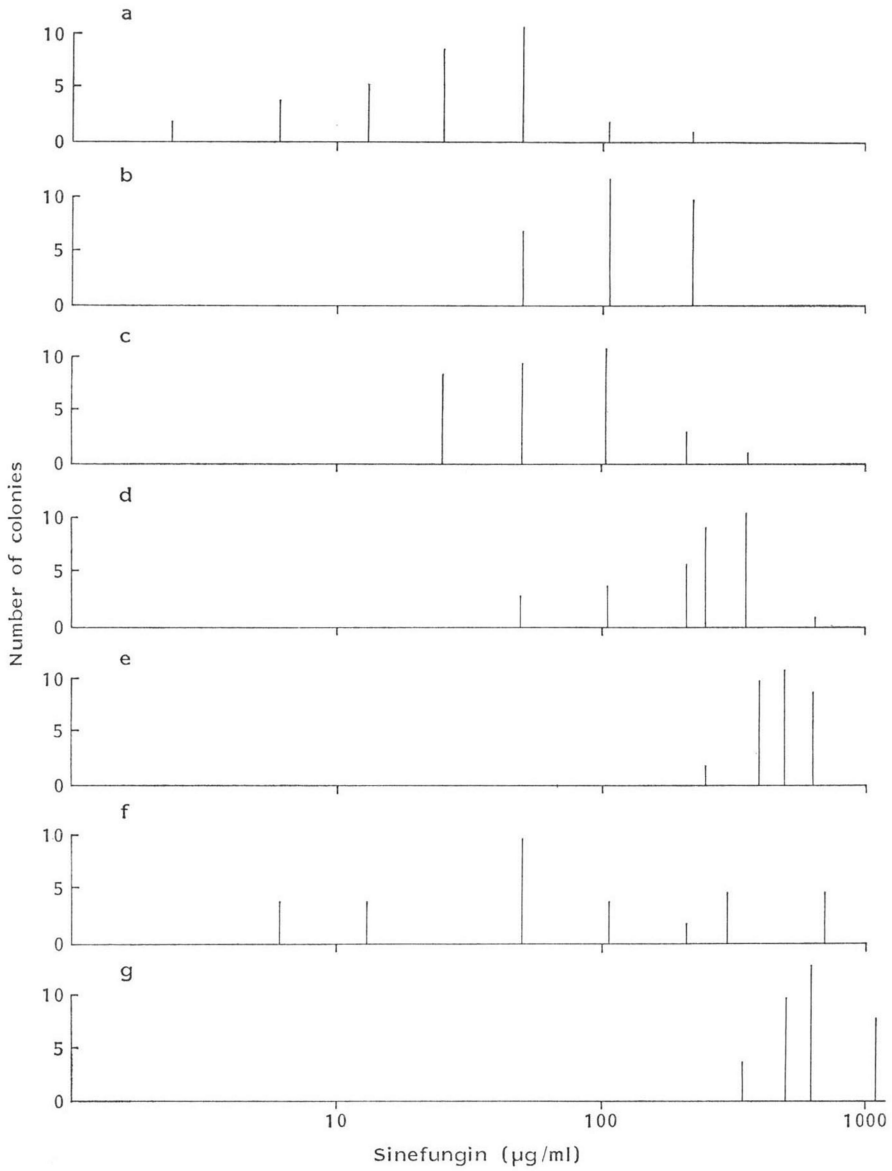
Improvement of the Antibiotic Production by *S. incarnatus* after a Two-step Fermentation in Complex Media

The original strain NRRL 8089, in medium B, originally published for *S. incarnatus*, produced very low amounts of antibiotic (0~10 μg/ml). To determine whether the strain contains more active variants, a spore suspension of NRRL 8089 was spread on N_8-agar medium, and the antibiotic activity of 35 colonies checked on medium N_1. The distribution of the activity is shown on Fig. 1-a. The variant LB-12 producing 216 μg/ml of the antibiotic was choosen for further studies.

Table 1 shows the composition of the media with the antibiotic production and the amount of biomass after 6 days cultivation in these media. The results were compared with those obtained in medium B. In this medium the antibiotic yield was as low as 40 μg/ml accompanied by a melanin-like pigment production. The replacement of soya-oil and corn steep liquor in medium B by soybean-meal resulted in a significant increase of the sinefungin production (medium N_1, N_2, N_5 and N_6 in Table 1). The highest yield was obtained with medium N_5 and N_2. The disadvantage with these two media however was the abundant mycelia production. When the carbon sources dextrin and glucose or maltose alone were added with Bacto-peptone as in medium N_3 and N_4 the antibiotic production decreased and the pigment production increased. Since the ratio of antibiotic production *versus* mycelial growth was the most favorable in N_1, this medium was used further. The distribution of the antibiotic production by the progeny strains of LB-12 is shown on Fig. 1-b. Those progenies produced 50~216 μg/ml of the antibiotic, and the mean activity was 126 μg/ml. Thus 3-fold increase of the yield over the 40 μg/ml was achieved in medium B.

Fig. 1. Distribution of sinefungin productivity.

a) Original strain *S. incarnatus* NRRL 8089; b) Second generation of the variant LB-12 obtained by mono-colony isolation from the original strain; c) after mutagenesis of LB-12 by NTG; d) after mutagenesis of the variant N-4 by ethyleneimine; e) tenth generation of the high yielding variant EI-132; f_1) third generation of regenerated protoplasts of LB-12; g) tenth generation of the high-yielding variant P-12.



Sinefungin Production after Mutagenesis

The variant LB-12 producing 216 μg/ml of the antibiotic was subjected to chemical mutagenesis by NTG. Results are shown in Fig. 1-c. Fifty three percent of the colonies produced less sinefungin than the parental LB-12 and the mean activity was 86.7 μg/ml. The only variant with increased activity, NTG-4 (354 μg/ml) showed different morphology from the parental strain (Table 2). The

Table 2. Characterization of the variants of *S. incarnatus* LB-12 after mutagenesis and protoplast regeneration*.

Designation	Aerial mycelium		Substrat mycelium		Activity (μg/ml)
	Morphology	Color	Color	Pigment production	
LB-12	*Rectus*	Light brown	Yellow	—	216
NTG-4	〃	White	〃	—	354
EI-131	*Spira*	〃	〃	—	501
EI-132	*Rectus-flexibilis*	Brown	Brown	+	630
EI-133	*Spira*	White	〃	+	501
EI-134	*Spira*	White-rose	Yellow	—	354
EI-135	RA**	〃	〃	—	354
P-1	RA	Beige	〃	—	354
P-2	*Spira*	〃	〃	—	250
P-4	No aerial mycelium		〃	—	354
P-7	*Spira*	Beige	〃	—	250
P-8	No aerial mycelium		〃	—	250
P-10	*Spira*	Beige	Brown	+	530
P-12	RA	〃	〃	+	707
P-14	*Rectus*	White	〃	+	618
P-16	RA	Beige	〃	+	250

* According to SHIRLING and GOTTLIEB[15]. ** *Retinaculum-Apertum.*

LB-12 variant of the original strain *S. incarnatus* NRRL 8089, EI-131～135 mutants isolated upon mutagenesis of NTG-4 by ethyleneimine.

NTG-4 mutant isolated upon mutagenesis of LB-12 by NTG.

P-1～P-16 obtained by regenerating protoplasts of LB-12.

variant NTG-4 was further treated with ethyleneimine. The resulting colonies could be classified into five morphologically different types, as shown in Table 2. The distribution of the antibiotic productivity among one mutagenized strains of NTG-4 is presented in Fig. 1-d. One of the resulted variants EI-132 producing 630 μg/ml sinefungin showed different morphology from the parental LB-12. The distribution of the antibiotic activity among one progeny strains of EI-132 determined after ten times successive transfer and cultivation in medium N_1 is presented in Fig. 1-e. The mean activity is 466 μg/ml corresponding to a 2-fold increase with respect to the yield of LB-12.

Sinefungin Production by Regenerated Protoplasts of *S. incarnatus*

As in the case of mutagenesis, the variant LB-12 was chosen for protoplast preparation and regeneration.

The mycelium of LB-12 incubated in medium S_2[12] with 0.1% to 0.6% of glycine showed high resistance to lysozyme action. When glycine concentration was raised to 0.7% and the mycelium incubated in medium P[13] with 1 mg/ml of lysozyme, the process of protoplast liberation was observed to occur within 90 minutes by a phase-contrast microscope. The purified protoplast suspension diluted in medium PWP[14] was plated on various regeneration media such as the medium R_4[12] containing sucrose, maltose or lactose and medium R_3[14] with 1.8% agar. No regeneration was observed in medium R_4[12] with sucrose or maltose, and only 3% of regenerated colonies were obtained with lactose. On medium R_3 at 28°C the extent of regeneration was 28%. The regenerated strains grew as non-sporulating bacillus-like colonies on medium R_3. The aerial mycelium was restored on medium 2[15]. As shown in Table 2, the morphology and color of the aerial and substrate mycelia as well as the pig-

ment production of the regenerated colonies were different from the parental strain LB-12. Two variants did not produce aerial mycelium nor pigment. The best variant after protoplast regeneration was P-12 (Fig. 1-f). When colonies of this variant were screened for sinefungin production (Fig. 1-g), 8 colonies of 35 examined produced 1,081 μg/ml antibiotic on N_1 medium, representing a 27-fold increase with respect to the production by LB-12 in medium B.

Discussion

Two strains are known to produce sinefungin. *S. griseolus* NRRL 3739[2] and *S. incarnatus* NRRL 8089[1]. Fermentation studies with the first strain were carried out at Eli Lilly Research Laboratories and were published by BOECK *et al.*[16]. Concerning *S. incarnatus*, no published results beside the patent data[1] are available.

With *S. griseolus* the highest yield of sinefungin production (520 μg/ml) was obtained with a fermentation medium containing cotton-seed oil 4%, $CaCO_3$ and phosphate 0.02%[1]. According to our results sinefungin production by *S. incarnatus* was the highest when the culture consisted of sugar 2% with soybean-meal 2%, phosphate 0.2% and cobalt ions 25 μg/ml. Thus, to stimulate the antibiotic production of *S. incarnatus*, about 10-fold more phosphate are necessary than for *S. griseolus*.

Marked improvement of antibiotic production was achieved by genetic variations induced by chemical mutagenesis or protoplast regeneration. IKEDA *et al.*[17] observed a 2- to 3.6-fold increase of macrolide antibiotic production with regenerated protoplasts of *S. ambofaciens* and *S. fradiae*. On the contrary, BALTZ[18] reported that regenerated protoplasts of *S. fradiae* produced very low level of tylosin. In the case of *S. incarnatus*, most of the progeny after regeneration of the protoplasts were different in morphology and antibiotic productivity from the original strain. They also produced more aerial mycelium. These differences suggest that pleiotropic genetic variations occurred during regeneration. Protoplast regeneration seems to be more suitable than chemical mutagenesis in the case of *S. incarnatus* to obtain high producers of sinefungin.

Acknowledgments

Dr. HALINA MALINA is recipient of a fellowship from World Health Organization.

This work was supported by grants from the Ministère de l'Industrie et de la Recherche: grant 50345; from the Institut National de la Santé et de la Recherche Médicale: grant 508325; the World Health Organization UNDP/World Bank/WHO Special Programme for Research and Traning in Tropical Diseases, from the Fondation de la Recherche Médicale Française and la Ligue Nationale Française contre le Cancer.

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