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Culture Parameters for Nonactin Production by Streptomyces viridochromogenes JM-4151

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Abstract Nonactin is the parent compound of a group of ionophore antibiotics, that known as the macrotetrolides. In previous report, in the course of screening superoxide radical-generating compounds from microbial sources, we first screened *Streptomyces viridochromogenes* JM-4151 that produces nonactin. It was proved that nonactin is superoxide radical-producing compound. In present study, we examined the optimal culture conditions of nonactin. The optimal culture conditions for nonactin production were as follows: 1% soluble starch, 1% yeast extract, 0.2% ammonium nitrate, 0.06% magnesium sulfate, 0.2% calcium carbonate, initial pH 7.0 at 28℃ for 96 h. The highest nonactin production was achieved in the production medium of initial pH 7.0 at 28℃ for 96 h. The threshold level of dissolved oxygen was found to be above 33.2% at 28℃ when 1% soluble starch was used as a carbon source. These results suggest that *S. viridochromogenes* JM-4151 might be a possible strain for industrial nonactin producer.

Key words: nonactin, *Streptomyces viridochromogenes* JM-4151, culture parameters

Introduction

Nonactin is the parent compound of a group of ionophore antibiotics, known as the macrotetrolides, produced by *Streptomyces griseus* [1]. Nonactin showed to inhibit the p-glycoproteinmediated efflux of 4-O'-tetrahydropyranyldoxorubicin in multidrug-resistant erythroleukemia K562 cells [2]. In the course of screening and developing superoxide radical-generating compounds from microbial sources, we first screened *Streptomyces viridochromogenes* JM-4151 that produces nonactin [3]. Because most of macrotetrolide antibiotic producers excrete antibiotic complexes into culture medium [4], the product yield of each pure form is relatively low. Therefore, if we get an improved strain by mutagenesis, the mutant strain will be very important for nonactin production, especially, when we need a bulk of pure nonactin for industrial application. Alternative is to optimize the productivity of nonactin by *S. viridochromogenes* JM-4151. In this study, therefore, we show that nonactin is a potent major antibiotic from the culture broth of *S. viridochromogenes* JM-4151. We also examine some culture parameters for the high productivity of nonactin.

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Materials and Methods

Used strains and assay methods

Streptomyces viridochromogenes JM-4151 was isolated from a soil sample collected at Mt. Palgong, Taegu, Korea [3]. The antibiotic production was determined by an agar diffusion method [3,5] using *Bacillus subtilis* KCTC 3069 (rec^-) and *B. subtilis* ATCC 6633 (rec^+) as assay strains. The MIC of nonactin against test strains was determined by the conventional two-fold serial agar dilution method using Mueller Hinton agar. After following the inoculation of the test organisms (10^7 CFU/ml), observation on the bacteria was made after 18 h at 37℃. Antibacterial activity was measured by the paper disc method on nutrient agar using *B. subtilis* KCTC 3069 (rec^-). Melted agar medium preincubated at 60℃ with spore suspensions of the wild type strain (rec^+) and the supersensitive mutant strain (rec^-) at 10^4 spores/ml was poured into a plastic plate and solidified at room temperature. After placing paper discs (d, 8 ㎜) containing samples on agar plates, the plates were incubated for 18 h at 37℃, and inhibition diameters of the plates were measured. For the identification of nonactin from the culture

broth, the active fraction extracted with ethyl acetate was applied to a HPLC column (3.9×300 mm reverse phase-C^{18} μ Bondapak) with a mobile phase of 95% acetonitrile. The peak was confirmed by a detector (L-4200, Hitachi, Japan) at 254 nm with flow rate of 1 ml/min and assayed against *B. subtilis* ATCC 3069.

Production media

The strain was grown on a Bennett's agar slant for 7 days, and the surface growth of the slant was swabbed into 100 ml of medium F2 in a 500-ml Erlenmeyer flask. Medium F2 contained the following per liter of distilled water: yeast extract 1.0g, soybean meal 1.0g, K_2HPO_4 0.5g, $MgSO_4 \cdot 7H_2O$ 0.5g, $CaCO_3$ 2.0g. The pH of the medium was adjusted to 7.0 prior to sterilization. Frozen vegetative preparations were prepared by mixing a culture grown for 3 days in medium F2 with an equal volume of 50.0% (w/v) glycerol, and aliquots frozen in a dry ice-acetone bath, and stored at -70.0℃. From the frozen stock, 4.0 ml was used as an inoculum into 100.0 ml of medium F2. The culture was grown for 3 days and then 4.0% (v/v) of the culture was used to inoculate medium F2, which was also used as the production medium. All cultures were grown in liquid media at 28.0℃ by shaking at 200 rpm unless stated otherwise. Cultures were grown in a 30 L-fermentor (San Ace 25, Sanyo Denki, Japan) containing 20 L of medium F2 supplemented with soluble starch. The pH of the medium was adjusted to 7.0 prior to sterilization. The antifoam agent polypropylene glycol P-2000 was added to a final concentration of 0.01% (v/v) prior to sterilization. The cultures were grown at 28.0℃, with agitation of 400 rpm, aeration of 1.0 vvm, and back pressure of 0.4 bar unless stated otherwise. Each fermentor was equipped with an oxygen probe, and measurements of dissolved oxygen were taken throughout the fermentation. The fermentors were inoculated with 800 ml of culture broth that had been grown in flasks by shaking for 3 days.

Cell growth determination

Cell growth was measured by dry cell weight (DCW). To determine mycelia growth, the mycelia were harvested by centrifugation. After removal of insoluble soybean meal and calcium carbonate by centrifugation, the pellet was dried in an oven and then weighed [6].

Sample preparation and nonactin activity

Samples from the culture were mixed with an equal volume of ethyl acetate for 1 h. After centrifugation, 1.0 ml of the ethyl acetate layer was removed and dried under nitrogen, and stored at -20℃ until use as described previously [3]. The nonactin activity was determined by agar disk technique using *B. subtilis* ATCC 3069 as a test organism, and indicated as relative activity between samples.

Results and discussion

In order to develop potent superoxide radical-generating compounds from microbial sources, we already isolated a *Streptomyces* sp. by an antagonism assay [3] and designated as *Streptomyces viridochromogenes* JM-4151. The isolated strain produced JM-1 [7]. We further investigated JM-1's structure by various chemical analyses, and finally identified that the compound is nonactin (Fig. 1A) [3,7]. Interestingly, high-pure form of nonactin produced by the strain. Conventional nonactin producer only excreted complexes into cultutre medium [4]. We confirmed that this strain produce highly pure form of nonactin by HPLC (Fig. 1B). The strain would be very promising one for industrial application, if we obtain more advanced mutant by mutagenesis and/or get high yield by culture optimization.

In previous reports, we confirmed that the antimicrobial action of nonactin might be correlated with its action as active oxygen radical generator [8]. In order to obtain high yield for nonactin production, we investigated the culture parameters for hishest nonactin productivity. The selection of suitable nutrients for fermentation media is one of impor-

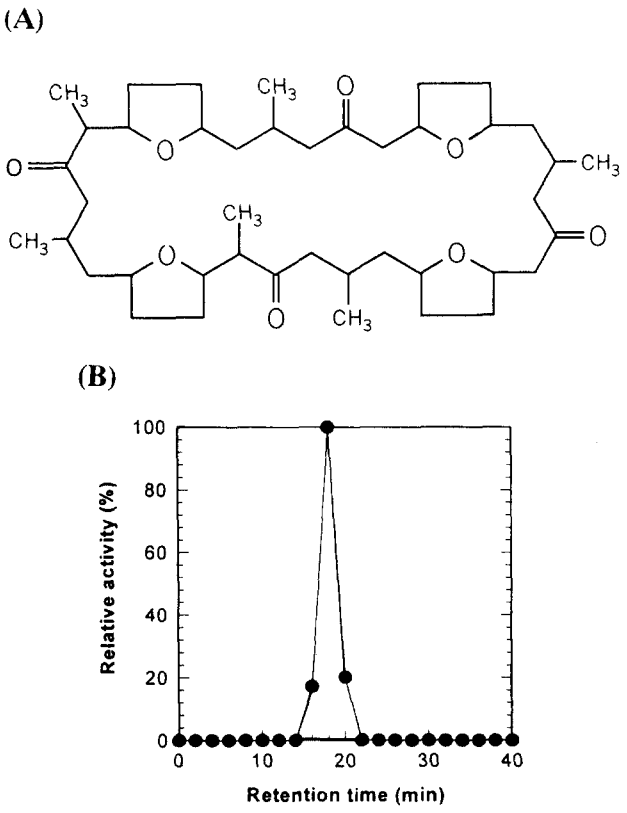

Fig. 1. Structure, and identification of nonactin produced by *S. viridochromogenes* JM-4151. (A), Structure of nonactin. (B), The identification of active component by *S. viridochromogenes* was assayed against *B. subtilis* ATCC 3069. The active fraction extracted with ethyl acetate was applied to a HPLC column (3.9 ×300 mm reverse phase-C^{18} μ Bondapak) with a mobile phase of 95% acetonitrile. The peak was confirmed by a detector (L-4200, Hitachi, Japan) at 254 nm with flow rate of 1ml/min and assayed against *B. subtilis* ATCC 3069.

tant determinants to improve the antibiotic production. Various carbon sources that were fixed at the concentration of 1% were supplemented with medium F2. The culture was grown by shaking for 120 h at 28℃ in an Erlenmeyer flask (500 ml) containing 100 ml of medium. The productivity of antibiotic (P/X) was good when we add soluble starch as a carbon source, whereas lactose and xylose showed lower activity (data not shown). It is well known that the production of antibiotics is promoted when readily utilizable sugars such as glucose and glycerol have been entirely consumed [9]. However, the inhibition of production by glucose did not show on nonactin production by the strain (data not shown). With a fixed condition of agitation of 400 rpm and an aeration of 1.0 vvm, the production of nonactin in media containing 1.0% and 3.0% soluble starch was examined (Fig. 2A and B). Similar amount of cell mass was formed under these conditions. The pH slightly increased at stationary stage. Dissolved oxygen level in the fermentor with 1.0% soluble starch was decreased to 83.0%. This depletion in oxygen coincided with the onset of stationary phase, and with the initiation of nonactin production. The optimum production of nonactin was reached after 84 h of incubation with 1.0% soluble starch as a carbon source. In the fermentor with 3.0% soluble starch, the lowest level of oxygen (85.0%) occurred after the beginning of stationary phase. It is well known that the aeration is most important factor for antibiotic production [10]. Up to 75% of dissolved oxygen level achieved 100% relative activity (Table 1) whereas the repression of nonactin synthesis resulted from decreased dissolved oxygen levels (below 33%; Table 1). Temperature has been shown to be important parameter in the synthesis of a number of secondary metabolites [11]. Incubation of *S. viridochromogenes* JM-4151 in fermentor with 1.0% soluble starch at 28.0℃ resulted in the highest production of nonactin (Fig. 2). Nonactin production, however, reduced to 35.7% of the control when the temperature was elevated to 31.0℃. We found that *S. viridochromogenes* JM-4151 produced high-pure form of nonactin at 28.0℃ on an optimized fermentation medium (Table 2). The optimized fermentation medium is as follows: 1.0% soluble starch, 1.0% yeast extract, 0.2% ammonium nitrate, 0.06% MgSO4.7H2O, 0.2% calcium carbonate, initial pH 7.0 (Table 3).

In summary, the highest productivity was achieved at 28 ℃ for 96 h under the 75% of dissolved oxygen (Table 4).

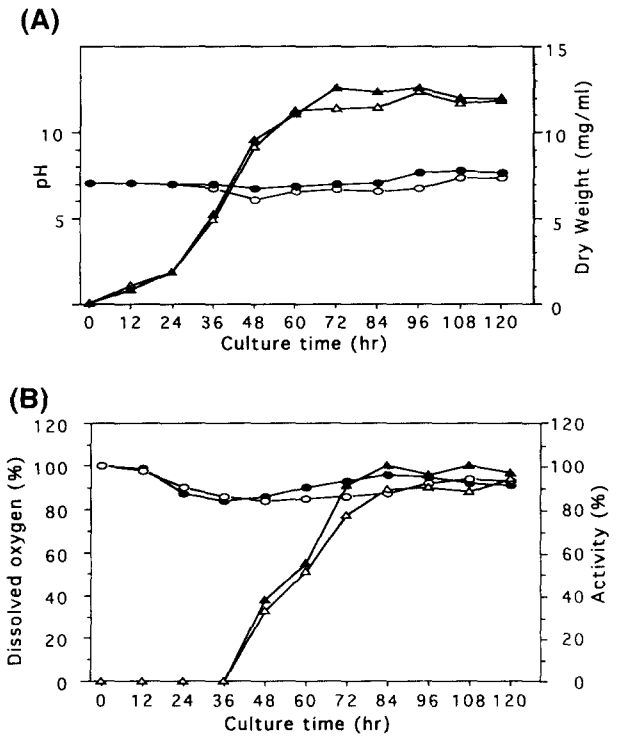

Fig. 2. Effects of soluble starch on nonactin production by *S. viridochromogenes* JM-4151 in the fermentor. (A), Cultivation was carried out with 1.0% (circles) or 3.0% (triangles) soluble starch by monitoring cell mass levels (open symbols) and pH (closed symbols). (B), Dissolved oxygen levels (open symbols) and nonactin activity (closed symbols) with 1.0% (circles) or 3.0% (triangles) soluble starch were also shown. Relative activity was measured as described in Table 1. Data are one of three independent experiments.

Table 1. Effect of dissolved oxygen on nonactin production by *S. viridochromogenes* JM-4151 during fermentation with 1.0% soluble starch at 28℃ by agitation of 400 rpm and aeration of 1 vvm, and back pressure of 0.4 bar using a 30 L-fermentor.

Dissolved oxygen (%)*	Relative activity (%)**
0	0
33	24
61	88
75	100

*The lowest level of dissolved oxygen was detected during cultivation in the fermentor.
**Highest level of nonactin detected during the fermentation.

Table 2. Effect of temperature on nonactin production by *S. viridochromogenes* JM-4151 during cultivation with 1.0% soluble starch in a 30 L-fermentor.

Temperature (℃)	Relative activity (%)*
25	35.7
28	100.0
31	82.5

*Highest level of nonactin detected during cultivation.

Table 3. Optimized medium composition and production condition of nonactin by *S. viridochromogenes* JM-4151.

Medium composition		Culture condition	
Ingredients opt.	%	Parameters opt.	condition
Soluble starch	1.0%	Initial pH	7.0
Yeast extract	1.0%	Temperature	28℃
Ammonium nitrate	0.2%	Dissolved oxygen	75%
MgSO4 · 7H2O	0.06%	Culture time	96 h
CaCO3	0.2%		

The threshold level of dissolved oxygen was found to be above 33.2% at 28℃ when 1% soluble starch was used as a carbon source. Further investigations on the specific functions, for example, the putative circumvention of p-glycoproteinmediated multidrug resistance, when we use nonactin and other anticancer drugs for clinical use could be rewarding, in that p-glycoprotein and/or multidrug resistance protein (MRP) are the main obstacle in cancer chemotherapy for cancer patients.

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