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Strain Improvement for High Gentamicin Production Using *Micromonosporas purpurea*

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*Micromonosporas purpurea*로부터 효율적 gentamicin 생산을 위한 균주 개발

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요 약

*Micromonosporas purpurea*로부터 효율적 gentamicin 생산을 위해 protoplast fusion와 protoplast mutagenesis 방법이 검토 되었다. $C0^{60}$ irradiation (2.3×10^5 units, UV 3 min) 방법에 의해서 MP3-112, MP3-141, MP3-143을 분리 했다. 특히 MP3-143 균주는 최대 gentamicin 생산량이 얻어졌다. 개량된 MP3-143 균주를 이용해서 탄소원 소비, 균체성장, 그리고 gentamicin 생산량이 batch culture에서 비교되었다. MP3-413와 parent 균주의 glucose 소비는 배양 2일과 3일 후에 각각 완전히 이루어졌다. 그러나 균체성장과 Soybean oil 소비는 비슷한 결과 얻어졌다. Gentamicin 최대 생산량은 배양 5일 후 29756 U/m/였다. 이 결과는 parent 균주에 비해 생산량이 5.6배 증가했다.

주요어: *Micromonosporas purpurea*, protoplast mutagenesis, gentamicin

I. Introduction

Gentamicin is known to be a stable, basic, and water-soluble aminoglycoside antibiotic. Gentamicin has a highly effective antibiotic, which is active against both gram-positive and gram-negative microorganisms such as the *Staphylococcus*, *Klebsiella*, *Pseudomonas*, and *Proteus* species. Therefore, it became highly efficient from an economic viewpoint to try and discover methods for enhancing gentamicin production that would be industrially feasible. A particularly advantageous gentamicin-producing strain of Micromonospora is *M. purpurea* NRRL 2953. Other available species and strains are *M. echinospora* NRRL 2985, *M. echinospora* var. *pallida* NRRL 2996 and *M. echinospora* var. *ferruginea* NRRL 2995.[1-3] Although many researchers have tried to enhance gentamicin productivity from *Micromonosporas echinospora* through mutation techniques, gentamicin titer is lower than other antibiotics such as streptomycin and erythromycin, which are produced by Streptomyces group strains.[4-6] Choi *et al.* have previously isolated *Streptomyces fradiae* using vegetable oils as the sole carbon source for efficient tylosin using protoplast mutagenesis with UV-irradiation. There was about 3.0-fold increase in tylosin production compared to that of parent strain[7] and for effective lincomycin production from *Streptomyces lincolnensis*, the protoplast mutagenesis method was also applied.[8]

This study examined effective gentamicin production using *Micromonosporas purpurea*, via the

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use of protoplast fusion and protoplast mutagenesis with UV irradiation along with spore mutagenesis using a combination of UV irradiation. Additionally, batch cultures were performed in a flask in order to compare the growth pattern and consumption of glucose and soybean oil and gentamicin production using *Micromonosporas purpurea* MP3-143 and its parent strain.

II. Material and Methods

1. Strain, media, and culture

The strain used in this study was *Micromonosporas purpurea* (mark MP1, MP2, and MP3). The composition of the agar slant medium was as follows (g/*l*): glucose, 5; starch, 10; yeast extract, 5; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, o.5; and agar 10. The composition of the basal medium was as follows (g/*l*): starch 10; KNO_3, 1.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5, $K_2HPO_4 \cdot 3H_2O$, 0.5; $CaCO_3$, 1.0; and $FeSO_4$, 0.01. The composition of the protoplast regeneration medium was as follows (g/*l*): added sucrose 70, $MgCl_2 \cdot 6H_20$, 10.2 into 1000 m*l* of basal medium. The composition for the mycelium medium was as follows (g/*l*): beef yeast extract 1.0; yeast extract, 1.0; tryptone, 3.0; glucose, 10; aspartic acid, 0.2; KNO_3, 0.5; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5, $K_2HPO_4 \cdot 3H_2O$, 0.5; $CaCO_3$, 1.0; and $FeSO_4$, 0.01. The composition of trace elements solution was as follows (mg/*l*): $ZnCl_2$, 40; $FeCl_3$, 200; $CaCl_2 \cdot H_2O$, 10; $MnCl_2 \cdot 4H_2O$, 10; $(NH_4 \cdot)_2MoO_4 \cdot H_2O$, 10; and $Na_2CO_4 \cdot 10H_2O$, 10. The composition of P solution was as follows (g/*l*): sucrose 100; $K_2HPO_4 \cdot 3H_2O$, 0.5; K_2SO_4, 0.25; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 2.77, aspartic 0.02; Tris buffer (pH 7.2, 0.025 M) 100 m*l*, and microelement solution 2 m*l*. The water purified by Mega pure 3a water system (Barnstead Co.) and UF & RO water system in this study was used.

The composition of fusion agent was as follows (g/*l*): PEG 4000, 420 added into 1000 m*l* of P solution. The composition of the spore medium was as follows (g/*l*): starch, 10; wheat bran, 20; KNO_3, 1.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5, $K_2HPO_4 \cdot 3H_2O$, 0.5; $CaCO_3$, 1.0; $FeSO_4$, 0.01, aspartic acid, 0.02; $CaCO_3$, 1.0; and agar, 20. The composition of the seed medium was as follows (g/*l*): starch, 10; corn meal, 15; soybean meal, 10; tryptone 2, glucose, 10; KNO_3, 0.5, $CaCO_3$, 5.0; and $CoCl_2$, 2 ppm. All the media components were sterilized at 121°C and 1.2 atm for 20 min. The pH of the media was adjusted to 7.0 before sterilization. One looful of *Micromonosporas purpurea* was transferred to the slant medium and cultured at 34°C for three days. Then, one looful of the *Micromonosporas purpurea* slant culture was inoculated into a 500 m*l* Erlenmeyer flask containing 50 m*l* of the seed medium. It was cultured at 34°C for one day on a reciprocating shaker at 120 rpm. For the production of gentamicin, 5% of the seed was inoculated into a 500 m*l* Erlenmeyer flask containing 50 m*l* of the production medium (g/*l*) containing glucose, 50; soybean oil, 20; soybean meal, 35; tryptone, 5; yeast extract, 5; KNO_3, 0.5; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5, $K_2HPO_4 \cdot 3H_2O$, 0.5; $CaCO_3$, 1.0; $FeSO_4$, 0.01; and trace elements solution, 2m*l*. and cultured at 34°C for 5 days.

2. Gentamicin concentration

Gentamicin concentration was assayed by agar diffusion method using the filtered culture broth.

3. Protoplast fusion

Fresh spores for protoplast fusion were inoculated in a 250 m*l* flask containing 30 m*l* of mycelium medium and culture at 34°C for 72 hrs. A rotary shaker at 240 rpm was used to collect mycelia by centrifugation. The procedure to prepare the protoplasts was as follows: the mycelia was washed twice with P solution, centrifuged at 3000 rpm for 15 min, resuspended in 3 m*l* of P solution, and treated with 15 mg/1 m*l* of enzyme for 2 hrs at 37°C. The precipitated protoplasts and mycelia were resuspended in 5 m*l* of P solution, and centrifuged at 3000 rpm for 15 min. The protoplasts were inoculated with a protoplast regeneration medium at 34°C for 15 days. The procedure of protoplast fusion was follows: Each 5 m*l* of protoplast solution was mixed and fused at 30°C for 5 min after addition of 5 m*l* of fusion agent. Next, the fusion agent was washed out with P solution twice. The fusion cell was chosen by using a regeneration medium with culture for 20 days at 34°C.

4. Mutagenesis

UV Irradiation: A fresh spore suspension was

filtered through cotton, and irradiated several minutes using a 15 W UV-Lamp. A protoplast suspension mutagened uses the same methods. Co^{60} Irradiation: A fresh spore suspension was filtered through cotton, and irradiated 23×10^4 units or 12×10^4 units of Co^{60}. The treated spores or protoplasts were grown in complete darkness on a basal medium or protoplast regeneration medium at 34°C for 7 or 15 days.

III. Results and Discussion

In this study, in order to improve the gentamicin producing *Micromonosporas purpurea* (mark MP1, MP2, and MP3), protoplast fusion and protoplast mutagenesis with UV irradiation and spore mutagenesis was tried using a combination of UV irradiation. It was determined that the MP1 and MP2 strains were resistant to streptomycin and chloromycin by using a gradient screen. MP1 was resistant to 30 U/m*l* of chloromycin and streptomycin. On the other hand, MP2 was resistant to 50 U/m*l* of chloromycin and streptomycin (data not shown). When the protoplast formation at 72 hrs of the mycelia age, 5 mg/m*l* of enzyme, and 2 hrs of enzyme treatment were performed, optimum conditions for protoplast formation were obtained (data not shown). For protoplast formation and regeneration, MP1 and MP2 strain were used. The results are shown in Table 1. The protoplast concentration ranged from 6.9 or 6.8×10^6/m*l*. The regeneration ratio was 16.2 and 14.9%, respectively. This regeneration ratio was enough to complete the fusion experiment.

For the MP1 and MP2 protoplast fusion, 45% of polyethylene glycol (PEG) as promoted agent was used. The fused protoplasts were selected on the regenerated solid medium including 50 or 30 U/m*l* of streptomycin and chloromycin. The results are shown in Table 2. When the fusion mixture without dilution was used, the fused frequency was approximately 10^{-7}. On the other hand, when the fusion mixture with dilution (×10) was used, the fused frequency was approximately 10^{-5}-10^{-6}. This phenomenon was the same as in other *Streptomycetes*. It may be shown that the unfused protoplast secretes some substance, which inhibits the growth of the fused protoplast. The fused cells were inoculated into a spore medium and grown at 34°C for 7 days. Then, the fermented titer of the fused strain was determined by two-step fermentation. The results are shown in Fig. 1. In total,

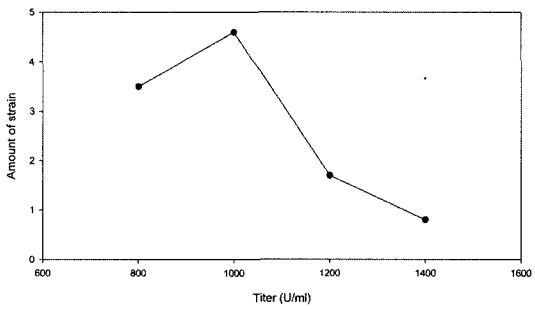

Fig. 1. Titer of distribution of fused cells.

Table 1. Protoplast formation and regeneration

Strains	Amount of protoplast formation	Amount of protoplast regeneration	Regeneration ratio
MP1	6.9×10^6	11.2	16.2
MP2	6.8×10^6	10.6	14.9

Table 2. Fusion on the fused protoplast and the fused frequency

Amount of protoplast	Amount of fused protoplast		Fused frequency	
	0	×10	0	10
1.0×10^7	1	4	5.9×10^{-7}	2.35×10^{-5}
4.0×10^7	1	2	2.5×10^{-7}	5.0×10^{-6}

Amount of the fused protoplasts and fused frequencies are calculated from the data of 0.1 m*l* of the plated fused mixture with dilution (×10) and without dilution.

about 72% of the fused strain showed a higher fermented titer than that of parent strain. This indicates that the protoplast fusion was an effective method for improving strains, which produces gentamicin.

Using the protoplast mutation method, 1 m*l* of protoplast suspension of MP1, which the concentration of protoplast arrived 10^7/m*l*, was irradiated for seven minutes with 115 W UV-Lamp, dilution were performed 10 times, and grown on the regeneration solid medium at 34°C in the complete darkness for 14 days. The amount of the regeneration strain was 100, which fermented titer was determined (data not shown). Of the regenerated strain, 10% showed a higher fermented titer than its parent. Our research successfully screened one strain (mark #194), with a fermented titer of 1854 U/m*l*, which was approximately 3.1 fold higher than that of he parent strain. This result showed that the protoplast indicated the possibility of higher sensitivity on mutagenic treatment because there were no cell walls. The frequency of positive mutation was 10%. Hence, mutagenesis protoplast will be a valuable method on mutation breeding.

In order to compare the mutagenesis of protoplasts, spore mutagenesis was tested via the two methods as follows: single mutation with $C0^{60}$ irradiation (2.3×10^5 unit) and second, a two-step mutation with a combination of $C0^{60}$ irradiation (1.2×10^5 unit) and UV irradiation (15 W, 3 min). The results are shown in Table 3. With the former mutation, mutant #112 and #141 were obtained from #194 and MP3, respectively. Regarding the latter, mutant #143 was obtained from MP3. The productivity of gentamicin of #112, 141 and 143 was 5747, 7416, and 11124 U/m*l*. These were approximately 3.1, 4.0, and 6.0 fold higher than the parent strain, respectively. These results indicate that the combined mutagenic treatment seemed to be preferable for strain breeding than single mutagenic treatment.

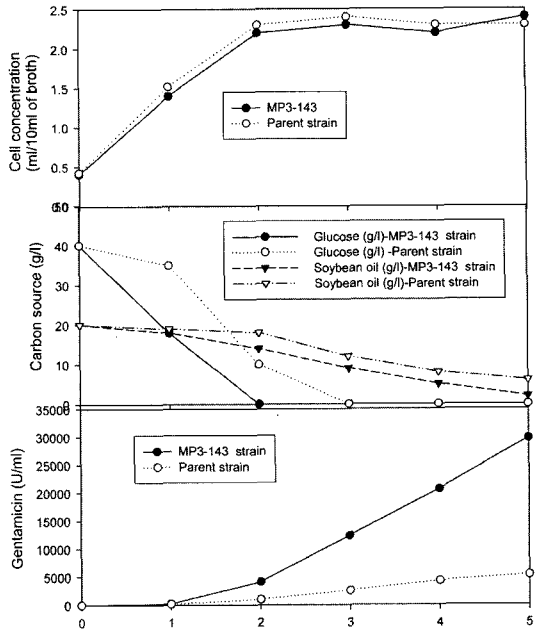

Fig. 2. Comparison of *Micromonosporas purpurea* MP3-143 and the parent strain on the growth pattern, the consumption of carbon source and gentamicin production.

In order to compare growth patterns, the consumption of glucose, soybean oil, and gentamicin production using of *Micromonosporas purpurea* MP3-143 and the parent strain, batch cultures were performed. The culture was performed at 34°C for five days at 220 rpm. The results are shown in Fig. 2. The cell was exponentially grown from the initial culture. However, cell growth increased slowly after 24 hrs of culturing. A maximum concentration of 2.3 m*l*/10 m*l* of culture broth was reached. This result was similar to that of the parent strain, *Micromonosporas purpurea* MP3-143. The pH of the culture broth ranged from 6.5 to 7.6 9 (data not shown). The apparent viscosity ranged from 120 to 150 cp (data not shown). These

Table 3. Effect of spore mutation on gentamicin production

Parent strain	Mutagenetic treatment	Mutation		Rate of increase (fold)
		Mark	Fermented titer (U/m*l*)	
194	$C0^{60}$ (2.3×10^5 units)	#112	5747	3.1
MP3	$C0^{60}$ (2.3×10^5 units)	#142	7416	4.0
MP3	$C0^{60}$ (2.3×10^5 units) UV 3 minutes	#143	11124	6.0

results were similar to that of the parent strain. The glucose using *Micromonosporas purpurea* MP3-143 after 2 days of culture was consumed perfectly. On the other hand, the parent strain, it was consumed after 3 days of culture. The consumed concentration of soybean oil using *Micromonosporas purpurea* MP3-143 after five days of culturing was 18 g/*l*, which was approximately 1.4 fold higher than the parent strain. The production of gentamicin was started after 1 day of culture while the maximum gentamicin concentration was 29756 U/m*l* after 5 days of culture. This was approximately 5.6 fold higher than the parent strain. The product yield from glucose and soybean oil was 5130 U/m*l*/g consumed carbon source, which was roughly 5.2 fold higher than the parent strain.

IV. Summary

This study was performed to develop gentamicin producing *Micromonosporas purpurea* using the protoplast mutagenesis with UV irradiation and spore mutagenesis, using a combination of UV irradiation. Using the protoplast mutation method, we successfully screened one strain (mark #194), whose fermented titer was 1854 U/m*l*. This was approximately 3.1 fold higher than the parent strain. Using a two-step mutation with a combination of $C0^{60}$ irradiation (1.2×10^5 unit) and UV irradiation (15 W, 3 min), mutant MP3-143 was obtained from MP3. The productivity of gentamicin was 11124 U/m*l*, which was approximately 6.0 fold higher than that of the parent strain, respectively. This result indicates that the combined mutagenic treatment seemed to be more preferable for strain breeding than single mutagenic treatment. In order to compare growth patterns, the consumption of glucose, soybean oil, and gentamicin production using of *Micromonosporas purpurea* MP3-143 and the parent strain, batch cultures were performed. The cell concentration, pH, and apparent viscosity levels were similar to the parent strain. The glucose, using *Micromonosporas purpurea* MP3-143, after two days of culture, was consumed perfectly. On the other hand, in the case of the parent strain, it was consumed after three days of culturing. Product yields from glucose and soybean oil were 5130 U/m*l*/g consumed carbon source, which was approximately 5.2 fold higher than the parent strain. This indicates that the mutation using a combination of UV irradiation was an effective method for improving strains, which produces gentamicin.

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