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Mutagenesis of *Streptomyces kasugaensis* for Kasugamycin Production

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Abstract

This study was performed to develop mutant strain using a combination of UV irradiation procedures with protoplast mutagenesis in order to achieve an effective kasugamycin production from *Streptomyceskasugaensis.* Whenlessthan1.0g/lofLinoleic acid was used, the cell growth was not inhibited. On the other hand, the cell growth was greatly inhibited when more than 1.6 g/l of linoleic acid was used. Among the various mutant strains, SK-12 was obtained in medium containing 1.6g/l of linoleic acid, showing the highest rate of both cell growth and kasugamycin production. In order to compare kasugamycin production with the SK-12 and the parent strain using soybean oil, cultures were performed in a flask. The production of kasugamycin was increased with the increase time. The maximum kasugamycin concentration was 1.2g/l after 6 days of culture. The product yield from soybean oil was 0.05g/l/g consumed carbon source, which was roughly 5.0 fold higher than the parent strain. These results show that it was effective method for obtaining a mutant resistant to linoleic acid for the effective production of kasugamycin from soybean oil.

Key words : : kasugamycin, *Streptomyces kasugaensis,* Linoleic acid.

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I. INTRODUCTION

Many researchers have tried to enhance antibiotic productivity through mutation techniques from *Streptomyces* group strains.[1~3] Choi *et al* isolated *Streptomyces fradiae* 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 [4] and for effective lincomycin production from *Streptomyces lincolnensis*, the protoplast mutagenesis method was also applied.[5] A combination of UV irradiation procedures using protoplast fusion and protoplast mutagenesis was systematically examined in order to achieve an effective gentamicin production from *Micromonos poraspurpurea*.[6] Choi *et al* also applied the natural nitrogen sources such as gluten meal and pharmamedia, in an air-lift bioreactor in order to effectively produce tylosin from *Streptomyces fradiae* T1555. This was achievable because the natural nitrogen sources contained a variety of aminoacids.[7] However, when the high concentration of rapeseed oil was used in culture media, tylosin production decreased because fatty acids such as oleic acid, linoleic acid, and linolenic acid from byproducts of the hydrolysis of rapeseed oil were accumulated in the culture.[8] Recently, we found that linoleic acid inhibits the growth and kasugamycin production of *Streptomyces kasugaensis*. Therefore, it is necessary to obtain a resistant strain to linoleic acid to improve kasugamycin productivity.

This study was examined for effective kasugamycin production from *Streptomyces kasugaensis*, a mutant resistant to linoleic by means of protoplast mutagenesis with UV irradiation along with spore mutagenesis. Additionally, batch cultures were performed in a flask in order to compare the growth pattern and kasugamycin production using mutant and its parent strain.

II. MATERIALS AND METHODS

1. Strain, media, and culture

The strain used in this study was Streptomyces kasugaensis SK-12. The composition of the agar medium was as follows(g/l): glucose 5, maltose 5, peptone 5, yeast extract 5 and agar 10. The composition of seed medium was as follows (g/l): soybean oil 2; maltose 10, soybean flour 2.5, peptone 5, yeast extract 10, $MgSO_4 \cdot 7H_2O$,0.5. For production of kasugamycin, the following medium was used(g/l); soybeanoil 3.0, soybean flour 5.0, glycine 5.5, K2HPO4 0.5 KH2PO4 0.2, NaCl 1.6, and a trace mineral salt solution 10 mL. A solution of trace elements contained the following ingredients (ppm): $FeCl_3$,500; $ZnCl_2$,600; $MnCl_2$,100; $CoCl_2$,300. Then, one looful of the slant culture of *Streptomyces kasugaensis* SK-12 was inoculated in to a 500ml Erlenmeyer flask containing 50ml of the seed medium and cultured at 30℃for one day on a reciprocating shaker at 120rpm. For the production of kasugamycin, 10% of the seed was inoculated into a

500ml Erlenmeyer flask containing 50ml of the production medium and cultured at 30℃.

2. Kasugamycin and Cell concentration

The kasugamycin, concentration was assayed by agar diffusion method using the filtered culture broth. The cell concentration was determined from the intracellular nucleic acid (INA) concentration using Schneider's method[9] because the natural nitrogen source contained insoluble components that do not completely dissolves in the fermentation broth, the optical density or dry cell weight can not be used.

3. Mutagenesis

Fresh spores for protoplast fusion were inoculated in a 250 ml flask containing 30 ml of mycelium medium and culture at 34℃ 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 3ml of P solution, and treated with 15 mg/ 1 ml of enzyme for 2 hrs at 37℃. The precipitated protoplasts and mycelia were resuspended in 5ml of P solution, and centrifuged at 3000 rpm for 15 min. The protoplasts were inoculated with a protoplast regeneration medium at 34℃ for 15 days. The procedure of protoplast fusion was follows: Each 5ml of protoplast solution was mixed and fused at 30℃ for 5 min after addition of 5ml of fusion agent. Next, the fusion agent. Saline water (40ml) containing spores was irradiated by ultraviolet light for 200 sec. Spore viability was 0.5% under these conditions. The irradiated spores were spread on to agar plates containing 0.1g/l of linoleic acid. Colonies that formed on the agar plates were screened through three selection steps in a liquid media. First, the colonies were inoculated into 5 ml of liquid medium containing 0.3 g/l of linoleic acid. Second, cells that grew in 0.4 g/l of linoleic acid were transferred to an agar medium containing 0.4 g/l of linoleic acid. Finally, the colonies resistant to 0.4 g/l of linoleic acid were inoculated into an agar medium containing 0.5 or 0.6 g/l of linoleic acid, respectively.

Ⅲ. RESULTS AND DISCUSSION

Some mutagenic strategies were applied to many microbial strains to produce their mutants capable of producing higher levels of secondary metabolites. For example, fungal strain *Aspergillus niger* UMIP 2564, producing citric acid, was randomly mutated by different doses of UV-Irradiation and chemical mutagens. Another fungus *Penicillium Janthinellum* NCIM 1171 was subjected to a treatment of ethyl methane sulfonate and UV-irradiation, leading to several mutants producing a higher amount of cellulose.[10] Generally, fatty acids are produced from the hydrolysis of vegetable oil by enzyme, while at the same time, mycelia

consume a portion of the fatty acids. The consumed fatty acids are then transformed into an antibiotic. Linoleic acid, which accounts for roughly 50-60% of total fatty acid content in soybean oil, enables the greatest inhibitory effect on cell growth among fatty acids. The composition of fatty acids, which accumulated in the culture broth, was similar to that of soybean oil. Therefore, there is not much specificity with the increase of the fatty acids consumption by the strains. This demonstrates that the more soybean oil is consumed, the more fatty acid accumulates in the culture broth. Therefore, the consumption of soybean oil results in the accumulation of fatty acids in the culture, while cell growth is simultaneously inhibited by the accumulated fatty acids. In order to overcome this inhibitory effect, it is necessary to have a mutant strain in which the fatty acid uptake rate is higher than those of the parent strain. In this study, antibiotic production can be improved using a mutant strain resistant to fatty acid by means of mutagenesis.

Table 1. 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^{8}	1	5	5.9×10^{-9}	2.35×10^{-8}
5.0×10^{8}	1	3	2.5×10^{-7}	5.0×10^{-9}

For the 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/ ml of streptomycin and chloromycin. The results are shown in Table 1. When the fusion mixture without dilution was used, the fused frequencies were approximately was 5.9×10^{-9} and 2.5×10^{-7}, respectively. On the other hand, when the fusion mixture with dilution(×10) was used, they were approximately 2.35×10^{-8} and 5.0×10^{-9}, respectively. This phenomenon was similar to that of other *Streptomycetes* sp. 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 30℃ for 6 days. Then, the fermented titer of the fused strain was determined by fermentation. In total, about 85% 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, 1ml of protoplast suspension, which the concentration of protoplast arrived 10^{8}/ml, wasirradiated for seven minutes with 115 WUV-Lamp, dilution were performed 10 times, and grown on the regeneration solid medium at 30℃ in the complete

darkness for 8-10 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, was approximately 4.0 fold higher than that of the 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.

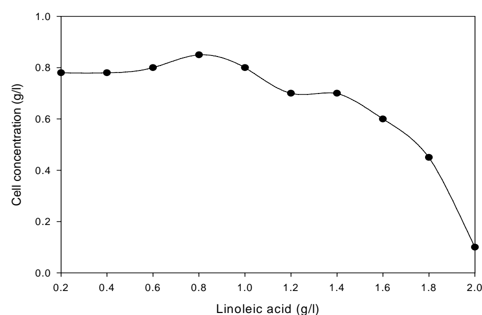

Fig. 1. Inhibitory effects of linoleic acid on growth *Streptomyces kasugaensis*.

In order to investigate the effects of fatty acids, which decompose by lipase on cell growth, linoleic acid was used in flasks for one days. Linoleic acid was added to a concentration of 0.2-2.0 g/l. The results are shown in Fig.1. When linoleic acid was added below 1.0 g/l, the cell growth was not inhibited. On the other hand, cell growth was greatly inhibited when levels were 1.6 g/l of linoleic acid. On agar plates containing 0.4, 0.8, 1.0, and 1.2 g/l of linoleic acid, no colonies formed on plates containing linoleic acid at concentration levels of 1.0g/l. Therefore, 1.2 g/l of linoleic acid was established as the selective minimal inhibitory concentrations in liquid and solid media.

Table 2. Growth, soybean oil consumption and kasugamycin production of mutant strains in flask cultures.

Strains	Consumed oil (g/l)	Cell concentration (g/l)	kasugamycin concentrati on (g/l)
SK-12	20.4	0.75	0.72
SK-27	18.1	0.70	0.61
SK-32	17.1	0.70	0.69
SK-39	19.9	0.68	0.48
SK-56	16.4	0.81	0.59

Serial selections were performed to acquire mutant strains on an agar solid medium containing 1.2 g/l of linoleic acid. Since only a few mutant cells were on 1.2 g/l of linoleic acid, a second selection was performed using a liquid medium containing 1.2 g/l of linoleic. The colonies, which grew on 1.2 g/l of linoleic medium, were transferred to liquid media containing 0.8, 1.0, 1.2, and 1.4 g/l of linoleic acid. Through such a series of experiments, the following strains were acquired: SK-12 containing 1.4 g/l of linoleic acid, SK-27 contains 1.2 g/l of linoleic acid, SK-32 and SK-39 in 1.0 g/l of linoleic acid, and SK-56 in 0.8 g/l of linoleic acid. Flask cultures with liquid medium containing 30 g/l of soybean oil as the sole carbon source were performed to evaluate the five mutant strains for 4days. The results are shown in Table 2. Based on soybean oil consumption and

kasugamycin production, SK-12 showed the highest cell growth and kasugamycin production rates among the five mutant strains.

Table 3. Comparison of SK-12 and the parent strain on the growth pattern, the consumption of oil and kasugamycin production.

	pH	Cell (g/l)	Consumed oil (g/l)	Kasugamycin (g/l)	Viscosity (cP)
SK-12	7-8	0.79	23.2	1.2	123-150
Parent strain	7-8	0.82	20.4	0.3	110-120

In order to compare growth patterns, the consumption of soybean oil, and kasugamycin production in the culture of *Streptomyces kasugaensis* SK-12and the parent strain, batch cultures were performed. The culture was performed at 30 ^{O}C for 6 days at 220 rpm. The results are shown in Table 3. The cell was exponentially grown from the initial culture. However, cell growth increased slowly after 24 hrs of culturing. A maximum concentration of 0.79 was reached. This result was similar to that of the parent strain, *Streptomyces kasugaensis* SK-12. The pH of the culture broth ranged from 7.0 to 8.0. The apparent viscosity ranged from 123 to 150 cP. These results were similar to that of the parent strain.
The consumed concentration of soybean oil using *Streptomyces kasugaensis* SK-12 after 6days of culturing was 23.2g/l, which was similar to that of the parent strain. The production of kasugamycin was started after 1 day of culture while the maximum kasugamycin concentration was 1.2g/l after 6days of culture. This was approximately 4.0 fold higher than the parent strain. The product yield from soybean oil was 0.052g/l/g consumed carbon source, which was roughly 5.0 fold higher than the parent strain.

Ⅳ. CONCLUSIONS

It was very sensitive to fatty acid when soybean oil as the carbon source was used for Kasugamycin production from *Streptomyces kasugaensis*. Mutant strain SK-12 resistant to linoleic acid was obtained by screening in solid and liquid media containing linoleic acid. The uptake rate of linoleic acid by SK-12 was approximately 2.0 fold higher than the parent strain. For comparing the SK-12 and the parent strain, batch cultures were carried out in a flask. The production of kasugamycin was started after 1 day of culture while the maximum kasugamycin concentration was 1.2g/l after 6 days of culture. This was approximately 4.0 fold higher than the parent strain. From these results, it

was effective method for obtaining a mutant resistant to linoleic acid for the effective production of kasugamycin from soybean oil. The ultraviolet induced mutation method is a simple means with which to screen mutant strains resistant to linoleic acid. Furthermore, we are attempting to obtain mutant strains resistant to high concentrations of linoleic acid, and increase kasugamycin production by improving the soybean oil consumption rate.

REFERENCES

1. Berdy, J and Jarl, M.: Micromonospora produced aminoglyoside antibiotics. *Process Biochem.*, 21: 103-106, 1986.
2. Zheng, Y. Gentamicin production from *Micromonosporas echinospora. Chinese J. Biotech.*, 3: 115-120, 1982.
3. Ryn, D.D.: Antibiotic production by Streptomyces group strains. *Appl. Environmental Microbiol.,* 45: 1854-1860 , 1983.
4. Choi, D. B., Tamura, S., Park, Y. S., Okabe, M., Seiue, Y., and Takeda, S.: Efficient tylosin production from *Streptomyces fradiae* using rapeseed oil. *J. Ferment. Bioeng.*, 82: 183-186, 1996.
5. Choi, D. B. and Cho, K. A.: Effect of carbon source consumption rate on lincomycin production from *Streptomyces lincolnensis. J. Microbiol. Biotechnol.* ,14: 532-541, 2004.
6. Eiki, H., Gushima, H., Saito, T., Ishida, H., Oka, Y. and Oono. T.: Product inhibition and its removal on josamycin fermentation by *Streptomyces narbronesis var josamyceticus. J. Ferment. Technol.* ,66: 559-565, 1988.
7. Pan, C. H., Speth, S. V., Mckillip, E. and Nash, C. H.: Methyloleate base medium for cepalosporin C production. *Dev. Ind. Microbiol.*, 23: 315-323, 1982.
8. Choi, D. B., Park, Y. S. and Okabe, M.: Improvement of tylosin production from *Streptomyces fradiae* culture by decreasing the apparent viscosity in an air-lift bioreactor. *J. Ferment. Bioeng.* ,86: 413-417, 1998.
9. Scheider, W.: Phosphorous compounds in animal tissues. I. Extraction and estimation of deoxpentose nucleic acid. *J. Biol. Chem.*, 161: 293-295, 1945.
10. Kim, S. J., Lee, H. K., Lee, Y. K., and Yim, J. H.: Mutant selection of Hahella chejuensis KCTC 2396 and statistical optimization of medium components for prodigiosin yield up*, J. Microl.,* 46: 183-188, 2008.