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Tylosin Production by Mutant Resistant to Oleic Acid

DuBok Choi · On You Choi* · Ok-Ran Moon · Mi-Ran Yoon** · Sung-Nam Ji** · Dae-Yewn Shin**†**

Biotechnology Laboratory, B-K Company Ltd., 228-1 Soryong-dong, Gusan-si, Jeonlabuk-do 573-879, South Korea
**United Graduate School of Agricultural Science, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan*
***Department of Environmental Engineering, Chosun University, Gwang-ju 501-759, South Korea*
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Oleic Acid 내성균주로부터 Tylosin 생산

최두복 · On You Choi* · 문옥란 · 윤미란** · 지성남** · 신대윤**†**

B-K Company 생명공학 연구실, *United Graduate School of Agricultural Science Shizuoka University,
**조선대학교 환경공학과

요 약

*Streptomyces fradiae*로부터 Tylosin을 효율적으로 생산하기 위해 지방산 내성균주을 분리 했다. 여러 지방산중에서 oleic acid 1.6 g/*l* 이상이 첨가될 때 세포 성장이 완전히 저해 되었다. 그러나 oleic acid 1.2 g/*l*에서 얻어진 TM-224-1 균주는 최대 균체농도와 tylosin 생산이 얻어졌다. 또한 oleic acid 소비속도는 parent strain 비해 3.8배 증가했다. Oleic acid 내성균주, TM-224-1 균주와 parent strain을 이용해서 jar fermentor에서 균체농도, tylosin 생산, 그리고 rapeseed oil 소비를 표준조건하에 5일동안 비교하였다. TM-224-1 균주을 이용할 경우 균체 농도는 초기에 parent strain비해 증가했으나 배양중반부터는 감소하기 시작했다. rapeseed oil 소비의 경우는 거의 비슷했다. 그러나 Tylosin 생산 수율은 parent strain 비해 약 3.2배 증가했다.

주요어 : 타이로신, 올레인산 내성균, 유체유

I. Introduction

Microbial reactions in the antibiotic synthesis are enhanced or inhibited by various reaction mechanisms.[1] Tylosin, a macrolide antibiotic, produced by *Streptomyces fradiae*[2], *S. rimosus*[3], and *S. hygoscopicus*[4], is used by pharmaceuticals for the treatment of gram-positive bacterial infections and mycoplasma[2]. The biosynthesis of tylosin and the regulation of its biosynthesis have been of interest in the biochemistry field for more than a decade.[5,6] Choi *et al.* have investigated various vegetable and animal oils as the sole carbon source for efficient tylosin production in the culture of *Streptomyces fradiae* T1555. There was a 1.6 or 7.0 fold increase in tylosin production when rapeseed oil was used, compared with using starch and glucose in the same concentration levels.[7] This enabled the identification of rapeseed oil effects on tylosin productivity, including methylmalonyl-CoA carboxyltransferase activity in the biosynthesis of the tylosin precursor protylonolide in batch culture in *Streptomyces fradiae* T1555.[8] 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 amino acids.[9] 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.[10] Among these fatty acids, oleic acid inhibits the growth of *Streptomyces fradiae*

†Corresponding author : Department of Environmental Engineering, Chosun University
Tel : 82-62-230-7153, Fax : 82-62-230-7216
E-mail : dysin@chosun.ac.kr

T1555. Therefore, it is necessary to obtain a resistant strain to oleic acid to improve tylosin productivity using rapeseed oil as the sole carbon source.

This study was designed to improve tylosin production using a mutant strain resistant to oleic acid by means of an ultraviolet-induced mutation. Other objectives included comparing growth patterns, the consumption of rapeseed oil, and tylosin production using mutant and the parent strains.

II. Material and Methods

1. Strain, media, and culture

The strain used in this study was *Streptomyces fradiae* TP-1239. The composition of the agar medium was as follows (g/*l*): starch, 10; yeast extract, 5; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.5; and agar 10. The composition of seed medium was as follows (g/*l*): rapeseed oil 5; starch, 5: yeast extract, 5; soybean meal, 5; $NaNO_3$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5. For production of tylosin, the following medium was used (g/*l*); rapeseed oil, 50; pharmamedia, 5; soybean meal, 5; KOH, 0.5; K_2HPO_4, 0.25; $MgSO_4 \cdot 7H_2O$, 0.5; a solution of trace elements, 3 m*l*. A solution of trace elements contained the following ingredients (ppm): $FeCl_3$, 500; $ZnCl_2$, 600; $MnCl_2$, 100; $CoCl_2$, 300. 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 *Streptomyces fradiae* TP-239 was transferred to the slant medium and cultured at 30°C for five days. Then, one looful of the slant culture of *Streptomyces fradiae* TP-1239 was inoculated into a 500 m*l* Erlenmeyer flask containing 50 m*l* of the seed medium and cultured at 30°C for one day on a reciprocating shaker at 120 rpm. For the production of tylosin, 10% of the seed was inoculated into a 500 m*l* Erlenmeyer flask containing 50 m*l* of the production medium and cultured at 30°C.

2. Cell growth

The cell concentration was measured using the packed cell volume (PCV) method, because natural nitrogen sources such as soybean meal and pharmamedia contain insoluble components that do not dissolve completely in the fermentation broth. Hence, it is not useful to use the optical density or dry cell weight.

3. Oil concentration

As described by Kim *et al.*, and Choi *et al.*, the rapeseed oil concentration was measured by a solvent extraction method. Three milliliters of culture broth were mixed with 6 m*l* of n-hexane and the mixture was vigorously shaken for 2 min in a capped Erlenmeyer flask and then centrifuged at 3,000 rpm for 15 min. The upper hexane layer was removed and dried at 80°C for 3 hr, and the residue was weighed to determine the extracted oil weight.[11,12]

4. Tylosin and fatty acid concentration

Tylosin and fatty acid concentration was measured by using a HPLC and GC, respectively. Fatty acids were measured as follows: 3 m*l* of culture broth were mixed with 6 m*l* of n-hexane and vigorously shaken for 3 min in a capped tube. After 2 m*l* of the hexane layer was dried in a vacuum desiccator, 3 m*l* of BF_3-MeOH were added before the mixture was boiled in a water bath for 3 min. After cooling, 3 m*l* of water and 2 m*l* hexane were added to the mixture before it was vigorously shaken for 3 min in a capped tube. A 2 m*l* of sample was taken and fatty acids were analyzed by gas chromatography. The conditions used to measure the tylosin concentration were as follows. Hitachi 163 GC equipped with a flame ionization detector: column, glass column (3 mm × 2 m); packed with 10% PEG-20M on 60/80 uniport HP; column temperature, 210°C; injection temperature, 240°C. Gas pressures of nitrogen, hydrogen and air were 1.0, 1.0, and 1.2 kg/cm^2, respectively.

5. Ultraviolet-induced mutation

Saline water (40 m*l*) containing pregerminated 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 0.1 g/*l* of oleic 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 m*l* of liquid medium containing 0.3 g/*l* of oleic acid. Second, cells that grew in 0.4 g/*l* of oleic acid were transferred to an agar medium containing 0.4 g/*l* of oleic acid. Finally,

the colonies resistant to 0.4 g/*l* of oleic acid were inoculated into an agar medium containing 0.5 or 0.6 g/*l* of oleic acid, respectively.

III. Results and Discussion

The toxicity of unsaturated fatty acids in gram-positive cells has been reported elsewhere. The toxicities resulting from structural changes in the cell membrane, inhibit amino acid uptake and spore germination, decreasing activity of membrane-bound enzymes.[11] Fatty acids are produced from the hydrolysis of rapeseed 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. Oleic acid, which accounts for roughly 62% of total fatty acid content in rapeseed 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 rapeseed oil (62% oleic acid, 20% linoleic acid, and 10% linolenic acid). Therefore, there are not much specificities Ambiguous-clarify what you mean by "specificity" with the increase of the fatty acids consumption by the strains. This demonstrates that the more rapeseed oil is consumed, the more oleic acid accumulates in the culture broth. Therefore, the consumption of rapeseed 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, tylosin production can be improved using a mutant strain resistant to oleic acid, by means of an ultraviolet-induced mutation. In order to investigate the effects of fatty acids, which decompose by lipase on cell growth, oleic acid, linoleic acid, linolenic acid, palmitic acid, and stearic acid were used in flasks for two days. Each fatty acid was added to a concentration of 0.2-1.6 g/*l*. The results are shown in Fig. 1. When concentration levels were below 0.4 g/*l*, oleic acid was added and cell growth was not inhibited. On the other hand, cell growth was greatly inhibited when levels were 1.6 g/*l* of oleic acid. On agar

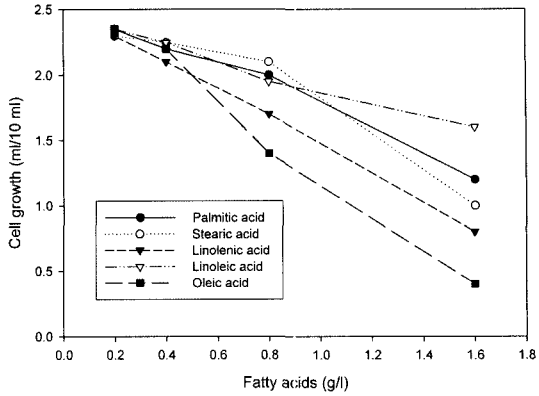

Fig. 1. Inhibitory effects of fatty acids on growth *Streptomyces fradiae* TP-1239.

plates containing 0.04, 0.08, 0.1, and 0.12 g of oleic acid, no colonies formed on plates containing oleic acid at concentration levels of 0.12 g/*l*. Therefore, 1.6 g/*l* and 0.12 g/*l* of oleic acid were respectively established as the selective minimal inhibitory concentrations in liquid and solid media.

Serial selections were performed to acquire mutant strains on an agar solid medium containing 0.12 g/*l* of oleic acid. Since only a few mutant cells were on 0.8 g/*l* of oleic acid, a second selection was performed using a liquid medium containing 0.6 g/*l* of oleic acid. The colonies, which grew on 0.6 g/*l* of oleic acid medium, were transferred to liquid media containing 0.8, 1.0, 1.2, and 1.4 g/*l* of oleic acid. Through such a series of experiments, the following strains were acquired: TM-224-0 containing 1.4 g/*l* of oleic acid, TM-224-1 containing 1.2 g/*l* of oleic acid, TM-224-3 and TM-224-2 in 1.0 g/*l* of oleic acid, and T-224-4 in 0.8 g/*l* of oleic acid. Flask cultures with liquid medium containing 60 g/*l* of rapeseed oil as the sole carbon

Table 1. Growth, rapeseed oil consumption and tylosin production of mutant strains in flask cultures

Strains	Consumed oil (g/*l*)	Cell growth (ml/10 m*l*)	Tylosin concentration (g/*l*)
TM-224-0	42.9	15.7	3.1
TM-224-1	48.3	25.5	6.5
TM-224-2	36.5	12.5	2.4
TM-224-3	35.1	21.8	4.5
TM-224-4	40.5	22.5	5.5

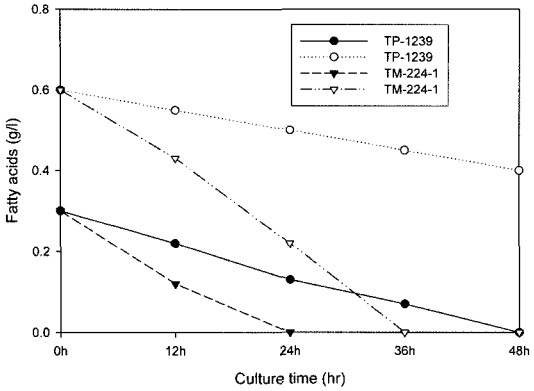

Fig. 2. Comparison of oleic acid uptake by parent strain and TM-224-1.

source, were performed to evaluate the five mutant strains. The results are shown in Table 1. Among the five mutant strains only the TM-224-1 strain consumed 48.3 g/*l* of rapeseed oil after five days of culturing. Although TM-224-0 resisted oleic acid the most, its consumption of rapeseed oil and tylosin production were the lowest among the five mutant strains. This indicates that the pathways for oil consumption in TM-224-0, TM-224-2, TM-224-3, and TM-224-4 may be blocked or damaged. Based on rapeseed oil consumption and tylosin production, TM-224-1 showed the highest cell growth and tylosin production rates among the five mutant strains.

The most important difference between the parent and TM-224-1 strains is the uptake of oleic acid. Since oleic acid is the inhibitory fatty acid in the TP-1239 culture, the effects of oleic acid uptake were investigated, which ranged from 0.3 g/*l* to 0.6 g/*l* on cell growth. The results are shown in Fig. 2. When 0.3 g/*l* of oleic acid was added, its concentration in the culture of TM-224-1 decreased to zero after 24 hrs of culturing, while after 48 hrs in the parent strain. When 0.6 g/*l* of oleic acid was added, the parent strain did not grow any further and the oleic acid concentration unchanged. On other hand, in the case of TM-224-1, it was decreased to zero after 36 hrs of culturing. The uptake rate of oleic acid for TM-224-1 was approximately 3.8 fold higher than the parent strain.

A comparison of tylosin production, cell growth, and oil consumption with an initial rapeseed oil

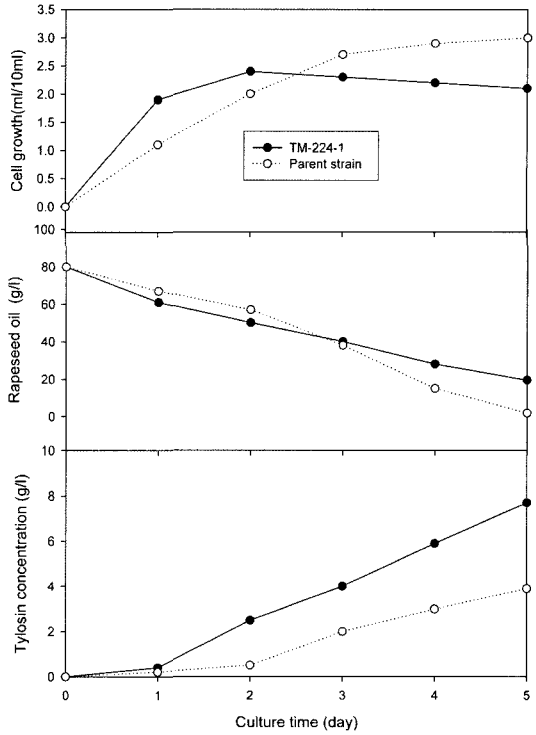

Fig. 3. A comparison of tylosin production, cell growth, and rapeseed oil consumption in the TM-224-1 and the parent strain.

concentration of 80 g/*l* in the TM-224-1 and the parent strain is presented in Fig. 3. The cultures were performed in a jar fermentor for five days. Cell growth of TM-224-1 strain was higher than the parent strain after two days of culturing. However, after four days of culturing, it was similar to that of the parent strain. The amount of rapeseed oil consumed by TM-224-1 and the parent strain were 60.5 and 78.2 g/*l*, respectively. The maximum tylosin production in the TM-224-1 was 7.7 g/*l* after five days of culturing, which was approximately 2.0 fold higher than the parent strain. The production yield of tylosin was 0.127 and 0.04 g/g consumed oil in the TM-224-1 and the parent strain, respectively. From these results, it was effective method for obtaining a mutant resistant to oleic acid for the effective production of tylosin from rapeseed oil. The ultraviolet induced mutation method is a simple means with which to screen mutant strains resistant to oleic acid. Furthermore, we are attempting to obtain mutant strains resistant

to high concentrations of oleic acid, and increase tylosin production by improving the rapeseed oil consumption rate.

IV. Summary

When rapeseed oil as the carbon source was used for tylosin production from *Streptomyces fradiae* TP-1239 was very sensitive to oleic acid. Cell growth was restrained by adding 0.8 g/*l* of oleic acid to the culture broth. Mutant strain TM-224-1 resistant to 1.2 g/*l* of oleic acid was obtained by screening in solid and liquid media containing oleic acid. The uptake rate of oleic acid by TM-224-1 was approximately 3.8 fold higher than the parent strain.

For comparing the TM-224-1 and the parent strain, batch cultures were carried out in a jar fermentor. Cell growth of TM-224-1 strain was higher than the parent strain after two days of culturing. However, after four days of culturing, it was similar to that of the parent strain. The amount of rapeseed oil consumed by TM-224-1 and the parent strain were 60.5 and 78.2 g/*l*, respectively. The production and yield of tylosin was approximately 2.0 and 3.2 fold higher than the parent strain, respectively. From these results, it was concluded that this mutant, which was resistant to oleic acid, has improved tylosin production.

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