Higher-level Production of Ascomycin (FK520) by *Streptomyces hygroscopicus* var. *Ascomyceticus* Irradiated by Femtosecond Laser

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**Abstract** Femtosecond laser irradiation technology was employed for the first time to improve the ascomycin (FK520) yield of *Streptomyces hygroscopicus* var. *ascomyceticus* NT2-11, which is an N-methyl-N-nitro-N-nitrosoguanidine (NTG)-induced strain derived from *S. hygroscopicus* (ATCC14891). The mutant FS35 with high and stable FK520 production capacity was then obtained in the optimal irradiation conditions (25 mW for 6 min) by the Titanium sapphire laser system (810 nm, 76 MHz, 150 fs). The FK520 production capacity of FS35 was 45% higher than that of the parental strain NT2-11. Moreover, under the optimal fermentation conditions, FK520 fermentation titer of FS35 reached 300 mg/L and the intrinsic kinetics of FS35 and NT2-11 were investigated comparatively in 3 phases. The mathematical models provided a good description of FK520 fermentation process for both strains and valuable information for optimizing operation and pilot-plant enlargement research. The comparative studies on parameters of the models confirmed the advantages in production and the decrease of substrate inhibition through femtosecond laser irradiation. Therefore, femtosecond laser irradiation provides a promising way to enhance the production of FK520 in *S. hygroscopicus*.

**Keywords:** FK520, *Streptomyces hygroscopicus* var. *ascomyceticus*, femtosecond laser irradiation, fermentation, intrinsic kinetics

1. **Introduction**

Ascomycin (FK520) is a macrolide compound produced by *Streptomyces hygroscopicus* No.KK317 and *S. hygroscopicus* subsp. *yakushimaensis* No.7238. FK520 was first isolated as a kind of antifungal drug [1], and later was identified the immunosuppressive activity with lower toxicity than FK506 [2]. FK520 and FK506 have similar pharmacological effects and both of them can be used to treat autoimmune diseases, skin diseases and prevent the organ transplant rejection [3]. In addition, a series studies showed that FK520 and its derivatives exhibit the activity of anti-malarial [4], nerve protection, and nerve regeneration [5].

Due to the important medical value and market prospect, it is of great significance to improve fermentation titer of FK520 by strain evolution and fermentation optimization. The latter mainly included improving the medium components and optimizing process parameters [6]. For example, the titer of 13-desmethoxy-13-methyl-FK520 was tripled by selecting an excellent fermentation medium and decreasing the dissolved oxygen (DO) [7]. However, for substantially improving the target product yield, the strain evolution is the most critical factor. Molecular breeding and mutation are two mainly effective methods [8]. To date, the biosynthesis of FK520 has been extensively researched and the biosynthetic gene cluster has been cloned and characterized [9]. However, many elements of FK520 biosynthetic machinery remain obscure and the key steps and key enzymes cannot be located accurately in the FK520 biosynthetic pathway. The genetic engineering
required to raise the FK520 titer has not been achieved yet. Meanwhile, there have been relatively few studies examining FK520 production, and the information is limited. Therefore, a powerful and effective method of elevating the production level of FK520 is important.

Compared with conventional UV and NTG mutagenesis, laser irradiation is safer and causes less radiation damage. Furthermore, femtosecond laser possesses ultrahigh temporal and spatial resolutions, showing numerous advantages over continuous-wave or long-pulse lasers [10]. In particular, high instantaneous power (10^{12} W) with high-energy photons can be absorbed by microbial cells or intracellular molecules in a nonlinear manner, which produces highly excited plasma. The reactive free radicals that are simultaneously generated, are easily reactive with cellular proteins and DNA, affecting the biological metabolism and enhancing the effect of the variation. Gong [11] induced Phaffia rhodozyma cell fusion by a femtosecond laser and improved its efficiency by 80%. Manevitch [12] found Trichophyton rubrum can be successfully killed when exposing onychomycosis to a femtosecond laser. However, there has been no report on femtosecond laser irradiation technology to improve FK520 production.

Kinetic modeling is regarded as an indispensable step in developing a fermentation process, since the models can be used to determine an optimal operation condition for the production of a target metabolite [13,14]. In addition, the quantitative understanding of production kinetics is necessary for rational design and optimization of the reactor. Therefore, accurate mathematical models for intrinsic kinetics are valuable towards improving the production of FK520. However, few studies on the intrinsic kinetics of FK520 production have been performed to date.

In this work, the objectives included: To determine the optimal femtosecond laser irradiation conditions for S. hygroscopicus NT2-11 and to obtain a positive mutant with stable heredity and high-yield of FK520; to develop the intrinsic kinetic models of cell growth, total sugar consumption and FK520 production in 3 phases; and to compare quantitatively the performance of FK520 production of the mutant and parental strain.

2. Materials and Methods

2.1. Bacterial strains

The original strain, S. hygroscopicus NT2-11, was an (NTG)-induced strain derived from S. hygroscopicus (ATCC 14891). The spores from this strain were suspended in glycerol (20%, by volume) and stored at -70°C in our laboratory.

2.2. Culture media and cultivation

The solid plate medium (agar slant) contained 20 g/L soybean meal, 20 g/l mannitol, and 20 g/L agar. The seed medium consisted of 10 g/L soluble starch, 30 g/L glucose, 6 g/L peptone, 6 g/L yeast powder, and 2 g/L CaCO\(_3\). Initial fermentation medium contained 20 g/L soluble starch, 40 g/L dextrin, 10 g/L peptone, 10 g/L yeast powder, 10 g/L corn steep powder, 5 g/L soybean extract, 1 g/L K\(_2\)HPO\(_4\), 3H\(_2\)O, 1 g/L (NH\(_4\))\(_2\)SO\(_4\), 1 g/L MgSO\(_4\)·7H\(_2\)O, and 1 g/L CaCO\(_3\). The optimal fermentation medium consisted of 24 g/L soluble starch, 40 g/L dextrin, 5 g/L peptone, 7 g/L yeast powder, 2 g/L corn steep powder, 1.5 g/L shikimic acid, 2.5 mL soybean oil, 1 g/L K\(_2\)HPO\(_4\)·3H\(_2\)O, 1.5 g/L (NH\(_4\))\(_2\)SO\(_4\), 1 g/L MgSO\(_4\)·7H\(_2\)O, and 1 g/L CaCO\(_3\). The pH level was maintained at 7.2 above by adding NaOH or HCl prior to sterilization.

The S. hygroscopicus NT2-11 was cultivated on the solid plate medium at 28°C and 60 ~ 80% relative humidity for 6 ~ 8 days to isolate single colony. The isolated pure strain was then grown on the agar slant medium for 10 days to harvest spores and 10^6/mL single spore suspension was made with sterile saline. A 0.2 mL of the spore suspension was inoculated into a 250 mL Erlenmeyer flask containing 50 mL seed medium and incubated at 28°C on a rotary shaker (200 rpm) for 48 h. A 20 mL aliquot of the culture was transferred into 220 mL initial (optimal) fermentation medium in a 1 L baffled flask and incubated at 28°C, 200 rpm (220 rpm) for 7 days (6 days). The resulting fermentation broth was processed and subjected to metabolite analysis of titer determination.

2.3. Optimization of the femtosecond laser irradiation conditions

Femtosecond generating laser supplemented with a Ti:sapphire laser excitation source (Verdi-V10+Mira900, Coherent Inc, America) was used in this work. The excitation laser was fixed at a wavelength of \(\lambda=800\) nm, repetition rate of 76 MHz, pulse width of FWH \(\leq150\) fs, and beam diameter of 4 mm. The 10^8 /mL single spore suspension was made and the temperature was controlled at 25±1°C. In order to avoid the influence of photo-repair, the femtosecond laser irradiation was carried out in darkroom. In addition, under the irradiation of 30 mW for 10 min, the increase in temperature was less than 0.4°C. In this work, the average heating volume change caused by temperature was neglected by strain mutation [15].

The effect of exposure time and output power of femtosecond laser irradiation on the mortality rate and positive mutation rate were investigated by varying the exposure time (1 ~ 10 min) and the output power (10 ~ 30 mW). The experimental protocol for the mutagenesis