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ORIGINAL PAPER

Strain development of *Streptomyces* sp. for tacrolimus production using sequential adaptation

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Abstract An immunosuppressant tacrolimus-producing strain of *Streptomyces* sp. TST8 was isolated and developed by the TS corporation in Korea using the sequential adaptation of media containing tacrolimus (600–1600 mg/l). The aim of the tacrolimus sequential adaptation protocol was to select those cells with tacrolimus resistance and to reduce product inhibition of the tacrolimus-producing strain. The developed strains produced more tacrolimus than the original strain. In particular, the TST10 strain adapted in the medium containing 900 mg/l of tacrolimus produced 972 mg/l of tacrolimus in the final titer after 7 days of cultivation in a 5-l jar fermenter. This is the largest final titer of tacrolimus produced by a specific strain to date. Because the sequential adaptation protocol is limited by the solubility of tacrolimus in water, the final tacrolimus titer of TST11 adapted in the medium containing 1600 mg/l of tacrolimus was lower than that of TST10. The developed strains and the development method using sequential adaptation can facilitate the efficient and economical production of tacrolimus.

Keywords Fermentation · Product inhibition · Sequential adaptation · Strain development · *S. tsukubaensis* · Tacrolimus

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Introduction

Tacrolimus (also FK-506 or Fujimycin) is an immunosuppressant that was discovered and reported by Kino et al. [1, 2] under the auspices of the Fujisawa Pharmaceutical Co. in 1987, which was merged to form Astellas Pharma in 2004. The annual sales of Prograf, the commercial product name of tacrolimus, in 2007 was US$ 2030 million, with Prograf ranked as the 52nd world's best-selling medicine [3].

In the initial studies, Fujisawa scientists isolated a tacrolimus-producing strain of *Streptomyces tsukubaensis*, no. 9993, and established fermentation and purification processes [1]. The final tacrolimus titer of *S. tsukubaensis* no. 9993 in the early days of the investigation was about 10 mg/l [1]. Many scientists have subsequently attempted to develop novel strains and fermentation processes for a more efficient and economical production of tacrolimus.

Kim and Park [4] isolated a novel tacrolimus-producing microorganism, *Streptomyces clavuligerus* CKD1119, from soil samples in Korea whose final tacrolimus titer was 58 mg/l in a 7-l jar fermenter after 8 days of cultivation. The same authors also optimized the carbohydrates and oils in the tacrolimus production medium and produced a final titer of 495 mg/l tacrolimus after 8 days of cultivation in a 200-l pilot fermenter [5]. Kumar et al. [6] improved the fermentation process for the production of tacrolimus under submerged aerobic conditions using fed-batch processing at a high aeration rate and *Streptomyces* sp. ATCC no. 55098, producing a final titer of 300–310 mg/l tacrolimus. Vaid and Narula [7] used vegetable oil as the sole carbon source for producing tacrolimus and produced a final titer of 80–330 mg/l tacrolimus after 6–8 days of cultivation. Other researchers have attempted to find a biosynthetic mechanism of antibiotic production in *Streptomyces* sp., but the results have not

yet been applied for increasing the tacrolimus productivity of *Streptomyces* sp. [8–11].

We optimized the production medium based on the alterations to the medium suggested by previous nutrient studies [12, 13] and developed a wild strain by selection, UV mutation, and tacrolimus resistance selection on agar plates containing 600 mg/l tacrolimus. This protocol for tacrolimus resistance selection was effective in increasing tacrolimus production and resulted in about a 100% increase in the final titer: from 242 to 503 mg/l tacrolimus after 7 days of fermentation in a 5-l jar fermenter. However, optimization of the production medium and fermentation conditions, selection, and UV mutation were not as effective in further increasing tacrolimus production. From these results, product inhibition was thought to be the major barrier to any further increase in tacrolimus production.

Through tacrolimus resistance selection, we isolated *Streptomyces* sp. TST8, which produced a final titer of about 500 mg/l tacrolimus after 7 days of fermentation. We then attempted to adapt the TST8 strain to medium containing a high concentration of tacrolimus by sequential adaptation with the aim of increasing tacrolimus production. Here, we report on the development of strain *Streptomyces* sp. TST8 using sequential adaptation and the fermentation results of the developed strains.

Materials and methods

Microorganism

The tacrolimus-producing strain, *Streptomyces* sp. TST8, isolated and developed at TS Corporation in Korea, was used in this study. Selection, UV mutation, and tacrolimus resistance selection on agar plates were used to develop a wild strain in a previous study.

Sequential adaptation

Streptomyces sp. TST8 was adapted through subculture in a medium containing tacrolimus. The frozen working stock of *Streptomyces* sp. TST8 was thawed and inoculated into a 500-ml flask containing medium initially supplemented with a tacrolimus concentration of 600 mg/l; this was eventually increased to a final concentration of 1600 mg/l. The tacrolimus concentration was increased by 100 mg/l at each subculture and was added to the subculture medium as a 100 g/l ethanol solution prior to bacterial inoculation. The inoculation volume at each subculture was determined by the cell density, measured at an optical density (OD) of 600 nm. After each subculture, the broth

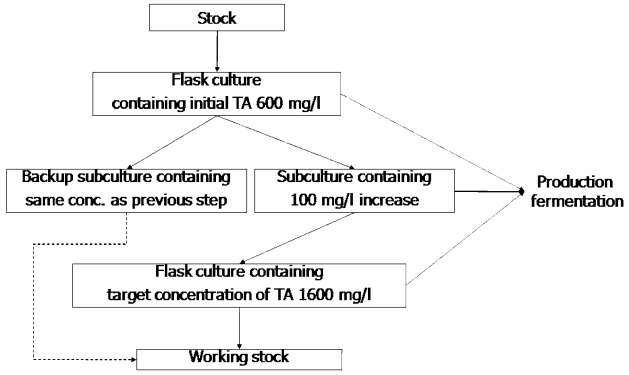

Fig. 1 Strategy of tacrolimus (*TA*) sequential adaptation

was inoculated into a 500-ml flask containing the production medium, for tacrolimus production culture. Working stocks were made from subcultures containing a tacrolimus concentration of 600, 900, and 1600 mg/l and labeled TST9, TST10, and TST11, respectively. The strategy of sequential adaptation is shown in Fig. 1. The subculture flask contained 50 ml medium and was incubated at 27°C on a 2-inch pitched reciprocal shaker at 240 rpm. The culture time was varied according to cell growth. The subculture medium (with the exception of the supplemented tacrolimus) consisted of 10 g/l soluble starch, 10 g/l bactopeptone, 7 g/l glycerol, 3 g/l yeast extract, and 3 g/l malto extract and was adjusted to pH 7.0 before sterilization. The tacrolimus production culture flask contained 30 ml medium and was incubated at 27°C for 7 days on a 2-inch pitched reciprocal shaker at 240 rpm. The production medium consisted of 70 g/l oxidized starch, 17 g/l inactive dry yeast, 5 g/l soybean meal, 1 g/l $(NH_4)_2SO_4$, 1 g/l $CaCO_3$, and 1 g/l surfactant GE304 and was adjusted to pH 6.2 before sterilization.

Flask culture

The flask culture consisted of a seed culture and a production culture. Working stocks of TST9, TST10, and TST11 were each thawed and inoculated into a 500-ml flask containing 30 ml seed medium, which was then incubated at 27°C for 1 day on a 2-inch pitched reciprocal shaker at 240 rpm. The seed medium consisted of 10 g/l oxidized starch, 10 g/l glycerol, 10 g/l soybean meal, 5 ml/l 45% corn steep liquor, 2 g/l $CaCO_3$, and 0.5 g/l surfactant GE304 and was adjusted to pH 5.8 before sterilization. The seed broth was inoculated in a 500-ml flask containing the production medium. The inoculum size was 3 ml, and the other conditions of the production culture were the same as those described above for tacrolimus production culture in a flask.

Five-liter jar fermentation

The jar fermentation consisted of a first seed culture, a second seed culture, and a production culture. The first seed culture was the same as the seed culture of the flask culture. A 20-ml sample of the first seed broth was inoculated into a 5-l fermenter containing 4 l of the second seed medium, with growth conditions set at 27°C, with 500 rpm agitation and 0.5 vvm aeration. The second seed medium consisted of 20 g/l oxidized starch, 10 g/l glycerol, 20 g/l soybean meal, 2 g/l inactive dry yeast, 5 ml/l 45% corn steep liquor, 2 g/l $CaCO_3$, and 0.5 g/l surfactant GE304 and was adjusted to pH 6.5 before inoculation. The second seed culture was ended at a dissolved oxygen level of between 30 and 40%, and 350 ml of the broth was then inoculated into a 5-l fermenter containing 3.15 l of the production culture medium and cultured for 7 days at 27°C, with 500 rpm agitation and 0.5 vvm aeration. The production culture medium consisted of 70 g/l oxidized starch, 5 g/l soybean meal, 17 g/l inactive dry yeast, 1 g/l $(NH_4)_2SO_4$, 1 g/l $CaCO_3$, and 1 g/l surfactant GE304 and was adjusted to pH 6.5 before inoculation.

Analysis

Broth samples were extracted for 1 h with the same volume of acetone to analyze the tacrolimus, and the harvested broth was also extracted with acetone for purification. The tacrolimus concentration of the final sample was corrected relative to that of the totally extracted harvest. The extracted tacrolimus was analyzed by high-performance liquid chromatography (HPLC) on a Synergi Polar RP (4 μm, 250 × 4.6 mm; Phenomenex, Macclesfield, UK) column with 50% acetonitrile effluent and a UV detector. Cell growth was measured by the packed mycelium volume (PMV). However, the PMV included not only the mycelium but also the non-soluble components of the inactive dry yeast and soybean meal in the medium and could not be used to determine cell growth directly. The Bertrand method was used to analyze the total reducing sugar in the broth after acid hydrolysis of the broth sample. The glucose in the supernatant of the broth sample was analyzed with a Glucose-E kit (YD Diagnostics, Korea).

Results and discussions

Sequential adaptation

Tacrolimus productivity of the strain *Streptomyces* sp. TST8 is thought to be mainly limited by product inhibition because tacrolimus is an antibiotic. *Streptomyces* sp. TST8 was subcultured in the media containing stepwise increased concentrations of tacrolimus and was ultimately able to grow in the medium containing 1600 mg/l tacrolimus (Fig. 2). By using this tacrolimus sequential adaptation protocol, we obtained strains that are less affected by the antibiotic tacrolimus.

The cell growth of each subculture varied between an OD of 2 and 12 at 600 nm, which correlates to 1.3 and 7.5 g/l, respectively, in dry cell weight. The final broth of each subculture was first observed under a microscope to confirm cell growth and then inoculated into the production medium in a flask. The tacrolimus production flask culture of the subcultured broth produced a final tacrolimus titer of between 400 and 700 mg/l (Fig. 3), which was almost same as that of the original strain *Streptomyces* sp. TST8.

Because the production seed culture step was omitted, the results of the tacrolimus production culture of each subculture did not give a true measurement of tacrolimus production by each adapted strain, but it did confirm a successful adaptation of *Streptomyces* sp. TST8 in the medium containing a high concentration of tacrolimus. Tacrolimus productivity in the flask culture was tested by flask culture with the working stocks of TST9, TST10, and TST11.

Flask culture

Flask cultures of the developed strains, TST9, TST10, and TST11, were performed under optimized conditions for

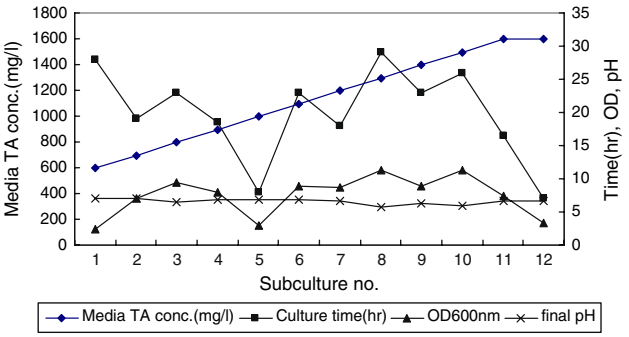

Fig. 2 Results of TA sequential adaptation. *OD* Optical density

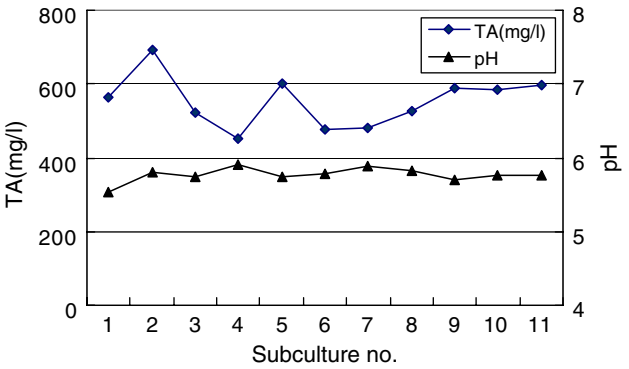

Fig. 3 Results of TA production flask culture of the subcultured broth

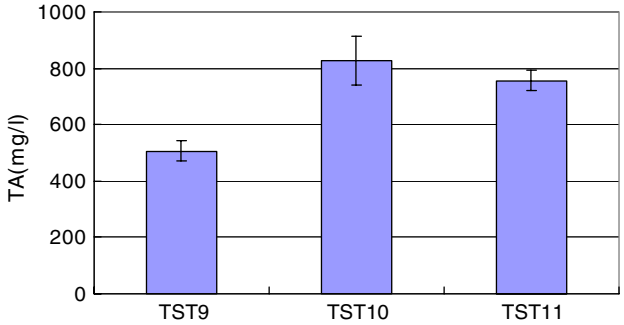

Fig. 4 Tacrolimus production flask culture of developed strains TST9, TST10, and TST11

tacrolimus production. The results are shown in Fig. 4 in which the tacrolimus concentration of each flask culture is given as the average value of six flasks in two independent cultures. The final tacrolimus titers of the developed strains of TST9, TST10, and TST11 were 507, 827, and 757 mg/l, respectively.

The final tacrolimus titer of TST9 was almost the same as that of the original strain, TST8, while the final tacrolimus titers of TST10 and TST11, which were adapted in medium containing a high concentration of tacrolimus, were 63 and 49%, respectively, higher than that of TST9. The tacrolimus sequential adaptation was supposed to reduce the product inhibition, and the adapted strains TST10 and TST11 could produce tacrolimus after a somewhat level of tacrolimus was produced. However, the adaptation concentration of tacrolimus was not proportional to the final tacrolimus titer of the adapted strain.

The tacrolimus productivities of the developed strains were not determined in flask cultures because aerobic flask cultures have some limitations in terms of agitation and aeration. Only a relative comparison among the developed strains was possible in the flask cultures.

Five-liter jar fermentation

The original strain, *Streptomyces* sp. TST8, produced a final titer of about 500 mg/l tacrolimus after 7 days of fermentation in a 5-l fermenter with optimized medium and fermentation conditions. The developed strains TST9, TST10, and TST11 obtained final tacrolimus titers of 562, 972 and 761 mg/l, respectively, in the 5-l jar fermentation system (Fig. 5), which are higher than those of the original strain and of any other reported strain [5–7]. The final tacrolimus titers of the developed strains in the 5-l jar fermentation system had the same trends as those in the flask culture. With optimized fermentation conditions in the 5-l jar fermenter, the results would reflect tacrolimus productivities of the developed strains as it is. The strains were developed using a tacrolimus sequential adaptation protocol

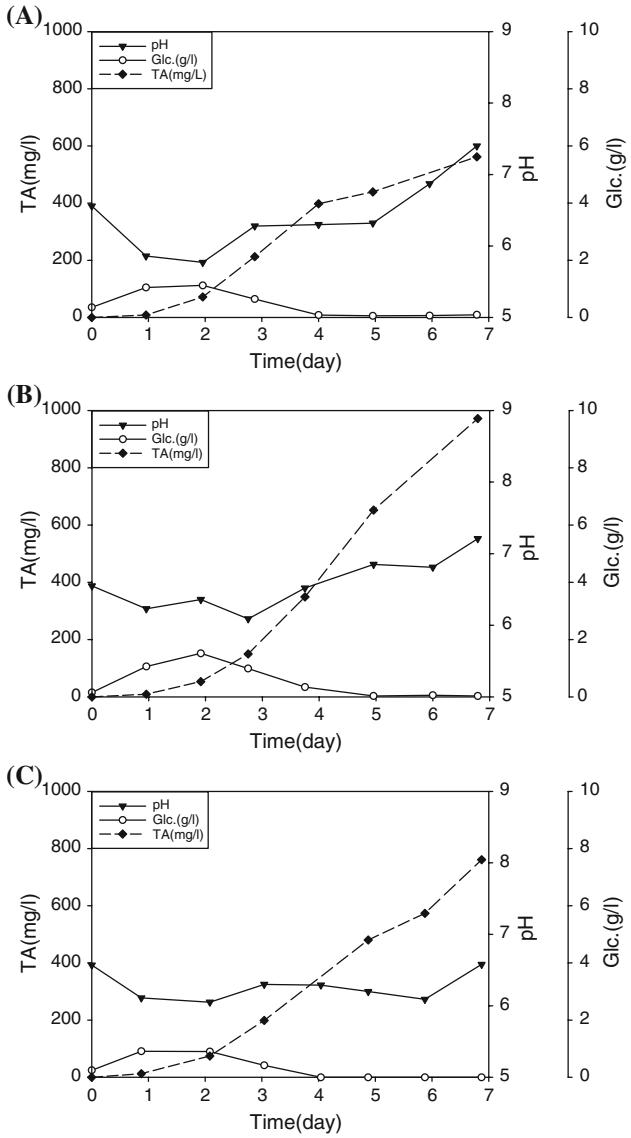

Fig. 5 Tacrolimus production from the 5-l jar fermentation of the TST9 (**a**), TST10 (**b**), and TST11 (**c**) developed strains. *Glc* Glucose

and produced higher concentrations of tacrolimus than has previously been obtained.

The objective of using the tacrolimus sequential adaptation protocol was to select cells with tacrolimus resistance, and this effect would be limited by the solubility of tacrolimus in the medium. TST11, adapted at a high concentration of tacrolimus, produced less tacrolimus than TST10, which was adapted at a moderate concentration of tacrolimus. Knowing that the solubility of tacrolimus in water at 20°C is 0.074 g/100 ml [14], it is possible that some of the tacrolimus in subculture media containing more than 740 mg/l tacrolimus could have recrystallized and no long affect the bacterial cells. The ethanol added together with the tacrolimus to the subculture medium may have increased the solubility of tacrolimus in the subculture medium, but this

increase is not believed to have been substantial. The adaptation in the subculture medium containing 1600 mg/l of tacrolimus was therefore not more effective than that in the subculture medium containing 900 mg/l of tacrolimus due to the limitations of tacrolimus solubility in the subculture medium and the added ethanol.

The original strain, *Streptomyces* sp. TST8, selected from a colony on a agar plate containing 600 mg/l tacrolimus is not likely to be clonal because filamentous *Streptomyces* cannot be plated as an individual microbe without sporulation. The developed strains also may not be clonal, and the fermentation results are probably those of mixed cultures. Further selection of the developed strains by sporulation will be needed in order to obtain stable clonal strains with a high productivity.

Overview

A tacrolimus-producing strain, *Streptomyces* sp. TST8, was developed using a tacrolimus sequential adaptation protocol that was able to produce a final tacrolimus titer of 972 mg/l. This is about twofold higher than the final titer produced by the original strain. The volumetric production rate of the developed strain, TST10, adapted in the subculture medium containing 900 mg/l of tacrolimus was 143 mg/l/day and is higher than that of any other reported strain.

The developed strains and method of sequential adaptation can facilitate the efficient and economical production of tacrolimus.

References

1. Kino T, Hatanaka H, Hashimoto M, Nishiyama M, Goto T, Okuhara M, Kohsaka M, Aoki H, Imanaka H (1987) FK-506, a novel immunosuppressant isolated from a *Streptomyces*: I. fermentation, isolation, and physico-chemical and biological characteristics. J Antibiot 40:1249–1255
2. Kino T, Hatanaka H, Miyata S, Imaura N, Nishiyama M, Yakima T, Goto T, Okuhara M, Kohsaka M, Aoki H, Ochiai T (1987) FK-506, a novel immunosuppressant isolated from a *Streptomyces*: II. immunosuppressive effect of FK-506 in vitro. J Antibiot 40:1256–1265
3. Med Ad News (2008) 200 World's best-selling medicine. Med Ad News 27: 12–33
4. Kim H, Park Y (2008) Isolation and identification of a novel microorganism producing the immunosuppressant Tacrolimus. J Biosci Bioeng 105:418–421
5. Kim H, Park Y (2007) Lipase activity and Tacrolimus production in *Streptomyces clavuligerus* CKD 1119 mutant strains. J Microbiol Biotechnol 17:1638–1644
6. Kumar P, Malviya HK, Maurya RK, Shukla A (2007) Fermentation processes for the preparation of tacrolimus. International publication patent no. WO 2007/039816 A2
7. Vaid S, Narula P (2006) Novel process for producing tacrolimus (FK-506) using vegetable oil as sole source of carbon. International publication patent no. WO 2006/011156 A2
8. Garrity GM, Heimbuch BK, Motamedi H, Shafiee A (1993) Genetic relationships among actinomycetes that produce the immunosuppressant macrolides FK506, FK520/FK523 and rapamycin. J Ind Microbiol 12:42–47
9. Khaw LE, Bohm GA, Metcalfe S, Staunton J, Leadlay PF (1998) Mutational biosynthesis of novel rapamycin by a strain of *Streptomyces hygroscopicus* NRRL 5491 disrupted in *rapL*, encoding a putative lysine cyclodeaminase. J Bacteriol 180:809–814
10. Motamedi H, Shafiee A, Cai S, Streicher SL, Arison BH, Miller RR (1996) Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK506 and FK520. J Bacteriol 178:5243–5248
11. Motamedi H, Shafiee A (1998) The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK506. Eur J Biochem 256:528–534
12. Lee HH (2003) Enhanced production of immunosuppressant FK-506 by *Streptomyces* sp. in a defined medium by release from the regulation of ammonium ion. Seoul National University, Seoul
13. Yoon YJ, Choi CY (1997) Nutrient effects on FK-506, a new immunosuppressant, production by *Streptomyces* sp. in a defined medium. J Ferment Bioeng 83:599–603
14. Weizheng WP, Kusters E, Lohse O, Mak CP, Wang Y (1999) Application of centrifugal counter-current chromatography to the separation of macrolide antibiotic analogues: I. selection of solvent systems based on solubility and partition coefficient investigations. J Chromatogr A 864:69–76