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Effects of Glycerol and Shikimic Acid on Rapamycin Production in *Streptomyces rapamycinicus*

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Rapamycin, derived from *Streptomyces rapamycinicus*, is an important bioactive compound having a therapeutic value in managing Parkinson's disease, rheumatoid arthritis, cancer, and AIDS. Because of its pharmaceutical activity, studies over the past decade have focused on the biosynthesis of rapamycin to enhance its yield. In this study, the effect of *rapG* on rapamycin production was investigated. The *rapG* expression vector was constructed by utilizing the integration vector pSET152 under the control of the erythromycin resistance gene (*ermE), a strong constitutive promoter. The rapamycin yield of wild type (WT) and WT/*rapG* overexpression mutant strains, under fermentation conditions, was analyzed by high-performance liquid chromatography (HPLC). Our results revealed that overexpression of *rapG* increased rapamycin production by approximately 4.9-fold (211.4 mg/l) in MD1 containing 15 g/l of glycerol, compared to that of the WT strain. It was also found that *Illicium verum* powder (10 g/l), containing shikimic acid, enhanced rapamycin production in both WT and WT/*rapG* strains. Moreover, the amount of rapamycin produced by the WT/*rapG* strain was statistically higher than that produced by the WT strain. In conclusion, the addition 15 g/l glycerol and 15 g/l *I. verum* powder produced the optimal conditions for rapamycin production by WT and WT/*rapG* strains.**

Keywords: **Rapamycin, WT/*rapG*, *Illicium verum* extract, *Streptomyces rapamycinicus***

Introduction

Rapamycin is an important bioactive compound derived from *Streptomyces rapamycinicus*, which was first isolated from a soil sample in 1975 [1]. Rapamycin is a macrolide antibiotic with antifungal and immunosuppressive properties [2]. Also, recent reports indicated that rapamycin has therapeutic value in managing Parkinson's disease [3], rheumatoid arthritis [4], cancer [5], and AIDS [6]. Based on a sequence analysis and genetic engineering study, the function of rapamycin's synthetic *rapG* gene was identified as an important positive regulatory gene in rapamycin biosynthesis. The sequence analysis showed that *rapG* is similar to other positive regulatory proteins, such as the SoxS and Rob proteins of *Escherichia coli* [7]. The *rapG* overexpression under ActII-ORF4/P_{actI} promoter in *S. rapamycinicus* significantly enhanced rapamycin production under specific fermentation conditions [8].

Glycerol is not only abundant and inexpensive, but can also facilitate maximum yield of antibiotics; therefore, the optimization of glycerol-based media seems to be an economical means of rapamycin production [9, 10]. Rapamycin can be classified as a cyclohexane carboxylic acid antibiotic. The biosynthesis of rapamycin begins with a unique cyclohexane unit derived from the shikimic acid pathway [11]. It has previously been reported that shikimic acid enhances rapamycin production; however, it is strain-dependent [10].

Most efforts have focused on the optimization of physi-

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ological factors for better rapamycin production from wild type strains of *Streptomyces* sp. [9, 12–15]. A few articles have reported that the optimal fermentation process for the production of rapamycin from mutant strains can be obtained by UV mutagenesis [10]. In this study, the effects of glycerol and shikimic acid on the rapamycin biosynthesis of *S. rapamycinicus* wild type and *rapG* overexpression mutant strains, in several rapamycin fermentation media, were investigated. Moreover, *Illicium verum* powder, used for obtaining shikimic acid, was optimized for industrial-scale production of rapamycin.

Materials and Methods

Bacterial strains

All strains and plasmids that were used in this study were described in Table 1. *Streptomyces rapamycinicus* DSM 41530 (WT strain) was used in the study. The WT and its recombinant strains were cultured at 28℃ on yeast extract-starch soluble (YS) agar plates. *E. coli* DH5α was used for the propagation of plasmids using a standard protocol [16]. The pGEM T-Easy vector system (Promega, US) was used for the subcloning process. *E. coli* ET12567, consisting of plasmid pUZ8002, was used to conjugate and transfer the target plasmid to *S. rapamycinicus* spores [17]. *S. rapamycinicus* mutant strain was cultured in media containing 50 μg/ml apramycin. *E. coli* ET12567/pUZ8002 strains were cultured in Lysogeny Broth (LB) containing 50 μg/ml rapramycin, 50 μg/ml chloramphenicol, and 50 μg/ml kanamycin.

Table S1. Primers used in this study.

Primer	Sequence
For Mutagenesis	
rapGF	GG**TCTAGA**ACCAACGGCGCTGGAGCGGAG
rapGR	GG**TCTAGA**GGTCAGCTGTCGGTCAGCCCGGTTG
For real-time PCR	
rrsA1090F	GGTCAACTCGGAGGAAGGTG
rrsA1306R	ACGTATTCACCGCAGCAATG
rapG84F	AGGATTCTTCCAGGCCTTCC
rapG293R	TACATCCGTACCCGGTCCTC

Construction of *rapG* overexpression mutant strain

The *rapG* overexpression mutant strain was constructed by using the method described by Kuscer with some modifications [8]. Briefly, the *rapG* gene was amplified from the genomic DNA of *S. rapamycinicus* DSM 41530 by means of polymerase chain reaction (PCR) method using the primers (Table S1). The PCR products of *rapG* were purified and cloned into a pGEM T-Easy vector to produce pGEM-*rapG*. The sequence-verified fragments, which were obtained by digesting pGEM-*rapG* with XbaI enzyme, were then joined with pSET152 to produce pRapG, which was then sequenced. Thereafter, pRapG was introduced into *S. rapamycinicus* by conjugation using *E. coli* ET12567/pUZ8002, as described by Kurniawan [17], to yield the WT/*rapG* overexpression mutant strain. The genotypes of WT and WT/*rapG* overexpression mutant strain were verified using Southern blot hybridization. For southern blot hybridization, *rapG* was used as a probe and the genomic DNAs were digested

Table 1. Bacterial strains and plasmids used in this study.

Strain/plasmid	Characteristics	Reference/Source
Bacterial strains		
Escherichia coli DHα	F– *recA lacZ*ΔM15	[16]
Escherichia coli ET12567/pUZ8002	Non-methylating ET12567 containing non-transmissible RP4 derivative plasmid pUZ8002, Cmlr, Tetr, Kanr	[17]
Streptomyces rapamycinicus DSM 41530	Wild-type strain	[1]
WT/*rapG*	Wild-type with integrative plasmid pRapG04, Apar^r	This study
Plasmids		
pGEM T-Easy		Promega
pSET152 (*ermEp**)	Integrative plasmid containing *ermEp**, *oriT*, *attP*, *ΦC31 int* and *aac(3)IV*	[23]
pRapG	pSET152 (*ermEp**)-based integrative plasmid containing a single copy of *rapG*, Apar^r	This study

by BamHI and KpnI.

Analysis of gene expression

For the study of gene expression, cells harvested at 48 h were subjected to total RNA extraction by using TRIzol™ reagent (ThermoFisher Scientific, USA). Genomic DNA was digested with RNase-free DNase I (ThermoFisher Scientific) and RNA concentration was measured by using the NanoDrop 2000 Spectrophotometer (Thermo). Reverse transcription to cDNA was conducted with a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFisher Scientific). The *rrsA* (16s rRNA) gene was employed as a reference for the real-time PCR assay [18]. Specific primers were listed in Table S1. Real-time PCR was performed with a Mastercycler® RealPlex^2 (Eppendorf, USA) by using GoTaq® qPCR Master Mix (Promega, USA) under the following conditions: 2 min at 95℃, 40 cycles of 15 s at 95℃, and final extension for 1 min at 60℃. For each cDNA sample, a cycle threshold value (C_t) of the reference gene was subtracted from that of the target gene to obtain ΔC_t, and the expression of each gene was quantified by $2^{-\Delta Ct}$.

Analysis of rapamycin production

The WT and WT/*rapG* overexpression mutant strains were cultivated in YS medium. At first, seed cultures were cultivated at 28℃ on an YS agar plate for 96 h, thereafter 10 cubes of agar containing the mycelia were transferred to 75 ml of fresh medium and shaken at 200 rpm for 72 h at 28℃. The media used for rapamycin production were as follows: MD1 containing 40 g/l dextrin, 30 g/l soybean meal, 5 g/l NaCl, 3 g/l Na_2HPO_4, and 2 g/l L-Lysine HCl and had a pH of 5.0 [10]; MD2 containing 20 g/l glucose, 5 g/l KH_2PO_4, and 20 g/l soybean meal and had a pH of 6.0 [19]; MD3 containing 6 g/l glucose 6, 10 g/l soybean meal, 0.025 g/l K_2HPO_4, 8 g/l sorbitol, and 0.2 g/l $(NH_4)_2SO_4$ and had a pH of 6,5 [20]; and MD4 containing 22 g/l glucose, 10 g/l malt extract, 0.3 g/l casein, 5.3 g/l $(NH_4)_2SO_4$, 5 g/l NaCl, 0.3 g/l Na_2SO_4, 4 g/l KH_2PO_4, 3 g/l $CaCO_3$, 0.06 g/l $ZnSO_4 \cdot 7H_2O$, 0.06 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l $FeSO_4 \cdot 7H_2O$, and 0.012 g/l $MnSO_4 \cdot H_2O$ and had a pH of 7.2 [21]. Either glycerol or *I. verum* extract was then added into the fermentation media. To obtain the extract, *I. verum* powder was placed in a teabag, dipped into 50 ml reverse osmosis (RO) water and autoclaved at 121℃. In order to analyse the rapamycin, the culture broth was separated from the mycelia. The mycelia were mixed with 7.5 ml methanol and then shaken intensively for 4 h at 30℃. After centrifugation at 4000 ×*g* for 20 min, the suspension was transferred to a culture broth, which was extracted with an equal volume of ethyl acetate, and then filtered. A fraction of this solution was then subjected to HPLC analysis. An Agilent 1260 series HPLC system equipped with an Eclipse XDB- C18 (4,6 × 150 mm, 5 µm) reversed phase analytical column was used. The mobile phase consisted of a mixture of acetonitrile and water (Table 2) and the eluate was monitored at 280 nm. At least three separate cultivations and extractions were carried out to obtain an average production yield of rapamycin.

Table 2. Mobile phase of the HPLC analysis.

Time (min)	H_2O (%)	Acetonitrile (%)
0.00	85	15
3.00	85	15
25.00	15	85
29.00	15	85
32.00	85	15
35.00	85	15

Results and Discussion

Overexpression of *rapG* gene in *S. rapamycinicus*

To investigate the functions of *rapG* gene in the regulation of rapamycin production in *S. rapamycinicus*, the gene was introduced and overexpressed in the *S. rapamycinicus* wild-type strain. The vector for overexpression of *rapG* was constructed by employing the integration vector pSET152 as described in Materials and Methods. The φC31-based integrating plasmid pRapG, under the control of a strong constitutive *ermE** promoter, was introduced into the wild-type *S. rapamycinicus* strain at the bacteriophage φC31 attachment site (*attB*) generating WT/*rapG*. The pRapG plasmid can be maintained in the chromosome of overexpression mutant strains at integrated sites. A fragment of 993 bp of the *rapG* gene, obtained by PCR, was digested by XbaI, after which it was added to pSET152 to generate the pRapG plasmid (Fig. 1A). The overexpression mutant was confirmed by Southern blot hybridization (Fig. 1B). A hybridizing band of approximately 4.0 kb was found for the WT strain whereas in the *rapG* overexpression mutant, 2

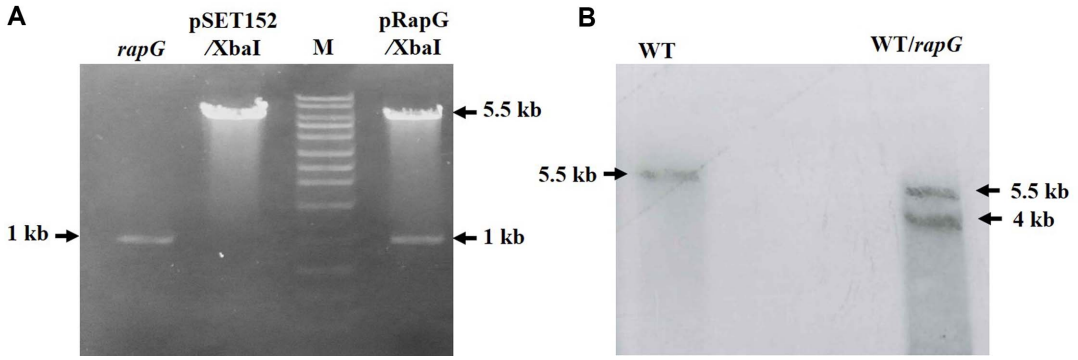

Fig. 1. (A) Gel electrophoresis of the PCR of the *rapG* gene, pSET152 digested by XbaI, Marker 100 bp DNA ladder and pRapG digested by XbaI, respectively. (B) Southern blot analysis of mutants with one or two *rapG* genes deleted. The DNA from the WT strain and WT/*rapG* strain were digested by BamHI and KpnI and hybridized with the probe covering the *rapG* region.

hybridizing bands of approximately 4.0 and 5.5 kb were observed, indicating that pRapG was introduced at a site different from that of the WT genome.

Enhanced production of rapamycin with glycerol and *Illicium verum* powder extract

To evaluate the effect of overexpression of *rapG* on rapamycin yield, fermentations using several media were performed and the yield of rapamycin monitored to evaluate the fermentation process. The fermentation media MD1, MD2, MD3 and MD4 were selected from previous studies. Both the WT and *rapG* overexpression mutant strains produced the highest quantity of rapamycin in the MD1 medium, compared to the other media. The yield of rapamycin of the WT and *rapG* overexpression mutant strains were 39.0 ± 4.0 and 54.5 ± 15.0 mg/l, respectively (Fig. 2A). However, there was no significant difference between the rapamycin yield of the WT and WT/*rapG* strains cultured in the MD1 medium.

Glycerol is an economical feedstock for fermentation processes, and it is reported to increase the yield of rapamycin biosynthesis. The addition of 36.2 g/l of glycerol yielded the maximum rapamycin production of *Streptomyces hygroscopicus* wild-type strain [9]. In this study, the effect of glycerol on rapamycin production was tested using several fermentation media and the data were shown in Fig. 2B. Our results confirmed that glycerol facilitated rapamycin production of the WT strain. For example, the rapamycin production of the WT strain in the MD1-glycerol medium was slightly higher than that

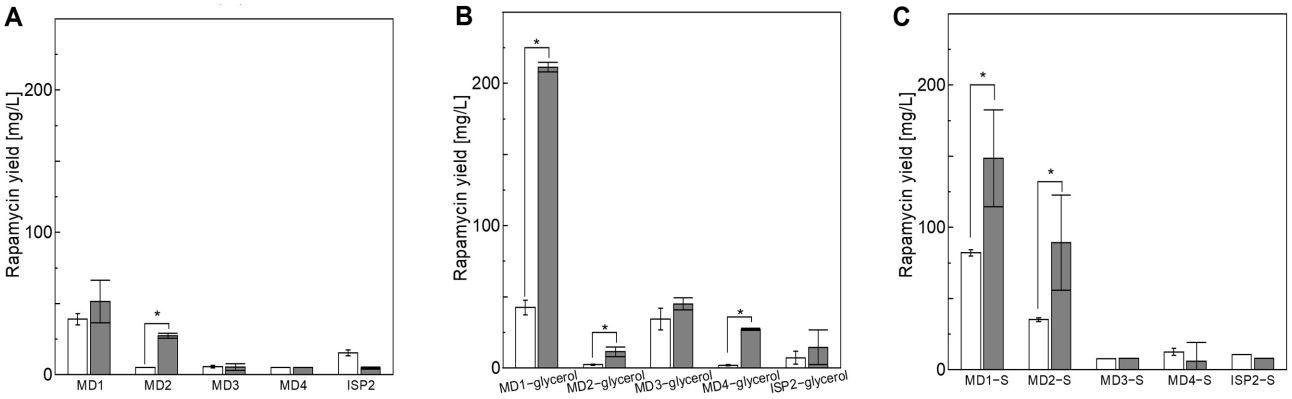

Fig. 2. Variability of rapamycin yields from *S. rapamycinicus* WT (open column) and WT/*rapG* (closed column) cultured in fermentation media (A), media containing 15 g/l glycerol (B) and media containing 10 g/l *Illicium verum* powder. The data are the means of measurements obtained from at least three independent experiments. The statistical significance among the data sets was assessed by Student's t-test (* $p < 0.05$).

in the original MD1 medium. Moreover, the rapamycin production of the WT strain in the MD3-glycerol medium was significantly higher than that in the original MD3 medium. For rapamycin production of the *rapG* overexpression mutant strain, the addition of 15 g/l glycerol significantly enhanced the production of rapamycin in MD1, MD2 and MD4 media compared to those media without added glycerol. For example, the yield of rapamycin in MD1-glycerol of the WT/*rapG* strain was 4.1 fold higher than that in the original MD1 medium. Interestingly, overexpression of *rapG* increased rapamycin production by approximately 4.9 fold to 211.4 mg/l in MD1 containing 15 g/l glycerol compared to that of the WT strain. Thus, the MD1-glycerol is chosen to use in further experiments.

The elucidation of rapamycin biosynthesis indicates that intracellular supplies of shikimic acid [10–12] are vital to the construction of the rapamycin scaffold. However, shikimic acid is an expensive material that cannot be adopted in large-scale fermentation. It was reported that shikimic acid could easily be extracted from *I. verum* powder using hot water at 120℃ [22]. Thus, it was hypothesized that the addition of *I. verum* extraction liquid positively affects rapamycin production. The results showed that both the WT and *rapG* overexpression mutant strains produced plentiful rapamycin in the MD1 and MD2 media which contained 10 g/l of *I. verum* extraction (Fig. 2C). Moreover, the amount of rapamycin produced by the WT/*rapG* strain was statistically higher than that produced by the WT strain, especially in the MD1-shikimic acid medium.

In order to understand the effect of both glycerol and shikimic acid on rapamycin production, extraction liquid of 5, 10, and 15 g/l *I. verum* was added to the MD1-glycerol media. For the WT strain, 130.5 ± 5.1, 120.0 ± 15.1, and 149.0 ± 24.1 mg/l rapamycin was produced in media containing 5, 10, and 15 g/l *I. verum* powder, respectively (Fig. 3). Our result revealed that the combination of glycerol and shikimic acid, found in *I. verum* extraction liquid, increased the rapamycin production of WT strain by approximately 3 fold, compared to that in the original medium. We observed an increase in the rapamycin production of WT/*rapG* of by 1.3 to 1.4-fold compared to the WT strain. Besides, the addition of 15 g/l glycerol and 15 g/l *I. verum* powder enhanced the rapamycin production of WT/*rapG* compared to those in MD1-glycerol and MD1-shikimic acid.

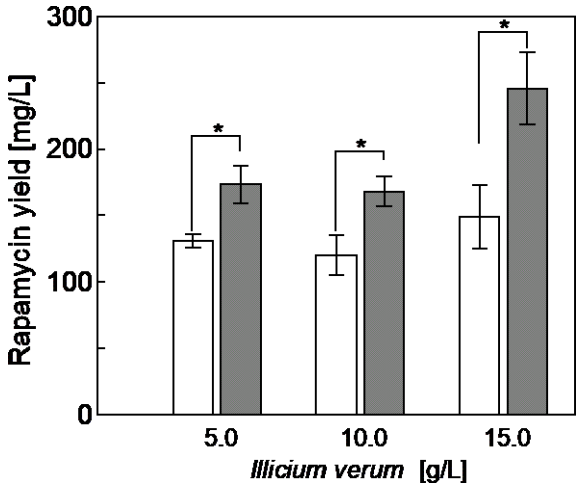

Fig. 3. Variability of rapamycin yields from *S. rapamycinicus* WT (open column) and WT/*rapG* (closed column) cultured with MD1 containing 15 g/l glycerol and *Illicium verum* powder. The data are the means of measurements obtained from at least three independent experiments. The statistical significance among the data sets was assessed by Student's t-test (* $p < 0.05$).

In a previous study, under flask cultivation conditions, 76.2 ± 4.7 mg/l of rapamycin was obtained from the WT strain in an optimized glycerol-based medium [9]. In our study, WT strain produced 149.0 ± 24.1 mg/l in MD1-glycerol containing 15 g/l *I. verum* powder. Moreover, the real-time PCR showed that *rapG* expression in WT/*rapG* was significantly higher than WT in MD1-glycerol medium (Fig. 4). As the result, the overexpression of *rapG,* under the control of the *ermE** promoter, significantly enhanced the biosynthesis of rapamycin compared to the WT strain in the MD1-glycerol medium. Also, the overexpression of *rapG* in WT/*rapG* strain resulted in the higher rapamycin yield of the WT/*rapG* strain in MD1-shikimic acid (Fig. 4). In the medium containing 15 g/l glycerol and 15 g/l *I. verum* powder, the *rapG* expression in WT/ *rapG* strain was 6.1-fold higher than in WT strain. Thus, it was suggested that the MD1-glycerol, MD1-shikimic acid and MD1-glycerol and shikimic acid media, combined with *rapG* overexpression, increased rapamycin production by 4.4, 4.3 and 6.3 fold, respectively, compared to that of the WT strain in the MD1 original medium. Therefore, it is evident that *rapG* plays a role in rapamycin biosynthesis of both the glycerol and the shikimic acid pathway. The *I. verum* powder is abundant and economic, therefore, it is believed

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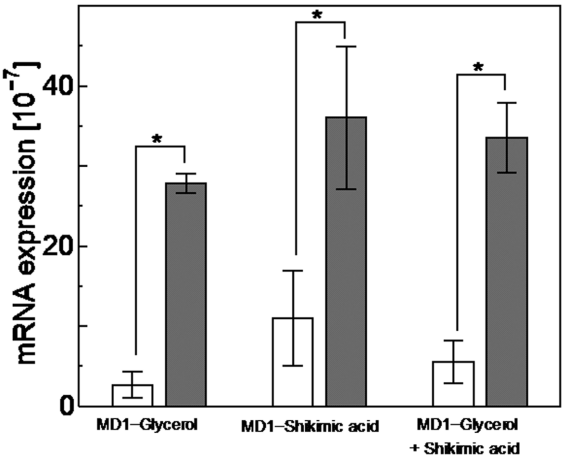

Fig. 4. Expressions of *rapG* in respective cells of WT and WT/*rapG* strains at 48 h in MD1-glycerol, MD1-shikimic and MD1-glycerol with 15 g/l *Illicium verum* powder media. The data are the means of measurements obtained from three independent experiments. The statistical significance among the data sets was assessed by the Student's t-test (*$p < 0.05$).

that *I. verum* powder could be used for obtaining shikimic acid for industrial-scale production of rapamycin.

In conclusion, *rapG* overexpression facilitated rapamycin biosynthesis in presence of the glycerol and the shikimic acid. This research provided information on the systematic optimization of rapamycin production from the WT and WT/*rapG* overexpression mutant strain. The addition 15 g/l glycerol and 15 g/l *I. verum* powder was the optimal condition for rapamycin production of WT and WT/*rapG* strains.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Vezina C, Kudelski A, Sehgal SN. 1975. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J. Antibiot.* **28**: 721-726.
2. Park SR, Yoo YJ, Ban YH, Yoon YJ. 2010. Biosynthesis of rapamycin and its regulation: past achievements and recent progress. *J. Antibiot.* **63**: 434-441.
3. Tain LS, Mortiboys H, Tao RN, Ziviani E, Bandmann O, Whitworth AJ. 2009. Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss. *Nat. Neurosci.* **12**: 1129-1135.
4. Shao P, Ma L, Ren Y, Liu H. 2017. Modulation of the immune response in rheumatoid arthritis with strategically released rapamycin. *Mol. Med. Rep.* **16**: 5257-5262.
5. Zou CY, Smith KD, Zhu QS, Liu J, McCutcheon IE, Slopis JM, *et al.* 2009. Dual targeting of AKT and mammalian target of rapamycin: a potential therapeutic approach for malignant peripheral nerve sheath tumor. *Mol. Cancer Ther.* **8**: 1157-1168.
6. Nicoletti F, Lapenta C, Donati S, Spada M, Ranazzi A, Cacopardo B, *et al.* 2009. Inhibition of human immunodeficiency virus (HIV-1) infection in human peripheral blood leucocytes-SCID reconstituted mice by rapamycin. *Clin. Exp. Immunol.* **155**: 28-34.
7. Molnar I, Aparicio JF, Haydock SF, Khaw LE, Schwecke T, Konig A, *et al.* 1996. Organisation of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of genes flanking the polyketide synthase. *Gene.* **169**: 1-7.
8. Kuscer E, Coates N, Challis I, Gregory M, Wilkinson B, Sheridan R, *et al.* 2007. Roles of rapH and rapG in positive regulation of rapamycin biosynthesis in *Streptomyces hygroscopicus*. *J. Bacteriol.* **189**: 4756-4763.
9. Kim YH, Park BS, Bhatia SK, Seo HM, Jeon JM, Kim HJ, *et al.* 2014. Production of rapamycin in *Streptomyces hygroscopicus* from glycerol-based media optimized by systemic methodology. *J. Microbiol. Biotechnol.* **24**: 1319-1326.
10. Zhu X, Zhang W, Chen X, Wu H, Duan Y, Xu Z. 2010. Generation of high rapamycin producing strain via rational metabolic pathway-based mutagenesis and further titer improvement with fed-batch bioprocess optimization. *Biotechnol. Bioeng.* **107**: 506-515.
11. Paiva NL, Roberts MF, Demain AL. 1993. The cyclohexane moiety of rapamycin is derived from shikimic acid in *Streptomyces hygroscopicus*. *J. Ind. Microbiol. Biotechnol.* **12**: 423-428.
12. Fang A, Demain AL. 1995. Exogenous shikimic acid stimulates rapamycin biosynthesis in *Streptomyces hygroscopicus*. *Folia Microbiol. (Praha)* **40**: 607-610.
13. Lee MS, Kojima I, Demain AL. 1997. Effect of nitrogen source on biosynthesis of rapamycin by *Streptomyces hygroscopicus*. *J. Ind. Microbiol. Biotechnol.* **19**: 83-86.
14. Yen HW, Hsiao HP. 2013. Effects of dissolved oxygen level on rapamycin production by pellet-form of *Streptomyces hygroscopicus*. *J. Biosci. Bioeng.* **116**: 366-370.
15. Yen H-W, Hsiao H-P, Chen L-J. 2013. The enhancement of rapamycin production using *Streptomyces hygroscopicus* through a simple pH-shifted control. *J. Taiwan Inst. Chem. E.* **44**: 743-747.
16. Hanahan D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**: 557-580.
17. Kurniawan YN, Kitani S, Iida A, Maeda A, Lycklama a Nijeholt J, Lee YJ, *et al.* 2016. Regulation of production of the blue pigment indigoidine by the pseudo gamma-butyrolactone receptor FarR2

in *Streptomyces lavendulae* FRI-5. *J. Biosci. Bioeng.* **121**: 372-379.
18. Dai D, Pu T, Liang J, Wang Z, Tang A. 2018. Regulation of dndB gene expression in *Streptomyces lividans*. *Front. Microbiol.* **9**: 2387.
19. Sallama LAR, El-Refaia AF, Osmanb ME, Hamdya AA, Ahmeda EM, Mohameda MA. 2010. Some physiological factors affecting rapamycin production by *Streptomyces hygroscopicus* ATCC 29253. *Am. J. Sci.* **6**: 188-194.
20. More AS, Gadalkar S, Rathod VK. 2017. Extraction of rapamycin (sirolimus) from *Streptomyces rapamycinicus* using ultrasound. *Prep. Biochem. Biotechnol.* **47**: 627-632.
21. Dutta S, Basak B, Bhunia B, Chakraborty S, Dey A. 2014. Kinetics of rapamycin production by *Streptomyces hygroscopicus* MTCC 4003. *3 Biotech.* **4**: 523-531.
22. Ohira H, Torii N, Aida TM, Watanabe M, Smith RL. 2009. Rapid separation of shikimic acid from Chinese star anise (*Illicium verum* Hook. f.) with hot water extraction. *Sep. Purif. Technol.* **69**: 102-108.
23. Bierman M, Logan R, O'Brien K, Seno ET, Rao RN, Schoner BE. 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116**: 43-49.

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