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Metabolic Engineering of Rational Screened *Saccharopolyspora spinosa* for the Enhancement of Spinosyns A and D Production

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Spinosyns A and D are potent ingredient for insect control with exceptional safety to non-target organisms. It consists of a 21-carbon tetracyclic lactone with forosamine and tri-*O*-methylated rhamnose which are derived from S-adenosyl-methionine. Although previous studies have revealed the involvement of *metK1* (S-adenosylmethionine synthetase), *rmbA* (glucose-1-phosphate thymidylyltransferase), and *rmbB* (TDP-D-glucose-4, 6-dehydratase) in the biosynthesis of spinosad, expression of these genes into rational screened *Saccharopolyspora spinosa (S. spinosa* MUV) has not been elucidated till date. In the present study, *S. spinosa* MUV was developed to utilize for metabolic engineering. The yield of spinosyns A and D in *S. spinosa* MUV was 244 mg L^{-1} and 129 mg L^{-1}, which was 4.88-fold and 4.77-fold higher than that in the wild-type (50 mg L^{-1} and 27 mg L^{-1}), respectively. To achieve the better production; positive regulator *metK1-sp, rmbA* and *rmbB* genes from *Streptomyces peucetius,* were expressed and co-expressed in *S. spinosa* MUV under the control of strong *ermE promoter, using an integration vector pSET152 and expression vector pIBR25, respectively. Herewith, the genetically engineered strain of *S. spinosa* MUV, produce spinosyns A and D up to 372/217 mg L^{-1} that is 7.44/8.03-fold greater than that of wild type. This result demonstrates the use of metabolic engineering on rationally developed high producing natural variants for the production.**

INTRODUCTION

Spinosyn A and spinosyn D together known as spinosad; a bio-insecticide derived from the fermentation of the soil microorganism *Saccharopolyspora spinosa*, discovered by Lilly in 1997 and is now widely used as a field pest control agent on many crops (Jin et al., 2009). Structurally, Spinosyns are macrolides consisting of a 21-carbon, tetracyclic lactone, to which two deoxysugars: tri-*O*-methylated rhamnose and forosamine are attached (Kirst et al., 1992). The most active and major components of the spinosyn family are spinosyns A and D which differs from each other by a single methyl substituent at position 6 of the polyketide; other factors in spinosyn family have different levels of methylation and are significantly less active (Madduri et al., 2001). Spinosad are highly effective against target insects and has an excellent environmental and mammalian toxicological profile (Crouse and Sparks, 1998; Sparks et al., 1998). Incorporation studies with ^{13}C-labeled acetate, propionate, butyrate, and methionine established that spinosyns are assembled by a polyketide pathway and that the two N-methyl groups of forosamine and the three *O*-methyl groups of tri-*O*-methylrhamnose are derived from S-adenosyl-methionine. Most of the genes involved in spinosad biosynthesis are clustered in a 74-kb region of the *S. spinosa* genome. The spinosad biosynthetic gene cluster contains five large genes encoding a type I polyketide synthase and 14 genes involved in sugar biosynthesis, sugar attachment to the polyketide, or cross-bridging of the polyketide. Four rhamnose biosynthetic genes, two of which are also necessary for forosamine biosynthesis, are located outside the spinosyn gene cluster (Waldron et al., 2001a; 2001b).

Emerging tools for synthetic biology and recombinant DNA technology has led to a number of strategies for metabolic engineering (Chaudhary et al., 2013; Koffas et al., 1999; Lee et al., 2013; Nielsen, 2001). The potential productivity of the actinomycetes is controlled by its genome and, therefore, the genome must be modified to increase the yield. Genome modification in *Saccharopolyspora spinosa* was achieved by duplication of gene cluster (18 kb) (Tang et al., 2011), genome shuffling (Jin et al., 2009) and overexpression of clustered genes (Luo et al., 2011; Xue et al., 2013) respectively. Heterologous expressions of the positive regulator S-adenosylmethionine synthetase (*metK1*) is found to be involved in enhancement of various secondary metabolites from different actinomycetes. (Jha et al., 2014; Kim et al., 2003; Lee et al., 2002; Okamoto et al., 2003; Wang et al., 2007; Zhao et al., 2006). The *spnH, spnI,* and *spnK* are the respective rhamnose 4′-, 2′-, and 3′-*O*-methyltransferase

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Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Sources
Bacterial strain		
S. spinosa ATCC83543.1	Wild type	ATCC
S. spinosa MUV	Rational strain obtain by UV mutagenesis into *S. spinosa* 83543.1	This study
S. spinosa MUV IBR25	Expression of pIBR25 vector into *S. spinosa* MUV	This study
S. spinosa MUV SIBR	pIBR25 with *metK1-sp* expressed into *S. spinosa* MUV	This study
S. spinosa MUV RMBIBR	pIBR25 with *rmbA* and *rmbB* expressed into *S. spinosa* MUV	This study
S. spinosa MUV SAM152	pSET152 with *metK1-sp* integrated into *S. spinosa* MUV	This study
S. spinosa MUV RMB152	pSET152 with *rmbA* and *rmbB* integrated into *S. spinosa* MUV	This study
E. coli		
XL1 Blue	General cloning host	Stratagene PBL company
JM110	Demethylation host	Stratagene Lajolla, USA
Plasmid		
pIBR25	Streptomyces expression vector, with *ermE** promoter and Tsr^r	SunMoon University
pSIBR	pIBR25 based recombinant plasmid harboring *metK1-sp*	This study
pRMBIBR	pIBR25 based recombinant plasmid harboring *rmbA* and *rmbB*	This study
pSET152	Streptomyces integration vector, with *ermE** promoter and Apr^r	SunMoon University
pSAM152	pSET152 based recombinant plasmid harboring *metK1-sp*	This study
pRMB152	pSET152 based recombinant plasmid harboring *rmbA* and *rmbB*	This study

which are responsible for the final rhamnosyl methylation step in the biosynthesis of spinosyn (Huang et al., 2008; Kim et al., 2010). Moreover, glucose-1-phosphate thymidylyltransferase (*rmbA)* and TDP-D-glucose-4,6-dehydratase (*rmbB*) were also found to be overexpressed for altering the metabolic flux distribution in TDP-4-keto-6-deoxy-D-glucose, which is the common intermediate in TDP-deoxysugar biosynthetic pathways (Madduri et al., 2001; Pan et al., 2011). Similarly, UV irradiation is one of the strain improvement strategies through random mutation. UV radiation, in the range of 200-300 nm, produces thymidine dimers and increases probability of deletion during the duplication process. UV is a very convenient and relatively safe mutagen (Kieser et al., 2000), however very few researchers have used this technique to overproduce spinosad (Jin et al., 2006; Liang et al., 2009). These examples imply that introduction of extra copies of biosynthetic gene clusters into a wild-type strain might be an effective approach to improve the yield of the corresponding product. Although these techniques have succeeded in generating many industrial strains, they never use natural variants, developed by UV irradiation for genetic manipulation.

In present study, the rational screened *Saccharopolyspora spinosa* MUV, a high producing strain was developed by UV mutagenesis, which was further modified by metabolic engineering with *metK1-sp* and combination of *rmbA* and *rmbB* from *S. peucetius* ATCC 27952 to study their effects on spinosyn A and D production.

MATERIALS AND METHODS

Micro-organisms and vectors

The bacterial strains and plasmids used in this study are listed in Table 1. The pGEM®-T Easy vector (Promega, USA) was used to clone the polymerase chain reaction (PCR) products. pSET152 (Bierman et al., 1992) was used as an integrative vector, and pIBR25 (Sthapit et al., 2004) was used as the expression vector. DNA manipulation was carried out in *Escherichia coli* XL1-Blue (MRF) (Stratagene, USA), a host cell used for the preparation of recombinants plasmids, was grown in Luria-Bertani (LB) broth and maintained on LB agar medium at 37°C; *E. coli* JM110 was used to propagate non-methylated DNA. For the selection and maintenance of plasmids, Luria-Bertani (LB) broth and agar plates were supplemented with the appropriate amount of antibiotics (apramycin, 100 mg ml^{-1} and ampicillin 100 mg ml^{-1}) to grow *E. coli*. *S. spinosa* was used for the development of rational strain *S. spinosa* MUV and *S. spinosa* MUV was further used for the metabolic engineering.

Culture conditions

S. spinosa, S. spinosa MUV and recombinants were cultured in different media for different purposes. For regeneration of spore it was cultured on regeneration medium (sucrose 10%, glucose 1%, yeast extract 0.5%, peptone 0.01%, $MgCl_2 \cdot 6H_2O$ 1%, KH_2PO_4 0.025%, $CaCl_2 \cdot 2H_2O$ 0.3%, agar 2%, pH 6.5 before autoclave and after autoclave 0.2% of trace element along with 2% TES buffer was added). The broth cultures were grown at 28°C in seed medium (starch 2%, glucose 1%, enzyme hydrolyzed casein 3%, yeast extract 0.3%, $MgSO_4 \cdot 7H_2O$ 0.2%, KH_2PO_4 0.05%, and pH 7.2 before autoclave). For spinosyn A and D production, the media was composed of glucose 4.5%, soybean meal 1%, yeast extract 1.4%, canola oil 2% and corn steep liquor 0.8% in distilled water and pH 6.8 was adjusted before autoclave; followed by inoculation with 5% seed of *S. spinosa* MUV and their recombinants into a baffled 500-ml flask containing 50 ml of the production medium and grown at 28°C on a rotatory shaker at 215 rpm for 8 days.

DNA manipulation and sequence analyses

PCR premix (Genotech, Korea) or TaKaRa *LA Taq*™ (Takara, Japan) was used according to the manufacturer's instructions to amplify the target DNA fragments. PCR was performed in a Thermal Cycler Dice (Takara). The amplification conditions for PCR were: initial denaturation at 94°C for 7 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 65-70°C for 1 min, polymerization at 72°C for 1 min; and gap filling at 72°C for 7 min. The PCR products were purified and cloned into the pGEM®-T Easy vector for DNA amplification and sequencing. DNA preparation, digestion, ligation and other DNA manipulations were performed using standard techniques for *E. coli* (Sambrook and Russell, 2001). The chemicals and enzymes used in this study were purchased from Sigma (USA). *In silico* analyses and comparisons of nucleotide and protein sequences were performed using the BLAST, FASTA, CLUSTALW, and GENEDOC programs.

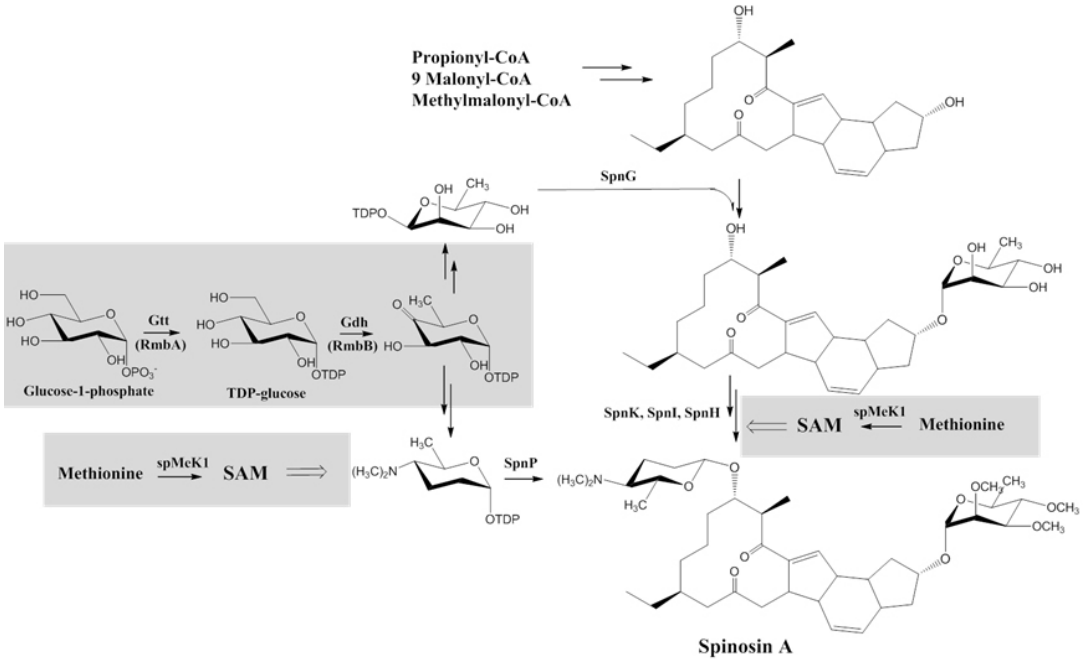

Fig. 1. Schematic diagram of approaches used for enhancement of the spinosyn production in *S. spinosa* MUV

Construction of the recombinant

The expression vector, pIBR25 (Sthapit et al., 2004) and integrative vector pSET152, (Bierman et al., 1992) under the control of strong *ermE** promoter, were used for construction of recombinant strains of *S. spinosa* MUV; using *metK1-sp*, *rmbA*, and *rmbB* for overproduction of spinosad. Oligonucleotides used to amplify *metK1-sp* (1,209 bp): *metK1-sp*-F- 5′GGA TCC AAG GAG GAT TCA TGT CCC GTC GTC TCT TCA 3′; *metK1-sp*-R- 5′AAG CTT TCA TGC CGC CAC CGC CTT GCG CTT AT 3′, *rmbA* (1,090 bp): *rmbA*-sp-F- 5′GGA TCC AAG GAG GTC TAC GGT GAA GGC ACT CGT ACT CTC T 3′; *rmbA*-sp-R- 5′GAA TTC TCA TGA GGA GAT CTG CAC CTT GCT 3′, and *rmbB* (1,010 bp): *rmbB*-sp-F-5′GAA TTC AAG GAG GTC CAA CAT GAC CAC GAA GAT CCT GGT G 3′; *rmbB*-sp-R-5′AAG CTT TCA CAG CGC CGC ACG CTC CTT CAG 3′ from genomic DNA of *S. peucetius* for expression vector “pIBR25”. Similarly for integrative vector “pSET152”**,** oligonucleotides used to amplify *metK1-sp*: *metK1-sp*-F-5′GGA TCC AAG GAG GAT TCA TGT CCC GTC GTC TCT TCA 3′; *metK1-sp*-R-5′AGA TCT TCA TGC CGC CAC CGC CTT GCG CTT AT 3′, *rmbA: rmbA*-sp-F-5′GAA TTC AAG GAG GTC TAC GGT GAA GGC ACT CGT ACT CTC T 3′; *rmbA*-sp-R- 5′ACT AGT TCA TGA GGA GAT CTG CAC CTT GCT 3′, and *rmbB: rmbB*-sp-F-5′ACT AGT AAG GAG GTC CAA CAT GAC CAC GAA GAT CCT GGT G 3′; *rmbB*-sp-R-5′GGA TCC TCA CAG CGC CGC ACG CTC CTT CAG 3′ from genomic DNA of *S. peucetius*. PCR was performed in a thermocycler (Takara, Japan). The PCR products were purified and cloned into pGEM-T Easy vector and sequenced prior to cloning in the expression vector and integrative vector to verify that no mutation had been introduced during the PCR amplification. After the sequence was analyzed, *metK1-sp* from *S. peucetius* was cloned into the *Bam*HI and *Hin*dIII sites of pIBR25 and *Bam*HI and *Bgl*II site of pSET152 to form the recombinant plasmid pSIBR and pSAM152, respectively. Similarly, combination of *rmbA* and *rmbB* from *S. peucetius* was cloned into the *Bam*HI/*Eco*RI and *Eco*RI/*Hin*dIII sites of pIBR25 and *Eco*RI/*Spe*I and *Spe*I/*Bam*HI site of pSET152 to form the recombinant plasmid pRMBIBR and pRMB152, respectively.

UV mutagenesis

High producing strain of *Saccharopolyspora spinosa* was obtained by rational screening procedure of UV mutagenesis. The spores were scraped from the agar surface, using a sterile loop. After filtering through cotton wool, the spore suspension was serially diluted and 5×10^{-5} cfu ml^{-1} of this suspension was poured and spread onto sterile glass plate containing regeneration medium. The plate without cover was exposed to UV irradiation for 22 s at the distance of 60 cm from UV lamp with wavelength of 254 nm and 40 W. After the UV irradiation, the plates were wrapped with aluminum foil to avoid photoreactivation and incubated at 28°C for 8 days. After 8 days of incubation, 99.9% of the spores were found to be dead, and the remaining colonies were transferred to new plate for stabilization and heavy growth of mutants. Mutants were cultured in seed medium followed by production medium to select the high producing rational strain of Saccharopolyspora spinosa. The high producing rational strain of Saccharopolyspora spinosa was obtained by HPLC analysis and designated as *S. spinosa* MUV.

Transformation and generation of recombinant strains

Protoplast transformation was done as previously described (Kieser et al., 2000). The recombinant pIBR25, pSIBR, pRMBIBR, pSAM152 and pRMB152 were propagated in *E. coli* JM110 to obtain demethylated DNA for transformation in the rational *S. spinosa* MUV. For protoplast transformation, *S. spinosa* MUV was cultured in 50 ml of seed medium and pH7.2 was adjusted before autoclave. After 60 h, the mycelium was harvested by centrifuging (3,200 RPM and 4°C for 12 min) and washed with 15 ml of sucrose solution (10.3%) , then centrifuged (3,200RPM and 4°C for 12 min), further washed with 15 ml of P-buffer. Finally, 10 ml of Lysozyme solution (2 mg ml^{-1} made in P-buffer) was added to cell pellets and incubated the content for 1 h

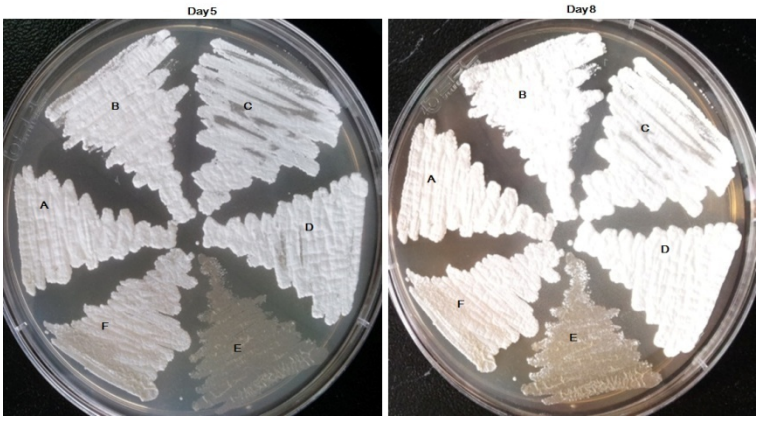

Fig. 2. Morphology of (A) *S. spinosa* ATCC83543.1, (B) *S. spinosa* MUV RMB152, (C) *S. spinosa* MUV RMBIBR, (D) *S. spinosa* MUV, (E) *S. spinosa* MUV SAM152, and (F) *S. spinosa* MUV SIBR on 5 and 8 days.

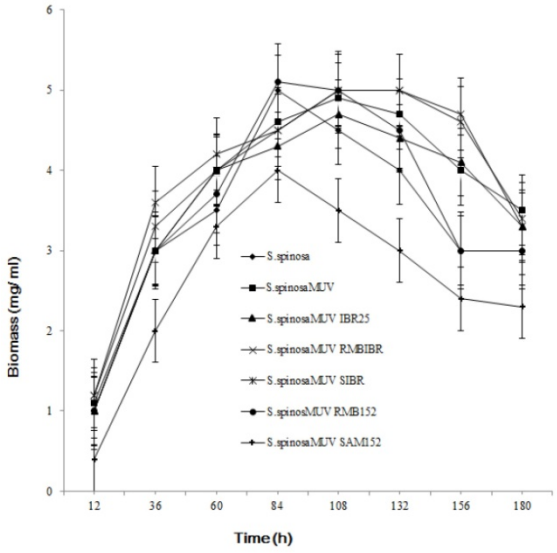

Fig. 3. Time course profiles of biomass obtained by introduction of pIBR25, pSIBR, pRMBIBR, pSAM152, and pRMB152 in *S. spinosa* MUV along with *S.spinosa* MUV and *S. spinosa* ATCC83543.1.

and 45 min at 37°C. After incubation, it was filtered and centrifuged for 12 min at 6,000 rpm, then washed with P-buffer twice, and mixed with 1 ml P-buffer. From the resulting mixture, 100 μl was mixed with 20 μl of plasmid DNA and 200 μl of 40% PEG1000 and centrifuged for 1min to discard the supernatant partially and mixed with 100 μl of P-buffer, finally it was plated on R2YE plate. The plates were incubated at 28°C for 24 h and then overlaid with 0.3% agar solution containing10 μg ml^{-1} thiostreptone and 60 μg ml^{-1} apramycin to select *S. spinosa* MUV recombinant with expression and integrative vector, respectively. After 1 week, thiostreptone and apramycin-resistant colonies were selected and transferred to regeneration medium for further conformation and heavy growth of colonies, then cultured in seed liquid media. Transformation was confirmed by isolation, PCR and restriction enzyme digestion of plasmid from each strain (data not included). The transformants were designated as, *S. spinosa* MUV IBR25, *S. spinosa* MUV SIBR, *S. spinosa* MUV RMBIBR, *S. spinosa* MUV SAM152, and *S. spinosa* MUV RMB152 (Table 1). Finally, for the integration of pSAM152 and pRMB152 in chromosomal DNA of *S. spinosa* MUV SAM152 and *S. spinosa* MUV RMB152, respectively, it was cultured up to 4 generation in seed medium with antibiotic. Integration was confirmed by isolation of total DNA of *S. spinosa* MUV SAM152 and *S. spinosa* MUV RMB152, followed PCR of *metk1-sp* and *rmbB*.

Analysis of growth on plate and in liquid medium

Growth and morphology of all the transformant including *S. spinosa* MUV and wild type was found to similar on regeneration plate media except *S. spinosa* MUV SAM152 which was found to be growing slower (Fig. 2). To analyze the growth in liquid, fermentation production of Spinosyns was monitored daily, once wild type, *S. spinosa* MUV and all transformants were cultured with 5% of 36 h old mycelia harvested from seed medium in different baffled 2 L flasks containing 0.15 L of production medium at 28°C and 200 rpm for 8 days. After 24 h of incubation, the cell pellets were collected at 24 h intervals until 180 h by centrifuging 10 ml of culture broth at 3,000 rpm for 15 min. The cell pellets were collected, washed with deionized water, and centrifuged twice. They were then placed at 80°C until their mass was constant to analyze the growth rate (Fig. 3), which was employed to prove best time for withdrawing spinosyn A and D.

Morphological analysis by field emission electron microscope

Wild type, *S. spinosa* MUV and their transformants was collected after 36 h from seed medium in different eppendorf tube. The cell pellets were collected by centrifuging 0.5 ml of culture broth at 3,000 rpm for 5 min. The cell pellets were collected, washed with 0.2 M phosphate buffer, pH 7.4, and centrifuged for 2 min thrice. They were prefixed with 2% glutaraldehyde at room temperature for 1 h and washed with 0.2 M phosphate buffer, pH 7.4, by centrifuge for 5 times. The prefixed and washed cell was mounted on SAM tube for dehydration and fixation. Dehydration was carried out with increasing concentration of ethyl alcohol 50%, 60%, 70%, 80%, 90%, 95% (4 min each) and 100% (8 min). The dehydrated cells were overlaid with ethanol denaturant (tert-butyl alcohol) at room temperature for 30 min, and were freeze-dried for 45 min. The resultant freeze-dried sample were coated with platinum at low temperature and examined in high performance field emission electron microscope “JSM-6700F” (CFE SEM) at × 5,000 and × 10,000 (Fig. 4).

Production, extraction and quantification of compound

To analyze the production, 5% seed of wild type, *S. spinosa* MUV and their transformants were cultured in production media at 28°C

Table 2. Production of spinosyn A and D

Bacterial strain	Spinosyn type A (mg L^{-1})	Spinosyn type D (mg L^{-1})
S. spinosa ATCC83543.1	50	27
S. spinosa MUV	244	129
S. spinosa MUV IBR25	246	129
S. spinosa MUV RMBIBR	372	217
S. spinosa MUV SIBR	351	220
S. spinosa MUV RMB152	337	217
S. spinosa MUV SAM152	230	121

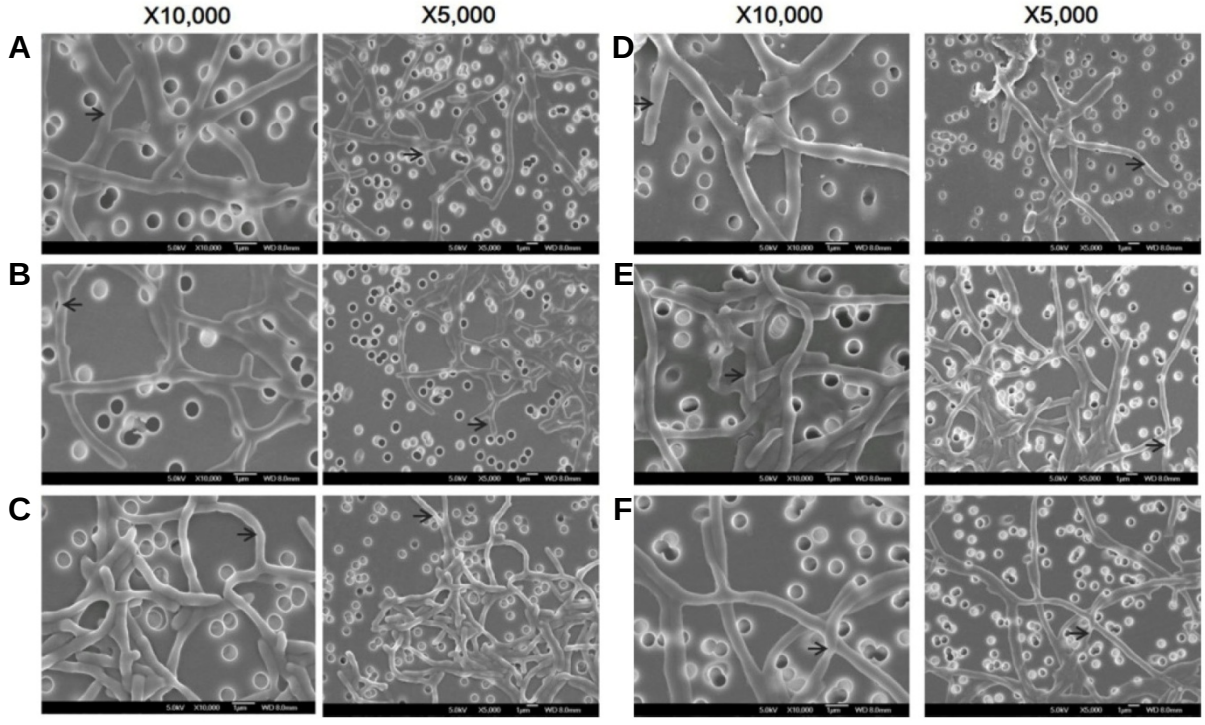

Fig. 4. SEM microscopy of (A) wild type strain, (B) *S. spinosa* MUV, (C) *S. spinosa*MUV SIBR, (D) *S. spinosa* MUV RMBIBR, (E) *S. spinosa* MUV SAM152, and (F) *S. spinosa* MUV RMB152. Bars = 1 μm. The arrows indicate the sites of enhanced spore separation.

for 8 days. One ml of culture broth of each strain was mixed with 2 ml of acetonitrile; vortexed for 5-8 seconds and left at room temperature for 30 min and then centrifuged at 12 K for 10 min to remove cell palates. The supernatant was filtrated using 0.22 μm syringe filter. The resulting filtrate was injected onto a C_{18}-reversed phase HPLC column (4.6 × 250 mm, 50 μm KANTO Reagents, Japan) under isocratic condition at 245 nm with flow rate 1 ml/min and the solvent system used was acetonitrile: methanol: 2% ammonium acetate buffer (2:2:1, v/v/v). Spinosyns yields in *S. spinosa* MUV and in their recombinant were determined by HPLC and comparison with a standard spinosyn A and D.

RESULTS AND DISCUSSION

Construction of recombinant vector

The *rmbA* (glucose-1-phosphate thymidylyltransferase), and *rmbB* (TDP-D-glucose-4,6-dehydratase) from *S. peucetius* are the enzymes that synthesized TDP-4-keto-6-deoxyglucose as the common intermediate for forosamine and tri-*O*-methylated rhamnose**.** Similarly, *metK1-sp* from *S. peucetius* plays an important role in the conversion of ATP and L-methionine to S-adenosyl-L-methionine (SAM) and may act as a methyl donor for the transmethylation reaction in the spinosyn gene cluster. Therefore, *metK1-sp,* and *rmbA* along with *rmbB* cloned into pIBR25 and pSET152 under the control of a strong promoter *ermE** to construct the recombinant plasmid pSIBR, pSAM152, pRMBIBR and pRMB152, respectively.

Development rational *S. spinosa*

Based on earlier studies, UV radiation, in the range of 200-300 nm, produces thymidine dimers and increases probability of deletion during the duplication process; therefore, *S. spinosa* was exposed to UV at 254 nm which led to the overproduction of spinosyn A and D. The resultant overproducing UV mutant was determined by HPLC analysis and finally, designated as *S. spinosa* MUV.

Construction of recombinant strain of *S. spinosa* MUV

We developed the recombinant strain of *S. spinosa* MUV by heterologous expression of *metK1-sp*, and *rmbA* along with

rmbB. MetK1-sp was used to make pSAM152 and pSIBR recombinant vector whereas *rmbA* along with *rmbB* used to make pRMBIBR and pRMB152. The pSAM152 and pSIBR were transformed into *S. spinosa* MUV via PEG-mediated protoplast transformation method. The transformed colonies were selected with appropriate concentration of antibiotic (apramycin 60 μg ml^{-1} and thiostrepton10 μg ml^{-1}), and plasmid DNA was isolated from selected colonies to conform the transformation. The resultant recombinant of *S. spinosa* MUV was designated as *spinosa* MUV SAM152 and *S. spinosa* MUV SIBR, respectively, which plays an important role in the conversion of ATP and L-methionine to S-adenosyl-L-methionine (SAM) and may act as a methyl donor for the transmethylation reaction in the spinosyn gene cluster that may include the two N-methyl groups of forosamine by *spnS*, and the three *O*-methyl groups of tri-*O*-methylrhamnose by *spnK, spnI and spnH* (Fig. 1). Similarly, *rmbA* and *rmbB* from *S. peuticus* was also amplified and the recombinant plasmid pRMBIBR and pRMB152 were constructed under the control of a strong promoter *ermE**. Introduction of pRMBIBR and pRMB152 into *S. spinosa* by protoplast transformation generated *S. spinosa* MUV RMBIBR and *S. spinosa* MUV RMB152, respectively, which may play the major role to alter the metabolic flux distribution in TDP-4-keto-6-deoxy-D-glucose (Fig. 1), which is the common intermediate for forosamine and tri-*O*-methylated rhamnose. We also found that chromosomal integration is congenitally stable and remains unchanged through many generations. Indeed, *rmbA* and *rmbB* may directly integrate into the promoters of the spinosyns gene cluster; hence strongly activate the genes involved in the spinosad biosynthesis, with an additional stimulatory effect on transcription.

Effect of growth and morphology on production

Surprisingly, we observed that the morphology of *S. spinosa* MUV SAM152 was drastically different from that of all the recombinant, *S. spinosa* MUV, and wild type on regeneration plate (Fig. 2). *S. spinosa* MUV SAM152 was found to be growing very slowly on plate and in liquid medium which may in turn has insignificant production of spinosyn type A and D in *spinosa* MUV SAM152, whereas *S. spinosa* MUV, *S. spinosa* MUV SIBR, *S. spinosa* MUV RMBIBR *S.* and *spinosa* MUV RMB152 exhibited a similar growth curve to the wild type (Fig. 3). Scanning electron microscope (SEM) was used to compare spores of wild-type and mutant strains (Fig. 4). Sample was coated with platinum in place of gold as its molecular mass is smaller than that of gold, which made it easier to study the morphology of cell. We found *S. spinosa* MUV SIBR, *S. spinosa* MUV RMBIBR and *S. spinosa* MUV RMB152 spores were cylindrical like *S. spinosa* MUV and were smaller in size and larger in number than that of *S. spinosa* MUV and wild type, which helps to maintain and increase mycelium life sustainability and cell mass, respectively. A good cell mass can be another cause for increased of spinosyn type A and D. Spore observed from *spinosa*MUV SAM152 was irregular and not distinct which made limited formation vegetative and aerial mycelium, therefore it was found to be slower in growth and lesser in production, whereas wild type spores were round and uniform in size.

Production of spinosyn A and D in recombinant strains

Recently, it was reported 405 mg L^{-1} spinosad can be produced where they didn't determine the type of spinosyn (till date) (Xue et al., 2013), but in our investigation we improve the production as well as determine the level of their type. To improve the production of spinosyn A and D, we develop *S. spinosa* MUV. Production of spinosyn A and D in *S. spinosa* MUV was 244 mg L^{-1} and 129 mg L^{-1}, which was 4.88 and 4.77-fold higher than that of wild-type (Table 2). Further production was carried out in *S. spinosa* MUV via efflux the spinosyn through *metK1-sp* which in turn found to be producing a higher amount after 6 days in culture and reached in stationary stage at 8 days. The production of spinosyn type A and D was found to be increased by 7.02 and 8.14-fold (351 and 220 mg L^{-1}) in *S. spinosa* MUV SIBR to that of wild type, respectively, whereas *spinosa* MUV SAM152 was not significant at 8 days, to that of *S. spinosa* MUV (Table 2). Similarly, *rmbA* with *rmbB* play the major role to alter the metabolic flux distribution in TDP-4-keto-6-deoxy-D-glucose (Fig. 1), which is the common intermediate for forosamine and tri-*O*-methylated rhamnose followed by highest yield of spinosyn A and D production at 8 days in compared to that of wild type, i.e. increased by 7.44/8.03-fold (372/217 mg L^{-1}) and 6.74/8.03-fold (337/217 mg L^{-1}) (Table 2). On the basis of the result, *S. spinosa* MUV RMBIBR was consider being the highest producing strain which produce 589 mg L^{-1} of spinosad (spinosyn A 372 mg L^{-1} and spinosyn D 217 mg L^{-1}) (Table 2).

In conclusion; industrially, spinosyn A and D are the most important polyketide. To improve the production of spinosyn A and D, people focused on random mutagenesis (Liang et al., 2009), duplication of gene cluster (Tang et al., 2011), genome shuffling (Jin et al., 2009) and overexpression of clustered genes (Luo et al., 2011; Xue et al., 2013). These techniques have succeeded in generating many industrial strains. Recently, overexpression of clustered genes enhances the production up to 405 mg L^{-1} spinosad (Xue et al., 2013). Still In terms of the industrial scale, this production is very small and comes with a high production cost. For the same purpose we focuse on strain improvement through random mutagenesis followed by genetic manipulation of the regulatory network. The results showed that application of metabolic engineering on rational strain of *S. spinosa* for the development of recombinant strain; favored as best way to obtain maximum spinosad production (589 mg L^{-1}). On the basis of these results, we conclude that the increased production of spinosad is due to metabolic regulation of genes, which favored as the best way to obtain maximum spinosad production till date and it could be harnessed for the feasible and cost effective production of spinosad at the industrial scale.

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