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Improvement of spinosad producing *Saccharopolyspora spinosa* by rational screening*

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Abstract: Spinosad (spinosyns A and D) is a mixture of secondary metabolites produced by *Saccharopolyspora spinosa*. It is used in agriculture as a potent insect control agent with exceptional safety to non-target organisms. Spinosyns are macrolides with a 21-carbon, tetracyclic lactone backbone to which the deoxysugars forosamine and tri-O-methylrhamnose are attached. According to the pathway and regulation of spinosad biosynthesis, a rational selection procedure with u.v. mutation was performed to obtain high spinosad producing strain. The spinosad resistant mutants, the rhamnose resistant mutants, the 2-deoxygen-D-glucose resistant mutants were selected, successively. Eventually, a strain *S. spinosa* 4~6 was obtained, with its production of spinosad reaching 268 mg/L, which is increased by 121% in comparison with that of the parent strain *S. spinosa* 1~5. The subculture experiments indicated that the hereditary character of high production of *S. spinosa* 4~6 is stable. The spinosad fermentation with *S. spinosa* 4~6 was scaled up in a 10 L fermentor, and a production of 458 mg/L was obtained, which was 71% higher than the production with shaking-flask fermentation.

Key words: Spinosad, *Saccharopolyspora spinosa*, Strain improvement, Rational screening
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INTRODUCTION

The spinosyns are novel macrolides produced by *Saccharopolyspora spinosa* (Kirst *et al*., 1991; Mertz and Yao, 1990). Spinosyns are comprised of a tetracyclic macrolide containing forosamine and tri-O-methylrhamnose, with different degrees of methylation on the polyketide or deoxysugars. The two major factors in *S. spinosa* fermentation, spinosyn A and spinosyn D, differ from each other by a single methyl substituent at position 6 of the polyketide (Fig.1). Spinosad, a combination of spinosyn A and spinosyn D, is used for control of agricultural insect pests, and is highly effective against target insects and has an excellent environmental and mammalian toxicological profile (Sparks *et al.*, 1998a; 1998b). Incorporation studies with ^{13}C-labelled acetate, propionate, 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 (Waldron *et al*., 2001a; 2001b). The polyketide portion of spinosyns differs from more common type I polyketides (e.g. erythromycin, rapamycin, or tylosin) in that it contains three intramolecular carbon-carbon bonds (Fig.1). Rhamnose biosynthesis is the limiting step in spinosad biosynthesis (Madduri *et al*., 2001a; 2001b).

Strain improvement by rational screening is an efficient method for enhancing antibiotics production (Jin *et al*., 2002a; 2002b; 2006). Based on the above analysis on the pathway and the metabolic regulation of spinosyns biosynthesis, improvement of *S. spinosa* 1~5 was performed by rational screening strategy to increase its production.

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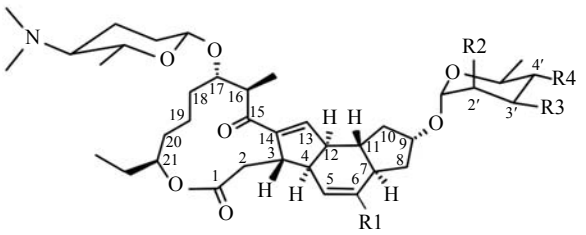

Factor	R1	R2	R3	R4
A	H	OCH_3	OCH_3	OCH_3
D	CH_3	OCH_3	OCH_3	OCH_3
H	H	OH	OCH_3	OCH_3
J	H	OCH_3	OH	OCH_3
K	H	OCH_3	OCH_3	OH
P	H	OCH_3	OH	OH

Fig.1 Structure of spinosyns. Forosamine and tri-O-methylrhamnose are attached at positions 17 and 9, respectively

MATERIALS AND METHODS

Microorganism

Saccharopolyspora spinosa 1~5 is the ancestor of all mutant strains described in this study.

Media and cultural conditions

Plate medium (g/L): enzyme-hydrolyzed casein, 30; yeast extract, 3; $MgSO_4 \cdot 7H_2O$, 2; glucose, 10; agar, 20; and adding distilled water to make up 1 L. The pH value of the medium was adjusted to pH 7.0~7.2 before autoclaving.

Slant medium (g/L): starch, 20; glucose, 5; yeast extract, 3; corn syrup, 10; $MgSO_4 \cdot 7H_2O$, 2; agar, 20; and adding distilled water to make up 1 L. The pH value of the medium was adjusted to pH 6.5 before autoclaving.

Slant and plate culture was incubated at 28 °C and 30%~60% relative humidity for 7~8 d.

The spores from slant culture were inoculated into a 250 ml Erlenmeyer flask containing 25 ml of seed medium (2% starch, 1% glucose, 3% enzyme-hydrolyzed casein, 0.3% yeast extract, 0.2% $MgSO_4 \cdot 7H_2O$, and 0.05% KH_2PO_4). After incubation at 28 °C on a rotary shaker at 220 r/min for 60 h, a 2 ml portion of the seed culture was used to inoculate 25 ml of production medium into a 250 ml Erlenmeyer flask.

The production medium contained: 6% glucose, 2% starch, 2% soybean meal, 1% fish meal, 1% corn syrup, 0.3% glutamine, 1% soybean oil, and 0.3% $CaCO_3$. The production culture was incubated under the same conditions as the seed culture, except that the cultivation period was extended to 7 d.

Mutation

We took 5 ml of spore suspension from slant culture of *S. spinosa*, and then transferred it to an aseptic plate. The plate, with cover removed, was exposed to u.v. irradiation for 40 s at a distance of 30 cm from u.v. lamp with wavelength of 253.7×10^{-10} m and power of 30 W.

Screening agents and screening for agent resistant mutants

Fifty mg/L spinosad, 1 mg/L spinosad, 1 mg/L rhamnose, 5 mg/L rhamnose, 1.7 mg/L 2-deoxy-D-glucose (2-DOG) and 5 mg/L 2-DOG were used as screening agents, respectively. The spore suspension of the parent strains after u.v. irradiation (the survival ratio was about 19.0%) was spread on medium containing one of the above agents, and the agent resistant mutants were screened from the above medium. The spore suspension with or without u.v. irradiation was also spread on agar medium without screening agent for comparison. Fifty colonies were isolated from each group, and after shaking flask fermentation, spinosad concentration was measured and compared.

Analytical method

Reduced sugar was measured by Fehling's reagent method (Chen and Xu, 1991). Amino nitrogen was analyzed by formaldehyde titration method (Chen and Xu, 1991). The dried cell weight (DCW) was determined according to the method described by Leblihi *et al*.(1987). Spinosad was assayed by HPLC method (Zhang *et al*., 2003).

RESULTS AND DISCUSSION

Screening strategy

Spinosad was produced by *S. spinosa*. In order to increase its production, a new screening procedure was tried. Spinosad is the secondary metabolite produced by *S. spinosa* and causes feedback inhibition and repression on spinosad biosynthesis. According to the biosynthetic pathway of spinosad, rhamnose

biosynthesis is the limiting step in spinosad biosynthesis (Madduri *et al*., 2001a; 2001b). Catabolite repression is a common cause of inhibition of cell growth and antibiotic production. So we examined the tolerance of the original strain, *S. spinosa* 1~5, to spinosad, rhamnose and 2-DOG for cell growth. The results indicated that *S. spinosa* 1~5 is susceptible to spinosad, rhamnose and 2-DOG, and that when the initial concentration of these agents in agar medium is 50 mg/L, 1 mg/L and 1.7 mg/L, respectively, the inhibitory ratio for cell growth is 92%, 96.7% and 97.8%. The above experimental results explained that a high concentration of these agents in the medium was harmful for cell growth; and that therefore, spinosad synthesis would certainly be affected. It is necessary to screen for strains resistant to these agents.

Spinosad resistant mutant strains

Spinosyns is a mixture of secondary metabolites produced by *S. spinosa*. Accumulation of spinosyns in the fermentation process is not advantageous to the growth of its producing strain, and will also inhibit the pathway of spinosyns biosynthesis. Based on such consideration we designed a rational screening process in which we use spinosad as the screening agent to get the spinosad resistant strain.

After u.v. irradiation, the spinosad resistant mutant strains were screened. The abilities of these strains to produce spinosad were examined, and the spinosad productions of strains without or with u.v. irradiation grown on the spinosad-free medium were also examined. After testing 100 spinosad resistant mutants, 50 u.v. mutants, 50 natural isolates, it was found that the spinosad productions of the spinosad resistant mutants were higher than those of the natural isolates and u.v. mutants. Among the spinosad resistant mutants, we obtained a mutant whose spinosad production was 184 mg/L, which is 52% more than the parental strain. This mutant was named *S. spinosa* 2~7.

Rhamnose resistant mutant strains

After u.v. mutation, the rhamnose resistant mutants were selected from *S. spinosa* 2~7. After analysis of 100 rhamnose resistant mutants, 50 u.v. mutants, and 50 natural isolates, it was found that the spinosad productions of the rhamnose resistant mutants were higher than those of the natural isolates and u.v. mutants. Among them, a rhamnose resistant mutant *S. spinosa* 3~9, whose spinosad prduction was 206 mg/L, an increase of 12% over that of the parental strain *S. spinosa* 2~7, was obtained.

According to the biosynthetic pathway of spinosad, rhamnose biosynthesis is the limiting step in spinosad biosynthesis. So rhamnose is a significant precursor in spinosad biosynthesis. The rhamnose resistance brought higher production, which suggested that the regulation in rhamnose biosynthesis by a rhamnose resistant mutant might be altered so that more rhamnose could be produced. The abundant supply of rhamnose would cause higher production.

2-deoxy-D-glucose resistant mutant strains

The biosynthesis of the microbial secondary products is often repressed by the catabolite of the fast-consumed carbon sources, for instance the glucose. In industry, glucose is often added at low concentration to avoid such inhibition. 2-DOG is the analog to the glucose. The 2-DOG resistant mutants can alleviate the catabolite repression caused by the fast-consumed carbon sources, therefore they cause the higher spinosad production.

After u.v. mutation, the 2-DOG resistant mutants were screened from *S. sprnoa* 3~9. The abilities of these strains to produce spinosad were examined, and the spinosad productions of strains without or with u.v. irradiation grown on the 2-DOG-free medium were also analysed for comparison. Analysis of 100 2-DOG resistant mutants, 50 u.v. mutants, 50 natural isolates revealed that the spinosad productions of the 2-DOG resistant mutants were obviously higher than those of the natural isolates and u.v. mutants; and that the spinosad productions of the 5 mg/L 2-DOG resistant mutants were higher than those of the 1.7 mg/L 2-DOG resistant mutants. Among the 5 mg/L 2-DOG resistant mutants, a mutant *S. spinosa* 4~6, whose spinosad production was 268 mg/L with an increase of 43% compared to *S. spinosa* 3~9, was obtained.

As described above, the strain *S. spinosa* 4~6 was characterized by spinosad^r, rhamnose^r and DOG^r respectively. It is inferred that *S. spinosa* 4~6 can alleviate catabolite repression caused by carbon sources, feedback repression and inhibition to spinosad biosynthesis by spinosad itself, and provide more rhamnose for spinosad biosynthesis, all of which are

favorable for spinosad production. The genealogical tree of *S. spinosa* 4~6 is shown in Fig.2. The spinosad production of *S. spinosa* 4~6 reaches 268 mg/L, an increase of 121% over that of the initial strain *S. spinosa* 1~5.

S. spinosa	Spinosad production (mg/L)
1~5	121
↓ u.v.+50 mg/L spinosad	
2~7	184
↓ u.v.+5 mg/L rhamnose	
3~9	206
↓ u.v.+5 mg/L 2-DOG	
4~6	268

Fig.2 The genealogical tree of *S. spinosa* 4~6

Hereditary stability of *S. spinosa* 4~6

The hereditary stability of *S. spinosa* 4~6 was examined by a colonial subculture method. There was little fluctuation in production among five generations (Table 1). This showed that the genetic stability of the high spinosad producing strain *S. spinosa* 4~6 was very good.

Table 1 Production of slant of each generation of *S. spinosa* 4~6

Generation	Production (mg/L)
1	268
2	255
3	300
4	263
5	249

Spinosad fermentation in 10 L fermentor with *S. spinosa* 4~6

Batch fermentation of spinosad with *S. spinosa* 4~6 was performed in a 10 L fermentor, and fermentation with *S. spinosa* 1~5 was also performed in a 10 L fermentor for control. The time courses of batch fermentation with *S. spinosa* 4~6 and 1~5 are shown in Fig.3.

Fig.3 shows that spinosad is only produced at the later stage of exponential growth phase and increases rapidly at stationary phase. Therefore the fermentation is biphasic. In the fermentation process with *S. spinosa* 4~6, the mycelium concentration is higher than that with *S. spinosa* 1~5, and the spinosad biosynthesis more earlier than that with *S. spinosa* 1~5. The production of spinosad fermentation in 10 L fermentor with *S. spinosa* 4~6 can reach as high as

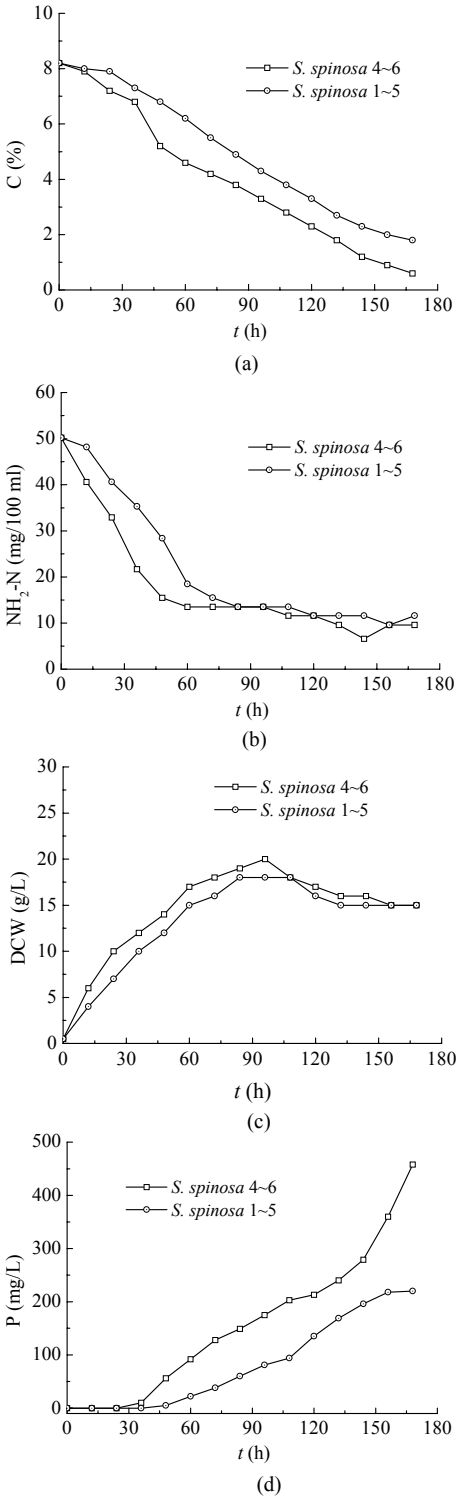

Fig.3 Batch fermentation process of spinosad with *S. spinosa* 4~6 and 1~5. (a) Reduced sugar; (b) Amino nitrogen; (c) Dried cell weight; (d) Product

458 mg/L after 7-days fed-batch fermentation, which was 71% higher than that with flask fermentation, while the production of spinosad fermentation in 10 L fermentor with *S. spinosa* 1~5 can reach only 220 mg/L. The proportion of spinosyn A and spinosyn D in the fermentation broth with *S. spinosa* 4~6 and *S. spinosa* 1~5 is both about 85:15.

Sugar is consumed rapidly in the exponential growth phase and the stationary phase. In the stationary phase the mycelium growth almost stopped, so in this phase the consumed sugar was primarily used for spinosad biosynthesis and mycelium maintenance. The amino nitrogen is consumed fast in the first 48 h of cultivation, which corresponds with the decrease in the pH value of the fermentation broth. After that the amino nitrogen remains relatively stable and the pH value is stable. In the fermentation process with *S. spinosa* 4~6, sugar and the amino nitrogen are consumed more rapidly than with *S. spinosa* 1~5.

CONCLUSION

According to the pathway and regulation of spinosad biosynthesis, a rational selection procedure with u.v. mutation was performed to obtain high spinosad producing strain. A strain *S. spinosa* 4~6 with spinosad^r, rhamnose^r and DOG^r was obtained, and its production of spinosad reached 268 mg/L which is 121% higher than that of the initial strain *S. spinosa* 1~5. The hereditary character of high production of *S. spinosa* 4~6 is stable. The spinosad fermentation with *S. spinosa* 4~6 was scaled up in a 10 L fermentor, and a production of 458 mg/L was obtained, which was 71% higher than the production with the shaking-flask fermentation.

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