Appl Biochem Biotechnol (2009) 159:655–663
DOI 10.1007/s12010-008-8500-0

Enhanced Production of Spinosad in *Saccharopolyspora spinosa* by Genome Shuffling

Z. H. Jin · B. Xu · S. Z. Lin · Q. C. Jin · P. L. Cen

Received: 1 August 2008 / Accepted: 17 December 2008 /
Published online: 9 January 2009

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. In this study, we applied genome shuffling of *S. spinosa* to achieve a rapid improvement of spinosad production. Ten strains with subtle improvements in spinosad production were obtained from the populations generated by the mutation with nitrosoguanidine and ultraviolet irradiation, and then they were subjected for recursive protoplast fusion. After four rounds of genome shuffling, a high yielding strain, designated as *S. spinosa* 4-7, was successfully isolated. Its production reached 547 mg/L, which was increased by 200.55% and 436.27% in comparison with that of the highest parent strain and the original strain, respectively. The subculture experiments indicated that the high producer of *S. spinosa* 4-7 was stable. Spinosad fermentation experiments by *S. spinosa* 4-7 were carried out in a 5-L fermentor, and its production of spinosad reached 428 mg/L after 168 h of fermentation.

Keywords Genome shuffling · Protoplast fusion · Spinosad · *Saccharopolyspora spinosa*

Introduction

Spinosad (spinosyns A and D), a bioinsecticide derived from the fermentation of the soil microorganism *Saccharopolyspora spinosa*, was first registered in 1997 and is now widely used as a field pest control agent on many crops [1–4]. Spinosyns comprised a tetracyclic macrolide containing forosamine and tri-*O*-methyl rhamnose, with different degrees of methylation on polyketide or deoxysugars. The two major components in the *S. spinosa*

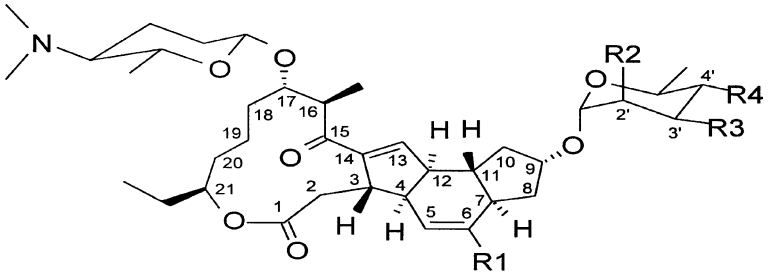
Z. H. Jin (✉) · B. Xu · S. Z. Lin · Q. C. Jin
Department of Biochemical and Pharmaceutical Engineering, Ningbo Institute of Technology, Zhejiang University, 315100 Ningbo, People's Republic of China
e-mail: zhking@nit.zju.edu.cn

Z. H. Jin · B. Xu · P. L. Cen
Institute of Bioengineering, College of Materials Science and Chemical Engineering, Zhejiang University, 310027 Hangzhou, People's Republic of China

fermentation, spinosyn A and spinosyn D, differ from each other by a single methyl substituent at position 6 of the polyketide (Fig. 1). Spinosad is highly effective against target insects and has an excellent environmental and mammalian toxicological profile [5, 6]. Incorporation studies with ^{13}C-labeled 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 [7, 8]. 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). According to the pathway and regulation of spinosad biosynthesis, a rational section procedure with UV mutation was performed and the increase of 121% in spinosad production was obtained [9]. Most of the genes involved in spinosyn biosynthesis are clustered in a 74-kb region of the *S. spinosa* genome. The spinosyn 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 [7, 8]. Duplication of the polyketide synthase genes stimulated the final step in the biosynthesis—the conversion of the pseudoaglycones with less forosamine to the end products. Duplication of genes involved in the early steps of deoxysugar biosynthesis also increased spinosyn yield significantly [10].

Strain improvement has been the hallmark of all industrial fermentation processes [11]. At present, strain improvement has been achieved chiefly through classical strain improvement technology and modern genetic engineering technology. Although mutation and random selection methods have succeeded in generating many industrial strains, it is a time-consuming and high-cost process. Rational metabolic and cell engineering approaches have also been successful in improving strain performance in several cases [12, 13]. However, such attempts were limited to the manipulation of only a handful of genes encoding enzymes and regulatory proteins selected from available information and research experience.

Genome shuffling is one of the most efficient methods for evolution of strains toward desirable phenotypes. The different genes associated with production can be recombined



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

during several rounds of genome shuffling and the good phenotypes can be obtained. Genome shuffling has been used successfully to improve the production of the polyketide antibiotic tylosin in *Streptomyces fradiae* [14], to improve acid tolerance in *Lactobacillus* [15, 16], to enhance resistance to the toxicity of pentachlorophenol in *Sphingobium chlorophenolicum* [17], and to increase resistance to (2S, 3R)-hydroxycitric acid in *Streptomyces* sp. [18]. However, so far, there seems to be no report to improve spinosad-producing strain by genome shuffling.

In the present study, genome shuffling was used to increase yield of spinosad in the fermentation with *S. spinosa*. By comparing the overproducing strain with the parent strain in fermentation experiment, some information about spinosad fermentation process was also acquired, which would be useful for scale-up of spinosad fermentation.

Materials and Methods

Microorganism

S. spinosa y-2 was used as the initial strain and was cryopreserved in 40% (*v*/*v*) glycerol at −80 °C.

Media and Cultural Conditions

Plate medium (in grams per liter): enzyme-hydrolyzed casein, 30; yeast extract, 3; $MgSO_4 \cdot 7H_2O$, 2; glucose, 10; agar, 20. The pH of the medium was adjusted to 7.0–7.2 before autoclaving.

Slant medium (in grams per liter): starch, 20; glucose, 5; yeast extract, 3; corn syrup 10; $MgSO_4 \cdot 7H_2O$, 2; agar, 20. The pH of the medium was adjusted to 6.5 before autoclaving. Slant and plate culture was incubated at 28 °C and 30–60% relative humidity for 7–8 days.

The spores from slant culture were inoculated to 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 rpm for 60 h, a 2-mL portion of the seed culture was used to inoculate 25 mL of production medium in a 250-mL Erlenmeyer flask.

The regeneration plate medium contains (per liter) 100 g sucrose, 10 g glucose, 5 g yeast extract, 0.1 g peptone, 10 g $MgCl_2 \cdot 6H_2O$, 0.25 g KH_2PO_4, 3 g $CaCl_2 \cdot 2H_2O$, 2 mL trace element solution, 20 mL TES buffer (5.73%, adjusted to pH 7.2), and 20 g agar. The pH value was adjusted to 6.5 before autoclaving.

The production medium contains 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 periods extended to 7 days.

Preparation of the Starting Mutants of Genome Shuffling

Ten milliliters of spore suspension of *S. spinosa* y-2 was transferred to an aseptic plate with a rotor. The plate, with the cover removed, was exposed to UV radiation for 90 s at a distance of 30 cm from a UV lamp with a wavelength of 253.7 nm and a power of 15 W. Then, 0.01% NTG was added into the suspension and treated for 45 min. The suspension of surviving spores was diluted and spread onto agar medium and cultured at 28 °C for 8–10 days.

Single colonies were transferred into a 250-mL Erlenmeyer flask with 25 mL seed medium. After incubation, a 2-mL portion of the seed culture was added to a 250-mL Erlenmeyer flask containing 25 mL of production medium and cultured on a rotary shaker at 220 rpm and 28 °C for 7 days.

After fermentation tests, the spinosad yield of each colony was analyzed by high-performance liquid chromatography (HPLC) and ten mutants with high production were used in the first round of genome shuffling.

Genome Shuffling

Genome shuffling was carried out using modified methods [14, 18, 19]. Spore suspension of the starting strains were cultured at 25 mL of seed medium in a 250-mL Erlenmeyer flask, respectively. After incubation, cells were harvested by centrifugation at 4,000×*g* for 10 min at 4 °C, washed twice with 10 mL of protoplast (p) buffer [20], and treated with lysozyme (2 mg/mL in p buffer) at 35 °C for 2 h. Once protoplast formation was observed with a phase-contrast microscopy, lysozyme was removed and protoplasts were fused by suspension in 5 mL of p buffer containing 40% PEG 4000 for 5 min. After washing twice with 10 mL of p buffer, diluted properly, 100 μL suspensions was spread onto regeneration plate medium. The plates were incubated at 28 °C and 30–60% relative humidity. After 10- to 12-day incubation, the colonies which appeared were isolated to carry out fermentation tests in shaking flask. After fermentation tests, the spinosad production of each colony was analyzed by HPLC and ten mutants with the highest production were selected as the starting strains of the next round genome shuffling. The detailed procedure of genome shuffling was illustrated in Fig. 2.

Spinosad Fermentation of High-Producing Recombinants in 5-L Fermentor

A loop of slant culture of the high-yield strain or the initial strain *S. spinosa* y-2 was inoculated into 100 mL of seed medium in a 500-mL Erlenmeyer flask. After incubation for 60 h, 100 mL seed broth was inoculated to 3-L production medium in a 5-L stirred-tank fermentor (Fermentor, Korea) and incubated at 28 °C at an agitation speed of 400 rpm and an aeration rate of 1 vvm.

Analytical Method

Glucose was measured by Fehling's reagent method [21]. Amino nitrogen was analyzed by the formaldehyde titration method [21]. Biomass was determined gravimetrically as dry cell weight by filtering the sample on a pre-weighed filter paper and drying at 70 °C. Spinosad was assayed by HPLC [22].

Results

Selection of Starting Strains for Genome Shuffling

Genome shuffling requires a diverse population of mutants that already show some improvement compared with the initial strain [14, 18]. In this study, *S. spinosa* y-2, with spinosad production of 102 mg L^{-1}, was mutated with UV and NTG treatment to obtain the starting strains for genome shuffling. After mutation, 210 colonies were screened for high

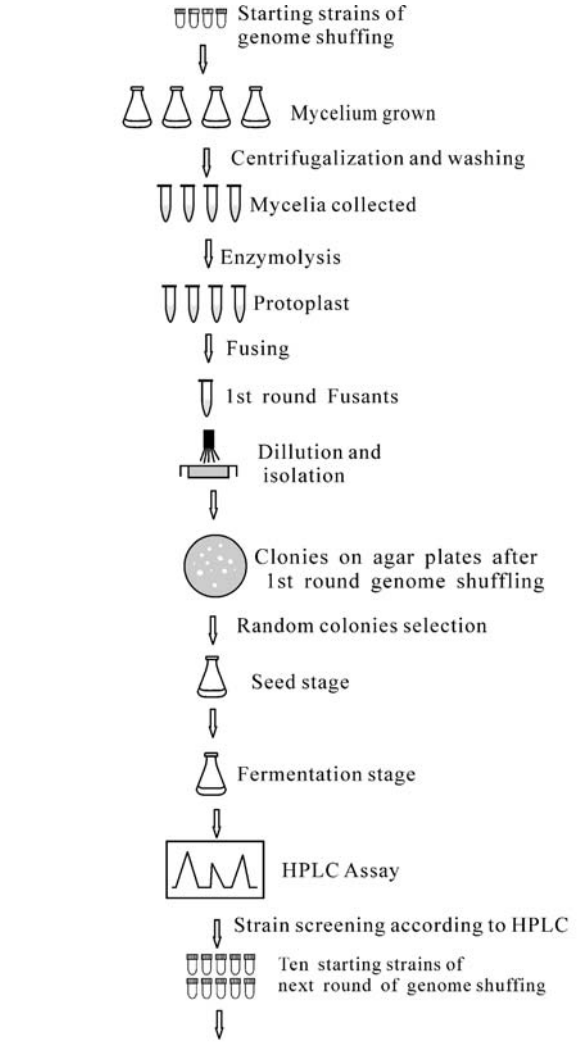


Fig. 2 A procedure of enhancing spinosad production by genome shuffling

spinosad production by the primary and secondary screening. The primary screening was carried out in a single-shake flask fermentation test, while the secondary screening was performed in a triplicate-shake flask fermentation test. Ten mutants with the highest spinosad production were selected. As shown in Table 1, the spinosad yield of the top ten mutants was in range of 136 to 182 mg L^{-1}. The highest production with the mutant UN-90 was an increase of 78.43% compared with the parent strain y-2. The ten mutants were then selected as starting strains for genome shuffling.

Improvement of Spinosad Production During Four Rounds of Genome Shuffling

Four rounds of genome shuffling were carried out according to the procedure shown in Fig. 2, and the results showed that the spinosad production increased gradually. As shown

Table 1 Spinosad production of top ten mutants or recombinants after mutation and genome shuffling in each screening.

UV and NTG mutagenization		First round genome shuffling		Second round genome shuffling		Third round genome shuffling		Fourth round genome shuffling	
Strain	Yield (g/L)	Strain	Yield (g/L)	Strain	Yield (g/L)	Strain	Yield (g/L)	Strain	Yield (g/L)
UN-3	0.148	1–12	0.347	2–5	0.390	3–7	0.442	4–7	0.547
UN-23	0.142	1–15	0.386	2–25	0.416	3–16	0.421	4–12	0.493
UN-31	0.136	1–16	0.359	2–43	0.409	3–19	0.459	4–16	0.519
UN-45	0.153	1–36	0.399	2–54	0.378	3–23	0.472	4–28	0.486
UN-61	0.155	1–42	0.345	2–72	0.364	3–28	0.476	4–29	0.496
UN-82	0.147	1–55	0.348	2–85	0.356	3–33	0.467	4–36	0.482
UN-87	0.151	1–76	0.389	2–92	0.373	3–39	0.453	4–37	0.513
UN-90	0.182	1–83	0.373	2–96	0.383	3–47	0.462	4–42	0.498
UN-135	0.149	1–112	0.362	2–103	0.385	3–51	0.478	4–46	0.504
UN-166	0.156	1–146	0.392	2–114	0.393	3–76	0.462	4–48	0.487

in Table 1, after the first round of genome shuffling, the spinosad production of the top ten strains reached 0.345 to 0.399 g L^{-1}. Among them, the highest increase was found in the recombinant 1-36, and it was 291.1% higher than the initial strain y-2. After the second round of genome shuffling, the spinosad production of the top ten strains reached 0.356 to 0.416 g L^{-1}. Among them, the highest increase was found in the recombinant 2-25, and it was 307.8% higher than the initial strain y-2.

In the third and fourth rounds of genome shuffling, spinosad production of the top ten recombinants reached 0.421 to 0.478 g L^{-1} and 0.482 to 0.547 g L^{-1}, respectively. Among them, the highest increase was found in the recombinant 4-7 and its production reached 0.547 g L^{-1}, which was increased by 200.55% and 436.27% in comparison with the highest parent strain UN-90 and the original strain y-2, respectively.

As a control, the spore suspension of mutant UN-90 was treated by four rounds of UV and NTG compound mutation. After each round, about 200 colonies on the agar plates were screened for high spinosad production to determine whether additional mutation could lead to further improvement of spinosad production. The results showed that there was only a little increase in spinosad production and that the highest production was only 0.276 g/L, an increase of 51.65% compared with the parent strain UN-90. This indicates that genome shuffling is much more efficient for evolution of high-producing strains than mutation. As another control, mutant UN-90 was treated by four rounds of protoplast preparation and regeneration without fusion. After each round, about 200 colonies on the agar plates also were screened for high spinosad production to determine whether protoplast formation and regeneration could lead to great improvement of spinosad production. The results showed that the highest production was only 0.258 g/L, an increase of 41.76% compared with the parent strain UN-90. It indicates that in genome shuffling process, genomes from ten mutants might have recombined, resulting in great enhancement of yield.

Stability of High-Yield Recombinants

The genetic instability is a very important issue for the high-producing strains originated from various treatments of mutation or recombination. The genetic stability of high spinosad production of *S. spinosa* 4-7 was evaluated by five successive sub-cultivation tests. The result showed that the spinosad yield was 0.556, 0.541, 0.543, 0.549, and

0.552 g L^{-1}, respectively, suggesting that the high-spinosad-producing recombinant *S. spinosa* 4-7 was stable.

Spinosad Fermentation by the High-Producing Recombinant 4-7 in a 5-L Fermentor

Experiments of spinosad fermentation by the high-producing recombinant 4-7 and the initial strain y-2 were carried out in a 5-L fermentor. As shown in Fig. 3, in the fermentation process with *S. spinosa*, spinosad is only produced at the later stage of exponential growth phase and at stationary phase. Therefore, the fermentation is biphasic. In the fermentation process with *S. spinosa* 4-7, the mycelium concentration is higher than that with *S. spinosa* y-2. The production of spinosad fermentation in the 5-L fermentor with *S. spinosa* 4-7 can reach as high as 428 mg/L after 7-day fed-batch fermentation, while the production of spinosad fermentation in the 5-L fermentor with *S. spinosa* y-2 can reach only 101 mg/L.

From Fig. 3a, b, 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 a pH decrease. In the fermentation process with *S. spinosa* 4-7, sugar and the amino nitrogen are consumed more rapidly than those with *S. spinosa* y-2.

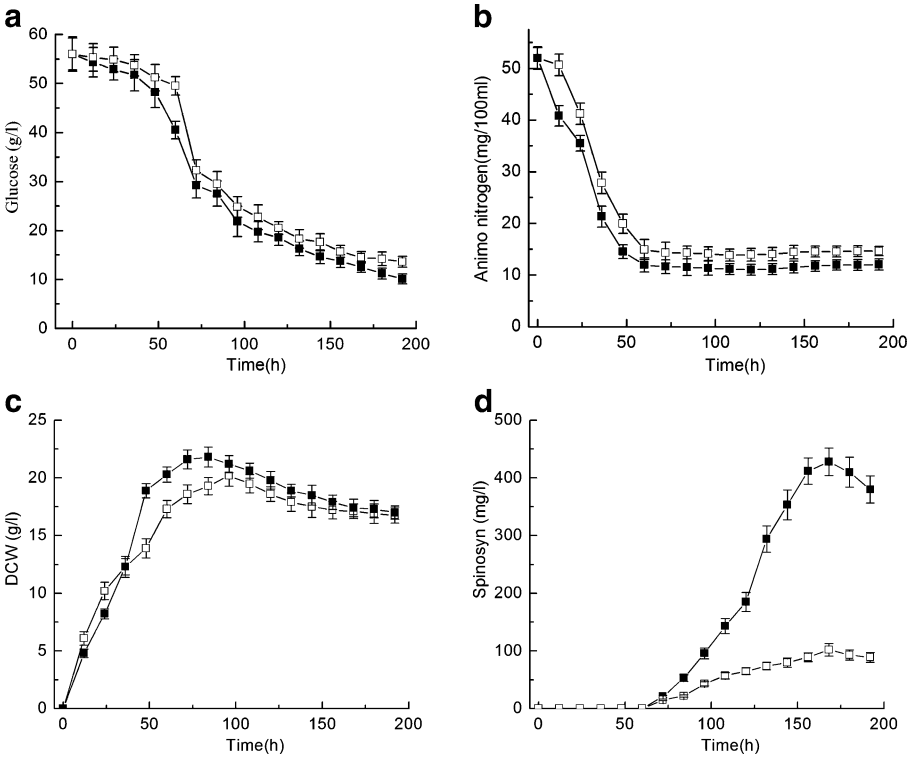


Fig. 3 Time courses of spinosad fermentation by recombinant *S. spinosa* 4-7 and the initial *S. spinosa* y-2. *Open symbols*, strain *S. spinosa* y-2; *closed symbol*, strain *S. spinosa* 4-7. *Error bars* represent standard deviations

Discussion

Conventionally, strain improvement has been achieved through mutation and selection or genetic recombination. Overproduction of primary and secondary metabolites is a complex process, and successful development of improved strains requires knowledge of physiology, pathway regulation, and control. The high-producing strains are generally obtained by various methods of chemical and physical mutations as well as direct gene manipulation. Because of the complexity in the pathway of antibiotic biosynthesis, the antibiotics production is seldom enhanced by applying direct gene manipulation. The chemical and physical mutation strategies are still the most commonly applied in the antibiotics production industry, but it is tedious and with low efficiency.

In this study, the initial strain *S. spinosa* y-2 was derived from a wild-type strain by working with various mutagens for several years. The result indicated that only a slight improvement was achieved by traditional mutation strategies, such as shown in Table 1. The highest mutant UN-90, derived from the UV and NTG compound mutation, was only an increase of 78.43%. Even through four rounds of UV and NTG compound mutation, there was only an increase of 51.65% compared with the mutant UN-90.

Genome shuffling is based on the genome recombination without the knowledge of detailed genome information. The application of protoplast fusing technique allows genomes from different genetic backgrounds to recombine, resulting in great enhancement of yield. As shown in Table 1, after four rounds of genome shuffling, the recombinant *S. spinosa* 4-7 was screened out and its spinosad production reached 0.547 g L^{-1}, which was increased by 200.55% and 436.27% compared with the parent mutant UN-90 and the initial strain *S. spinosa* y-2, respectively. The results indicated that genome shuffling is a much more powerful and efficient means for breeding of improved organisms, especially for production strains that underwent classic strain improvement many times.

Conclusion

Genome shuffling was used for the enhancement of spinosad production in *S. spinosa*. After four rounds of genome shuffling, a high-producing recombinant *S. spinosa* 4-7 was obtained. Its production reached 547 mg/L, which was increased by 200.55% and 436.27% in comparison with that of the highest starting strain UN-90 and the original strain y-2, respectively. The hereditary character of high production of *S. spinosa* 4-7 was stable. The production of spinosad fermentation by *S. spinosa* 4-7 in a 5-L fermentor could reach 428 mg/L after 168 h of fermentation.

Acknowledgments This work was financially supported by the Natinal Basic Research Program (973) of China (no. 2007CB714305), the National Natural Science Foundation of China (no. 20576122), and the Natural Science Foundation of Zhejiang Province, China (no. Y404291).

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