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ORIGINAL ARTICLE

Improvement of the ability to produce spinosad in *Saccharopolyspora spinosa* through the acquisition of drug resistance and genome shuffling

Hang Wang · Wei Xue · Yong-Mei He · Ri-He Peng · Quan-Hong Yao

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Abstract Spinosyns, a secondary metabolite from the fermentation of *Saccharopolyspora spinosa*, exhibits evident insecticidal activity. The most active components of the spinosyns family are spinosyns A and D, which are macrocyclic lactone antibiotics. Spinosad is a defined combination of the two principal fermentation factors, spinosyns A and D. Spinosad is used on grain storage, vegetable and fruit crops, ornamentals, and turf for pest control because it is toxic to many insects, but relatively nontoxic to mammals. In this study, we combined drug resistance screening and genome shuffling to achieve rapid improvement of spinosad yield of *S. spinosa*. The starting mutant population was generated by UV irradiation of *S. spinosa* ATCC 49460 protoplasts, which were then screened for erythromycin or neomycin resistance. Two mutant strains, Ery-13 (erythromycin resistant) and Neo-127 (neomycin resistant), were selected according to their spinosad yield. The highest titers of Ery-13 and Neo-127 strain reached 188 μg/ml and 165 μg/ml, respectively, which are 3.7-fold and 3.3-fold higher than that of the parental strain ATCC 49460. After four rounds of genome shuffling, an improved recombinant EN4-33 with both erythromycin and neomycin resistance was obtained. The highest spinosad yield of the recombinant EN4-33 reached 332 μg/ml, which is 6.6-fold higher than that of ATCC 49460. Results demonstrated that combining genome shuffling with antibiotics resistance screening is an effective approach for the molecular breeding of high-producing strains.

Keywords Erythromycin · Genome shuffling · Neomycin · Spinosad · *Saccharopolyspora spinosa*

Introduction

Spinosyns are highly effective insecticides derived from a naturally occurring Actinomycetes bacterium, *Saccharopolyspora spinosa*. Spinosad, a mixture of spinosyn A and D, which are the two most active components in the family of spinosyn, is produced by fermentation of *S. spinosa* (West and Turner 2000). Spinosyns comprise a tetracyclic macrolide containing forosamine and tri-O-methyl rhamnose, with different degrees of methylation on polyketides or deoxysugars (Fig. S1). The two major components of *S. spinosa* fermentation, spinosyns A and D, differ by a single methylsubstituent at position 6 of polyketides (Jin et al. 2009). Other components with different methylation levels during fermentation are less active. Spinosad has relatively low toxicity on predatory insects, mammals, and aquatic animals. A chronic toxicity test in mammals showed that spinosad has no carcinogenic, teratogenic, mutagenic, or neurotoxic effects (Breslin et al. 2000; Stebbins et al. 2002; Hanley et al. 2002; Yano et al. 2002). Furthermore, spinosad has a short half-life and cannot persist for long periods in the environment (Salgado and Sparks 2005).

The advantages and excellent environment profile of spinosad have driven the use of spinosad-based insecticides for pest control (Huang et al. 2009). Nevertheless, the production of spinosad from original strains is extremely low (Kirst 2010).

In this study, the neomycin resistance screening is used as an effective approach for high productive strains. Introduction of combined drug-resistance mutations was found to be quite effective in increasing antibiotic production (Tamehiro et al.

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H. Wang · W. Xue · Y.-M. He · R.-H. Peng · Q.-H. Yao (✉)
College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, China
e-mail: quanhongyao502@hotmail.com

2003), so we also chose erythromycin as another resistance. Through genome shuffling, we combined these two resistances and achieved rapid improvement of spinosad production. Our results demonstrate that resistance to erythromycin and neomycin is related to improved spinosad production in mutants. Moreover, combining drug resistance screening with genome shuffling is a powerful tool for strain improvement.

Materials and methods

Microbial strains

The *S. spinosa* ATCC 49460 strain was a low producer of spinosyns obtained from the American Type Culture Collection (ATCC), and its production of spinosad was 50 μg/ml. ATCC 49460, which was used as the ancestral strain of all mutant strains described in this study, was cryopreserved at −80 °C.

Medium and cultural conditions

The seed medium contained 30 g/l trypticase soy broth, 3 g/l yeast extract, 2 g/l $MgSO_4$, 5 g/l glucose, 4 g/l maltose, and 0.2 % glycine. The pH of the medium was adjusted to 7.0 to 7.2 before autoclaving.

The plate medium contained 2 g/l enzyme-hydrolyzed casein, 1 g/l yeast extract, 10 g/l glucose, 1 g/l beef extract, and 15 g/l agar. The pH of the medium was adjusted to 7.0 to 7.2 before autoclaving.

S. spinosa was incubated on the seed and plate media at 28 °C for 3 days.

The regeneration plate medium contained 103 g/l sucrose, 10 g/l glucose, 5 g/l yeast extract, 0.1 g/l peptone, 10.12 g/l $MgCl_2{\cdot}6H_2O$, 0.25 g/l K_2SO_4, 10 ml 5 M $CaCl_2{\cdot}2H_2O$, 10 ml TES buffer, 10 ml 5 % KH_2PO_4, 7 ml 1 M NaOH, and 6 g/l agar. The pH of the medium was adjusted to 7.0 before autoclaving. The regeneration plate medium was incubated at 28 °C for 5 days.

The fermentation medium contained 2 g/l yeast extract, 30 g/l glucose, 40 g/l starch, 0.2 g/l $K_2HPO_4{\cdot}3H_2O$, 3 g/l NaCl, 1 g/l $CaCO_3$, and 0.05 g/l $FeSO_4{\cdot}7H_2O$. The fermentation medium was incubated under the same conditions as the seed culture, except that the cultivation period was extended to 7 days.

Minimum inhibit concentration

The minimum antibiotic concentration that was able to fully inhibit growth was defined as the minimum inhibitory concentration (MIC) (Liu et al. 2013). To determine the MIC of the parental strain ATCC 49460, spore solution was spread onto agar plates that contained different concentrations of the antibiotics erythromycin (10, 20, 30, and 40 μg/ml) and neomycin (5, 10, 20, and 30 μg/ml). All agar plates were incubated at 28 °C for 7 days, and the antibiotic concentration was obtained according to colony growth rate.

Protoplast formation

The incubated spore suspension of the ATCC 49460 strain was harvested by centrifugation at 9,000 rpm for 10 min at 4 °C, washed twice with 10 ml of protoplast (P) buffer (Baltz 1999), treated with lysozyme (2 mg/ml in P buffer) for 1 h at 35 °C, and then washed twice with 10 ml of P buffer again. Finally, the protoplast was diluted with 10 ml of P buffer, filtered in 400 mesh gauze, and then added with 0.6 % LiCl.

Preparation of the starting strains for genome shuffling

Approximately 10 ml of the *S. spinosa* protoplast was irradiated for 70 s under a 15 W UV lamp at a distance of 30 cm (Zheng et al. 2010; Lv et al. 2013). The treated protoplast was kept in the dark for 2 h to avoid photoreactivation repair. Afterward, approximately 0.2 ml of the protoplast suspension was spread onto the surface of the regeneration plate medium containing erythromycin (40 μg/ml) and neomycin (30 μg/ml), respectively. Mutant strains that grew on the regeneration plate medium were transferred onto plate medium containing the corresponding antibiotics to stabilize them at 28 °C for 5 days.

Genome shuffling

Two hundred mutant strains showing resistance were analyzed by high-performance liquid chromatography (HPLC). Mutant strains exhibiting higher spinosad yield than the parental strain ATCC 49460 were selected as the starting strains for the first round of genome shuffling. Based on the protoplast formation, we obtained the protoplasts of the starting strains. Equal volumes of protoplasts from different resistant strains were mixed and then centrifuged for 5 min at 5,000 rpm. The mixed protoplasts were fused for 5 min in 1 ml of 40 % PEG 6000, washed once with 1 ml of P buffer, and then centrifuged for 5 min at 5,000 rpm. The precipitation was transferred onto the surface of the regeneration plate medium containing erythromycin and neomycin. After incubation at 28 °C for 10 day, the colonies that appeared were isolated, and their productions were evaluated with a fermentation test in a shaking flask. The selected mutant strains with resistance to both erythromycin and neomycin and with high spinosad yield were used as the starting strains for the next round of genome shuffling. Four successive rounds of genome shuffling were performed by increasing the concentrations of erythromycin and neomycin. The concentrations of erythromycin and neomycin were 40+30 μg/ml, 80+60 μg/ml, 120+100 μg/ml, and 160+

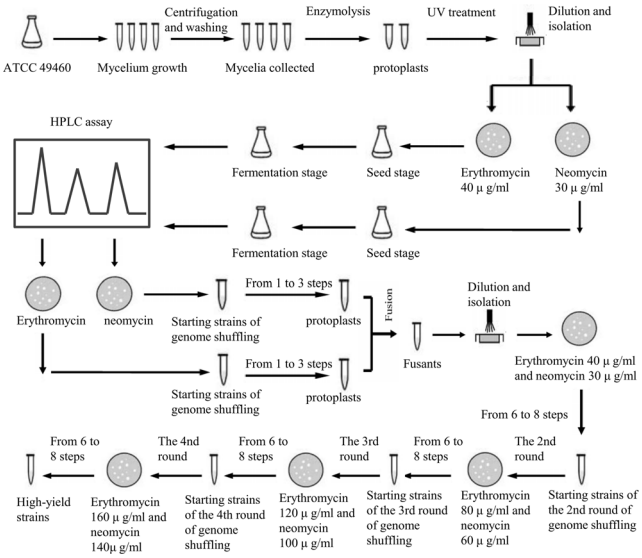

Fig. 1 Course of genome shuffling and drug resistance screening

140 μg/ml, from the first to the fourth round. Detailed procedures of screening of drug resistance and genome shuffling are illustrated in Fig. 1.

Fermentation methods

The selected mutant strains of *S. spinosa* were grown in 2 ml of the seed media containing the corresponding antibiotics, at 28 °C on a rotary shaker at a rotation rate of 200 rpm. After 3 days, 2 ml of the seed medium was inoculated in 20 ml of the fermentation medium, and then incubated for another 7 days at the same culture conditions.

Analytical methods

Twenty milliliters of methanol was added into approximately 20 ml of the fermentation broth to extract the spinosad at 28 °C on a rotary shaker at a rotation rate of 200 rpm for 24 h, and then centrifuged at 5,000 rpm for 5 min. The supernatant liquid was measured by high-performance liquid chromatography (HPLC) (Agilent 1100). Quantitative HPLC was accomplished with a C-18 reversed-phase column (4.6× 150) at a flow rate of 1 ml/min for 15 min. The mobile phase contained acetonitrile, methanol, and 0.05 % (w/v) aqueous ammonium acetate (45:45:10). Spinosad were monitored at 250 nm by a UV detector.

Glucose was measured by Fehling's reagent method (Chen and Xu 1991). The dry cell weight (DCW) was measured according to the method described by (Lebrihi et al. 1987). Amino nitrogen analysis was performed by the formaldehyde titration method (Chen and Xu 1991).

Table 2 Results of strains mutagenesis by drug resistance screening

Strains	Concentration of erythromycin and neomycin (μg/ml)	Frequency of Positive mutation (%)[a]	Highest production value (μg/ml)[b]
Ery-13	40	7.5 (15/200)	188
Neo-127	30	5.5 (11/200)	165

[a] Numbers in parentheses represent the number of mutant strains producing more spinosad and the number of colonies screened, respectively

[b] The highest production value of spinosad by the mutant strains Ery-13 and Neo-127

Results

Minimum inhibit concentration for screening of drug resistance

As shown in Table 1, the spore suspension of the parental strain ATCC 49460 could grow on the plates containing erythromycin (10 μg/ml to 30 μg/ml). The growth rate was suppressed with increasing antibiotic concentration. The mycelium growth was fully suppressed when erythromycin concentration reached 40 μg/ml. Similarly, the growth of the parental strain ATCC 49460 was suppressed when neomycin concentration was more than 30 μg/ml. Thus, the starting antibiotic concentrations were determined to be 40 and 30 μg/ml for erythromycin and neomycin, respectively.

Selection of starting strains for genome shuffling

Genome shuffling can promote directed evolution through recursive genetic recombination. However, the starting strains require a diverse population of mutants that exhibit

Table 1 Minimum inhibition concentration

Concentration of antibiotics	Erythromycin (μg/ml)				Neomycin (μg/ml)			
	10	20	30	40	5	10	20	30
Growth situation	+ + +	+ +	+	−	+ + +	+ +	+	−

+ indicates that the initial strain can grow. The more + symbols there are, the better it grows

− indicates that the initial strain has been fully inhibited

Table 3 Strain screening during four rounds of genome shuffling

Rounds	Concentration of erythromycin and neomycin (μg/ml)	Frequency of recombinants producing increased spinosad (%)[a]	Highest production value (μg/ml)[b]
Ck-1	0	0 (0/200)	50
Ck-2	0	6 (12/200)	193
1st round	40+30	19.5 (39/200)	249
2st round	80+60	22.8 (32/140)	280
3st round	120+100	26.0 (25/96)	314
4st round	160+140	35.0 (14/40)	332

Ck-1 is the fusion of parental strains that have no resistance

Ck-2 is the fusion of strains Ery-13 and Neo-127, which have slightly enhanced resistance

[a] Recombinants producing more spinosad than the starting strains Ery-13 and Neo-127. Numbers in parentheses represent the number of mutant strains producing more spinosad and the number of colonies screened, respectively

[b] The value of production of the recombinant producing the highest amount of spinosad

improvement in the trait of interest (Zhang et al. 2002; Petri and Schmidt-Dannert 2004). In the present study, erythromycin resistance and neomycin resistance were used to screen the strains that produce spinosad with high efficiency. After the initial mutagenesis, 200 of the colonies showing resistance to erythromycin or neomycin were isolated for primary and secondary screening. The shake flask fermentation test was performed only once for the primary screening, whereas it was performed thrice for the secondary screening. Finally, strains Ery-13 and Neo-127, showing resistance to erythromycin and neomycin, respectively, were obtained. The highest spinosad yields of Ery-13 and Neo-127 were 188 μg/ml and 165 μg/ml, respectively (Table 2). Thus, Ery-13 and Neo-127 were used as the starting strains for genome shuffling.

Improvement of spinosad production during the four rounds of genome shuffling

Before genome shuffling, we selected a number of fusions with no resistance and slightly enhanced resistance to spread on regeneration plates without antibiotics as controls (ck-1, ck-2). Ck-1 is the fusion of parental strains that have no resistance. Ck-2 is the fusion of strains Ery-13 and Neo-127, which have slightly enhanced resistance. There was no improvement of spinosad production in Ck-1. Compared with Ck-1, the frequency of positive mutants in Ck-2 was increased by 6 % (Table 3). Four successive rounds of genome shuffling were carried out using the two mutants (Ery-13 and Neo-127) as the starting strains. From the first to the fourth round of genome shuffling, the concentrations of erythromycin and neomycin in the regeneration plates were 40+30 μg/ml, 80+60 μg/ml, 120+100 μg/ml, and 160+140 μg/ml (Table 3). All colonies in each round were selected for a fermentation test and production assay. After the fourth round of protoplast fusion, *S. spinosa* EN4-33 produced the highest spinosad yield of 332 μg/ml, which is 6.6-fold higher than the spinosad yield of the parental strain ATCC 49460 (Fig. 2). The improved production of spinosad was consistent with the increase in resistance to erythromycin and neomycin (Fig. S2).

The genetic stability of the *S. spinosa* strain EN4-33 was evaluated by five successive subcultivation tests. The spinosad yield produced by the five generations ranged from 329 μg/ml to 332 μg/ml. This result suggests that the hereditary character of the high spinosad-producing recombinant (*S. spinosa* EN4-33) is stable.

Sensitivities of the mutant and shuffled recombinant strains to antibiotics

In order to avoid the occurrence of cross-drug resistance and the range of antibiotic resistance of some clones overlapping

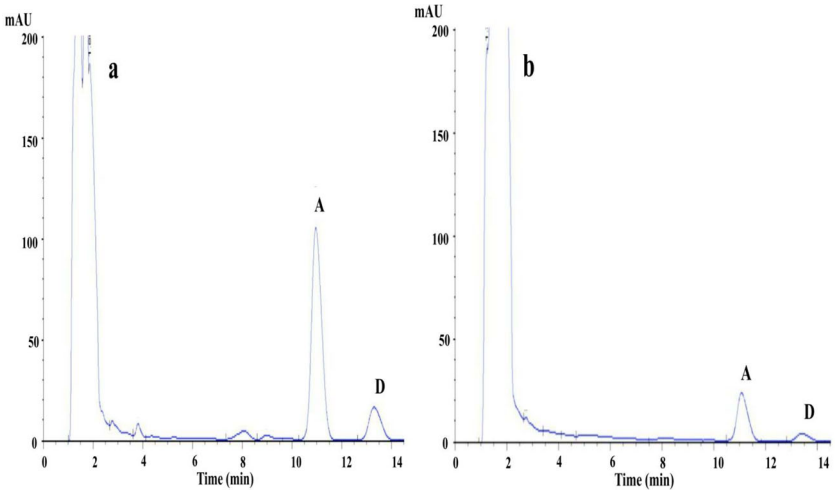

Fig. 2 HPLC analysis of antibiotic production. (**a**) HPLC chromatogram of antibiotics from recombinant strain EN4-33. **b** HPLC chromatogram of antibiotics from the parental strian ATCC 49460. A represents spinosyn A and D represents spinosyn D

with other clones from the next round, we detected sensitivities of all mutant and shuffled recombinant strains to the four kinds of antibiotic concentrations used in the present study. ATCC 49460, Ery-13, Neo-127, and EN1-70 grew on agar plates without antibiotics. Only Ery-13 and EN1-70 grew on agar plates containing 40 μg/ml of erythromycin. This finding suggests that Neo-127 has no cross-resistance to erythromycin (40 μg/ml). Similarly, only Neo-127 and EN1-70 grew on agar plates containing 30 μg/ml of neomycin. This result indicates that Ery-13 has no cross-resistance to neomycin (30 μg/ml). Only the EN4-33 strain grew on agar plates containing both 40 μg/ml erythromycin and 30 μg/ml neomycin. This result suggests that ATCC 49460, Ery-13, and Neo-127 are sensitive to 40 μg/ml erythromycin and 30 μg/ml neomycin (Fig. 3a). All generations were sensitive to the antibiotic concentrations in the next round of genome shuffling (Fig. 3b). In the fifth round of genome shuffling, no colonies appeared on the agar plates containing 200 μg/ml erythromycin and 180 μg/ml neomycin. Therefore, the antibiotic concentrations selected in this study were suitable for improving spinosad yield. The highest spinosad productivities of all antibiotic resistance mutants are listed in Table S1.

Spinosad fermentation properties of the recombinant EN4-33 and the parental strain ATCC 49460

The spinosad production and cell growth of the parental strain ATCC 49460 and high-producing recombinant EN4-33 were carried out in a shake flask. No significant differences in glucose and amino nitrogen consumption in the first 10 h were observed between ATCC 49460 and EN4-33. The consumption rate of the glucose and amino nitrogen of EN4-33 became higher than that of ATCC 49460 as fermentation was prolonged. When mycelium was grown into the stationary phase, the consumed sugar and amino nitrogen were mainly used for spinosad biosynthesis and mycelium maintenance (Fig. 4a and b).

The DCW of ATCC 49460 and EN4-33 rapidly increased in the first 40 h of cultivation; however, EN4-33 exhibited better growth. The DCW of EN4-33 and ATCC 49460 peaked at 50 and 70 h. The DCW of ATCC 49460 and EN4-33 indicated that these strains entered the stationary phase at 120 h. The growth characterizations of these two strains were consistent with spinosad biosynthesis (Fig. 4c).

In the fermentation of *S. spinosa*, spinosad production commenced after the onset of the exponential growth phase

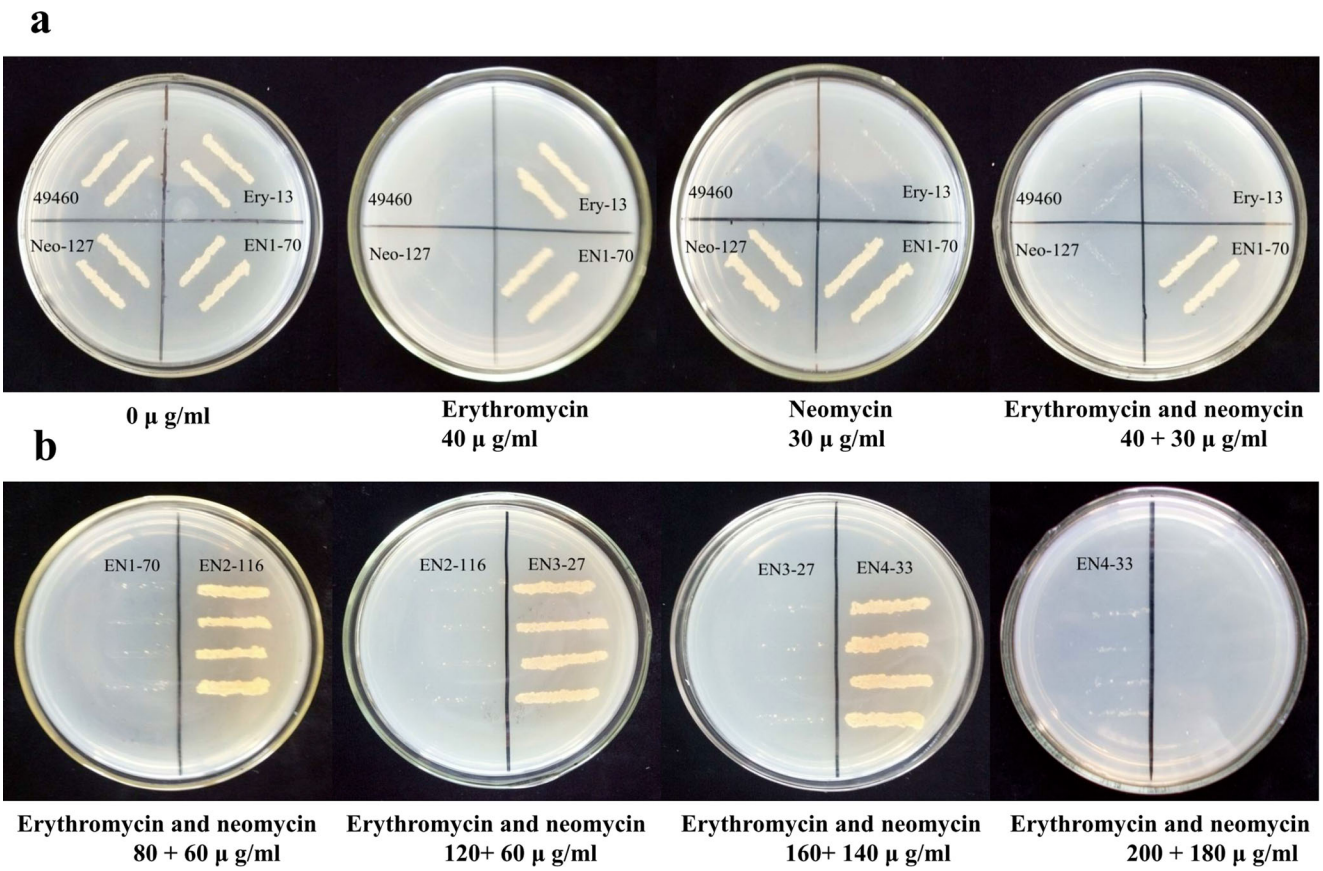

Fig. 3 Sensitivities of the mutant and shuffled recombinant strains to antibiotics. (**a**) Strain 49460 represents the parental strain. Strain Ery-13 and Neo-127 were obtained from screening of erythromycin resistance and neomycin resistance. Strain EN1-70 was obtained from the first round of genome shuffling. **b** Strain EN2-116, EN3-27, and EN4-33 were obtained from the second, third, and fourth rounds of genome shuffling, respectively

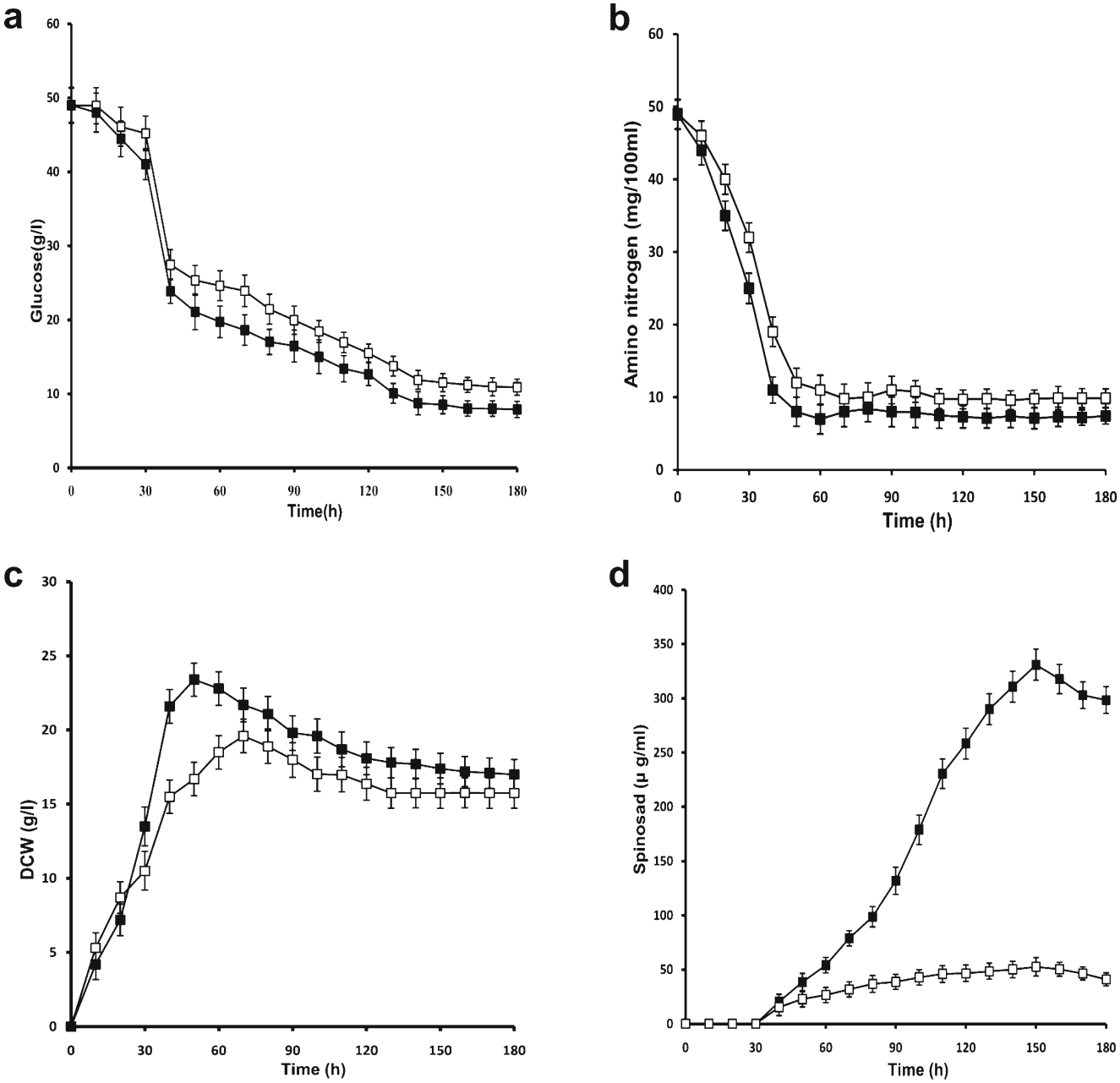

Fig. 4 Time curves of spinosad fermentation by recombinant EN4-33 and parental strain ATCC 49460. Open symbols represent the strain ATCC 49460; closed symbols represent the recombinant strain EN4-33. *Error bars* represent standard deviations

and peaked at the stationary phase. After 7 days of fermentation, the curve of spinosad production of EN4-33 is much higher than that of ATCC 49460 (Fig. 4d).

Discussion

Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all industrial commercial fermentation processes (Parekh et al. 2000). Although classical strain improvement methods have been successful in improving the target bioproducts, they are time consuming and labor intensive. Modern genetic engineering has been successful in generating many industrial strains. However, these attempts have been limited to the manipulation of only a handful of genes that encode enzymes and regulatory proteins selected using available information and research experience (Luo et al. 2012). In the present study, we successfully combined drug resistance screening and genome shuffling to significantly improve production of spinosad in *S. spinosa.*

Drug resistance screening was used to screen the starting strains for genome shuffling. By using this method, we obtained two mutant strains, Ery-13 and Neo-127, which are resistant to erythromycin and neomycin, respectively. The highest spinosad yields of Ery-13 and Neo-127 were 3.7-fold and 3.3-fold higher than that of the parental strain ATCC 49460 (Table 2). The results suggest that inducing resistance to erythromycin and neomycin can highly extend the selection scope of the parental strain and provide more opportunities for obtaining the desired phenotype for genome shuffling.

The simultaneous introduction of several resistant mutations can increase the production of antibiotics, such as actinorhodin, salinomycin, and thiazolyl peptide GE2270 in *Streptomyces coelicolor*, *Streptomyces albus*, and *Planobispora rosea*, respectively (Wang et al. 2008). Therefore, Ery-13 (erythromycin resistant) and Neo-127 (neomycin resistant) were selected as the starting strains for genome shuffling. After four rounds of genome shuffling and increasing antibiotic concentrations, the spinosad yield of the recombinant *S. spinosa* strain EN4-33 increased by 6.6-fold compared to that of the parental strain ATCC 49460 (Fig. 2). These results illustrate that the desired phenotypes are gradually enhanced from the cycles of genome shuffling without the need for genome sequence information. In addition, parallel to Ck-1 and Ck-2, it is clear that these antibiotics are indeed the driving force that can highly promote the efficiency of screening (Table 3). Compared with the parental strain ATCC 49460, the recombinant strain EN4-33 presented more rapid cell growth and better assimilation of carbon and nitrogen sources. Spinosad production of strain EN4-33 peaked at 150 h, and it was much higher than that of parental strain (Fig. 4d). These traits powerfully demonstrate that EN4-33 is a high-yield strain.

In conclusion, combining drug resistance screening and genome shuffling is a very effective method for improving spinosad production in *S. spinosa*.

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