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Improvement of *Saccharopolyspora spinosa* and the Kinetic Analysis for Spinosad Production

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Abstract In this paper, a new spinosad-producing mutant UV-42-13 was obtained by employing rhamnose and sodium propionate resistant selection strategies in series with UV irradiation. Spinosad production of the mutant was 125.3 mg/L, improved 285.5% compared with that of the wild strain (32.5 mg/L).The results of experiment on tolerance of propyl alcohol addition showed that the tolerant ability to precursor was higher. The precursor-resistant ability of the mutant improved through tolerance experiment by adding propyl alcohol, and the spinosad production was greatly increased. The kinetic models for biomass, substrate consumption, and spinosad production of mutant strain and wild strain were studied by conducting batch fermentation in the shaking flask. The result showed that the kinetic models could describe the fermentation process of spinosad producing well.

Keywords *Saccharopolyspora spinosa* · Spinosad · Fermentation · Rational selection · Kinetic models

Introduction

Spinosad, a combination of spinosynA and spinosynD, which are two most active components in the family of spinosyn [1, 2], is a novel tetracyclic macrolide produced by fermentation of *Saccharopolyspora spinosa* [3, 4]. It is highly effective against target insects and has an excellent environmental and mammalian toxicological profile [5].

Incorporation studies with ^{13}C-labeled acetate, propionate, and methionine established that spinosyns are synthesized through a polyketide pathway [6]. Cloning and analysis of the spinosad biosynthetic gene cluster of *Saccharopolyspora spinosa* proved that polyketide portion of spinosyn differs from type I polyketide [7]. In the biosynthesis process of

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spinosyn, the polyketide chain extension starts from a unit of propionic acid, and then acetyl and propionyl group are sequentially added in. The quantity of acetate and propionate is important to spinosad biosynthesis; to improve the production of spinosad, we usually add these precursors into medium, but a high ability of tolerance to precursor is necessary.

Rhamnose, an important intermediate, is proved to be not only a component of *S. spinosa* cell walls for cell integrity and morphology, but also playing a critical role in the spinosyn biosynthesis. It is a precursor for the biosynthesis of spinosad [8]. Therefore, the quantity of rhamnose supplied in the cell in the improvement of spinosad production is important because it usually becomes a limiting step in spinosad biosynthesis. It is important to change the regulation mechanism in the microorganism to accumulate rhamnose for spinosad biosynthesis.

Classical mutation is still an effective approach to change the gene and characteristics of microorganism [9]. However, obtaining improved mutants is a challenge in the screening program. In this paper, on the basis of understanding the metabolism and pathway regulation, rational selection was employed to screen mutant with a high capability of spinosad production. Therefore, a method of rhamnose-resistant and sodium propionate-resistant rational selection with UV mutation was developed.

Addition of precursors may enhance the production of spinosad, but in many instances, precursors are toxic and repress expression of relevant enzyme [10, 11]. It is, therefore, necessary to select mutant with high tolerance to high concentration of precursors. After a precursor-resistant mutant was obtained, in this study, its tolerance ability to precursor was researched, and the result indicated that the production increased greatly after precursors were added in the medium.

To control and optimize the fermentation process of spinosad, it is necessary to develop mathematical models to predict the effect of fermentation operating parameters on cell growth rate, spinosad fermentation rate, and substrate utilization rate. Kinetic model describing the behavior of microbiological systems can be a highly appreciated tool and can be reduce tests to eliminate extreme possibilities [12]. In this study, the models employed to predict the cell, substrate, and spinosad concentrations over time in batch fermentation were developed and evaluated using the experiment data. The objective of this work is to offer data and reference for reducing the cost and improving the productivity in industrial production.

Materials and Methods

Microorganism and Spores Suspensions

Saccharopolyspora spinosa (NRRL 18395) culture stocks were maintained as suspension containing 15% glycerol at −70 °C. Thawed stock 0.1 ml was used to inoculate 30 ml of vegetative medium in a 250-ml shake flask. Spores on slant was inoculated into 20 ml of vegetative medium in a 150-ml shake flask and incubated in an orbital shaker at 30 °C and 250 rpm for 72 h. Spores suspension harvested was filtrated by sterile pledget and counted microscopically (10^7~10^8 spores ml^{-1})

Medium and Cultural Conditions

Plate and slant medium consisted of the following ingredients (g/l): glucose, 10; N-Z-amine A, 30; yeast extract, 3.0; agar 15, with pH adjusted to 7.3, incubated at 30 °C up to 8 days.

Vegetative medium was used for increasing the count and activity of maintained strain containing (g/l): glucose, 10.0; N-Z-amine A, 30.0; yeast extract, 3.0, and $MgSO_4.7H_20$, 2.0 in deionized water with no pH adjustment.

Fermentation medium (g/l): glucose, 50; cotton seed flour, 20; yeast extract, 2; maltose, 10; corn steep liquor, 15; $(NH_4)_2SO_4$, 1; Zn_2SO_4, 0.2; $CaCO_3$ (sterilized separately), 5; with pH adjusted to 7.0, using 250-ml conical flasks containing 30 ml medium. The strains were incubated in an orbital shaker at 30 °C and 250 rpm for 7 days. The experiments were carried out in triplicate.

Selective isolation medium (SIM) was utilized to isolate rhamnose-resistant and sodium propionate-resistant mutants, and were prepared by adding rhamnose(2 mg/l) or sodium propionate(1.5%), respectively, in plate medium.

UV Mutagenesis

About 5 ml of the spore suspension of *S. spinosa* was irradiated for 10, 20, 40, and 50 s at a distance of 30 cm from a UV-30-W-254-nm lamp. The treated spores were kept in the dark for 2 h to avoid photoreactivation repair. The suspension was then diluted and spread onto the surface of the plate medium. Mutants with resistant were then selected on SIM containing rhamnose or sodium propionate subsequently. Strains on plate medium grown were transferred onto slants for further studies.

Minimum Inhibition Concentration (MIC)

One milligram per liter, 2 mg/l, 5 mg/l rhamnose, 1.0%, 1.5%, 2.0% sodium propionate were used as screening agents. The spore suspension of mutants with UV irradiation for 40 s was spread on the plate medium containing one of the above screening agents.

Mutants Isolation

Fifty mutants screened in each exposed time of UV irradiation were inoculated on every SIM (30 °C, 8 days). Resistant mutants were then isolated and spinosad concentration was determined and compared after shaking flask fermentation.

Experiment on Tolerance of Propyl Alcohol

Propyl alcohol 0.05%, 0.1%, and 0.5% was added into fermentation medium after cultivating 72 h. Spinosad production was measured and the concentration of propyl alcohol used determined.

Analytical Methods

Spinosad concentration was measured by high-performance liquid chromatography (HPLC). The quantitative HPLC was accomplished with a C-18 reversed-phase column (LabAlliance, USA, 250×4.6 mm). The flow rate was 1.0 ml/min. Twenty microliters of samples were eluted with a mobile phase consisting of acetonitrile, methanol, and 1.0% (*w*/*v*) aqueous ammonium acetate (54:40:6). The eluent was monitored at 246 nm with a UV detector (Model 500, LabAlliance, USA). Total sugars of the supernatants by centrifuging were analyzed by the hot anthrone method. The dried cell weight (DCW) was determined by centrifuging and then drying.

All experiments were repeated three times. The data shown in the portion of "Results and Discussion" are the mean values of thrice-conducted experiments.

Results and Discussion

Determination of Minimum Inhibition Concentration

According to the method described in "Minimum Inhibition Concentration", the plate is cultivated and the number of colonies grown is calculated. Finally, 2 mg/l rhamnose and 1.5% sodium propionate were selected as the minimum inhibition concentrations, respectively.

Mutagenesis and Mutants with Resistant Screening

After UV irradiation at different exposure times and incubation on SIM, mutants resistant to rhamnose were isolated. Of 200 UV-treated colonies screened, 19 expressed resistance to rhamnose.

Then the fermentation result showed that of the 19 rhamnose-resistant mutants obtained, only two could not produce spinosad, and three produced less than their parental strain. The spinosad production of the other 14 mutants increased. The highest concentration of spinosad produced by mutant U-42 was 70.4 mg/l (216.6% production compared with the original strain NRRL 18395).

In the following experiments, the results showed that six among 30 rhamnose-resistant mutants tested expressed resistance to sodium propionate and produced more spinosad than the original strain U-42. The highest spinosad level was produced by the mutant U-42-13, with spinosad concentration of 125.3 mg/l, 178.0% production compared with parental strain U-42, whereas 385.5% compared with the original strain NRRL 18395.

Experiment on Tolerance of Propyl Alcohol

The results of the tolerance experiment showed that relative spinosad production increased when adding propyl alcohol into the medium, and the highest level of both wild-type and mutant U-42-13 was obtained when the addition of propyl alcohol was 0.1%. The spinosad production decreased when the addition of propyl alcohol was either excessive or deficient. However, in every condition when adding propyl alcohol, the spinosad production was higher in the fermentation of mutant than in the fermentation of wild-type strain. The result indicated that precursor-resistant mutant enhanced the tolerance to precursor, so spinosad producing ability was improved. However, we find that the growth of cell was inhibited when the addition of propyl alcohol was excessive and the precursor could not play a role of inducement to synthetic enzyme when deficient, so it is important to control the quantity of precursor in the bioprocess.

From the above results, it could be concluded that increased resistant to precursor can be used as a successful selective procedure to change the regulation mechanism and improve the ability of enzymatic synthesis in the original strain. The comparison of the cell concentration, spinosad production, and substrate consumption between the mutant strain UV-42-13 and wild-strain NRRL 18395 is shown in Fig. 1

From Fig. 1, spinosad was produced at the final stage of exponential phase at first, and then increased rapidly at the stationary phase. Therefore, the fermentation of spinosad is

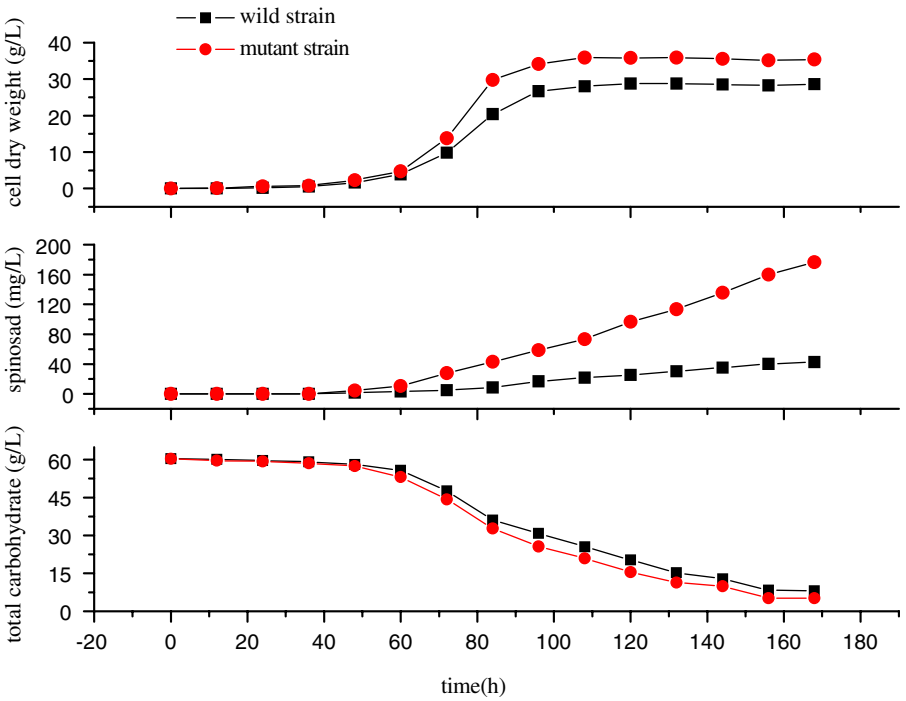

Fig. 1 Comparison of cell growth, spinosad production, and substrate consumption between the mutant strain and wild strain

biphasic. The maximum cell concentration was higher in the fermentation with the mutant strain than that with the wild strain, and the maximum spinosad production was higher, with a concentration of 176.6 mg/l of mutant strain compared with 42.7 mg/l of wild strain, which increased by 313.6%. The result also showed that sugar was consumed rapidly in the exponential phase and stationary phase. In the stationary phase, cell growth almost ceased; the sugar was used primarily for the synthesis of spinosad. Sugar was consumed more rapidly and sufficiently in the fermentation of mutant strain than in the fermentation of wild strain. The results indicated that the mutant strain showed better characteristic in cell growth and spinosad production than the wild strain.

Models for Fermentation Kinetics

Model for Growth Kinetics

Logistic equation is considered capable to reflect the rule of fermentation well [13, 14, 15]. In many antibiotic fermentation systems by various types of microorganisms, logistic model is used to describe the kinetics of cell growth. The logistic equation is given by Eq. 1:

$$\frac{dx}{dt} = \mu_m(1 - x/x_m)x, \tag{1}$$

where μ_m is the maximum specific growth rate (h^{-1}) and x_m is defined as the maximum biomass concentration (g dry weight l^{-1}), whereas x is shown as biomass concentration (g dry weight l^{-1}). Integrating the Eq. 1 with the initial condition: $X = X_0$ at t=0, gives a

sigmoidal variation of $X(t)$ that may empirically represent both an exponential and a stationary phase (Eq. 2):

$$x(t) = \frac{x_0 e^{\mu_m t}}{\{1 - (x_0/x_m)(1 - e^{\mu_m t})\}} \tag{2}$$

Model for Product Formation Kinetics

The kinetics of product formation can be described by Leudeking–Piret model [16], which suggests that the product formation rate linearly depends on both the instantaneous biomass concentration (x) and growth rate dx/dt:

$$\frac{dP}{dt} = \alpha \frac{dx}{dt} + \beta x \tag{3}$$

Where α represents growth-associated product formation constant (gram spinosad/gram cell mass); β represents nongrowth-associated product formation constant (gram spinosad per gram cell mass per hour). They are both empirical constants that may vary with fermentation conditions. And constant β can be obtained from the data at stationary phase when dx/dt=0 and $x = x_m$, using the following equation:

$$\beta = \frac{(dp/dt)_{st}}{x_m} \tag{4}$$

Substituting Eq. 1 into Eq. 3 and then integrating yields the following equation:

$$p(t) = p_0 + \alpha[x(t) - x_0] + (\beta x_m/\mu_m) \ln\{1 - (x_0/x_m)(1 - e^{\mu_m t})\} \tag{5}$$

Model for Substrate Utilization Kinetics

It is necessary to establish the model of substrate consumption kinetics in the process of spinosad synthesis. Carbon resource consumption is important for spinosad formation. In this paper, the modified Leudeking–Piret model was used to describe the substrate utilization kinetics as following:

$$-\frac{dS}{dt} = k_1(dx/dt) + k_2(dp/dt) + k_3 x \tag{6}$$

The equation suggests that substrate consumption depends on three terms: the instantaneous cell growth rate, the instantaneous product formation rate, and a cell mass maintenance function. Where k_1 is a constant that carbohydrate used in cell growth, k_2 is a constant that carbohydrate utilized in product formation, k_3 is a constant that carbohydrate consumed to maintain the biomass which can be attributed to k_1 because the magnitude of substrate is small consumed by strain breath. Then Eq. 7 was obtained by integrating Eq. 6:

$$s(t) = s_0 - k_1 x(t) - k_2 p(t) \tag{7}$$

Determination of Kinetic Parameters and Model Evaluation

The model parameters were determined by nonlinear regression to experimental values at the basic of Eqs. of 1–7 [17], using a search method of minimizing the sum of squares of the difference between the predicted and observed values [18]. The predicted values were then used to simulate the profiles of cell, substrate, and product concentration during the fermentation.

Table 1 gave the kinetic parameter values of the mutant strain and the wild strain in batch fermentation. The result showed that the maximum cell concentration X_{max} and the maximum specific growth rate μ_{max} obtained from the mutant strain were higher than that of wild strain. This means that mutant cell was more active than the wild strain, and the active and quantity of important enzyme used in the biosynthesis of spinosad improved. A growth-associated rate constant α and nongrowth-associated rate constant β obtained from fermentation of mutant strain are two times and four times higher than that of wild strain, respectively. Moreover, the values of α and β were neither zero in the fermentation of two strains, so it could be concluded that the fermentation of spinosad was half-growth-associated. In the fermentation of mutant strain, the constant for carbohydrate used in cell growth k_1 was higher than the constant for carbohydrate utilized in product formation k_2, whereas in the fermentation of wild strain, both constant k_1 and k_2 were almost similar.

Figures 2 and 3 show the simulation curves calculated according to the proposed kinetic models and the measured values of cell, substrate, and spinosad concentration of the mutant strain and the wild strain. The solid curves represented the predicted values and the symbol represented measured values. A good agreement between the model predictions and the experimental data was obtained of the mutant strain and the wild strain, respectively. The correlation coefficient (R^2) was 0.9961, 0.9955, and 0.9944 for cell, spinosad, and carbohydrate of wild strain, whereas the correlation coefficient (R^2) was 0.9984, 0.9934, and 0.9938 for cell, spinosad, and carbohydrate of mutant strain. This confirmed that the kinetic models were capable to predict the experiment results with high accuracy and could express well the process of spinosad fermentation.

Conclusions

A high spinosad production strain UV-42-13 was obtained using rhamnose and sodium propionate-resistant screening and the spinosad concentration was up to 125.3 mg/l. The

Table 1 Parameter values used for the kinetic model.

Kinetic Parameter	Mutant Strain	Wild Strain
X_{max} (g/l)	35.9	28.9
X_0 (g/l)	0.0005	0.0005
μ_{max} (h^{-1})	0.1502	0.1423
P_0 (mg/l)	0	0
S_0 (g/l)	60.4	60.4
α(mg/g)	0.7555	0.3368
β(mg/g h)	0.04382	0.0130
K_1 (g/g)	0.7641	0.7094
K_2 (g/mg)	0.1730	0.7635

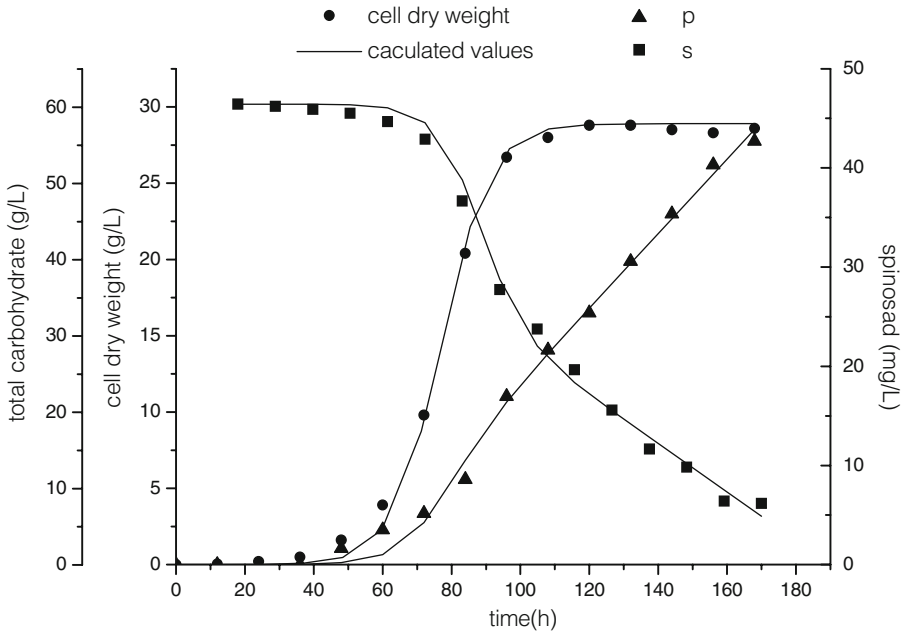

Fig. 2 The predicted and measured values of cell, spinosad, and carbohydrate concentration in the fermentation of wild strain

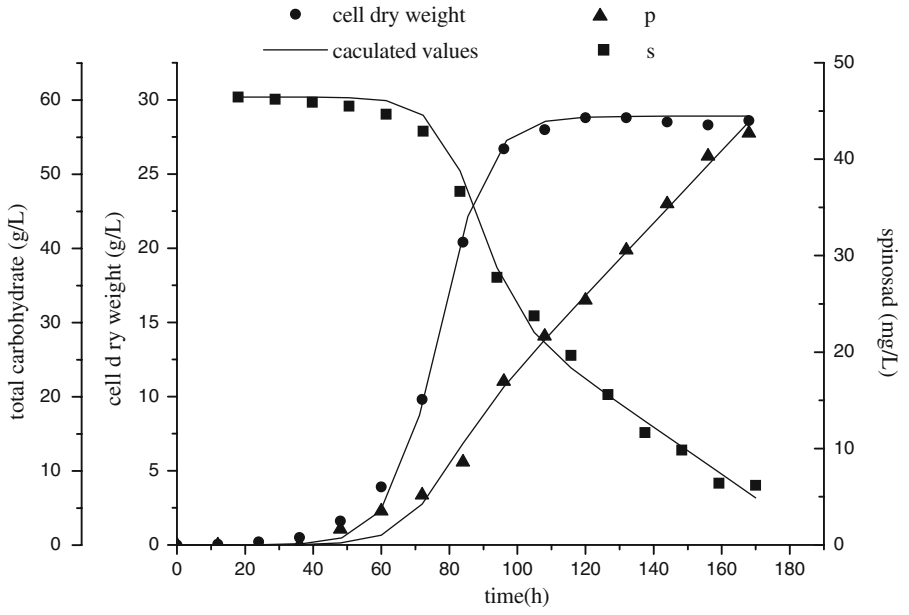

Fig. 3 The predicted and measured values of cell, spinosad, and carbohydrate concentration in the fermentation of mutant strain

production of spinosad was increased when adding 0.1% propyl alcohol to fermentation medium and 177.8 mg/l of spinosad was obtained. This result suggests that precursor-resistant screening and precursor addition with suitable concentration was effective to improve the production of spinosad.

According to the Logistic model and Leudeking–Pire model, kinetic models for batch fermentation were developed. The models performed well in predicting the cell, spinosad, and carbohydrate concentrations of both wild strain and mutant strain with high accuracy. So the models developed could express the process of spinosad fermentation well.

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