Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

Improved production of spiramycin by mutant *Streptomyces ambofaciens*

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Received July 11, 2003; revision accepted Aug. 21, 2003

Abstract: Strain improvement and medium optimization to increase the productivity of spiramycin were carried out. Of oil tolerant mutant strains screened, one mutant, *Streptomyces ambofaciens* XC 2-37, produced 9% more spiramycin than the parent strain *S. ambofaciens* XC 1-29. The effects of soybean oil and propyl alcohol on spiramycin production with *S. ambofaciens* XC 2-37 were studied. The potency of *S. ambofaciens* XC 2-37 was improved by 61.8% with addition of 2% soybean oil in the fermentation medium and 0.4% propyl alcohol at 24 hours after incubation. The suitable time for feeding propyl alcohol is at 24 hours after incubation in flask fermentation and at 20 hours after incubation in fermentor fermentation. The new process with *S. ambofaciens* XC 2-37 was scaled up for industrial scale production of spiramycin in a 60 m^3 fermentor in Xinchang Pharmaceutical Factory, Zhejiang Medicine Company, Ltd., China, and the potency and productivity of fermentation were improved by 42.9%.

Key words: Spiramycin, *Streptomyces ambofaciens*, Strain improvement, Fermentation
Document code: A **CLC number:** TQ927

INTRODUCTION

Spiramycin belongs to the macrolide antibiotics group. The molecular structure of spiramycin consists of a 16-membered branched lactonic ring with eight precursors for its biosynthesis: five acetates, one proprionate, one butyrate and another unidentified precursor made up of two carbons (Omura *et al.*, 1977). It possesses two amino sugars (mycaminose and forosamine) and one neutral sugar (mycarose). The methoxy carbon of the aglycone, the dimethyl-amino carbons of mycaminose and forosamine and the C-3″ methyl carbon of mycarose are derived from methionine (Inoue and Deguchi, 1983). There are three components of spiramycins, namely spiramycin I which has a hydroxyl group at C-3 of aglycone, spiramycin II in which the hydroxyl group is acetylated, and spiramycin III in which the same position is propionylated (Omura *et al.*, 1979). The addition of short-chain fatty acids stimulates the production of spiramycin by *Streptomyces ambofaciens* cultivated on dextrins and ammonium chloride (Khaoua *et al.*, 1992). Previous research work indicated that spiramycin production with *Streptomyces spiramyceticus* can be enhanced by screening and using the spiramycin resistant mutant strains, the valine resistant mutant strains and the α-aminobutyric acid resistant mutant strains (Jin *et al.*, 1992; Jin, 1995). And there is little increase in spiramycin potency of *Streptomyces spiramyceticus* by adding sodium acetate, ethanol, *n*-propanol or *n*-butanol at 48 hours after cultivation, but significant increase by adding soybean oil either at the beginning or at 48

hours after cultivation (Zhang and Jin, 1993).

This paper reports the screening for the oil-tolerant mutant strains and the effects of soybean oil and propyl alcohol on spiramycin production with *Streptomyces ambofaciens*. Scale-up of the new fermentation process will also be explored.

MATERIALS AND METHODS

Spiramycin-producing strain and solid culture media

Streptomyces ambofaciens XC 1-29, provided by Xinchang Pharmaceutical Factory, Zhejiang Medicine Company Ltd., China, was the parent strain of all mutant strains described in this study. Solid medium consisted of the following (per litter): 20 g starch, 10 g soybean meal, 0.5 g $MgSO_4$, 3 g NaCl, 5 g $CaCO_3$, 20 g agar, and adding distilled water to 1 litter. Solid culture was incubated at 28 °C and 40%−50% relative humidity for 10 days.

Spiramycin fermentation in flask

The mycelium and spores from the solid culture were inoculated into a 250 ml Erlenmeyer flask containing 25 ml of seed medium (4% starch, 1% glucose, 2% soybean meal, 0.5% peptone, 0.05% KH_2PO_4, 0.3% $CaCO_3$). After incubation at 28 °C for 48 hours on a rotary shaker at 220 rpm, 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 was comprised of: 7% starch, 2% soybean meal, 0.5% fish meal, 0.8% NH_4NO_3, 0.05% KH_2PO_4, 0.1% $MgSO_4$, 0.3% NaCl, 0.5% $CaCO_3$. The production culture was incubated under the same conditions as for the seed culture, but only for 4 days.

Isolation of oil tolerant mutant strains

A 5 ml sample of spore suspension from solid culture of *S. ambofaciens* XC 1-29 was transferred to an aseptic plate. The plate with cover removed was exposed to ultraviolet (UV) irradiation for 60 seconds at a distance of 30 cm from the UV lamp with wavelength of 3537 Å and power of 30 W. After UV irradiation (the survival ratio was about 9.2%) the spore suspension of the parent strain was spread onto the agar medium containing 2% soybean oil instead of starch. Then the oil tolerant mutants (designated as oil^t) were isolated from the plate. Spore suspension with or without UV irradiation was also spread onto the agar medium without soybean oil for comparison. A total of 50 colonies was isolated from each group, and after the flask fermentation, spiramycin concentration was measured and compared.

Spiramycin fermentation in 15 L fermentor

A fermentor (15 L) containing 8 L of production medium was inoculated with 800 ml of broth cultured in flask. The fermentation was completed after 108 hours cultivation at 28 °C.

Spiramycin fermentation in 60 m^3 fermentor

A seed fermentor (400 L) containing 220 L of seed medium was inoculated with 480 ml of broth cultured in flask. After 40 hours of incubation at 28 °C, the seed broth was used to inoculate a preculture fermentor (4 m^3) containing 2.4 m^3 of seed medium. After 24 hours of incubation at 28 °C, the seed broth was transferred into the production fermentor (60 m^3) containing 28 m^3 of production medium. After 108 hours' fermentation at 28 °C, the fermentation broth was harvested.

Analytical method

Reduced sugar was determined by Fehling's reagent method. Amino nitrogen was determined by the formaldehyde titration method (Chen and Xu, 1991).

Mycelial growth was estimated by measuring the packed mycelium volume. After centrifugation of 10 ml fermentation broth at 3000 rpm for 15 min, the packed mycelium volume was obtained.

Determination of spiramycin was done by a conventional disc diffusion method using *Sarcina lutea* 28001 as the sensitive strain.

RESULTS

Isolation of oil tolerant mutant strains

S. ambofaciens XC 1-29 is susceptible to soybean oil. In the medium containing 2% soybean oil instead of starch, *S. ambofaciens* XC 1-29 growth was slower and was inhibited by 81.2%.

After UV mutation, the oil tolerant mutant strains were screened. The abilities of these strains to produce spiramycin were examined. The results are listed in Table 1, where the relative spiramycin potencies of natural isolates, UV mutants and oil tolerant mutant strains are listed for comparison.

From Table 1 it was obvious that the spiramycin potencies of the oil tolerant mutants were higher than those of natural isolates and UV mutants. Among the oil tolerant mutants, an oil^t mutant XC 2-37, with maximum potency increase of 9% over that of the natural isolates, was obtained.

Influence of soybean oil on spiramycin production

In order to study the influence of soybean oil on the production of spiramycin by *S. ambofaciens* XC 2-37, we designed eleven kinds of media for flask fermentation experiment. The measured potencies of spiramycin fermentation are listed in Table 2.

The data in Table 2 showed that the potency of spiramycin fermentation with *S. ambofaciens* XC 2-37 could be increased by the addition of soybean oil, and that the potency of spiramycin fermentation with medium No.7 was higher than that with other medium. The potency of spiramycin with medium No.7 was 24% higher than that of the contrast medium No.1.

Effect of propyl alcohol on spiramycin production

Among the above eleven kinds of media, four kinds of media were selected for studying the effect of propyl alcohol on spiramycin production by *S. ambofaciens* XC 2-37. Propyl alcohol was added at 24 hours after incubation. The measured potencies of spiramycin fermentation with the four media are listed in Table 3, which indicates that the addition of propyl alcohol can increase spiramycin potency

Table 1 Comparison of relative spiramycin potency among the natural isolates, UV mutants and the oil tolerant mutant strains

	Relative spiramycin potency	
	Average	Highest
Natural isolates	100	100
UV mutants	102	105
The oil^t mutants	106	109

Table 2 Effect of soybean oil on the production of spiramycin by *S. ambofaciens* XC 2-37

	Medium No.		Mycelium concentration (%)	Potency (mg/L)	Increased (%)
	Starch (%)	Soybean oil (%)			
1	0	7	45	2045	Contrast
2	0.5	7	44	2231	9.1
3	1	6	43	2312	13.1
4	1.5	6	43	2237	9.4
5	1.0	5	42	2353	15.1
6	1.5	5	42	2424	18.5
7	2	5	40	2537	24.0
8	1.5	4	40	2407	17.7
9	2	4	39	2437	19.2
10	2.5	4	38	2408	17.8
11	3	4	38	2368	15.8

Table 3 Effect of propyl alcohol on spiramycin production by *S. ambofaciens* XC 2-37

Medium No.	Mycelium concentration (%)	Potency (mg/L)	Increased (%)
1*	45	2173	
1	44	2280	5.2
2	43	2347	8
3	43	2437	12.1
7	40	3277	50.8
9	39	3516	61.8

* No additional propyl alcohol was added

a little in the soybean oil-free medium and the low-soybean oil medium, but increase greatly in the production medium containing more soybean oil. The potency of spiramycin fermentation by *S. ambofaciens* XC 2-37 was improved by 61.8% with 2% soybean oil in the production medium and addition of 0.4% propyl alcohol during the fermentation process.

The propyl alcohol concentration was further optimized. Propyl alcohol with different concentration was added at 24 hours after incubation in flask fermentation. The result (Table 4) showed that the optimum concentration of propyl alcohol was 0.4%–0.5%.

In order to increase the potency of spiramycin fermentation further, the influence of propyl alcohol feeding time on spiramycin production was also studied. Spiramycin fermentation experiments were performed both in flask and in fermentor, and propyl alcohol was added at different time during the fermentation process. The results (Fig.1) showed that the suitable time for adding propyl alcohol in flask fermentation is at 24 hours after incubation, and that the suitable time of feeding propyl alcohol in fermentor fermentation is at 20 hours after incubation. The mycelia grew faster in fermentor fermentation than in flask fermentation. Therefore the time of adding propyl alcohol in fermentor fermentation should be earlier than that in flask fermentation.

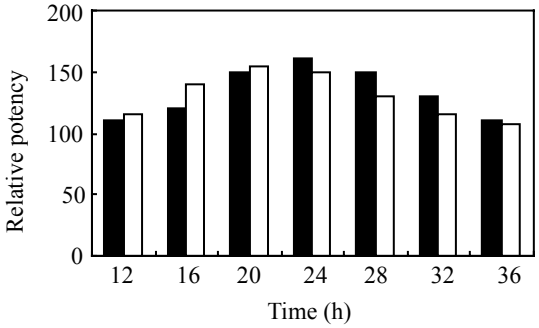

Fig.1 Effect of propyl alcohol feeding time on spiramycin production both in flask (■) and in fermentor (□)

Spiramycin fermentation in 15 L fermentor

The fermentation experiment with soybean oil in place of partial starch in the production medium and feeding propyl alcohol during the fermentation process by *S. ambofaciens* XC 2-37 was performed in a 15 L fermentor. The data measured during the fermentation process were compared with those of the original process by the original strain *S. ambofaciens* XC 1-29, see Fig.2.

Table 4 Effect of propyl alcohol concentration on spiramycin production by *S. ambofaciens* XC 2-37

Propyl alcohol concentration (%)	Mycelium concentration (%)	Potency (mg/L)
0	39	2437
0.2	40	2889
0.3	39	3326
0.4	39	3516
0.5	38	3509
0.6	38	3318
0.8	36	2827

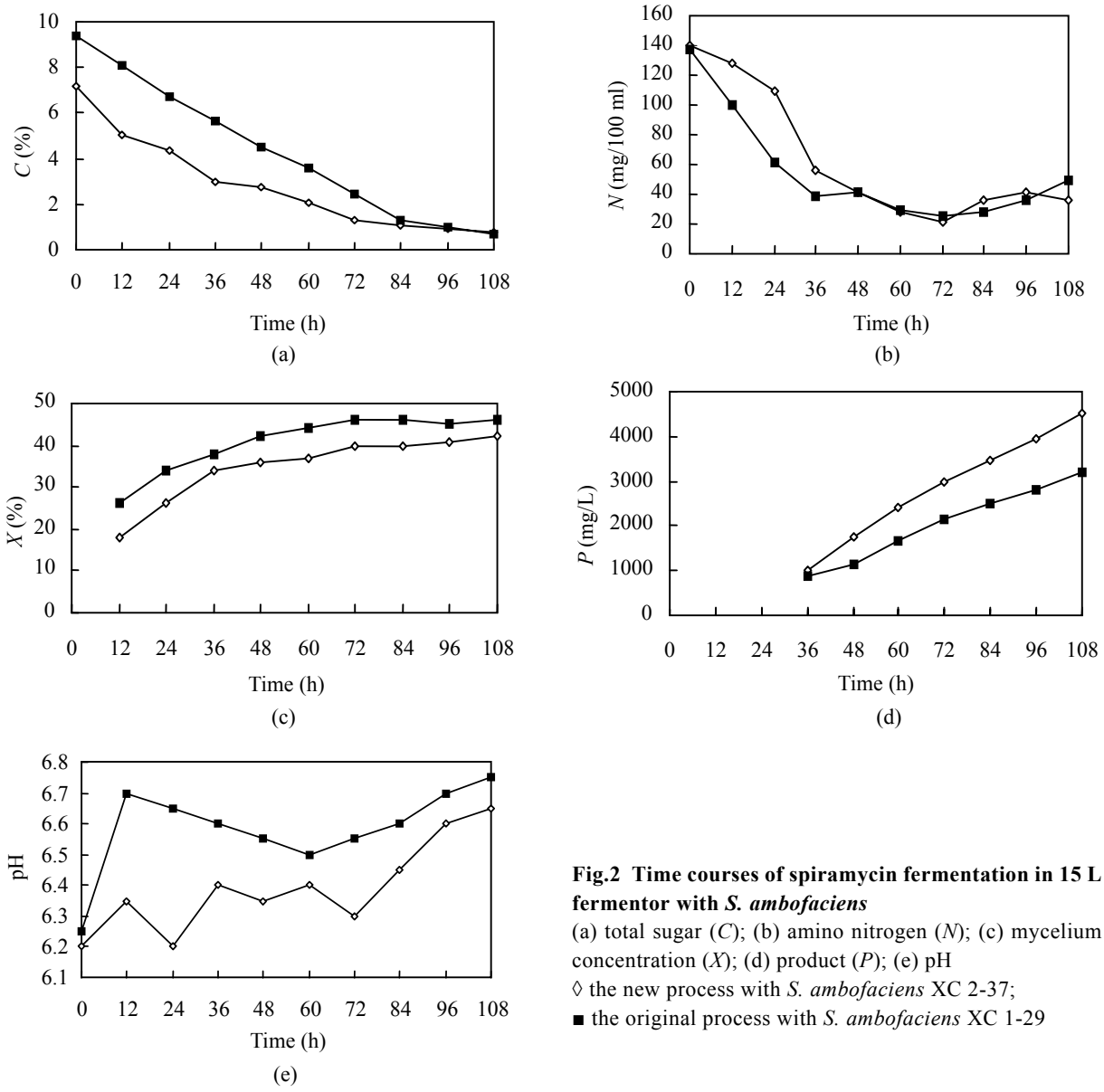

Fig.2 Time courses of spiramycin fermentation in 15 L fermentor with *S. ambofaciens*

(a) total sugar (*C*); (b) amino nitrogen (*N*); (c) mycelium concentration (*X*); (d) product (*P*); (e) pH

◊ the new process with *S. ambofaciens* XC 2-37;

■ the original process with *S. ambofaciens* XC 1-29

Fig.2 shows that the spiramycin potency of the new process with *S. ambofaciens* XC 2-37 (the new strain) reached 4498 mg/L, which was 40% higher than that of the original process with *S. ambofaciens* XC 1-29 (the old strain). Compared with the original process with *S. ambofaciens* XC 1-29, the mycelia grew more slowly and the mycelium concentration was lower in the new process with *S. ambofaciens* XC 2-37, which was beneficial in product separation. In the new process with *S. ambofaciens* XC 2-37, sugar was less consumed in the stationary phase, because soybean oil was consumed in place of sugar. The pH value during the fermentation process of the new process with *S. ambofaciens* XC 2-37 was about 6.4, while the pH value in the original process with *S. ambofaciens* XC 1-29 was higher.

Scale-up of spiramycin fermentation

Dissolved oxygen is liable to become a control factor during the fermentation of antibiotics. The critical value of dissolved oxygen for spiramycin is above 10% of saturation (Li *et al.*, 1988). The dissolved oxygen during the fermentation process of spiramycin with the new process by using *S. ambofaciens* XC 2-37 in a 15 L fermentor was above

15% of saturation, therefore, oxygen was not the limiting factor for cell growth and spiramycin accumulation.

The new process by *S. ambofaciens* XC 2-37 was scaled up for spiramycin production in a 60 m^3 fermentor in Xinchang Pharmaceutical Factory, Zhejiang Medicine Company Ltd., China. The main scale-up parameters from the 15 L fermentor to the 60 m^3 fermentor are listed in Table 5.

From Table 5, it is obvious that the aerated agitation power per unit volume as well as the oxygen transfer coefficient (k_La) in the 60 m^3 fermentor were much higher than those in the 15 L fermentor. So the dissolved oxygen during the fermentation process of spiramycin in the 60 m^3 fermentor should not be a limiting factor for spiramycin fermentation.

The spiramycin fermentation in the 60 m^3 fermentor in the new process by *S. ambofaciens* XC 2-37 and the original one by *S. ambofaciens* XC 1-29 are listed in Table 6, showing that spiramycin potency with the new process by *S. ambofaciens* XC 2-37 in the 60 m^3 fermentor was kept the same as that in the 15 L fermentor; and that applying the new process and the new strain *S. ambofaciens* XC 2-37 in the 60 m^3 fermentor increased the spiramycin potency and productivity by 42.9%.

DISCUSSION

According to the pathway of spiramycin biosynthesis, the acetate, proprionate, and butyrate are the precursors for spiramycin biosynthesis (Omura *et al.*, 1977; Li and Chen, 2002). The addition of short-chain fatty acids can stimulate the production of spiramycin by *Streptomyces ambofaciens* (Khaoua *et al.*, 1992; Laakel *et al.*, 1994). Short-chain fatty acids not only can supply the precursors for spiramycin biosynthesis but also can induce the synthesis of acylkinases and acylphosphotransferases, by which the short-chain fatty acids are activated.

The potency of spiramycin fermentation by *Streptomyces spiramyceticus* can be increased greatly by adding soybean oil either at the beginning or at 48 hours after cultivation (Zhang and Jin, 1993). Soybean oil can supply both the energy and carbon source needed for the organisms. In spiramycin fermentation, soybean oil is an ideal carbon source since it gradually supplies the short-chain fatty acids during the fermentation process. During the process of spiramycin fermentation, the intracellular ATP content was lower in the medium containing oil than in the medium without oil (Li *et al.*, 1990). The lower content of ATP favors spiramycin biosynthesis (Li *et al.*, 1990; Lounes *et al.*, 1995).

S. ambofaciens XC 1-29 is susceptible to soybean oil. The oil tolerant mutant strains can grow on agar medium containing soybean oil as the only resource of carbon, which means that the oil tolerant mutant strains can produce more lipase to ca-

Table 5 Parameters of fermentor scale-up

Fermentor	15 L	60 m^3
Agitation speed (rpm)	600	130
Air flow rate (m^3/h)	0.4	2300
Non-aerated agitation power per unit volume (KW/m^3)	3.3	4.5
Aerated agitation power per unit volume (KW/m^3)	1.41	2.78
Liquid phase oxygen transfer coefficient (h^{-1})	3637	13988

Note: fomulae (Gao, 1989): $P_0/V=N_Pd^5N^3\rho/V$; $Pg/V=2.25(P_0^2ND^3/Q^{0.08})^{0.39}\times10^{-3}/V$; $k_La=(2.36+3.3Ni)(Pg/V)^{0.56}v_S^{0.7}(60\times N)^{0.7}\times0.286$

Table 6 Comparison of spiramycin fermentation levels between the new process and the original one

	The new process with *S. ambofaciens* XC 2-37	The original process with *S. ambofaciens* XC 1-29	Increased (%)
Potency (mg/L)	4500	3150	42.9
Productivity (mg/L·h)	41.67	29.17	42.9

talyze soybean oil into short-chain fatty acids. An abundant supply of short-chain fatty acids would cause higher spiramycin potency during the fermentation process with the oil tolerant mutant strains. It is supposed that the addition of soybean oil can enhance the spiramycin potency of the oil tolerant mutant strains further. Our experiments showed that there was 24% increase in spiramycin potency by the addition of soybean oil, and that there was 61.8% increase in spiramycin potency by the addition of both soybean oil and propyl alcohol. It is inferred that catalytic decomposition of soybean oil can produce many even fatty acids and few odd fatty acids. Biosynthesis of spiramycin requires both even fatty acids and odd acids as precursors. Therefore simultaneous addition of soybean oil and propyl alcohol can increase the spiramycin potency largely, while the addition of soybean oil alone or propyl alcohol alone can increase spiramycin potency a little only.

In the scale-up of spiramycin fermentation from 15 L to 60 m^3, the spiramycin potency with the new process by the new strain *S. ambofaciens* XC 2-37 in industrial fermentator is the same as that in laboratory fermentor, which explains that the new process by the new strain has great importance in industrial application.

CONCLUSION

A new strain, *Streptomyces ambofaciens* XC 2-37, which produced 9% more spiramycin than the parent strain *S. ambofaciens* XC 1-29, was obtained by screening the oil tolerant mutant strains. Study of the effect of soybean oil and propyl alcohol on spiramycin production with *S. ambofaciens* XC 2-37 showed that the potency of spiramycin fermentation can be improved by 61.8% with addition of 2% soybean oil in the fermentation medium and 0.4% propyl alcohol at 24 hours of incubation. After the new process by *S. ambofaciens* XC 2-37 was scaled up for spiramycin production on industrial scale, the potency and productivity of spiramycin fermentation was increased by 42.9%.

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