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

Scale-up of rifamycin B fermentation with *Amycolatoposis mediterranei*

JIN Zhi-hua (金志华)[1,2], LIN Jian-ping (林建平)[1], CEN Pei-lin (岑沛霖)[†1]

([1]*Institute of Bioengineering, School of Materials Science and Chemical Engineering, Zhejiang University, Hangzhou 310027, China*)

([2]*Department of Bioengineering and Pharmaceutical Engineering, Ningbo Institute of Technology, Zhejiang University, Ningbo 315100, China*)

[†]E-mail: cenpl@cmsce.zju.edu.cn

Received Aug. 8, 2003; revision accepted Oct. 22, 2003

Abstract: Study of the effect of dissolved oxygen and shear stress on rifamycin B fermentation with *A. mediterranei* XC 9-25 showed that rifamycin B fermentation with *Amycolatoposis mediterranei* XC 9-25 needs high dissolved oxygen and is not very sensitive to shearing stress. The scale-up of rifamycin B fermentation with *A. mediterranei* XC 9-25 from a shaking flask to a 15 L fermentor was realized by controlling the dissolved oxygen to above 25% of saturation in the fermentation process, and the potency of rifamycin B fermentation in the 15 L fermentor reached 10 g/L after 6-day batch fermentation. By continuously feeding glucose and ammonia in the fermentation process, the potency of rifamycin B fermentaion in the 15 L fermentor reached 18.67 g/L, which was 86.65% higher than that of batch fermentation. Based on the scale-up principle of constantly aerated agitation power per unit volume, the scale-up of rifamycin B fed-batch fermentation with continuous feed from a 15 L fermentor to a 7 m^3 fermentor and further to a 60 m^3 fermentor was realized successfully. The potency of rifamycin B fermentation in the 7 m^3 fermentor and in the 60 m^3 fermentor reached 17.25 g/L and 19.11 g/L, respectively.

Key words: *Amycolatoposis mediterranei*, Fermentation, Rifamycin B, Scale-up
doi:10.1631/jzus.2004.1590 **Document code:** A **CLC number:** TQ 927

INTRODUCTION

Development of fermentation process is usually carried out in three steps: (1) flask scale to screen strains and evaluate medium composition; (2) pilot scale to establish optimal fermentation condition; (3) industrial scale process to produce desired product economically. Usually, the productivity of the desired product is high in flask scale, and will be gradually reduced as the scale is enlarged because of the complexity of fermentation process. This will affect the efficiency of industrial fermentation process. So it is important to study scale-up of the fermentation process and adopt suitable strategy of scale-up in order to increase the productivity of the desired product on the industrial level.

The method for scaling-up a fermentation system is usually based on empirical criteria such as constant power input per unit volume, a constant mass transfer coefficient, constant mixing time and constant impeller tip velocity (Shuler and Kargi, 1992). Among these criteria, the agitation power per unit volume is the most frequently used parameter for scale-up. As sensor technologies developed for dissolved oxygen (DO) concentration, scale-up method to maintain DO (Oosterhuis and Kossen, 1983) at a constant level became popular. The scale-up method related to oxygen transfer is established by controlling the DO concentration at a suitable level. When filamentous microorganisms, such as fungi or actinomycetes, are cultured in a

large fermentor, cells are damaged by shear stress (Ziegler *et al*., 1980), and product yield is reduced. Shear tress is considered to be proportional to the tip speed of the agitation impeller. A microorganism that is sensitive to shear stress should be cultured with large-diameter impellers or multiple impellers (Bader, 1986), to maintain a suitable DO level with low agitation speed.

The rifamycins (Oppolzer and Prelog, 1973), exemplified by rifamycin B, are a family of ansamycin antibiotics (Rinehart and Shield, 1976) with pronounced anti-mycobacterial activity that are extensively used in the clinical treatment of tuberculosis, leprosy and AIDS-related mycobacterial infections (Sepkowitz *et al*., 1995). The rifamycins are produced by *Amycolatoposis mediterranei* (Sensi *et al*., 1959), and their biosynthesis involves a polyketide intermediate synthesized with chain extension of an unusual starter unit, 3-amino-5-hydroxybenzoic acid (AHBA) (Kibby *et al*., 1980; Ghisalba and Nüesch, 1981), and its assembly with two acetate and eight propionate units on a type I polyketide synthase (PKS) (Lancini and Carvelieri, 1997). According to the pathway and the metabolic regulation of rifamycin B biosynthesis, improvement of a rifamycin B producing *Amycolatoposis mediterranei* XC-102 was realized by rational screening strategy with which a strain *A. mediterranei* XC 9-25, whose rifamycin B potency in shaking flask reached 10 g/L, was obtained (Jin *et al*., 2002).

In order to increase the productivity of *A. mediterranei* XC 9-25 on industrial scale, the effect of dissolved oxygen and shear stress on rifamycin B fermentation with *A. mediterranei* XC 9-25 was studied, and the scale-up strategy of rifamycin B fermentation with *A. mediterranei* XC 9-25 from a shaking flask to a 15 L fermentor, to a 7 m^3 fermentor and further to a 60 m^3 fermentor was evaluated.

MATERIAL AND METHOD

Microorganism

Amycolatoposis mediterranei XC 9-25 (Jin *et al*., 2002) was employed in this study. Slant medium consisted of the following (per litter): 4 g yeast extract, 4 g malt extract, 4 g glucose, 20 g oatmeal, 20 g agar, and 1 litter distilled water. Slant culture was incubated for 7–8 days at 28 °C and 40%–50% relative humidity.

Rifamycin B fermentation in flask

The mycelium from slant culture was inoculated into a 250 ml Erlenmeyer flask containing 25 ml of seed medium (2% starch, 1.5% glucose, 1% soybean meal, 1% peptone, 0.05% KNO_3, 0.03% KH_2PO_4, 0.3% $CaCO_3$). After incubation at 28 °C for 48 hours on a rotary shaker at 220 r/min, a 2 ml portion of the seed culture was used to inoculate 25 ml of production medium into 250 ml Erlenmeyer flask.

Production medium was comprised of 12% glucose, 2% soybean meal, 1% peptone, 0.5% fish meal, 0.8% KNO_3, 0.05% KH_2PO_4, 0.0001% $CoCl_2$, 0.5% $CaCO_3$. Production culture was incubated under the same conditions for the seed culture, except that the cultivation period was extended to 7 days.

Rifamycin B fermentation in a 15 L fermentor

The fermentor (15 L) containing 8 L of production medium was inoculated with 800 ml of broth cultured in shaking flasks. After 144 hours cultivation at 28 °C, the fermentation was completed.

For fed-batch fermentation, glucose and ammonia were fed continuously during the fermentation process in order to maintain the reduced sugar concentration at 1.0%−1.5% and the pH value at 6.4−6.6. After 9 days cultivation at 28 °C, the fermentation broth was harvested.

Rifamycin B fed-batch fermentation in a 7 m^3 fermentor and in a 60 m^3 fermentor

Three-stage fermentation procedure was adopted in industrial fermentation: first-seed culture, second-seed culture and industrial fermentation. The temperature for seed culture and industrial fermentation was 28 °C, and the cultivation time for first and second seed culture was 72 and 48 hours, respectively. The inoculum ratio for sec-

ond-seed culture and industrial fermentation was about 10%. During fed-batch fermentation, glucose and ammonia were fed continuously in order to maintain the reduced sugar concentration at 1.0%–1.5% and the pH value at 6.4–6.6. After 9 days cultivation at 28 °C, the fermentation broth was harvested.

Analytical method

Reduced sugar was measured by Fehling's reagent method. Amino nitrogen was analyzed by the formaldehyde titration method (Chen and Xu, 1991). The dried cell weight (DCW) was determined according to the method described by Leblihi *et al*.(1987). Rifamycin B was assayed by the spectrophotometric method (Sensi and Thiemann, 1967).

RESULT AND DISCUSSION

Effect of dissolved oxygen on rifamycin B fermentation

The medium volume in a shaking flask and the rotating rate of a shaking bed can affect the dissolved oxygen level of the fermentation broth in a shaking flask. The smaller the medium volume in a shaking flask, the higher is the dissolved oxygen. Also, higher rotating rate of a shaking bed is favorable for enhancing oxygen mass transfer. When the rotating rate of the shaking bed was fixed at 220 r/min, the effects of medium volume in a 250 ml shaking flask on rifamycin B fermentation with *A. mediterranei* XC 9-25 are listed in Table 1.

The experimental data in Table 1 indicates that the medium volume played a very important role in rifamycin B fermentation. If the medium volume in a 250 ml shaking flask was less than 25 ml, the rifamycin B potency was almost the same as that of 25 ml medium volume. With the increase of medium volume, the rifamycin B potency was reduced so quickly that no rifamycin B was synthesized when 50 ml medium was added into a 250 ml shaking flask.

When the medium volume in a 250 ml shaking flask was fixed at 25 ml, the effect of different rotating rate of a shaking bed on rifamycin B fermentation with *A. mediterranei* XC 9-25 was as shown in Table 2. From Table 2, it was obvious that rotating rate was also a very important factor for rifamycin B fermentation. If the rotating rate of a shaking bed was decreased from 260 r/min to 180 r/min, the rifamycin B potency was reduced 41%.

These results illustrated that the dissolved oxygen had significant role in rifamycin B fermentation.

Effect of shear stress on rifamycin B fermentation

Effect of shear stress on rifamycin B fermentation was evaluated by adding glass beads into a shaking flask. The experimental data in Table 3 revealed that the addition of glass beads in a shaking flask had little effect on rifamycin B fermentation.

Table 1 Effect of medium volume in a 250 ml shaking flask on rifamycin B production with *A. mediterranei* XC 9-25

Medium volme (ml)	pH	Dried cell weight (g/L)	Residual sugar (%)	Residual amino nitrogen (mg/L)	Relative potency (%)
20	8.3	16	1.08	8.96	100
25	8.4	17	1.16	9.52	96
35	8.5	15	3.50	8.96	46
50	8.8	5	8.42	10.08	0

Table 2 Effect of rotating rate of a shaking bed on rifamycin B production with *A. mediterranei* XC 9-25

Rotating rate (r/min)	pH	Dried cell weight (g/L)	Residual sugar (%)	Residual amino nitrogen (mg/L)	Relative potency (%)
260	8.2	18	1.08	8.96	100
220	8.3	17	1.16	9.52	95
180	8.4	16	3.14	8.96	59

Table 3 Effect of the number of glass beads in a shaking flask on rifamycin B production with *A. mediterranei* XC 9-25

Number of beads	pH	Dried cell weight (g/L)	Residual sugar (%)	Residual amino nitrogen (mg/L)	Relative potency (%)
0	8.3	18	1.08	8.96	100
1	8.4	17	1.16	9.52	98
2	8.5	15	1.08	9.52	95
3	8.5	15	1.16	10.08	90

Even if three glass beads were added in a shaking flask, the rifamycin B potency was only reduced 10%. The results suggested that rifamycin B fermentation was not very sensitive to shear stress.

Rifamycin B batch fermentation in a 15 L fermentor

The shaking flask experimental data showed obviously that rifamycin B fermentation with *A. mediterranei* XC 9-25 needed high amount of dissolved oxygen and was not very sensitive to shear stress, so the dissolved oxygen was used as the main basis for scale-up of rifamycin B fermentation.

Rifamycin B fermentation experiment with *A. mediterranei* XC 9-25 in a 15 L stirred tank was performed. Varying agitation rate (400 r/min, 500 r/min, 550 r/min and 600 r/min, respectively), varied the dissolved oxygen in the fermentation broth accordingly. The relationship between the lowest amount of dissolved oxygen and rifamycin B production is shown in Fig.1.

Fig.1 shows that if the agitation rate was 550 r/min and 600 r/min, the lowest amount of dissolved oxygen was 25% and 40% of saturation, respectively; and that the rifamycin B potency was about 10 g/L. If the agitation rate was reduced to 500 r/min and 400 r/min, the lowest amount of dissolved oxygen decreased to 5% and 0% of saturation, respectively, and then the rifamycin B potency was only 5 g/L and 2 g/L, accordingly. These results illustrated that rifamycin B fermentation was dependent on high amount of dissolved oxygen, and that the dissolved oxygen must be kept above 25% of saturation to meet the oxygen requirement for mycelium growth and rifamycin B production.

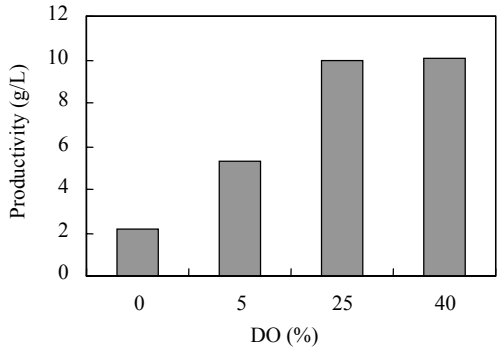

Fig.1 Relationships between dissolved oxygen and rifamycin B production

On condition that the dissolved oxygen was maintained above 25% of saturation, the time courses of fermentation with *A. mediterranei* XC 9-25 in a 15 L fermentor are shown in Fig.2.

Fig.2 shows that the rifamycin B potency in a 15 L fermentor could reach 10 g/L after 6-day batch fermentation. Compared to the fermentation in shaking flasks (Jin *et al*., 2002), the period of rifamycin batch fermentation in a 15 L fermentor was shortened from 7 days to 6 days because of improvement in oxygen mass transfer.

Rifamycin B fed-batch fermentation in a 15 L fermentor

Fed-batch fermentation is an important operation mode to increase productivity for secondary metabolites, such as antibiotics. For achieving fed-batch fermentation for rifamycin B production with *A. mediterranei* XC 9-25 in a 15 L fermentor, the following feeding method was adopted: after cultivation for 72 hours, glucose was added continuously to maintain the reduced sugar at 1%–1.5% while ammonia was also added automatically to complement nitrogen source and to maintain the pH value of the fermentation broth at 6.4–6.6. The time courses of fed-batch fermentation with *A. mediterranei* XC 9-25 in a 15 L fermentor are shown in Fig.3.

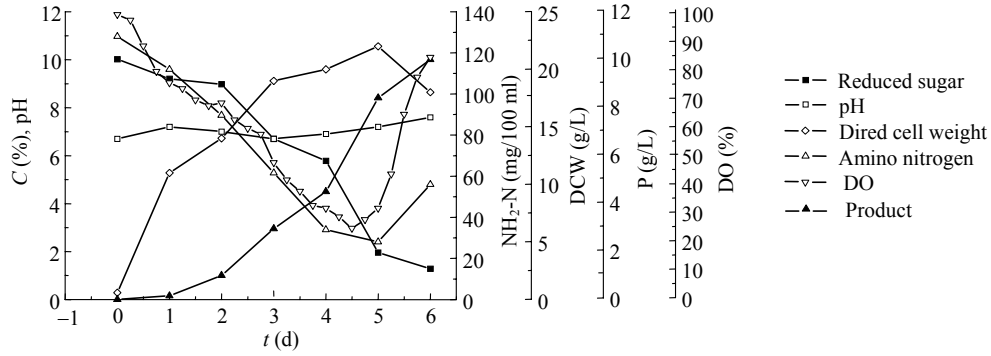

Fig.2 Batch fermentation process of rifamycin B with *A. mediterranei* XC 9-25 in a 15 L fermentor

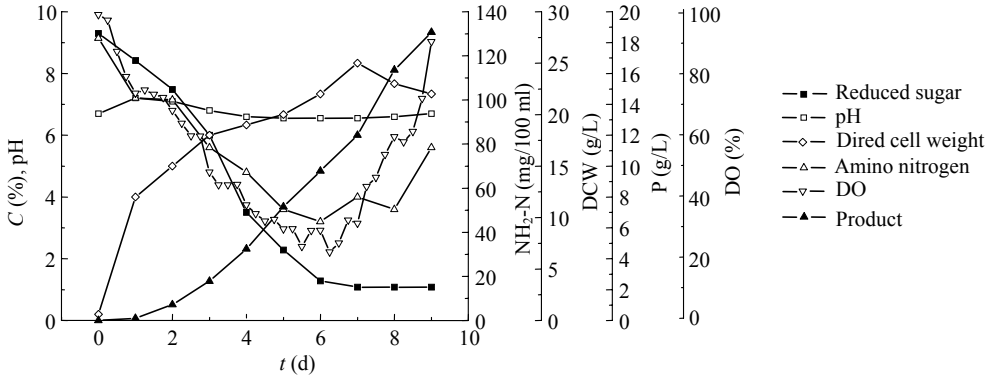

Fig.3 Rifamycin B fed batch fermentation with continuous feed in a 15 L fermentor

Fig.3 shows that the rifamycin B concentration at the end of fed-batch fermentation was much higher than that of batch fermentation. The rifamycin B potency could reach 12 g/L after 7-day fermentation and 18.67 g/L after 9-day fermentation. The above results indicate that the fed-batch method is favorable for rifamycin B fermentation, because rifamycin B production with *A. mediteranei* XC 9-25 is a non-growth associated process (Jin *et al.*, 2002). The feeding rate of glucose was under control according to the glucose concentration in the fermentation broth, so that the mycelium growth rate and mycelium concentration could be in favor of rifamycin B formation.

Fermentor scale-up criteria

Rifamycin B fermentation was scaled up for rifamycin B production in a 7 m^3 fermentor and further in a 60 m^3 fermentor, respectively, in Xinchang Pharmaceutical Factory, Zhejiang Medicine Company Ltd., China. The calculated main parameters for scale-up from a 15 L fermentor to a 7 m^3 fermentor and a 60 m^3 fermentor are listed in Table 4.

Table 4 shows obviously that the aerated agitation power per unit volume in the 60 m^3 fermentor was almost the same as that in the 7 m^3 fermentor and was higher than that in the 15 L fermentor. However, the oxygen transfer coefficient (k_La) in the 60 m^3 fermentor was much higher than that in the 7 m^3 fermentor and in the 15 L fermentor. Although the 7 m^3 fermentor and the 60 m^3 fermentor were not designed specially for rifamycnin B fermentation, they accorded with the scale-up principle of constant aerated agitation power per unit volume.

Rifamycin B fed-batch fermentation in a 7 m^3 fermentor

The time courses of fed-batch fermentation in the 7 m^3 fermentor with *A. mediterranei* XC 9-25are shown in Fig.4 showing that the rifamycin B potency of fed-batch fermentation in the 7 m^3 fermentor with *A. mediterranei* XC 9-25 could reach 17.25 g/L, which was only about 7% lower

Table 4 Parameters of fermentor scale-up

Fermentor	N (r/min)	Q (m^3/h)	P_0/V (kW/ m^3)	P_g/V (kW/ m^3)	k_La (h^{-1})
15 L	600	0.4	3.3	1.41	3637
7 m^3	220	260	4.4	2.70	8378
60 m^3	130	2300	4.5	2.78	13988

Note: fomulae (Gao, 1989):

$P_0/V=N_pD^5N^3\rho/V$; $P_g/V=2.25(P_0^2ND^3/Q^{0.08})^{0.39}\times10^{-3}/V$; $k_La=(2.36+3.3N_i)(P_g/V)^{0.56}v_s^{0.7}(60\times N)^{0.7}\times0.286$

*where D is impeller diameter (m), k_La is liquid phase oxygen transfer coefficient (h^{-1}), N is agitation speed (r/min), N_i is number of impeller, N_p is agitation power number, P_0 is non-aerated agitation power (kW), P_0/V is non-aerated agitation power per unit volume (kW/m^3), P_g is aerated agitation power (kW), P_g/V is aerated agitation power per unit volume (kW/m^3), Q is air flow rate (m^3/h), v_s is linear air flow rate through the inner section of fermentor (m/s), V is broth volume in fermentor (m^3), and ρ is broth density (kg/m^3)

than that in the 15 L fermentor.

Rifamycin B fed-batch fermentation in 60 m^3 fermentor

The time courses of fed-batch fermentation in the 60 m^3 fermentor with *A. mediterranei* XC 9-25 are shown in Fig.5 showing that:

(1) The metabolic statue of fed-batch fermentation in the 60 m^3 fermentor was similar to that in the 15 L and 7 m^3 fermentors.

(2) Amino nitrogen concentration during the stationary phase of fed-batch fermentation in the 60 m^3 fermentor was higher than that in the 15 L and 7 m^3 fermentors, due to the more rapid consumption of glucose. The faster glucose consumption rate will result in increased glucose feeding, which will decrease the pH in the fermentation broth. In order to keep the pH at 6.4–6.6, more ammonia must be fed, which results in higher concentration of amino nitrogen.

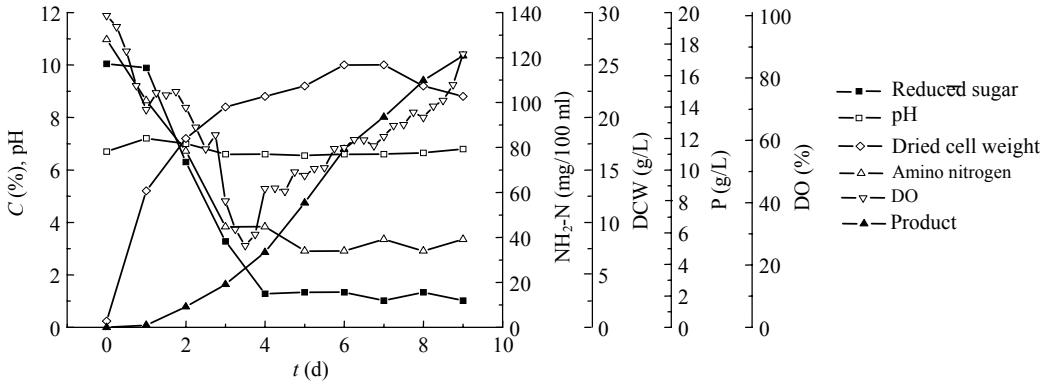

Fig.4 Rifamycin B fed-batch fermentation with continuous feed in a 7 m^3 fermentor

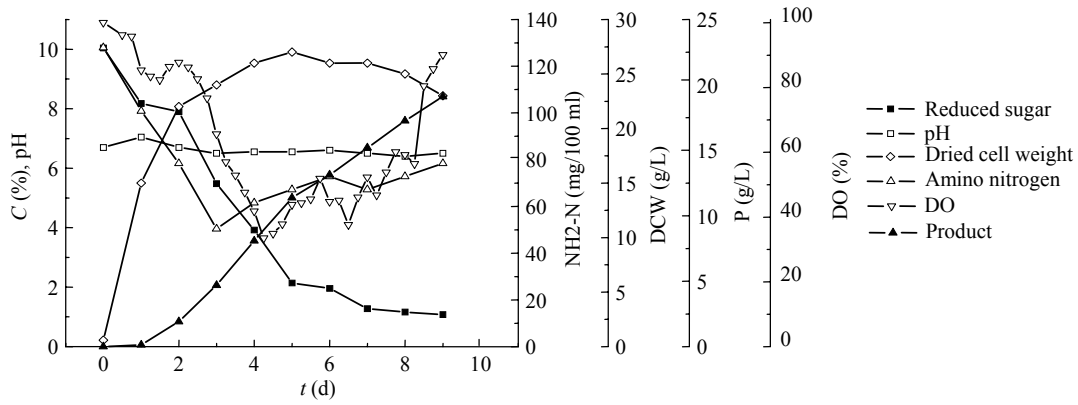

Fig.5 Fed-batch fermentation of Rifamycin B in a 60 m^3 fermentor with *A. mediterranei* XC 9-25

(3) The dried cell weight of fed-batch fermentation in the 60 m^3 fermentor could reach 25 g/L, which was higher than that in either the 15 L or 7 m^3 fermentor.

(4) The rifamycin B potency of fed-batch fermentation in the 60 m^3 fermentor could reach 19.11 g/L, which was 3% and 10% higher than that in the 15 L and 7 m^3 fermentors, respectively. The main reason was the further improvement of oxygen mass transfer (k_La) in the 60 m^3 fermentor.

CONCLUSION

Shaking flask experiments data indicated that rifamycin B fermentation with *Amycolatoposis mediterranei* XC 9-25 needed high amount of dissolved oxygen and was not very sensitive to shearing stress. By controlling the dissolved oxygen to above 25% of saturation in the fermentation process, the scale-up of rifamycin B fermentation with *A. mediterranei* XC 9-25 from a shaking flask to a 15 L fermentor was realized. The potency of rifamycin B fermentation in the 15 L fermentor could reach 10 g/L after 6-day batch fermentation.

Fed-batch fermentation with continuous feed method favors increasing production of non-growth associated product. By continuously feeding glucose and ammonia in the fermentation process, the potency of rifamycin B fermentation in a 15 L fermentor could reach 18.67 g/L, which was 86.65% higher than that of batch fermentation.

For shear stress-insensitive fermentation process, the scale-up principle of constant aerated agi tation power per unit volume can be applied. And the rifamycin B fed-batch fermentation with continuous feed was scaled up successfully from a 15 L to a 7 m^3 fermentor and further to a 60 m^3 fermentor. The potency of rifamycin B fermentation in the 7 m^3 and 60 m^3 fermentors could reach 17.25 g/L and 19.11 g/L, respectively.

References

Bader, F.G., 1986. Modeling mass transfer and agitator per-formance in multiturbine fermentor. *Biotechnol Bioeng*, **30**:37-51.

Chen, J.M., Xu, L.T., 1991. Analysis of Antibiotic Industry. Chinese Press of Pharmaceutical Science, Beijing, p. 109-111 (in Chinese).

Gao, K.N., 1989. Fermentation Engineering and Equipment. Press of Light Industry, Beijing, p.188-222 (in Chinese).

Ghisalba, O., Nüesch, J., 1981. A genetic approach to the biosynthesis of the rifamycin-chromophore in *Nocardia mediterranei*. IV Identification of 3-amino-hydroxybenzoic acid as a direct precursor of the seven-carbon amino starter-unit. *J Antibiot*, **34**:67-71.

Jin, Z.H., Lin, J.P., Xu, Z.N., Cen, P.L., 2002. Improvement of industry-applied rifamycin B producing strain, *Amycolatopsis mediterranei*, by rational screening. *J Gen Appl Microbiol*, **48**:329-334.

Kibby, J.J., McDonald, I.A., Richards, W.R., 1980. 3-Amino-5-hydroxybenzoic acid as a key intermediate in asamycin and maytansinoid biosynthesis. *J Chem Soc Chem Commun*, **1980**:768-769.

Lancini, G., Carvelieri, B., 1997. Rifamycins. *In*: Strohl, W. R. (Ed.), Biotechnology of Antibiotics, vol. 2. Dekker, New York, p.521-549.

Leblihi, A., Germain, P., Lefebvre, G., 1987. Phosphate repression of cephamycin and clavulanic acid production by *Streptomyces clavuligerus*. *Appl Microbiol Biotechnol*, **28**:44-51.

Oosterhuis, N.M.G., Kossen, N.W.F., 1983. Dissolved oxygen concentration profiles in a production-scale bioreactor. *Biotechnol Bioeng*, **26**:546-550.

Oppolzer, W., Prelog, V., 1973. On the constitution and cofiguration of rifamycin B, O, S and SV. *Helv Chim Acta*, **56**:2287-2314.

Rinehart, K.L.Jr., Shield, L.S., 1976. Chemistry of the ansamycin antibiotics. *Fortschr Chem Org Naturst*, **33**: 231-307.

Sensi, P., Thiemann, J.E., 1967. Production of rifamycins. *Prog Ind Microbiol*, **6**: 21-59.

Sensi, P., Margalis, P., Timbal, M.T., 1959. Rifamycin, a new antibiotic: preliminary report. *Farmaco Ed Sci*, **14**: 146-147.

Sepkowitz, K.A., Rafalli, J., Riley, L., Kiehn, T.E., Armstrong, D., 1995. Tuberculosis in the AIDS era. *Clin Microbiol Revs*, **8**:180-199.

Shuler, F.L., Kargi, F., 1992. Bioprocess Engineering: Basic Concepts. Prentice-Hall, Englewood Cliffs, NJ.

Ziegler, H., Dunn, I.J., Bourine, J.R., 1980. Oxygen transfer and mycelial growth in a tubular loop fermentor. *Biotechnol Bioeng*, **22**:1613-163.