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Effects of cultivation conditions on the production of natamycin with *Streptomyces gilvosporeus* LK-196

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Abstract

Natamycin is a very attractive antifungal agent with wide applications in medical and food industries. In order to improve the productivity of natamycin, the effects of cultivation conditions were investigated with *Streptomyces gilvosporeus* LK-196 in the shake flasks and 30-L fermentors. The results showed that dissolved oxygen and shear force would affluence the biosynthesis of natamycin significantly. The high concentration of natamycin (2.03 g/L) was achieved under the suitable culture conditions in the shake flask scale. Further investigations in 30-L fermentors showed that the optimal pH was controlled at 6.0 during the whole bioprocess, and the dissolved oxygen level should be more than 30% by adjusting the aeration and agitation rates for high production of natamycin. Under these optimal conditions the high concentration of natamycin (3.94 g/L) was achieved with *Str. gilvosporeus* LK-196 in the 30-L fermentor. Finally, the high-level fermentation process was successfully scaled up to 1000-L fermentors and 18,000-L fermentors in the pilot plant.

Keywords: Natamycin; *Streptomyces gilvosporeus*; Cultivation conditions

1. Introduction

Natamycin, also known as pimaricin, is a polyene macrolide antibiotic produced in submerged culture of some *Streptomyces* strains such as *Str. natalensis*, *Str. chattanoogensis* and *Str. gilvosporeus* [1]. It is a very attractive antifungal agent. The importance of this antibiotic lies in its broad-spectrum activity against yeasts and molds with low toxicity against mammalian cells [2]. Natamycin is used to treat fungal Keratitis because it is especially effective against *Aspergillus* and *Fusarium* corneal infections [3]. Besides its medical applications, natamycin is also used in food preservation and approved as a Generally Regarded As Safe (GRAS) product by Food and Drug Administration (FDA) for use in food manufacturing. Therefore, natamycin is widely used in many food industries to increase the shelf time without any effect on flavor or appearance [4,5].

Although many efforts were paid to clone the biosynthetic gene clusters and investigate the pathways of natamycin biosynthesis in *Str. natalensis* [6,7], few reports were found to study the fermentation process for enhanced production of natamycin [8,9]. The effects of inoculum types and cultivation conditions on natamycin production were examined with *Str. natalensis* NRRL 2651 [8], and one optimized medium was formulated to support high production of natamycin [9]. However, under the above optimal conditions, the volumetric productivity of natamycin was still very low (1.5 g/L), and the investigation on the effects of culture conditions on natamycin biosynthesis in *Str. gilvosporus* was never reported. In order to meet its increasing market, it is very demanding to improve the productivity of natamycin by strain improvement or bioprocess optimization. In our previous work, we selected one genetic-improved mutant (LK-119) of *Str. gilvosporus* after space-flight as a potential high natamycin producer, and one optimal medium was formulated for enhanced production of natamycin with this strain [10]. Several high-yield strains including *Str. gilvosporus* LK-196 were further separated from LK-119 culture in our laboratory. The objectives of this work were: (1) to investigate the effects of different culture conditions for natamycin produced by *Str. gilvosporus* LK-196 in shake flasks and 30-L bench-top fermentor and (2) to scale up the fermentation process both in 1000-L and 18,000-L fermentors by properly adjusting fermentation conditions. High production

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of natamycin (3.94 g/L) was achieved with *Str. gilvosporus* LK-196 in the 30-L bench-top fermentor by optimizing fermentation parameters systematically. Then, according to the optimized culture conditions, this natamycin fermentation bioprocess was successfully scaled up to 1000-L and 18,000-L fermentors.

2. Materials and methods

2.1. Microorganisms

Str. gilvosporeus LK-196, a high-yield mutant derived from *Str. gilvosporeus* ATCC 13326, was used in this study.

2.2. Media and culture conditions

Slant medium (g/L): Glucose, 10.0; malt extract, 3.0; yeast extract, 3.0; peptone, 5.0 and agar, 20.0. The pH of the medium was pre-adjusted to 7.2–7.4 before sterilization. Slant culture was incubated for 8–12 days at 28 °C and 30–60% humidity.

The arisen spores from slant culture were inoculated into a 500 mL Erlenmeyer flask containing 40 mL of seed medium (glucose, 20.0 g/L; malt extract, 6.0 g/L; peptone, 6.0 g/L; NaCl, 0.2 g/L and pH 7.0–7.2 before sterilization). After incubation at 28 °C for 48 h on a rotary shaker at 220 rpm, a 5 mL portion of the seed culture was used to inoculate 40 mL production medium in 500 mL Erlenmeyer flask. The production medium was composed of (g/L): glucose, 60; soybean cake meal, 10.0; peptone, 5.0; yeast extract, 5.0; beef extract, 5.0; NaCl, 2.0; $CaCO_3$, 5.0; $MgSO_4$, 1.0 and pH 7.6 before sterilization. The production culture was incubated under the same conditions as the seed culture, except the investigated conditions.

2.3. The investigation of culture conditions in 30-L fermentors

According to the above protocol, about 240 mL seeds were prepared and inoculated into 30-L fermentor containing 20-L production medium. The pH, temperature, agitation and foam level were automatically controlled. During all tests, the agitation speed and airflow rate were adjusted for the investigation of their effects on natamycin production. Samples were taken at various intervals to measure cell concentration, natamycin concentration and residue glucose level. The samples were centrifuged at 12,000 × *g* for 5 min to prepare cell supernatants.

2.4. The upscaled production of natamycin in the pilot plant

The vegetative 30-L fermentor containing 20 L of seed medium was inoculated with 240 mL of broth cultured in the flasks. After 48 h incubation at 28 °C, the vegetative broth was used to inoculate 1000-L fermentor containing 700-L production medium. During the fermentation, the agitation and aeration were adjusted to maintain a high DO level (more than 30%), and the fermentation was ended after 96 h cultivation.

The seeds in 30-L fermentor could be transferred to 1000-L fermentors containing 700-L seed medium. After 48 h incubation at 28 °C, the vegetative broth was used to inoculate 18,000-L fermentor containing 12,000-L production medium. During the fermentation, the agitation and aeration were adjusted to maintain a high DO level (more than 30%), and the fermentation was ended after 96 h cultivation.

2.5. Analytic method

The dried cell weight (DCW) was determined according to the method described by Leblihi et al. [11]. Glucose concentration in the supernatant was measured with DNS method [12]. The concentration of natamycin in the broth was determined by HPLC according to the method in the United State Pharmacopoeia edition 29 [13].

3. Results and discussions

3.1. Primary studies on the effects of shearing force and dissolved oxygen in the shake flasks

The effects of shearing force on the productivity of natamycin were investigated by adding different numbers of glass beads into the shake flasks. As shown in the Table 1, the addition of glass beads obviously harmed the mycelial growth of *Str. gilvosporeus* LK-196 and reduced natamycin concentration in the broths. Even when only one glass bead was added to 500 mL flask containing 40 mL production medium, after 4 days fermentation, the dry mycelial weight and the volumetric productivity of natamycin would be reduced by 7.2% and 31.3%, respectively. The more the number of glass beads added, the worse the mycelial growth and the production of natamycin. It indicated that the growth of this strain was very sensitive to shearing force and high shearing force would reduce the production of natamycin in the fermentation process seriously.

The effects of dissolved oxygen on the productivity of natamycin were investigated by adding different quantity of production medium into 500 mL flasks. Similarly, the increase of medium volume greatly influenced the mycelial growth and the biosynthesis of natamycin by *Str. gilvosporeus* LK-196 (Table 1). When medium volume was changed from 40 mL to 100 mL, the productivity of natamycin was reduced by 95.1%, although the mycelial weight did not decrease so obviously. According to the above investigation, it is essential to maintain the enough supply of dissolved oxygen to improve the production of natamycin. Further comparison of the decrease of natamycin concentrations in the broths suggested that the influence of shearing force was less serious than that of dissolved oxygen during the fermentation process. Compared with the original productivity of natamycin (1.8 g/L) with this strain, about 17% improvement of natamycin production was achieved by adding suitable quantity of medium in the shake flask scale.

Table 1
The effects of glass bead addition and medium volume on the production of natamycin

Items	Number of glass beads					Medium volume (mL)			
Number	0	1	2	3	6	40	60	80	100
DCW (g/L)	18	16.7	16	15.4	14.3	16.8	15.6	14.9	14.5
Natamycin (g/L)	2.01	1.38	1.34	1.23	1.11	2.03	1.53	0.59	0.10

The dried cell weight and natamycin concentration were determined at the end of the 96 h cultivation period. The reported values are averages from triplicate flasks.

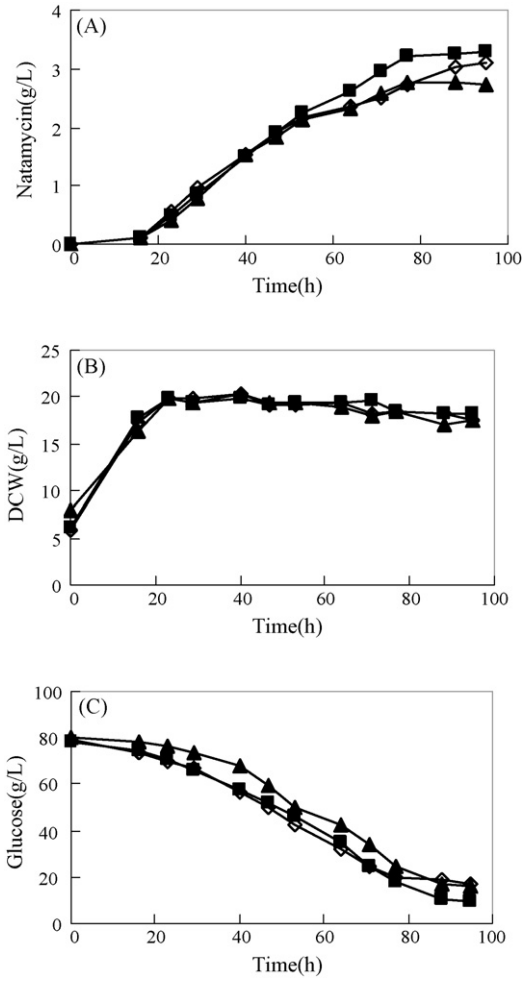

Fig. 1. The effects of different pH controls on mycelial growth, glucose consumption and natamycin biosynthesis during the cultivation of *Str. gilvosporeus* LK-196. The DO levels in 30-L fermentors were controlled over 30% by adjusting agitation (100–400 rpm) and aeration (~1 VVM) rates. Different pH arranges ((▲) pH 5.5 ± 0.2; (■) pH 6.0 ± 0.2 and (◊) pH 6.5 ± 0.2) in the broths were controlled by automatic addition of H_2SO_4 or NaOH.

3.2. The investigation of the effects of different culture conditions in 30-L fermentors

3.2.1. Effects of different pH control strategies

During the whole fermentation process with *Str. gilvosporeus* LK-196 in the 30-L fermentor, different pH arranges (5.5 ± 0.2, 6.0 ± 0.2 and 6.5 ± 0.2) were controlled by automatically feeding the alkaline or acidic solution, respectively. As shown in Fig. 1, the highest productivity of natamycin (3.3 g/L) was achieved when the medium pH was controlled at 6.0 ± 0.2, although the mycelial growth of *Str. gilvosporeus* LK-196 was not affected significantly by different pH control strategies. It was found that the consumption rate of glucose under pH control of 6.0 ± 0.2 was higher than those under pH control of 5.0 ± 0.2 or 6.5 ± 0.2, and the lowest concentration of residual glucose (10 g/L) was observed at 95 h under the pH control of 6.0 ± 0.2 among all these fermentation processes. It was concluded that the enhancement of glucose metabolism under the pH control of 6.0 ± 0.2 might be contributed to the increase of natamycin productivity in *Str. gilvosporeus* LK-196.

3.2.2. Effects of culture temperature

Under the suitable culture conditions, the effects of different culture temperatures were examined by carrying out the fermentation processes at 26 °C, 28 °C and 30 °C, respectively. As shown in Fig. 2A, the average rate of glucose consumption was almost same (0.58 g/L h) during these 96 h fermentation processes under different temperature controls. However, the profiles of glucose consumption during the cultivation process at 30 °C can be divided obviously by two phases, from 0 h to 65 h and 65 h to 96 h. And the consumption rate of glucose (0.65 g/L h) at first phase was much higher than those (0.47 g/L h) at the second phase. Accordingly, the highest dry mycelial weight (23.0 g/L) was obtained at 54 h, and further extension of culture time would lead to the decrease of cell mass seriously (Fig. 2B). At this temperature the biosynthesis of natamycin was not maintained for the whole fermentation

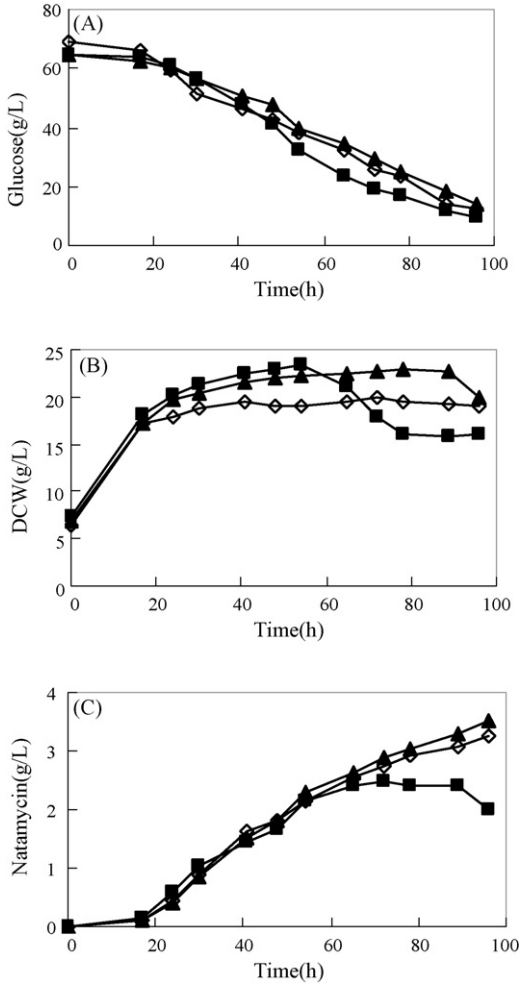

Fig. 2. The effects of different culture temperature on mycelial growth, glucose consumption and natamycin biosynthesis during the cultivation of *Str. gilvosporeus* LK-196. The DO levels in 30-L fermentors were controlled over 30% by adjusting agitation (100–400 rpm) and aeration (~1 VVM) rates. The pH in the broths was controlled at an arrange of 5.5 ± 0.2 by automatic addition of H_2SO_4 or NaOH ((◊) 26 ± 0.5 °C; (▲) 28 ± 0.5 °C and (■) 30 ± 0.5 °C).

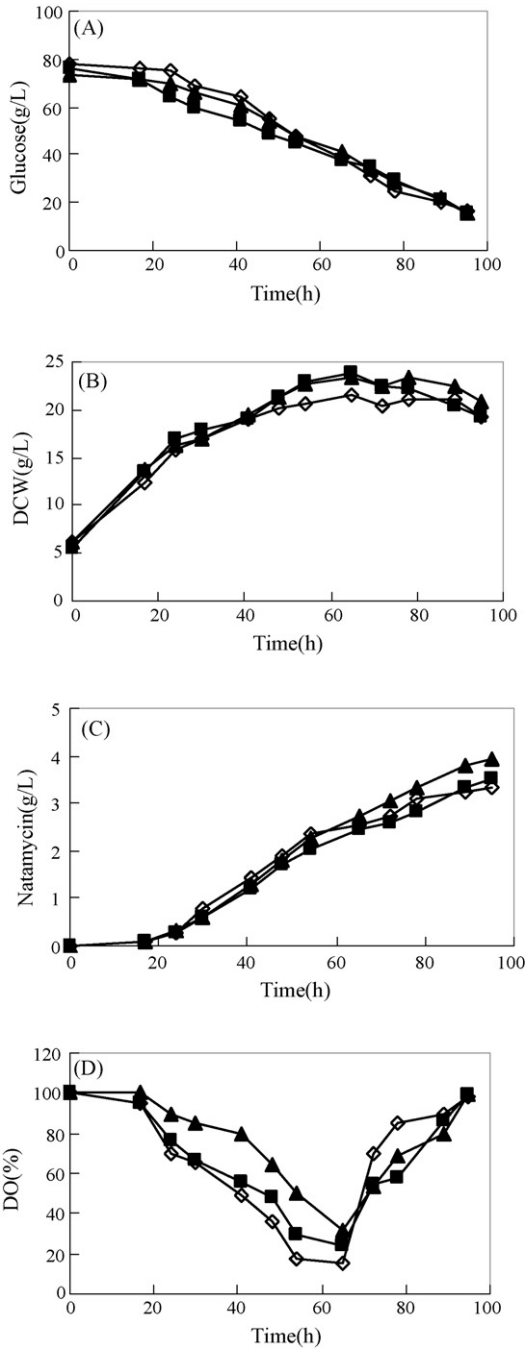

Fig. 3. The effects of different aeration rates on mycelial growth, glucose consumption and natamycin biosynthesis during the cultivation of *Str. gilvosporeus* LK-196 at 28 °C. The pH in the broths was controlled at an arrange of 5.5 ± 0.2 by automatic addition of H_2SO_4 or NaOH. The agitation in this 30-L fermentor was fixed at a rate of 400 rpm, and different aeration rates ((◊) 0.5 VVM; (■) 0.7 VVM and (▲) 1.0 VVM) were applied to this natamycin fermentation, respectively.

process, and the volumetric productivity of natamycin did not rise after 65 h cultivation even decreased at 90 h. At lower temperature (26 °C or 28 °C) the profiles of glucose consumption and mycelial growth were more stable than those at 30 °C. This might contribute the higher productivity of natamycin at lower temperature with regard to that achieved at 30 °C. The highest concentration of natamycin (3.52 g/L) was achieved when the 96 h fermentation was carried out at 28 °C. Further decrease of temperature to 26 °C would not improve the productivity of natamycin, thus the optimal temperature for natamycin fermentation is 28 °C with *Str. gilvosporeus* LK-196.

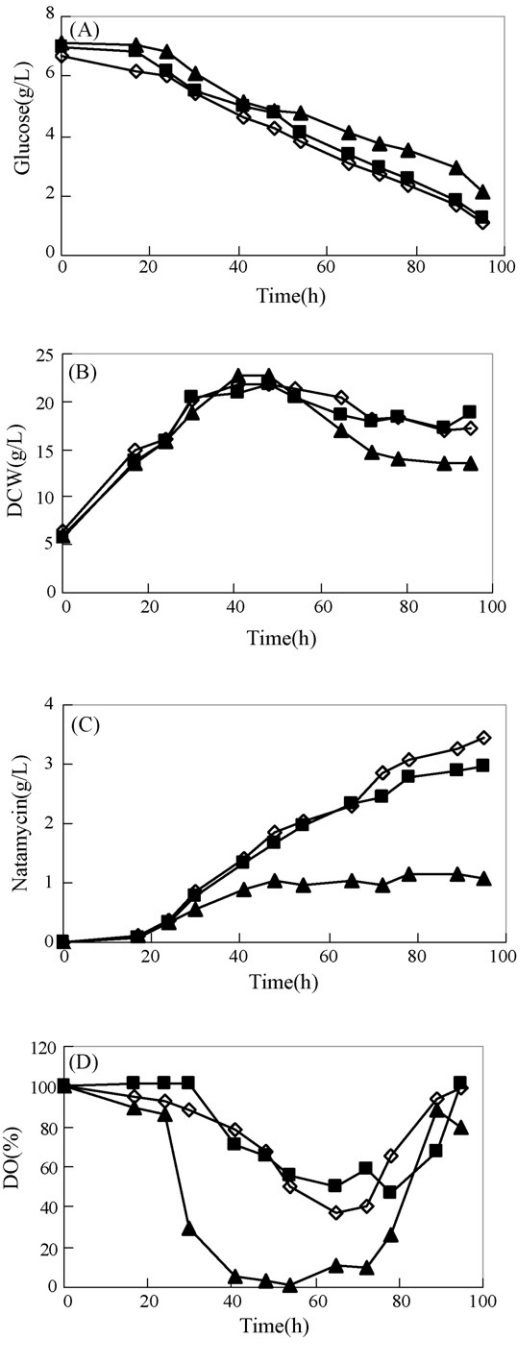

Fig. 4. The effects of different agitation on mycelial growth, glucose consumption and natamycin biosynthesis during the cultivation of *Str. gilvosporeus* LK-196 at 28 °C. The pH in the broths was controlled at an arrange of 5.5 ± 0.2 by automatic addition of H_2SO_4 or NaOH. The aeration rate in this 30-L fermentor was fixed at a rate of 0.8 VVM, and different agitation rates ((◊) 400 rpm; (■) 350 rpm and (▲) 300 rpm) were applied to this natamycin fermentation, respectively.

Table 2
The controlled parameters for natamycin production at different fermentor scales

Fermentor (L)	Stirring rate (rpm)	Aeration (VVM)	The lowest DO level (%)	Timing of the lowest DO (h)
30	400	1.00	37.0	65
1000	220	1.11	68.3	58
18000	100	0.74	30.5	64

The standard stirred-tank fermentors with the above volume were employed to scale up the production of natamycin in the pilot plant at Shangdong Lukang Pharmaceutical Group Co., Ltd. (Shandong, China). All the fermentation processes were carried out at 28 °C. The stirring rate and aeration were fixed at the indicated values in the table during the whole fermentation process. The profile of dissolved oxygen (DO) level was automatically determined with DO electrodes for the evaluation of cellular physiology.

3.2.3. *Effect of agitation speed and aeration rate*

As shown before, high-level maintaining of the dissolved oxygen was critical for high production of natamycin in the shake flask scale. The dissolved oxygen in the fermentor was affected by many culture parameters, such as aeration, agitation rate, fermentor pressure, the viscosity of the fermentation broth, etc. The effects of aeration and agitation rate on natamycin biosynthesis were investigated in the present work.

As shown in Fig. 3, the profiles of dissolved oxygen concentration were changed by different aeration rates. The growth and the metabolism of the microorganism would bring about the decrease of DO rapidly before 65 h of cultivation. The minimum levels of DO at 65 h were 15%, 24% and 32%, respectively, when the aeration rate was set at 0.5 VVM, 0.7 VVM or 1.0 VVM. Although the profiles of glucose consumption were almost the same in these different fermentations, the profiles of mycelial weight had a relatively obvious difference. Especially, the higher biomass between 48 h and 96 h were maintained at a high aeration of 1.0 VVM, which might benefit high production of natamycin during late phase of cultivation. At the end of the fermentation, the highest concentration of natamycin (3.94 g/L) was achieved when the aeration of 1.0 VVM was maintained during the whole bioprocess. This result indicated that the aeration rate could influence the dissolved oxygen concentration, which then affected the production of natamycin significantly.

The effects of agitation rate on natamycin biosynthesis were further examined in the 30-L fermentor. As shown in Fig. 4, the agitation rate affected the DO level obviously during the whole culture process of *Str. gilvosporeus* LK-196. Apparently, a low stirring rate of 300 rpm would lead to very low DO in the broth for more than 55 h after 24 h cultivation. This brought about a low consumption rate of glucose and low biomass at late phase of this fermentation, and a very low productivity of natamycin (1.08 g/L) was observed at this situation. According to the above investigation, the DO level should be controlled over 30% during the whole process by adjusting aeration rate and agitation rate for the purpose of high production of natamycin.

3.3. *The upscaled production of natamycin in 1000-L and 18,000-L fermentors*

On the basis of the results of 30-L bench-top fermentors, we scaled up the production of natamycin from 30-L fermentor to 1000-L and 18,000-L fermentors with *Str. gilvosporeus* LK-196. The controlled parameters for natamycin production at different bioreactor scales are summarized in Table 2. Although the lowest DO points and the timing of their appearance were quite variant under different controlled conditions, the dissolved oxygen level was maintained to be over 30% saturation in three different fermentors. Table 3 summarizes and compares the performance of various fermentation processes obtained in this and previous studies. In this study, about double production of natamycin (3.94 g/L) was achieved after the optimization of culture conditions in 30-L bench-top fermentors, as compared with the highest concentration of natamycin (about 2.0 g/L) in the shake flask. Even with some differences among the profiles of DO level and natamycin production in different-scale fermen-

Table 3
Comparison of natamycin production in various fermentation processes

Microorganism	Bioreactor	Fermentation time (h)	Natamycin titer (g/L)	Natamycin productivity (mg/L h)	Reference
Str. natalensis NRRL 2651	250 mL shake flasks	96	1.50	15.6	[9]
Str. natalensis NRRL 2651	250 mL shake flasks	96	1.70	17.7	[8]
Str. gilvosproeus 9–6	250 mL shake flasks	120	3.00	25.0	[1]
Str. gilvosporeus LK-119[a]	500 mL shake flasks	120	1.90	15.8	[10]
Str. gilvosporeus LK-196[b]	500 mL shake flasks	96	2.03	21.1	
	30-L fermentor	95	3.94	41.5	This study
	1000-L fermentor	94	3.91	41.6	
	18000-L fermentor.	99	3.81	38.5	

[a] *Str. gilvosporeus* LK-119 is a metabolite repression resistant mutant derived from *Str. gilvosporeus* ATCC 13326.
[b] *Str. gilvosporeus* LK-196 is a high-yield mutant derived from *Str. gilvosporeus* LK-119.

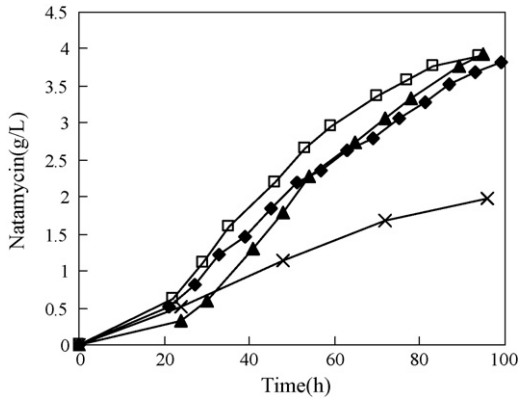

Fig. 5. Kinetics of natamycin fermentations by *Str. gilvosporeus* LK-196 at different fermentor scales. All the tests were carried out at the optimal culture conditions (28 °C, pH 6.0). The dissolved oxygen levels were controlled over 30% by adjusting agitation (100–400 rpm) and aeration (~1 VVM) rates in different-scale fermentors at Shangdong Lukang Pharmaceutical Group Co., Ltd (Shandong, China) ((×) 500 mL Erlenmeyer flask; (▲) 30-L fermentor; (□) 1000-L fermentor and (♦) 18,000-L fermentor).

tors (Fig. 5), but both natamycin concentration and productivity obtained in 1000-L and 18,000-L fermentors were comparable to the best values obtained in the 30-L fermentor, suggesting a successful scale up of the natamycin fermentation process by properly controlling a DO range of over 30% saturation. The highest natamycin concentration of 3.81 g/L and productivity of 38.5 mg/L h in the 18,000-L fermentor were much better than that obtained with a mutant of *Str. gilvosporeus* ATCC 13326 grown in shake flasks [1], which gave the highest natamycin concentration of 3.0 g/L and productivity of 25.0 mg/L h previously reported in the literature.

Among all the natamycin-producing strains, more efforts are paid to improve the production of natamycin with *Str. Natalensis* in the previous literature [8,9]. However, the present work showed that *Str. gilvosporeus* might have the higher ability of synthesizing natamycin after rational strain screening and culture condition optimization. The high-yield strain (*Str. gilvosporus* LK-196) was derived from one carbon metabolite regression resistant mutant (LK-119), which was screened from space-flight treated culture of *Str. gilvosporeus* ATCC 13326 and has almost the same natamycin production ability either in the medium where glucose was used as the only carbon source or in the medium where starch is used as the main carbon source [10]. According to the findings that high-level production of natamycin was achieved at large scale by utilizing high concentration of glucose (60 g/L) as the sole carbon source, it will be promising to further improve the productivity of natamycin by developing some novel fed-batch fermentations in the future.

4. Conclusion

The production of natamycin in *Str. gilvosporeus* LK-196 was significantly affected by the shear force and dissolved oxygen in the shake flask scale. By adding suitable quantity of production medium, the high productivity of natamycin (2.03 g/L) was achieved with this strain. Further tests in 30-L fermentors showed that the highest productivity (3.94 g/L) could be achieved by investigating the effects of different pH control strategies and temperature, and high dissolved oxygen level would improve natamycin production greatly. This strategy of maintaining high DO level (over 30%) was still very effective to achieve high productivity of natamycin in 1000-L fermentors and 18,000-L fermentors in the pilot plant.

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