JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Natamycin Production by *Streptomyces gilvosporeus* Based on Statistical Optimization

GUAN-QUN CHEN,*[,†] FU-PING LU, AND LIAN-XIANG DU

Tianjin Key Laboratory of Industrial Microbiology, College of Biotechnology, Tianjin University of Science and Technology, 29 Thirteenth Avenue, TEDA, Tianjin 300457, China

Natamycin has been widely used as a natural preservative to prevent mold contamination in food. In this study, statistically based experimental designs were employed for the optimization of medium components for natamycin production by *Streptomyces gilvosporeus*. After glucose, yeast extract, and soy peptone were screened as suitable carbon and nitrogen sources, a full factorial design was used to evaluate the effects of various factors on natamycin production. Glucose and pH were identified as having significant effects (with confidence level >90%). Glucose concentration and initial pH were subsequently optimized by use of a central composite design. The result indicated that glucose and pH had a significant interactive effect on natamycin production. The optimal glucose concentration and initial pH value were 38.2 g/L and 7.8, respectively. This optimization strategy led to a natamycin yield of 2.45 g/L, which was nearly 90% higher than that in the original medium.

KEYWORDS: Natamycin; optimization; experimental design; *Streptomyces gilvosporeus*

INTRODUCTION

Natamycin (**Figure 1**), a polyene macrolide antibiotic, is a potent antifungal compound that can effectively prevent the growth of yeasts and inhibit aflatoxin formation in molds (*1*). As one of the few antibiotics being recommended by FDA as a food additive and classified as a GRAS (Generally Regarded As Safe) compound, natamycin is widely used in the food industry as a natural food preservative for the prevention of mold contamination of beverages, cheese, fruits, and other nonsterile foods (i.e., cured meats, sausages) (*2, 3*). Moreover, because of its low toxicity to mammalian cells, natamycin plays an important role in antifungal therapy by working synergistically with other antifungal or antitumor compounds (*4*).

In nature, natamycin is mainly produced by actinomycetes such as *Streptomyces gilvosporeus* and *Streptomyces natalensis* (*5*). The regulation of antibiotic biosynthesis in *Streptomyces* can be achieved at extracellular and intracellular levels, including metabolism of nutrients, the onset of transcription and post-transcriptional processes, and translational and posttranslational control (for detailed reviews, see refs *6–8*). Similar to the biosynthesis of other polyene macrolide antibiotics, natamycin production is regulated by culture medium and environmental conditions, in which the metabolic pathway of the strain can be shifted between the production of primary and secondary antibiotics (*1, 6, 9*).

The carbon and energy source is the most important medium component for growth and antibiotic formation (*7*). Among the carbon sources, glucose is most frequently utilized for fermentation. High concentration of glucose, however, often represses antibiotic formation through carbon catabolite repression (*6, 7*). The choice of nitrogen source and its concentration are also of crucial importance for antibiotic production, though not much is known about the molecular mechanisms behind the observed effects on antibiotic formation (*7*). The pH of the growth medium is also important in influencing antibiotic production, because the activity of several major enzymes that catalyze metabolic reactions for cell growth and antibiotic formation are affected by pH (*10, 11*). In general, the onset of antibiotic biosynthesis occurs in response to the deprivation of carbon and/or nitrogen sources (*12*).

Due to the important commercial value of natamycin, it is necessary to look for a strategy for the optimization of growth condition for natamycin production. Until now, few studies focusing on the production of natamycin have been conducted (*5, 11, 13*). For example, the influences of inoculum type and nutrient and oxygen limitation on natamycin production were briefly examined in *S. natalensis* (*5, 13*). The effects of cultivation conditions were investigated with a mutant strain of *S. gilvosporeus* (*11*). The culture conditions in these studies, however, were optimized by the one-at-a-time strategy: varying one factor while keeping

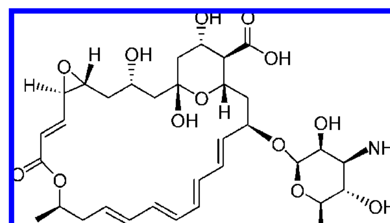

Figure 1. Chemical structure of natamycin.

* Author to whom correspondence should be addressed: telephone 852-3411-2530; fax 852-3411-5995; e-mail gchen@hkbu.edu.hk.

[†] Present address: Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong.

10.1021/jf800479u CCC: $40.75 © 2008 American Chemical Society
Published on Web 06/07/2008

all others constant. This strategy is simple and easy, but the disadvantage is that a relatively large number of experiments is required and the interaction among factors is often ignored. Although statistically based experiment design is widely known to be more efficient than the one-at-a-time method in value prediction and the estimation of interactions among different variables (*14, 15*), systematic investigation on the influence of culture conditions upon natamycin fermentation using statistically based experimental designs has not been reported.

The purpose of this study was to assess the influence of culture conditions on natamycin production by *S. gilvosporeus* by use of statistically based experimental designs. Specifically, several carbon and nitrogen sources were initially screened. The effects of the best carbon and nitrogen sources and initial pH for natamycin production were determined and compared by using full factorial design. The levels of the culture factors with significant effects on natamycin were optimized by using a central composite design and response surface analysis.

MATERIALS AND METHODS

Organism and Culture Conditions. *Streptomyces gilvosporeus* TUST24 was provided by the Culture Collection at Tianjin University of Science and Technology, China. The strain was maintained on a slant with an International Streptomyces Project (ISP) 2 medium, which contained (per liter) 4.0 g of glucose, 4.0 g of yeast extract, 10.0 g of malt extract, and 20.0 g of agar (pH 7.0). The strain grew well after 6 days' cultivation at 29 °C and the resulting spores were collected in saline solution (0.9% NaCl, w/v) for inoculation. Thirty milliliters of seed medium, consisting of (per liter) 20.0 g of glucose, 6.0 g of soy peptone, and 10.0 g of NaCl (pH 7.0) in 250 mL Erlenmeyer flasks, were inoculated with spores to the final concentration of 10^8 spores/mL. This preculture was incubated in an orbital shaker (at 200 rpm) at 29 °C for 24 h. For natamycin production, 500 mL Erlenmeyer flasks, each containing 50 mL of natamycin production medium consisting of (per liter) 30.0 g of glucose, 4.5 g of yeast extract, and 20.0 g of soy peptone (pH 7.0) (unless otherwise stated), were inoculated with 2% (v/v) of an exponentially growing inoculum and incubated at 29 °C with orbital shaking at 200 rpm for 96 h.

Determination of Cell Dry Weight. A 3-mL aliquot of the fermentation broth was periodically sampled to determine the cell dry weight. The sample was centrifuged at 3000 rpm for 5 min, and the cell pellet was washed with distilled water twice. Cell dry weight was determined by filtering the fluid through a preweighed filter paper (Whatman GF/C), which was dried to constant weight at 80 °C in a vacuum oven.

Determination of Natamycin and Glucose. Natamycin analysis was performed on a Biotronik HPLC system (Maintal, Germany), equipped with a Phenomenex prodigy ODS3 100 A column (5 μm, 250 × 4.6 mm) (Phenomenex Australia, Lane Cove, NSW, Australia). The mobile phase composition was methanol−water−phosphoric acid (85:15:0.15 v/v/v) and set at a flow rate of 1 mL/min. Detection of natamycin was performed at 303 nm and quantified with an external standard (Sigma−Aldrich, St. Louis, MO) (*16*). Glucose was quantified with a glucose enzymatic detector (SBA Institute Inc., Jinan, China).

Experimental Design (1): Full Factorial Design. After the suitable carbon and nitrogen sources were identified (**Figure 2**), each variable was selected at high (+), control (0), and low (−) levels (**Table 1**). The low level of each variable was set at a point much lower than the control used in the original natamycin production medium. A high level of each factor was set far apart from the low level so that possible significant effects could be detected. The natamycin concentration in the fermented broth after 96 h of fermentation was used as the response value (**Table 2**). Student's *t*-test was applied to determine the significance of each variable with confidence levels above 90% ($p < 0.1$).

Experimental Design (2): Central Composite Design. After the variables having the greatest influence on the responses were identified, a 13-run CCD experiment was used to optimize the levels. The design matrix and true values are shown in **Table 3**. The relationship between the natamycin concentration and the independent variables is expressed in the following second-order polynomial model:

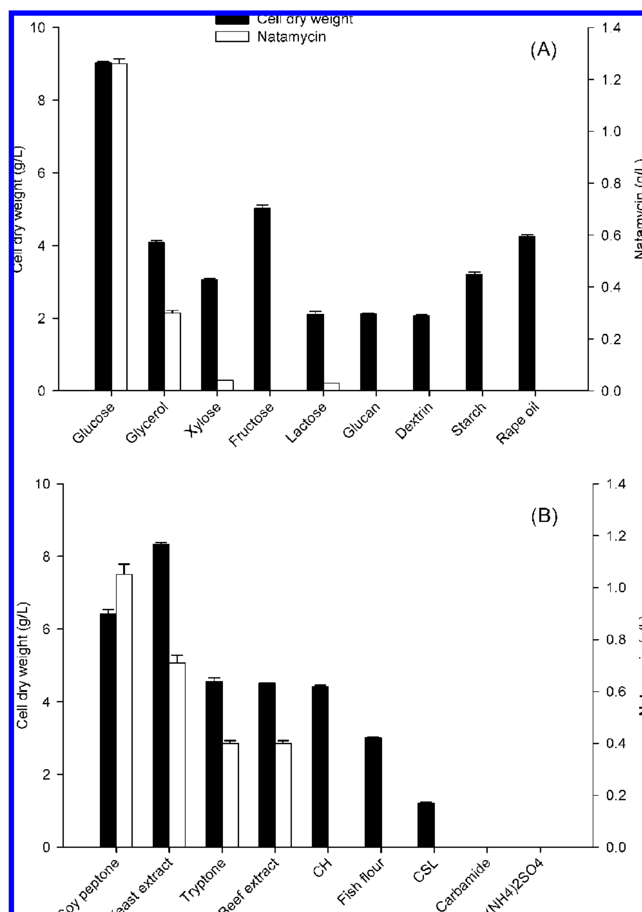

Figure 2. Effects of carbon and nitrogen sources on cell growth and natamycin production of *S. gilvosporeus*. (**A**) Carbon sources (30 g/L). (**B**) Nitrogen sources (20 g/L). CH, casein hydrolysate; CSL, corn steep liquor. Data are means of triplicates, and the error bars show standard deviations.

$$Y = \beta_0 + \sum \beta_i x_i \sum \beta_{ii} x_i^2 \sum \beta_{ij} x_i x_j \quad (1)$$

where Y is the predicted response, natamycin concentration, β_n are coefficients of the equation, and x_i and x_j are the coded levels of the variables i and j.

The correlation coefficients of the above model were obtained by multiple nonlinear regression analysis of the response (JMP software V7.0, SAS Institute Inc., Cary, NC). The estimates of the coefficients with confidence levels above 90% ($p < 0.1$) were accepted in the final models, and the F-test was employed to evaluate the significance of the model.

RESULTS AND DISCUSSION

Optimization of Carbon and Nitrogen Sources. As shown in **Figure 2A**, *S. gilvosporeus* grew effectively on all carbon sources tested but accumulated high levels of natamycin only in the medium containing glucose as the sole carbon source. Glucose was the best carbon source for both cell growth and natamycin production. This result was consistent with a previous report investigating *S. natalensis* (*13*). The best nitrogen source for cell growth was yeast extract, but the best for natamycin production was soy peptone (**Figure 2B**). Inorganic nitrogen, as $(NH_4)_2SO_4$, did not support cell growth and natamycin production, whereas complex nitrogen sources were generally suitable for large-scale antibiotic production. In fact, a mixture

Table 1. Variables Screened in Full Factorial Design and Their Effects[a]

	variables	low level (−)	control (0)	high level (+)	effects	*t*-value	*p*-level
A	yeast extract, g/L	3.0	4.5	6.0	0.0571	1.55	0.1422
B	glucose, g/L	15.0	30.0	45.0	−0.0904	−2.46	0.0268
C	soy peptone, g/L	15.0	20.0	25.0	0.0402	1.09	0.2925
D	initial pH	6.0	7.0	8.0	0.3898	10.58	<0.0001

[a] The significance of variables was determined by Student's *t*-test. The variables with *p* -values <0.1 were accepted to be significant.

Table 2. Full Factorial Design of Variables with Natamycin Concentration as Response

	variables				
run	A	B	C	D	natamycin (g/L)
1	−1	−1	−1	−1	0.97
2	1	−1	−1	−1	1.11
3	−1	1	−1	−1	0.83
4	1	1	−1	−1	0.83
5	−1	−1	1	−1	1.09
6	1	−1	1	−1	1.08
7	−1	1	1	−1	1.11
8	1	1	1	−1	1.04
9	−1	−1	−1	1	1.60
10	1	−1	−1	1	2.25
11	−1	1	−1	1	1.50
12	1	1	−1	1	1.76
13	−1	−1	1	1	1.90
14	1	−1	1	1	1.90
15	−1	1	1	1	1.71
16	1	1	1	1	1.67
17−20	0	0	0	0	1.29

Table 3. Central Composite Design of Variables with Natamycin Concentration as Response

	variables[a]		
run	glucose	initial pH	natamycin (g/L)
1	−1 (20.0 g/L)	−1 (7.5)	1.83
2	−1	1 (8.5)	1.83
3	1 (40.0 g/L)	−1	2.47
4	1	1	2.07
5	−1.414 (15.9 g/L)	0 (8.0)	1.88
6	1.414 (44.1 g/L)	0	2.24
7	0 (30.0 g/L)	−1.414 (7.3)	1.93
8	0	1.414 (8.7)	1.68
9−13	0	0	2.37

[a] Variables are presented in coded levels, with real values in parentheses.

containing a non-yeast-protein nitrogen compound and a yeast protein was recommended to improve the production of natamycin in a previous study (*17*). Therefore, a combination of soy peptone and yeast extract was selected for further optimization.

Full Factorial Design. Full factorial design has been proved effective for identification of significant variables, especially for a small number of potential variables (≤4) (*14*). In this study, an FFD experiment was applied to identify which of the factors glucose, yeast extract, soy peptone, and initial pH, was significant in natamycin production. As shown in **Table 2**, natamycin production in the control experiments reached 1.29 g/L (run 17−20). Glucose and initial pH over the range tested were shown to have significant effects on natamycin biosynthesis (**Table 1**).

The significance of glucose identified by the FFD experiments may be attributed to the requirement of glucose for cell growth and natamycin formation in *Streptomyces* (*13*). The inhibitory effects of glucose at high concentrations on the accumulation of antibiotics have been reported frequently and are associated with inhibition of the secondary metabolite accumulation by carbon catabolites (*7, 18*). For this reason, glucose concentration in the medium was further optimized in CCD experiments.

pH is an essential parameter in affecting the cell growth and antibiotic production in actinomycetes and could be influenced by the metabolism of medium components (i.e., carbon source) during cell growth (*15*). A relatively high initial pH was reported to benefit both cell growth and natamycin production of another *Streptomyces* strain (*19*). The positive effect of initial pH in the range of 6.0−8.0 on natamycin production obtained from this study was consistent with the previous findings (**Table 2**).

Soy peptone and yeast extract had no significant influence on natamycin production. This might be because these two factors were tested at their optimal concentrations during the screening experiment. Therefore, the levels of glucose and initial pH were further optimized by using CCD experiments with soy peptone and yeast extract set at their corresponding low levels.

Central Composite Design. The coded and actual values of glucose concentration and initial pH for the CCD experiment are listed in **Table 3**. Estimates of the coefficients, the associated *t*-values, and the significant levels are shown in Table 4 (in Supporting Information). All the estimates had significance levels higher than 90% ($p < 0.1$) and thus were included in the final second-order polynomial model:.

$$\text{natamycin (g/L)} = 2.3716 + 0.1732[\text{Glu}] - 0.0922[\text{pH}] - 0.1262[\text{Glu}]^2 - 0.0995[\text{Glu}][\text{pH}] - 0.2535[\text{pH}]^2 \quad (R^2 = 0.93) \quad (2)$$

In this equation, [Glu] and [pH] represent the glucose concentration and initial pH value, respectively. The *F*-value for this model was high (17.51) and a significance level of >99.9% was obtained. The optimal values of glucose and initial pH (38.2 g/L and 7.8, respectively) are clearly shown in the three-dimension surface plot (**Figure 3**). The predicted value of natamycin production was determined to be 2.46 g/L.

To verify the predicted result, the cells were subsequently cultured under these optimized conditions. **Figure 4** shows the time course of cell dry weight, glucose consumption, pH variation, and natamycin production of *S. gilvosporeus* in the optimized medium. After a 24 h lag phase, the cells started to grow rapidly with a specific growth rate of 0.056 /h. The cell dry weight reached a maximum of 9.84 g/L at 80 h. The pH value in the medium decreased gradually from 7.8 to near 6.0 in the initial 48 h, where rapid cell growth was observed. From 48 to 96 h, the culture arrived at late log phase and the pH of the medium ranged from 6.0 to 5.6. During this period, rapid accumulation of natamycin was observed (**Figure 4**). Natamycin production achieved a maximum of 2.45 g/L at 96 h, which was very close to the predicted value. The optimized production was nearly 90% higher than that achieved in the original medium (**Table 2**). The result proved the validity of the statistically based experimental designs.

The second-order polynomial model indicated that there was significant interaction between glucose concentration and initial

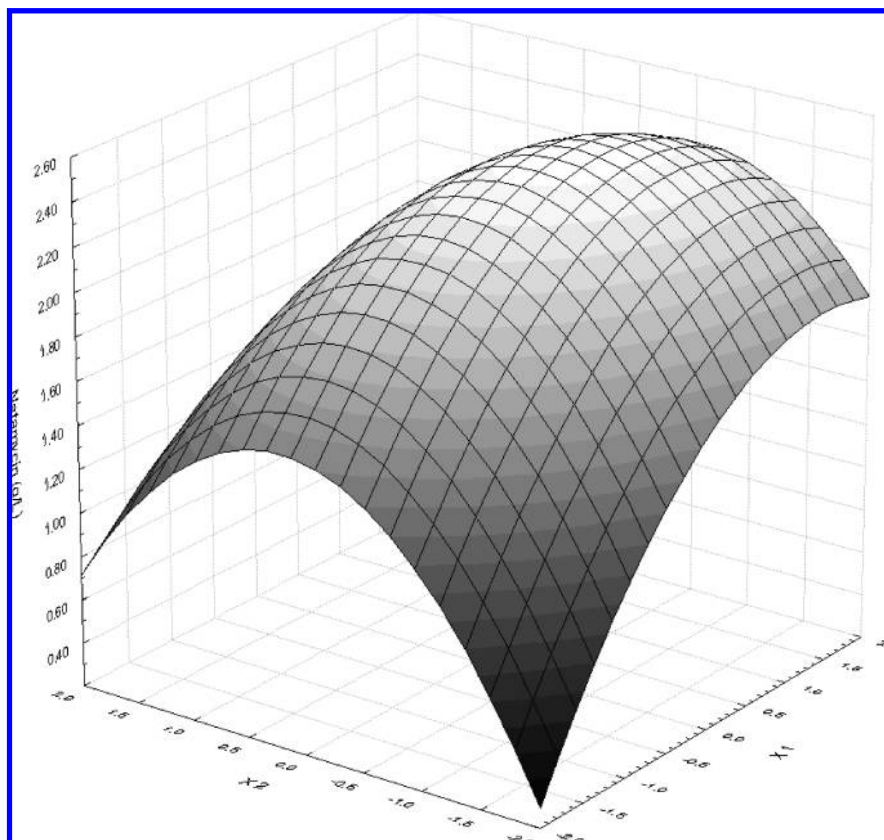

Figure 3. Three-dimensional surface plot of natamycin yield as the function of glucose ($X1$) and initial pH ($X2$) in coded units.

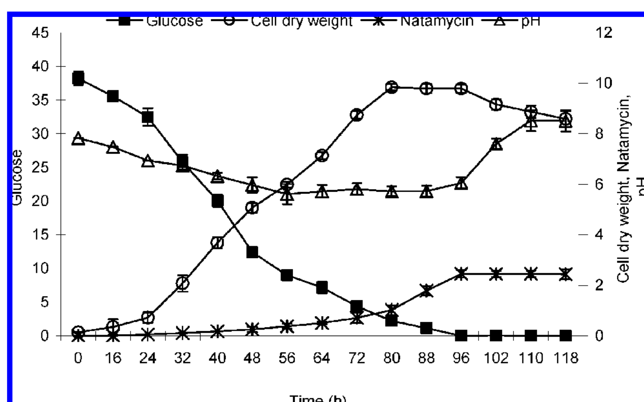

Figure 4. Time course of natamycin production (grams per liter), cell dry weight (grams per liter), pH, and consumption of glucose (grams per liter). Data are means of triplicates and the error bars show standard deviations.

pH in determining natamycin production (**Figure 3**). Most of the polyene-antibiotic-producing actinomycetes utilize glucose through glycolysis and the tricarboxylic acid cycle (TCA cycle), which could generally result in a decrease of pH in the fermentation broth (*15*). Moreover, as a typical secondary metabolite, natamycin mainly accumulated after the rapid growth phase, and the actual pH value and glucose concentration suitable for growth were both higher than for natamycin formation in *Streptomyces* spp (*19*). In this study, the high concentration of glucose (38.2 g/L) and initial pH (7.8) might benefit cell growth in the initial growth phase. Subsequently, the pH of the broth decreased concomitantly with glucose consumption (**Figure 4**) and thus could be suitable for natamycin biosynthesis. This might be the major reason for the interactive effect of glucose and initial pH for natamycin production in *S. gilvosporeus*.

In this study, the medium for natamycin production of *S. gilvosporeus* was optimized with a statistical method. Natamycin production reached a maximum of 2.45 g/L (90% higher than that in the original medium) in the optimal medium containing (per liter) 38.2 g of glucose, 3.0 g of yeast extract, and 15.0 g of soy peptone (pH 7.8). A yield of 0.25 g of natamycin/g of cell dry weight was achieved, which was 56% higher than that observed in the original medium. Initial glucose concentration, initial pH, and their interaction had significant influence on the production of natamycin, probably because of the different requirements for cell growth and natamycin accumulation. Moreover, the results clearly demonstrated that the glucose concentration and pH for natamycin production were lower than required for cell growth. The cessation of natamycin production after 96 h of culture might result from exhaustion of the carbon source (glucose) in the medium. The results from this study form the foundation for future production studies using various fermentation techniques, such as the use of fed-batch technique in a fermenter using the optimized medium with pH being regulated at 5.7 during natamycin accumulation.

Supporting Information Available: Table 4: Regression Coefficients of the Variables in Central Composite Design in Response to Natamycin Concentration. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Aparicio, J. F.; Mendes, M. V.; Anton, N.; Recio, E.; Martin, J. F. Polyene macrolide antibiotic biosynthesis. *Curr. Med. Chem.* **2004**, *11*, 1645–1656.

(2) Cong, F. S.; Zhang, Y. G.; Dong, W. Y. Use of surface coatings with natamycin to improve the storability of Hami melon at ambient temperature. *Postharvest Biol. Technol.* **2007**, *46*, 71–75.

(3) Delves-Broughton, J.; Thomas, L. V.; Williams, G. Natamycin as an antimycotic preservative on cheese and fermented sausages. *Food Aust.* **2006**, *58*, 19–21.

(4) Malecha, M. A. Fungal keratitis caused by *Scopulariopsis brevicaulis* treated successfully with natamycin. *Cornea* **2004**, *23*, 201–203.

(5) El-Enshasy, H. A.; Farid, M. A.; El-Sayed, E. S. A. Influence of inoculum type and cultivation conditions on natamycin production by *Streptomyces natalensis*. *J. Basic Microbiol.* **2000**, *40*, 333–342.

(6) Bibb, M. J. Regulation of secondary metabolism in streptomycetes. *Curr. Opin. Microbiol.* **2005**, *8*, 208–215.

(7) Rokem, J. S.; Lantz, A. E.; Nielsen, J. Systems biology of antibiotic production by microorganisms. *Nat. Prod. Rep.* **2007**, *24*, 1262–1287.

(8) Martin, J. F.; Demain, A. L. Control of antibiotic biosynthesis. *Microbiol. Rev.* **1980**, *44*, 230–251.

(9) Martin, J. F.; McDaniel, L. E. Production of polyene macrolide antibiotics. *Adv. Appl. Microbiol.* **1977**, *21*, 2–52.

(10) Guimaraes, L. M.; Furlant, R. L. D.; Garrido, L. M.; Ventura, A.; Padilla, G.; Facciotti, M. C. R. Effect of pH on the production of the antitumor antibiotic retamycin by *Streptomyces olindensis*. *Biotechnol. Appl. Biochem.* **2004**, *40*, 107–111.

(11) Liang, J. G.; Xu, Z. N.; Liu, T. F.; Lin, H. P.; Cen, P. L. Effects of cultivation conditions on the production of natamycin with *Streptomyces gilvosporeus* LK-196. *Enzyme Microb. Technol.* **2008**, *42*, 145–150.

(12) Mendes, M. V.; Tunca, S.; Anton, N.; Recio, E.; Sola-Landa, A.; Aparicio, J. F.; Martin, J. F. The two-component *phoR*−*phoP* system of *Streptomyces natalensis*: Inactivation or deletion of *phoP* reduces the negative phosphate regulation of pimaricin biosynthesis. *Metab. Eng.* **2007**, *9*, 217–227.

(13) Farid, M. A.; El-Enshasy, H. A.; El-Diwany, A. I.; El-Sayed, E. S. A. Optimization of the cultivation medium for natamycin production by *Streptomyces natalensis*. *J. Basic Microbiol.* **2000**, *40*, 157–166.

(14) Harrison, W. *Handbook of statistical methods for engineers and scientists*; McGraw-Hill: New York, 1998; p 688.

(15) Xiong, Z. G. *Fermentation: process and theory*; China Medical Science and Technology Press: Beijing, 1995; p 428.

(16) Chen, G. Q.; Yang, D. J.; Du, L. X. Determination of natamycin in fermentation broth by reversed-phase high performance liquid chromatography. *J. Tianjin Univ. Light Ind.* **2003**, *18*, 9–11.

(17) Eisenschink, M. A.; Olson, P. T. U.S. Patent 5,231,014, 1993.

(18) Fjaervik, E.; Zotchev, S. B. Biosynthesis of the polyene macrolide antibiotic nystatin in *Streptomyces noursei*. *Appl. Microbiol. Biotechnol.* **2005**, *67*, 436–443.

(19) He, Y. L.; Wu, J. G.; Lu, F. P.; Du, L. X. Fed-batch fermentation to improve the yield of natamycin. *Med. Biotechnol.* **2002**, *9*, 224–226.

Received for review February 15, 2008. Revised manuscript received April 28, 2008. Accepted April 29, 2008. This research was supported by a Natural Science Grant of Tianjin, China (013609511).

JF800479U