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ORIGINAL ARTICLE

Production of lovastatin by a self-resistant mutant of *Aspergillus terreus*

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Abstract A lovastatin-resistant mutant of *Aspergillus terreus* designated as LA4414 was rationally screened by multiple cycles of UV mutation by using the characteristic of the lovastatin resistance to its own biosynthesis. The production ability was approximate 3 times that obtained from the parent strain *A. terreus* ATCC 20542. The optimal and economical carbon and nitrogen sources were soluble starch and peanut meal, respectively, for hyper-production of lovastatin by the selected mutant. The final lovastatin production reached 883.2 ± 19.5 mg l^{-1} in the optimized medium was also 3-fold that obtained from the unoptimized medium. This may be helpful to further improve the production ability of the lovastatin-producing strain and to supply a novel idea for the hyper-production of lovastatin or many other antibiotics from bacteria and filamentous fungi.

Keywords Lovastatin · *Aspergillus terreus* · Mutation · Resistance screening · Hyper-production

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Introduction

Lovastatin ($C_{24}H_{36}O_5$, Mevinolin, Monacolin K, and Mevacor™) is a fungal secondary metabolite produced by filamentous fungi such as *Aspergillus terreus* via the polypeptide synthase (PKS) pathway (Alberts et al. 1980; Chan et al. 1983; Endo 1980; Yoshizawa et al. 1994). It is a pharmaceutically important compound in treating hypercholesterolemia because of its potent inhibitory activity toward hydroxymethylglutaryl-coenzyme A (HMG-CoA) reeducates, which catalyzes the rate-limiting step in cholesterol biosynthesis.

In the basic investigations and large-scale processes, *A. terreus* and its mutants are the most applicable lovastatin-producer. The wild-type strains isolated from nature produce very low levels of lovastatin and thus some typical strain improvement programs such as chemical and physical mutagenesis, protoplast fusion and in vitro recombinant DNA technology are broadly applied to improve the production of lovastatin (Vilches Ferron et al. 2005; Kumar et al. 2000a, b; Lai et al. 2003; Park et al. 2004). Many antibiotics participate in feedback regulation on their related biosynthetic enzymes or inhibit the growth of their producer. Thereby, it is possible to screen an antibiotic-resistant mutant for its hyper-production. The increase of its resistance may improve the production ability of the antibiotic-producer (Demain and Adrio 2008). It is well known that the biosynthetic genes of antibiotic are clustered generally. The lowest concentration which inhibits growth of the parent strain is thus used as the primary screening concentration in the improvement program of the antibiotic-resistant mutant. It has been identified that a feedback regulation exists in lovastatin biosynthesis from *A. terreus*, i.e., lovastatin inhibits its own biosynthesis (Casas López et al. 2004; Jia et al. 2010). It is the foundation of a strain

improvement program for hyper-production of lovastatin. To our knowledge, no such investigation is reported as currently taking place.

In the current work, a lovastatin-resistant mutant of *A. terreus* for hyper-production of lovastatin is rationally screened by multiple cycles of UV mutation by using the characteristic of the lovastatin resistant to its own biosynthesis. The culture conditions were also optimized in submerged cultivation. This result may be helpful for the hyper-production of lovastatin or many other antibiotics from bacteria and filamentous fungi.

Materials and methods

Microorganism and seed culture

Strain improvement was initiated on *A. terreus* ATCC 20542 by multiple random mutations. Spores of *A. terreus* ATCC 20542 were germinated in the germination medium at 28°C for 6–8 h. The germination medium contained 10 g l^{-1} of malt extract, 0.5 g l^{-1} of peptone, and 0.1 g l^{-1} of yeast extract powder with pH of 7.0 before sterilization. After being dispersed, filtrated, centrifuged (~4,000 *g*, 5 min), and resuspended in sterile germination medium, the spore suspension was exposed to UV (15 W, 254 nm, 25 cm) in turn according to the conditions listed in Table 1. Then the spores were diluted and poured onto the plates containing the exogenous sterilized β-hydroxyl acid form of lovastatin (mevinolinic acid). The plate was prepared by supplementing sterilized ethanol solution of mevinolinic acid to the pre-melted plate medium. The concentrations of exogenous mevinolinic acid are also listed in Table 1. The plates were covered in black polythene bags and incubated at 28°C for 5 days. Primary screening of the strains were carried out using a novel agar plug method (Kumar et al. 2000a, b). This method utilizes the anti-fungal property of lovastatin to produce a zone of inhibition against *Neurospora crassa*. Strains with improved yields were further subjected to the treatments listed in Table 1 for four cycles in turn.

The slants and plates medium all contain 20 g l^{-1} of glucose, 20 g l^{-1} of malt extract, 1 g l^{-1} of peptone, and 20 g l^{-1} of agar. After inoculation from the original slant, the inoculated slants were incubated at 28°C for 5 days, and then used for inoculation of seed culture. Conidiospores were harvested by washing the slant cultures with sterile distilled water, filtrated, centrifuged (~4,000 *g*, 5 min), and resuspended in sterile distilled water. The spore concentration was adjusted to about 5×10^7 spores ml^{-1} by haemacytometer.

The seed culture medium consisted of the following components per liter: 40 g tomato paste, 10 g oat meal, 10 g glucose, 5 g corn steep liquor, 10 ml trace element solution, with pH 6.8. The trace element solution per liter contained: 1 g $FeSO_4 \cdot 7H_2O$, 1 g $MnSO_4 \cdot 4H_2O$, 0.025 g $CuCl_2 \cdot 2H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.056 g H_3BO_3, 0.01 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.2 g $ZnSO_4 \cdot 7H_2O$. Two milliliters of the above spore resuspensions (approximate 10^8 spores) were inoculated in a 100-ml shake flask containing 20 ml of seed culture medium and were shaken for 24 h at 220 rpm (28°C).

Table 1 Conditions for UV mutation[a]

Run of mutagenesis[b]	UV mutation (min)	Dark reparation	Lov-resistant level (g l^{-1})
1	3	28°C, 150 rpm, 24 h, dark	0.25
2	1	28°C, 150 rpm, 24 h, dark	0.5
3	1	28°C, 150 rpm, 24 h, dark	1.0
4	1.5	28°C, 150 rpm, 8 h, dark	4.0

[a] Each run of mutagenesis were all started from 45 s of UV irradiation, followed by 30 min of light reparation before UV mutation

[b] Each cycle of mutation was originated from the former strain

Culture conditions

A second culture was conducted in a 100-ml conical flask containing 20 ml of the production medium. The seed cultures were filtrated and washed several times to remove the residual medium with sterile deionized water, then resuspended in the fresh medium. The 10% of seed resuspensions (v/v) were inoculated. All fermentations were carried out for 8 days at 28°C on a rotary shaker (220 rpm). Multiple flasks at least in triplicate were run at the same time to ensure reproducibility. The production medium contained 45 g l^{-1} of soluble starch, 10 g l^{-1} of yeast extract powder, 2.5 g l^{-1} of polyethylene glycol 2000, 2 g l^{-1} of KH_2PO_4, 1 g l^{-1} of $(NH_4)_2SO_4$. The original pH of the medium was adjusted to 6.5 before sterilization.

Analytical methods

The biomass concentration was measured by filtering a culture broth, and the cake was washed with distilled water, and then dried at 60°C until constant weight.

The culture broths were extracted with ethyl acetate. Thin layer chromatography (TLC) was used for initial screening the extracts by drying a sample of the organic phase on a silica gel $60F_{254}$ thin layer plate. The developing solvent was dichloromethane/acetic acid (85:15, v/v), and visualization was by UV light (λ=254 nm) (Jia et al. 2010). The desired products contents were determined by HPLC using a Kromasil C18 (4 mm × 150 mm, 4 μm particle

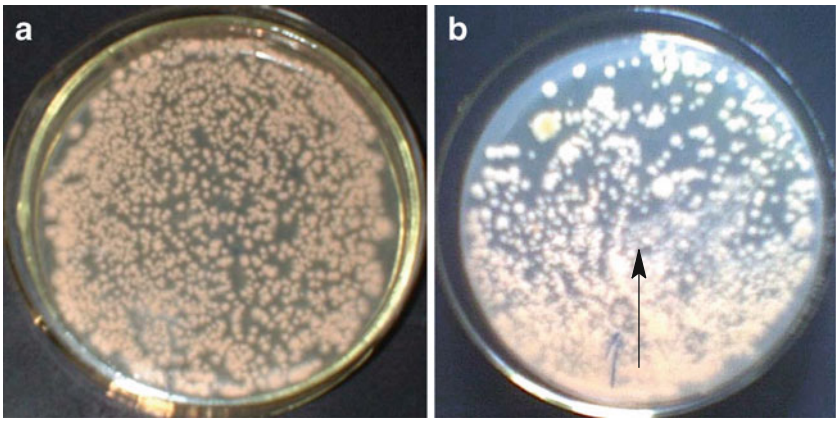

Fig. 1 Morphology of *A. terreus* ATCC 20542 growing on control plate (**a**) and lovastatin grads plate (**b**) at day 5. The *arrow* directs the grads increase of lovastatin concentration (0~100 mg l^{-1})

sizes) of Eliter (Liaonin, China) column with an eluent comprising methanol/0.1% aqueous phosphoric acid (77.5: 22.5, v/v) (Konfino et al. 1993). The injection volume was 5 μl. Elution was performed at a flow rate of 1 ml min^{-1} at 25°C. A tunable absorbance detector with UV wavelength of 238 nm was used for monitoring eluate. Samples were centrifuged at 5,000 *g* for 10 min before the injection. The known standard sample of lovastatin was applied for comparison qualitatively and quantitatively. The final lovastatin product was detected as the sum of its lactones and β-hydroxyl acid forms. The latter was prepared by dissolving its lactones form (gifted by Dr. X.B. Mao from Chong-qing Academy of Chinese Materia Medica, China) in a mixture of 0.1 mol l^{-1} NaOH and ethanol (1:1, v/v), heating at 50°C for 20 min, and neutralizing with 1 mol l^{-1} HCl (Michael and Joel 1985).

Results and discussion

Resistance of lovastatin to *A. terreus* ATCC 20542

It is known that feedback inhibition of desired metabolites on their own biosynthesis occurs in many antibiotics productions. Supplementation of exogenous lovastatin to the culture of *A. terreus* ATCC 20542 will inhibit its own biosynthesis to some extent. Increase of supplemental lovastatin level will lag the startup of its own biosynthesis. However, it does not affect the cell growth in the submerged cultivation (Casas López et al. 2004; Jia et al. 2010). Therefore, the resistance of lovastatin to its parent producer *A. terreus* ATCC 20542 was investigated on the plate containing exogenous lovastatin (Fig. 1). During the plate cultivation, the growth of aerial hypha and the maturation of conidium on the lovastatin grads plate (the level of exogenous lovastatin from 0 to 100 mg l^{-1}) were markedly slower than those on the control plate. After growing for 5 days, colonies with abundant mature spores appeared to uniform and gray-yellow on the control plate. However, the growth of colonies and the maturation of spores were obviously lagged with the increase of the levels of exogenous lovastatin on the lovastatin grads plate (Fig. 1). Cultivating this grads plate for 3 days continuously, the colonies grown in the area with higher level of lovastatin would tardily spread and produce numerous mature spores (data not shown). Cell growth was not influenced by supplementation of lovastatin. However, supplementing exogenous lovastatin to the culture would obviously lag the cell growth of its producing strain. This phenomenon has been identified in submerged cultivation of *A. terreus* (Jia et al. 2010). Therefore, it is possible to obtain a self-resistant mutant for hyper-production of lovastatin. This is the theoretical foundation for screening the lovastatin-resistant mutant of *A. terreus*.

Screening of lovastatin-resistant mutant for hyper production

The original strain *A. terreus* ATCC 20542 was processed in turn by four cycles of UV mutation before being screened by the culture of plate containing exogenous lovastatin. A total of 30 mutants were selected to detect the yields of lovastatin by shake flask culture in each cycle. The yield distribution of mutants is depicted in Fig. 2. The original strain only produced 168 ± 11 mg l^{-1} of lovastatin. After being screened by 0.25 g l^{-1} of exogenous lovastatin in the first cycle of mutation, the mutants led to 20% of positive mutation. With the rise of exogenous lovastatin concentration in the latter process (from 0.25 to 4 g l^{-1}), the positive mutation declined, compared with its own original strain. The final lovastatin productions of mutants obtained from each cycle of mutation are shown in Table 2. Compared with the parent strain of *A. terreus*, the mutant L2144 resulted in an increase of 86% for lovastatin production. However, the mutants obtained from the other three cycles of mutation improved the lovastatin production only by 8, 19, and 20% respectively, compared with their

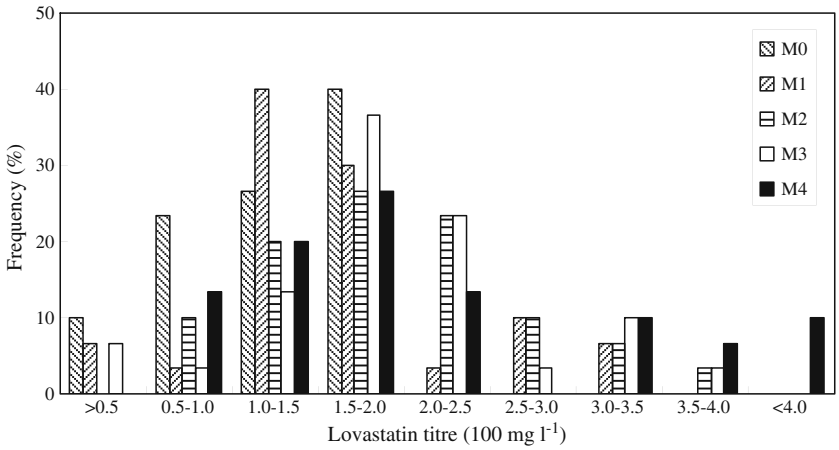

Fig. 2 Yield distribution of mutants

original strains. The final resistant mutant LA4414 screened by four times of mutation produced 483 ± 21 mg l^{-1} of lovastatin, which increased by approximately twice compared with the parent strain ATCC 20542. The morphology of the mutant varied markedly. After growing for 5 days on the plate, the lawn of colonies appeared to be thinner than the parent strain and changed from yellow-brown to damask. Germination mainly occurred in the center of the colony. The aerial hyphae gradually spread around in the culture and appeared as a distinct zone in which fewer spores were formed. Being sub-cultured for five generations, the mutant LA4414 displayed a better heredity for lovastatin production. A decrease of only 7% in lovastatin production was produced.

The good understanding of the lovastatin biosynthetic pathway and the related genes reveals that self-resistance to lovastatin is provided by ORF1 or the *lvrA* gene (Kennedy et al. 1999). The former encodes an esterase-like protein while the latter encodes a protein very much like the known HMG-CoA reductases. Introduction of *lvrA* into the lovastatin sensitive *Aspergillus nidulans* confers high-level lovastatin resistance (Hutchinson et al. 2000). Mutagenesis of the parent strain probably affects the expression of the above two resistant genes, which result in different genotypes of mutants. Furthermore, the feedback regulation of lovastatin is also possibly related to the inhibition of a certain enzymes (LovA or LovC) which is involved in the formation of monacolin J (Jia et al. 2010). The mutant might be partially released from the inhibition.

Table 2 Results of multiple cycle UV mutation of the lovastatin-resistant mutant[a]

Run of mutagenesis	Strain	Lovastatin production (mg l^{-1})	Relative production (%)
0	ATCC 20542	168 ± 11[b]	100
1	L2144	312 ± 7	186
2	L4404	337 ± 15	201
3	L44041	401 ± 17	239
4	LA4414	483 ± 21	288

[a] Each cycle of mutation was originated from the former strain

[b] The maximum errors were calculated from 3 independent samples

Production of lovastatin in submerged cultivation of the lovastatin-resistant mutant

As one of the most significant components in the medium, the carbon source plays a critical role as the source of precursors and energies for synthesis of biomass building blocks and secondary metabolite production. In addition, the nature and concentration of the carbon source could regulate lovastatin biosynthesis on the level of catabolite repression and signaling of growth or substrate limitations (Hajjaj et al. 2001; Casas Lo´pez et al. 2003). A slowly

Table 3 Optimization of carbon sources for cell growth and lovastatin production by submerged cultivation of the parent strain and the mutant of *A. terreus*[a]

Carbon source[b]	ATCC 20542		LA4414	
	Biomass (g DCW l^{-1})	Lovastatin production (mg l^{-1})	Biomass (g DCW l^{-1})	Lovastatin production (mg l^{-1})
Glycerol	12.68 ± 0.18	448.3 ± 9.7	11.52 ± 0.45	935.3 ± 21.4
Glucose	14.15 ± 0.09	177.5 ± 12.4	5.65 ± 0.10	112.3 ± 11.8
Sucrose	13.48 ± 0.22	243.2 ± 15.3	7.10 ± 0.20	218.8 ± 6.3
Lactose	4.29 ± 0.13	305.0 ± 6.4	2.10 ± 0.25	212.4 ± 11.6
Soluble starch	17.48 ± 0.32	165.2 ± 14.3	10.70 ± 0.10	502.5 ± 22.4

[a] 10 g l^{-1} of yeast extract powder was regarded as the sole complex nitrogen source in all experiments

[b] The 20 g l^{-1} of carbon content for each carbon source was used. The actual concentrations were 51.2, 55, 47.5, 50, and 45 g l^{-1}, respectively

[c] The maximum errors were calculated from 3 independent samples

Table 4 Optimization of complex nitrogen sources for cell growth and lovastatin production by submerged cultivation of the parent strain and the mutant of *A. terreus*[a]

Nitrogen source[b]	ATCC 20542		LA4414	
	Biomass (g DCW l^{-1})	Lovastatin production (mg l^{-1})	Biomass (g DCW l^{-1})	Lovastatin production (mg l^{-1})
Yeast extract	17.48 ± 0.32[b]	165.2 ± 14.3	10.70 ± 0.10	502.5 ± 22.4
Peptone	14.32 ± 0.11	166.4 ± 10.7	13.51 ± 0.22	278.9 ± 14.7
peanut	12.65 ± 0.08	314.1 ± 12.4	10.45 ± 0.16	883.2 ± 19.5
soybean meal	18.21 ± 0.15	351.5 ± 16.4	16.95 ± 0.27	352.1 ± 21.3
Corn steep liquor	13.46 ± 0.05	257.6 ± 9.8	12.65 ± 0.18	154.3 ± 7.5
Oat meal	11.34 ± 0.12	132.2 ± 11.4	8.50 ± 0.04	683.2 ± 18.6

[a] 45 g l^{-1} of soluble starch was regarded as the sole carbon source in all experiments

[b] The initial level of nitrogen source was 10 g l^{-1} in each experiments

[c] The maximum errors were calculated from 3 independent samples

utilized carbon source such as lactose or glycerol was better assimilated for lovastatin biosynthesis than readily metabolized glucose (Hajjaj et al. 2001; Casas Lo´pez et al. 2003; Szakács et al. 1998). Simultaneously, nitrogen source mainly participates in construction of the basic molecules (amino acid, protein and nucleic acid) and formation of nitrogen-containing metabolites during the period of growth. It is usually regarded as a factor of nutrient limitation to determine directly the growth of microorganisms and affect the biosynthesis of many secondary metabolites. Barrios-González and Miranda (2010) have reported that the carbon source (lactose, glycerol, and fructose) and the nitrogen source (yeast extract, corn steep liquor, and soybean meal) were more beneficial to the production of lovastatin. The use of a slowly metabolized carbon source (lactose) in combination with either soybean meal or yeast extract under N-limited conditions gave the highest titers and specific productivity.

In order to identify a suitable carbon source for lovastatin production by submerged cultivation of its self-resistant mutant of *A. terreus*, different carbohydrates such as glycerol, glucose, sucrose, lactose, and soluble starch were used, while 10 g l^{-1} of yeast extract powder was regarded as the sole complex nitrogen source in all experiments. Simultaneously, the parent strain of *A. terreus* was also cultivated in the same conditions. Table 3 shows the comparison of the final biomass (dry cell weight, DCW) and lovastatin titer between the parent strain and the mutant under various carbon sources after 8 days of cultivation. Apparently, the cell growth of the mutant was slower than that of the parent strain while the ability of lovastatin production was markedly affected by mutagenesis. The maximal biomass and lovastatin production were all obtained from glycerol medium. The lowest biomass was attained in lactose medium and the smallest lovastatin titer was produced in glucose medium. However, the highest yield of lovastatin production on biomass (101.1mg g^{-1} DCW) was obtained in lactose medium, followed by glycerol medium (81.2 mg g^{-1} DCW) and soluble starch medium, which were 5.1-, 4.1- and 2.4-fold that obtained from glucose medium, respectively. Compared with the parent strain, the yield of lovastatin production on biomass obtained in soluble starch medium increased 4-fold while others only improved from approximately 50 to 100%. As a result, soluble starch was economically preferred to cell growth and lovastatin production.

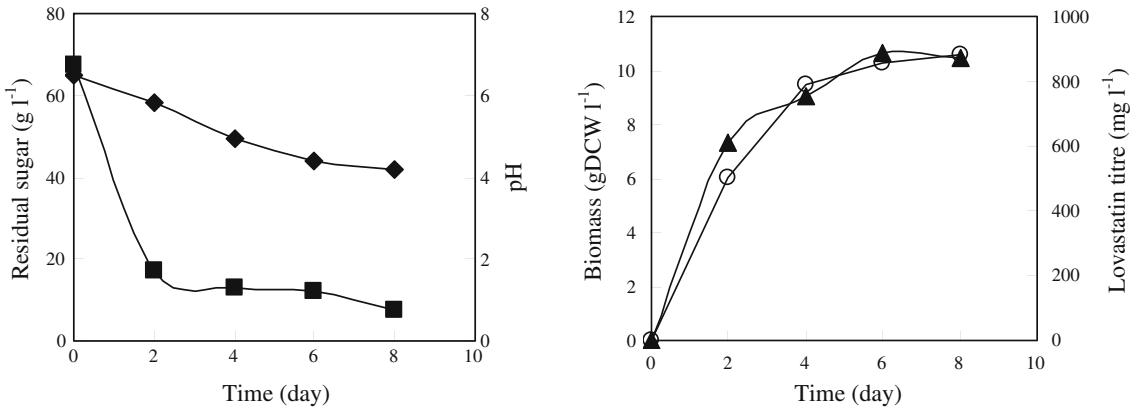

Fig. 3 Batch cultures of the lovastatin-resistant mutant. Symbols: pH (♦), residual sugar (■), biomass (▲), lovastatin titre (○); 45 g l^{-1} of soluble starch was regarded as the sole carbon source and 10 g l^{-1} of peanut meal was regarded as the sole complex nitrogen source

Six complex nitrogen sources including yeast extract powder, peptone, peanut meal, oat meal, corn steep liquor, and soybean meal were investigated in submerged cultivation of *A. terreus* ATCC 20542 and of its mutant LA4414 when soluble starch was regarded as the sole carbon source. After being processed by mutagenesis, the mutant appeared to have a strong ability for lovastatin production in the medium containing many complex nitrogen sources except for corn steep liquor compared with the parent strain. All the cell growth decreased to some extent (Table 4). Of all the investigated nitrogen sources, soybean meal was the best complex one for the mutant, which resulted in the highest biomass at the end of fermentation. However, oat meal went against the cell growth. Simultaneously, the maximal lovastatin titer was produced in the presence of peanut meal while corn steep liquor led to the smallest lovastatin titer. These results indicated that the smallest yield of lovastatin on biomass (12.2 mg g^{-1} DCW) was obtained when corn steep liquor was regarded as the sole complex nitrogen source. The other five nitrogen sources all resulted in increases of these values to some extent compared with corn seep liquor, i.e. 1.7-fold for peptone; 3.8-fold for yeast extract powder; 6.9-fold for peanut meal; 6.6-fold for oat meal; and 1.7-fold for soybean meal. Peanut meal was thus indentified to be most beneficial for lovastatin biosynthesis.

Rollini and Manzoni (2006) revealed that the best lovastatin yields (280 mg l^{-1}) were obtained employing soybean peptone when the carbon and energy sources were the combination of glycerol, glucose and lactose in the base medium. However, lower yields were obtained with cotton (207 mg l^{-1}) and peanut (250 mg l^{-1}) with *A. terreus* at 14 days fermentation. In our study, an optimal culture medium contained 45 g l^{-1} of soluble starch as the sole carbon source and 10 g l^{-1} of peanut meal as the sole complex nitrogen source was used for the batch cultivation of the selected lovastatin-resistant mutant. More than 7 g l^{-1} of residual sugar existed in the cultures at harvest (Fig. 3a). The pH of the broth appeared to a linear decline, and the final pH reached 4.18 ± 0.02. Furthermore, the highest biomass reached 10.65 ± 0.62 g DCW l^{-1} at day 6 and the maximal lovastatin titre produced was 883.2 ± 19.5 mg l^{-1} at day 8 (Fig. 3b). This result was over 2.5-fold higher than that obtained from the work by Rollini and Manzoni (2006). The specific growth rate of 1.92 day^{-1} and the specific lovastatin productivity of 1.44 mg g^{-1}DCW h^{-1} were obtained during the fermentation. These indicate that large quantities of organic acids such as a−ketoglutarate, citric acid and malonate were probably produced in the cultures. The accumulation of these organic acids further prevent the tricarboxylic acid cycle from advancing, which creates abundant acetyl-CoA and malonyl-CoA for supplying enough precursors to the biosynthesis of lovastatin (Wu et al. 2008).

Conclusion

The resistance of lovastatin to its parent producer *A. terreus* ATCC 20542 was investigated on the plate containing exogenous lovastatin. The quantities of colonies and spores decreased with the increase of the levels of exogenous lovastatin. Based on this result, a self-resistant mutant of *A. terreus* for hyper-production of lovastatin was screened by four cycles of UV mutation and by using plate cultures containing exogenous lovastatin. With the rise of screening lovastatin concentration, the positive mutation declined compared with its own original strain. The production ability of final resistant mutant LA4414 was approximate 3-fold that obtained from the parent strain. The optimal and economical carbon and nitrogen sources were soluble starch and peanut meal for hyper production of lovastatin by the selected mutant, respectively. The final lovastatin production reached to 883.2 ± 19.5 mg l^{-1} in the optimized medium, which was also 3-fold that obtained from the un-optimized medium.

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