Biotechnology and Bioprocess Engineering **2005**, 10: 78-84

Enhanced Lovastatin Production by Solid State Fermentation of *Monascus ruber*

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Abstract The purpose of this study was to optimize the solid state cultivation of *Monascus ruber* on sterile rice. A single-level-multiple-factor and a single-factor-multiple-level experimental design were employed to determine the optimal medium constituents and to optimize carbon and nitrogen source concentrations for lovastatin production. Simultaneous quantitative analyses of the β-hydroxyacid form and β-hydroxylactone for of lovastatin were performed by the high performance liquid chromatography (HPLC) method with a UV photodiode-array (PDA) detector. The total lovastatin yield (4~6 mg/g, average of five repeats) was achieved by adding soybean powder, glycerol, sodium nitrate, and acetic acid at optimized levels after 14 days of fermentation. The maximal yield of lovastatin under the optimal composition of the medium increased by almost 2 times the yield observed prior to optimization. The experimental results also indicated that the β-hydroxylactone form of lovastatin (LFL) and the β-hydroxyacid form of lovastatin (AFL) simultaneously existed in solid state cultures of *Monascus ruber*, while the latter was the dominant form in the middle-late stage of continued fermentation. These results indicate that optimized culture conditions can be used for industrial production of lovastatin to obtain high yields.

Keywords: *Monascus ruber*, lovastatin, solid state fermentation

INTRODUCTION

Lovastatin (Mevinolin, Monacolin K and Mevacor®) is a potent drug for lowering blood cholesterol. It is a competitive inhibitor of 3-hydro-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) [1], which catalyzes the rate-limiting step of cholesterol biosynthesis. Lovastatin can be produced by *Aspergillus terreus* [1], *Penicillium* species [2] and *Monascus ruber* [3]. A number of studies attempting to increase lovastatin production through more efficient processes have been documented [4-9]. Most of these studies have focused on the optimization of *A. terreus* cultivation. In comparison to *A. terreus*, the lovastatin production from red rice fermented by *M. ruber* has been seldom reported [10,11]. Since *A. terreus* is not considered edible, tedious downstream procedures for the extraction and purification of lovastatin from the broth are needed, whereas *Monascus* species are non-pathogenic and extensively used in Chinese foods and traditional Chinese medicines. In addition, a pharmaceutical study has demonstrated that red yeast rice reduces blood cholesterol in three animal models of hypercholesterolemia [12]. Furthermore, the use of red yeast rice as a dietary supplement in a human clinical trial has shown significant reductions of cholesterol levels in 83 tested individuals [13]. These facts indicate that the production of lovastatin by *Monascus* spp. might be advantageous with an increased saving in cost, and it may be used directly as a health functional food as long as it proves to be nontoxic. However, medium optimization experiments with *M. ruber* for lovastatin production have not been extensively studied, especially for solid state fermentation (SSF). The present study aimed to optimize the SSF conditions of *M. ruber* on steamed rice for improving lovastatin production. A traditional single-level-multiple-factor experimental design was applied to study the effect of medium constituents on lovastatin production, and a single-factor-multiple-level experimental design was applied to study the effect of medium concentration on lovastatin production.

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MATERIALS AND METHODS

Chemicals and Media

Lovastatin (Mevinolin) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the solvents

Table 1. Screening for optimum lovastatin production with six strains of *M. ruber*.

Strains	Source	Color value (U/g)	AFL content (mg/g)	LFL content (mg/g)	TL content (mg/g)
FM000	KFRI, Korea	0	2.320 ± 0.091	0.468 ± 0.033	2.789 ± 0.092
FM008	KFRI, Korea	321.2	1.075 ± 0.066	0.276 ± 0.032	1.352 ± 0.067
FM012	KFRI, Korea	67.25	0.455 ± 0.088	0.079 ± 0.092	0.543 ± 0.096
AS3.4633	CTCCCAS, China	43.25	0.674 ± 0.045	0.345 ± 0.026	1.019 ± 0.048
GMA7	Hangzhou, China	252.5	1.840 ± 0.102	0.400 ± 0.098	2.240 ± 0.099
GM011	Hangzhou, China	80.75	2.636 ± 0.159	0.810 ± 0.135	3.446 ± 0.154

The results are presented as mean ± S.E. of five replicates. AFL, acid form lovastatin; LFL, lactone form lovastatin; TL, total lovastatin.

used in chromatography were of HPLC purity (Duksan Pure Chemical Co., Ltd., Ansan, Korea), and solvents used for spectroscopy were of spectroscopic grade. Trifluoroacetic (TFA) was purchased from Fluka (Buchs, Switzerland). HPLC grade water was prepared using a compact ultra-pure water system (Compact Co. Ltd., Iowa, USA). $NaNO_3$, KH_2PO_4, $MgSO_4$, glycerol, acetic acid and other nutrient sources were supplied by Duksan (Duksan Pure Chemical Co., Ltd., Ansan, Korea). Yeast extract, and peptone were from Bacto (Bacto™, Sparks, MD, USA).

Microorganism and Growth Conditions

In our study, the screening for lovastatin production was carried out with six strains of *Monascus* species (Table1.). The strains were obtained from CTCCCAS (The Committee on Type Culture Collection of Chinese Academy of Science) and KFRI (Korea Food Research Institute). Among these, the strain GM001 (strain marker) employed in the production of functional foodstuff, contained Monacolin K, from Dbio Inc., Korea, which was isolated from a food sample from China. It was classified as *M. ruber* and proved to have 100% similarity to the strain of AS3.549 *M. ruber* (Type Culture Collection of Chinese Academy of Science) [14]. This strain was selected for further optimization because of its highest total lovastatin (TL) production (3.446 mg/g) and desirable color value among the tested strains.

The stock culture was periodically maintained on a DIFCO potato dextrose agar (PDA) slant. Spores were prepared by growing on PDA petri dish cultures for 8 days at 30°C. A suspension of spores was obtained by washing the petri dish cultures with a sterile aqueous solution of 2% Tween-20. The resulting suspension was centrifuged (3,000 × g, 5 min) and the solids were resuspended in sterile distilled water. A suspension of 10^8 spores was used to inoculate a 250-mL baffled Erlenmeyer flask containing 100 mL of synthetic medium. The synthetic liquid culture medium was used for the preparation of seed for a solid state culture. The composition of the medium was 50 mL/L glycerol, 30 g/L glucose, 10 g/L yeast extract, 8 g/L peptone, 2 g/L $NaNO_3$, 1 g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 3.5 mL/L acetic acid, and 20 g/L rice powder. The initial pH value of the cultures was adjusted to 5.0 before sterilization. The cultures were incubated on a shaking incubator at 250 rpm at 30°C for 6 days.

Before SSF, 150 g of soaked rice (equivalent to 100 g of dry rice) with additive carbon and nitrogen sources were autoclaved for 30 min. The steamed rice was inoculated with 10% (w/v) liquid seed of *M. ruber*. The SSF of lovastatin on steamed rice was routinely carried out in a plastic mushroom bottle for 2 weeks. For the first 3 days, the culture was maintained at 32 to 30°C, and shaken three to five times per day according to the growth status. Subsequently, the temperature was reduced to 28°C for 1 to 3 days, and then to 26°C for 8 to 10 days. After 14 days, the cultured rice was autoclaved and dried for quality analysis. Prior to optimization, the basal solid medium consisted of 100 g of rice, 14 g of soybean powder, 2 g of sucrose, and 1 g of yeast extract. It was also used for the control experiment. For comparison, various carbon sources (sucrose, glucose, lactose, glycerol) (Fig. 5A), and nitrogen sources (yeast extract, peptone, sodium nitrate, ammonium sulfate) (Fig. 5B) at various concentration levels (Fig. 6) were used for the optimization study.

Experimental Design and Statistical Analysis

A traditional single-level-multiple-factor and a single-factor-multiple-level experimental design were used to optimize the medium constituents. Each treatment was repeated five times. Results are expressed as Mean ± S.E. The statistical analysis software SPSS was used for all data process. The significance of differences between the control group and the optimization treatment group means was determined by one-way ANOVA with Post Hoc Tests. P values less than 0.05 were considered statistically significant.

Quantitative Analyses of Lovastatin by HPLC

The quantitative analyses of lovastatin were carried out by the Beckman HPLC system. It was System Gold ® (Beckman, USA) equipped with a 128 Solvent Module, a 168 Detector, a 7725 auto sampler and an on-line degassing instrument. An ODS column (250 × 4.6 mm *i.d.*, 5 μm) from Sphenomenex was used. The 168 detector was equipped with a photo-diode array (PDA) detector. A Gold™ Nouveau Chromatography Station was used for system control, data collection and analysis. The mobile phase consisted of acetonitrile and water contained 0.05% TFA (62:38 by volume). The eluent flow rate was

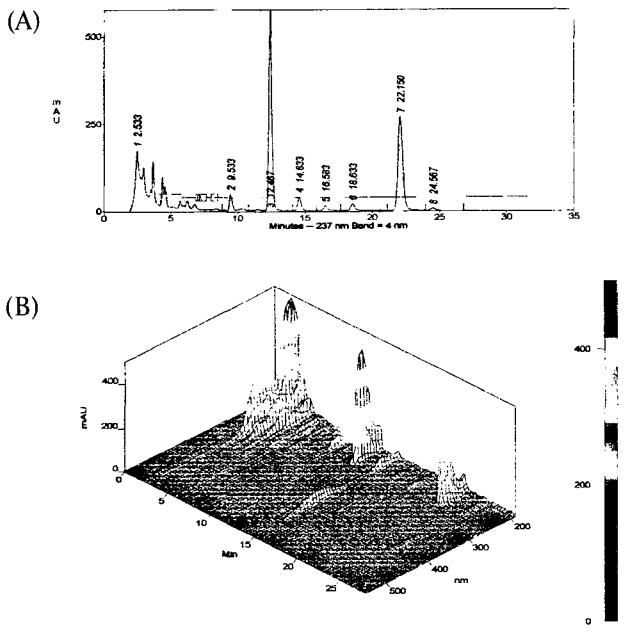

Fig. 1. Typical HPLC chromatogram (A) and 3D plot (from 190 to 600 nm) (B) of metabolites from the SSF of *M. rubber*

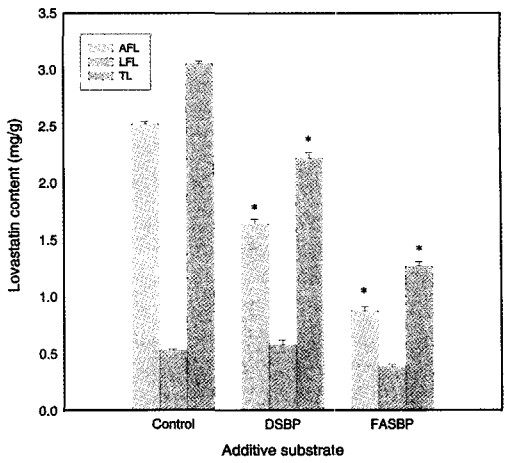

Fig. 2. Effect of the properties of soybean flour on lovastatin production in SSF. Control, soybean powder; DSBP, defatted soybean powder; FASBP, feed additive. Each bar represents the mean ± S.E. of five replicates. * $P< 0.05$, difference between treatment groups and the control. AFL, acid form lovastatin; LFL, lactone form lovastatin; TL, total lovastatin.

0.8 mL/min. Since lovastatin exhibits UV absorption (lovastatin: λ_{Max}^{CH3CN} (nm) = 237), the detection was monitored at 237 nm, and the UV photo diode array (PDA) detection range was set from 200 to 600 nm. The sample injection volume was 5 μL, and the run time was 30 to 40 min. In addition, the color value was determined by optical densities of pigments on a spectrophotometer (Varian Cary 100 Conc system, Varian, Australia) at 510 nm. In the current study, the preparation of sample and standard solutions for HPLC analysis was processed in the same manner as done in our previous study [15].

RESULTS AND DISCUSSION

Typical HPLC Chromatogram Analysis of SSF of *M. ruber*

A reversed-phase HPLC system was carried out for the quantification of lovastatin in the SSF of *M. ruber*. First, two linear fit external standard curves of AFL and LFL were obtained by HPLC analysis. For samples analysis, after HPLC separation, eluting substances were monitored with a photodiode array detector. Seven peaks were obtained with full baseline separation. A typical HPLC trace showing the profile of the metabolites in the SSF of *M. ruber* is shown in Fig. 1A. Meanwhile, an HPLC 3D plot (Fig. 1B) of the SSF was achieved to monitor UV absorption information of every baseline separation peak; furthermore, it verified the simultaneous existence of AFL and LFL in the SSF. After the quantitative assay procedure was established, fermentation experiments were carried out by monitoring with HPLC.

Since the previously described HPLC procedure [16], which was determined only for LFL, an accurate quantification of total lovastatin has not been achieved. To ensure correct quantification, the present study employed a simultaneous analysis procedure of AFL and LFL, which followed our previous study [15]. Although the chromatogram of the solid cultures was complicated, a well resolved chromatogram of the lovastatin sample was achieved based on the screened chromatographic conditions. Both the AFL (R.T. 12.4 min) and LFL (R.T. 22.1 min) peaks of lovastatin (Fig. 1A) were well separated from all the other peaks, which made the quantification of lovastatin easy and accurate.

Screening of Optimal Strain for Lovastatin Production

In the first part of the research, a screening work for lovastatin production was carried out by employing *M. ruber* strains. Lovastatin production was comparatively evaluated on 14-day-old cultures grown on steamed rice additive with a base medium as described above. All of the six test strains of the *M. ruber* produced lovastatin in yields ranging from 0.534 to 3.446 mg/g. The strains AS3.549, FM000, GMA7, and GM011 showed good lovastatin yield (Table 1). Among these, the *M. ruber* GM011 strain was selected for further optimization study because of its highest total lovastatin production (3.446 mg/g) and fine color value among the tested strains.

Effect of Substrate on Lovastatin Production

The composition of a solid substrate is an important factor that affects fungi growth and productivity. For the SSF of *M. ruber*, rice is a common substrate, and soybean flour is a common additive substrate. In this study, the properties of soybean flour were considered as important factors, and the effect of the properties of soybean flour on lovastatin production are illustrated in Fig. 2.

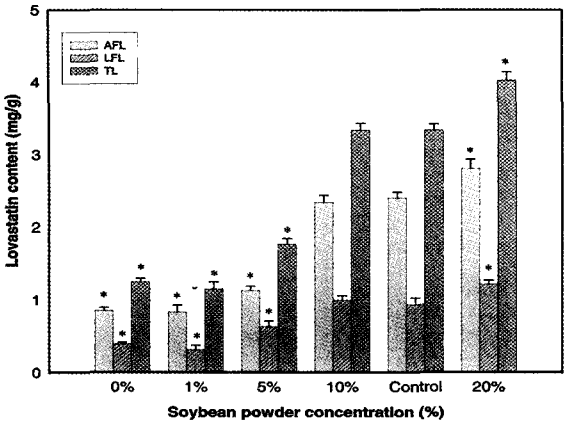

Fig. 3. Effect of soybean powder content on lovastatin production in SSF. Control, 14% soybean powder. Each bar represents the mean ± S.E. of five replicates. * $P< 0.05$, difference between treatment groups and the control. AFL, acid form lovastatin; LFL, lactone form lovastatin; TL, total lovastatin.

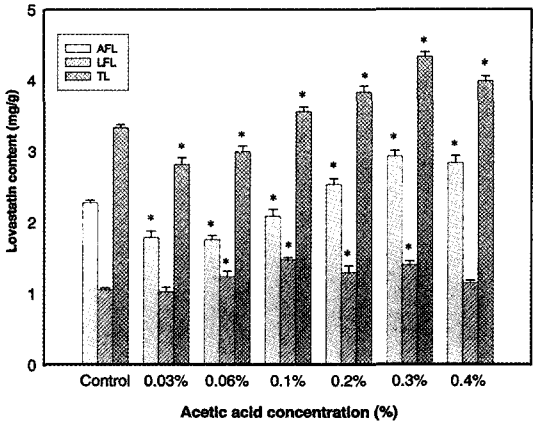

Fig. 4. Effect of acetic acid addition on lovastatin production in SSF. Control, without an acetic acid addition. Each bar represents the mean ± S.E. of five replicates. * $P< 0.05$, difference between treatment groups and the control. AFL, acid form lovastatin; LFL, lactone form lovastatin; TL, total lovastatin.

The results suggest that three kinds of soybean flour could produce lovastatin, in yields ranging from 1.26 to 3.05 mg/g, with significant differences between the experiment groups. Soybean powder contributed the highest lovastatin yield, compared with defatted soybean powder (DSBP) and mixture feed additive (FASBP) (contained defatted soybean powder), and differences reached to a significant level ($P<0.05$). Therefore, soybean powder was used as the basic additive substrate for the optimizing experiments. In order to further explore the optimal soybean powder additive proportion, a single-factor-multiple-level experiment of soybean powder was done. The results (Fig. 3) showed that a 10% addition, a 14% addition (as the control), and a 20% addition could result in high level lovastatin yields: yields ranging from 3.33 to 4.02 mg/g. These results were significantly different from those at other levels (0, 1, and 5%), and this fact further verified that soybean powder is an important additive substrate for the SSF of *M. ruber.* The results also illustrated that the soybean powder addition at the 20% level generated the highest yield among the present experiment levels, and lovastatin yields at this level were significantly different from the control group yield.

As with any solid state fermentation product, the culture medium has a significant influence on the yield of a metabolite, thus the screening and optimization of substrate constituents are a key steps in industrial scale fermentation. The selection and composition optimization of a suitable substrate are also important for lovastatin production from the SSF of *M. ruber.* In the present study, soybean powder was found to be the best additive substrate among the different properties of soybean flour. Defatted soybean flour and feed additive soybean flour achieved lower lovastatin yields that significantly differed with that of soybean powder. These facts partly differ with those of a previous study [17] that tested on the liquid fermentation of 10 tested strains of the *Monascus* genus. This could be attributed to a different fermentation style or strain property. Due to different protein and lipid proportions among the three kinds of soybean flour, as an additional carbon source and nitrogen source with a different C/N ratio, different lovastatin productions should be achieved. In the addition tests with various concentration levels of soybean, we found that lovastatin production increased with an increase in soybean powder content. This might be directly attributed to the proportion variance of the substrate rice (about 75% carbohydrate) and additive substrate soybean (40~41% protein, 22~24% lipid), therefore the carbon source and nitrogen source proportions changed. Upon continued increasing soybean powder content, we found that fermentation was easily contaminated by bacteria, so we concluded that 14~20% soybean powder additions were proper for the production of high quality functional red rice.

Effect of Acetic Acid on Lovastatin Production

So far, lovastatin from the fungus *A. terreus* (ATCC 20524) has been well-known to derived from acetate *via* a polyketide pathway [18-20], whereas a biosynthesis pathway of lovastatin from *Monascus* spp. is not very clear. The present study also presumed the lovastatin biosynthesis pathway of *M. ruber* is derived from acetate via a polyketide pathway. In addition, acetic acid, as a special carbon source and pH regulating reagent, is extensively used for industrial fermentation. Taking the above-mentioned factors into account, acetic acid was considered an important additive substance to use for the experimental test. The data indicated that 0.1, 0.2, 0.3, and 0.4% additions of acetic acid significantly increased total lovastatin production greater than the control group production (without an acetic acid addition), while 0.03, and 0.06% additions of acetic acid decreased lovastatin production significantly (Fig. 4).

As for the acetic acid addition tests, we found that 0.1, 0.2, and 0.3% acetic acid additions contributed to higher

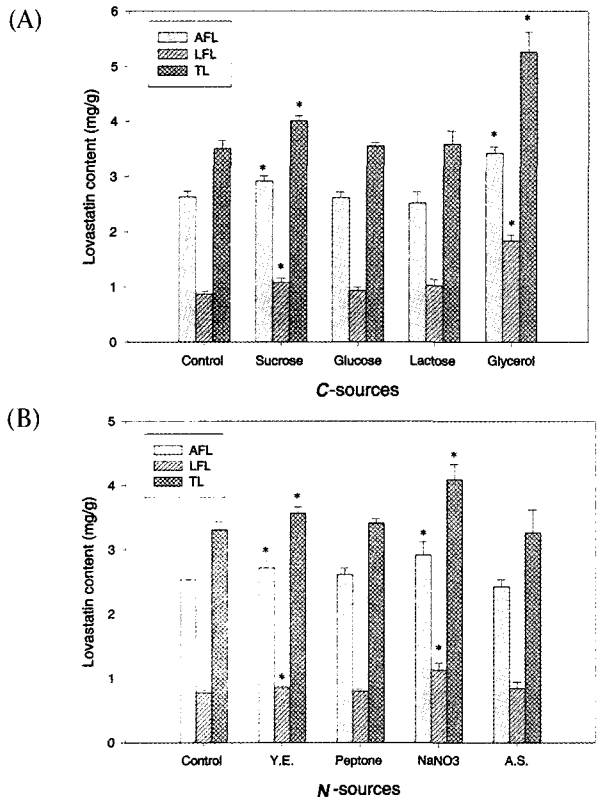

Fig. 5. Effects of the additions of carbon and nitrogen sources on lovastatin production in SSF. Each nitrogen source was added at 0.2%, each carbon source was added at 3%. (A), The effect of different carbon sources; (B), The effect of different nitrogen sources. Y.E., yeast extract; A.S., $(NH_4)_2SO_4$. Each bar represents the mean ± S.E. of five replicates. * $P< 0.05$, difference between treatment groups and the control. AFL, acid form lovastatin; LFL, lactone form lovastatin; TL, total lovastatin.

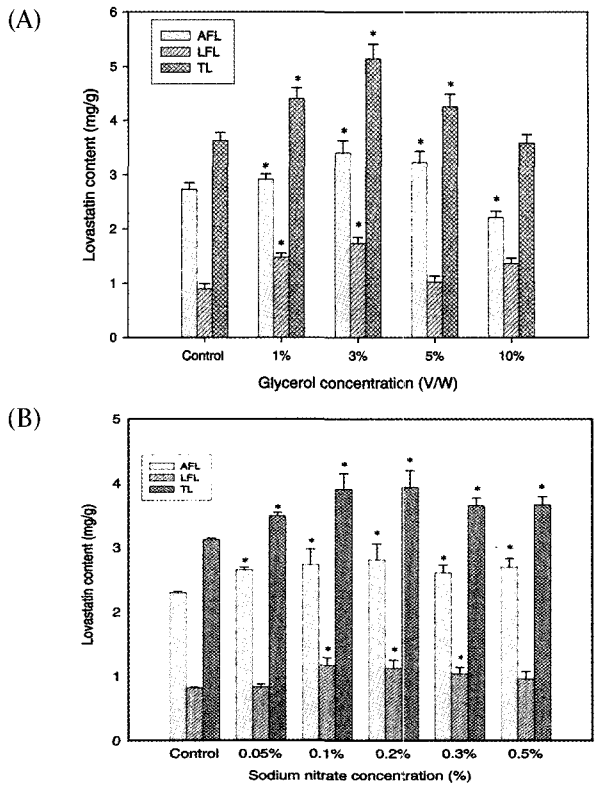

Fig. 6. Effect of various levels of glycerol and sodium nitrate on lovastatin production in SSF. (A), The effect of glycerol. Control, without glycerol; (B), The effect of sodium nitrate. Control without sodium nitrate. Each bar represents the mean ± S.E. of five replicates. * $P< 0.05$, difference between treatment groups and the control. AFL, acid form lovastatin; LFL, lactone form lovastatin; TL, total lovastatin.

lovastatin yields. 0.4% acetic acid also contributed to a higher yield, but at this level, the lovastatin yield took on a decreasing trend. Because of the complex biosynthesis pathway of lovastatin from *Monascus* spp., it is not very clear as to the effect of acetic acid on lovastatin production. In this respect, additional studies on biosynthesis mechanisms are needed as a basis for further research.

Effect of Carbon and Nitrogen Additives on Lovastatin Production

Of the major culture nutrients, carbon and nitrogen sources generally play a dominant role in fermentation productivity because these nutrients are directly linked with the formation of the biomass and the metabolite. Also, the biosynthesis of lovastatin has been found to depend on carbon and nitrogen sources. During our preliminary experiments, the effects of various carbon, and nitrogen sources on lovastatin production were evaluated, and the data are shown in Fig. 5. Among the different carbon source tests, sucrose and glycerol significantly enhanced lovastatin production when compared to the control and other nutrient sources (Fig. 5A). However, the glucose and lactose additions had no significant effect on lovastatin production. Because glycerol contributed to the highest lovastatin level in the current tests, glycerol as an additive carbon source was further used for the single-factor-multiple-level experimental test (Fig. 6A) to evaluate the optimal concentration level of glycerol. The results showed that the 1, 3, and 5% glycerol additions demonstrated significant differences ($P<0.05$) from the control group (without glycerol addition), but no significance was found in the 10% glycerol addition tests. Among the glycerol addition tests, the 3% glycerol addition achieved the highest lovastatin production and with a significant difference ($P<0.05$) from the other additions. For the different nitrogen source tests, the organic nitrogen sources, such as yeast extract, and peptone, and the inorganic nitrogen sources such as sodium nitrate, and ammonium sulfate were used for evaluation. The results showed that the organic nitrogen source, yeast extract, and the inorganic nitrogen source, sodium nitrate, achieved relatively highter lovastatin productions than the control (without the nitrogen addition test) and other nutrient

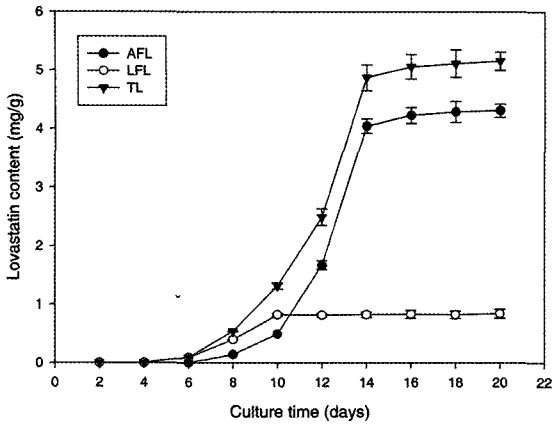

Fig. 7. Kinetic variation of lovastatin between LFL and AFL in SSF. ● AFL, acid form lovastatin; ○ LFL, lactone form lovastatin; ▼ TL, total lovastatin.

sources (Fig. 5B) with significance. No significance was found in the peptone and ammonium sulfate addition tests. Because sodium nitrate contributed to the highest lovastatin production in the current tests, sodium nitrate as an additive nitrogen source was further used for the single-factor-multiple-level experimental test (Fig. 6B) to evaluate the optimal concentration level of sodium nitrate. The results showed that all of the various levels of the sodium nitrate additions (0.05, 0.1, 0.2, 0.3, 0.5%) generated significant differences ($P<0.05$) from the control group (without sodium nitrate addition). Among the sodium nitrate addition tests at different levels, the 0.1, and 0.2% additions achieved relatively higher lovastatin production values with significant differences ($P<0.05$) from the other additions.

For the carbon and nitrogen additives tests, the addition of 3% glycerol and 0.2% inorganic nitrogen ($NaNO_3$) contributed towards higher lovastatin yields. Among the glycerol concentration level tests, when increasing the glycerol level over 3% to the 5, and 10% levels, we found that the lovastatin yield decreased along with the increases in glycerol level. This might be attributed to the decrease in fungal cell permeability. Increasing the content of organic nitrogen (yeast extract), lead to an increase in lovastatin yield, and subsequently this also increased the chances of bacterial contamination.

Lovastatin Time Course Production

After optimizing the lovastatin production conditions, we investigated the time course of lovastatin production from *M. ruber* GM011. Fermentation was performed by employing the optimized medium. Lovastatin production kinetics was monitored by HPLC. The *M. ruber* GM011 fermentation kinetics profile is illustrated in Fig. 7.

The profile showed that lovastatin was not produced in the first 4 days of cultivation. At the initial stage of lovastatin production, only LFL was detected, then both LFA and AFL could be detected simultaneously at the early-middle-stage. This indicated that lovastatin was first synthesized in its lactone-form (LFL) rather than in its acid-form (AFL), then LFL was partly transferred into AFL. The kinetics profile also indicated that LFL and AFL simultaneously existed in solid state cultures of *M. ruber*, while the latter was the dominant form in the middle-late stage of continued fermentation. Furthermore, lovastatin production was generally increased slowly after 14 days of cultivation.

In conclusion, the production of lovastatin was influenced by the compositions and concentration levels of medium constituents. The lovastatin yield increased significantly through the addition of soybean powder, glycerol, sodium nitrate under optimal concentration levels. The maximal yield of lovastatin under the optimal composition of the medium increased by almost 2 times prior to optimization. These results indicate that optimized culture conditions can be used for industrial production of lovastatin to obtain high yields.

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[Received November 15, 2004; accepted February 12, 2005]