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Lovastatin production by *Aspergillus terreus* in solid-state fermentation*

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Abstract: Lovastatin production by *Aspergillus terreus* ATCC 20542 in solid-state fermentation (SSF) was studied. Various substrates were used to evaluate the ability of *A. terreus* to produce lovastatin. The results showed that either rice or wheat bran was suitable substrate for lovastatin production in SSF. The maximum yield of lovastatin (2.9 mg/g dry substrate) using rice as substrate was achieved after incubating for 11 d at the following optimized process parameters: 50%~60% initial moisture content, pH 5.5, incubation temperature 28 °C.

Key words: Solid-state fermentation (SSF), Rice, Lovastatin, *Aspergillus terreus*
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INTRODUCTION

Solid-state fermentation (SSF) processes can be defined as "the growth of microorganisms (mainly fungi) on moist solid materials in the absence of free-flowing water". It has been known and applied successfully for the production of oriental foods for centuries. More recently, the SSF processes have regained attraction because of its advantages compared with submerged fermentation (SmF). In the SSF processes, the substrate costs little and is widely available; the water and energy consumptions are less than that in SmF process, whereas the product yield is generally higher. The main problems existing in the SSF processes are the design and operation of SSF reactors related to the solid substrate treatment. Generally speaking, the SSF process offers a better opportunity for the biosynthesis of low-volume-high-cost products (Balakrishnan and Pandey, 1996; Cen and Xia, 1999; Xia and Cen, 1999; Hölker and Lenz, 2005; Hölker *et al.*, 2004). Presently, the SSF processes have been widely studied in the production of microbial enzymes (Gombert *et al*., 1999; Abdel-Fattah and Olama, 2002), fine chemicals (Shojaosadati and Babaeipour, 2002), antibiotics (Ohno *et al*., 1993; Adinarayana *et al*., 2003) and immunosuppresants (Ramana Murthy *et al*., 1999), etc.

Lovastatin, also known as Monacolin K, is a kind of fungal metabolite, serving as a competitive inhibitor of 3-hydroxyl-3-methylglutaryl coenzyme A reductase (HMG-CoA), the rate-limiting enzyme in cholesterol biosynthesis (Endo, 1979). It can effectively reduce plasma cholesterol levels in various mammalian species including human, and is thereby effective in the therapy of hypercholesterolemia (Frishman *et al*., 1989). Nowadays, lovastatin and its semisynthetic derivatives are very important drugs since the mortality of heart disease becoming relatively high.

Currently, lovastatin is mainly produced by submerged fermentation of *A. terreus* (Casas López *et al*., 2003; Sitaram Kumar *et al*., 2000). As a kind of secondary metabolite of fungi, lovastatin is an intracellular product and mostly accumulated in mycelia.

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In submerged fermentation, its yield is proportional to the amount of biomass, with the high cell density causing the increase of the fermentation broth viscosity and the difficulty in stirring and oxygen mass transfer; an alternative strategy to produce lovastatin is by SSF process. Except the low substrate cost and low energy consumption, the SSF process can offer a good environment for fungi to grow, therefore high mycelia density and high lovastatin production can be expected. The SSF fermentation for lovastatin production was studied by Valera *et al*.(2005) with *Aspergillus flavipes* and Xu *et al*.(2005) with *Monascus rubber* and solid-liquid fermentation by Chang *et al*.(2002) with *Monascus rubber*. The lovastatin yields were 4~6 mg/g of dry solid, 16.78 mg/g of dry solid and 131 mg/L respectively.

The aims of this work are to evaluate the feasibility of SSF process for lovastatin production by *A. terreus* ATCC 20542 and to optimize the SSF process to increase the yield of lovastatin.

MATERIALS AND METHODS

Culture

Strain *A. terreus* ATCC 20542 was maintained on malt-extract agar slants at 4 °C.

Inoculum preparation

A. terreus was grown on malt-extract agar slants at 30 °C for 7 d until complete sporulation. Five ml sterile water was added to the slant and the spores were scraped and transferred into a 150 ml Erlenmeyer flask containing 50 ml 0.05% Tween-80 and several glass beads. The spore suspension was shaken evenly and then used as inoculum.

Solid-state fermentation

Solid-state fermentation was conducted in 500 ml Erlenmeyer flasks containing 50 g moistened substrate. The flasks containing substrate were autoclaved for 40 min at 121 °C and the moisture content of the substrate was measured and adjusted with sterile distilled water to produce a level of 60%. After cooling the flasks to room temperature, 5 ml inoculum (with spore concentration of about 10^7~10^8 ml^{-1}) was added and the contents of the flasks were thoroughly mixed by shaking. The flasks were incubated at 28 °C for 11 d. At the end of SSF, lovastatin was extracted by methanol. After centrifugation to remove solid residue, the supernatant was collected and the concentration of lovastatin was analyzed by HPLC (high performance liquid chromatography). All experiments were done in triplicate and average values were reported.

Optimization of SSF process

Various solid substrates such as wheat bran, rice, rice husk, soybean cake particle and corn particle were used as solid substrate to perform SSF for lovastatin production to screen the most suitable substrate; and then the effects of the particle size, moisture content, initial pH value and incubation temperature on the lovastatin production was examined to determine the optimized operation conditions of the SSF process.

Analytical methods

The concentration of lovastatin was assayed with a HPLC system, in which a Novapak C18 column was applied. Mobile phase consisted of methanol and 18 mmol/L phosphoric acid with a volume ratio of v/v=75:25 and with the flow rate of 0.6 ml/min. UV detector was used with the wave length of 237 nm. At the end of SSF, the solid culture was dried at 60 °C for 12 h and 0.5 g culture was weighed and extracted with 10 ml methanol for 12 h; after filtration with filter paper, the filtrate was assayed by HPLC system.

The moisture content in the SSF process was measured as follows. At different time of the fermentation cycle, about 2 g fermented substrate was withdrawn and weighed (W_1), and then dried at 60 °C until constant weight (W_2). Moisture content of the fermented substrate can be calculated as follows:

$$\text{Moisture content}=(W_1-W_2)/W_1\times 100\%.$$

In SSF, it was difficult to determine the biomass concentration directly. Because the content of intracellular nucleic acid is relatively constant in mycelia (Arima and Uozumi, 1967; Koliander *et al*., 1984), therefore, the intracellular nucleic acid was measured to represent the mycelial concentration in this work. The intracellular nucleic acid was extracted by 5% trichloro acetic acid and assayed by spectrometer at wavelength of 260 nm (Liu *et al*., 2000).

The concentration of total sugars and reduced sugars in the medium were assayed by DNS (3,5-dinitrosaligenin) method.

RESULTS AND DISCUSSION

Screening of solid substrates in SSF for lovastatin production

From preliminary experimental data, the optimal moisture content, initial pH value and fermentation temperature for lovastatin production with *A. terreus* ATCC 20542 in the SSF process are 50%~60%, pH 5.5 and 28 °C, respectively. These operation conditions were used in the following experiments.

In order to screen out the best solid substrates for *A. terreus* growth and lovastatin accumulation, the following substrates including wheat bran, rice, soybean cake, rice husk and corn particles were evaluated to study their effectiveness for lovastatin production in SSF process. Rice, wheat bran and rice husk were used directly; whereas, soybean cake and corn particle must be ground and sieved to reduce their particle size. Each substrate was added into three flasks in parallel, without any addition of other nutriments. The SSF was run as described earlier. The experimental results were the average of three flasks and shown in Table 1.

Table 1 Effect of substrates on lovastatin production in SSF process

Substrate	Lovastatin yield (mg/g dry substrate)
Soybean cake particle	1.0±0.039
Rice	2.2±0.085
Corn particle	1.2±0.047
Wheat bran	2.0±0.038
Rice husk	0.6±0.030

From Table 1, it is obvious that rice and wheat bran are better substrates for lovastatin production via SSF process than the other ones. The observation of the mycelia growth (Fig.1) also indicated that rice and wheat bran were more suitable for *A. terreus* growth. Because lovastatin is an intracellular product, better growth of mycelia means more mycelia and higher lovastatin yield. Rice husk was the worst substrate for cell growth and lovastatin accumulation due to its lowest nutriments content. When soybean cake was used as substrate, the relatively low productivity of lovastatin explained that higher protein content in soybean cake is not necessary and that starch content was more important for cell growth and lovastatin accumulation. In the following experiments, rice was used as substrate.

Fig.1 *Aspergillus terreus* ATCC 20542 growth on rice medium

Effect of rice particle size on lovastatin production

Before sterilization (cooking), rice was grounded and sieved. The effects of rice particle size on lovastatin production in the SSF process are shown in Fig.2.

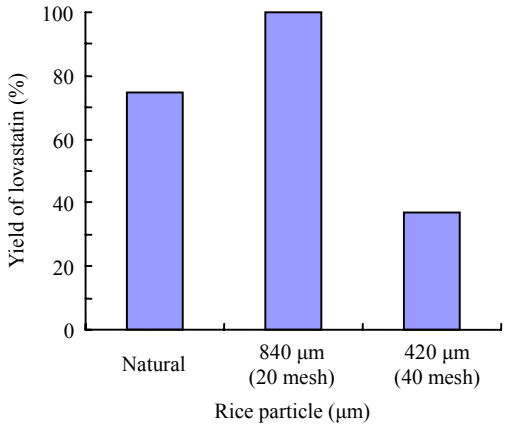

Fig.2 Effect of particle size on lovastatin production

The results indicated that the rice particle ground into 20 mesh (840 μm) was more suitable for lovastatin production than either natural rice grain or 40 mesh (420 μm) size. Generally speaking, smaller substrate particles will provide larger surface area for the attachment of microbes, which is favorable for

mycelia growth and product accumulation. However, if the substrate size is too small, it may result in substrate agglomeration after cooking in most cases, thus the reduction of inter-particle void space and the increase of oxygen mass transfer resistance are unfavorable for mycelia growth and lovastatin production.

Effect of additional nutrients on lovastatin production

The main composition of rice is starch. It must be hydrolyzed to glucose then can be metabolized by *A. terreus*. Although *A. terreus* can produce amylase to hydrolyze starch, in the beginning of the SSF process, it does not have enough amylase secreted by cells. Also rice is a poor nitrogen source with minimal protein content. In order to enhance cell growth and product accumulation, it is beneficial to supplement rapidly available carbon sources and nitrogen sources into the solid substrate. The effects of the additional carbon source (glucose) and/or nitrogen source (peptone) on the cell growth and lovastatin production were evaluated with the results being shown in Table 2.

Table 2 Effect of additional nutrients on lovastatin production

Additional nutrient	Yield of lovastatin (mg/g dry substrate)
Rice	2.2±0.085
Rice+5% glucose	2.4±0.037
Rice+0.5% peptone	2.7±0.065
Rice+0.5% peptone+5% glucose	2.9±0.049

Glucose can be directly utilized by microorganisms, therefore, the addition of glucose was favorable for the growth of mycelia in the beginning of the SSF process, and the lovastatin yield was increased slightly to 2.4 mg/g dry substrate. Because of low protein content in rice pudding, additional peptone improved the carbon/nitrogen ratio in the substrate, which promoted mycelia growth and lovastatin accumulation. The highest lovastatin yield was up to 2.9 mg/g dry substrate when both glucose and peptone were added into the solid substrate.

Production of lovastatin during fermentation cycle

The SSF process for lovastatin production with *A. terreus* at optimized parameters (50%~60% moisture level, pH 5.5, incubation temperature 28 °C, with addition of 5% glucose and 0.5% peptone) was studied. The cultivation lasted for a period of 15 d, with the results being shown in Figs.3 and 4.

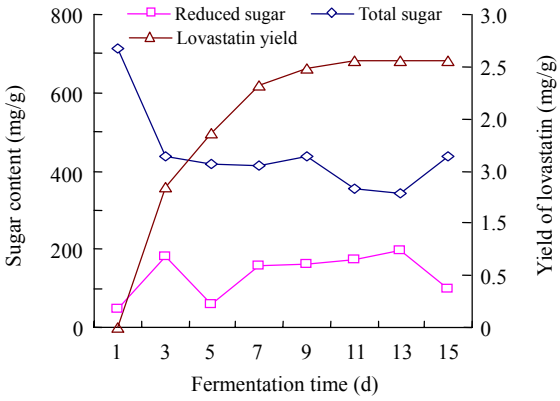

Fig.3 Time courses of total sugar, reduced sugar and lovastatin production during SSF process

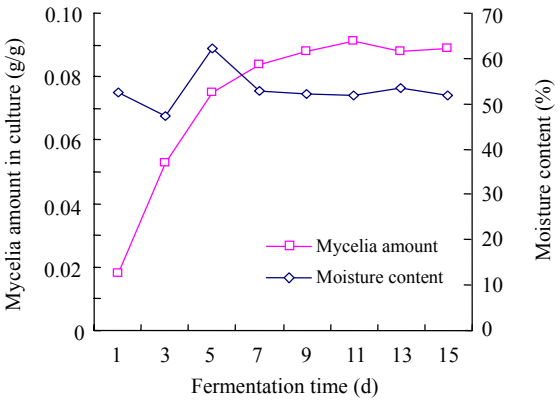

Fig.4 Time courses of mycelia amount and moisture content during SSF process

During the SSF process, the amount of total sugar decreased rapidly in the first five days, then kept stable in the following days. The amount of reduced sugar increased in the first three days because of the hydrolysis of starch by amylase secreted by fast growing fungi. After five days the reduced sugar was kept almost constant because of the dynamic balance between glucose formation and consumption.

During first six days, the *A. terreus* grew very fast, and the mycelia covered the rice substrate gradually. After then, the dried weight of mycelia was stabilized and slightly decreased in the final stage of the SSF process. During the whole process, the moisture content was kept almost constant, except in

the first few days, the fluctuation observed probably caused by mixing and sample taking.

The lovastatin yield increased fast from day 1 to day 5, which explained that although lovastatin is a kind of secondary metabolite, its accumulation in mycelia seems growth related, which is different with the phenomena in submerged fermentation. The reason should be explored further. The maximum lovastatin yield was achieved on day 11, after then, the lovastatin yield was almost unchanged. From the above results, it is obvious that the SSF can be ended at day 11. Because lovastatin is a kind of intracellular product, the product accumulation is almost simultaneously increased as cell growth.

CONCLUSION

In this work, rice and wheat bran were found to be suitable substrates for lovastatin production by *A. terreus* in the SSF process. The preferred substrate was rice grain ground into 20 mesh particles and supplemented with 5% glucose and 0.5% peptone. The optimized operation parameters for the SSF process were as follows: 50%~60% initial moisture content, pH 5.5, incubation temperature 28 °C and incubation time 11 d. Under the above conditions, the maximum lovastatin yield of 2.9 mg/g dry substrate was achieved. Compared with SmF by using the same strain conducted in our laboratory (Liu, 1997), in which the maximum yield was only about 2.3 mg/ml, it is clear that lovastatin productivity by the SSF process is equal to or even better than that by the SmF process.

Comparison with literature data showed that the lovastatin productivity in SSF process via *A. terreus* ATCC 20542 is lower than that via *A. flavipes* BICC 5174 (Valera *et al*., 2005) and similar to that via *M. rubber* (Xu *et al*., 2005), whereas the fermentation period is shorter than that by *M. rubber*.

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