

Evaluation of rice straw for purification of lovastatin

WEN-TA SU AND CHIN-CHUAN YI

ABSTRACT: Cholesterol synthesis in the human body can be catalyzed by the coenzyme HMG-CoA reductase, and lovastatin, a key enzyme inhibitor, can reduce hypercholesterolemia. Lovastatin can be obtained as a secondary metabolite of *Aspergillus terreus* ATCC 20542. In this study, rice straw of lignocellulose was used in aeration and agitation bath fermentation in a 1-L flask, and a maximal crude extraction rate of 473 mg/L lovastatin was obtained. The crude extract was treated with silica gel (230–400 mesh) column chromatography. Ethyl acetate/ethanol (95%) was used as the mobile phase, and isolation was performed through elution with various ethyl acetate/ethanol ratios. The highest production rate of 153 mg/L was achieved with ethyl acetate/ethanol in a ratio of 8:2. The lovastatin gained from the crude extract was added to 12 fractions treated with 0.001 N alkali, and acetone was then added. After 24 h of recrystallization at 4°C, the extract underwent high-performance liquid chromatography. The purity had increased from 25% to 84.6%, and the recovery rate was 65.2%.

Application: This study devised a process for producing lovastatin in its pure form from a culture of *Aspergillus terreus* ATCC 20542, and a new extraction procedure was designed for the isolation of lovastatin.

Rice straw is a major lignocellulosic residue produced worldwide, with an estimated availability of 685 million tons/year [1]. It has been a major agricultural byproduct in Asia, and researchers have shown an increasing interest in its liquid fermentation because agricultural residue such as rice straw is recognized as a type of economical substrate [2]. Thus, rice straw has a high content of lignin (5%–24%), cellulose (32%–47%), and hemicellulose (19%–27%), rendering it an attractive substrate for hydrolysis into simple sugars [3].

Lovastatin, a member of the drug class statins, is used for lowering cholesterol to prevent hypercholesterolemia. This drug is a potent inhibitor of HMG-CoA reductase, the rate-controlling enzyme in the cholesterol biosynthesis process [4–8]. Lovastatin is also a fungal metabolite produced during fermentation by filamentous fungal strains such as *Penicillium citrinum* [9], *Monascus ruber* [10], and *Aspergillus terreus* [11–13]. Among the statins, lovastatin was the first approved for hypocholesterolemia by the U.S. Food and Drug Administration [14,15].

Lovastatin is commercially produced mainly through submerged fermentation of *A. terreus* [16,17], and processes employing this method have been developed for large-scale lovastatin production [18]. High purity is vital for the manufacture of a safe and effective drug. Thus, recent research on secondary metabolites has focused on isolation and purification techniques such as liquid–liquid extraction, solid–liquid extraction, organic solvent extraction, membrane filtration, and column chromatography [19–22]. The recognized methods for isolating lovastatin from fermentation broth cannot reach a pharmaceutically accept-

able level of purity economically and on an industrial scale, or they require chromatographic isolation to achieve high purity [23].

Chromatographic purification can be achieved through adsorption chromatography using silica gel column chromatography or high-performance liquid chromatography (HPLC; **Fig. 1**). Each chromatography process has its own advantages and disadvantages, but adsorption chromatography using silica gel columns is widely used because of its simplicity and high purity [24]. Crystals are solid materials with high purity, precipitated from a pure substance of a homogeneous phase [25–30], and recrystallization, which can achieve even higher purity, was adopted in the study [31,32]. This study devised a process for producing lovastatin in its pure form from a culture of *A. terreus* ATCC 20542, and a new extraction procedure was designed for the isolation of lovastatin.

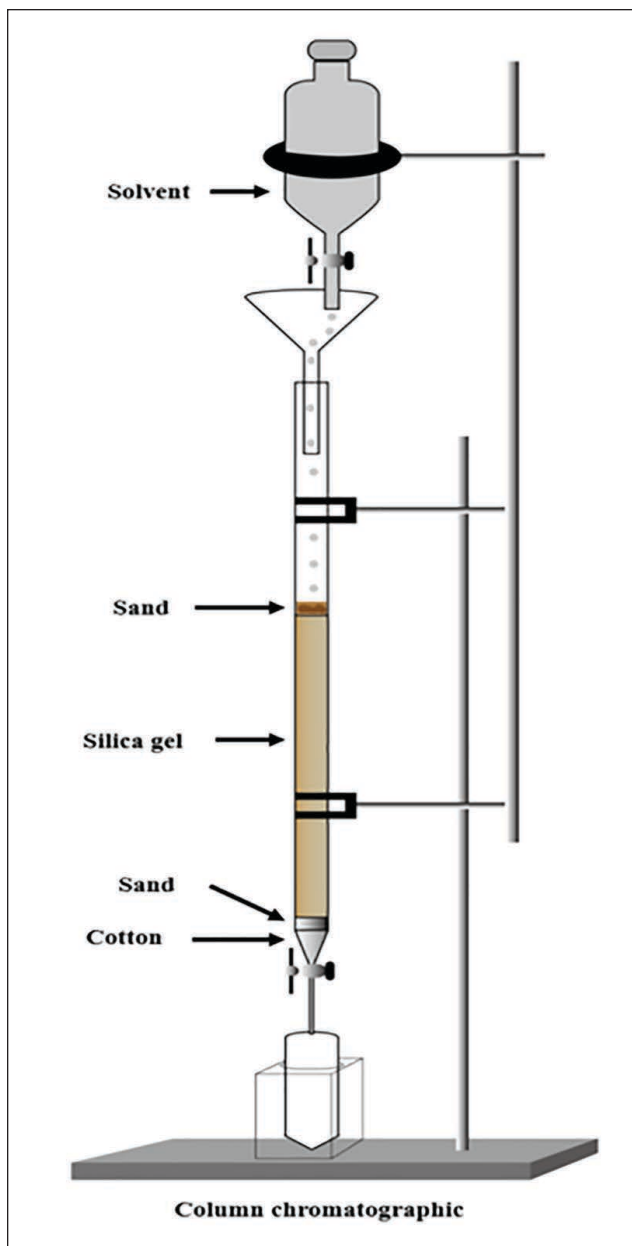
MATERIALS AND METHODS

Microorganisms, media, and culture conditions

A strain of *A. terreus* ATCC 20542 was grown on potato dextrose agar slants at 26°C. An initial spore concentration of 107 spores/mL was added to a 500-mL shake flask containing 100 mL of liquid medium, and the mixture was incubated for 2 days at 28°C, 180 rpm, and pH 6.5 in an oscillating incubator to serve as a source of inoculum for the liquid culture, with a two-stage fermentation culture and seed culture.

Inoculum preparation

The medium in the first stage of the fermentation process



1. Column chromatography.

comprised glucose (30 g/L), malt extract (5 g/L), yeast (5 g/L), peptone (1 g/L), K_2HPO_4 (1 mg/L), KH_2PO_4 (1 mg/L), NH_4SO_4 (1 mg/L), $FeSO_4 \cdot 7H_2O$ (1 mg/L), $CaCl_2 \cdot 2H_2O$ (1 mg/L), $MgSO_4 \cdot 7H_2O$ (1 mg/L), and citric acid (1 mg/L). The fermentation conditions for 2 days of seed culture were set as follows: pH 6.5, 180 rpm, and 28°C.

The medium in the second stage of the fermentation process comprised lactose (20 g/L), malt extract (5 g/L), yeast (5 g/L), peptone (1 g/L), K_2HPO_4 (1 mg/L), KH_2PO_4 (1 mg/L), NH_4SO_4 (1 mg/L), $FeSO_4 \cdot 7H_2O$ (1 mg/L), $CaCl_2 \cdot 2H_2O$ (1 mg/L), $MgSO_4 \cdot 7H_2O$ (1 mg/L), and citric acid (1 mg/L). The fermentation conditions for 7 days of culture were set as follows: pH 6.5, 180 rpm, and 28°C.

Measurement of dry cell weight, residual sugar, and pH

The fermentation broth was sampled daily. It was baked at 50°C for 24 h for the measurement of dry cell weight for 7 consecutive days. The pH value was measured daily for 7 days using a pH meter, and the residual sugar concentration was determined using the phenol-sulfuric acid method.

Analysis of lovastatin

HPLC was used to determine the amount of lovastatin in the samples of the fermentation broth. The C18-column HPLC with a diode array detector, acetonitrile, and water acidified with 1.1% phosphoric acid (60:40 v/v) was performed. The flow rate was set at 1 mL/min. A sample volume (20 μ L) was taken, and ultraviolet detection was carried out in the mobile phase. The ultraviolet detection wavelength at 28°C was 238 nm.

Acid treatment of pretreated rice straw

Washed rice straw was cut into 2.5 cm sections, mixed with 1% sulfuric acid (H_2SO_4), and left in an autoclave at a constant temperature of 30°C for 24 h. Next, the sections were taken out and washed in water until neutral. Finally, the sections were dried in an oven at 50°C to a constant weight, crushed in a pulverizer, and sieved using a 60-mesh sifter.

Fermenter operations

Fermenter operation proceeded as follows: 1% acid-treated rice straw culture was added to aerated and agitated fermentation broth with *A. terreus* ATCC 20542 in a 1-L flask for 7 days, with the settings of 6.5 pH, 200 rpm, and 28°C.

Isolation of lovastatin from the fermentation broth

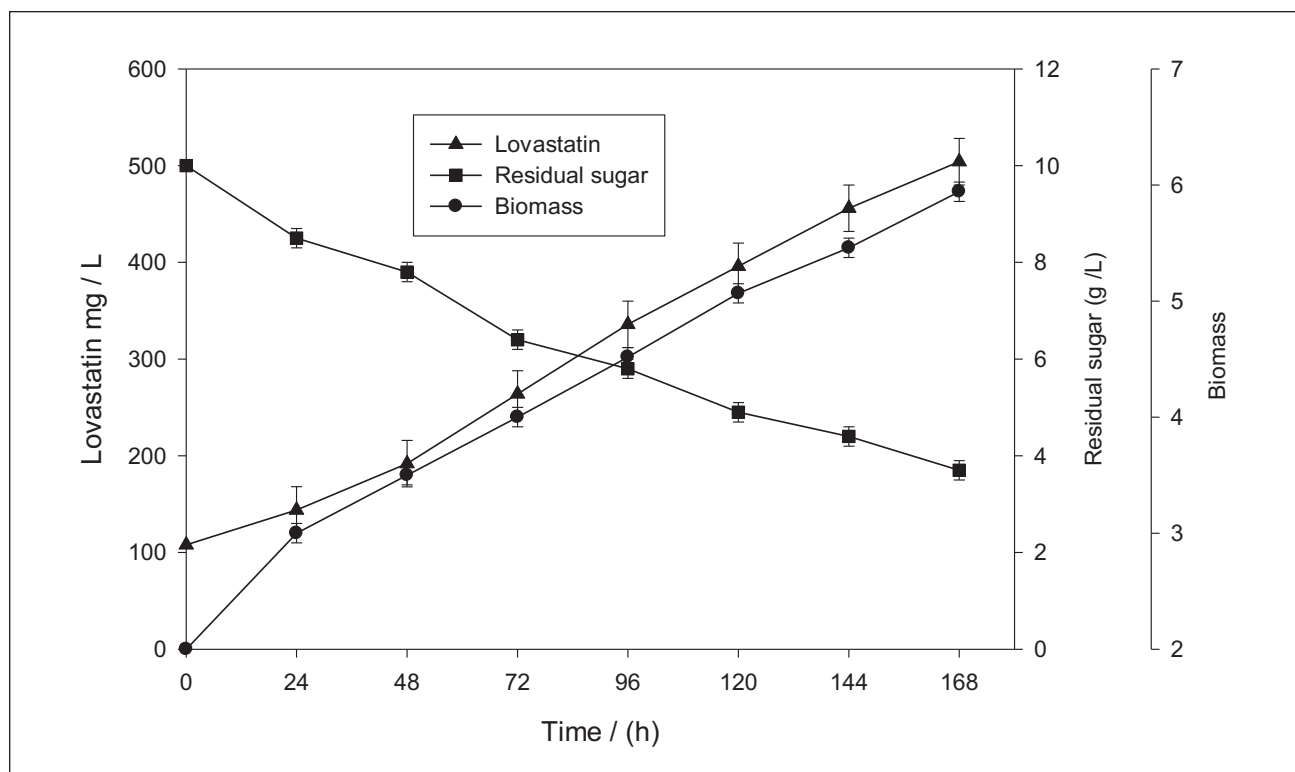
The fermentation broth was freeze-dried and operated in the liquid for column chromatography at a flow rate of 1:1 v/v. Ethyl acetate/ethanol (90%) was used as the mobile phase, and the elution buffer was separated using ethyl acetate/ethanol at the following ratios: 50%, 40%, 30%, 20%, and 10%.

Chromatographic purification of lovastatin

The lovastatin content was determined through column chromatography using ethyl acetate/ethanol at a ratio of 8:2 as the elution. For Fractions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12, every 25 mL was collected in a tube. All the tubes were centrifugally concentrated and baked at 50°C. After the baked contents were dissolved in methanol, they were filtered through a mesh of 0.45- μ m pores, and HPLC was conducted.

Recrystallization of lovastatin

Column chromatography was used to isolate and purify the lovastatin produced by the fermentation broth. At an



2. The time courses of mycelial growth and the production of lovastatin in the 1% acid-treated rice straw culture in aerated and agitated fermentation broth with *A. terreus* ATCC 20542 in 1-L flask for 7 days.

elution ratio (ethyl acetate/ethanol), Fractions 1–12 in tubes of 20 mL were collected and concentrated by centrifugation for 1 h 20 min at 1350 rpm and 65 °C. After centrifugation and filtration, the samples, with alkali added, were stored at 4°C, slowly crystallized, reassembled and rearranged in the refrigerator, and dried in the oven for 24 h for reconstitution for HPLC analysis. The fraction treated with 0.001 N alkali and acetone (or other organic solvents) was added, crystallized at -4°C for 24 h, and filtered for HPLC analysis.

Statistical analysis

All experiments were performed with three different samples. The data are expressed as means $\pm$ standard deviations calculated through analysis of variance. In statistical comparisons, *p* values <0.05 were considered significant.

RESULTS AND DISCUSSION

Shake flask experiments

Fermenter operation proceeded as follows: 1% acid-treated rice straw culture was added to aerated and agitated fermentation broth with *A. terreus* ATCC 20542 in a 1-L flask for 7 days, with the settings of 6.5 pH, 200 rpm, and 28°C.

By hour 168, the biomass of the fermentation broth had reached 5.9 g/L, reducing sugar had reached 3.8 g/L, and the crude lovastatin extract produced had reached 473.2 mg/L, as shown in **Fig. 2**; this extract was used for further isolation and purification.

Effect of ethyl acetate/ethanol in different ratios on lovastatin

Ethyl acetate/ethanol (95%) acted as the mobile phase for isolation using eluents of ethanol at 50%, 40%, 30%, 20%, and 10%. The lovastatin production reached 153 mg/L when an ethyl acetate/ethanol ratio of 8:2 was used, as shown in **Fig. 3**.

Effect of ethyl acetate/ethanol ratio of 8:2 on lovastatin

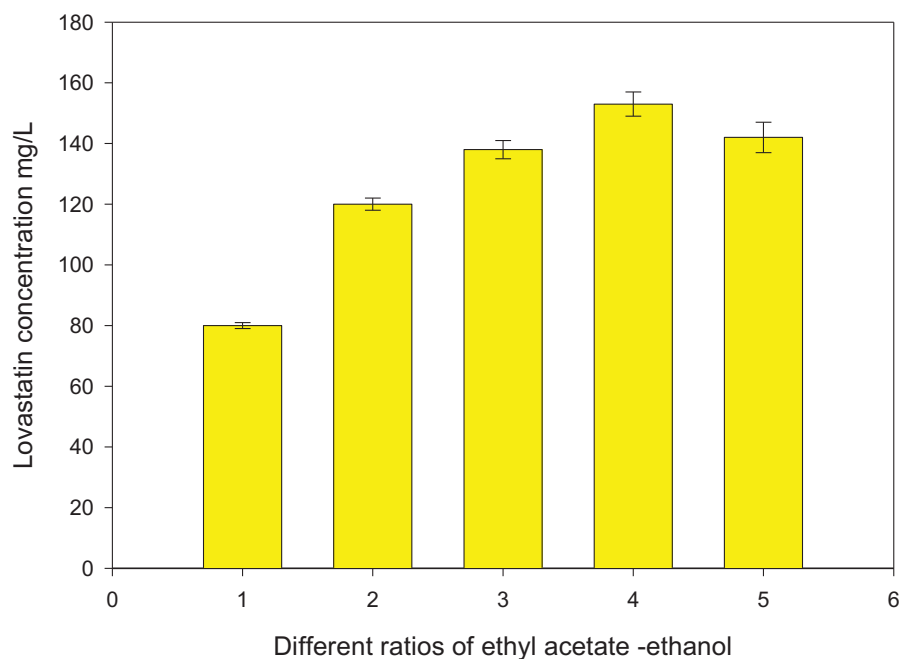
For column chromatography, an elution of ethyl acetate and ethanol at a ratio of 8:2 was used. Fractions 1–12 were collected in 25-mL tubes and concentrated through centrifugation at 50°C for drying in methanol and reconstituting 0.45 μ m filtration for HPLC analysis. The 12 fractions of lovastatin 125 mg/L eluted were purer, as shown in **Fig. 4**.

Effect of HPLC on crude extract of lovastatin obtained from fermentation broth

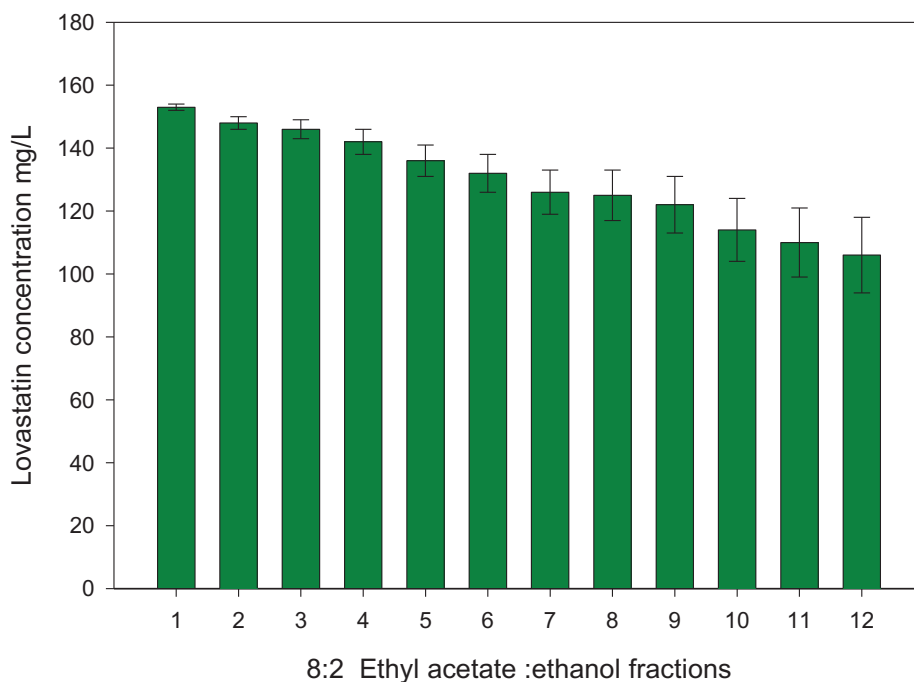
HPLC was conducted on the 473 mg/L crude lovastatin extract gained from the fermentation broth. The results indicated that its purity was 25%, as shown in **Fig. 5**.

Effects of different organic solvents on the purity and recovery rate of lovastatin

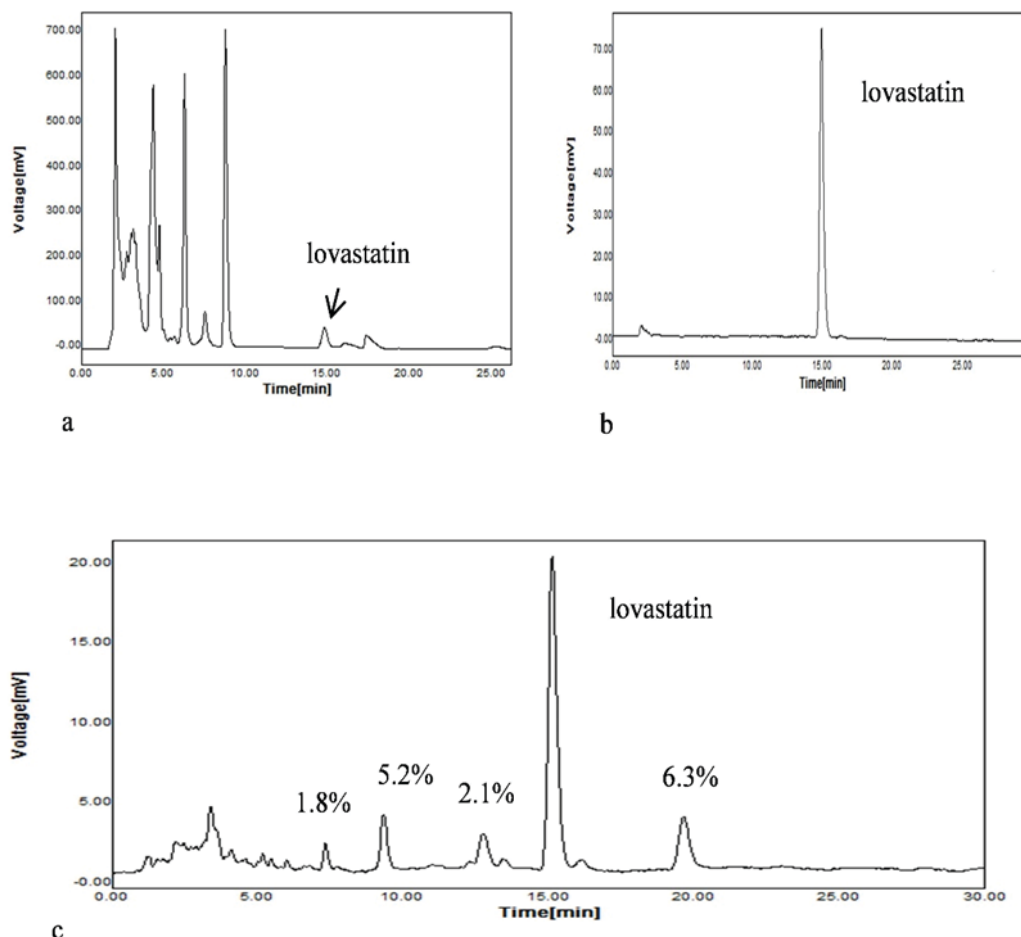
The crude extract of lovastatin was mixed with 12 0.001 N alkali-treated fractions and dried and reconstituted for HPLC analysis by removing the carboxyl group. The extract



3. For conducting column chromatography, ethyl acetate/ethanol (95%) acted as the mobile phase for isolation using eluents, such as at 50%, 40%, 30%, 20%, and 10% of ethanol.



4. For column chromatography, elution was made with an 8:2 ratio of ethyl acetate to ethanol. Fractions 1-12 were collected in tubes of 25 ml each and concentrated by centrifugation at 50°C for drying methanol and reconstituting 0.45 μ filtration for high-performance liquid chromatography (HPLC) analysis.



5. The HPLC profile of lovastatin: a) HPLC analysis of the crude extract of lovastatin; b) the standard spectrum of lovastatin was detected at 238 nm; and c) the HPLC results of the recrystallization of lovastatin.

No./Solvent	Purity of Lovastatin	Recovery Rate, %
1. ethanol	58.7	44.5
2. toluene	65.2	58.5
3. acetone	84.6	65.2

I. Effects of different organic solvents on the purity and recovery rate of lovastatin. Purity is the high-performance liquid chromatography (HPLC) peak to lovastatin standard. Recovery rate is lovastatin recovered after liquid-phase extraction relative to the original crude lovastatin extract.

was then placed in the refrigerator at -4°C . After crystallization, various solvents (ethanol, toluene, and acetone) were added, and the extract was reconstituted. The purity obtained was 58.7% with alcohol, 65.2% with toluene, and 84.6% with acetone. The recovery rate was 44.5% with alcohol, 58.5% with toluene, and 65.2% with acetone, as shown in **Table I** and Fig. 5.

CONCLUSIONS

In this study, ethyl acetate/ethanol (95%) was adopted as the mobile phase, and different ratios were used to isolate lovastatin. The optimal ratio, which produced 153 mg/L lovastatin, was 8:2 ethyl acetate/ethanol.

The crude extract of lovastatin was mixed with 12 0.001 N alkali-treated fractions and acetone, and it was left for 24 h for recrystallization. The HPLC revealed that the purity of lovastatin improved from 25% to 84.6%, and the recovery rate was 65.2%. **TJ**

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ABOUT THE AUTHORS

Rice straw has been a major agricultural byproduct, and lovastatin is a secondary metabolite obtained from the fermentation of the *Aspergillus terreus* ATCC 20542. Chemical treatment can be divided into two methods: dilute acid hydrolysis to remove lignin and reduce the crystallinity of cellulose, and use of dilute sulfuric acid to destroy cellulose.

Lovastatin is used to reduce blood cholesterol or certain blood lipid substances. The lovastatin content in this study was determined through column chromatography using ethyl acetate/ethanol at a ratio of 8:2 as the elution at which high-performance liquid chromatography was conducted. The present study concludes that the biotransformation of lignocellulose produced lovastatin with *Aspergillus terreus* ATCC 20542, and a new extraction procedure was designed for the purification of lovastatin.

The most difficult aspect of this research was determining the proportion of medicament for the column chromatography. To address this, we studied journals and experiments. We found that lignocellu-

lose from agricultural waste substances of rice straw is a very good carrier. It was interesting to find that lignocellulose and bacteria can be used to produce pharmaceuticals. The next step in this research is mass production of pharmaceuticals in reactors using lignocellulose and fungi.



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