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Original Article

Statistical optimization of lovastatin and confirmation of nonexistence of citrinin under solid-state fermentation by *Monascus sanguineus*

Rashmi Dikshit, Padmavathi Tallapragada *

Department of Microbiology, Centre for PG Studies, Jain University, Jayanagar, Bangalore 560011, Karnataka, India

ARTICLE INFO

Article history:
Received 6 February 2015
Accepted 5 November 2015
Available online 4 January 2016

Keywords:
citrinin
Fourier transfer infrared spectrometry
Monascus species
optimization
plasma cholesterol
statins

ABSTRACT

Lovastatin is a well-known natural statin, which is used for lowering plasma cholesterol levels by inhibiting 3-hydroxy-3-methyl glutaryl coenzyme A reductase. Different strains of *Aspergillus* and *Monascus* sp. have been exploited for statin production but *Monascus sanguineus* is still unexplored. In this study, lovastatin production from *Monascus sanguineus* under solid state fermentation was optimized using response surface methodology. The optimized value of the lovastatin yield was 20.04 mg/gds with soybean concentration of 20 g/L, $CaCl_2$ concentration of 2.5 g/L, acetic acid concentration of 25 μL and inoculum size of 3.4 mL. This study also documented spectrometric characterization and fragment pattern of lovastatin with the help of Fourier transfer infrared spectrometry and mass spectrometry. Citrinin was not detected in any of the samples used for this study.

1. Introduction

Coronary heart disease and most heart attacks are due to hypercholesterolemia, which is the accumulation of cholesterol in blood plasma that results in atherosclerosis (blockage of arteries). Almost one-third of the total body cholesterol comes from food intake and the other two-thirds is synthesised in the body [1]. A potent hypocholesterolemic agent, known as lovastatin (mevinolin and monacolin K) can inhibit the rate-limiting enzyme 3-hydroxy-3-methyl glutaryl coenzyme A reductase. This enzyme has a regulatory and rate-limiting function in cholesterol biosynthesis [2]. Lovastatin was the first natural statin; it is a fungal secondary metabolite and was approved by the US Food and Drug Administration in August 1987 [3,4]. The microorganisms used for statin production under solid-state fermentation (SSF) conditions mostly belong to *Aspergillus* and *Monascus* sp. There are also a few reports of statin production in rice fermented by *Penicillium* and *Monascus* sp., which is also called Angkak. It is known to contain several valuable secondary metabolites such as lovastatin, γ-aminobutyric acids, monascodilone, monascorubramine, monascin, ankaflavin, rubropunctatin and citrinin [5–7].

* *Corresponding author*. Department of Microbiology, Centre for PG Studies, Jain University, 18/3, 9th Main road, 3rd Block, Jayanagar, Bangalore 560011, Karnataka, India.
E-mail addresses: blrsn@rediffmail.com, vam2010tpraviju@gmail.com (P. Tallapragada).
http://dx.doi.org/10.1016/j.jfda.2015.11.008

Citrinin is a mycotoxin with nephrotoxic, hepatotoxic and carcinogenic activities. *Penicillium citrinum*, *Aspergillus* sp. and *Monascus* sp. along with other secondary metabolites are known to produce citrinin, which greatly limits the application of the *Monascus* fermented products. Due to a potential risk to livestock and human health, extensive research on citrinin biosynthesis is being conducted. Comprehensive details are not known about the biological background of citrinin biosynthesis [8].

Recent studies have mainly focused on the production of microbial secondary metabolites using SSF approaches. The development of good-quality fermenters along with temperature, humidity, aeration, and sterility control has accelerated research using SSF. However, research should also explore new strains for improved statin production [9]. Mass spectrometry (MS) with electrospray ionization is considered an expanding approach for the structural elucidation of organic species. It can provide molecular mass information along with fragment pattern. It is widely used for the identification of impurities and depredates in the pharmaceutical industry [10].

Response surface methodology (RSM) can be used to assess the relative significance of several affecting factors. It is an empirical statistical modeling technique used for multiple regression analysis using quantitative data. These data are obtained from appropriately designed experiments required to solve multivariable equations simultaneously. RSM is being increasingly used for optimization of the process in fermentation [11].

The purpose of present study was to determine suitable conditions for scale-up of lovastatin production, verification of the absence of citrinin, and spectrometric characterization of lovastatin. The experiments were carried out under SSF with *Monascus sanguineus* in order to achieve optimum SI yield. Characterization of lovastatin was done with UV spectrophotometry and Fourier transform infrared (FT-IR) techniques. Liquid chromatography (LC)-MS was used to study fragment ions and mass using an electrospray ionization approach.

2. Methods

2.1. Culture and inoculum preparation

Pomegranate (*Punica granatum*) was used to isolate the wild strain of *Monascus*, which was identified as *M. sanguineus*. The spores were scraped off under aseptic conditions to produce a spore suspension that was prepared in 0.9% saline water [12].

2.2. SSF

Ten grams of wheat bran as a substrate were taken and placed in a 250-mL conical flask to which 30 mL of basal medium was added. The basal medium composition included 100 g dextrose, 10 g peptone, 2 g KNO_3, 2 g NH_4 H_2 PO_4, 0.5 g $MgSO_4.7H_2O$, and 0.1 g in 1 L distilled water. The pH of the medium was adjusted to 6.0 [13].

2.3. Extraction of lovastatin

Acetonitrile was used as extraction solvent. One gram of fermented dry substrate was dissolved in 20 mL acetonitrile and kept in a shaker incubator for 2 hours at 180 rpm and 70°C. It was then filtered with Whatman filter paper (Sigma Aldrich, USA) and the filtrate was centrifuged at 3000 × *g* for 8 minutes [14].

2.4. Screening of citrinin

Fermented dry substrates were dissolved in ethyl acetate and acetified up to pH 5. The aqueous layer was removed and the organic layer was concentrated. Ten microliters of these samples was applied to Silica gel 60 F254 Aluminum sheets (Merck, Germany). Ethyl acetate: acetone: water (4: 4: 1, by volume) was used as the mobile phase. Subsequently, the drying plates were examined under UV light at 350 nm to observe fluorescent yellow bands [15].

2.5. Estimation of lovastatin by UV spectrophotometry

Lovastatin was purified from the sample with the help of thin layer chromatography (TLC). Purified spots from TLC were scraped and transferred into glass tubes and acetonitrile was added. The tubes were centrifuged, filtered, and the filtrate was estimated at 238 nm using UV-visible spectrophotometry. Lovastatin was estimated in its acid form (mevinolinic acid) [16].

2.6. Identification of lovastatin by FT-IR and LC-MS

For FT-IR analysis, the sample was kept in vacuum desiccators over solid KOH for 48 hours and IR analysis was carried out using Thermo-Nicolet 6700 Fourier Transfer Infrared spectrometer. LC-MS analysis was carried using a 250 mm × 4.6 mm internal diameter Lichrosper 100 C18 column of particle size 5 μm, loop injector of 20 μL, and Shimadzu CLASS-VP version 5.032 software (Shimadzu, Japan). The mobile phase used for this analysis was acetonitrile: water (65: 35 v/v and pH 3.5). The flow rate was set to 1.0 mL/min and the detection was carried out using a wavelength of 235 nm by UV detector SPD10A VP (Shimadzu Europe, Germany) [17].

2.7. Experimental design (RSM)

The experimental design was formulated according to the central composite design of RSM using MATLAB software for four selected parameters: soya bean meal, $CaCl_2$, acetic acid and inoculum size (Table 1). A set of 30 experiments was required with each variable being at five levels. All the flasks

Table 1 – Four variables in coded and natural units.

Variables with designate	Code	Actual factor level at coded factor levels of				
		−2	−1	0	1	2
Soybean meal (g/L)	X1	4	8	12	16	20
$CaCl_2$ (g/L)	X2	0.5	1	1.5	2	2.5
Acetic acid %(v/v)	X3	0	0.025	0.05	0.075	0.1
Inoculum size % (v/v)	X4	5	8	11	14	17