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Statistical optimization of anticholesterolemic drug lovastatin production by the red mold *Monascus purpureus*

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ABSTRACT

Lovastatin (HMG-CoA reductase inhibitors), is an important anticholesterolemic drug which inhibits the conversion of HMG-CoA to mevalonate in the biosynthesis of cholesterol. Plackett–Burman statistical screening of 12 media components and subsequent optimization of significant parameters by response surface methodology for the biotechnological production of lovastatin by *Monascus purpureus* MTCC 369 was studied. In this study, the statistical analysis of Plackett–Burman experimental results showed that the medium components glucose, peptone, $MnSO_4{\cdot}H_2O$, NaCl and NH_4Cl as the significant components influencing the lovastatin production. The most significant medium components, glucose, peptone and $MnSO_4{\cdot}H_2O$ which have confidence level of more than 95% were further optimized using a full factorial central composite design of the response surface methodology. Maximum lovastatin production of 97.5 mg l^{-1} was obtained after 14 days of fermentation period in the optimized medium containing, glucose, 52.61 g l^{-1} peptone, 16.65 g l^{-1}; NH_4Cl, 1 g l^{-1}; KH_2PO_4, 1 g l^{-1}; yeast extract, 3 g l^{-1}; K_2HPO_4, 1 g l^{-1}; KNO_3, 0.5 g l^{-1}; $MgSO_4{\cdot}7H_2O$, 0.2 g l^{-1}; $MnSO_4{\cdot}H_2O$, 0.418 g l^{-1}; NaCl, 0.5 g l^{-1}; $CaCl_2{\cdot}2H_2O$, 0.1 g l^{-1} and $FeSO_4{\cdot}7H_2O$, 0.001 g l^{-1} at 30 °C and 120 rpm. The production of lovastatin by *M. purpureus* MTCC 369 in the optimized medium was found to be four times higher than the basal medium in the submerged fermentation. The statistical experimental design serves as an efficient tool for screening large number of variables with minimum number of experiments and optimizing the significant variables for enhancing the production of lovastatin.

Keywords: Lovastatin; *Monascus purpureus*; Submerged fermentation; Plackett–Burman design; Response surface methodology

1. Introduction

Lovastatin, a specific and potent competitive inhibitor of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) is a powerful serum cholesterol-lowering drug in humans and other species (Alberts, 1988). It is formerly called as mevinolin; monacolin K, and mevacor® and it is a fungal secondary metabolite which inhibits HMG-CoA reductase (E.C 1.1.1.34), the first committed enzyme of cholesterol biosynthesis (Manzoni et al., 1998). The endogenous synthesis of cholesterol is carried out by the mevalonate pathway, in which the rate limiting reaction is the conversion of (S) HMG-CoA to (R) mevalonate, catalyzed by HMG-CoA reductase. The history of statin began in 1987 when the lovastatin received Food and Drug Administration (FDA) approval in the USA (Manzoni and Rollini, 2002). Mevastatin was the first statin to be reported as a fungal secondary metabolite followed by lovastatin (Endo, 1979). *Monascus ruber* produces an active methylated form of compactin known as monacolin K (lovastatin; mevinolin) in liquid fermentation (Endo, 1979). Lovastatin have revolutionized the treatment of hypercholesterolemia and it is proven that lovastatin is also therapeutically and preventatively effective in the treatment of major kind of diseases like atherosclerosis, sepsis, peripheral arterial disease, peripheral vascular disease, cerebro vascular disease, ischemic disease, and bone fracture.

Lovastatin is produced by a variety of filamentous fungi and some of the industrially important microbial sources for

Abbreviations: CCD, central composite design; PB, Plackett–Burman; RSM, response surface methodology; SE, standard error.

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Nomenclature

x_i	coded value of an independent variable
M_i^+	lovastatin production at high x_i concentrations
M_i^-	lovastatin production at low x_i concentrations
V_{eff}	variance of the concentration effect
E_d	concentration effect for the dummy variable
N	the number of trials
p	significance level
k	number of variables
X_i	real value of an independent variable
X_0	real value of an independent variable at the center point
R^2	coefficient of determination
ΔX_i	step change value
Y	predicted response (lovastatin production)
β_0	offset term
β_i	coefficient of linear effect
β_{ii}	coefficient of squared effect
β_{ij}	coefficient of interaction effect

the production of lovastatin are *Monascus* sp., *Aspergillus* sp. and *Pencillium* sp. In particular, *Monascus purpureus*, *M. ruber*, *Monascus pilosus*, *Monascus vitreus* and *Monascus pubigerus* were found to be the most significant producers of lovastatin (Negishi et al., 1986). The *Monascus* species is a Chinese traditional fermentation fungus used on food for over thousands of years and has also been considered an essential part of wine making and other fermented food products. Red mold rice which contains a large amount of γ-aminobutyric acid and possesses anti-hypertensive effects for humans is obtained usually by cultivation of *Monascus* species on the rice grain. *Monascus* species also produces pigments like: rubropunctatin (red color), monascorubrin (red color), monascin (yellow color), ankaflavin (yellow color), rubropunctamine (purple color) and monascorubramine (purple color) which is widely used to replace synthetic food dyes by natural colorants (Manzoni et al., 1998; Chang et al., 2002). There are different types of statins currently available, which can be classified broadly into natural statins (obtained directly by fermentation), semisynthetic and synthetic. Lovastatin and pravastatin are natural statins, while simvastatin is a semisynthetic and atorvastatin and fluvastatin are synthetic statins (Manzoni and Rollini, 2002).

The culture medium has a significant influence on any fermentation product and plays a vital role in the production of lovastatin. Conventional method of medium optimization involves changing one independent variable at a time while keeping the others at fixed level is time consuming and may lead to misinterpretation of results. However, statistical method offers several advantages over conventional methods being rapid and reliable, short lists significant nutrients, helps understanding the interactions among the nutrients of various concentrations and reduces the total number of experiments. As the number of nutrients to be screened and optimized is greater in fermentation processes; experiments based on multi-factorial design will be difficult. Plackett–Burman (PB) design, an effective technique for the nutrient component optimization screens the components which significantly influence the production and eliminate the insignificant components in order to obtain a smaller, more manageable set of factors.

Response surface methodology (RSM), one of the global optimization methods, has become more attractive in the process optimization due to its non-requirement on the calculation of the local sensitivity of each design variable and being effective for both the single- and multi-disciplinary optimization problems. RSM is a collection of statistical and mathematical techniques useful for developing; improving and optimizing processes. It also has important applications in designs, development and formulation of new products, as well as improvement of existing product designs (Myers and Montgomery, 1995). RSM has been successfully utilized to optimize the compositions of microbiological media (Oh et al., 1995), improving fermentation process (Lee and Chen, 1997) and product development (Gomes and Malcata, 1998). Although response surface methodology is widely used for other processes, there are only few examples in the literature involving RSM for the production of lovastatin using *M. purpureus* (Gomes and Malcata, 1998; Xin et al., 2005; Sayyad et al., 2007). The objective of the present study is to screen the nutritional parameters using PB two-level factorial design and to optimize the significant parameters using RSM to maximize lovastatin production by *M. purpureus* efficiently.

2. Materials and methods

2.1. Culture maintenance and microorganism

The fungal culture of *M. purpureus* MTCC 369 was obtained from the Institute of Microbial Technology, Chandigarh, India. The culture was maintained on the PDA slants at 4 °C, and subcultured every 30 days.

2.2. Culture conditions and inoculum preparation

Actively growing slants were taken and spore suspension of *M. purpureus* MTCC 369 was prepared using sterile water. 10% spore suspension was inoculated to conical flasks containing the basal medium: 100 g dextrose, 10 g peptone, 2 g KNO_3, 2 g $NH_4H_2PO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$ and 0.1 g $CaCl_2$ in 1000 ml of distilled water, adjusted to pH 6.0. These cultures were incubated at 30 °C for 48 h in a shaking incubator at 120 rpm. All the chemicals used were of analytical grade and obtained from Hi-media Laboratories Limited (Mumbai, India).

2.3. Submerged fermentation

10 ml of the inoculum was transferred to 250 ml Erlenmeyer flasks containing 100 ml of different production medium as per the statistical experimental designs. The fermentation period, temperature, initial pH of the fermentation medium and speed of agitation was maintained constant at 14 days, 30 °C, pH 6 and 120 rpm respectively for all the fermentation runs. The initial pH of media was adjusted to pH 6 and the production media was sterilized by autoclaving at 121 °C for 20 min. Since lovastatin is a secondary metabolite, samples were collected after 8 days for every 24 h of fermentation period till 14 days.

2.4. Extraction of lovastatin

After fermentation, the harvested broth was homogenized well to get the intracellular product. An equal volume of methanol was added and the suspension was shaken for 1 h on a rotary shaker at 200 rpm and 30 °C. The suspension was then