Original Article

Applied Food Biotechnology

APPLIED FOOD BIOTECHNOLOGY, 2015, 2(4): 21-26
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

Optimization of Monacolin Production in a Controlled System

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Abstract

Monascus purpureus is a fungus that had been cultured on the rice in eastern Asian countries since thousands years ago and used as a food for long years. The fungus produces very valuable metabolites with polyketide structure. The most important metabolite is Monacolin K, lovastatin or competitive inhibitor of 3-Hydroxy-3-Methylglutaryl-Coenzyme A reductase (an affective enzyme in cholesterol synthesis). This metabolite has various properties including reducing blood cholesterol, preventing infection, and treatment of progressive renal disease, a variety of tumors, vascular diseases and bone fractures. In this study, *Monascus purpureus* PTCC5303 has been used for lovastatin production in liquid fermentation. The nutritional concentrations that were significant in biomass and lovastatin production including maltose and $MgSO_4$ were optimized by Response Surface Methodology in a mili-bioreactor. The optimum concentration of maltose and $MgSO_4$ was obtained as 10 gl^{-1} and 0.78 gl^{-1}, respectively. According to our results, maximum lovastatin production under optimum conditions including maltose 10 gl^{-1}, peptone 5 gl^{-1}, $MgSO_4.7H_2O$ 0.78 gl^{-1}, $MnSO_4.H_2O$ 0.5 gl^{-1}, KH_2PO_4 4 gl^{-1}, thiamine 0.1 gl^{-1}, and pH=7 at 30°C, 130 rpm and flow rate 1.8 l min^{-1} was obtained to be 309 µg l^{-1} after 10 days of fermentation period.

Article Information

Article history:
Received 5 Jul 2015
Revised 25 Jul 2015
Accepted 19 Aug 2015

Keywords:
Bioreactor
Lovastatin
Monascus purpureus
Optimization
Oxygen Transfer Rate

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1. Introduction

Cardiovascular diseases (CVDs), particularly coronary heart disease (CHD), are among the major causes of mortality in the developed countries [1, 2]. Atherosclerosis is the underlying disorder in the majority of patients presenting CVDs. It is displayed when fat, cellular waste products, calcium, and other substances, particularly cholesterol, are deposited inside the artery, gradually reducing the inside diameter of the artery. This may cause blockage of large and medium sized arteries [1,3]. Numerous clinical and epidemiological trials have shown the efficacy of lowering cholesterol for reducing CHD risk. Therefore, for prevention of atherosclerosis blood cholesterol level should be controlled [4]. Endo [5] extracted monacolin K (also known as Mevinolin, lovastatin or Mevacor) from Monascus sp. in 1980. The metabolite is a powerful drug for reducing blood cholesterol level, and acts as a competitive inhibitor of 3-Hydroxy-3-Methylglutaryl-Coenzyme A (HMG-CoA) reductase. The enzyme catalyzes the rate-limiting step in

cholesterol biosynthesis in human liver [5-9]. Moreover, it has been proven that monacolin K is therapeutically and preventatively effective in the treatment of major kind of diseases like atherosclerosis, sepsis, peripheral arterial disease, peripheral vascular disease, cerebrovascular disease, ischemic disease, and bone fracture [9-13]. One of the industrially important microbial sources for lovastatin production is Monascus sp. Aspergillus sp., especially *Monascus purpureus* was found to be the most significant producers of lovastatin [14]. In many studies, *A. terreus* was used for lovastatin production. Monascus species are nonpathogenic, and are frequently used in food processing whereas Aspergillus species are pathogenic, and not safe to consume [11, 15]. Monacolin K ($C_{24}H_{36}O_5$) was the first statin drug, which was approved by the United States Food and Drug Administration in August 1987 [11, 16, 17].

Lovastatin should be produced under the controlled conditions because fermentation parameters are effective in production of Monascus-products. Carbon and nitrogen sources regulate the growth and production of secondary metabolites in fermentation process, whereas these factors' cost plays a major role in selection [18]. Hence, before isolation/extraction of lovastatin, optimization of different physical and nutritional factors to achieve maximum growth of the fungus and optimum lovastatin production is very important.

To decrease the time needed for optimization, the use of miniature bioreactors. Therefore, rapidly metabolizing, cell density in microbial cultivations using MBRs can be supported, and the amount of the products in oxygen-dependent bioprocesses can be increased. Also growth kinetics and product formation at miniature-scale can be scaled up quantitatively [19].

There are several techniques for optimization. Statistical method has more advantages than conventional method that includes less experiment numbers, is rapid and reliable, helps understanding the interactions between different concentrations of nutrients, finds the most suitable condition, and forecasts response [16-22].

In this study, significant factors effective on biomass and lovastatin production by *M. purpureus* were optimized. Response Surface Methodology (RSM) for lovastatin production was applied. A controlled mili-bioreactor was used for the first time.

2. Materials and Methods

2.1. Materials and Microorganism

All materials and culture media for cultivation such as potato dextrose agar were obtained from the Merck Company. Lovastatin standard was donated by PourSina Pharmaceutical Company. Culture of *M. purpureus* PTCC 5303 was purchased from Iranian Research Organization for Science and Technology, Iran, maintained on slants of potato dextrose agar at 4°C , and sub-cultured every 30 days.

2.2. Fermentation

All experiments were carried out in 100-ml mili-bioreactor containing 30 ml medium as per experimental design. A punched mycelium (r= 4.5 mm) of *M. purpureus* PTCC 5305 was transferred into each cell, and then incubated at 30°C on a rotary shaker at 130 rpm with air flow rate of 1.8 $Lmin^{-1}$ for 10 days. At the end of the fermentation period, lovastatin and biomass production was measured. To measure the dry cell weight, the entire volume of the fermented broth was filtered through a filter membrane (Whatman No. 41). The remaining biomass was freeze dried. Biomass was measured till the weight was constant.

2.3. Central Composite Design (CCD)

The variables concentrations, which were jointly significant and effective on the biomass and lovastatin production, were optimized according to the CCD using DESIGN EXPERT software 6.0.10 trial version (Stat-Ease, Minneapolis, USA) (Table 1). The relative effects of two variables on response were identified from dimensional plots. An optimum value of the variables for maximum production of lovastatin was determined by point prediction tool of the software. Dry cell weight of biomass (R_1) and lovastatin production (R_2) was measured as a response in each trial, and the obtained data were analyzed.

2.4. Mili-bioreactor

Designed tests were carried out in a mili-bioreactor in the Research Center for New Technologies in Life Science Engineering, University of Tehran, Iran. It consists of two parts: the shaker-incubator part with the main function of mixing and controlling the temperature. The mixing rate could be adjusted from 100 to 500 rpm, and temperature is controlled with PT100 sensor around the ambient temperature to 40°C with an accuracy of 0.1 centigrade. For lower temperatures, the apparatus was equipped with chiller model IKA KV 600; thus, temperatures lower than the ambient temperature (down to -20°C) can be achieved. Time can be controlled with the same sensors, which were used in the first section.

The second part consists of glass parts, cells, cables and connectors. Four tests could be done simultaneously by the four independent cells. Each cell has four entrance parts: Part one (sampling section), which was used for inoculating and adding materials to sterilized medium. Part two, which is connected to aeration tube and has gas filter. Part three also has gas filter, and is connected to sucking gas tube. Part four contains Oxygen Transfer Rate sensor.

2.5. Measurement of Lovastatin

Procedure given by Su et al. [22] for extraction of lovastatin was slightly modified. After pre-separating the biomass from the culture using a filter paper, a certain amount of supernatant was harvested, adjusted

to pH 3.0 using 2N H_3PO_4, and extracted with an equal volume of ethyl acetate on a rotary shaker 180 rpm at 60°C for 2h. The mixture was centrifuged at 3000 ×g for 10 min. The organic phase was collected, lactonized with 1% trifluoroacetic acid and concentrated. Finally, 95% methanol was added for HPLC analysis using spectrophotometric method. All samples were filtered through 0.22 µm millexLH (Millipore corp., Bedford, MA 01730) before injection [22].

Table 1. Full factorial central composite design matrix of two variables with the observed responses in a mili-bioreactor (SD: ±5%)

	Significant factors		Cell mass (gl^{-1})		Lovastatin ($OD_{238\ nm}$)	
Run no.	Maltose	$MgSO_4.7H_2O$	Actual	Predict	Actual	Predict
1	-1	-1	1.00	2.76	0.143	0.147
2	+1	-1	5.20	5.24	0.190	0.186
3	-1	+1	5.86	4.92	0.221	0.210
4	+1	+1	7.36	7.4	0.193	0.172
5	-1	0	4.66	3.82	0.164	0.101
6	+1	0	6.40	6.30	0.155	0.179
7	0	-1	3.66	2.26	0.140	0.127
8	0	+1	3.10	3.34	0.133	0.152
9	0	0	3.33	3.34	0.139	0.140
10	0	0	3.33	3.34	0.139	0.140
11	0	0	3.33	3.34	0.139	0.140
12	0	0	3.33	3.34	0.139	0.140
13	0	0	3.33	3.34	0.139	0.140

For analysis of lovastatin concentration, we used HPLC (KNAUER) equipped with a C18 column of particle size 5 µm and 250 mm×4.6 mm I.D. and UV detector. The chromatogram was monitored at 238 nm. The mobile phase used acetonitrile and 0.1% wv^{-1} trifluoroacetic acid in the ratio of 70:30, respectively. The eluent was pumped at a flow rate of 1mL min^{-1}. The injection volume was 20 µL. The lovastatin peak was so well separated from the other peaks, making reading the quantity of lovastatin very easy.

3. Results and Discussion

3.1. Optimization of lovastatin production in a mili-bioreactor

According to the previous results, Mansoori et al. determined maltose and magnesium sulfate as significant factors on biomass and lovastatin production by Monascus [23]. Therefore, in order to optimize the concentration of these variables in the mili-bioreactor, 13 experiments were designed. The effect of two factors, i.e. maltose and $MgSO_4.7H_2O$ concentrations, their interaction, and the importance of each of the mentioned factors were evaluated using Minitab software for lovastatin production in a mili-bioreactor. According to the CCD, 13 tests with five central points were designed. The results of these experiments are presented in Table 1.

3.2. Analysis of the data obtained from optimizing the biomass production

The p-values provide evaluation of the significance of each variable. The p-values <0.05 were considered as assurance of effective coefficient. p-values less than 0.05 represent the significance of the model terms [24]. The results of analysis are presented in Table 2.

Among the test variables used in the study, according to p-values, A (maltose), B ($MgSO_4.7H_2O$) and A^2 were significant model terms, and are presented in a quadratic equation. As a result, optimum biomass production is a function of mentioned terms. AB and B^2 terms with P-values more than 0.05 were deleted by the software. Also, in order to better analysis of the proposed model, R-squared is presented. Normally, R^2 over 0.7 indicate a relatively good correlation coefficient; the closer the R^2 value is to 1, the fitter is the model to the experimental data. R^2 value of 0.81 showed appropriate overlap of experimental data, and predicted the values by the model proposed for biomass production. In this case, the value of the determination coefficient (R^2= 0.81), as shown in Figure 1, indicates that the sample variation of 81% for cell mass production is attributed to the independent variables, and only 19% of the total variations are not explained by the model.

The final proposed model for optimum biomass production is as Eq.1:

$$R_1 = 3.34 + 1.24\ A + 1.08\ B + 1.74\ A^2 \qquad \text{Eq. 1}$$

Where, A and B are the values of the test variables, maltose and $MgSO_4.7H_2O$, respectively.

The effectiveness of maltose and $MgSO_4.7H_2O$ concentrations on biomass production is showed in Figure 2.

Table 2. Results of software analysis for optimization of biomass production in a milibioreactor

Term	Sum of squares	Mean square	F-value	p-value Prob>F
A	9.23	9.23	9.92	0.0117
B	6.96	6.96	7.48	0,0230
A^2	9.73	9.73	10.47	0,0102

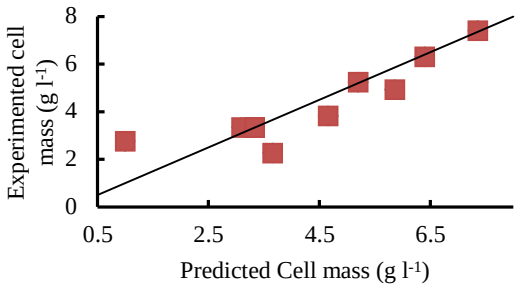

Figure 1 Experimental data and the predicted values by the proposed model for cell mass production

As shown, by increasing the maltose and $MgSO_4.7H_2O$ concentrations towards +1 code, the rare of biomass production is increased. The optimum value of maltose and $MgSO_4.7H_2O$ was determined as 10 gl^{-1} 0.78 gl^{-1}, respectively. *M. purpureus* produced the maximum biomass under the optimum condition including maltose 10 gl^{-1}, peptone 5 gl^{-1}, $MgSO_4.7H_2O$ 0.78 gl^{-1}, $MnSO_4.H_2O$ 0.5 gl^{-1}, KH_2PO_4 4 gl^{-1}, thiamine 0.1 gl^{-1}, and pH=7 for 10 days. Under these conditions, biomass production was predicted to be 4.91 gl^{-1}.

3.3 Analysis of the data obtained from optimizing the lovastatin production

The results of analysis are presented in Table 3. A and B represent maltose and magnesium sulfate concentrations, respectively .

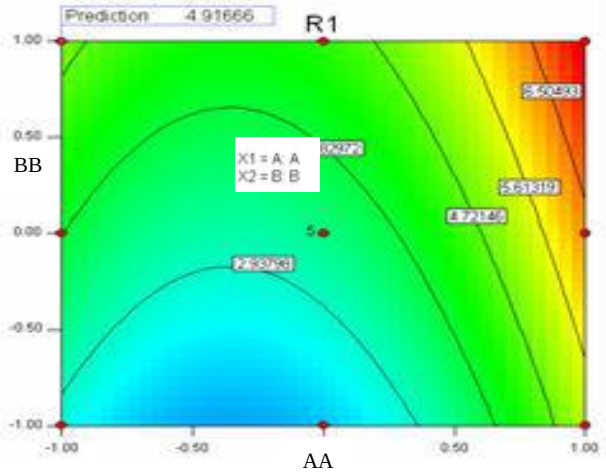

Figure 2. Response surface and contour plots of maltose (A) and $MgSO_4.7H_2O$ (B) on biomass production by *M. purpureus*

Table 3. Results of software analysis for optimization of lovastatin production in a milibioreactor

Term	F-value Sum of squares	Mean square	F-value	P-value Prob>F
B	004-9.127E	004-9.127E	4.36	0.0663
AB	003-1.406E	003-1.406E	6.73	0.0291
A^2	003-5.010E	003-5.010E	23.96	0.0009

The experimental data using the software fitted into a multiple nonlinear regression model proposes Eq. 2 for lovastatin production (R^2):

$$R^2(abs) = 0.14 + 0.0124\ B - 0.0194\ AB + 0.039\ A^2 \quad \text{Eq. 2}$$

According to Table 3, p-value is less than 0.05 for quadratic term of maltose concentration (A^2) and the simultaneous impact of maltose and $MgSO_4.7H_2O$ concentrations. In cases where the value of p is between 0.05 to 0.1. it is probably significant; so B term is provided in the model too. As a result, optimum monacolin K production is a function of the mentioned terms. R^2 value of 0.80 was obtained for this equation. Figure 3 shows appropriate overlap of the experimental data and the predicted values by the proposed model for lovastatin production. The effectiveness of maltose and $MgSO_4.7H_2O$ concentrations on lovastatin production is considered as well. By increasing of the $MgSO_4.7H_2O$ concentration and decreasing the maltose concentration, lovastatin production is increased. All the response surfaces/contours could be analyzed for determining the optimized value of the factors, but it was difficult to analyze all of these simultaneously. Hence, point prediction of the design expert software was used to determine the optimum values of the factors for maximum lovastatin production. The maximum production of lovastatin under the optimum conditions was predicted as 0.208 abs. In order to proving the authenticity of optimal conditions, it was repeated in the mili-bioreactor again. Amount of lovastatin absorbance in 238 nm, 0.221 absorbance and biomass 5.86 g l^{-1} was obtained.

4. Conclusion

Few studies are available about increasing of lovastatin production efficiency by *M. purpureus* in the liquid fermentation. Since optimization of numerous variables is time consuming and costly, other part of mentioned articles is focused to the use of statistical designs and data analysis software and predict of finally production in different concentration of effective factors. Good correlation between the predicting and experimental models confirms the value and importance of statistical methods for decreasing the number of experiments.

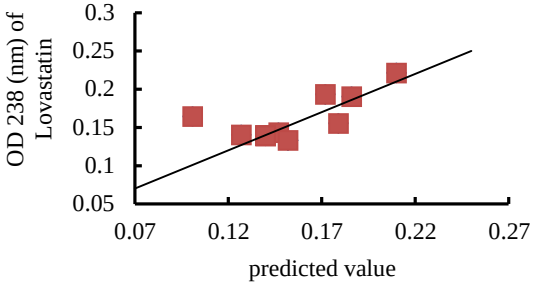

Figure 3. Experimental data and the predicted values by the proposed model for lovastatin production

In the present study, optimization of lovastatin production was carried out by *M. purpureus* in the mili-bioreactor. Maximum lovastatin yield can be achieved by reduction of maltose and increasing $MgSO_4.7H_2O$ whereas mycelia production is not maximum. The mycelium production increase leads to rise of the medium viscosity, reduces ORT, and finally, reduces the desired metabolite production. In this study, we achieved the maximum production of lovastatin under optimal conditions including maltose 10 gl^{-1}, peptone 5 gl^{-1}, $MgSO_4.7H_2O$ 0.78 gl^{-1}, $MnSO_4.H_2O$ 0.5 gl^{-1}, KH_2PO_4 4 gl^{-1}, thiamine 0.1 gl^{-1}, and pH=7, air flow rate 1.8 $Lmin^{-1}$ after 10 days.

5. Acknowledgments

The authors thank Ms. Oroojalian for her guidance.

6. Conflict of interest

No conflict was stated by authors.

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