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Original Article

Semi-industrial Production of Lovastatin with a Standard Amount of Mycotoxin Citrinin from *Monascus purpureus*

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ABSTRACT

Lovastatin is a statin used to treat hypercholesterolemia and cardiovascular diseases (CVDs). It is produced by several medicinal non-pathogenic fungi, including *Monascus purpureus* (MP). While CVDs account for a high percentage of deaths in Iran, few studies have investigated lovastatin production in the country. This study aimed to examine the semi-industrial production of lovastatin from MP by controlling the amount of mycotoxin citrinin. Eleven treatments containing varying levels of several sources of carbon, nitrogen, and several physical factors of the fungus culture medium were tested simultaneously using a Plackett–Burman screening design. Building on Pareto chart, the Plackett–Burman test determined the contribution of each factor to the production of lovastatin and mycotoxin citrinin. As a carbon source, higher barley concentration increased lovastatin production. Lovastatin production required an increased aeration rate with a further rise in barley content. The maximum production of lovastatin (318 mg/L) and lower content of mycotoxin citrinin (48 ppb) occurred under optimized conditions (20 g/L of barley and 8 L/min of aeration). Barley and aeration rates contributed significantly to higher lovastatin and lower mycotoxin citrinin production. These findings can be used in the semi-industrial production of lovastatin with low levels of mycotoxin citrinin (less than the allowable limit).

INTRODUCTION

Cardiovascular disease (CVD) is a general term for diseases that affect the heart or blood vessels. Physical inactivity, high-calorie diets, and saturated fats contribute to the incidence of atherosclerosis and other metabolic disorders such as metabolic syndrome, diabetes mellitus, and hypertension, thus leading to CVD development [1]. Specific genetic mutations also play a role in CVD development. Atherosclerosis is a prevalent CVD characterized by the accumulation of oxidized cholesterol, fat, calcium, waste, and other molecular substances in the arteries. As undesirable molecules, atherosclerosis-related plaques reduce the artery diameter and thicken the arterial wall, thus disrupting blood and oxygen supply [2]. Atherosclerosis can be prevented by lowering low-density lipoprotein cholesterol (LDL-C) or raising high-density lipoprotein cholesterol (HDL-C).

Physicians have long used statins to treat hypercholesterolemia, hyperlipoproteinemia, and hypertriglyceridemia. Lovastatin is a statin applied to treat hypercholesterolemia and CVD. Similar to a cholesterol-synthesis pathway precursor called mevalonate, lovastatin can competitively inhibit HMG-CoA reductase. It is a secondary metabolite synthesized by microorganisms such as *Aspergillus terreus*, *Monascus ruber*, some of which are pathogenic [3]. Among the above microorganisms, *M. ruber* and MP are two fungi of the *M.* genus that are widely used to produce lovastatin. *Monascus* also produces the toxic mycotoxin citrinin [4]. Mycotoxin citrinin has antibiotic activity against gram-positive bacteria. Nonetheless, its nephrotoxic properties have limited its use.

Mycotoxin citrinin causes nephropathy, hepatotoxicity, and kidney adenomas in various cellular and animal models. Hence, its fungal-based production should be reduced. The extant literature indicates that few studies in Iran have examined lovastatin production using a standard amount of mycotoxin citrinin [5]. Therefore, the present study

aimed to explore the semi-industrial production of lovastatin by controlling the amount of mycotoxin citrinin from the liquid culture of MP. The ultimate intention is to maximize lovastatin production by optimizing the culture medium.

MATERIALS AND METHODS

For in vitro study, MP (PTCC5330) was purchased from the fungal center affiliated with the Iranian Research Organization for Science and Technology (IROST). The mycelium of MP was cultured on a Potato Dextrose Agar (PDA) slant and subsequently placed at 4 °C. The culture was reiterated on the PDA medium every 30 days. The fungus was incubated in a Petri dish at 30 °C for seven days to grow and was subsequently transferred to the medium at 4 °C. This medium was used as the primary seed culture [6]. Eleven treatments were used in the present study to investigate the effects of carbon source, nitrogen source, potassium phosphate, temperature, aeration rate, stirring speed, and ambient pH (Table 1).

Carbon and Mineral Sources

Carbon sources (wheat, barley) were soaked in water, germinated, and subsequently dried in an oven at 50 °C. On the flame, wheat, barley (previous step), and rice were pulverized and mixed with distilled water in different proportions. Potatoes were washed, added to boiling distilled water, and crushed in water after cooking. After the carbon sources were boiled in distilled water for a few minutes, they were passed through a 100 µm filter using a vacuum pump. The first 100 cc of the extract was removed for its concentration to homogenize, and its dry weight was assessed (drying process at 60 °C for 24 hours). Later, the desired concentrations were prepared and applied. Nitrogen was supplied using ammonium nitrate and urea. Phosphorus and potassium were also supplied using potassium phosphate. After preparing the media, culture was performed, and then sampling was performed during culture. Sampling was carried out as per the aeration and stirring rates in different stages of culture. A key priority in the present research was the inexpensive and competitive production of secondary metabolite lovastatin.

Bioreactor Design

Essential factors for successful fungal culture include the aeration and stirring rates of materials, acidity control, temperature control, ventilation of gases from the culture medium, access to the culture medium for analysis, and the possibility of long-term evaluation of the culture process. After preliminary examinations and using response surface methodology (RSM), the key factors that significantly impacted the production of secondary metabolites were selected, and effective elements were incorporated to design an applied bioreactor. First, a one-liter bioreactor with 12 separate units was made, which could ventilate the culture medium, create a dark environment, and control the sterile aeration, stirring speed, and temperature. In this reactor, various factors were investigated. Moreover, after the contributory factors to increasing lovastatin production and decreasing mycotoxin citrinin production were selected, the bioreactor capacity was first raised to 2-liter units and subsequently to 20-liter units to better resemble semi-industrial production. Besides, 0.5 µm filters were installed on the inlet valve of the aeration pump for initial filtration, and to increase the life of the main filter, 0.22 µm filters were installed on the outlet valve of the aeration pump as main filters.

Table 1 Levels of nutritional and physical factors used in the experimental design in the Plackett–Burman test

Treatments		Carbon sources (g/L)	Nitrogen sources (g/L)	Nutrient elements (g/L)	Temperature (°C)	Stirring (L/Min)	pH	Aeration (L/Min)
		4	2	3	3	3	3	3
Materials		Wheat extract	Ammonium nitrate	Mixed of (KH_2PO_4 40%, $MgSO_4.7H_2O$ 40%, KCl 19% and $FeSO_4.7H_2O$ 1%)	Culture media	Water jet	HCL-NAOH	-
		Barley extract	Urea	-	-	-	-	-
		Potato extract	-	-	-	-	-	-
		Rice extract	-	-	-	-	-	-
Levels	+1	18	5	4	30	6	7	2
	0	10	3	2	25	4.5	5	1.5
	-1	2	1	1	20	3	3	1

The filters were replaced periodically. The sterile air pump outlet was connected to the water pump inlet. The water pump started the flow of the culture medium and mixed the air with the culture medium in the best way.

The culture medium and autoclavable containers were sterilized through autoclave sterilization for 20 minutes at 121 °C and a pressure of 1.2 atm. Other non-autoclave equipment, including a 20-liter tank, air pumps, water, pipes, and fittings, among others, were sterilized using 10% sodium hypochlorite. Other components were connected to the device, and 10% hypochlorite solution flowed through the pumps. All components were sterilized simultaneously after they were assembled and installed. Sodium hypochlorite solution was removed from the drain valve 24 hours later, and the tank was rinsed with sterile distilled water several times. After sterilization of other components, the autoclaved culture medium was introduced into the tank, and a 10 mm diameter mycelium punch was transferred from each 7-day MP colony to each culture chamber (Figure 1).

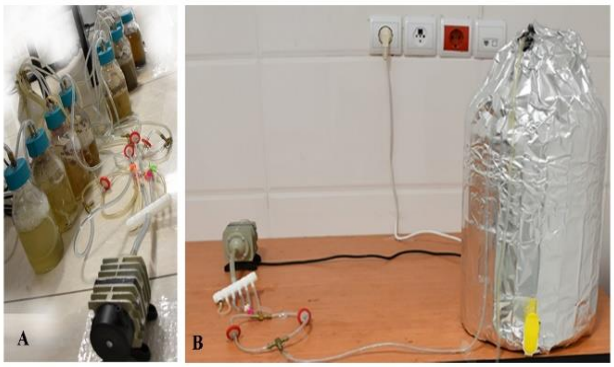

Fig. 1 A: View of the one-liter bioreactor with aeration pump and sterile inlet and outlet air filters and fittings B: The 20-liter bioreactor with aeration pump, sterile inlet, and outlet air filters, electric agitator, and fittings.

Screening of Factors Affecting the Process

Significant factors are screened and optimized when many factors affect biological processes [7]. In the present study, Plackett–Burman Design (PBD) was employed to screen the factors affecting the production of lovastatin and mycotoxin citrinin by MP in liquid culture. Eleven treatments with different levels were selected (Table 1). Twelve experiments with three central points were designed by Plackett–Burman in the DESIGN EXPERT 12.0 (Stat-Ease, USA).

Central Composite Design Test

According to the results of PBD screening and the Pareto chart, we selected two factors that led concurrently to the increased production of lovastatin and reduced production of mycotoxin citrinin. Optimization was performed using the central composite design (CCD) [8] and RSM [9] in the DESIGN-EXPERT program ver. 12 (Stat-Ease, USA). Process prediction (post-analysis) and DESIGN EXPERT ver. 12 (Stat-Ease, USA) helped determine the interaction between the two variables on the response. As such, the optimal values of factors for maximum lovastatin and minimum mycotoxin citrinin production were identified.

Measurement of Lovastatin and Mycotoxin Citrinin Levels

Extraction steps were performed according to a modified form of the method proposed by Baneshi *et al*. (2014) [10]. After separating the biomass from the culture medium using Whatman grade 41 quantitative filters, the supernatant was removed, and its pH was adjusted to 3 using H_3PO_4 (2N). For extraction, ethyl acetate was added to the supernatant on a shaker at 1000 rpm. The addition was done at a ratio of 1:5 for lovastatin and 1:1 for mycotoxin citrinin at 30 °C for 2 hours. The mixture was centrifuged at 4000 rpm for 15 minutes. Finally, each sample was treated with 95 percent methanol, and the organic phase was separated using a 0.22 µm filter. The lovastatin and mycotoxin citrinin standards were specified by Tehran Shimi Pharmaceutical Company. We employed HPLC (KNAUER) with a C18 column with a particle size of 5 µm and a 250 mm×4.6 mm I.D. and a UV detector to determine the concentrations of lovastatin and citrinin. At 238 and 330 nm, the chromatograms of lovastatin and the mycotoxin citrinin were monitored, respectively. Acetonitrile and 0.1 percent wv^{-1} trifluoroacetic acid were utilized in the mobile phase in a 70:30 ratio. The eluent was pumped at a 1mL min^{-1} flow rate. The injection volume was 20 µL. The peaks of lovastatin and citrinin were so far off from the other peaks, making it simple to understand the amount of lovastatin and citrinin.

Statistical Calculations, Mathematical Model, and Correlation

Laboratory data were compared with the data obtained from the statistical model (post-analysis)

using DESIGN EXPERT 12.0 software (Stat-Ease, USA). Moreover, after examining various linear equations, e.g., quadratic equations, the best equation that could yield maximum correlation (R^2) with the laboratory data was selected. A one-way analysis of variance (ANOVA) was employed to assess the impact of treatments on lovastatin and mycotoxin citrinin production. The significance level was considered at $p \leq 0.05$ in all tests.

RESULTS

Data Screening Analysis with PBD

Culture compounds and physical factors can significantly affect lovastatin and mycotoxin citrinin production. The Pareto chart clearly shows the impact of each factor involved in this process. The charts indicate that barley, aeration, rice, temperature, stirring, and pH have the highest importance and positively impact lovastatin production (Figure 2).

All of these factors have a positive effect on lovastatin production. Other factors such as ammonium nitrate and potassium phosphate have a positive but insignificant impact on lovastatin production. Urea, wheat, and potatoes may be detrimental to lovastatin production, with urea having a more negative effect than other factors.

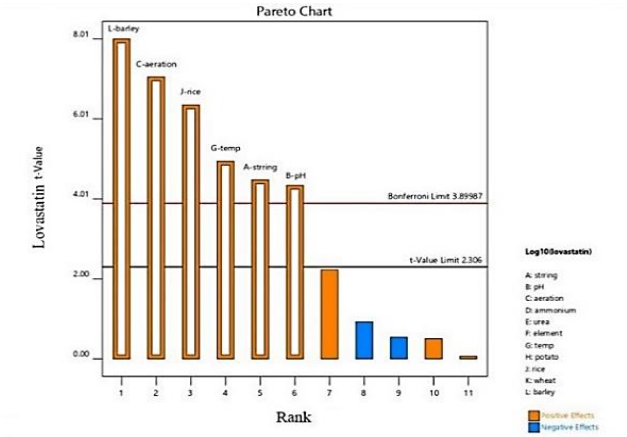

Fig. 2 Pareto chart of lovastatin production from *M. purpureus*. Important factors are listed at the top of each column.

The most significant factors affecting mycotoxin citrinin production included potatoes, wheat, and barley as carbon sources; stirring rate; aeration rate; temperature; and rice as a carbon source, respectively (Figure 3). According to the charts, potato, wheat, rice, and temperature have the highest importance and positively affect lovastatin production, respectively. Moreover, barley, stirring, and aeration factors have the most substantial adverse effects on mycotoxin citrinin production. These factors can be categorized into two groups. The potato, wheat, temperature of the culture medium, and rice are among the most critical factors with an increasing effect on mycotoxin citrinin production. Rice is less effective than other factors in this regard. The three factors of barley, stirring, and aeration rate exerts the most negative, decreasing impact on mycotoxin citrinin production. Other factors, such as urea, ammonium nitrate, potassium phosphate, and pH, did not significantly affect mycotoxin citrinin production.

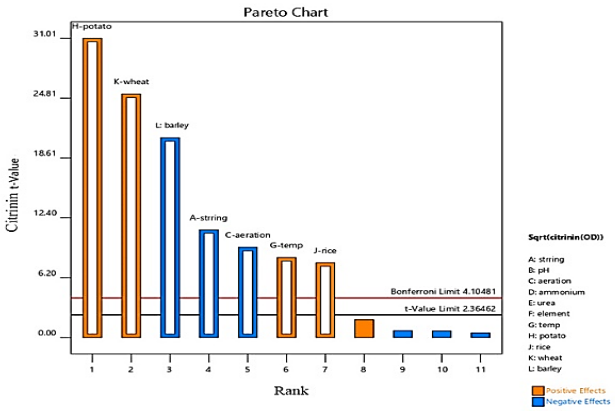

Fig. 3 Pareto chart of mycotoxin citrinin production from *M. purpureus* medium. Significant factors are listed at the top of each column.

Analysis of Data Obtained from Optimization of Medium and Culture Conditions via the RSM Method

Analysis of data obtained from optimization of medium and culture conditions via the RSM method in lovastatin production

Based on the Pareto chart, we selected factors typically contributing to maximum lovastatin and minimum mycotoxin citrinin production. They included barely, as a carbon source, and aeration rate. The experimental design was performed by the CCD method in RSM using barely as a carbon source (0-20 g/L) and aeration rate (0-8 L/min) in alpha after 13 experiments and in five central points (Table 2).

Experiments were designed according to the CCD method in a laboratory setting. The effect of variables and their interactions was investigated randomly during the fermentation process. The lovastatin and mycotoxin citrinin concentrations, as fermentation process responses, were analyzed using DESIGN-EXPERT ver. 12 software (Stat-Ease, USA). The software proposed a nonlinear regression model

using laboratory data obtained for lovastatin. A mathematical model was then developed for lovastatin using the coefficients that were estimated by the software.

R1 (abs) = 0.41 + 0.042A + 0.287B + 0.028AB + 0.061A2 + 0.043B2

In model R1 (OD)[1], A is aeration (L/min), and B is a carbon source (barely) (g/L). Given the p-values being smaller than 0.05 for the terms A, B, AB, A2, and B2, these terms are cited in the equation and are significant. Therefore, lovastatin production depends on the above items.

The software also introduced the variable R^2 for better analysis. R2> 0.7 indicates a good correlation. Notably, at higher values (closer to 1), experimental data is more likely to match the regression model, suggesting more accuracy of the model. R^2 is 0.996, adjusted R^2 is 0.994, and predicted R^2 is 0.98. The Lack of Fit test is 0.49, which is indicative of the good fit of the model. The model fit test should not be significant. The "Adeq Precision" indicates the difference between the predicted response and the average prediction error rate, and an Adeq precision value higher than four indicates the appropriate fit of the model. Here, the Adeq precision value was 64.19 (Table 3a).

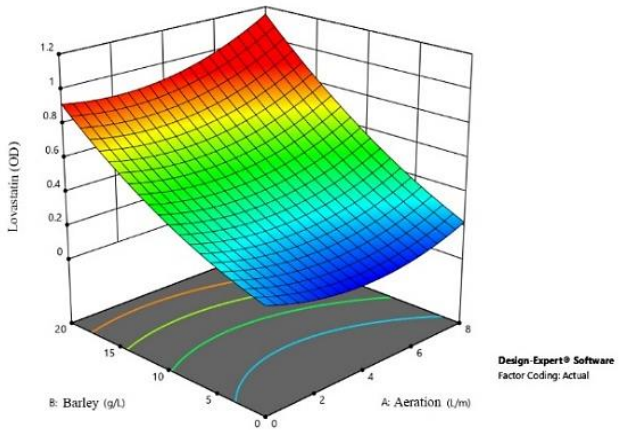

Fig. 4 Three-dimensional display of the effect of carbon material (barley) (g/L) and aeration (L/min) on lovastatin production from *M. purpureus*. An increase in barley content increases lovastatin production. Increased lovastatin production at higher barley levels requires increased aeration.

Figure 4 displays lovastatin production from the medicinal fungus, i.e., MP. Increased barley content led to higher lovastatin production from MP. With a further increase in barley, lovastatin production requires an increased aeration rate. At average aeration rates, lovastatin production does not follow the linear function and decreased. The maximum lovastatin production (318 mg/L) occurred at barley content of 20 g/L and aeration of 8 L/min. The lowest lovastatin production (35 mg/L) was observed at the barley content of 0 g/L and aeration rate of 4 L/min.

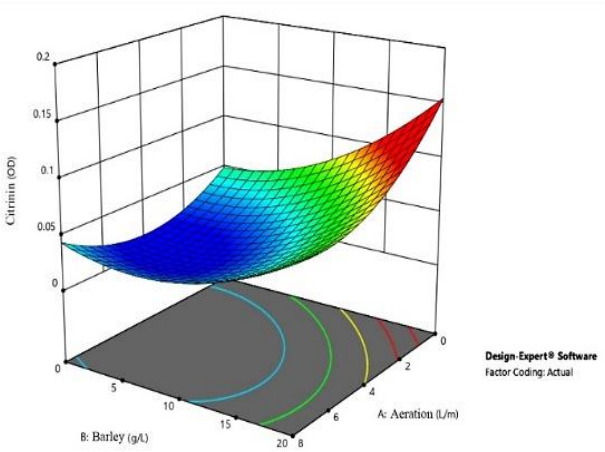

Fig. 5 Three-dimensional display of the effect of carbon (barley) (g/L) and aeration levels (L/min) on mycotoxin citrinin production from *M. purpureus*. Higher barley content with minimal aeration led to increased mycotoxin citrinin production.

Analysis of data obtained from optimization of medium and culture conditions by RSM method in mycotoxin citrinin production

The software proposed a nonlinear regression model using laboratory data obtained for mycotoxin citrinin. The coefficients estimated by the software were used to yield a mathematical model for mycotoxin citrinin. R^2 is the equation for mycotoxin citrinin production from MP.

R^2 (abs) = 0.026 + 0.014A + 0.024B-0.009AB + 0.014A2 + 0.012B2

In the R^2 (OD) model, aeration is in L/min, and B (barley), the carbon source, is in g/L. Since the p-values obtained for the terms A, B, AB, A2, B2 are smaller than 0.05, these terms are significant and mentioned in the equation. The mycotoxin citrinin production depends on the above items.

The software also introduced the variable R^2 for better analysis. R^2> 0.7 represents a good correlation, and at higher values (closer to 1), experimental data are more likely to match the regression model, and the model is considered more accurate. R^2, adjusted R^2, and predicted R^2 are equal to 0.99, 0.98, and 0.96, respectively. The Lack of Fit test is equal to 0.42, which denotes the good fit of the model. The model fit test should not be significant. The Adeq precision indicates the difference between the predicted

response and the average prediction error rate. An Adeq precision value greater than 4 denotes the appropriate fit of the model. Here, the Adeq precision value was 35.3 (Table 3b).

Figure 5 demonstrates mycotoxin citrinin production from MP. As this diagram depicts, higher barley content increases mycotoxin citrinin production from MP. More barley use leads to higher mycotoxin citrinin production. The maximum mycotoxin citrinin production was observed at the barley level of 20 g/L and aeration of 0 L/min. When barley content increased from 0 to 10 g/L, there was a greater need to increase the aeration rate gradually. Also, when the barely exceeded 10 g/L, the need for aeration increased at a higher slope. The lowest mycotoxin citrinin production was observed when the barely content and aeration rate were 0 g/L and 4 L/min, respectively (Fig. 4).

Table 2 Central composite design arrangement as per the alpha and the results obtained under laboratory conditions and mathematical model prediction

Standard Order	Run Order	Factor 1 Aeration (L/Min)	Factor 2 Barley extract (g/L)	Lovastatin (OD)* Actual Value	Lovastatin (OD) Predicted Value	Citrinin (OD) Actual Value	Citrinin (OD) Predicted Value
4	1	6.8284	17.0711	0.896	0.883	0.056	0.054
12	2	4	10	0.398	0.419	0.024	0.026
5	3	0	10	0.498	0.482	0.074	0.76
9	4	4	10	0.406	0.419	0.029	0.026
7	5	4	0	0.101	0.098	0.019	0.017
2	6	6.8284	2.9289	0.259	0.249	0.021	0.023
13	7	4	10	0.446	0.419	0.023	0.026
6	8	8	10	0.587	0.602	0.036	0.034
3	9	1.1715	17.0711	0.731	0.740	0.0106	0.102
8	10	4	20	0.91	0.912	0.084	0.087
10	11	4	10	0.419	0.419	0.025	0.026
1	12	1.1715	2.9289	0.209	0.222	0.035	0.034
11	13	4	10	0.428	0.419	0.031	0.026

OD*= Optical density

Table 3 a One-way analysis of variance (ANOVA) for lovastatin production by *M. purpureus*

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.7158	5	0.1432	410.63	0.0001 **	significant
A- Aeration	0.0145	1	0.145	41.66	0.0001 **	
B- Barley	0.6630	1	0.6630	1901.98	0.0001 **	
AB	0.0033	1	0.0033	9.48	0.0178 *	
A^2	0.263	1	0.0263	75.45	0.0001**	
B^2	0.0129	1	0.0129	36.88	0.0005 **	
Lack of Fit	0.0010	3	0.0003	0.9592	0.4932	not significant

R^2=0.996 $R^2_{Adjusted}$=0.994 $R^2_{Predicted}$= 0.98 Adeq Precision=64.19

Table 3 b One-way analysis of variance (ANOVA) for the production of mycotoxin citrinin by *M. purpureus*.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0093	5	0.0019	147.35	0.0001 **	significant
A- Aeration	0.0017	1	0.0017	137.45	0.0001 **	
B- Barley	0.0049	1	0.0049	388.41	0.0001 **	
AB	0.0003	1	0.0003	25.70	0.0014 **	
A^2	0.0015	1	0.0015	117.18	0.0001 **	
B^2	0.0012	1	0.0012	91.29	0.0001 **	
Lack of Fit	0.0000	3	0.000	1.16	0.4282	not significant

R2=0.99 $R2_{Adjusted}$=0.98 $R2_{Predicted}$= 0.96 Adeq Precision=35.3

When the aeration rate exceeded 5 L/min, the mycotoxin citrinin level was below the standard set in Japan. Indeed, it was equal to 48 ppb in the optimal treatment where the maximum lovastatin level was produced (barley=20 g/L and aeration=8 L/min).

DISCUSSION

M. purpureus is a species of red mold, and the rice fermented by this fungus (red yeast rice) is consumed as food or natural dietary supplement [11]. This fungus produces a set of pharmaceutically active ingredients, of which lovastatin is best known and most examined. Also known as monacolin K (lovastatin), produces different pigments (for example, "red rice" (ang-kak or anka) as food dyes and similar bioactive polypeptides that regulate cholesterol levels in humans. It is the most significant secondary metabolite of *M.* and a crucial enzyme in cholesterol synthesis. This is because of its potent cholesterol-lowering functions resulting from the inhibition of enzyme 3-hydroxy-3-methylglutaryl A reductase [10, 12].

Lovastatin is biosynthesized by several other *Monascus* species, such as *M. ruber*, *M. pilosus*, *M. vitresus*, *M. anka*, *M. serorubescens*, and *M. pubigerus*. *Monascus* pigments can be divided into three major groups: yellow (ankaflavin, monascin-C, and monascidin A), orange (rubropunctatin, monascorbin), and red pigments (rubropunctamine, monascorubramine). In nature, *Monascus* grows on solid substrates. In most commercial environments, rice is used to grow a solid substrate. However, MP can be grown in other substrates containing starch such as wheat, corn, soybeans, cassava, potatoes, corn flour, peanut flour, barley, and sorghum [13]. The quality and amount of secondary metabolites in plants and also in fungi depend on the type of culture medium, plant and fungi strain, access to oxygen, nutrient, temperature, culture duration, inoculation volume (i.e., number of spores), substrate pH, and moisture [14,15].

Monascus species produce mycotoxin citrinin (also known as monascidine A), which is known to cause nephropathy [16]. Mycotoxin citrinin has a bactericidal effect on genera *Bacillus*, *Streptococcus*, and *Pseudomonas*. Previous studies have shown the potential use of mycotoxin citrinin as a tentative substitute for nitrate/nitrite salts in preserving meat and poultry products in Europe. However, it is still an undesirable *Monascus*-derived product due to its toxic effects. Accordingly, the European Commission (EC) has allowed for a maximum of 2 μg of mycotoxin citrinin/g of the food product. Some Asian countries, such as South Korea and Japan, have introduced 0.05 μg and 0.2 μg of mycotoxin citrinin/g of the food product as the permissible limit, respectively.

The production of mycotoxin citrinin-free *M.* pigments has received substantial attention in the food industry. Yet, few studies have explored lovastatin production from the genus *Monascus* [16, 17]. In particular, there is a scarcity of studies that have used standard statistical methods to optimize lovastatin production and reduce mycotoxin citrinin production. In reality, all associated factors must be studied simultaneously to optimize mycotoxin citrinin production and achieve the desired outcome. For this purpose, Plackett–Burman screening method and RSM are beneficial. Recently, several studies have focused on producing increased levels of lovastatin and pigments at lower levels of citrinin. This purpose can be reached through (1) selecting non-toxic strains, (2) correcting environmental factors, and (3) correcting feeding conditions.

Plackett-Burman screening identified barely and aeration as the primary factors in the present study. Barley malt is a good source of carbon for the growth of MP. Therefore, barley malt gained from the malting process was selected as a carbon source. Malt is a germinated cereal that provides an acceptable source of proteins, carbohydrates, minerals, and various B vitamins [16, 18]. Malting comprises a set of biotechnological processes, including soaking, germination, drying of germinated grain, and temperature and humidity control. Cereal malt contains 60-70 g/L/maltose, 2% nitrogen, and other useful salts and elements [16,19].

When cereals germinated and dried in the present study, they were used as a malt source and added to the culture medium. These complete food sources are substantially less expensive than conventional laboratory food media and can produce low-cost and competitive lovastatin. As the carbon content increases, the fungus growth amplifies, and as the density of the culture medium augments, anaerobic conditions and stress occur. These conditions add to the mycotoxin citrinin concentration[20]. Therefore, increasing the aeration rate helps reduce mycotoxin citrinin concentration.

The present study aimed to explore the semi-industrial production of lovastatin from liquid MP culture by controlling mycotoxin concentration. The results showed that increasing barley content increases lovastatin production. When there is a further increase in barley, lovastatin production requires an increased aeration rate. Under optimized conditions (20 g/L of barley carbon source and 8 L/min of aeration), maximum lovastatin and lower mycotoxin citrinin productions were 318 mg/L and 48 ppb, respectively. Therefore, the present study showed the significant effect of barley and aeration rate on lovastatin and mycotoxin citrinin production. Thus far, many studies have inquired into increased rates of lovastatin production and decreased rates of mycotoxin citrinin production from the MP medium. Ajda Marič *et al*. compared the production of lovastatin, mycotoxin citrinin, and other pigments from the MP medium in rice and millet media. They cultured six MP strains on brown rice and millet as substrate. MOPU GS1 was the only strain to produce lovastatin (1.3 mg lovastatin mg/g of substrate's dry weight). In other strains, mycotoxin citrinin production ranged from 0.3 to 18.2 mg/g dry weight of the substrate. In this study, millet displayed good potential as a substitute substrate for rice as it led to higher lovastatin and lower mycotoxin citrinin production [21].

In another study, Lee *et al*. used the Dioscorea root as a liquid culture medium for MP NTU 568. They reported that monoculin K and mycotoxin citrinin formation rates from *M. purpureus* NTU 568 in immersed Dioscorea medium were 148% and 147%, respectively. Besides, optimal culture conditions (pH = 5.7, 1% dioscorea concentration, 0.5% ethanol concentration) increased monacolin K level to 27.9 mg/g (47%) and decreased the mycotoxin citrinin level to 2.15 µg/g (54%) compared to control conditions (pH = 3.5, 5% dioscorea, and no ethanol) [22]. Sani *et al*. reported a decrease in mycotoxin citrinin concentration and an increase in lovastatin production from MP under solid-state fermentation conditions. The optimum culture conditions in this study were 2% glycerol (volume/w), 0.14% methionine (w/w), and 0.01% $NaNO_3$ (w/w) for 16 days at 25 °C. The obtained mycotoxin citrinin concentration was 0.26 mg/kg [23].

In the present study, the carbon source was critical in lovastatin and mycotoxin citrinin production. The results showed that barley has the most reducing effect on mycotoxin citrinin production. In a study, Zhou et al. also identified the carbon source of the culture medium as the most influential factor. They found that corn powder, followed by maltose and rice flour as carbon sources, could produce the highest lovastatin concentration. As a carbon source, maltose exhibited the highest mycotoxin citrinin gain (1.23 mg/L). On the other hand, mycotoxin citrinin was not produced when millet flour and soluble starch were used as carbon sources [24]. This finding is inconsistent with our results, which may be attributed to the strain effect. In another study, obtained the maximum lovastatin yield (1475.30 µg/L) after five days of culture when maltose was used as the only carbon source. Moreover, maltose led to better intracellular function of pigments, higher lovastatin production, and lower mycotoxin citrinin production during the ten-day culture period [25], which confirms our results.

Culture duration is also another factor influencing mycotoxin citrinin production in *Monascus*. According to studies, the fungus grows up to 10 days after culture, while the production of yellow pigments (ankaflavin and monascin) increases up to 24 days after culture. In contrast, orange pigments (rubropunctatin and monascorbin) are synthesized only up to 14 days after culture. The mycotoxin citrinin concentration increases up to 14 days after culture but then decreases when the mycotoxin citrinin breaks down. It has been shown that mycotoxin citrinin decomposes to mycotoxin citrinin H_2 at pH>7.0. Both mycotoxin citrinin's breakdown and the reduction of orange pigments are associated with possible mycelial lysis [26]. Consistent with our finding, however, studies have shown a decrease in mycotoxin citrinin concentration on day 14. Research findings indicate that the nitrogen source in the culture medium affects the final pH and regulates the final pH of mycotoxin citrinin biosynthesis.

Temperature is another crucial factor affecting the metabolic activity and growth of *Monascus* and mycotoxin citrinin production. Studies have referred to 30-37 °C as optimal temperatures for different strains of *Monascus* [27]. High temperatures reduce mycotoxin citrinin production. Alongside this, the mycotoxin citrinin production decreased significantly when the culture temperature increased from 27 °C to 37 °C.

Studies have demonstrated that high temperatures, similar to high pH values, produce a new compound

called mycotoxin citrinin H2, which exerts weaker cytotoxic effects than mycotoxin citrinin[28]. Saithong *et al*. found that the production of monoculin K by *M. purpureus* strain IFRPD 4046 increased with temperature (25-30 °C), room temperature, and time (0-24 days). Higher room temperatures, i.e., 32-35 °C, and lower than 25 °C can reduce mycotoxin citrinin concentrations[29].

Sterile aeration also plays a significant role in reducing mycotoxin citrinin. According to the results of a study, when the initial ambient pH, stirring speed, and the aeration rate were set respectively to 6.2, 350 rpm, and 2 vvm, the M011 strain showed the highest production of yellow pigments and a minimal amount of mycotoxin citrinin (<2 ppm) [30]. Hajjaj *et al*. showed that red pigments and mycotoxin citrinin production is related to oxygen supply conditions. The study revealed that increasing the stirring speed from 100 to 250 rpm improved red pigment production, although further increase (400 rpm) led to no better production. Additionally, while increasing the aeration rate to 2 vvm did not cause a further rise in pigment concentration, it led to a higher mycotoxin citrinin concentration [31]. Yang *et al*. stated that the most suitable conditions for the fermentation process in a 10-liter fermenter involved a three-phase aeration step (0-48 hours at 300 L/H, 48-96 hours at 500 L/H, 96-120 hours at 200 L/H) at a stirring speed of 300 rpm at 30 °C for 120 hours. In this study, compared to fixed culture aeration (500 L/H), pigment and mycotoxin citrinin production decreased by 29.6% and 79.5%, respectively [32].

While secondary metabolites by medium factors such as carbon, nitrogen, temperature, light, and pH sources have been of interest to researchers [33], direct gene manipulation has also been extensively studied [34]. The genes responsible for synthesizing mycotoxin citrinin and pigments have recently been identified. Gene manipulation has been extensively studied as a measure to reduce mycotoxin citrinin production. *M. purpureus* mutation is made by substituting the ctnA regulator gene, which produces low mycotoxin citrinin concentrations and high pigment concentrations [35]. Similarly, the disruption of the pksCT gene of *Monascus* results in the production of *Monascus* free-citrinin pigments [36]. While these facts suggest that reducing mycotoxin citrinin biosynthesis through gene manipulation is somewhat successful, genetically engineered foods have not yet been widely accepted. Therefore, industrial and semi-industrial culture methods with inexpensive materials are still needed to reduce mycotoxin citrinin further and increase lovastatin production.

Overall, the present study found aeration, rice, and barley as carbon sources, temperature, stirring, and pH as effective enhancers of lovastatin production. Also, potato and wheat as carbon sources, a higher temperature of the culture medium, and rice increased mycotoxin citrinin production. Lastly, the three factors of barley, stirring, and aeration reduced mycotoxin citrinin production.

CONCLUSION

The results indicated the significant effects of barely, as a carbon source, and aeration rate on increased lovastatin and reduced mycotoxin citrinin production in MP. Our findings have implications for the semi-industrial production of lovastatin with low levels of mycotoxin citrinin (less than the allowable limit). This is especially true in cases where economic and competitive priorities are considered for lovastatin production.

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Conflict of Interest

The authors have no conflict of interest to declare in the present study.

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