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Optimization of divalent metal cations for maximal concentration of Monacolin K in *Monascus* M1 by response surface methodology

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Abstract: Inorganic salts are important factors in the growth and secondary metabolites production of microorganisms. This study investigated the influences of divalent metal cations, Mn^{2+}, Zn^{2+}, and Mg^{2+} on the cell growth and Monacolin K production in *Monascus* M1. Then the concentration of the three kinds of divalent metal cations was optimized by response surface methodology, and the optimum conditions for the highest production of Monacolin K were determined. The optimum concentrations of the three divalent metal ions were selected as follow: Mn^{2+} 0.33%, Zn^{2+} 0.16%, and Mg^{2+} 1%. In this condition the concentration of Monacolin K reached 9.57mg/g which was close to the predicted values, indicating that the model was adequate for the Monacolin K production. The yield of Monacolin K in *Monascus* can be increased by adding metal ions during industrial production.

Keywords: divalent metal cations; Monacolin K; *Monascus* M1; response surface methodology

Monascus species are traditional food in Asia that produce various useful secondary metabolites (SMs) including pigments (natural colouring agents), γ-aminobutyric acid (GABA, a hypotensive agent), and monacolins (a group of anti-hypercholesterolemic agent) (Shi & Pan 2011; Patakova 2013; Wang *et al.* 2015). Monacolin K (MK), is a strong inhibitor of HMG-CoA reductase, which can mediate the rate-limiting reaction of cholesterol biosynthetic (Wang *et al.* 2003; Seraman *et al.* 2010; Yang & Mousa 2012; Mulder *et al.* 2015). MK can also suppress the growth of human malignant glioma cell (Valera *et al.* 2005) and reduce the tumor progression and metastatic ability of murine lewis lung carcinoma cells (Ho & Pan 2009). To date, MK has been widely used as a potent drug for lowering blood cholesterol, and it is one of the best-selling pharmaceuticals in the USA (Panda *et al.* 2010; Zhang *et al.* 2015). Furthermore, *Monascus* fermented rice has also developed into a functional food for cholesterol lowering agents (Lin *et al.* 2011; Priatni *et al.* 2014). To enhance the production of MK, some researches focused on gen-

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eral culture conditions, such as different culture media, temperature and pH (Lee *et al.* 2007; Sang *et al.* 2015). And some researches focused on the effects of nature and concentrations of both carbon and nitrogen sources on the biosynthesis of lovastatin (Hajjaj *et al.* 2001; López *et al.* 2003). Inorganic salts are the other important factors in the growth of microorganisms. Fe^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+} and Mn^{2+} are some essential divalent metal cations which have been proved to be necessary to the growth of fungal cell and markedly affect the production of metabolite (Haq *et al.* 2002; Chu *et al.* 2004).

The divalent metal cations are important to support the growth of cell and the production of lovastatin in *Aspergillus terreus* (Hajjaj *et al.* 2001; López *et al.* 2003; Porcel *et al.* 2008). Jia *et al.* (2009) showed that all of Fe^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+} could promote the cell growth and lovastatin biosynthesis in different extents. Zn^{2+} could enhance the biosynthesis of lovastatin and increasing 17.6 mg/l per mM. Cu^{2+} could inhibit the cell growth, but they had no influence on biosynthesis of lovastatin in *A. terreus*. MK is a fungal polyketide produced by *Monascus* spp. and its chemical structure is the same as that of lovastatin produced by *A. terreus* (Endo 1980; Yoshizawa *et al.* 2002). However, there are a few reports focus on the effect of the divalent metal cations on MK production in *Monascus* spp.

The objective of this work is to investigate the influence of three kind of the divalent metal cations, Mn^{2+}, Zn^{2+}, and Mg^{2+} on the production of MK and the optimized formula of bivalent metal cations for food and pharmaceutical industry was established to improve the maximum yield of MK from *Monascus* spp.

MATERIAL AND METHODS

Strains and culture conditions. *Monascus buliginosus* M1 strain isolated from rice and preserved in our laboratory was used in this study. It was activated on malt extract agar for 7 days at 30°C. Spores were harvested with sterile water (2 ml) and inoculated into 100 ml of seed medium (6 g of glucose, 2 g of peptone, 1 g of KH_2PO_4, 1 g of $NaNO_3$, and 0.5 g of $MgSO_4 \cdot 7H_2O$) in 250 ml flask. The cultures were incubated at 30°C for 48 h with shaking at 180 rpm. The basic medium was the seed medium agar (SMA) contained 0.3% $ZnSO_4$, 0.5% $MgSO_4$, and 0.2% $MnSO_4$. For MK production, 1 ml of the seed fungus liquid was inoculated on SMA covered by cellophane and incubated at 30°C for 6 days, and then 25°C for 20 days.

Determination of the biomass. The biomass of the mycelium at different kinds of SMA rich of Zn^{2+}, Mg^{2+} and $Mn^{2+}$was determined by lifting up the cellophane to obtain the mycelia. The biomass yield was determined by gravimetric method after drying at 50°C overnight to a constant weight. The biomass was measured every 3 days. All samples were determined at least three times.

Extraction and HPLC analysis of MK. The spore suspension of *Monascus* M1 was inoculated on SMA and incubated at 30°C for 6 days, and then 25°C for 20 days. Then the sample was dried by oven (DGG-101-2B; Tianjin Tianyu experimental instrument Co.;) heating at 50°C and ground into powder by mortar and pestle. About 0.5 g of the powder was transferred into a 10 ml centrifuge tube. The preparations were extracted in triplicate with 3 ml of 75% ethanol for 30 min on an ultrasonic bath (KQ8200B; Kunshan ultrasonic instrument Co.,China) and subsequently centrifuged at 2150 *g* for 15 min (model Anke TDL-5-A; Shanghai Anting Scientifi c Instrument Factory Co., China). The total suspension was merged and passed through regenerated cellulose 0.22-μm filters (ANPEL Laboratory Technologies Inc., China).

MK was analysed by High Performance Liquid Chromatography (HPLC). Eclipse XDB-C18 column (5 μm, 4.6 ×150 mm; Agilent, USA) was used at 25°C, and isocratic elution was performed for 30 min using 0.05% methanoic acid in water/acetonitrile (40/60, v/v) as the mobile phase at 1 ml/min. Samples were monitored by a DAD detector at 270 nm. Sigma standard was adopted.

Single factor experiment. On the basis of the single factor experiment, the experimental design and study were carried out with Box-Benhnken Design (BBD). The Zn^{2+}-SMA was the SMA containing different concentration (0, 0.1, 0.2, 0.3, 0.4, and 0.5%) of $ZnSO_4$. The Mg^{2+}-SMA was the SMA containing different concentration (0, 0.25, 0.5, 0.75, and 1%) of $MgSO_4$. The Mn^{2+}-SMA was the SMA containing different concentration (0, 0.1, 0.2, 0.3, 0.4, and 0.5%) of $MnSO_4$. The concentration of MK was determined every 3 days. All samples were determined at least three times.

Box-Behnken design (BBD). Box-benhnken design method can be used to obtain the regression equation and analyze the optimal combination through experimental design, thus the statistical method for solving the multivariable problem is obtained. Taking Zn^{2+}, Mn^{2+}, Mg^{2+} three factors as independent vari-

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ables, in order to get a combination of metal ions, the response surface of three factors and three levels was designed by using the Design Expert 8.0.6 (Stat-Ease, Inc., USA), the optimum solution was added to the greatest degree of increase production of MK.

RESULTS AND DISCUSSION

Effect of divalent metal ions on biomass. The effect of Mn^{2+} on biomass of *Monascus* presented in Figure 1A. The effect of Zn^{2+} on biomass of *Monascus* is presented in Figure 1B. The effect of Mg^{2+} on biomass of *Monascus* is presented in Figure 1C. The results showed that at the range of 0–5% of Mn^{2+}, the biomass of *Monascus* M1 was increased with the increase of content of Mn^{2+}. The biomass weight of *Monascus* M1 was the heaviest (1.81 g/dish) when the concentration of Mn^{2+} reached 0.5%. The biomass weight of *Monascus* M1 was the lightest when there was no Mn^{2+}. These results indicated that Mn^{2+} could promote the growth of *Monascus* M1. Battah *et al.* (2015) showed that Mn^{2+} can increase the biomass by increasing the lipids of the green microalga *Chlorella vulgaris*.

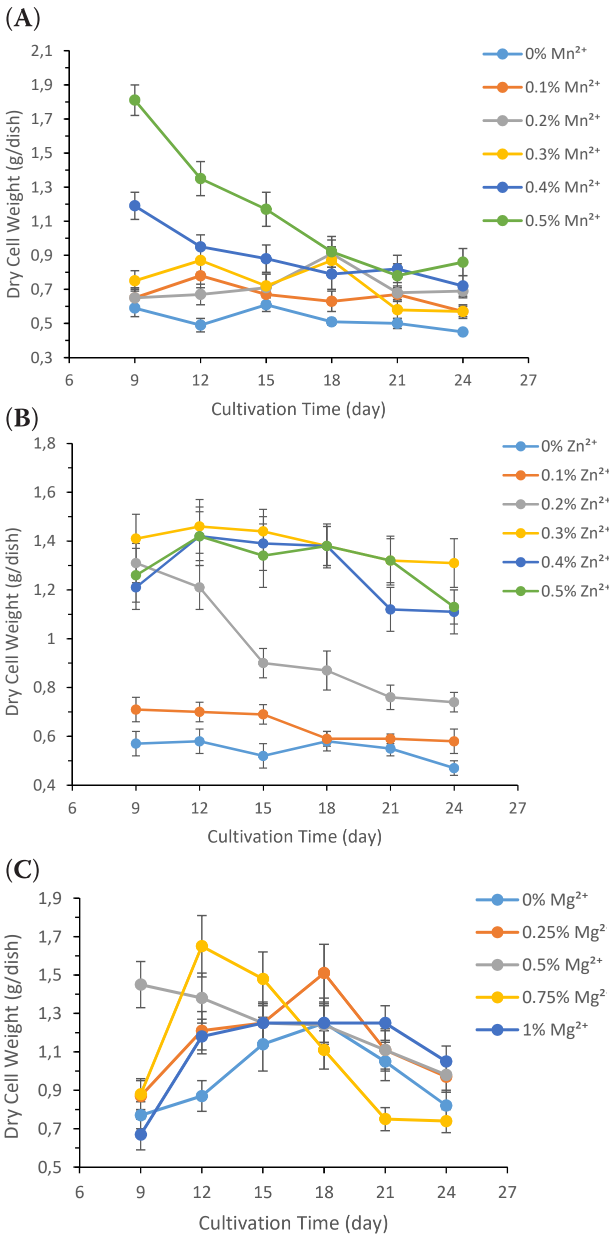

Figure 1. Effects of Mn^{2+} (**A**), Zn^{2+} (**B**), and Mg^{2+} (**C**) on *Monascus* biomass

Figure 1B shows an important relationship between the biomass weight and the concentration of Zn^{2+}. When the concentration of Zn^{2+} was increased from 0.3% to 0.5%, the biomass weight of *Monascus* M1 increased significantly. The biomass weight of *Monascus* M1 increased not significantly when the concentration of Zn^{2+} was increased from 0% to 0.2%. When the concentration of Zn^{2+} reached at 0.3%, the biomass weight of *Monascus* M1 was the heaviest (1.46 g/dish) at the 12th day. These results indicated that Zn^{2+} could promote the growth of *Monascus* M1 within a certain concentration range. Sun and Wang (2009) showed that the Zn^{2+} concentration at 10 μmol/l was essential to maintain optimal growth of the cells, but a higher concentration of Zn^{2+} inhibited the growth of *Isochrysis galbana*. The experimental results showed that the biomass increases to a certain concentration range of Mn^{2+}, Zn^{2+}, which was consistent with our experimental results.

The biomass of *Monascus* M1 showed a trend of first increasing and then decreasing in the concentration of Mg^{2+} (Figure 1C). But there was no significant effect between the biomass of *Monascus* M1 and the concentration of Mg^{2+}. Tang *et al.* (2018) showed that Mg^{2+} stress significantly reduced the total biomass in a dose-dependent manner within a certain concentration range in *Acidithiobacillus ferrooxidans*. But in our present study, we did not find any correlation between the biomass and the concentration of Mg^{2+} in *Monascus* M1 (Figure 1C).

Effect of divalent metal ions on the concentration of MK. The effect of divalent metal ions on the concentration of MK is presented in Figure 2, the effect of Mn^{2+} on the concentration of MK (Figure 2A), and the effect of Zn^{2+} and Mg^{2+}on the concentration of MK is presented in Figure 2B and Figure 2C, respectively. Figure 2 present, that the concentration of MK increased first and then decreased later. The Figure 2A showed the concentration of MK was the highest

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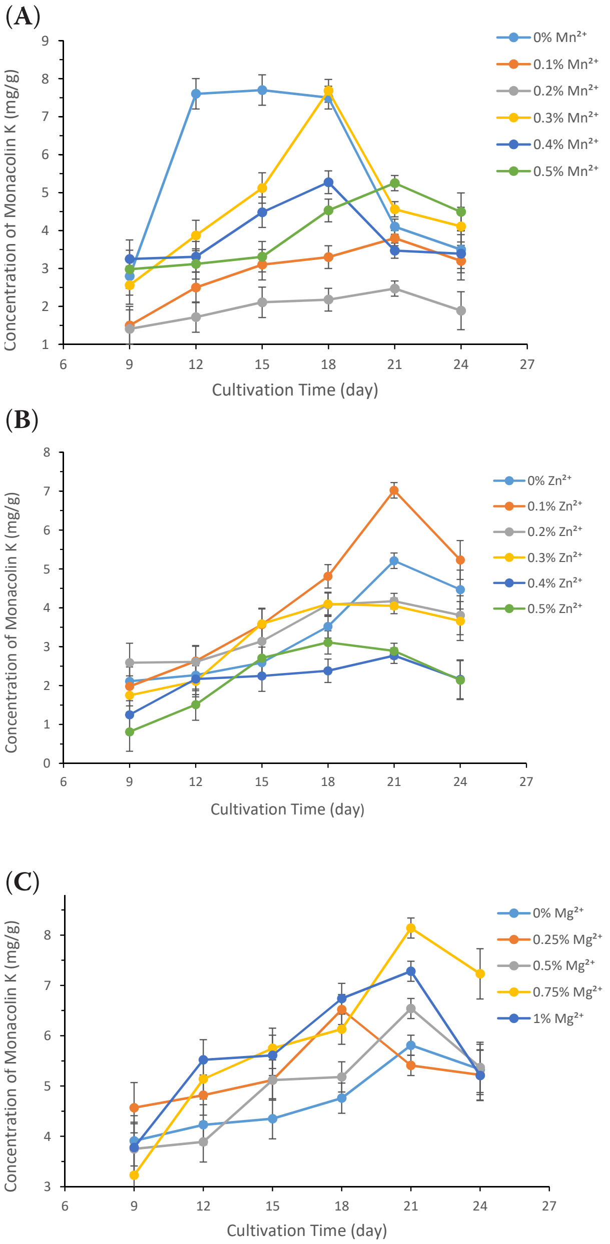

Figure 2. Effects of Mn^{2+} (**A**), Zn^{2+} (**B**), and Mg^{2+} (**C**) on Monacolin K production in *Monascus*

when the concentration of Mn^{2+} reached 0.3% at the 18th day, which can be 7.68 mg/g dry cell weight (DCW). These results indicated that Mn^{2+} can promote MK production of *Monascus* M1 within a certain concentration range. The Figure 2B showed the concentration of MK was the highest when the concentration of Zn^{2+} reached 0.1% at the 21th day, which can be 7.02mg/g DCW. These results indicated that Zn^{2+} can promote MK production of *Monascus* M1 within a certain concentration range. The Figure 2C shows that the concentration of MK was the highest when the concentration of Mg^{2+} reached 0.75% at the 21th day, which can be 8.14mg/g DCW. Jia *et al.* (2009) found that Mn^{2+}, Zn^{2+}, and Mg^{2+} can promote the production of lovastatin in *Aspergillus terreus*, which is consistent with our experimental results. It can be inferred that Mn^{2+}, Zn^{2+}, and Mg^{2+} can promote the MK production by promoting the accumulation of precursors in MK biosynthesis, Monacolin J and 2-methylbutyric acid (Jia *et al.* 2009).

Box-Behnken design (BBD) experiment. The BBD experiments were performed to evaluate the significance of three parameters (concentration of Mn^{2+}, Concentration of Zn^{2+}, and concentration of Mg^{2+}). Table 1 shows the three factors and their levels. The BBD experimental design and results sre shown in Table 2.

Table 1. Evaluated factors and their levels in Box-Behnken design

Coded value	Factor (%)		
	A	B	C
–1	0.2	0	0.5
0	0.3	0.1	0.75
1	0.4	0.2	1

A – Mn^{2+}; B – Zn^{2+}; C – Mg^{2+}

Table 2. Box-Behnken experimental design and results

Run	A	B	C	$Y(M)^a$ (mg/g)
	(%)			
1	0	–1	1	6.62
2	1	–1	0	6.89
3	0	–1	–1	8.51
4	0	1	1	9.20
5	–1	0	–1	8.19
6	1	1	0	8.02
7	1	0	1	8.54
8	0	0	0	8.97
9	–1	0	1	7.77
10	0	0	0	8.89
11	1	0	–1	8.42
12	–1	1	0	7.21
13	0	0	0	8.99
14	0	0	0	9.01
15	0	1	–1	7.11
16	0	0	0	9.11
17	–1	–1	0	6.62

[a]concentration of Monacolin K; for abbreviations see Table 1

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An initial response surface model of the Monacolin K concentration from the percentage composition of divalent metal ions was generated by the Design-Expert 8.0.6 software according to the following Equation (1):

$$Y(M) = 4.52850 + 40.37A - 8.485B - 4.582C + 13.5AB + 5.4AC + 39.8BC - 71.95A^2 - 108.95B^2 - 0.712C^2 \quad (1)$$

where: A – efect of Mn^{2+}; B – efect of Zn^{2+}; C – efect of Mg^{2+}; AB – interaction of Mn^{2+} and Zn^{2+}; AC – interaction of Mn^{2+} and Mg^{2+}; BC – interaction of Zn^{2+} and Mg^{2+}; A^2 – quadratic term of Mn^{2+}; B^2 – quadratic term of Zn^{2+}; C^2 – quadratic term of Mg^{2+}

Analysis of variance (ANOVA) was then performed to retain the significant terms ($P < 0.05$) and exclude the insignificant terms ($P > 0.05$). In general, variables of a large coefficient with a small P (< 0.05) were considered as significant influence. So, C^2 ($P = 0.4557 > 0.05$) was excluded and the optimized results of statistical regression analysis was shown in Table 3. This result indicated that A, B, AB, AC, BC, A^2, B^2 were significant. Consequently, a simplified is expressed by following Equation (2):

$$Y(M) = 4.88684 + 40.51053A - 8.43816B - 5.65C + 13.5AB + 5.4AC + 39.8BC - 72.18421A^2 - 109.18421B^2 \quad (2)$$

where: A – efect of Mn^{2+}; B – efect of Zn^{2+}; C – efect of Mg^{2+}; AB – interaction of Mn^{2+} and Zn^{2+}; AC – interaction of Mn^{2+} and Mg^{2+}; BC – interaction of Zn^{2+} and Mg^{2+}; A^2 – quadratic term of Mn^{2+}; B^2 – quadratic term of Zn^{2+}

The model F was 130.7 which indicated that the model is significant ($P < 0.0001$). The lack of fit ($P = 0.1522$) suggested that it was an adequate model to accurately predict the response variable. The regression coefficient $R^2 = 0.9924$ also indicated that the resulting model to be a good fit for MK production. According to the F of response surface analysis, the order of divalent metal ions significantly affects the MK production is: $Zn^{2+} > Mn^{2+} > Mg^{2+}$.

Figure 3 is the 3D surface and planar contour plots between every two independent variables on the basis of Equation 2. Figure 3A shows the effect of Mn^{2+} and Zn^{2+} on the yield of MK. When Zn^{2+} fixed, the yield of MK increased with the increase of Mn^{2+} until reaching a maximum and then decreased. Similarly, Zn^{2+} caused an initial increase and then decrease in the yield of MK. This result indicated that Mn^{2+} and Zn^{2+} were important variables for MK production. It can be seen from the variation density of planar contour plots, the effect of Zn^{2+} on the production of MK was stronger than the one of Mn^{2+}. Figure 3B shows the effect of Mn^{2+} and Mg^{2+} on the yield of MK. When Mn^{2+} was fixed, the yield of MK increased with the increasing of Mg^{2+} until reaching a maxi-

Table 3. Results of statistical regression analysis

Source	Sum of squares	df	Mean square	F	P prob > F	Significance
Model	13.33	8	1.67	130.70	< 0.0001	significant
A	0.54	1	0.54	42.43	0.0002	significant
B	1.05	1	1.05	82.49	< 0.0001	significant
C	1.250E-003	1	1.250E-003	0.098	0.7622	–
AB	0.073	1	0.073	5.72	0.0437	significant
AC	0.073	1	0.073	5.72	0.0437	significant
BC	3.96	1	3.96	310.72	< 0.0001	significant
A^2	2.20	1	2.20	172.62	< 0.0001	significant
B^2	5.03	1	5.03	394.94	< 0.0001	significant
Residual	0.10	8	0.013	–	–	–
Lack of fit	0.077	4	0.019	3.06	0.1522	n.s.
Pure error	0.025	4	6.280E-003	–	–	–
Cor total	13.43	16	–	–	–	–
R-squared	0.9924	–	–	–	–	–
Adj R-squared	0.9848	–	–	–	–	–
Pred R-squared	0.9411	–	–	–	–	–
Adeq precision	33.053	–	–	–	–	–

A – efect of Mn^{2+}; B – efect of Zn^{2+}; C – efect of Mg^{2+}; AB – interaction of Mn^{2+} and Zn^{2+}; AC – interaction of Mn^{2+} and Mg^{2+}; BC – interaction of Zn^{2+} and Mg^{2+}; A^2 – quadratic term of Mn^{2+}; B^2 – quadratic term of Zn^{2+}

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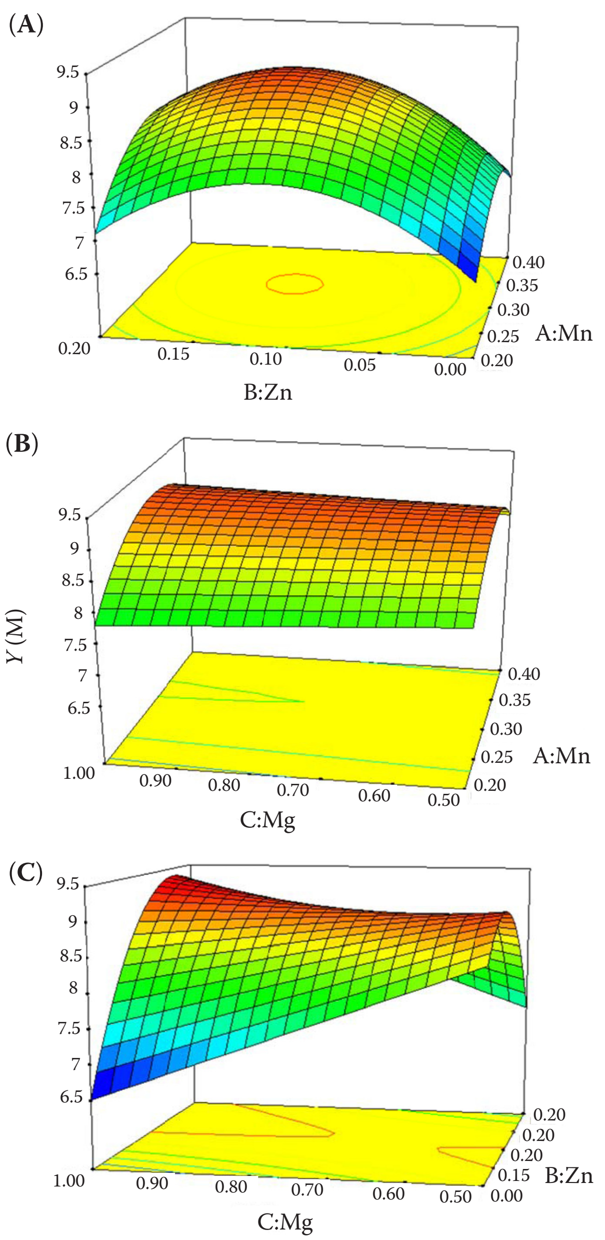

Figure 3. Three-dimensional contour plots showing the experimental factors and their mutual interactions: effect of Mn^{2+} and Zn^{2+} (**A**), $Mn^{2+}$and Mg^{2+} (**B**), Zn^{2+} and Mg^{2+} (**C**) on the yield of Monacolin K

mum and then decreased. It can be seen from the variation density of planar contour plots, the effect of Mn^{2+} on the production of MK was stronger than the one of Mg^{2+}. Figure 3C shows the effect of Zn^{2+} and Mg^{2+} on the yield of MK. When Mg^{2+} was fixed, the yield of MK increased with the increasing of Zn^{2+} until reaching a maximum and then decreased. It can be seen from the variation density of planar contour plots, the effect of Zn^{2+} on the production of MK was stronger than the one of Mg^{2+}. The maximum yield of MK was calculated as 9.46461 mg/g in the following optimum divalent metal ions percent: Mn^{2+} of 0.33%, Zn^{2+} of 0.16% and Mg^{2+} of 1%. In the validation, the yield of MK reached 9.57 mg/g (n = 3) under this condition (Mn^{2+} 0.33%, Zn^{2+} 0.16%, and Mg^{2+} 1%) which were close to the predicted values, indicating that the model was adequate for the MK production.

CONCLUSION

Many factors could influence secondary metabolites' biosynthesis in fungi including medium components, pH and temperature. Mineral salts, especially divalent metal cations, as crucial environmental signals, could regulate the growth and secondary metabolites' biosynthesis in fungi. In this study the three kinds of divalent metal cations, Mn^{2+}, Zn^{2+}, and Mg^{2+}, were selected to study the effect on biomass and MK production of *Monascus* M1. Then the concentration of the three kinds of divalent metal cations were optimized by response surface methodology, and the optimum conditions for the highest yield of MK were determined.

The results indicated that $Mn^{2+}$and Zn^{2+} could promote the cell growth of *Monascus* M1. The Mn^{2+}, Zn^{2+} and Mg^{2+} could enhance MK production of *Monascus* M1 within a certain concentration range. The optimum concentrations of the three divalent metal ions for MK production were selected as follow: Mn^{2+} 0.33%, Zn^{2+} 0.16% and Mg^{2+} 1%. In the validation, the yield of MK was 9.57 mg/g. It indicated that the yield of MK in *Monascus* can be increased by adding metal ions during industrial production.

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