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Improving the Ratio of Monacolin K to Citrinin Production of *Monascus purpureus* NTU 568 under Dioscorea Medium through the Mediation of pH Value and Ethanol Addition

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Using dioscorea root as substrate of *Monascus* species was found to stimulate monacolin K (cholesterol-lowering agent) formation in our previous study, but the mycotoxin−citrinin has never been studied. This study used dioscorea root as the liquid medium to culture *Monascus purpureus* NTU 568 using a 6.6 L jar fermentor. Culture pH value, dioscorea concentration, and ethanol concentration were used as the factors of response surface methodology (RSM) to investigate the optimal culture condition for high monacolin K production and low citrinin formation. Monacolin K and citrinin formation of *M. purpureus* NTU 568 under submerged dioscorea medium were respectively found to be significantly formed by 148% and 147%, as compared to that under submerged rice medium. The reason is due to the pH value (3.5) of dioscorea medium involved in the formation of *Monascus* cell amount and secondary metabolite. RSM results further indicated that lowering the pH value to 2.5 would result in high monacolin K and citrinin concentrations as well as high biomass in fixed dioscorea amount, implying that pH value may stimulate the formation of monacolin K and citrinin through increasing *Monascus* cell amount. Lowering dioscorea and ethanol concentration was able to increase the ratio of monacolin K level to citrinin level. The optimal culture condition (pH 5.7, 1% dioscorea concentration, and 0.5% ethanol concentration) would increase monacolin K levels to 27.9 mg/g (by 47%) and decrease citrinin level to 2.15 μg/g (by 54%), as compared to control conditions (pH 3.5, 5% dioscorea, and ethanol free).

KEYWORDS: ***Monascus*; dioscorea; monacolin; citrinin; response surface methodology**

INTRODUCTION

Monascus species has been used as the traditional food fungus in Eastern Asia for several centuries. Since the worthful secondary metabolite, monacolin K, was found to inhibit the biosynthesis of cholesterol, *Monascus*-fermented rice know as red mold rice (RMR) was gradually developed as the popular functional food for hypolipidemia (*1*, *2*). However, red mold dioscorea (RMD) known as *Monascus*-fermented dioscorea is a novel and worthful *Monascus*-fermented product. Our previous study found that higher monacolin K production could be obtained in the RMD. In addition, monascin, a *Monascus* yellow pigment with anti-inflammatory potential, would be significantly formed and substitute for the red pigment as the major pigment of RMD (*3*). Although dioscorea substrate causes *Monascus* species to form higher monacolin K production, another secondary metabolite, citrinin, a mycotoxin with hepatotoxicity and nephrotoxicity, is probable to be formed with the increase of monacolin K and pigment (*4*, *5*). The investigation of citrinin formation of RMD has never been mentioned in current studies. Monacolin K production is the marker for the worth of *Monascus* product, but citrinin concentration is related to the food safety. Investigating a culture condition for obtaining the RMD with high monacolin K and low citrinin levels should be an important topic in the development of *Monascus* functional food.

The composition of starch or the type of carbon source would directly affect the growth of *Monascus* species (*6*, *7*). Dioscorea species is a member of the monocotyledonous family *Dioscoreaceae*. Over 70% of starch was included in the dried dioscorea. Amylose is the major type of starch in dioscorea, and its content in the dioscorea is more than that in the rice grain (*8*). The amylose starch is also suggested as the suitable carbon source to be used by microorganism (*3*). In general, amylose molecules consisting of single mostly unbranched chains with 500−20 000 α-(1−4)-D-glucose units decomposed easily and were used by microorganism (*9*). This characteristic may result in a comfortable environment for *Monascus* species to grow. In addition, mucilage is an important material in dioscorea, which includes various kinds of amino acids (aspartic acid, glutamic acid, leucine, glycine, etc.) and carbohydrates (mannose, arabinose,

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glucose, xylose, and rhamnose) (*10*, *11*). Therefore, a large amount of carbon and nitrogen sources in the mucilage of dioscorea is enough to maintain the growth of *Monascus* species.

The above description indicated that many components of dioscorea were the suitable carbon or nitrogen source for the use of *Monascus* species (*3*). Although dioscorea could be the suitable substrate for the growth of *Monascus* species, culture condition would significantly affect protein expression and the levels of the secondary metabolite of *Monascus* species (*12*). Moreover, the different substrates would use significantly different culture conditions and form different metabolites for the fermentation. Even though the culture condition of *Monascus* species under rice substrate has been studied by several researchers (*4*, *10*, *11*), the different substrate should lead to significantly different results on the metabolism and production of monacolin K and citrinin.

Citrinin, monacolin K, and pigments all were derived from polyketide, so these metabolites levels should be easily affected by influencing the polyketide biosynthesis pathway (*13*, *14*). Our previous studies found that RMD includes high levels of yellow and orange pigment, however, that resulted from the dioscorea substrate with lower pH value (3.0) (*3*). The commercial dry dioscorea have a lower pH value, because the fresh dioscorea have been immersed with citrate solution for the prevention of browning (*15*). Although the dioscorea have a lower pH value, the acidic dioscorea is suitable for the growth of *Monascus* species. *Monascus* species grow at the pH value from 2.5 to 10.0, but the various pH values would result in the formation of various metabolite (*16*). RMR and RMD have been found to have a significant difference on the formation of pigments because of pH value. However, it should be further investigated whether the enhanced monacolin K level caused by dioscorea substrate is associated with low pH value or not.

In addition to the pH value, many culture conditions are also proven to affect citrinin and monacolin K formation of *Monascus* species (*4*). Various species of dioscorea have different character, so the optimal dioscorea species for the fermentation of *Monascus* species should be studied. Further, substrate concentration would affect the concentration of target product if that is too much to be completely consumed or not enough for the growth of *Monascus* species. Regarding the condition for lowering citrinin levels, ethanol added has been used to repress citrinin formation under the solid fermentation of *Monascus* species (*4*, *17*). According to the above description, dioscorea species, substrate concentration, pH value, and ethanol added are regarded as the influential factors for altering monacolin K and citrinin formation of *Monascus* species under submerged culture. *Monascus purpureus* NTU 568 was selected as the strain to ferment dioscorea medium using a jar fermentor. This study first examined the effect of these condition factors on monacolin K and citrinin levels of RMD, respectively, and further used response surface methodology (RSM) to investigate the optimal culture condition for obtaining the RMD with higher monacolin K and lower citrinin levels.

MATERIALS AND METHODS

Chemicals. Monacolin K (mevanolin) and citrinin were purchased from Sigma Chemical Co. (St. Louis, MO). LC grade acetonitrile was purchased from Merck Co. (Darmstadat, Germany). Tryptone, yeast extract, peptone, malt extract, PDA broth, and Bacto-agar were purchased from Difco Co. (Detroit, MI).

Microorganism and Seed Cultures. *M. purpureus* NTU 568 is a mutant with high monacolin K production. Its fermented RMR has been proven to have a hypolipidemic effect in hyperlipidemic hamster model by our previous studies (*1*). The other strains including *Monascus* sp. CA 505, *Monascus* sp. CH 001, *M. purpureus* NTU 601, *M. purpureus* NTU 301, *M. anka* M13, and *Monascus* sp. KT were isolated from red mold rice. The culture strains were maintained on Potato Dextrose Agar (PDA) slanted at 10 °C and transferred monthly.

Table 1. Process Variables and Levels in the Three-Variables Three-Levels Response Surface Design

variable	symbol	coded-variable level –1	0	1
pH value	X_1	2.5	5.0	7.5
dioscorea concentration (%)	X_2	1	3	5
ethanol concentration (%)	X_3	0.5	1.0	1.5

Table 2. Process Variables and Levels in the Three-Factor Three-Levels Response Surface Design of Secondary Metabolites Experiment

runs	independent variables (coded-level) pH value	dioscorea (%)	ethanol (%)
1	7.5 (1)	5 (1)	1.0 (0)
2	7.5 (1)	1 (–1)	1.0 (0)
3	2.5 (–1)	5 (1)	1.0 (0)
4	2.5 (–1)	1 (–1)	1.0 (0)
5	7.5 (1)	3 (0)	1.5 (1)
6	7.5 (1)	3 (0)	0.5 (–1)
7	2.5 (–1)	3 (0)	1.5 (1)
8	2.5 (–1)	3 (0)	0.5 (–1)
9	5.0 (0)	5 (1)	1.5 (1)
10	5.0 (0)	5 (1)	0.5 (–1)
11	5.0 (0)	1 (–1)	1.5 (1)
12	5.0 (0)	1 (–1)	0.5 (–1)
13	5.0 (0)	3 (0)	1.0 (0)
14	5.0 (0)	3 (0)	1.0 (0)
15	5.0 (0)	3 (0)	1.0 (0)

Source of Dioscorea and Rice. Dry dioscorea root slice (*Dioscorea batatas* Dence, *D. alata* L Chungkuochang, *D. alata* L Kaotien, *D. alata* L Shansan, and *D. alata* L var. purpurea) were provided by the Taichung District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan (DARES, Taichung, Taiwain). The long-grain rice (*Ipomoea batatas*) was purchased from a local supermarket (Taipei, Taiwan).

Seed Culture. Dry dioscorea root or rice was milled into powder (60−80 mesh) and used as the substrates of seed culture and submerged fermentation. The media of seed culture and submerged fermentation were prepared with the same species of dioscorea and rice. Seed cultures were prepared by transferring a loopful of spore from the PDA agar slant into a 500 mL Hinton flask containing 100-mL of 5% dioscorea medium or rice medium. The cultures were incubated at 30 °C for 48 h at 130 rpm.

Preparation of Culture Medium with or without Fixed pH Value. Four liters of medium including 5% substrate powder was prepared with or without mediation of pH value according to the pH condition of fermentation. The mediation of pH value of dioscorea (3.0, 7.0, or 9.0) or rice medium (3.0) was adjusted with 2.0 M citrate solution or 1.0 N sodium hydroxide.

Fermentation Using a Jar Fermentor. Five percent (v/v) spore suspension (10^7 spores/mL) was transferred into a 6.6 L jar fermentor (Firstek Co, Taipei, Taiwan) containing 4 L of medium. Fermentation with or without fixed pH value was carried out with aeration rate at 4 vvm, 30 °C, and agitation at 200 rpm for 12 days. The fixed pH value (3.0, 7.0, or 9.0) was maintained with 2.0 M citrate solution or 1.0 N sodium hydroxide during the whole stage of RMD and RMR fermentation. After fermentation, the RMD or RMR was collected and dried at 55 °C for 48 h and then weighted the total dry biomass for the calculation of the ratio of dry biomass weight to dioscorea added weight (B/D ratio).

Determination of Monacolin K and Citrinin Levels. One gram of dried RMR or RMD samples was extracted with 10 mL of ethanol

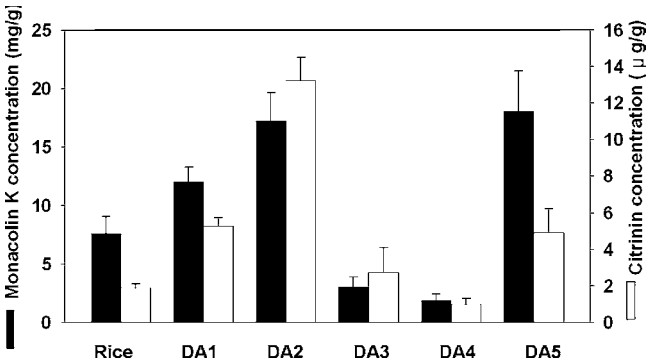

Figure 1. The effect of various species of dioscorea on monacolin K and citrinin levels by *M. purpureus* NTU 568. Fermentation was carried out using a 6.6 L jar fermentor containing 4 L of dioscorea medium (5%) at 30 °C, 4 vvm, and 200 rpm for 12 days ($n = 3$). DA1, *D. alata* L var. purpurea; DA2, *D. alata* L Kaotien; DA3, *D. alata* L Shansan; DA4, *D. alata* L Chungkuochang; DA5, *Dioscorea batatas* Dence.

at 65 °C for 30 min (*18*). The extracts (10% w/v) were further filtered with 0.45 μm filter and analyzed by HPLC. HPLC was performed according to the method described previously (*18*) and carried out on an HPLC system PU2089 plus (Jasco Co., Tokyo, Japan). A Discovery C_{18} column, 25 cm × 4.6 mm i.d., 5 μm (Bellefonte, PA), was used as the analytical column. The mobile stage consisting of 45% water, 55% acetonitrile, and 0.5% trifluoroacetate was eluted at a flow rate of 1.0 mL/min. Total monacolin K was detected using a UV detector UV2075 plus (Jasco Co.) set at 238 nm. For citrinin analysis, the fluorescence detector FL-1 (Rainin Co, Wobum, MA) was set with an excitation wavelength of 330 nm and an emission wavelength of 500 nm.

Pigment Estimation. Pigment concentrations are estimated using a spectrophotometer set at 400 nm for yellow pigment and 500 nm for red pigment. The results are expressed as optical density units per gram of dried medium multiplied by dilution factor (*19*).

Design of Experiments. To identify the optimum conditions, a Box-Behnken design (*20*) was selected. The crucial factors involved are pH value (X_1), dioscorea concentration (X_2), and the ethanol concentration (X_3). These factors, and the level at which the experiments were carried out, are given in **Tables 1**and **2**. A total of 15 runs with center points were generated. The central point of the design arrangement decided on was: pH value 5.0; dioscorea concentration 3%; ethanol added 1%. Control conditions were: pH value 3.0; dioscorea concentration 5%; ethanol free.

Response Surface Methodology. The analysis of data was carried out using response surface regression (RSREG) in Statistical Analysis System (SAS, Cary, NC). A second-order model was employed to fit the data individually for the responses Y_1 (monacolin K level), Y_2 (citrinin level), Y_3 (B/D ratio), and Y_4 (monacolin K level (mg/g)/citrinin level (μg/g) ratio, M/C ratio) by the general mode (*21*), with three factors, each factor coded to be in the range of −1, 0, +1.

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{11}(X_1)^2 + A_{22}(X_2)^2 + A_{33}(X_3)^2 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3$$

The coded points for this experimental design are given in **Tables 1** and **2**.

The model was evaluated in terms of statistically significant coefficient, r^2, and p values.

RESULTS AND DISCUSSION

Effect of Various Species of Dioscorea on Monacolin K and Citrinin Levels. Dioscorea may provide a good environment for monacolin K formation of *Monascus* species. In this study, various species of dioscorea were used as the medium of *M. purpureus* NTU 568 to test the monacolin K and citrinin production. The results were shown as **Figure 1**; various dioscorea species would result in a significant difference in monacolin K and citrinin levels. As compared to rice substrate, monacolin K production can be increased under DA1 (by 57.7%), DA2 (by 125.7%), and DA5 (by 148.3%) substrate, and decreased under DA3 (60.5%) and DA4 (75.3%) substrate. Various dioscorea species caused *M. purpureus* NTU 568 much formation of monacolin K, but also resulted in different increase levels on citrinin formation. Citrinin levels would be significantly increased by 180% and 600% under DA1 and DA2 substrate, and the increased ratio was higher than monacolin K. However, the results clearly clarified that DA5 (*Dioscorea batatas* Dence) was the most suitable substrate for obtaining higher monacolin K level and lower citrinin level.

Monacolin K Level of Various *Monascus* Species under Submerged Fermentation. Our previous study proved that using the solid fermentation method to produce RMD leads to higher monacolin K and monascin production (*3*). The medium of submerged culture only consisted of 5% dioscorea or rice powder to use a simple composition of medium to find out the reason why dioscorea stimulates monacolin K formation. This study investigated whether submerged culture using a jar fermentor was also able to exhibit the tendency toward the formation of monacolin K and monascin. As shown in **Table 3**, submerged culture exhibited higher monacolin K and citrinin production as well as yellow pigment under dioscorea medium than that under rice medium. Furthermore, submerged culture of *Monascus* species would perform stable and higher reproducibility on the production of secondary metabolites than solid culture and so was more suitable for the investigation of culture condition.

Effect of Dioscorea Medium and Rice Medium on the Change of Monacolin K Formation and pH Value. This study

Table 3. Production of the Secondary Metabolites on Rice Medium and Dioscorea Medium by Different *Monascus* Species

species	substrate	monacolin K (mg/g)	citrinin (μg/g)	red pigment (A_{500}/g)	yellow pigment (A_{400}/g)
M. sp. CA 505	rice	2.42	0.20	112	98.4
	dioscorea	8.45	0.93	105	132
M. purpureus	rice	7.62	1.89	143	120
NTU 568	dioscorea	18.92	4.67	115	135
M. sp. CH 001	rice	5.92	2.52	140	110
	dioscorea	17.65	8.70	120	149
M. purpureus	rice	0.58	0.46	72.0	60.0
NTU 601	dioscorea	3.54	1.57	61.1	88.3
M. purpureus	rice	0.51	0.37	93.5	77.6
NTU 301	dioscorea	3.08	0.76	110	93.0
M. anka M 13	rice	0.11	0.48	93.4	80.1
	dioscorea	0.63	0.37	50.6	63.1
M. sp. KT	rice	0.36	0.86	85.5	70.2
	dioscorea	3.89	0.97	66.9	93.5

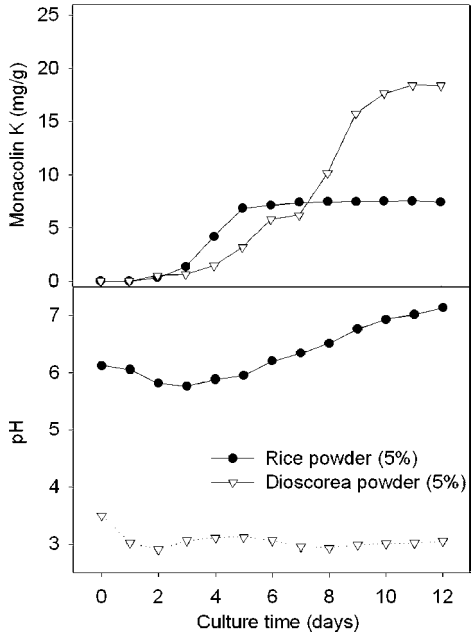

Figure 2. The comparison of the effect of dioscorea medium and rice medium on monacolin K formation and the change of pH value by *M. purpureus* NTU 568. Fermentation was carried out using a 6.6-L jar fermentor containing 4 L of medium including 5% substrate at 30 °C, 4 vvm, and 200 rpm for 12 days ($n = 3$).

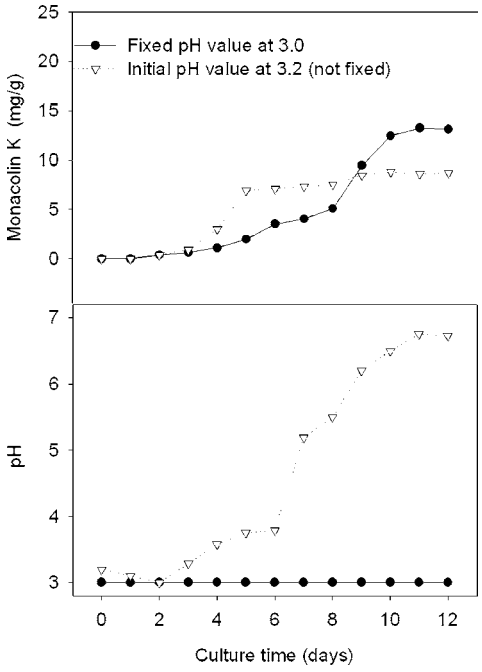

Figure 3. The comparison of the effect of rice medium with or without fixed pH value at 3.0 on monacolin K formation and the change of pH value by *M. purpureus* NTU 568. Fermentation was carried out using a 6.6-L jar fermentor containing 4 L of medium including 5% substrate at 30 °C, 4 vvm, and 200 rpm for 12 days ($n = 3$).

used a jar fermentor to continuously monitor the difference on the change of secondary metabolite production and pH condition between using rice medium and dioscorea medium during whole *Monascus* fermentation. As shown in **Figure 2**, the two media resulted in a significant difference in the change of pH value during the submerged fermentation. The pH value of liquid rice medium without adjustment is measured among 5.6−6.6, which ranged over the general pH condition for the culture of *Monascus* species in common with many studies (*4*, *6*). During submerged culture of *M. purpureus* NTU 568 under rice medium, the pH value would be decreased to 5.0−5.5 by organic acid formation in the initial stage and then gradually increased to neutral pH value in a later stage. However, dioscorea medium modulated the pH value toward a significantly different trend during submerged culture because of the citrate pretreatment of dioscorea. The initial pH value of the dioscorea medium is measured at 3.5 before inoculation. After inoculation, the pH value was decreased to 3.0 during the initial stage and then only exhibits a slight variation until the end of fermentation. *Monascus* species is able to grow under a wide range of pH values between 2.5 and 10.0. Previous researchers usually cultured *Monascus* species under pH 6.0−7.0 (*4*, *12*, *22*); an extreme culture condition at pH 3.0 has never been used to culture *Monascus* species for produce monacolin K. Therefore, pH value may be an important factor for stimulating monacolin K and monascin.

Effect of Rice Medium with or without Fixed pH Value at 3.0 on the Change of Monacolin K Formation. To investigate whether rice medium with acid condition as dioscorea medium can stimulate monacolin K formation or not, the pH value of rice medium was acidified to 3.2 before inoculation but not modulated during whole fermentation process. As shown in **Figure 3**, the pH value would be slightly decreased in the initial stage and observably increased from the middle stage to later stage. Finally, the pH value of acidified rice medium would be increased to neutral 6.8, which also led to less monacolin K production than RMD. *M. purpureus* stops forming monacolin K at a level about 6.8 mg/g as the pH value is increased over 5.0 during the later stage. This study further fixed the pH value of rice medium at 3.0 by a jar fermentor during whole fermentation to parallel a fixed acidic pH condition of dioscorea medium. In the results, monacolin K formation would be increased to 13.3 mg/g under rice medium with a fixed pH value at 3.0 until the later fermentation stage, and its trend was similar to that under the dioscorea medium (**Figure 3**). In addition to monacolin K, the appearance of the yellow pigments under acidic rice medium was also similar to that under dioscorea medium (**Figure 4**). In contrast, rice medium with neutral pH value would result in the appearance of the red pigments.

According to the above results, the acidic fermentation condition was one of the probable reasons why dioscorea resulted in higher monacolin K level. However, the monacolin K level formed under acidic rice medium was still less by 64.3% than that formed under dioscorea medium. The composition and character of dioscorea should make up for the lack of monacolin K formation.

Effect of Various pH Values of Dioscorea on Monacolin K and Citrinin Production. Although the pH value of dioscorea was an important factor to stimulate monacolin K and monascin formation in *Monascus* species, its influence on citrinin formation has never been reported by *Monascus*-related studies. The acidic (pH 3.0), neutral (pH 7.0), and alkaline (pH 9.0) dioscorea media were used to culture *M. purpureus* NTU 568 to investigate the effect of pH value of dioscorea medium on monacolin K and citrinin formation. As shown in **Figure 5**, monacolin K and citrinin formation under various pH values had a similar tendency toward positive production with decreasing pH value. Both monacolin K and citrinin levels produced under acidic dioscorea medium (pH 3.0) are higher by 102% and 114% than that produced under alkaline dioscorea medium (pH 9.0).

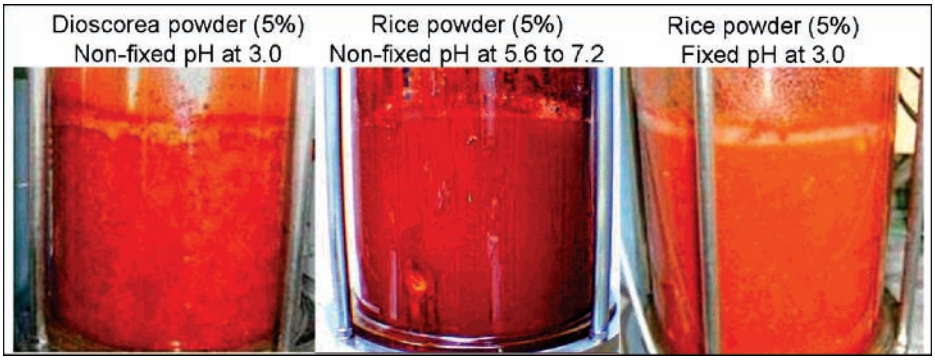

Figure 4. The comparison of the effect of dioscorea medium and rice medium with or without fixed pH value at 3.0 on the outward appearance by *M. purpureus* NTU 568. Fermentation was carried out using a 6.6-L jar fermentor containing 4 L of medium including 5% substrate at 30 °C, 4 vvm, and 200 rpm for 12 days ($n = 3$).

Table 4. Analysis of Variance for the Production of Citrinin, Monacolin K, B/D Ratio, and M/C Ratio with Various Culture Conditions[a]

		sum of square			
source	df[b]	monacolin K	citrinin	B/D ratio[c]	M/C ratio[d]
regression	9	15 658 439	10 124 580	3.38	120.15
residual	5	306 989	1 942 694	0.02	10.07
lack of fit	3	306 981	1 942 674	–0.04	8.65
pure error	2	7.84	19.71	0.06	1.42
variability explain (r^2)		0.98	0.84	0.99	0.92

[a] df: degree of freedom. [b] Analysis of variance from SAS statistics system. [c] The ratio of dry biomass to added dioscorea weight. [d] The ratio of monacolin K to citrinin level.

Optimum Culture Conditions Based on RSM. *Design of Experiments and Model.* This study used RSM to investigate optimum culture conditions taking account of three factors: culture pH value, dioscorea concentration, and ethanol concentration. The factors and coded values are given in **Tables 1** and **2**.

Regression Equation, r^2 Value of Model. Data from 15 experiments were used. The following equations, where the factors take their coded value, were obtained from regression analysis for the secondary metabolite concentrations:

$$\text{monacolin K (mg/g)} = 15.92 - 23.25X_1 - 28.06X_2 - 8.66X_3 - 24.63(X_1)^2 + 16.03(X_2)^2 + 1.50(X_3)^2 - 0.11X_1X_2 + 11.81X_1X_3 + 11.86X_2X_3$$

$$\text{citrinin } (\mu\text{g/g}) = 18.26 - 5.25X_1 - 22.13X_2 - 5.05X_3 + 7.13(X_1)^2 + 4.59(X_2)^2 - 3.52(X_3)^2 - 27.96X_1X_2 + 8.42X_1X_3 + 15.57X_2X_3$$

$$\text{B/D ratio} = 0.819 - 0.042X_1 - 0.578X_2 + 0.033X_3 - 0.064(X_1)^2 + 0.409(X_2)^2 - 0.056(X_3)^2 + 0.058X_1X_2 - 0.005X_1X_3 - 0.003X_2X_3$$

$$\text{M/C ratio} = 0.895 - 1.498X_1 + 0.174X_2 + 1.027X_3 - 0.493(X_1)^2 - 1.492(X_2)^2 + 0.146(X_3)^2 + 3.653X_1X_2 - 2.114X_1X_3 + 1.974X_2X_3$$

The variability in the responses accounted for by the factors (r^2 value) for the models is given in **Table 4**. The r^2 values of monacolin K level, citrinin level, B/D ratio, and M/C ratio were 0.98, 0.84, 0.99, and 0.92, respectively. Also, the test statistics p value for the overall regression is significant at the 5% level, which further supports that the model is adequate in approximating the response surface of the experimental design.

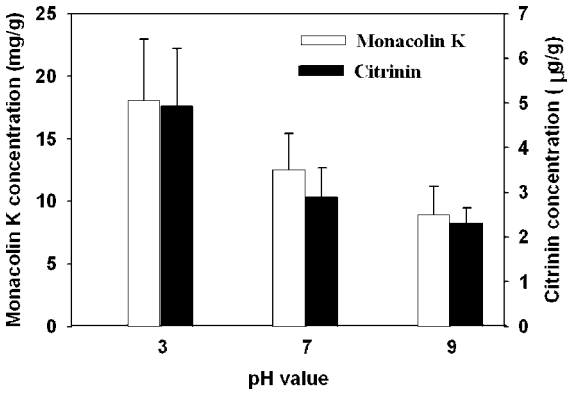

Figure 5. The effect of dioscorea medium with various pH values on monacolin K and citrinin formation by *M. purpureus* NTU 568. Fermentation was carried out using a 6.6-L jar fermentor containing 4 L of medium including 5% substrate at 30 °C, 4 vvm, and 200 rpm for 12 days ($n = 3$).

Effect of the Condition Factors on Monacolin K Formation. As shown in the **Figure 6**, monacolin K production would be gradually increased with the decreasing pH value under lower dioscorea concentration. Although dioscorea is a suitable substrate for monacolin K formation, increasing dioscorea concentration contrarily gained lower monacolin K concentration. The reason is that *Monascus* species was not able to completely consume and use up too much dioscorea substrate during the fermentation stage of 12 days. The monacolin K concentration of total solid material would be decreased if too much unfermented dioscorea substrate remained in the medium after fermentation. However, monacolin K formation would be repressed by increasing ethanol concentration. According to the effects of the three factors, the curve surface figure indicates that a higher monacolin K level could be obtained under pH 2.5 condition including 1% dioscorea and 0.5% ethanol.

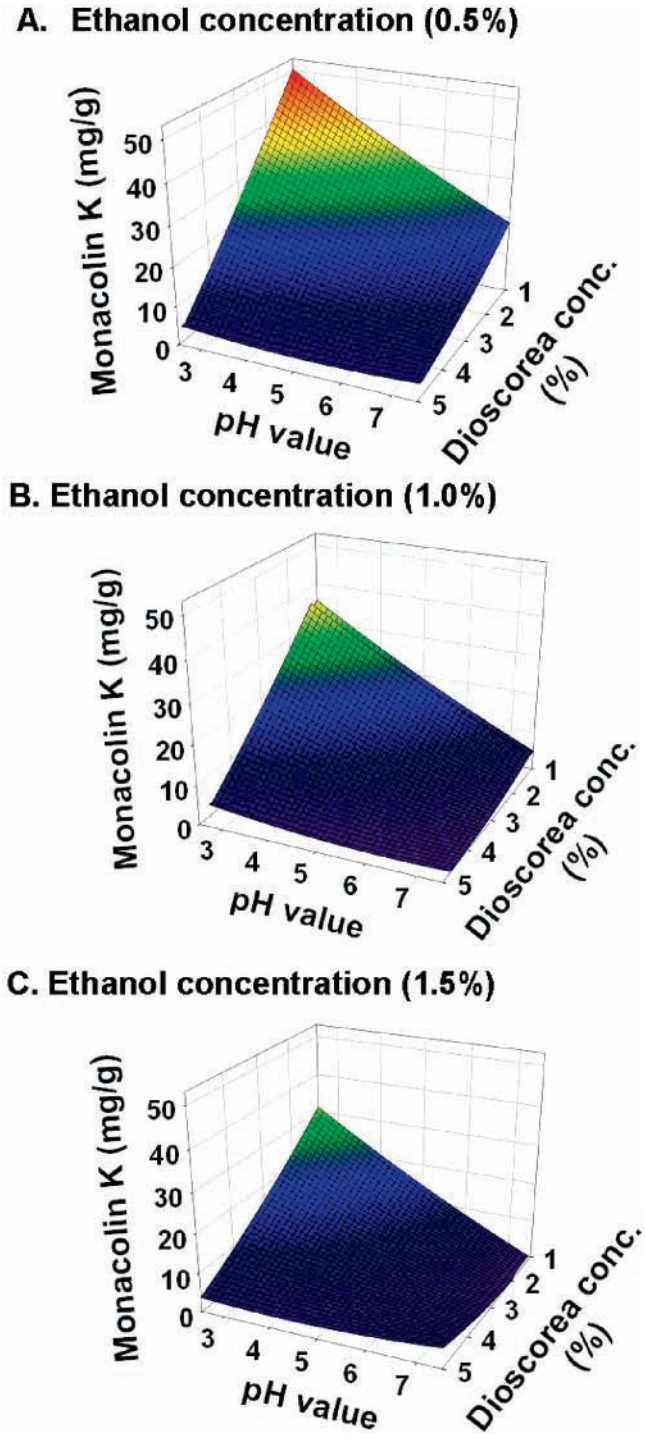

Figure 6. The response surface for the production of monacolin K at various pH values and dioscorea concentrations. Fermentation was carried out using a 6.6-L jar fermentor containing 4 L of medium at 30 °C and 200 rpm for 12 days.

Effect of the Condition Factors on Citrinin Formation. The effect of factors on citrinin formation is shown in **Figure 7**. The trend of citrinin formation affected by pH condition was similar to that of monacolin K formation. Citrinin level of RMD would be increased by decreasing pH value. Dioscorea concentration led to different results between monacolin K and citrinin formation. Citrinin level was stimulated to arrived at the most accumulation under 3% dioscorea concentration. Increasing ethanol concentration would also repress citrinin formation as the pH value was mediated between 4.0 and 7.5.

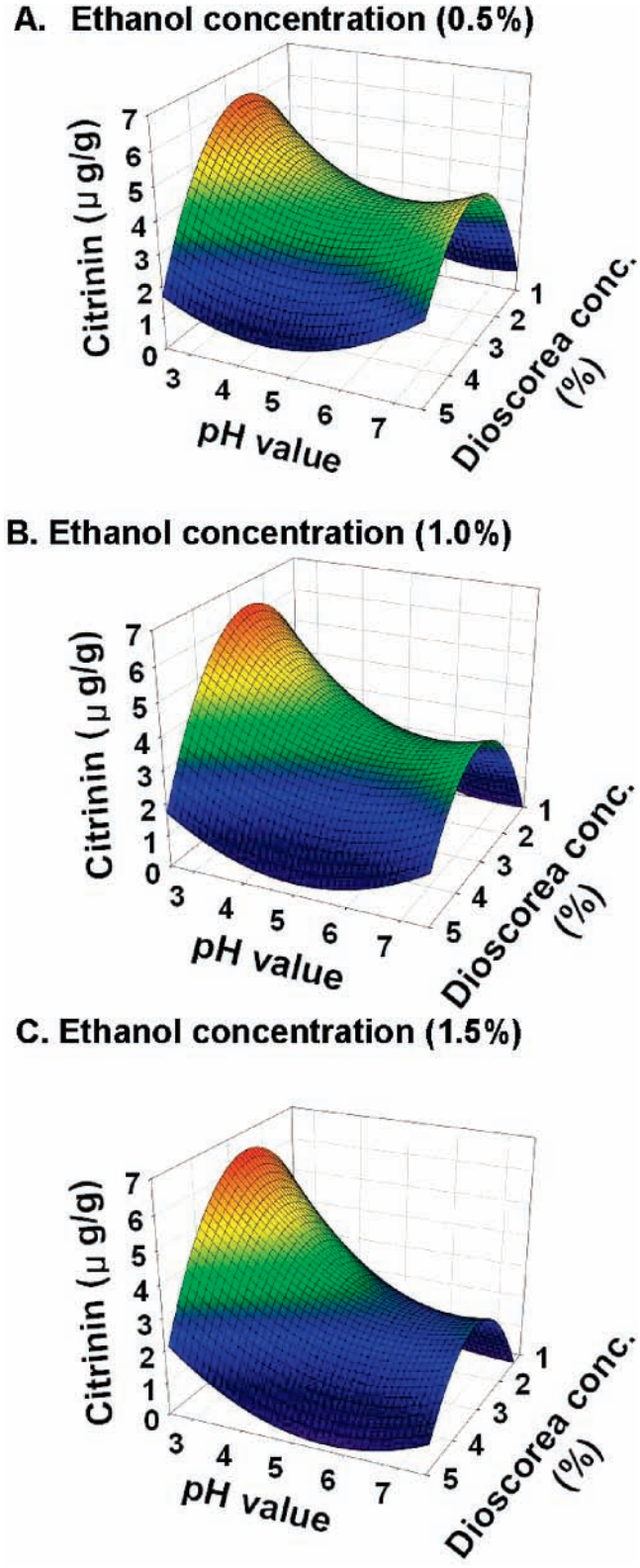

Figure 7. The response surface for the production of citrinin at various pH values and dioscorea concentrations. Fermentation was carried out using a 6.6-L jar fermentor containing 4 L of medium at 30 °C and 200 rpm for 12 days.

Lower citrinin level was also obtained as 1.0% or 5% dioscorea concentration was used as the submerged culture medium.

Effect of the Condition Factors on the Ratio of Dry Biomass to Dioscorea Added. The effect of culture condition on the formation of total pellet per fixed dioscorea added was also mentioned in this study. The ratio of dry biomass weight

to added dioscorea weight (B/D ratio) was used as the marker to evaluate the formation of *Monascus* cell amount. The increased ratio implied that more *Monascus* cell amount could be formed under the same amount of dioscorea added. The surface figure (**Figure 8**) clearly indicates that the ratio related to the formation of *Monascus* cell amount would be enhanced by decreasing pH value but not affected by the change of dioscorea concentration. According to these RSM results, monacolin K and citrinin formation stimulated by acidic pH value were due to the increase of the cell amount. In addition, ethanol added would not lead to a significant change of the ratio, suggesting that the ethanol-mediated monacolin K and citrinin formation may not associate with the change of *Monascus* cell amount, but the biosynthesis mediation of secondary metabolite.

Effect of the Condition Factors on the Ratio of Monacolin K Level to Citrinin Level. A well culture condition for *Monascus*-fermented product should result in higher monacolin K and lower citrinin concentration. In this study, the ratio of monacolin K level (mg/g) to citrinin level (μg/g) (M/C ratio) was calculated and used as the marker for screening the optimal culture condition. The RSM surface figures for M/C ratio are shown as **Figure 9**. Dioscorea concentration resulted in a more significant difference on the change of M/C ratio than pH value. Lower dioscorea concentration led to higher M/C ratio, which indicated that higher monacolin K and lower citrinin levels would be gained under this culture condition. Lower pH value would stimulate the formation of *Monascus* cell amount and result in higher monacolin K according to the above results, but the M/C ratio was still decreased because of the formation of higher citrinin levels (**Figure 9**). In addition, 0.5% ethanol concentration was the most suitable condition to gain higher M/C ratio.

Selection for the Optimal Culture Condition through the Comprehensive Conclusion of RSM Results. To obtain the optimal culture condition, the vertical views of three surface figures including **Figure 6A**, **7A**, and **9A** were overlapped and then used to select the optimal culture condition for obtaining higher monacolin K and lower citrinin levels. The overlap figure was shown as **Figure 10**. Higher monacolin K level could be obtained under lower dioscorea concentration and lower pH value. However, to obtain RMD including lower citrinin level, both higher pH value and lower dioscorea concentration should be used as the culture condition. Lower dioscorea concentration was an important condition for increasing the M/C ratio. According to these trends, l% dioscorea concentration was selected as one of the optimal culture conditions. However, pH value should not be modulated too low or too high for fear of resulting in high citrinin levels or low monacolin K levels. According to the surface figure, the pH value at 5.7 was a suitable condition for obtaining higher M/C ratio. The optimal culture condition was shown in **Figure 10**; culture medium including 1.0% dioscorea and 0.5% ethanol at pH 5.7 may result in the optimal fermented product including 29.14 mg/g monacolin K and 1.91 μg/g citrinin. The optimal culture condition based on RSM result was further used to verify the production of monacolin K and citrinin. As shown in **Table 5**, high monacolin K level at 27.90 mg/g and low citrinin level at 2.15 μg/g would be obtained in the RMD fermentation using the RSM condition. The M/C ratio was also increased from 4.05 to 12.98 using RSM condition.

The formation of citrinin is always a troublesome problem for the development of *Monascus* functional food (*23*). The safety of *Monascus* product containing high citrinin level was

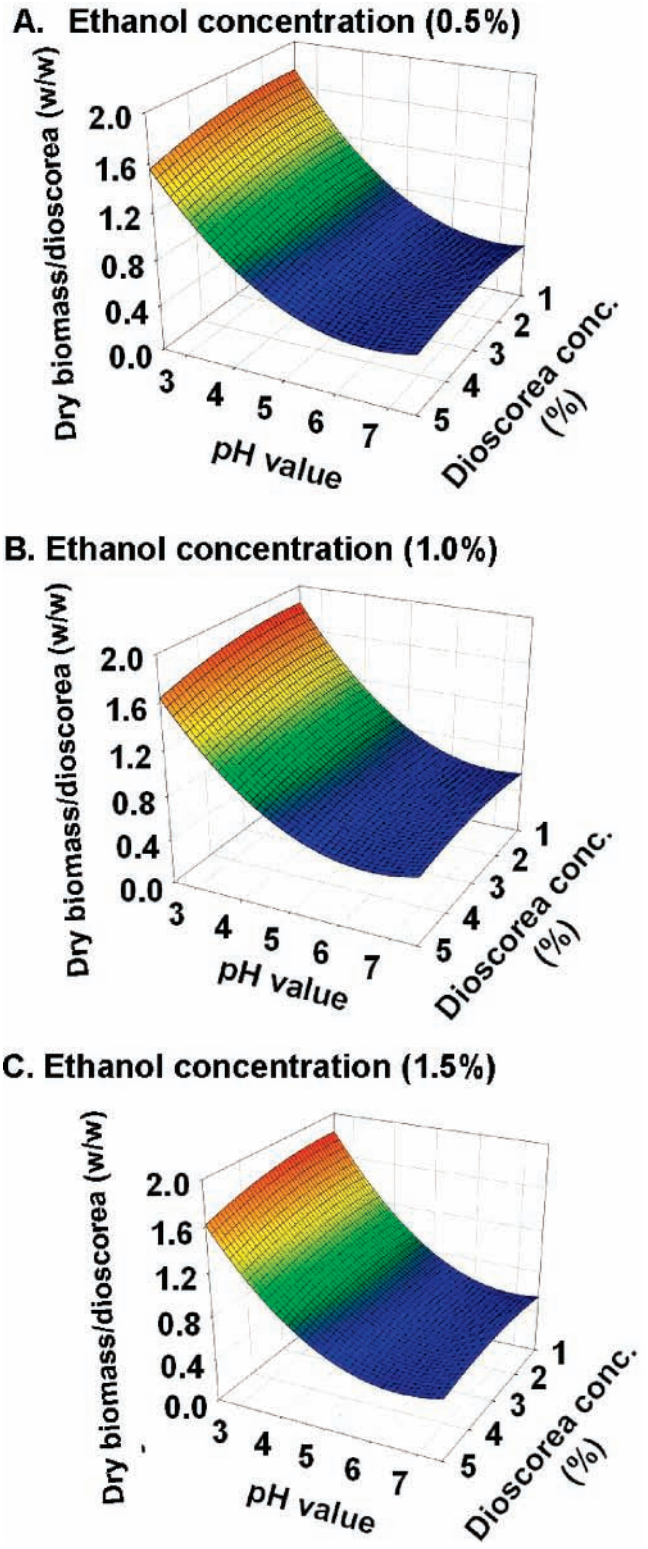

Figure 8. The response surface for the ratio of dry biomass to dioscorea weight at various pH values and dioscorea concentrations. Fermentation was carried out using a 6.6-L jar fermentor containing 4 L of medium at 30 °C and 200 rpm for 12 days.

a concern even though many health functions including hypolipidemia, hypotensive, anti-fatigue, and anti-cancer have been reported by many researchers and developed as the popular commercial product (*1*, *24*−*26*). Although previous studies have proven that monacolin K and monascin production would be increased under solid dioscorea substrate (*3*), more citrinin formation was found to be stimulated under submerged dioscorea

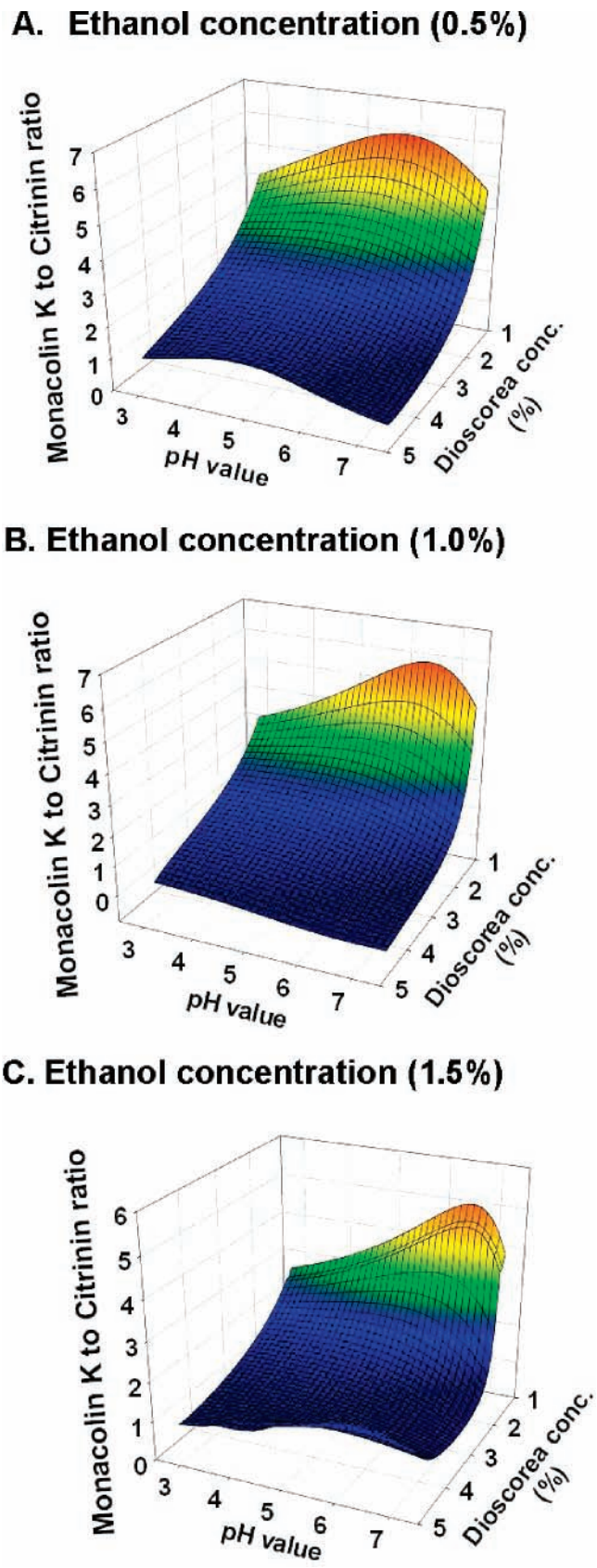

Figure 9. The response surface for the ratio of monacolin K to citrinin levels at various pH values and dioscorea concentrations. Fermentation was carried out using a 6.6-L jar fermentor containing 4 L of medium at 30 °C and 200 rpm for 12 days.

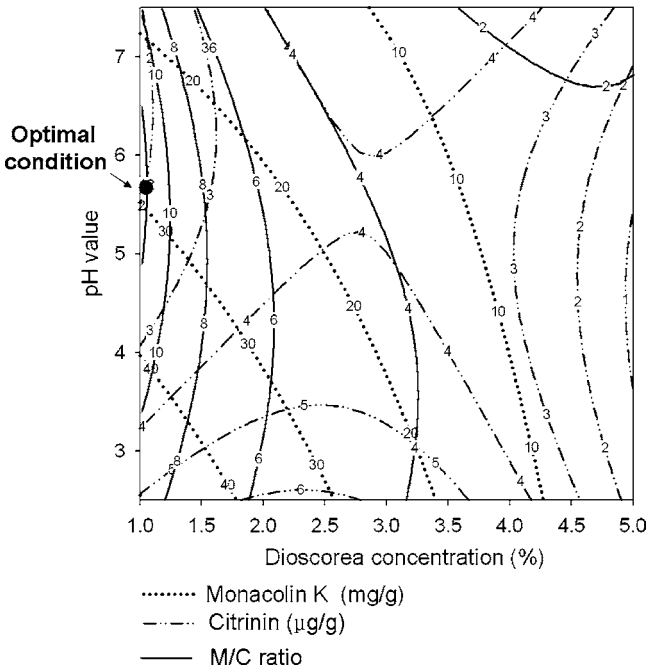

Figure 10. Overlap contour plot for monacolin K level, citrinin level, and for the ratio of monacolin K to citrinin level under various pH values and dioscorea concentrations (ethanol = 0.5%).

medium by this study (**Figure 1**). As compared to previous studies (*3*), the formation of secondary metabolites under solid culture and submerged culture has a similar trend and production. Furthermore, submerged culture using a jar fermentor is suitable to investigate the culture condition because stable and high reproducibility for the formation of secondary metabolite is easy to be gained.

RSM is a reliable and useful statistics methodology for the investigation of the optimal condition. RSM is usually used to investigate the effects of the condition factors on the changes of target product or markers through multiple views provided by the curve surface figure (*4*). This study uses RSM to investigate the effects of pH value, ethanol, and dioscorea concentration on the changes of monacolin K and citrinin levels, B/D ratio, and M/C ratio. The three factors (pH value, ethanol and dioscorea concentrations) were demonstrated to result in a significant change in monacolin K and citrinin levels by this study or our previous studies (*3*, *4*). Increasing ethanol concentration of medium would decrease the citrinin formation of *Monascus* species, but also reduce monacolin K level at the same time. According to the results of RSM, both citrinin and monacolin K levels would be decreased by increasing ethanol concentration. However, the ethanol-induced effects were not the cause of the reduction of cell amount but the interference of polyketide metabolism. Half percentage ethanol concentration is the most suitable condition for obtaining a higher M/C ratio.

Although dioscorea provides the source of carbon and nitrogen for the growth of *Monascus* species, monacolin K concentration would not be increased by increasing dioscorea concentration. In contrast, RSM results indicated that the monacolin K concentration of RMD would be diluted by the increasing dioscorea added because too much dioscorea was unable to be completely consumed by *Monascus* species. The biggest change caused by dioscorea concentration is M/C ratio in which higher monacolin K and lower citrinin levels could be obtained under 1% dioscorea concentration.

The commercial dioscorea root with low pH value (3.0) resulted from the pretreatment with citrate solution to prevent the browning (*15*). However, this food process may cause *M. purpureus* NTU 568 to produce high monacolin K production

Table 5. Effect of Culture Conditions on the Production of Monacolin K, Citrinin, and the Ratio of Monacolin K to Citrinin Level by *Monascus purpureus* NTU 568

culture condition				effector		
medium	pH range	substrate (%)	ethanol (%)	monacolin K (mg/g)	citrinin (μg/g)	M/C ratio[b]
rice	5.7–7.2	5		7.62	1.89	4.03
dioscorea	3.0–3.2	5		18.92	4.67	4.05
dioscorea[a]	5.7	1	0.5	27.90	2.15	12.98

[a] The optimal culture condition based on RSM. [b] The ratio of monacolin K to citrinin level.

at 18.92 mg/g under dioscorea medium. Fixing the pH value of rice medium at 3.0 also increased the monacolin K production to 13.3 mg/g. Fermentation condition with low pH value is probable to stimulate monacolin K formation even though it cannot be equal to that obtained under dioscorea medium. However, pH value condition is certainly one of the major reasons for modulating monacolin K production. The result of RSM clearly indicates that higher monacolin K formation induced by lower pH value was due to the increase of *Monascus* cell amount. *Monascus* species is able to grow at the pH value among 2.5−10.0. Previous researchers proved that the various types of *Monascus* pigments would be formed with the change of pH culture condition (*16*). Carels and Shepherd suggested that lowering the pH value to 2.5 would increase the formation of yellow and orange pigment (*27*). Monacolin K, citrinin, and the pigments are the derivatives of polyketide so the pH condition should also affect the formation of monacolin K and citrinin. Lower pH value under 4.5 was used as the culture condition to investigate the production of monacolin K and citrinin by *Monascus*-related studies. However, lowering the pH value resulted in higher monacolin K level, but also higher citrinin level. The optimal culture condition based on RSM results indicates that a pH value at 5.7 is a suitable condition for increasing the M/C ratio (**Figure 10**). This dioscorea medium without extreme pH value also led to higher monacolin K production than rice medium. This RSM result implies that the components and character of dioscorea are the important factors for stimulating monacolin K formation, and culture pH value should be considered as a sensitive factor for the modulation of *Monascus* cell amount and citrinin formation.

Amylose is the major type of starch in dioscorea (*8*). This character may result in a comfortable environment for *Monascus* species to grow. In addition to the composition of starch, mucilage of dioscorea includes various kinds of amino acids (aspartic acid, glutamic acid, leucine, glycine, etc.) and carbohydrates (mannose, arabinose, glucose, xylose, and rhamnose) (*10*, *11*). Therefore, the mucilage including enough carbon and nitrogen sources can be quickly consumed and used by *Monascus* species. Furthermore, the optimal culture condition without lower pH value under 3.0 still results in high monacolin K formation at 27.90 mg/g and low citrinin formation at 2.15 μg/g. Therefore, this study suggests that dioscorea stimulating monacolin K formation is probably due to its composition and character, but pH condition should be an important modulator for obtaining RMD with high M/C ratio.

Dioscorea medium with low pH value at 3.5 was found to significantly stimulate monacolin K formation by 148% but also increase citrinin level by 147%, as compared to rice medium. Culture pH value, dioscorea concentration, and ethanol concentration were used as the factors of RSM to investigate the optimal culture condition for high monacolin K production and low citrinin formation. In the results, the pH value of dioscorea medium was involved in the formation of *Monascus* cell amount and secondary metabolite. RSM results further indicated that lowering the pH value to 2.5 would result in high monacolin K and citrinin concentration as well as large biomass in fixed dioscorea amount, implying that pH value may stimulate the formation of monacolin K and citrinin through increasing *Monascus* cell amount. However, the composition and character of dioscorea should be the important factors for the mediation of monacolin K formation in addition to pH value. Lowering dioscorea and ethanol concentration was able to increase the ratio of monacolin K level to citrinin level. The optimal culture condition (pH 5.7, 1% dioscorea concentration, and 0.5% ethanol concentration) would increase monacolin K level to 27.9 mg/g (by 47%) and decrease citrinin level to 2.15 μg/g (by 54%), as compared to control conditions (pH 3.5, 5% dioscorea, and ethanol free).

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