

## CHAPTER 5

### **Statistical Optimization of Monacolin K Production by *Monascus purpureus* CMU002U under Solid State Fermentation using Purple Glutinous Rice Powder as Substrate**

#### **5.1 Introduction**

Red mold rice (RMR), fermented rice by *Monascus* species widely used in Asian cuisines for food coloring and preservative (Ma *et al.*, 2000). Especially in China during the Ming Dynasty (1368–1644), RMR has been used for improving the blood circulation and recorded on the books of Chinese medicine (Heber *et al.*, 2001).

Monacolin K is a competitive inhibitor of HMG-CoA reductase in cholesterol biosynthesis (Albert *et al.*, 1980). Monacolin K contain in RMR has been reported to be anticholesterol agent. Monacolin K is a member of the drug class of statins used for lowering cholesterol (Endo, 1979), tumor progression and metastasis of Lewis lung carcinoma cells (Ho and Pan, 2009).

Many strains of *Monascus* including *M. pilosus*, *M. ruber* and *M. purpureus* as well as other species of filamentous fungi such as *Aspergillus terreus* and *Penicillium funiculosum* can be produced monacolin K (Chang *et al.*, 2002; Lopez *et al.*, 2003; Miyake *et al.*, 2006; Sayyad *et al.*, 2007; Latha *et al.*, 2011). It is an intracellular product and mostly accumulated in mycelia (Wei *et al.*, 2007). Generally, monacolin K production can be affected by the type of carbon and nitrogen source as well as other parameters including strain of fungal, temperature, time and moisture (Panda *et al.*, 2009; Pansuriya and Singhal, 2010).

Many materials including rice, cereals or tuber crops (cassava, sweet potato, potato and yam) are generally used as substrate for produce monacolin K (Lee *et al.*, 2006; Chairote *et al.*, 2008). In addition, the agricultural waste such as banana peel,

orange or pineapple epicarp can also be used as a substrate for monacolin K production (Kavitha *et al.*, 2012).

Citrinin is another secondary metabolite may be found during RMR production or agricultural commodities. It has a negative impact on the acceptance of RMR product from nephrotoxic and hepatotoxic properties (Betina, 1989). Many countries concerned about citrinin contamination in the products, Japan has issued an advisory limit of 200 ppb; the Food and Drug Administration (FDA) action level of citrinin in agricultural products for sale is 20 ppb, and the European Union (EU) has a recommended a limit of 100 ppb (Shi and Pan, 2011). In 2014, the Commission Regulation under the EU No. 212/2014 accepted the limitation of citrinin concentration in rice fermented with *M. purpureus* increased from 100 to 2,000 ppb in the European Union (EU) (The European Commission 2014). Production of citrinin was depended on media composition, culture conditions or fungal strain. Several studies have shown the way to decrease citrinin level. The mutant strain that cannot produce citrinin could be obtained from the improvement of fungal strain by ultraviolet ray,  $\gamma$ -irradiation and chemical mutagen (Wang *et al.*, 2004; Suh *et al.*, 2007). Some studies used the statistical to find the optimum condition for no citrinin production including type of substrate, temperature, moisture and pH (Kang *et al.*, 2014).

There are several reports on monacolin K production that used the Response Surface Methodology (RSM) to obtain the optimal conditions (Panda *et al.*, 2009; Rajasekaran and Kalaivani, 2012; Feng *et al.*, 2014). In order to find the suitable condition, Plackett Burman design and RSM are the effective statistical technique that used to optimization for obtain high monacolin K yield (Bezerra *et al.*, 2008).

In the present study, we focused on the monacolin K production using purple rice powder as substrate and parameters of solid state fermentation were optimized by RSM to obtain optimal condition for high monacolin K yield.

## **5.2 Material and methods**

### **5.2.1 Rice powder preparation**

Purple glutinous rice was obtained from Lanna Rice Research Center, Chiang Mai University including Doi Muser, Doi Saked, Na, Nan, Phayao and

Hom CMU. Glutinous rice was ground and passed through a sieve 35 mesh size to make powder. Rice powder was oven dried at 60°C and kept at dry place.

### **5.2.2 Inoculum preparation**

*Monascus purpureus* CMU002U is a mutant strain with high monacolin K production ability which was obtained from the Sustainable Development of Biological Resources (SDBR) Laboratory, Faculty of Science, Chiang Mai University. It was maintained on potato dextrose agar (PDA) at 4°C and subcultured every month. Inoculum was prepared by fungal cultivation in PDB 3-4 days at 30°C 150 rpm.

### **5.2.3 Effect of glutinous purple rice varieties on monacolin K and citrinin production**

Packing 20 grams of purple rice powder in 250-ml Erlenmeyer flask and sterilized with steam at 121°C 15 minutes. The flasks contain rice powder were allowed to stand at room temperature until cool and then initially adjusted to moisture levels of 40% and inoculum size of 5% v/w. The inoculated flasks were incubated at 30°C for 14 days and the end products were oven dried at 60°C overnight and stored at dry place in darkness before analysis.

### **5.2.4 Effect of different nitrogen source on monacolin K and citrinin production**

Two percent of nitrogen source (corn meal, malt extract, oat meal, peptone, soybean meal and yeast extract) was mixed to rice powder. All other steps are the same as above.

### **5.2.5 Determination of monacolin K and citrinin**

Chromatographic separation was carried on a Mightysil C18 column (150 × 2.0 mm, 5 µm). For monacolin K analysis, Acetonitrile-0.5% Phosphoric acid (65:35 v/v) was used as the mobile phase. The eluent was pumped at a flow rate of 0.7 ml/min. UV detection was set at 238 nm. For citrinin detection, the

composition of water: acetonitrile: trifluoroacetate (450:550:0.5) was mobile phase. The flow rate was set at 0.6 ml/min, and the detector used was a fluorescence detector. The excitation and emission wavelength was 330 and 500 nm, respectively

## 5.2.6 Optimization of monacolin K production

### 5.2.6.1 Plackett-Burman experiment design

To determine the variables significantly on monacolin K production by *M. purpureus* CMU002U, a Plackett-Burman design (PBD) was formulated. An experimental design of 15 runs containing 3 central points and 5 independent variables were tested at 3 levels, high (+1), medium (0), low (-1) and range of different level for the variables were listed in Table 5.1. The temperature for SSF (30°C) was set at constant. Response value was measured in term of monacolin K yield.

**Table 5.1** Range of different factors studied in Plackett-Burman design

Factor	Code	Level		
		-1	0	+1
Moisture (%)	A	25	35	45
Fermentation time (day)	B	7	14	21
Corn meal (%)	C	0.75	2.25	3.75
Inoculum size (%)	D	5	7.5	10
Rice powder (g)	E	20	25	30

### 5.2.6.2 Central Composite design (CCD)

Based on the results from preliminary study of Plackett-Burman experiment design, three factors including initial moisture, fermentation time and corn meal were found to have influence on monacolin K production. These factors were selected for further optimization by response surface methodology using 5 levels of CCD. A total of 20 runs, including 15 factorial points and 5 replicates at the

center point for the estimation of the pure error sum of square were performed. The actual value of the factors at various levels was showed in Table 5.2. The model was represented by the following quadratic Equation 5.1,

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad (5.1)$$

where, Y is the predicted response,  $\beta_0$  is the offset term,  $\beta_i$  is the linear offset,  $\beta_{ii}$  is the squared offset,  $\beta_{ij}$  is the interaction effect and  $x_i$  is the dimensionless coded value of  $x_j$ . The relative effect of 3 factors on monacolin K production was identified from the contour and response surface plot. All experimental designs and statistical data were analyzed by using software Design-Expert® 7.0.0

**Table 5.2** Range of the independent variable used in CCD

Factor	Code	Level				
		$-\alpha$	-1	0	+1	$+\alpha$
Moisture (%)	A	19.77	30	45	60	70.23
Fermentation time (day)	B	9.23	14	21	28	32.77
Corn meal (%)	C	0.38	1.2	2.4	3.6	4.42

### 5.2.6.3 Validation of the model

Validation experiment was conducted to determine the maximum monacolin K production when the variables were set at the optimum levels established above, through Plackett-Burman experiment design and CCD. Percentage of validation was calculated according to Equation 5. 2,

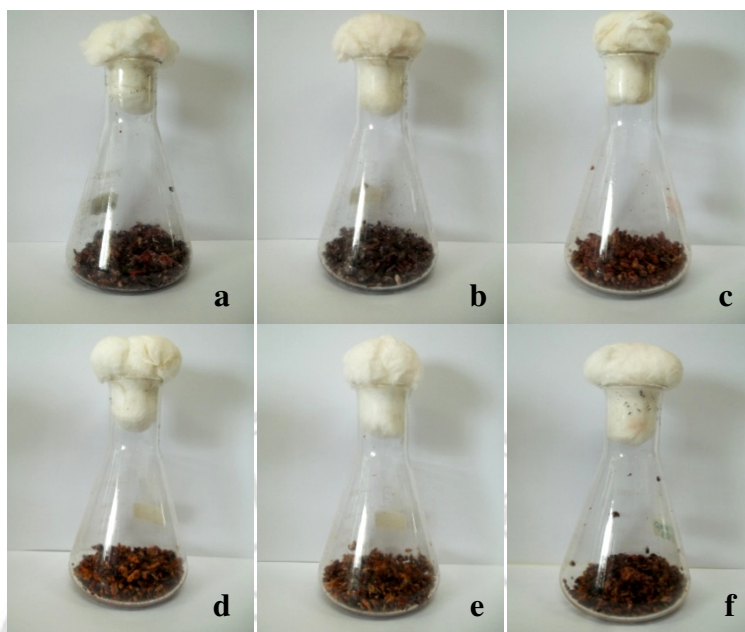
$$\% \text{ Validation} = \frac{\text{Actual value}}{\text{Predicted value}} \times 100 \quad (5.2)$$

## 5.3 Results and Discussion

### 5.3.1 Effect of glutinous purple rice varieties and nitrogen sources on monacolin K and citrinin production

From preliminary study, the optimal condition for the growth of *M. purpureus* CMU002U on rice powder as substrate was the moisture content of 40% and temperature of 30°C. These conditions were used in the following experiments. Purple glutinous rice (also known as black rice) is commonly grown in Northern Thailand and other countries such as China, Lao and Vietnam. It is a good source of nutrients, mineral, phytochemicals and antioxidants. The purple rice has the unique characteristics of containing high levels of gamma oryzanol and anthocyanin antioxidants. These substances enable the use of purple rice as in traditional herbal medicine to reducing plasma cholesterol, cholesterol absorption and decreasing early atherosclerosis and inhibition of the growth of Lewis lung carcinoma cells *in vivo* (Chen *et al.*, 2005; Saenjum *et al.*, 2012). After cooking, the texture of purple rice had crumbly and not sticky. Therefore, the rice grain was decreased to small particles and in this case rice powder was sieve through 35 mesh size (500 µm) and used instead their natural rice. Glutinous rice was found to be a popular substrate for RMR production and generally used it original size of grain for fungal cultivation. However, some researcher reported that particle of rice had affect on the monacolin k production. Smaller substrate particles will provide larger surface are for the attachment of microbes, which is likeable for mycelia growth and accumulation of their products (Wei *et al.*, 2007). If the rice was used in form of grain, the fungal was difficult to growth and its required high initial moisture and fermentation time will be increased to complete the fermentation process.

Each powder substrates were carried out without any addition. After fermentation, the colour and odor of RMR from purple glutinous rice powder did not different from previous study or commercial RMR. It had dark red colour and good odor from ester and alcohol (Figure 5.1).



**Figure 5.1** RMR from purple glutinous rice powder a) Doi Muser, b) Doi Saked, c) Na, d) Nan, e) Phayao and f) Hom CMU

From Table 5.3, it was obviously that Doi Muser glutinous rice powder is the best substrate than other glutinous rice to achieve high monacolin K yield. There are so many different types of substrate that used for monacolin K production. Each substrate could be affect on different monacolin K yields that caused by difference composition of nutrients.

In order to screen the best nitrogen sources for *M. purpureus* CMU002U growth and monacolin K production, the following nitrogen sources including corn meal, malt extract, oat meal, peptone, soybean meal and yeast extract were evaluated to study their effect on monacolin K production. Among all the nitrogen sources, corn meal was found to be better than other and had a positive effect on monacolin K production that increased to 159% compared with control (Table 5.4).

**Table 5.3** Effect of glutinous purple rice varieties on monacolin K and citrinin production

Purple glutinous rice	Monacolin K (ppm)	Citrinin (ppb)
Doi Muser	7404	848
Doi Saked	4125	970
Na	2668	455
Nan	2899	376
Phayao	2498	437
Hom CMU	4454	451

**Table 5.4** Effect of nitrogen source on monacolin K and citrinin production

Nitrogen Source	Monacolin K (ppm)	Citrinin (ppb)
Control	6360	891
Corn meal	10082	173
Malt extract	5130	383
Oat meal	4966	297
Peptone	1423	279
Soybean meal	6272	396
Yeast extract	3962	814

When 2% cornmeal was added to glutinous rice powder, the production of citrinin decreased from 891 to 173 ppb while monacolin K increased. The results were reported according to maximum yield of monacolin K was obtained from cultured the *A. terreus* in chemical medium supplement with corn meal as organic nitrogen source (Li *et al.*, 2011). All nitrogen supplementation decreased citrinin level in RMR. One research study (Xiong *et al.*, 2014) reported that the selection of agricultural products with low protein content such as rice meal and corn meal could inhibit the citrinin production. However, there are some nitrogen sources were favorable to the citrinin production such as  $\text{NH}_4\text{NO}_3$ ,  $\text{NH}_4\text{Cl}$  and monosodium glutamate (Blanc *et al.*, 1995). Carbon and nitrogen sources generally played a dominant role in fermentation productivity because these

nutrients are directly linked with the formation of the biomass and the metabolite. Also, the biosynthesis of monacolin K depended on carbon and nitrogen sources (Xu *et al.*, 2005; Miyake *et al.*, 2006).

### 5.3.2 Optimization of monacolin K production

The screening of parameters for the highest monacolin K production was carried out by Plackett-Burman design (Plackett and Burman, 1946). Many studies used the Plackett-Burman design to select the significant factors and improve the monacolin K yield (Panda *et al.*, 2009). The propose of screening were the investigation of the variables under studying influencing monacolin K production and selection of the important variables on the basis of their effects for further optimization.

**Table 5.5** Experimental design and response of Plackett-Burman study

Run	A	B	C	D	E	Monacolin K (ppm)
1	1	1	1	-1	-1	19710
2	-1	-1	-1	1	-1	3490
3	1	-1	-1	-1	1	11480
4	1	-1	1	1	1	12190
5	1	1	-1	-1	-1	16260
6	-1	-1	1	-1	1	5460
7	0	0	0	0	0	10230
8	-1	1	-1	1	1	6750
9	0	0	0	0	0	10070
10	1	1	-1	1	1	16420
11	-1	-1	-1	-1	-1	3690
12	-1	1	1	-1	1	7960
13	1	-1	1	1	-1	12690
14	-1	1	1	1	-1	7790
15	0	0	0	0	0	9120

The results of monacolin K production by a Plackett-Burman design were shown in Table 5.5. Maximum monacolin K yield was found in the experimental trial 1, whereas, minimum in trial 2. This fitting of the first-order model was calculated to be 0.9767 by the coefficient of determination  $R^2$ , which explained the 97.67% variability of the data (Table 5.6). From the first-order model equation, it was predicted that the increasing of initial moisture, fermentation time and corn meal that significant factors should enhanced monacolin K production. Amount of rice powder and inoculum size should be reduced; however the effect will be not being significant. There are some studies supported that higher inoculum size decreased the monacolin K production (Pansuriya and Singhal, 2010). Inoculum size was necessary for SSF because too few spore lead to insufficient biomass as well as too many spores lead to over production of biomass leading to quick depletion of nutrients (Lingappa and Babu, 2005).

**Table 5.6** Analysis of significant independent variables on monacolin K production using the Plackett-Burman design

Source	Sum of squares	df	F value	P > F
Model	3.04E+08	5	67.03	< 0.0001*
A-Moisture	2.4E+08	1	264.43	< 0.0001*
B-Time	55857675	1	61.67	< 0.0001*
C-Corn meal	4953675	1	5.47	0.0475*
D-Inoculum size	2279408	5	2.52	0.1513
E-Rice powder	946408.3	5	1.04	0.3366
Curvature	642735	1	0.71	0.4240
Residual	7245917	8		
Lack of Fit	6525850	6	3.02	0.2695
Pure Error	720066.7	2		
Cor Total	3.11E+08	14		
Statistical analysis				
Std. Dev.	951.70		R-Squared	0.977
Mean	10220.67		Adj R-Squared	0.962

\* Significant level at  $P \leq 0.05$

Based on the results from PBD, two factors including inoculum size and amount of rice powder did not show significant effect. However, these two factors could not be eliminated from the experiment so the low value of these two factors was used in the further experiment. On the other hand, the initial moisture content, fermentation time and corn meal supplementation were the significant factors that influencing monacolin K yield.

**Table 5.7** CCD of factors in coded levels with monacolin K as response

Run	A	B	C	Monacolin K (ppm)	
				Actual	Predicted
1	1.68	0.00	0.00	9130	10086
2	0.00	0.00	0.00	20350	22679
3	-1.00	-1.00	-1.00	4230	3987
4	0.00	0.00	0.00	20920	22679
5	1.00	-1.00	-1.00	11310	11227
6	0.00	0.00	-1.68	13470	13558
7	-1.00	1.00	1.00	7240	9515
8	0.00	0.00	0.00	23530	22679
9	0.00	-1.68	0.00	10870	11020
10	1.00	-1.00	1.00	13320	13587
11	-1.00	-1.00	1.00	5890	6348
12	0.00	0.00	1.68	18750	17528
13	0.00	1.68	0.00	17630	16347
14	0.00	0.00	0.00	23650	22679
15	1.00	1.00	-1.00	15240	14394
16	0.00	0.00	0.00	24160	22679
17	0.00	0.00	0.00	23270	22679
18	-1.00	1.00	-1.00	5480	7154
19	1.00	1.00	1.00	17050	16755
20	-1.68	0.00	0.00	0	2090

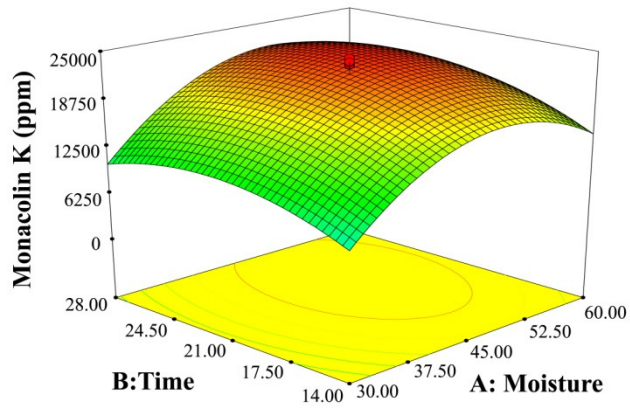
In this study, SSF was carried out using purple glutinous rice powder as substrate and RSM was used to predict the level of these parameters. The CCD was conducted in the optimum to predict the optimum levels of initial moisture content, fermentation time and corn meal for monacolin K production. The CCD design and the corresponding experimental data were shown in Table 5.7.

The experimental results of the CCD were fitted with a second order polynomial equation (in the coded factor) by a multiple regression obtained for monacolin K production (Equation 5.3);

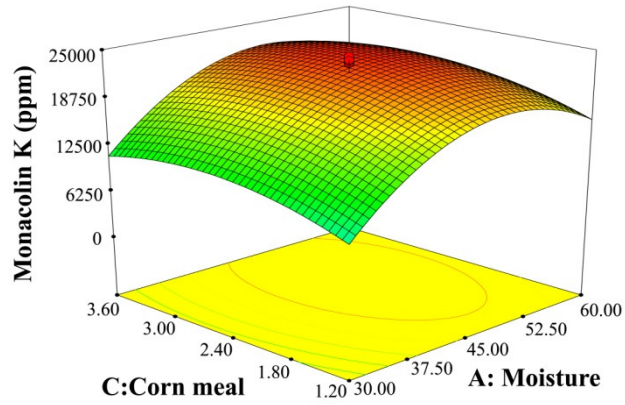
$$Y = 22679 + 3619(A) + 1583(B) + 1180(C) + 632(A)(B) + 50(A)(C) - 1250(B)(C) - 6605(A^2) - 3.181(B^2) - 2522(C^2) \quad (5.3)$$

where Y is the monacolin K produced (ppm), A, B and C are the moisture content, fermentation time, corn meal, respectively.

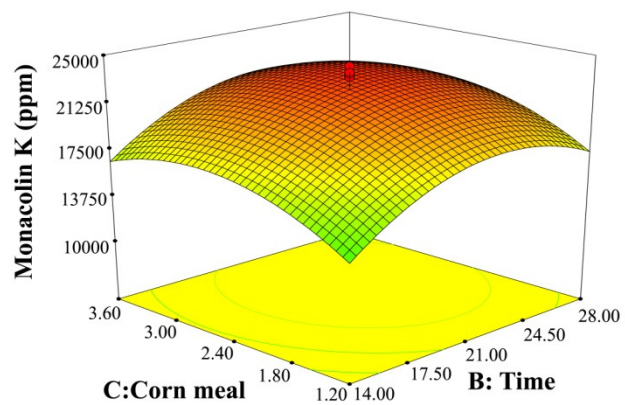
The relative effects of two parameters on monacolin K production while keep the other variable at its central level was described by three dimensional (3D) response surface graphs (Figure 5.2–5.4). The analysis of variance of regression for monacolin K production was summarized in Table 5.8. The fit of the model was checked by the coefficient of determination  $R^2$  which was calculated to be 0.9735, indicated that 97.35 of the variability in the response could be explained by the model.



**Figure 5.2** Effect of moisture (A) and fermentation time (B) on monacolin K production



**Figure 5.3** Effect of moisture (A) and corn meal (C) on monacolin K production



**Figure 5.4** Effect of fermentation time (B) and corn meal (C) on monacolin K production

**Table 5.8** Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental design

Source	Sum of squares	df	F value	P > F
Model	9.963E+008	9	40.87	< 0.0001*
A- Moisture	1.789E+008	1	66.06	< 0.0001*
B-Time	3.425E+007	1	12.65	0.0052*
C- Corn meal	1.903E+007	1	7.02	0.0243*
AB	3.200E+006	1	1.18	0.3025
AC	20000.00	1	7.384E-003	0.9332
BC	1250.00	1	4.615E-004	0.9833
A <sup>2</sup>	6.287E+008	1	232.09	< 0.0001*
B <sup>2</sup>	1.458E+008	1	53.82	< 0.0001*
C <sup>2</sup>	9.173E+007	1	33.87	0.0002*
Residual	2.709E+007	10		
Lack of Fit	1.436E+007	5	1.13	0.4486
Pure Error	1.272E+007	5		
Cor Total	1.023E+009	19		
Statistical analysis				
Std. Dev.	1645.79		R-Squared	0.9735
Mean	14274.50		Adj R-Squared	0.9497

\* Significant level at  $P \leq 0.05$

### 5.3.3 Validation of model

The combination of Plackett-Burman design and RSM was shown to be effective to select the significant factors and finding the optimal conditions of those factors for monacolin K production by *M. purpureus* under SSF. The optimal values of the selected variables were obtained by solving the regression equation. After calculation by Design Expert software, the optimal conditions of monacolin K production were moisture content of 49.3%, fermentation time of 22.9 days and 2.68% corn meal supplementation with the corresponding monacolin K = 23,560 mg/g. To confirm these conditions, experiments were

performed in triplicate under optimized conditions. The monacolin K yield was 23,172 ppm, significantly higher than non optimization (6,360 ppm).



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