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ORIGINAL PAPER

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Improvement of monacolin K, γ-aminobutyric acid and citrinin production ratio as a function of environmental conditions of *Monascus purpureus* NTU 601

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Abstract *Monascus*, a traditional Chinese fermentation fungus, is used as a natural dietary supplement. Its metabolic products monacolin K and γ-aminobutyric acid (GABA) have each been proven to be a cholesterol-lowering drug and a hypotensive agent. Citrinin, another secondary metabolite, is toxic to humans, thus lowering the acceptability of red mold rice to the general public. In this study, the influence of different carbon and nitrogen sources, and fatty acid or oils, on the production of monacolin K, citrinin and GABA by *Monascus purpureus* NTU 601 was studied. When 0.5% ethanol was added to the culture medium, the production of citrinin decreased from 813 ppb to 561 ppb while monacolin K increased from 136 mg/kg to 383 mg/kg and GABA increased from 1,060 mg/kg to 7,453 mg/kg. In addition, response surface methodology was used to optimize culture conditions for monacolin K, citrinin and GABA production, and data were collected according to a three-factor (temperature, ethanol concentration and amount of water supplemented), three-level central composite design. When 500 g rice was used as a solid substrate with 120 ml water and 0.3% ethanol, the production of monacolin K at 30°C increased from 136 mg/kg to 530 mg/kg, GABA production increased from 1,060 mg/kg to 5,004 mg/kg and citrinin decreased from 813 ppb to 460 ppb.

Keywords *Monascus* · Monacolin K · Citrinin · GABA · Functional food · Response surface methodology

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Introduction

Monascus spp. is a traditional fermentation fungus used on food for thousands of years in China; its special effect on, and application to, food was recorded in ancient records. The types of secondary metabolites produced by *Monascus* spp. include a group of yellow, orange and red pigments [2,28], a group of antihypercholesterolemic agents, including monacolin K and the hypotensive agent γ-aminobutyric acid (GABA) [1,15,24], and antibacterial compounds including pigments and citrinin (as monascidin) [3,4,28].

Monacolin K (also known as Lovastatin, Mevinolin and Mevacor) is a secondary metabolite of *Monascus* and *Aspergillus* species with the molecular formula $C_{24}H_{36}O_5$ and a molecular weight of 404.55 [9]. Monacolin K, a more active methylated form of compactin, is formed by *Monascus ruber* [9] and is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in cholesterol biosynthesis [1]. It not only inhibits cholesterol biosynthesis, but also lowers blood cholesterol level in both humans and animals. A clinical trial conducted using a dietary supplement with a standardized content of monacolins demonstrated an 18% decrease in total cholesterol, a 23% decrease in low-density lipoprotein cholesterol, and a 15% decrease in triglycerides [13], which can help in alleviating arteriosclerosis [6]. GABA has several physiological functions, including neurotransmitting, hypotensive, and diuretic effects [19,26]. GABA is produced by the decarboxylation of glutamic acid by glutamate decarboxylase [21]. GABA, with two receptors-$GABA_A$ and $GABA_B$, is the main suppressive nerve transmitter of the central nervous system [19]. *Monascus* has a prominent blood-pressure-lowering effect, the antihypertensive substance being GABA [16,25]. In a previous study, we found that different strains produced different amounts of GABA [24].

Citrinin, another secondary metabolite of *Monascus* spp., can limit the acceptability of the red mold rice product. Citrinin is a typical mycotoxin first found in

Penicillium citrinum, and later in *Aspergillum* and *Monascus* spp. [3]. Citrinin [$C_{13}H_{14}O_5$, IUPAC (3R, 4S-trans)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyrane-7-carboxylic acid] is an acidic lemon-yellow crystal with maximal absorption at 250 and 311 nm, LD_{50} values of 35 mg/kg (mouse) and 67 mg/kg (rat), and a specific rotation at $+217.1°$ [7]. Citrinin has nephrotoxic and hepatotoxic properties, and is found in both solid and submerged cultures in the 100–400 mg/l range. In 12 types of red mold rice on the market the content of citrinin varied from 0.2 to 17.1 ppm [20]. The range of citrinin in red mold rice on the Taiwanese market was 0.1–122 ppm [14]. Thus, reduction of citrinin is of great concern.

In general, monacolin K, citrinin and GABA production can be influenced by the medium composition, especially by the type of carbon and nitrogen source and other components [2,12,22]. In addition, production is differently affected by environmental parameters, such as agitation [11], temperature [18] and moisture content [8].

In this research, the effect on the production of citrinin, monacolin K and GABA of adding different substances to the medium was investigated. Response surface methodology (RSM) was used to follow the reduction of citrinin production while maintaining the best conditions for the formation of monacolin K and GABA.

Materials and methods

Chemicals

γ-Amino-*n*-butyric acid (GABA), monacolin K and citrinin were purchased from Sigma (St. Louis, Mo.). LC grade acetonitrile was from Merck (Darmstadt, Germany). Tryptone, yeast extract, peptone, malt extract, potato dextrose agar (PDA) broth and Bacto-agar were from Difco (Detroit, Mich.). Reagent grade ethyl acetate was purchased from ALPS (Taiwan, ROC)

Microorganism, seed culture and preparation of red mold rice

Citrinin, monacolin K and GABA production was carried out on strain *M. purpureus* NTU 601. The strain was maintained on PDA slants at 10°C and transferred monthly. The seed culture method and the preparation of red mold rice were described in our previous study [24].

Preparation of mold extracts and measurement of monacolin K, citrinin and GABA

Preparation of mold extracts and the concentration of monacolin K, citrinin and GABA was measured by the methods described by Wang et al. [27], Kycko et al. [17] and Rossetti and Lomband [23], respectively.

Effect of medium components on monacolin K, citrinin and GABA production

M. purpureus NTU 601 was fermented on solid-state medium. After the rice was sterilized and cooled, extra chemical compounds were added individually. The effect of the carbon source (glucose, acetate and ethanol), nitrogen source [ammonium chloride, monosodium glutamate (MSG), methionine and urea], and/or fatty acid and oil (octanoic acid, dodecanoic acid, soybean oil and corn oil) on monacolin K, GABA and citrinin production in solid-state culture was analyzed.

Design of experiments

In order to identify the optimum conditions, a Box-Behnken design [5] was selected. The crucial factors involved are temperature (X_1), ethanol concentration (X_2), and the amount of water supplemented (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: temperature 30°C; concentration of ethanol 0.5%; water supplement 120 ml/500 g rice (water was added on the 5th day, once every 12 h, a total of three times). Control conditions were: temperature 30°C, water added 100 ml, ethanol-free.

Response surface methodology

The analysis of data was carried out using RSREG in Statistical Analysis System (SAS, Cary, N.C.). A second order model was employed to fit the data individually for the responses Y_1 (monacolin K), Y_2 (citrinin) and Y_3 (GABA) by the general model [10], with three factors, each factor coded to be in the range of −1, 0, +1.

$$Y = A_0 + A_xX_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2$$

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

Variation of secondary metabolites in the solid culture process

Metabolite variation is shown in Fig. 1. Production of monacolin K, citrinin and GABA started to increase slowly between days 2 and 4. Monacolin K and citrinin are secondary metabolites derived from the polyketide pathway, and are therefore produced together in red mold rice, with their growth rate doubling on days 5–8 day, during the idiophase. Monacolin K reached maximum production on day 8, and leveled off thereafter. The growth rate of citrinin and GABA gradually in-

Table 1 Process variables and levels in the three-factor three-levels response surface design of secondary metabolites experiment. I Actual value of factors as per Box-Behnken designs

Factors	Symbol	Coded-variable level		
		−1	0	1
Temperature (°C)	X_1	25.0	30.0	35.0
Ethanol (%)	X_2	0.3	0.5	0.7
Amount of water added (ml)	X_3	80.0	120.0	160.0

Table 2 Process variables and levels in the three-factor three-levels response surface design of secondary metabolites experiment. II Observed experimental data. *GABA* γ-Aminobutyric acid

Run	Independent variables (coded-level)			Observed metabolite compound		
	Temperature (°C)	Ethanol (%)	Water added (ml)[a]	Monacolin K (mg/kg)	Citrinin (ppb)	GABA (mg/kg)
1	35 (1)	0.7 (1)	120 (0)	113	271	3,431
2	35 (1)	0.3 (−1)	120 (0)	120	293	3,132
3	25 (−1)	0.7 (1)	120 (0)	199	457	4,499
4	25 (−1)	0.3 (−1)	120 (0)	158	497	4,782
5	35 (1)	0.5 (0)	160 (1)	108	320	3,900
6	35 (1)	0.5 (0)	80 (−1)	92	278	3,983
7	25 (−1)	0.5 (0)	160 (1)	322	536	4,758
8	25 (−1)	0.5 (0)	80 (−1)	184	491	4,279
9	30 (0)	0.7 (1)	160 (1)	452	413	6,741
10	30 (0)	0.7 (1)	80 (−1)	317	289	6,897
11	30 (0)	0.3 (−1)	160 (1)	705	611	4,110
12	30 (0)	0.3 (−1)	80 (−1)	396	409	5,857
13[b]	30 (0)	0.5 (0)	120 (0)	351	334	5,669
14[b]	30 (0)	0.5 (0)	120 (0)	365	356	5,823
15[b]	30 (0)	0.5 (0)	120 (0)	333	342	6,210

[a]Adding different amount water content for every 500 g rice
[b]Runs 13–15 were thee replications of center points

creased from days 4–9, although stable production had not been reached by day 9.

Effect of carbon sources

Addition of 0.5% ethanol decreased production of citrinin from 813 ppb to 561 ppb, monacolin K increased from 136 mg/kg to 383 mg/kg, and GABA increased from 1,060 mg/kg to 7,453 mg/kg (Tables 1 and 2). Although citrinin production decreased with increasing ethanol concentration, the production of monacolin K and GABA also decreased. Addition of 2% glucose to the solid medium resulted in a significant decrease in citrinin and monacolin K production.

Effect of nitrogen sources

The only nitrogen source that had a positive effect on monacolin K production was 0.5% NH_4Cl. All other nitrogen sources decreased monacolin K production (Table 3). All the nitrogen additives increased GABA production to 3,564–4,722 mg/kg. Methionine and 0.5% urea also decreased citrinin production; however, they also suppressed the production of monacolin K.

Effect of fatty acids and oils

As shown in Table 3, octanoic acid and dodecanoic acid suppressed production of citrinin. Octanoic acid lowered citrinin production to 488 ppb, and dodecanoic acid

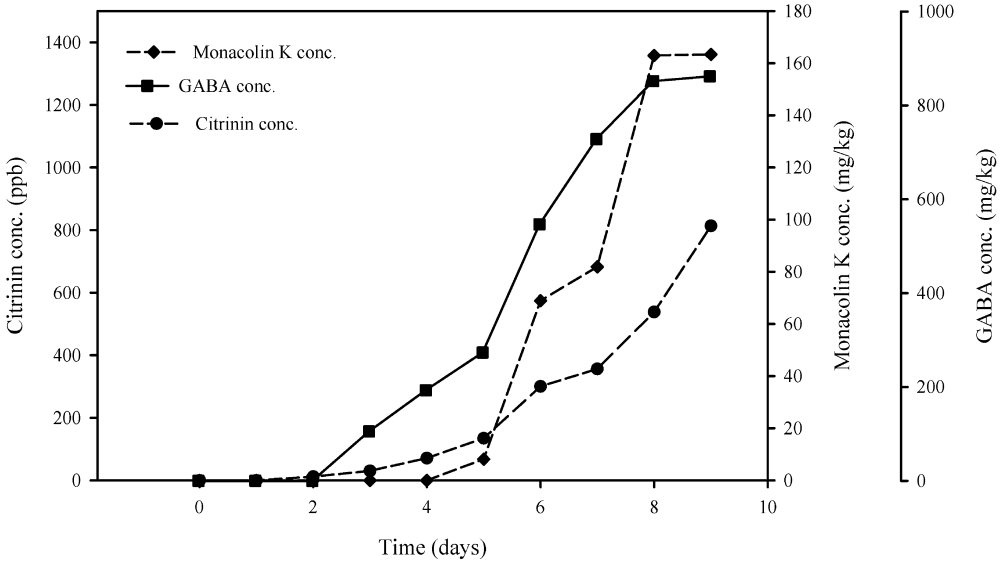

Fig. 1 Time course of monacolin K, citrinin and γ-aminobutyric acid (GABA) production

Table 3 Effect of medium components on monacolin K, citrinin and GABA production. *MSG* Monosodium glutamate

Items	Monacolin K (mg/kg)	Citrinin (ppb)	GABA (mg/kg)
Control	136	813	1,060
Carbon source			
Ethanol (0.5%)	384	561	7,453
Ethanol (1.0%)	123	131	5,872
Ethanol (2.0%)	1	103	2,632
Glucose (1.0%)	178	816	2,725
Glucose (2.0%)	4	38	3,110
Acetic acid (1.0%)	–[a]	621	1,601
Nitrogen source			
NH_4Cl (0.5%)	194	863	3,793
NH_4Cl (1.0%)	16	90	4,632
Methionine (0.5%)	4	81	3,934
Methionine (1.0%)	4	75	4,133
Urea (0.5%)	–[a]	55	4,722
MSG (1.0%)	–[a]	98	3,564
Fatty acid and oil			
Corn oil (0.5%)	103	4,037	763
Soybean oil (0.5%)	73	262	4,574
Soybean oil (1.0%)	19	1,659	714
Octanoic acid (0.3%)	51	580	7,621
Octanoic acid (0.5%)	46	488	7,322
Dedocanoic acid (0.3%)	–[a]	85	7,793
Dedocanoic acid (0.5%)	–[a]	70	1,525

[a]Under the detection limit

lowered citrinin production to 70 ppb, yet monacolin K also decreased. However, octanoic acid significantly increased production of GABA to 7,000 mg/kg or above. Corn oil (0.5%) and soybean oil (1%) together with long chain fatty acids also increased the growth of citrinin; 0.5% corn oil increased citrinin production to 4 ppm.

Optimum culture conditions based on RSM

Design of experiments and model

This research used RSM to investigate optimum culture conditions taking account of three factors: cultivation temperature, ethanol concentration and amount of water added. 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, was obtained from regression analysis for the secondary metabolite concentrations:

$$\text{Monacolin K (mg/kg)} = 349.6 - 53.8X_1 - 37.3X_2 + 74.74X_3 - 246.49X_1^2 + 44.29X_2^2 + 73.32X_3^2 - 12.04X_1X_2 - 30.56X_1X_3 - 43.43X_2X_3$$

$$\text{Citrinin (ppb)} = 343.87 - 102.37X_1 - 47.46X_2 + 51.73X_3 + 5.76X_1^2 + 30.01X_2^2 + 56.6X_3^2 + 4.57X_1X_2 - 0.8X_1X_3 - 19.74X_2X_3$$

$$\text{GABA (mg/kg)} = 5.901 - 0.484X_1 + 0.461X_2 - 0.188X_3 - 1.805X_1^2 - 0.134X_2^2 + 0.135X_3^2 + 0.146X_1X_2 - 0.14X_1X_3 + 0.398X_2X_3$$

The percentages of variability in the responses accounted for by the factors (R^2 value) for the models are given in Table 4. The R^2 values of monacolin K, citrinin and GABA production were 91.83%, 90.00% and 85.87%, 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 2 shows three-dimensional (3-D) response surface plots of the effect of cultivation temperature and water added on the production of monacolin K. As the ethanol concentration increased from 0.3% to 0.7%, the curve gradually decreased, i.e., the production of monacolin K gradually decreased. When the temperature was around 30°C, the production of monacolin K gradually increased along with water addition. Figure 3 shows 3-D response surface plots of the relationship between citrinin production, temperature, and water addition. The addition of ethanol lowered the production of citrinin, citrinin production went down when the temperature was above 30°C, and water addition increased the production of citrinin. In addition, when comparing Figs. 2 and 3, it is obvious that citrinin and monacolin K had similar trends under the same cultivation conditions; conditions that increased monacolin K also increased production of citrinin. Thus, production of monacolin K and citrinin is related.

Figure 4 shows 3-D response surface plots of the effect of cultivation temperature and water addition on the production of GABA. Addition of ethanol brought the curve upward, i.e., production of GABA gradually increased. Production is optimum at a temperature of around 30°C, and reduced water addition increased GABA content.

Table 4 Analysis of variance for the production of monacolin K, citrinin and GABA and various culture conditions[a]. *df* Degree of freedom

Source	Monacolin K		Citrinin		GABA	
	df	Sum of squares	*df*	Sum of squares	*df*	Sum of squares
Regression	9	358,781.83	9	139,187.91	9	16.96
Residual	5	31,930.08	5	15,749.40	5	2.79
Lack of fit	3	31,850.18	3	15,693.28	3	2.04
Pure error	2	79.91	2	56.12	2	0.74
Variability explain (r^2)		91.83		90.00		85.87

[a]Analysis of variance from SAS statistics system

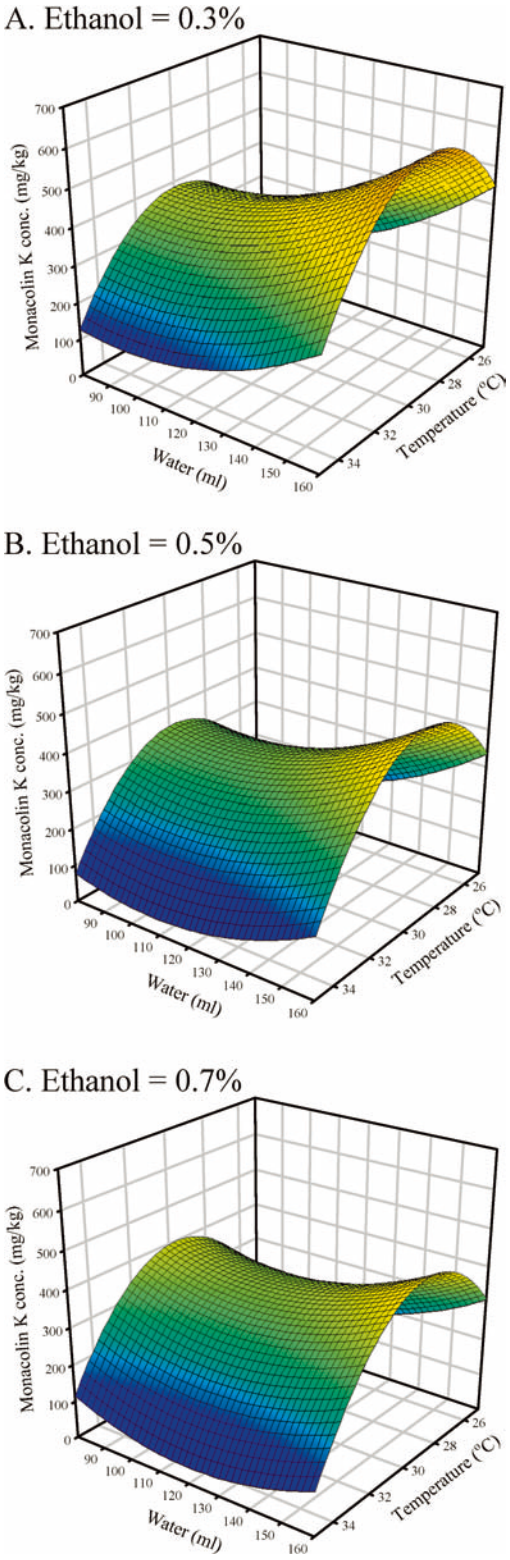

Fig. 2 A–C The response surface for the production of monacolin K at various temperatures and amount of water added

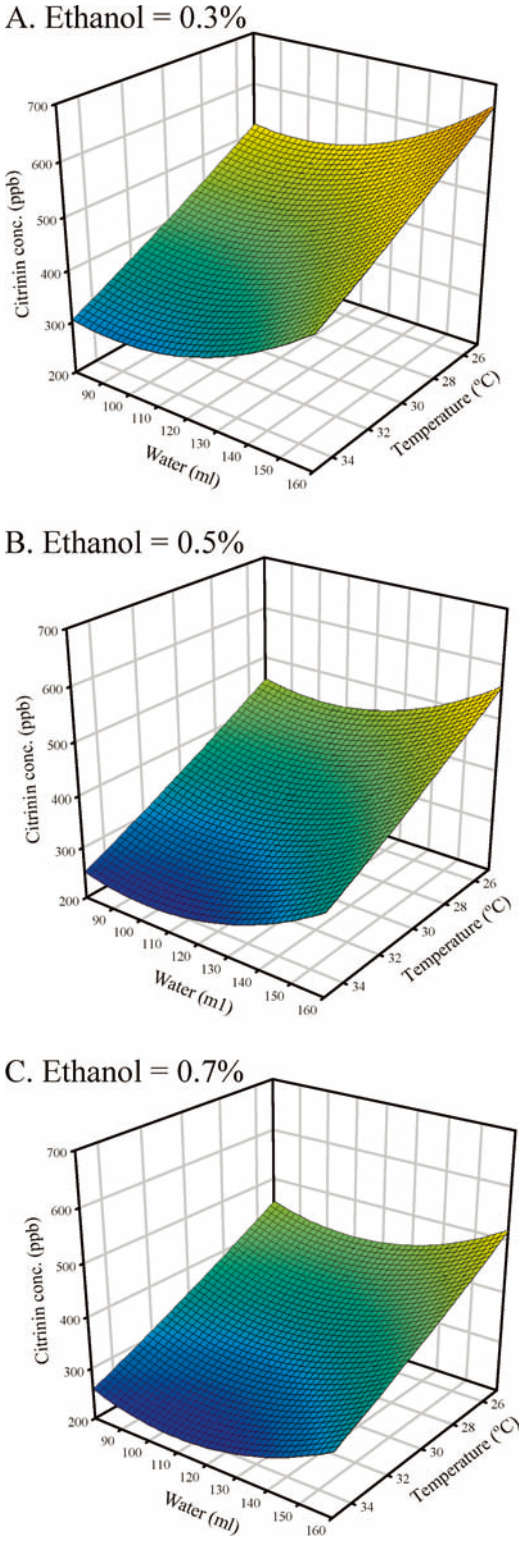

Fig. 3 A–C The response surface for the production of citrinin at various temperatures and amount of water added

Optimum conditions based on RSM

Since production of both monacolin K and citrinin may increase at the same time, any decision on optimum conditions should consider the relationship between these two elements. Comparing Figs. 2 and 3, when the ethanol concentration reached 0.3%, the production of both monacolin K and citrinin increased. Production of

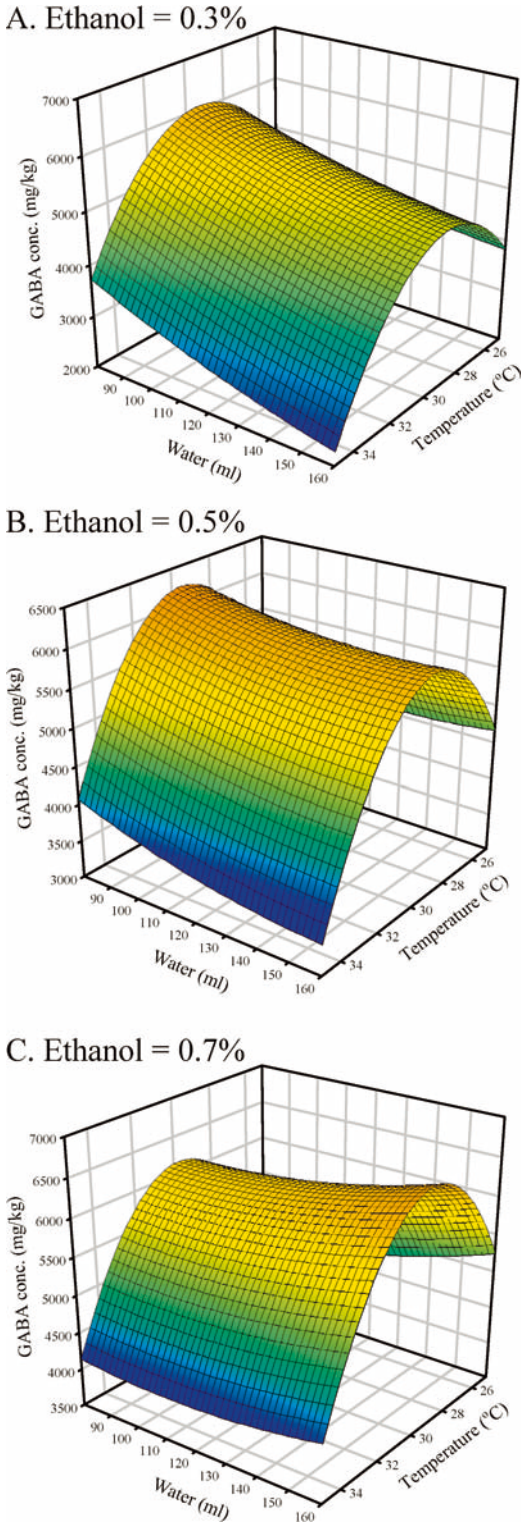

Fig. 4 A–C The response surface for the production of GABA at various temperatures and amount of water added

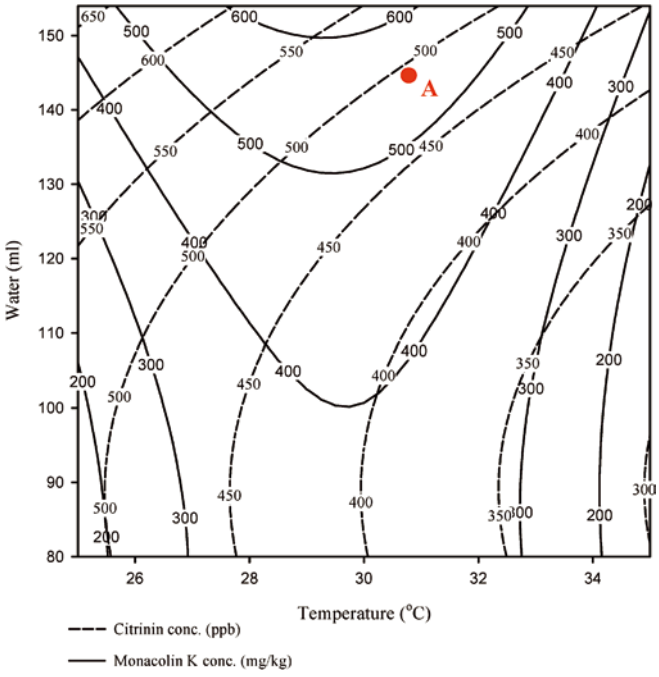

Fig. 5 Overlap contour plot for the production of monacolin K and citrinin under various temperatures and amount of water added (ethanol = 0.3%)

Table 5 Effect of culture conditions on the production of monacolin K, citrinin and GABA by *Monascus purpureus* NTU 601. *RSM* Response surface methodology

Items	Monacolin K (mg/kg)	Citrinin (ppb)	GABA (mg/kg)
Control	136	813	1,060
Ethanol 0.5%	385	561	7,453
RSM condition	530	460	5,004

monacolin K was higher than that of citrinin, so the most appropriate ethanol concentration would be 0.3%. The optimum conditions to increase monacolin K productivity while lowering citrinin can be found by overlapping Figs. 3A and 4A. Figure 5 shows that the optimum conditions (29.5–30.5°C, ethanol 0.3%, and water addition at 144–146 ml) would be expected to increase monacolin K productivity to 500–530 mg/kg and lower citrinin production to 460–480 ppb. The GABA content would be 4,800–5,200 mg/kg under optimum conditions.

Based on the above findings, RSM was used to verify the experimental results on red mold rice production. As shown in Table 5, the results and control group indicated that the production of monacolin K could be increased from 136 mg/kg to 530 mg/kg, citrinin lowered from 813 ppb to 460 ppb, and GABA content increased from 1,060 mg/kg to 5,004 mg/kg.

Discussion

Medium composition and culture conditions may affect the growth of filamentous fungi and alter secondary metabolite production. Addition of 0.5% ethanol to the medium gave the best result. Hajjaj et al. [12] suggested

that when glucose was used as the carbon source for submerged culture, the productivity of citrinin could be increased along with the increasing concentration of glucose. However, we obtained a different result. Different strains or culture methods (submerged or solid-state culture) may affect secondary metabolites. Addition of methionine and urea as nitrogen source suppressed formation of citrinin and monacolin K from *M. purpureus* NTU 601. This is consistent with the findings Blanc et al. [3]. Additional chemical compounds added to a solid medium, such as acetate, octanoic acid or dedocanoic acid, could result in a significant decrease in citrinin and monacolin K production, as proposed by Pimentel et al. [22] and Hajjaj et al. [12].

Production of citrinin and monacolin K follow similar trends under the same cultivation conditions: when monacolin K increased, so did citrinin. In addition, the synthetic pathways of monacolin K and red pigment from *Monascus* species were closely related as low monacolin K production tended to result in decreased red pigment [15]. However, both red pigment and citrinin are produced from a polyketide derivative [11]. Therefore, the synthetic pathways of monacolin K and citrinin from *Monascus* are closely related.

RSM uses experimental design, algorithm deduction, and system analysis to demonstrate the effect of individual factors on the results of the multi-factor experiment, and to seek the optimum condition for each variable, which can be expressed in terms of a mathematic function. The figures generated illustrate the effect of the variables on the products and the interaction between variables. RSM has several advantages, including requiring fewer experiments, suitability for multiple factor experiments, demonstration of relations between factors, determination of the most suitable conditions, and the ability to forecast responses. RSM revealed the following conditions to be optimal: 500 g substrate was inoculated with a 5% (w/v) spore suspension of *Monascus* and the inoculated substrate was cultivated at 30°C, with 0.3% ethanol as carbon source and water addition at 145 ml (water supplement was added on the 5th day, once every 12 h, a total of three times). The production of monacolin K could be increased from 136 mg/kg to 530 mg/kg, citrinin production could be decreased from 810 ppb to 460 ppb, and GABA content could be increased from 1,060 mg/kg to 5,004 mg/kg.

Although we were unable to significantly lower citrinin production by manipulating the fermentation conditions, in future research we could utilize mutations or genetic engineering to select strains with low, or no, production of citrinin. Since citrinin is the primary element lowering the acceptability of red mold rice, reduction of citrinin in red mold rice is currently the most important topic regarding this health food product. If we can eliminate or effectively reduce citrinin content, we will be able to promote the application red mold rice in the development of health food products.

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