JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Modified Mutation Method for Screening Low Citrinin-Producing Strains of *Monascus purpureus* on Rice Culture

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Monascus purpureus NTU 601 is a strain that produces monacolin K, γ-aminobutyric acid (GABA), and citrinin under solid culture conditions. Because citrinin is a mycotoxin and possesses nephrotoxic and hepatoxic effects, it has a negative impact on the acceptance of red mold rice by people. In this research, a simple and quick selection method for mutant strains with low citrinin production was designed based on the fact that citrinin possesses antibacterial activity for *Bacillus subtilis* and will form an inhibition zone around the colony of the *Monascus* strain. The mutant strain *M. purpureus* N 301 only produced 0.23 ± 0.01 ppm citrinin, which was 50% less than that of the parent strain, and the monacolin K production was 481.29 ± 7.98 ppm and maintained 91% productivity. *M. purpureus* N 310, the other mutant strain, produced 0.27 ± 0.01 ppm citrinin, which was 41% less than that of the parent strain, and the monacolin K production was 526.29 ± 5.54 ppm, which showed no significant changes when compared with the parent strain. The GABA content of the two strains was 5000 ppm, which is similar to that of the parent strain. The results showed that the method could be used to select red mold rice with low citrinin production.

KEYWORDS: ***Monascus*; monacolin K; citrinin; GABA; red mold rice**

INTRODUCTION

The *Monascus* species is a Chinese traditional fermentation fungus used on food for over thousands of years in China, and its special effects and application on food were recorded in ancient Chinese records. The types of secondary metabolites produced from the *Monascus* species include (i) a group of pigments (yellow pigment, ankaflavin and monascin; orange pigment, monascorubrin and rubropunctanin; and red pigment, monascorubramine and rubropuctamine) (*1*, *2*), (ii) a group of antihypercholesterolemic agents including monacolin K and a hypotensive agent, γ-aminobutyric acid (GABA) (*3*), (iii) antioxidant compounds including dimerumic acid (*4*) and 3-hydoxy-4-methoxy-benzoic acid (*5*), and (iv) an antibacterial activity compound including pigment and citrinin (also known as monascidin) (*2*, *6*, *7*).

Endo (*8*) discovered that a more active methylated form of compactin known as monacolin K would be formed in the broths of *Monascus ruber*. Monacolin K (also known as lovastatin, mevinolin, and mevacor) is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterol biosynthesis (*9*). It can not only inhibit cholesterol biosynthesis but also lower blood cholesterol levels in both humans and animals. Therefore, it was proven to have special effects when used as a cholesterol-lowering drug. The relationship between cholesterol and atherosclerosis has long been a concern, and an excessive concentration of cholesterol may lead to coronary artery disease or cause death (*10*). GABA has several physiological functions including neurotransmitting, hypotensive, and diuretic effects (*11*, *12*). GABA is produced from the decarboxylation of glutamic acid by catalyzing glutamate decarboxylase. Red mold rice contained a large amount of GABA and possessed antihypertensive effects for humans (*13*). The fact that red mold rice has a special effect on lowering cholesterol levels and that it can be used as a hypotensive agent makes it more popular in the fervent development of functional food.

Citrinin, one of the secondary metabolites of the *Monascus* species, has a negative impact on the acceptability of red mold rice product. Citrinin is a mycotoxin first found in 1931 from *Penicillium citrinum*; it was later found in *Aspergillum* spp. and *Monascus* spp. (*6*, *14*). Citrinin [$C_{13}H_{14}O_5$; IUPAC, (3*R*,4*S*-*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 a maximal absorption at 250 and 331 nm, LD_{50} values of 35 (mouse) and 67 mg/kg (rat), and a specific rotation of +217.1° (*15*). This metabolite exhibits a wide range of activities in diverse biological systems, including antimicrobial, phytotoxic, cytotoxic, hypocholesterolemic, and enzyme inhibitory effects (*14*).

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10.1021/jf049783o CCC: $27.50 © 2004 American Chemical Society
Published on Web 10/12/2004

The antibacterial activity of *M. purpureus* was first reported by Wong and Bau (*16*). The mutant strains isolated from *Monascus* wild-type species were shown to possess antibiotic properties for *Bacillus*, *Streptococcus*, and *Pseudomonas* species during bacteria tests. Citrinin has nephrotoxic and hepatotoxic properties, and it was found in both solid and submerged cultures in the 100−400 mg/L range. Monica et al. (*17*) conducted research on 12 types of red mold rice and found that the content of citrinin varied in the 0.2−17.1 ppm range. Hsieh and Pan (*18*) found that the citrinin content in red mold rice in a Taiwan market ranged from 0.1 to 122 ppm. Therefore, the reduction of citrinin draws a lot of attention by researchers throughout the world.

In general, microbiology always uses mutagenesis to increase the productivity and change the metabolism. However, in this study, we tried to reduce the concentration of citrinin in red mold rice for the sake of safety and to maintain the composition of the healthy monacolin K and GABA by using a mutation method to screen the strain with low citrinin. The screening process was developed based on the inhibition effect of citrinin on *Bacillus subtilis* and the formation of the inhibition zone size. In addition, this research compared the byproducts (monacolin K, pigment, and GABA) of the mutant strains before and after the mutation.

MATERIALS AND METHODS

Chemicals. GABA, monacolin K, citrinin, ethylmethanesulfonate (EMS), and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) were purchased from Sigma Chemical Co. (St. Louis, MO). Liquid chromatography (LC) grade acetonitrile was purchased from Merck Co. (Darmstadt, Germany). Tryptone, yeast extract, peptone, malt extract, potato dextrose agar (PDA) broth, and Bacto-agar were purchased from Difco Co. (Detroit, MI). Reagent grade ethyl acetate was purchased from ALPS Chem. Co. (Taiwan).

Microorganism and Seed Cultures. The microorganism used in this study included *M. purpureus* BCRC 31615, *M. ruber* BCRC 31538, *M. anka* M-13, *Monascus* sp. KT, *M. purpureus* NTU 601, and *B. subtilis*. These strains were either purchased from the Bioresources Collection and Research Center (BCRC, Taiwan) or isolated from red mold rice. The culture strains were maintained on a PDA slant at 10 °C and transferred monthly. In addition, *M. purpureus* NTU 601 was used for the generation of mutants. The seed culture was described in our previous study (*3*).

Red Mold Rice Preparation. The traditional red mold rice preparation method had been proposed in our previous studies (*3*). The whole long-grain rice (*Oryza sativa* L., Japonica) was purchased from a local supermarket and was used as the substrate for red mold rice production under solid state cultivation. Five hundred grams of long-grain rice was soaked in distilled water for 8 h. After that, the excess water was removed with a sieve. The soaked rice was autoclaved for 20 min at 121 °C in a "koji-dish" (the koji-dish is made of wood with dimensions of 30 cm × 20 m × 5 cm). After it was cooled, the substrate was inoculated with a 5% (v/w) spore suspension and 0.3% (v/w) ethanol. The inoculated substrate was cultivated at 30 °C for 10 days. At the end of the cultivation, the crushed and dried substrate with the mold (red mold rice) was used for the experiments (*3*). Besides, the optimum condition for the formation of red mold rice was also investigated by using the response surface methodology (RSM) reported in our previous study (*19*). The RSM applied in this research was stated as below: 500 g of long-grain rice was inoculated with a 5% (v/w) spore suspension and 0.3% (v/w) ethanol. The inoculated substrate was cultivated at 30 °C for 10 days. In addition, during the culturing stage, 145 mL of water was added once every 12 h a total of three times and the addition of water was started on the fifth day of cultivation.

Mutagenesis. *M. purpureus* NTU 601 was used as a parental strain. It was cultured at 30 °C for 3 days on PDA slants medium.

UV Irradiation. Four milliliters of cell suspension (10^6 spores/mL) of the starting strain was evenly spreaded on a Petri dish and was placed under an ultraviolet lamp (15 W) with a distance of 45 cm and was irradiated for 5 min. The viable cell (death rate 90%) was harvested by centrifugation, washed three times with sterile distilled water, and was suspended in fresh PDB medium. The cell was then allowed to grow for 6−8 h at 30 °C in dark and was subsequently plated on PDA agar plates and incubated for 72 h at 30 °C. The colonies were formed and were used for the preparation of the bioassay.

NTG or EMS Treatment. One milliliter of EMS (30 μL/mL, in 0.2 M, pH 5.0, citrate buffer solution) or NTG (100 μg/mL, in 0.2 M, pH 5.0, citrate buffer solution) was added to 1 mL of cell suspension of the starting strain (10^6 spores/mL). After it was incubated at 30 °C for different time intervals ranging from 15 to 60 min, the mixture was diluted 1000 times with sterile distilled water immediately. The viable cell (death rate 50%) was harvested by centrifugation, washed three times with sterile distilled water, and was suspended in fresh PDA medium. The cell was then allowed to grow for 6−8 h at 30 °C and was subsequently plated on PDA agar plates and was incubated for 72 h at 30 °C. The colonies formed were used for the preparation of the bioassay.

Bioassay and Isolation of Lower Citrinin Production Strains. *B. subtilis* was plated in PDB at 30 °C for 24 h. After that, an aliquot (200 μL) of the culture was spread on a PDA plate. The mutant strains were placed in the PDA culture for 3 days to observe whether an inhibition zone was formed. Then, the *Monascus* strain with low or no inhibition zone (with an inhibition rate greater than 99%) was selected and analyzed for citrinin content in the inhibition zone in order to determine the relationship between the inhibition zone size and the citrinin content. Finally, the most appropriate strain was selected to study the relationship among citrinin, monacilon K, and GABA productivities. The inhibition ratio was determined using the following formula

$$\text{inhibition ratio (\%)} = [(C - E)/C] \times 100$$

where C is the average diameter of the inhibition zone of the parental strain and E is the average diameter of the inhibition zone of the mutant strain.

Determination of Citrinin Concentration on the Inhibiting Zone and the Preparation of the Samples. The inhibiting zone formed on the medium was peeled off and was added into 5 mL of chloroform for homogeneity. After that, the mixture was shaken at 50 °C for 30 min and then centrifuged (1500*g*) for 10 min. One milliliter of the upper clear layer liquid was taken and dried before adding it into 200 μL of acetonitrile. The solution was then analyzed by using a high-performance liquid chromatography (HPLC) method.

Dose−Response Curve of Inhibition Zone of *B. subtilis* to Citrinin Concentration. The *Monascus* strains that could produce citrinin were put on the PDA plates and were incubated at 30 °C for 4 days. After that, the strain was transfered with a diameter of 0.5 cm to the PDA plates, which were evenly covered with *B. subtilis*. The circular *Monascus* strains were then incubated at 30 °C for 48 h. We then measured the dimension of the clear zone around the strain, analyzed the citrinin concentration on the inhibiting zone, determined the dose−response curve, and obtained the coefficients of linear regression analysis.

Determination of the Monacolin K Concentration. Red mold rice (1 g) was extracted with 5 mL of ethyl acetate at 70 °C for 1.5 h. The suspension was then filtered through filter paper. The filtrate was evaporated to dryness under vacuum. After lactonization, 1 mL of acetonitrile was added to the resulting mixture, followed by filtration with a 0.45 μm pore size filter and analyzed by HPLC (model L-6200, Hitachi Co., Japan) (*20*). Chromatographic separation was conducted on a Beckman Ultrasphere ODS column (150 mm × 4.6 mm i.d.). Acetonitrile−phosphoric acid (0.5%, 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.

Preparation of Mold Extract and Detection of Citrinin. The red mold rice (1 g) was extracted with 10 mL of acetonitrile at 50 °C for 1.5 h. The filtered extract was twice defatted with isooctane. After an equal volume of water was added and acidified to pH 4.5 with H_2SO_4 (50:50, v/v), the extract was partitioned with $CHCl_3$. The lower phase

Figure 1. Photograph of the *Monascus* mutant strain screening with *B. subtilis*.

was evaporated to dryness and was then dissolved in methanol, followed by filtering with a 0.45 μm pore size filter and analyzed by HPLC (*6*). Citrinin was determined by HPLC on a C_{18} column (LiChroCART 250-4, Merck) using the mobile phase with the composition water: acetonitrile:triflouacetate (450:550:0.5). The flow rate was set at 1.0 mL/min, and the detector used was a fluorescence detector (model FL-1, Rainin Co., Woburn, MA). The excitation and emission wavelength was 330 and 500 nm, respectively (*21*).

Determination of GABA Concentration. GABA was derivatized to phenylthiocarbamyl-GABA (PTC-GABA) according to the direction proposed by Rossetti et al. (*22*). Red mold rice (1 g) was extracted with 5 mL of water at 70 °C for 2 h. A 200 μL aliquot of supernatant (or standard solution of GABA) was dried under vacuum. The residue was dissolved in 40 μL of solution with a composition of ethanol−water−triethylamine (2:2:1), and the mixture was then evaporated to dryness under vacuum. Sixty microliters of ethanol−water−triethylamine−phenylisothiocyanate solution (7:1:1:1) was added to the residue, and the mixture was allowed to react for 20 min at room temperature to form PTC-GABA. The excess reagent was then removed under vacuum. The standard curve of GABA was determined by applying the same procedure as that for standard solutions of GABA.

HPLC Separation and Detection of PTC-GABA. The dry residue containing PTC-GABA was dissolved in 200 μL of mobile phase that consisted of 80% solution A (aqueous solution of 8.205 g of sodium acetate, 0.5 mL of triethylamine, 0.7 mL of acetic acid, and 5.0 mL of acetonitrile in 1000 mL of distilled water) and 20% solution B (acetonitrile−water, 60:40). The pH value for both solutions A and B was adjusted to 5.8. Chromatographic separation was conducted on a C_{18} column (LiChroCART 250-4, Merck). The eluent was pumped at a flow rate of 0.6 mL/min. UV detection was set at 254 nm (*22*).

Pigment Estimation. Pigment concentrations were estimated by a spectrophotometer at 500 nm. The results were expressed as optical density units per gram of dried medium multiplied by a dilution factor (*23*).

RESULT

Estimation on Citrinin Concentration−Relationship between the Size of Inhibition Zone and Citrinin Production. Wong and Bau pointed out that *B. subtilis* would be inhibited by the antibacterial activity produced by *Monascus* species (*16*), and an inhibition zone was formed around the colony of strain as shown in **Figure 1**. A bigger inhibition zone means a higher antibacterial activity of the *Monascus* species. Blanc et al.

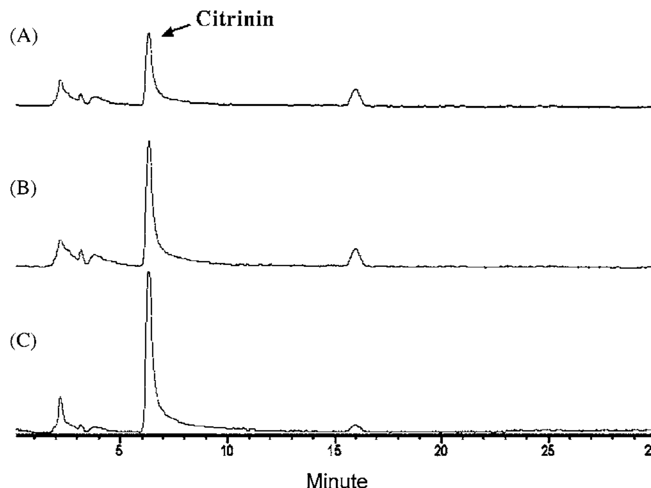

Figure 2. HPLC chromatography of the extract of citrinin in the inhibition zone. Inhibition zone diameter: (**A**) 1.5, (**B**) 3.0, and (**C**) 4.5 cm.

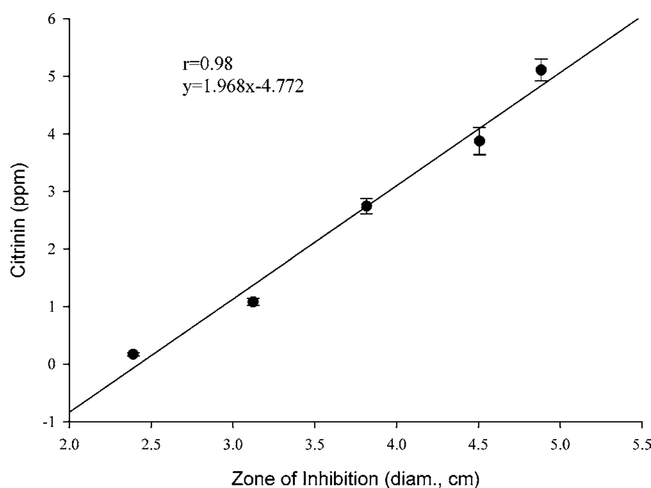

Figure 3. Dose−response curve of the inhibition zone of *B. subtilis* to the citrinin concentration. The values represent the mean ± SD (n = 3).

proved that the antibacterial substance monascidin A is citrinin (*6*). In this study, HPLC analysis was used to evaluate the content of citrinin in the inhibition zone produced by *Monascus* species, and the results are shown in **Figure 2**. It was found that a bigger inhibition zone indicated that more citrinin was produced. The regression coefficient was 0.98 (see **Figure 3**). In addition, a different *Monascus* fungus was used to prove the sensitivity of this method. The relationship between the diameter of the inhibition zone and the citrinin content in PDA medium or red mold rice is shown in **Table 1**. It can be seen that when the inhibition zone is bigger, the citrinin concentration in the red mold rice or PDA medium is higher and when the inhibition zone is smaller, the production of citrinin is relatively lower. Therefore, the result confirmed that the diameter of the inhibition zone was directly proportional to the citrinin concentration in previous studies. On the basis of the results mentioned above, we concluded that this method was feasible to screen the strain with low citrinin production.

Screening of Mutant Strains. Chemical mutagens and UV irradiation were applied to treat *M. purpureus* NTU 601 in order to obtain the *Monascus* strains with low citrinin productivities. After the strains were cultured in PDA, 1463 colonies were isolated. Eleven of these colonies showed that inhibition ratios were greater than 99%, which might indicate a low citrinin production. Besides, the citrinin productions of the 11 strains were also found to be lower than those of the parental strain.

Table 1. Relationship between the Clear Zone of *B. subtilis* and the Production of Citrinin by *Monascus* spp.[a]

species	inhibition zone diameter (cm)	citrinin concn (in clear zone, ppm)	citrinin concn (in red mold rice, ppm)
M. ruber BCRC 31538	5.0 ± 0.1 a	4.36 ± 0.14 a	4.02 ± 0.22 a
M. anka M-13	3.8 ± 0.2 b	2.74 ± 0.13 b	1.73 ± 0.20 b
M. purpureus BCRC 31615	2.9 ± 0.1 c	1.08 ± 0.05 c	0.81 ± 0.05 c
M. sp. KT	2.4 ± 0.3 d	0.17 ± 0.02 d	0.71 ± 0.02 d

[a] The values represent the mean ± SD ($n = 3$). The significant notation was denoted in the order of a–d, with a representing the least desired (least metabolite concentration) condition and d representing the most desired (most metabolite concentration) condition.

Table 2. Production of Citrinin and Monacolin K in PDA Medium by *Monascus* that Have Small Inhibition Zones in Primary Screening Tests[a]

strain nos.	citrinin concn (ppm)	monacolin K concn (ppm)
M. purpureus NTU 601	1.08 ± 0.02	251.16 ± 7.34
M. purpureus N 301[b]	0.51 ± 0.01	261.38 ± 9.23
M. purpureus N 302[c]	0.42 ± 0.01	100.54 ± 5.87
M. purpureus N 303[d]	0.61 ± 0.01	20.76 ± 1.12
M. purpureus N 304[b]	0.51 ± 0.01	15.25 ± 3.11
M. purpureus N 305[c]	0.73 ± 0.02	182.15 ± 7.12
M. purpureus N 306[d]	0.23 ± 0.01	12.26 ± 2.54
M. purpureus N 307[b]	0.65 ± 0.01	92.98 ± 9.12
M. purpureus N 308[c]	0.45 ± 0.01	80.45 ± 4.45
M. purpureus N 309[b]	0.21 ± 0.01	138.65 ± 4.48
M. purpureus N 310[b]	0.28 ± 0.01	201.97 ± 9.17
M. purpureus N 311[b]	0.16 ± 0.01	9.15 ± 1.32

[a] The values represent the mean ± SD ($n = 3$). [b] Mutant obtained from the NTG treatment. [c] Mutant obtained from the EMS treatment. [d] Mutant obtained from the UV irradiation.

Seeking Mutants with Low Citrinin and High Monacolin K and GABA Productions. This study used *M. purpureus* NTU 601 as the parental strain and used NTG, EMS, and UV radiation to select 11 mutant strains with low citrinin production (citrinin production was in the range of 0.16−0.73 ppm). Of the 11 strains, *M. purpureus* N 311 produced the least amount of citrinin in PDA and low monacolin K as well. As shown in the **Table 2**, the concentration of monacolin K produced by the 11 strains had no direct relationship with the concentration of citrinin. The target of this study was to screen out strains with low citrinin production and high monacolin K concentration. Examining the concentrations of monacolin K and citrinin of the 11 strains in the PDA medium, *M. purpureus* N 301 and *M. purpureus* N 310 were found to produce a significant amount of monacolin K, and, in the meantime, the concentrations of citrinin were reduced by 47−26% as compared to that of the parental strain. Therefore, these two strains were selected because they resulted in the best production of monacolin K and GABA.

Optimum Culture Condition for Monacolin K, GABA, and Citrinin Production. In a recent study (*19*), we applied RSM to study the use of *M. purpureus* NTU 601 for solid state fermentation on rice medium and found that the method could result in a higher production of monacolin K and GABA as well as a lower production of citrinin. In that study, when the RSM condition was used, we observed that the content of monacolin K was increased from 136.03 ± 8.23 to 530.61 ± 11.31 ppm, and the GABA concentration was increased from 1060 ± 180 to 5000 ± 221 ppm, while the citrinin content was decreased from 0.81 ± 0.01 to 0.46 ± 0.01 ppm.

In addition, *M. purpureus* N 301 and N 310 strains were used as above methods for solid state culture and were analyzed by using the RSM method. The result is shown in **Table 3**. It can be seen that the *M. purpureus* N 301 strain produced 0.23 ± 0.01 ppm citrinin, which was 50% less than that of the parental strain; the monacolin K production was 481.29 ± 7.98 ppm, which was 91% of that obtained by the parental strain. The *M. purpureus* N 310 strain produced 0.27 ± 0.01 ppm citrinin, which was 41% less than that of the parental strain, and the monacolin K production was 526.29 ± 5.54 ppm, which was similar to that produced by the parental strain. The GABA concentration of both strains was 5000 ppm, which was equivalent to that of the parental strain.

Estimation on the Pigmentation. The citrinin, monacolin K, and pigment of red mold rice came from the metabolic pathway of polyketide. The result of the test on the coloring ability of the strains was shown in **Table 3**. The red pigment produced by *M. purpureus* N 301 and *M. purpureus* N 310 was higher than that of the parental strain.

DISCUSSION

For all of the previous studies concerning the secondary metabolites of red mold rice, the pigment of red mold rice drew the most attention, and most researchers used physical or chemical mutagens to improve the production of red pigments. Mutagensis was found to be the most effective method to increase the hyperpigmentation (*24*, *25*). Pigment, monacolin K, and citrinin were chemical compounds of polyketide. Therefore, to obtain strains with low or zero production of citrinin, researchers used physical and chemical mutagens to induce the mutant and to screen out the strains with low or zero production of citrinin. Hiroi and Yongsmith pointed out that the improvement of strains by UV irradiation treatment was most effective (*25*, *26*). In this study, we used UV irradiation and chemical mutation techniques to improve the strains and found out that the outcome of chemical mutation was satisfactory. Although the citrinin of *M. purpureus* N 311 was 85% lower than that of the parental strain, the monacolin K concentration also decreased 96% when compared with that of the parental strain. It meant that the metabolic pathway of *M. purpureus* N 311 was significantly changed due to mutation.

The size of inhibition zone was a commonly used method to test the antibacterial activity of the strains. Previous studies showed that the antibacterial substances were produced by *Monascus* species (*6*). The antibacterial substance was identified as citrinin (monascidin A), which had an antibacterial effect to *B. subtilis*. The *M. purpureus* NTU 601 strain was proven to produce citrinin in our earlier study (*19*). Therefore, this study postulated that the antibacterial effect of citrinin on *B. subtilis* was related to the size of the inhibition zone. This study applied an HPLC technique and discovered that the size of the inhibition zone of the *Monascus* species was proportional to the citrinin content (**Figure 2**). Thus, the extent of the citrinin concentration could be sensed from the size of the inhibition zone. In addition, all mutants were analyzed in PDA after they were cultured in

Table 3. Production of Citrinin, Monacolin K, and GABA on Solid Culture by *Monascus* Mutant Strains[a]

strains	citrinin concn (ppm)	monacolin K concn (ppm)	GABA concn (ppm)	pigment (A_{500}/g)
M. purpureus NTU 601[b]	0.81 ± 0.01 a	136.03 ± 8.23 a	1060 ± 180 a	38.42 ± 2.22 a
M. purpureus NTU 601[c]	0.46 ± 0.01 b	530.61 ± 11.31 d	5000 ± 221 c	43.12 ± 3.12 b
M. purpureus N 301[c]	0.23 ± 0.01 d	481.29 ± 7.98 b	5210 ± 286 d	50.21 ± 2.39 d
M. purpureus N 310[c]	0.27 ± 0.01 c	526.29 ± 5.54 c	4720 ± 135 b	48.56 ± 1.87 c

[a] The values represent the mean ± SD (n = 3). The significant notation was denoted in the order of a–d, with a representing the least desired (least metabolite concentration) condition and d representing the most desired (most metabolite concentration) condition. [b] Traditional culture method. [c] RSM culture method.

PDA medium, and the concentration of citrinin was found to be proportional to the size of the inhibition zone. Although different strains were used in the sensitivity test, we found that all strains produced inhibition zones and the size of the inhibition zone was proportional to the citrinin concentration by solid state culture.

Eleven strains with smaller inhibition zones were used in this study. *M. purpureus* N 301 and *M. purpureus* N 310 were selected as the ideal strains for the production of monacolin K and GABA because they resulted in low citrinin and high monacolin K productions. Although *M. purpureus* N 301 had a higher concentration of citrinin in PDA than that of *M. purpureus* N 310 as shown in **Table 2**, **Table 3** showed that the citrinin concetraiton in *M. purpureus* N 301 was the same as that of *M. purpureus* N 310 when rice was used as the base material under solid state fermentation. The result showed that *M. purpureus* N 301 could reduce the citrinin concentration and increase monacolin K production if ethanol or a proper amount of water was added to the substrate.

Pigments and citrinin are secondary metabolites derived from the polyketide pathway. Hajjaj et al. (*27*, *28*) suggest that the precursor for both pigments and citrinin is tetraketide, which was formed by condensation of one acetyl-CoA molecule and three malonyl-CoA molecules. Thus, citrinin was formed by adding one acetyl-CoA molecule to tetraketide, followed by a series of reactions including methylation, condensation, reduction, O-alkylation, cleavage between C-1 and C-2 bonding, oxidation, and dehydration. On the other hand, pigments were formed by adding two malonyl-CoA molecules to tetraketide followed by estification. On the basis of the above reasoning, the pigment production is accompanied by the production of citrinin, synthesized by the polyketide pathway (*27*, *28*). In this study, the red pigment productivities of the parental strain and mutant strains were listed in **Table 3**. It was found that the red pigment produced by both *M. purpureus* N 301 and *M. purpureus* N 310 was higher than that of the parental strain. However, the citrinin content in the metabolites was decreased. This phenomena might be related to the difference in the metabolic pathway. In other words, the mutant operation conducted in this study might have already changed the metabolic pathway of the *M. purpureus* NTU 601.

Although this research did not screen out any strain that has zero production of citrinin, *M. purpureus* N 301 and *M. purpureus* N 310 did show much lower citrinin concentrations than that of the parental strain. Besides, this study also pointed out that mutation methods may be used to select a strain that has zero production of citrinin, and physical or chemical methods can be used to reduce the citrinin concentration in *Monascus* species while maintaining monacolin K and GABA productivities. If citrinin could be reduced in red mold rice, it may become an important functional health food to human beings.

LITERATURE CITED

(1) Blanc, P. J.; Loret, M. O.; Santerre, A. T.; Pareilleux, A.; Prome, D.; Prome, J. C.; Laussac, J. P.; Goma, G. Pigment of *Monascus. J. Food Sci.* **1994**, *59*, 862−865.

(2) Wong, H. C.; Koehler, P. E. Production and isolation of an antibiotic from *Monascus purpureus* and its relationship to pigment production. *J. Food Sci.* **1981**, *46*, 589−592.

(3) Su, Y. C.; Wang, J. J.; Lin, T. T.; Pan, T. M. Production of the secondary metabolites γ-aminobutyric acid and monacolin K by *Monascus*. *J. Ind. Microbiol. Biotechnol.* **2003**, *30*, 40−46.

(4) Aniya , Y.; Ohtani, I. I.; Higa, T.; Miyagi, C.; Gibo, H.; Shimabukuro, M.; Nakanish, H.; Taira, J. Dimerumic acid as an antioxidant of the mold, *Monascus anka*. *Free Radical Biol. Med.* **1999**, *286*, 999−1004.

(5) Wu, G. F.; Wu, X. C. Screening DPPH radical scavengers from *Monascus* sp. *Acta Microbiol. Sin.* **2000**, *40*, 394−399.

(6) Blanc, P. J.; Laussac, J. P.; Le Bars, J.; Le Bars, P.; Loret, M. O.; Pareilleux, A.; Prome, D.; Prome, J. C.; Santerre, A. L.; Goma, G. Characterization of monascidin Afrom *Monascus* as citrinin. *Int. J. Food Microbiol*. **1995**, *27*, 201−213.

(7) Blanc, P. J.; Loret, M. O.; Goma, G. Production of citrinin by various species of *Monascus*. *Biotechnol. Lett.* **1995**, *17*, 291−294.

(8) Endo, A. Monacolin K, a new hypocholesterolemic agent produced by *Monascus* species. *J. Antibiot.* **1979**, *32*, 852−854.

(9) Albert, A. W.; Chen, J.; Springer, J. Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol lowing agent. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3957−3961.

(10) Carlson, L. A. Serum lipids and atherosclerotic disease. In *Metabolic Risk Factors in Ischemia Cardiovascular Disease*; Carlson, L. A., Pernow, B., Eds.; Raven Press: New York, 1982; pp 1−16.

(11) Matheson, G. K.; Freed, E.; Tunnicliff, G. Novel GABA analogues as hypotensive agent. *Neuropharmacology* **1986**, *25*, 1191−1195.

(12) Ueno, Y.; Hayakawa, K.; Takahashi, S.; Oda, K. Purification and characterization of glutamate decarboxylase from *Lactobacillus brevis* IFO 12005. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 1168−1171.

(13) Kohama, Y.; Matsumoto, S.; Mimura, T.; Tanabe, N.; Inada, A.; Nakanishi, T. Isolation and identification of hypotensive principles in red-mold rice. *Chem. Pharm. Bull.* **1987**, *35*, 2484−2489.

(14) Deruiter, J.; Jacyno, J. M.; Davis, R. L.; Cutler, H. G. Studies on aldose reductase inhibitors from fungi. I. Citrinin and related benzopyran derivatives. *J. Enzyme Inhib.* **1992**, *6*, 201−210.

(15) Budavari, S.; Maryadele, J. O.; Smith, A.; Heckelman, P. E. *The Merck Index*; 1989; Vol. 11, pp 2330−2331.

(16) Wong, H. C.; Bau, Y. S. Pigmentation and antibacterial activity of fast neutron- and X-ray-induced strains of *Monascus purpureus* Went. *Plant Physiol.* **1977**, *60*, 578−581.

(17) Monica, S.; Roel, F. M.; Johanna, F. G. Mutagenicity of commercial *Monascus* fermentation products and the role of citrinin contamination. *Mutat. Res.* **1999**, *444*, 7−16.

(18) Hsieh, Y. T.; Pan, T. M. The analytic methods and assays for secondary metabolites of *Monascus* products. *J. Biomass Energy Soc. China* **2002**, *21*, 63−71.

(19) Wang, J. J.; Lee, C. L.; Pan, T. M. Improvement of monacolin K, γ-aminobutyric acid and citrinin production ratio as a function of environmental conditions of *Monascus purpureus* NTU 601. *J. Ind. Microbiol. Biotechnol.* **2003**, *30*, 669−676.

(20) Wang, U. L.; Houng, J. Y.; Chang, H. S.; Chien, H. C. R.; Hsu, W. H. Selection of drug-resistant mutants *Monascus pilosus* for enhanced monacolin K production. *J. Chin. Agric. Chem. Soc.* **1998**, *36*, 192−200.

(21) Kycko, S.; Shiho, S. S.; Yoko, K.; Tamio, M. Analytical method for citrinin in *Monascus* colour. *Jpn. J. Food. Chem.* **1998**, *5*, 64−68.

(22) Rossetti, V.; Lombard, A. Determination of glutamate decarboxylase by high-performance liquid chromatography. *J. Chromatogr. B* **1996**, *681*, 63−67.

(23) Lin, C. F.; Iizuka, H. Production of extracellular pigment by mutant of *Monascus kaoliang* sp. nov. *Appl. Environ. Microbiol.* **1982**, *43*, 671−676.

(24) Lin, C. F.; Suen, S. J. T. Isolation of hyperpigment productive mutants of *Monascus* sp F-2. *J. Ferment. Technol.* **1973**, *51*, 757−759.

(25) Yongsmith, B.; Krairak, S.; Bavavoda, R. Production of yellow pigments in submerged culture of a mutant of *Monascus* spp. *J. Ferment. Bioeng.* **1994**, *78*, 223−228.

(26) Hiroi, T.; Shima, T.; Suzuki, T.; Tsukioka, M.; Ogasawara, N. Hyperpigment productive mutant of *Monascus anka* for solid culture. *Agric. Biol. Chem.* **1979**, *43*, 1975−1976.

(27) Hajjaj, H.; Blanc, P. J.; Groussac, E.; Goma, G.; Uribelarrea, J. L.; Loubiere, P. Improvement of red pigment/citrinin production ratio as a function of environment conditions by *Monascus ruber*. *Biotechnol. Bioeng.* **1999**, *64*, 497−501.

(28) Hajjaj, H.; Blanc, P. J.; Groussac, E.; Uribelarrea, J. L.; Goma, G.; Loubiere, P. Kinetic analysis of red pigment and citrinin production by *Monascus ruber* as a function of organic acid accumulation. *Enzyme Microb. Technol.* **2000**, *27*, 619−625.

Received for review February 7, 2004. Revised manuscript received April 25, 2004. Accepted August 31, 2004.

JF049783O