Full Paper

Induction of mutation in *Monascus purpureus* isolated from Thai fermented food to develop low citrinin-producing strain for application in the red koji industry

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Red koji is produced from cultivating rice with *Monascus* strains that contain various types of fungal secondary metabolites, such as red pigments and monacolin K. *Monascus* strain also produces citrinin—a mycotoxin. In this study, *Monascus purpureus* KUPM5 isolated from the Thai fermented food, *sufu*, was mutagenized to reduce its citrinin production using UV irradiation, NTG treatment, and a combination of UV and NTG. Screening of the mutants using plate bioassay based on the inhibitory effect against *Bacillus subtilis* enables the selection of 10 mutants. The mutant strains KS301U and KS302U showed an 80% reduction in citrinin production in red koji compared with the wild type (wt), and maintained the ability to produce red pigments similar to the wild type. Activities of enzymes, α -amylase, protease, and lipase, from red koji extract produced by the mutant strain KS302U, were higher than those of the wt, whereas those of the mutant strain KS301U were similar to those of the wt. Consequently, strains KS301U and KS302U were successfully selected as strains suitable for producing red koji and fermented food.

Key Words: citrinin; fermented food; *Monascus purpureus*; mutagenesis; rkoji; secondary metabolites

Introduction

Red koji (red mold rice) is used as a food additive and colorant in the food industry. It is produced from *Monascus purpureus* and has been traditionally used to produce several fermented products, such as red soy cake, red *sufu*, sour pork, fish sauce, and rice fermented wine in east Asia (Chow, 2017). It has several beneficial effects such as cancer prevention and blood sugar level reduction as well as antiinflammatory and antitumor effects (Yasukawa et al., 1996).

However, certain *Monascus* sp. produce various amounts of citrinin—a secondary metabolite mycotoxin. Citrinin has negative effects in humans and animals, including nephrotoxic, hepatotoxic, and cytotoxic effects (Blanc et al., 1995; Liu et al., 2005; Liu et al., 2012). It also exhibits antibacterial activity (Wong and Bau, 1977). In recent years, there has been a greater awareness of the consequences of citrinin to human health. The European Food Safety Authority has established a maximum level of citrinin in food supplements, based on red koji, as 2,000 $\mu\text{g}/\text{kg}$ because of concerns that citrinin directly affects kidney toxicity (European Food Safety Authority, 2012). Therefore, the selection of *Monascus* strains is the first important step in the production of red koji with low amounts of citrinin. Citrinin biosynthesis occurs in *Monascus*, *Penicillium*, and *Aspergillus* species (Flajs and Peraica, 2009). The pathway involves the synthesis of an unreduced trimethylated pentaketide by a non-reducing polyketide synthase (nrPKS), known as CitS. *cit* cluster centered on the 7.9 kbp gene, which encodes an iterative type I non-reducing polyketide synthase (CitS), which was discovered in *M. purpureus*. (Sakai et al., 2008; Shimizu

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et al., 2007). The disruption of *citS* resulted in the complete elimination of citrinin production in *M. purpureus* (He et al., 2013). Thus, the construction of citrinin-nonproducing *Monascus* strains using gene recombinant technology is a powerful tool (Huang et al., 2017; Liang et al., 2018), although using classical mutagenesis might be more favorable for use in the fermented food industry. Isolation and screening of low citrinin-producing mutants of *M. purpureus* has been reported by Wang et al. (2004) and Putri et al. (2005). In addition to low citrinin-productivity, they intended to obtain the mutants showing a high productivity of monacolin K and γ -aminobutyric (Wang et al., 2004), and red pigments (Putri et al., 2005). In the present study, we induced mutations in *M. purpureus* KUPM5 isolated from Thai fermented food, *sufu* (Kanlayakrit et al., 2011) as a parental wild type (wt) strain, which was different from *Monascus* strains reported previously, to produce mutant strains with low citrinin production and maintaining the enzyme activities for *sufu* making. The selected mutant strains were characterized with respect to the citrinin production, red pigments, and enzyme activity to evaluate mutant strains suitable for production red koji in the food industry.

Materials and Methods

Microorganisms and culture media. *M. purpureus* strain KUPM5 isolated from Thai fermented food, *sufu* (Kanlayakrit et al., 2011), was obtained from the Faculty of Agro-industry, Kasetsart University, Thailand. *Bacillus subtilis* NBRC13719 was obtained from the National Bioresource Center, Japan. KUPM5 was maintained on potato dextrose agar (PDA) medium (Becton Dickinson company) at 30°C for 7 days. *B. subtilis* strain was maintained in Luria-Bertani (LB) broth.

Red koji production. Red koji was prepared by *Monascus* strains in triplicate according to the procedure described by Kanlayakrit et al. (2011). Fifty grams of long grain rice (*Oryza sativa*) were soaked in tap water for 8 h. Excess water was drained, and soaked rice was transferred into a 500-ml Erlenmeyer flask, after which 15 ml of distilled water was added and then autoclaved at 121°C for 30 min. After the steamed rice was cooled to room temperature, 2 ml of fungal spore suspension (10^6 spores/ml) was inoculated and incubated at 30°C for 7 d. The resultant red koji was stored at -80°C until use.

Mutagenesis. Ultraviolet (UV) irradiation: *Monascus* spore suspension was prepared from *Monascus* grown on PDA plates with 0.01% Tween80 solution, and the number of spores was adjusted to 10^6 spores/ml. Four milliliters of spore suspension was poured upto a dish (d.i. 90 mm), which was then placed under a UV lamp at a distance of 55 cm for 20, 30, and 40 min (Wang et al., 2004). The spores were collected by centrifugation and suspended in fresh potato dextrose broth (PDB) medium. The spores were incubated at 30°C in the dark for 6 h and then spread on PDA plates at 30°C for 72 h. Colonies developed, which were then screened by plate bioassay.

NTG (N-methyl-N'-nitro-N-nitrosoguanidine) treatment: The number of spores was adjusted to 10^6 spores/ml using 0.1M citrate buffer (pH 5.0). Two milliliters of an NTG

solution (50, 100, and 150 μ g/ml) was added to 2 ml of spore suspension. After incubation for 30 min, the spores were immediately collected by centrifugation and washed three times with sterilized distilled water. The spores were grown in fresh PDB medium at 30°C for 6 h and spread on PDA plates and then incubated at 30°C for 72 h. Colonies developed, which were then screened by plate bioassay.

Combination of UV irradiation and NTG treatment: Two milliliters of an NTG (100 μ g/ml) solution was added to 2 ml of spore suspension and placed under a UV lamp for 10, 20, and 30 min. The spore suspension was centrifuged and washed three times with sterilized distilled water. The spores were incubated in PDB medium at 30°C for 6 h and spread on PDA plates and then incubated at 30°C for 72 h. Colonies developed, which were then screened by plate bioassay.

Screening of *Monascus* mutants showing low citrinin production. Screening of *Monascus* mutants by a plate bioassay was performed according to the method described by Wang et al. (2004). Mutagenized *Monascus* cells were spread on PDA plates containing 0.01% TritonX-100 and incubated at 30°C for 72 h. *B. subtilis* strain NBRC 13719 was grown in LB medium at 30°C for 24 h. One hundred microliters of *Bacillus* culture was added to 8 ml of soft LB agar medium (0.7% agar) and then poured onto the *Monascus*-grown plate. After 72 h, mutant strains with low citrinin production were selected by observing bacterial inhibition zones around the fungal colonies. The inhibition ratio was calculated using the following formula (Wang et al., 2004):

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

where *C* is the average zone of inhibition of the wt and *E* is the average zone of inhibition of the mutant.

Determination of amounts of citrinin in the koji using HPLC. Red koji was prepared using the selected mutant strains. Citrinin produced by the *Monascus* strains in the koji were extracted by the method described by Putri et al. (2005). One gram of dried red koji was ground and extracted with 40 ml of 70% ethanol by shaking at 80 rpm at 30°C. After 3 h, the extracts were filtrated through a membrane (Dismic-13CP, 0.45 μ m pore size, Advantec Inc., Japan), and the amounts of citrinin in the red koji was determined by HPLC. HPLC was performed using a C18 column (TSKgel ODS 4.6 mm $\times$ 250 mm, Tosho, Japan) and 80% acetonitrile:0.1% phosphoric acid (60:40) as the mobile phase at a flow rate of 1.0 ml/min, with a fluorescence detector (excitation at 330 nm and emission at 500 nm).

Determination of the amount of red pigments. Red pigments in the koji were extracted according to the method described above in citrinin extraction. The amount of red pigments in the koji was estimated by measuring the absorbance at 500 nm. Results were expressed as absorbance unit per gram koji (Putri et al., 2005).

Enzyme assays. Water (150 ml) was added to the red koji (7 d growth), and enzymes were extracted at 4°C for 4 h.

Fig. 1. Zone of inhibition of *Monascus* mutant strains against *Bacillus subtilis*.

All mutant strains were cultured at 30°C for 5 d on PDA plate, and *Bacillus* culture (in soft LB agar medium, 0.7% agar) was poured onto the plate for screening. Mutant strains KS301U (A), KS1001C (B), KS1003C (C), and K1004C (D) showed a lower citrinin production because of the smaller zone of inhibition compared with the wt strain KUPM5 (E).

Table 1. Size of inhibition zone and inhibition ratio of *Monascus* mutants.

<i>M. purpureus</i> strains	Size of inhibition zone (cm)	Inhibition ratio (%)
KUPM5 (wt)	0.24	—
KS301U	0.15	37.5
KS302U	0.16	33.3
KS101N	0.12	50.0
KS102N	0.07	70.8
KS103N	0	100
KS104N	0.05	79.2
KS1001C	0.07	70.8
KS1002C	0.02	91.7
KS1003C	0.05	79.2
KS1004C	0	100

The resultant crude enzyme solution was filtrated through a membrane (Whatman No. 1, 125 mm) and stored at –20°C (Kanlayakrit et al., 2011).

α -Amylase assay: α -Amylase activity was determined using the method described by Onishi and Sonoda (1979). A substrate solution (3.9 ml of 1% gelatinized starch in 0.1 M McIlvaine buffer, pH 4.0) was preincubated at 50°C for 10 min and then added to 0.1 ml of the crude enzyme solution. After 10 min, 0.2 ml of the reaction mixture was added to 5 ml of 0.167 mM iodine solution. The α -amylase activity of the red koji was determined by measuring the absorbance at 770 nm. One unit (U) of α -amylase was defined as the change in absorbance per min.

Protease assay: Protease activity was estimated according to Chancharoonpong et al. (2012) using casein as a

Fig. 2. Chromatogram of citrinin in koji extracts from *Monascus* strains.

Citrinin in the red koji made by strains KUPM5 (A), KS104N (B), and KS301U (C) was separated by HPLC. The retention time of citrinin was detected at 13.7 min.

Table 2. Citrinin concentration and relative citrinin production of *Monascus* mutants.

<i>M. purpureus</i> strains	Citrinin concentration (ng/g)	Relative citrinin production to wild type
KUPM5 (wt)	174.3±1.5	100%
KS301U	32.0±0.3	18.4%
KS302U	33.2±0.2	19.1%
KS101N	5347.3±17.1	3067.7%
KS102N	3745.4±19.4	2148.7%
KS103N	3452.4±89.7	1980.6%
KS104N	2229.0±32.2	1278.8%
KS1001C	0	0
KS1002C	0	0
KS1003C	0	0
KS1004C	0	0

substrate. A substrate solution (1 ml of 1.5% casein in 0.1 M phosphate buffer pH 7.0) was preincubated at 37°C for 10 min. One milliliter of the crude enzyme solution was added, well shaken, and incubated at 37°C for 10 min. Two milliliters of 0.44 M trichloroacetic acid (TCA) solution was added to terminate the reaction and then incubated for 30 min. Two milliliters of the mixture was filtrated and then 2.5 ml of 0.4 M Na₂CO₃ solution was added and well shaken, and 0.5 ml of Folin reagent was added. Protease activity was determined by measuring the absorbance at 660 nm. One unit (U) of protease activity was defined as the amount of 1 μ g tyrosine released per minute. Lipase assay: Lipase activity was assayed using

Table 3. Enzyme activity and red pigment production of *Monascus* mutant strains.

<i>M. purpureus</i> strains	Enzyme activity (Unit/ml)			Red pigment (A ₅₀₀ /g)
	α -amylase	protease	lipase	
KUPM5 (wt)	4.89±0.65 ^{bcd}	2.63±0.67 ^f	5.39±0.99 ^g	9.17±0.41 ^b
KS301U	4.51±0.65 ^{bcd}	3.38±0.30 ^{ef}	9.21±0.73 ^f	6.95±0.30 ^c
KS302U	4.26±0.22 ^{cd}	6.43±0.72 ^d	12.72±0.27 ^c	9.05±0.80 ^b
KS101N	6.52±0.95 ^a	15.07±0.40 ^a	13.25±1.87 ^{de}	5.37±0.24 ^d
KS102N	3.51±0.95 ^d	13.20±1.05 ^b	16.89±1.06 ^{bc}	7.00±0.31 ^c
KS103N	5.76±0.78 ^{ab}	14.28±0.89 ^{ab}	18.95±3.03 ^{ab}	10.03±0.48 ^b
KS104N	4.39±0.22 ^{cd}	13.60±1.00 ^{ab}	20.09±1.06 ^a	14.27±0.86 ^a
KS1001C	4.39±0.57 ^{cd}	4.78±0.47 ^e	14.30±1.80 ^{cde}	1.72±0.24 ^e
KS1002C	3.76±1.36 ^{cd}	8.93±0.33 ^c	15.48±1.67 ^{cd}	2.78±0.25 ^e
KS1003C	4.01±0.22 ^{cd}	8.66±1.76 ^c	15.83±0.42 ^{cd}	1.67±0.33 ^e
KS1004C	5.14±0.43 ^{bc}	9.32±1.27 ^c	15.70±0.55 ^{cd}	2.15±0.40 ^e

Different superscript letters (a, b, c, d, e, f, g) in the same column represent significantly different values ($p \leq 0.05$) and preparation of koji were carried out in triplicate.

Stuckmann's method (Stuckmann and Winkler, 1979) with *p*-nitrophenyl palmitate (*p*-NPP) as a substrate. The substrate solution was prepared by mixing solution A (10 ml of 30 mg *p*-NPP in isopropanol) with solution B (90 ml of 0.05M phosphate buffer, pH 7.0, containing 100 mg arabic gum (acacia), 207 mg sodium deoxycholate, and 2 ml TritonX-100). The substrate solution (2.4 ml) was preincubated at 37°C for 10 min, and 0.1 ml of the crude enzyme solution was then added and incubated at 37°C for 15 min. Lipase activity in the red koji was determined by measuring the absorbance at 410 nm. One unit (U) of lipase activity was defined as 1 μ mol of *p*-nitrophenol released per minute.

Results

Selection of the low citrinin-producing mutants

M. purpureus KUPM5 (wt strain) was isolated from Thai fermented food, *sufu* (Kanlayakrit et al., 2011). To make this strain safe for producing red koji, we attempted to improve it by reducing the citrinin production. UV irradiation, NTG treatment, and the combination of both UV irradiation and NTG treatment were used to induce the mutation in wt KUPM5. The irradiation period of UV and the concentration of NTG treatment were determined by the survival rate of the wt as an index. After UV irradiation for 30 and 40 min, the survival rate of the wt strain was 10%, and 7%, respectively. Meanwhile, the spores treated with 50, 100, and 150 μ g/ml NTG showed a 50%, 10%, and 0% survival, respectively. The survival rate of spores after UV irradiation for 10 min in the presence of 100 μ g/ml of NTG was <10%, whereas spores after UV treatment for 20 and 30 min showed a 0% survival. We determined the mutagenic conditions to the wt strain KUPM5 with UV irradiation to be 30 min and with 100 μ g/ml NTG treatment to be 30 min.

In total, 2,420 colonies, including 983 colonies by UV irradiation (termed U), 850 colonies by NTG treatment (termed N), and 587 colonies by the combination of both UV and NTG (termed C), were screened by plate bioassay using *B. subtilis* NBRC13719. Ten colonies (U: 2, N: 4,

and C: 4) were selected as showing >80% of diameter of colony formed by the wt strain, and the size of bacterial inhibition zone was >30% smaller than that of the wt (Fig. 1, Table1). These 10 mutants were serially subcultured 5 times on the PDA medium to stabilize the mutation. The mutant strains KS301U (d.i. 20.2 mm), KS1001C (d.i. 18.7 mm), KS1003C (d.i. 17.3 mm), and KS1004C (d.i. 18.4 mm) formed smaller sizes of colonies than the wt (d.i. 21.4 mm) did, indicating that the growth of the selected mutants was slightly repressed on the PDA medium (Fig. 1).

Citrinin production in the koji produced by mutants

Red koji was individually produced by the selected 10 *Monascus* mutant strains. Citrinin was extracted from the red koji and analyzed by HPLC to confirm the citrinin production levels in the mutants (Fig. 2). Among these 10 mutants, all mutants KS1001C, KS1002C, KS1003C, and KS1004C obtained after the combination of UV and NTG treatments completely eliminated citrinin production in the red koji (Table 2). Strains KS301U and KS302U showed citrinin productivity of 32.0 ± 0.3 and 33.2 ± 0.2 ng/g koji, respectively, which was 80% lower than that of the wt (174.3 ± 1.5 ng/g koji). However, strains KS101N, KS102N, KS103N, and KS104N, selected after NTG treatment, produced higher amounts of citrinin in red koji than the wt (Table 2).

Pigment production

M. purpureus produces the red pigment, azaphilone, as a secondary metabolite (Chen et al., 2017). This property of *Monascus* allows it to be used as a food colorant. The result of red pigment production is shown in Table 3. Strains KS103N and KS104N produced red pigments at levels of 10.03 ± 0.18 and 14.27 ± 0.86 U/g koji, respectively, higher than the wt (9.17 ± 0.41 U/g koji). Strain KS302U maintained red pigment production with 9.05 ± 0.80 U/g koji, which was similar to the wt. KS301U produced 24% less red pigment than the wt at 6.95 ± 0.30 U/g koji, whereas all mutants KS1001C, KS1002C, KS1003C, and KS1004C obtained by the combination of UV and NTG showed more than 70% reduction in red pig-

Fig. 3. Red koji after 7 days cultivation at 30°C by the *Monascus* strains.

All red koji samples made by the wt KUPM5 (A) and mutant strains KS301U (B), KS104N, (C), and KS1003C (D) were dried at 60°C for 24 h.

ment production. Thus, the combination mutants almost lost the ability to produce red pigments and became albino mutants (Fig. 3).

Enzyme activities in red koji produced by *Monascus* mutant strains

The quality of Thai fermented food *sufu* is closely related with activities of α -amylase, protease, and lipase from red koji (Han et al., 2001). Thus, it is important to obtain mutants showing these enzymatic activities. To characterize the mutant strains, enzyme activities in the red koji extract of selected mutants were determined (Table 3). The red koji extract produced by strains KS301U and KS302U showed 4.51 ± 0.65 U/ml and 4.26 ± 0.22 U/ml of α -amylase, respectively, which were similar to the α -amylase activity of the wt (4.89 ± 0.65 U/ml). As compared with the enzyme activities of the wt (2.63 ± 0.67 U/ml of protease, 5.39 ± 0.27 U/ml of lipase), strains KS301U and KS302U exhibited enhanced activities with 3.38 ± 0.30 U/ml of protease, 9.21 ± 0.73 U/ml of lipase, 6.43 ± 0.72 U/ml of protease, and 12.72 ± 0.27 U/ml of lipase, respectively. The red koji made by the mutant strain KS104N showed 4.39 ± 0.22 U/ml of α -amylase and indicated high activities of lipase (13.60 ± 1.00 U/ml) and the highest activities of protease (20.09 ± 1.06 U/ml). The mutant strains KS102N, KS1002C, and KS1003C produced lower α -amylase activities and higher lipase activities than the wt.

Discussion

Red koji (red mold rice) is the product of *M. purpureus*, which has been widely used in the food industry. Citrinin, a secondary metabolite derived from the polyketide pathway of *M. purpureus*, is a known mycotoxin. Currently, there are concerns relating to citrinin contamination in red koji. Therefore, methods to reduce citrinin production by

Monascus have been studied. The most powerful technology to produce a citrinin non-producing mutant is to rely on genetic engineering (Huang et al., 2017; Liang et al., 2018). In fact, the loss of function of *citS* involved in the first step of the reaction of citrinin production leads to the complete elimination of citrinin production (He et al., 2013). However, in the field of food industry, classical mutagenesis using mutagens has been an effective technique to improve microorganisms. Wang et al. (2004) and Putri et al. (2005) have used UV irradiation as a physical mutagen, and NTG as a chemical mutagen, to induce the mutation of the *Monascus* strain to obtain a citrinin nonproducing strain showing a high productivity of monacolin K and γ -aminobutyric acid (Wang et al., 2004) and red pigments (Putri et al., 2005). In this study, we used UV irradiation and NTG treatment as mutagens to develop *Monascus* mutant strains with a low citrinin production, and screened them using plate bioassay. Citrinin production in the red koji produced by strains KS301U, KS302U, KS1001C, KS1002C, KS1003C, and KS1004C were 80–100% less than that in the parental strain. However, strains KS101N, KS102N, KS103N, and KS104N produced higher amounts of citrinin than the parental strain, which conflicted with the results of our plate bioassay. This might be due to the difference in culture conditions between plate bioassay and red koji production (Pattanagul et al., 2008; Zhou et al., 2015).

In this study, we used the *M. purpureus* strain KUPM5 isolated from the Thai fermented food *sufu*. Although *Monascus* strains that have been previously mutagenized are different from our *Monascus* strain, the present results are in agreement with those of previous researchs that UV irradiation efficiently induced mutation within genes related to citrinin production, but not within other genes, such as those related to red pigment production, enzyme activity, and fungi hyphal development (Kalaivani and Rajasekaran, 2014; Pattanagul et al., 2008; Wang et al.,

2004). Strains KS1001C, KS1002C, KS1003C, and KS1004C, selected after combined mutagenesis of UV and NTG treatments, eliminated citrinin production and simultaneously reduced red pigment production, leading to an albino koji (Fig. 3). A fungal regulator protein, *LaeA*, is an upstream key regulator for secondary metabolism and sporulation. Alteration of the *laeA* expression leads to simultaneous change in secondary metabolite production in *Monascus* (Lee et al., 2013; Liu et al., 2016). It is possible that these mutants have a mutation within the regulatory gene that functions at the upper level of secondary metabolism and commonly regulates each secondary metabolism. In contrast, strains KS301U and KS302U obtained by UV irradiation showed reduced citrinin production in red koji but maintained the ability to form red pigment similar to the parental strain (Table 3). It is likely that the mutation occurred only in genes involved in citrinin production. We are in the process of conducting a comparative genome analysis between the wt and mutant strains, which will allow us to identify the mutation point in mutant strains.

Reduced citrinin production while maintaining red pigment production in strains KS301U and KS302U make them favorable mutants to produce red koji. With regards to the Thai *sufu* production, α -amylase, protease, and lipase activities were closely associated with the quality of *sufu*, such as flavor, taste and texture (Han et al., 2001). Strains KS301U and KS302U have the possibility to produce another type of *sufu* because of their higher protease and lipase activities than the wt. Further studies examining *sufu* production using *Monascus* mutants should be undertaken to confirm the effect of each mutant and to determine the quality of the *sufu*.

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References

- Blanc, P. J., Laussac, J. P., Le Bars, J., Le Bars, P., Loret, M. O. et al. (1995) Characterization of monascidin A from *Monascus* as citrinin. *Intern. J. Food Microbiol.*, **27**, 201–213.
- Chancharoonpong, C., Hsieh, P. C., and Sheu, S. C. (2012) Enzyme production and growth of *Aspergillus oryzae* S. on soybean koji fermentation. *APCBEE Procedia*, **2**, 57–61.
- Chen, W., Chen, R., Liu, Q., He, Y., He, K. et al. (2017) Orange, red, yellow: biosynthesis of azaphilone pigments in *Monascus* fungi. *Chem Sci.*, **8**, 4917–4925.
- Chow, S. M. (2017) Red fermented rice and food safety. *Food Safety Focus*, **126**, 1–4.
- European Food Safety Authority (EFSA) (2012) Scientific opinion on the risks for public and animal health related to the presence of citrinin in food and feed. *EFSA J.*, **10**, 1–82.
- Flajs, D. and Peraica, M. (2009) Toxicological properties of citrinin. *Arh. Hig. Rada Toksikol.*, **60**, 457–464.
- Han, B. Z., Rombouts, F. M., and Nout, M. J. R. (2001) A chinese fermented soybean food. *Int. J. Food Microbiol.*, **65**, 1–10.
- He, Y., Liu, Q., Shao, Y., and Chen, F. (2013) Ku70 and Ku80 null mutants improve the gene targeting frequency in *Monascus ruber* M7. *Appl. Microbiol. Biotechnol.*, **97**, 4965–4976.
- Huang, Z., Su, B., Xu, Y., Li, L., and Li, Y. (2017) Determination of two potential toxicity metabolites derived from the disruption of the *pksCT* gene in *Monascus aurantiacus* Li As3.4384. *J. Sci. Food Agric.*, **97**, 4190–4197.
- Kalaivani, M. and Rajasekaran, A., (2014) Improvement of monacolin K/citrinin production ratio in *Monascus purpureus* using UV mutagenesis. *Nutrafoods*, **13**, 79–84.
- Kanlayakrit, W., Phromsak, K., Sirirote, P., and Parakulsuksatid, P. (2011) Development of red Tofu fermentation process by pure culture. *Proc. 49th Kasetsart Univ. Annu. Conf. Agro-Industry (in Thai)*, **49**, 532–540.
- Lee, S. S., Lee, J. H., and Lee, I. (2013) Strain improvement by overexpression of the *laeA* gene in *Monascus pilosus* for the production of monascus-fermented rice. *J. Microbiol. Biotechnol.*, **23**, 959–965.
- Liang, B., Du, X. J., Li, P., Sun, C. C., and Wang, S. (2018) Investigation of citrinin and pigment biosynthesis mechanisms in *Monascus purpureus* by transcriptomic analysis. *Front. Microbiol.*, **9**, 1374.
- Liu, B. H., Wu, T., Su, M. C., Chung, C. P., and Yu, F. Y. (2005) Evaluation of citrinin occurrence and cytotoxicity in *Monascus* fermentation products. *J. Agric. Food Chem.*, **53**, 170–175.
- Liu, Q., Cai, L., Shao, Y., Zhou, Y., Li, M. et al. (2016) Inactivation of the global regulator *LaeA* in *Monascus ruber* results in a species-dependent response in sporulation and secondary metabolism. *Fungal Biol.*, **120**, 297–305.
- Liu, S., Wang, D., Zhang, J., Zhang, D., Gong, M. et al. (2012) Citrinin reduces testosterone secretion by inducing apoptosis in rat Leydig cells. *Toxicol. Vitro.*, **26**, 856–861.
- Onishi, H. and Sonoda, K. (1979) Purification and some properties of an extracellular amylase from a moderate halophile, *Micrococcus halobius*. *Appl. Environ. Microbiol.*, **38**, 616–620.
- Pattanagul, P., Pinthong, R., Phianmongkhon, A., and Tharatha, S. (2008) Mevinolin, citrinin and pigments of adlay angkak fermented by *Monascus* sp. *Int. J. Food Microbiol.*, **126**, 20–23.
- Putri, S. P., Kinoshita, H., and Nihira, T. (2005) Mutagenesis of *Monascus purpureus* for higher/reduced red pigment production, and phenotypic/genetic analyses on the relevant mutants. *Annu. Rep. ICBiotech*, **27**, 745–754.
- Sakai, K., Kinoshita, H., Shimizu, T., and Nihira, T. (2008) Construction of a citrinin gene cluster expression system in heterologous *Aspergillus oryzae*. *J. Biosci. Bioeng.*, **106**, 466–472.
- Shimizu, T., Kinoshita, H., and Nihira, T. (2007) Identification and *in vivo* functional analysis by gene disruption of *ctnA*, an activator gene involved in citrinin biosynthesis in *Monascus purpureus*. *Appl. Environ. Microbiol.*, **73**, 5097–5103.
- Stuckmann, M. and Winkler, U. K. (1979) Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *J. Bacteriol.*, **138**, 663–670.
- Wang, J. J., Lee, C. L., and Pan, T. M. (2004) Modified mutation method for screening low citrinin-producing strains of *Monascus purpureus* on rice culture. *J. Agric. Food Chem.*, **52**, 6977–6982.
- Wong, H. C. and Bau, Y. S. (1977) Pigmentation and antibacterial activity of fast neutron- and X-ray-induced strains of *Monascus purpureus* Went. *Plant Physiol.*, **60**, 578–581.
- Yasukawa, K., Takahashi, M., Yamanouchi, S., and Takido, M. (1996) Inhibitory effect of oral administration of *Monascus* pigment on tumor promotion in two-stage carcinogenesis in mouse skin. *Oncology*, **53**, 247–249.
- Zhou, Z., Guo, H., and Xie, C. (2015) Effects of culture conditions on production of red pigment and citrinin by fermentation of *Monascus ruber*. *Chem. Eng. Trans.*, **46**, 1363–1368.