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 µg/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
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 acid (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 Institute of Agro-industry, Kasetsart University, Thailand. *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 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 500 ml. After the steamed rice was cooled to room temperature, 2 ml of fungal spore suspension (10⁶ spores/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 µg/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):

\[
\text{Inhibition ratio (\%) = } \frac{C - E}{C} \times 100
\]

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 filtered through a membrane (Dismic-13CP, 0.45 µm 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 × 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.
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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 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 µg tyrosine released per minute.

Lipase assay: Lipase activity was assayed using M. purpureus strains. Size of inhibition zone and inhibition ratio of Monascus mutants.

| M. purpureus 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                  |

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).

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

| M. purpureus strains | Citrinin concentration (μg/g) | Relative citrinin production to wild type |
|----------------------|-----------------------------|----------------------------------------|
| KUPM5 (wt)           | 174.3±1.5                   | 100%                                   |
| KS301U               | 32.0±0.3                    | 18.4%                                  |
| KS302U               | 39.2±0.2                    | 19.1%                                  |
| KS101N               | 3347.3±17.1                 | 3067.9%                                |
| KS102N               | 3745±19.4                   | 2148.7%                                |
| KS103N               | 3452±89.7                   | 1980.6%                                |
| KS104N               | 2229±32.2                   | 1278.8%                                |
| KS1001C              | 0                           | 0%                                     |
| KS1002C              | 0                           | 0%                                     |
| KS1003C              | 0                           | 0%                                     |
| KS1004C              | 0                           | 0%                                     |
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 arabinose (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 µmol 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 µg/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 µg/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 µg/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, 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, KS302U, KS101N, KS102N, KS103N, KS104N, KS1001C, KS1002C, KS1003C, and KS1004C 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 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, 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).
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Enzyme activities in red koji produced by Monascus mutant strains

The quality of Thai fermented food sufu is closely related with activities of \( \alpha \)-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 \( \alpha \)-amylase, respectively, which were similar to the \( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-amylase activities and higher lipase activities than the wt.

Discussion

Red koji (red mold rice) is the product of \textit{M. purpureus}, which has been widely used in the food industry. Citrinin, a secondary metabolite derived from the polyketide pathway of \textit{M. purpureus}, is a known mycotoxin. Currently, there are concerns relating to citrinin contamination in red koji. Therefore, methods to reduce citrinin production by \textit{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 \textit{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 \textit{Monascus} strain to obtain a citrinin nonproducing strain showing a high productivity of monacolin K and \( \gamma \)-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 \textit{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 \textit{M. purpureus} strain KUPM5 isolated from the Thai fermented food \textit{sufu}. Although \textit{Monascus} strains that have been previously mutagenized are different from our \textit{Monascus} strain, the present results are in agreement with those of previous researches 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.,

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Fig. 3. Red koji after 7 days cultivation at 30°C by the \textit{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.
undertaken to confirm the effect of each mutant and to examine the lipase activities than the wt. Further studies examining mutant strains, which will allow us to identify the mutation point 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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