Construction of a *Monascus purpureus* Mutant Showing Lower Citrinin and Higher Pigment Production by Replacement of *ctnA* with *pks1* without Using Vector and Resistance Gene

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Monascus products are used as both natural colorants and food additives all over the world. However, its safety is really an issue because of the presence of citrinin, which is considered to be hepatotoxic and nephrotoxic. Therefore, the objective of the present study was to develop an approach for using a fragment of pigment-related gene *pks1* to replace citrinin-activator gene *ctnA*. The character of citrinin antibacterial activity to *Bacillus subtilis* was used for primary screening of transformants based on the foundation that the inhibition zone formed around the mutant colonies with low citrinin products will become smaller. The selected mutants were then further confirmed by polymerase chain reaction and high-performance liquid chromatography methods. During all of the processes, antibiotic markers and vectors were avoided. The results showed that the citrinin products of mutants were reduced to 42%, while the pigment products were improved to 33.9%, respectively, over those of the wild-type strains.

KEYWORDS: Citrinin; *Monascus*; pigments; *pksCT*; *pks1*; gene replacement

INTRODUCTION

The filamentous fungus genus *Monascus* is known to produce various bioactive secondary metabolites, such as pigments, monacolin K (lavastation), and γ-aminobutyric acid (GABA) (*1*). The red pigments have been traditionally used as a colorant in Oriental foods and have been rediscovered as natural colorants by modern food industries (*2*). Monacolin K is also commercially known as lavastation, mevacor, and cholestin and regarded as a popular hypolipidemic functional food by blocking a key enzyme, named HMG−CoA, of cholesterol synthesis (*3*).

Another secondary metabolite citrinin ($C_{13}H_{14}O_5$), which has been found in *Aspergillus* and *Pencillium* (*4*), is known as a mycotoxin, reported to be hepatotoxic and nephrotoxic in mammals (*5*), and it has led to a controversy regarding the safety of *Monascus* fermentation production. To reduce citrinin production, there were some research groups focusing on the modification of *Monascus* culturing conditions (*6−9*), while some others investigated genes related to citrinin biosynthesis, including polyketide synthesis gene (*pksCT*) clusters (*10−14*). Now, it is clear that the full length of the *pks* gene is 7838 bp with a single intron of 56 bp and encodes a protein of 2593 amino acids (*10*). There are five open reading frames (ORFs) among the gene, and the activor *ctnA* is located between ORF1 and ORF3. The breakage of *ctnA* will lead to the lower production of citrinin, while the production of red pigments is not affected by the

absence of the gene (*12*). Another study also showed that the production of citrinin was independent of the pigment production by *Monascus* species (*15*) or that the pigment production can be improved (*16*).

Polyketide synthesis gene *pks1* is responsible for polyketide synthesis in *Alternaria alternata* and *Colletotrichum lagenarium* (*17*) and is also found in *Nodulisporium* sp. and *Ceratocystis* sp. (*18*), which contains one ORF with three exons separated by two short introns. The polypeptide encoded by *pks1* shows significant similarities with other polyketide synthases involved in conidial pigmentation, such as wA in *Aspergillus nidulans* (*17, 19*). The *pks1* was discovered by the mRNA/cDNA method in *Monascus*. Similar to *C. lagenarium*, the *pks1* in *Monascus* contains polyketide synthesis polymerases (PKSs), acetyl/malonyl transferase, and transport protein domains (GenBank AJ414729.1). Although there was no further report on the function of this *Monascus pks1* gene, because of the analogous structure of *Monascus pks1* with that of *C. lagenarium*, analogical function of this gene can be inferred in conidial pigmentation.

There were some studies on the gene breakage of *pksCT* and its activator to reduce the citrinin production; however, different vectors and antibiotics, such as hygromycin B and aureobasidin A (AbA), were always used. Thus, exogenous and antibiotic genes were transferred into the genome of *Monascus*, which resulted in the contamination of the genome and gave another concern of producing a safe product. On the other hand, exogenous genes have different GC contents compared to the host genome, which will not be beneficial for the genetic stability of transformants. Therefore, in this study, two sequences in addition to *ctnA* ORF

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Article

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Table 1. Primer Sets for PCR Amplification and Gene Fusion

F1	5′-GGCCAAGCTTCTTGCTCCTGACCAGATTGT-3′
R1	5′-ATTGACGAATGGCGGAATATCCCACTGACTTTGCGGACTTTAT-3′
F2	5′-TAAGAGCGTTTGGACCAGGCCGGCGTTCATAAGGTTGGTTA-3′
R2	5′-GGCCAAGCTTGTGAGCGAAGCCCTGTCT-3′
F3	5′-ATAAAGTCCGCAAAGTCAGTGGGATATTCCGCCATTCGTCAAT-3′
R3	5′-TAACCAACCTTATGAACGCCGGCCTGGTCCAAACGCTCTTA-3′

and *pks1* were cloned, respectively, and were further ligated with each other to form one fusion sequence with the splicing by overlapping extension polymerase chain reaction method (SOE PCR) (*20*). The fusion genes were then transferred into *Monascus* protoplasts with the restriction-enzyme-mediated-integration (REMI) approach to improve the transformation rate (*21*). The transformants with smaller inhibition zones were selected on the basis of the antibacterial activity of citrinin to *Bacillus subtilis* (*22*).

MATERIALS AND METHODS

Fungal Strain and Growth Stations. *Monascus purpureus* ZD19 is a wild-type strain with a high yield of red pigments and citrinin. The strain was maintained on slants (2% agar) and plates (2% agar) of PDA medium (DIFCO, Detroit, MI) at 30 °C for 7−10 days. Spore suspensions were obtained by washing the slants with distilled water, followed by filtering with eight layers of lens-cleaning tissue and washing with distilled water 2 times. For liquid cultivation, a mycelial mat about 1 cm^2 was taken from a PDA agar plate, transferred into a 500 mL Erlenmeyer flask with 100 mL of liquid rice powder medium (consisting of 6% rice powder, 3% soy beans, 0.3% NaNO$_3$, 0.3% KH$_2$PO$_4$, and 0.3% MgSO$_4$·7H$_2$O, and the initial pH was adjusted to 3.0 with 1 M lactic acid), and incubated at 30 °C for 7 days with shaking at 120 strokes per min (spm) on a rotary shaker (*23*).

Isolation of Genomic DNA. *Monascus* genomic DNA was extracted according to the method described by Bingle et al. (*24*), with the following modifications. Mycelium was filtered with distilled water and transferred onto filter paper, dried by squeezing. Then, it was separated to small parts of 0.3 g or so, packaged with aluminum foil, and frozen in liquid nitrogen for 20 min. Then, the frozen mycelia were ground to a fine powder under liquid nitrogen. The aqueous phase containing genomic DNA was obtained by centrifugation (10000*g*, 10 min) at room temperature. Proteins were digested with proteinase K and then removed by successive rounds of extraction with phenol and chloroform. The sample was cleaned with sodium acetate (3 M, pH 5.2) and 70% ethanol. Genomic DNA was dissolved in 50 *μ*L of TE buffer [50 mM Tris-HCl at pH 8.0 and 10 mM ethylenediaminetetraacetic acid (EDTA)].

SOE PCR for Splicing. Genomic PCR was used to amplify a portion of fragments from *Monascus* with several primer sets using a thermocycler (Whatman Biometra Co., Ltd.). The primer sets included *ctnA* left sequence primers (F1 and R1), *ctnA* right sequence primers (F2 and R2), and *pks1* fragment ones (F3 and R3) that are based on the consensus sequences of the *pksCT* and *pks1* genes, respectively (**Table 1**). The underlined letters of F1 and R2 indicate the *Hind*III sites inserted, which would help to improve the homologous recombination rate in REMI. The four bases "GGCC" before the enzyme sites at the 5′ terminal are protective bases. To carry out gene splicing by SOE PCR, letters underlined in R1 and F2 are complementary base sequences with F3 and R3, respectively.

Gene splicing was carried out as shown below (**Figure 1**). Primers F1 and R1 were used to amplify the fragment on the left side of *ctnA* with a product of 1960 bp, including additional bases on the 5′ ends of both primers. The amplified fragment mainly included ORF1 of the *pksCT* gene cluster. Primer sets F2 and R2 were used to clone the right side sequences of *ctnA* with a length of 1523 bp, including additional bases on the 5′ ends of both primers also, and the amplified sequence mainly included ORF3 and partial ORF4. The *pks1* fragment was cloned with primers F3 and R3 with a 2129 bp product, including additional bases on the 5′ ends, which is near to that of the *ctnA* gene (2006 bp) in length. Each fragment was amplified respectively, separated on 1% agarose gel, and purified by gel recovery before it was used for splicing. The expected sizes and sequences of the amplified fragments were confirmed by 1% agarose gel and PCR product sequencing.
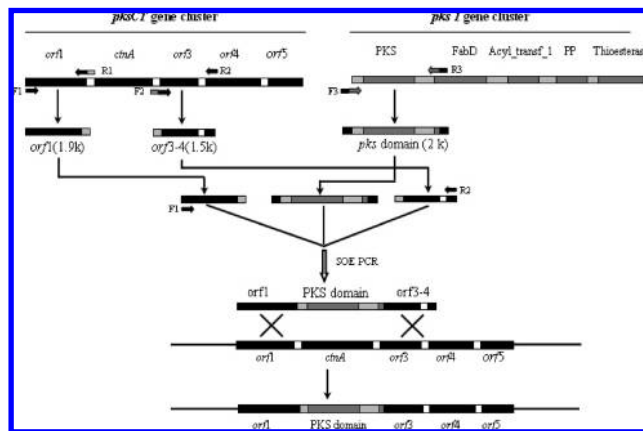

Figure 1. Outline of the preparation of the fusion gene with the SOE PCR method. Three fragments were amplified from *pksCT* clusters and spliced with the sequence amplified from *pks1*.

A total of 100 ng of diluted gemomic DNA was used as the template. The amplification conditions for the three fragments were denaturation at 95 °C for 4 min, which was followed by 30 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 2 min and 30 s), and finally with a single extension at 72 °C for 10 min. The PCR products were sequenced with an ABI Prism 3730 sequencer by Shenggong Co., Ltd., China.

The SOE PCR conditions for gene fusion were denaturation at 95 °C for 3 min followed by 30 cycles of 20 s in 94 °C, 6.5 min in 72 °C, and finally with a single extension at 10 min in 72 °C. PrimeSTAR HS DNA polymerase enzyme (Takara Co., Ltd., Japan) was used for all of the amplifications above. The PCR product was examined on 1% agarose gel, purified by gel recovery (TaKaRa agarose gel DNA purification kit, version 2.0), and sequenced.

Protoplast Preparation. The preparation of protoplasts was carried out as described by Chen et al. (*25*) and Zhou et al. (*26*). A spore suspension of *M. purpureus* was spread on a piece of cellophane in plates with PDA medium and cultured at 30 °C for 40−50 h. The mycelia were harvested and immersed in dithiothreitol (DTT) (5 mmol/L) for 30 min and then digested with mixed enzymatic solution (0.6:0.4:0.8% snailase/ lysozyme/cellulase at 1.0 mol/L MgSO$_4$ solution; snailase and cellulase were bought from Dingguo Co., Ltd., China, products DH314 and DH0521-1, and lysozyme was purchased from Sigma Chemical Co., St. Louis, MO, product L6876) for 2 h at 30 °C on a rotary shaker with 100 spm. The enzymatic hydrolysate was filtered with 6 layers of lens-cleaning tissue, washed 2 times, and finally resuspended using MgSO$_4$ solution (1.0 mol/L). The suspension was then divided into equal volumes and adjusted to 120 *μ*L with 1 M MgSO$_4$ solution on ice. To examine the digesting effect, 20 *μ*L of the protoplast suspension was separated on a regeneration PDA medium (0.6 mol/L MgSO$_4$ solution was added to PDA medium for osmotic pressure balance). Other suspensions were stored at 4 °C for the next steps.

REMI Transformation Protocol. The REMI transformation was performed as described by Zhou et al. (*26*), with some modifications. One tube of protoplast suspension was saved as the control, and another one was put on ice for 10 min for preparing transformation. Totally, 10 *μ*L of purified splicing genes (1.5 *μ*g) was added, followed by 11 *μ*L of endonuclease *Hind*III (100 units, Takara Co., Ltd., product D1060A). After the mixture was kept on ice for 30 min, it was added to 900 *μ*L of PTC (40% PEG4000, 50 mmol/L Tris at pH 8.0, and 50 mmol/L CaCl$_2$) and kept at room temperature for 45 min.

Transformants Culturing, Selecting, and Testing. *B. subtilis* B825 was cultured with PDB medium (PDA without agar) at 30 °C overnight with shaking at 120 spm, and 100 *μ*L of the bacteria was spread on the regeneration PDA medium plates (15 cm in diameter). The transformation solution was diluted 10^4 times with 1.0 M MgSO$_4$, and 150 *μ*L was spread on each of the plates. Then, these plates were inverted and incubated at 30 °C. Potential mutant colonies with smaller inhibition zones than others were examined after 24 h of incubation. The mutant clones were then transferred to new PDA medium plates (12 cm in diameter) without

B. subtilis to be cultured at 30 °C for 7−10 days and subcultured for 5 generations. Lastly, 0.5 cm mycelia mats of mutants and the control were picked from each of the clones with a punch and transferred to new PDA medium plates with *B. subtilis* to confirm inhibition zones and genetic stabilities.

Genotype Identification of Transformatants. Genomic DNA was extracted from *M. purpureus* mutants with the method described above, and genomic PCR was performed with primer sets as follows: 5′-AGATGGCTGGTTATTCG-3′ and 5′-ATGGCAACCTGGACT-AC-3′, which were used to amplify the joint of *pks1* with the ORF3−ORF4 fragment. The primer sets 5′-AAACTACGCTGTGACGGACA-3′ and 5′-TAACTGCACCAGACGAAACG-3′ from former studies were used to check the *ctnA* gene (*12*). The amplification conditions were both as follows: denaturation at 95 °C for 3 min, which was followed by 30 cycles of denaturation (94 °C for 30 s), annealing (52 °C for 30 s), and extension (72 °C for 1 min), and finally with a single extension at 72 °C for 10 min. The PCR product was gel-recovered, sequenced, and then analyzed by BLAST homology in the GenBank nt/nr database.

Analysis of Citrinin Products. The citrinin analysis was performed as described by Shimizu et al. (*10*) with some modifications. Mutant and wild-type mycelia were cultured in liquid rice powder medium following protocols described above, harvested by filtration, and dried with the qualitative filter paper. A total of 1 g of mycelium was finely ground with silicon dioxide, treated by sonication at 30 W/15 kHz (VC130PB, Sonics and Materials, Newtown, PA) for 10 min, and then extracted with 50 mL of 70% ethanol (pH 8.0) by stirring for 1 h at 60 °C. Then, the mixture was centrifuged at 10000g for 10 min at room temperature. The upper layer was passed through a 0.20 μm filter, analyzed by high-performance liquid chromatography (HPLC) on a C_{18} column with acetonitrile/water (50:50, adjusted pH to 2.5 with 1 M phosphoric acid) as the mobile phase at a flow rate of 1.0 mL/min, and detected by fluorescence (excitation at 325 nm and emission at 500 nm). Commercial citrinin (product C1017, Sigma Chemical Co., St. Louis, MO) was used as the standard.

Analysis of Red Pigments. The analysis of red pigments was carried out as described by Pattanagul et al. (*7*) and Babitha et al. (*9*). The mutants and wild-type strain were cultured in liquid rice powder medium for 9 days at 30 °C with shaking at 150 spm, and the color values were detected at daily intervals over 5−9 days. For every time, the mycelim was treated using the same methods as for citrinin analysis. Then, 10 mL mixtures were extracted with 90 mL of 70% ethanol and incubated at 60 °C for 30 min with rotary agitation at 200 spm. After that, the mixtures were centrifuged at 8000g for 2 min, and the upper layers were diluted with 70% ethanol to keep OD_{505} between 0.5 and 1. Then, the color values of soluble pigment extracts were measured by an Eppendorf spectrophotometer, at an absorbance of 505 nm for red pigments. Each sample was cultured in duplicate, and the average absorbance value of triplicate measurements was calculated for each of the color values after multiplying with a dilution factor in 70% ethanol.

RESULTS

Splicing of a Fusion Gene. The amplified fragments used for gene splicing are shown in **Figure 2** (lanes 1−3). In pre-experiments, it was found that, if the three fragments were mixed together and assembled in one tube, aggravated nonspecific amplifications were observed. To avoid the effects of mixing the three fragments, the *pks1* fragment was fused with the other two fragments separately for the first round (lanes 4 and 5 in **Figure 2**). After purification with gel recovery, the two fragments were fused for the second round to form the aimed splicing gene with primers F1 and R2 (lane 6 in **Figure 2**).

Selection and Genotyping Analysis of Transformants. Although obvious inhibition zones were observed around most of the clones, mutants with reduced ones were found and transferred to new PDA plates without *B. subtilis*. Then, they were incubated for 7−10 days and subcultured for 5 generations. Finally, a piece of 0.5 cm^2 mycelia mats of each of the fifth generation mutants was transferred to new PDA medium plates with *B. subtilis* again to examine the genetic stabilities. The results showed that there were no obvious increases of the inhibition zone (**Figure 3**).
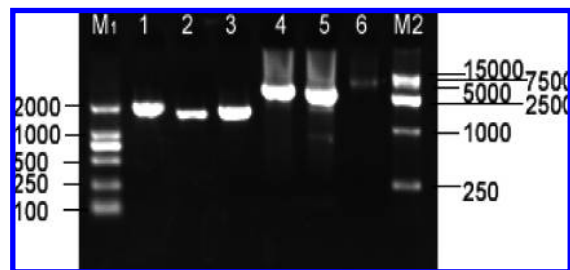

Figure 2. Process of gene fusion. M1, DL2000 marker; M2, DL15000 marker; lane 1, *pks1* DNA fragment prepared for the replacement of *ctnA* (A); lane 2, left part sequence of *ctnA* (B); lane 3, right DNA fragment of *ctnA* (C); lane 4, (A + B) fragment; lane 5, (A + C) fragment; and lane 6, (A + B + C) fragment, which was the spliced gene.
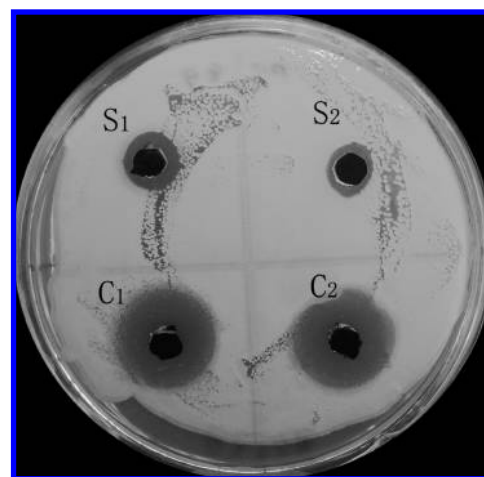

Figure 3. Purified clones and the comparison of the inhibition zone with wild-type strains. S showed engineering strains, and C represented wild-type strains.

To confirm that the *ctnA* in the mutant cells had been replaced by *pks1*, the genomic DNA from the fifth generation mutant cells was used as templates for PCR using two pairs of primers: one pair for the amplification of the fragment consisting of partial *pks1* and ORF3 and the other pair for the *ctnA* gene. The PCR products for the junction showed the predicted length (lane 2 in **Figure 4**), while it cannot be found in the wild type (lane 1 in **Figure 4**). The PCR product sequence showed the junction of *pks1* and ORF3 fragment by splicing with the primer F2. A BLAST homology search in the GenBank nt/nr database also showed that the amplified sequence consisted of two parts, which were of 99% identity to the *pks1* gene (AJ414729.1, from 2101 to 2222) and the ORF3 of *pksCT* (AB243687.1, from 4653 to 4909). On the contrary, the PCR products for the *ctnA* gene showed the predicted length in the wild type (lane 3 in **Figure 4**), while it cannot be found in the mutant (lane 4 in **Figure 4**). The BLAST homology search in the GenBank nt/nr database for the PCR product sequence of the wild type confirmed that it was a part of the *cntA* gene (AB243687.1, from 2699 to 3565). These results indicated that the *ctnA* was really replaced by the *pks1* fragment.

Analysis of Citrinin Production. Commercial citrinin was used as a standard, and citrinin from the wild type was used as a control in HPLC analysis. The citrinin retention time of the wild type was 18.221 min, detected by the fluorescence detector, with a calculated citrinin content of 3.0 μg/mL, and the citrinin retention time of the mutant was 18.360 min with a calculated content of 1.26 μg/mL (**Figure 5**). It showed that the citrinin content of the mutant was reduced to 42% compared to the wild-type

strain, which confirmed the result of the inhibition zone comparison.

Analysis of Red Pigments. The color values of the red pigment in the liquid fermentation cultural mixture were detected at daily intervals for 5−9 days (**Table 2**). It was found that red pigment production reached its peak at days 8−9 with an improved ratio of 33.5 and 33.9%, respectively, compared to that of the wild type, whereas only a 14.3% improvement was found at the fifth day. Totally, the results showed a slow color value improvement at the beginning, a more rapid increase in 6−7 days, finally reaching the peak at the eighth day, and then no obvious improvement after that at the ninth day.

DISCUSSION

For centuries, *Monascus* has been used for manufactured goods and as food additives. The concentration of citrinin is one of the most important quality indexes of the *Monascus* products, which bring potential safety problems, because of its nephrotoxic activity in mammals (5), with a median lethal dose (LD50) of 110 mg/kg in mice, which is at the same level with aflatoxin B1 (28), while the citrinin concentration of *Monascus* production is 211.61 mg/kg on average in China (29). Therefore, it is very important to obtain strains with low citrinin production for the commercial production of red pigments and other useful metabolisms, such as monacolin K.

The *M. purpureus* citrinin is mainly synthesized by the production of the *pksCT* gene cluster. It was reported that red pigments and citrinin begin with a common biosynthesis pathway and the same precursors (27). The blockage of the citrinin biosynthesis pathway would result in the decrease of citrinin production and the improvement of red pigments (15, 16). It has already been known that the biosynthesis of *pksCT* is controlled by the activator, *ctnA* (10), and the elimination of the *ctnA* gene or *pksCT* will greatly restrain *pksCT* transcription and the production of citrinin (14). It was reported also that gene knockout of *ctnA* or *pksCT* will not terminate the produce of citrinin (16).

Whether producing pigment production or not, all of the *Monascus* species produce citrinin. For example, *M. aurantiacus*, *M. sanguineus*, *M. pilosus*, *M. ruber*, and *M. purpureus* can produce red or orange soluble pigments, while *M. floridanus*, *M. lunisporas*, and *M. pallens* do not (15). It was also found that citrinin biosynthesis pathways were different between *M. ruber* with *Penicillium* and *Aspergillis* species (27). These revealed that the biosynthesis of citrinin is independent to that of the pigments, too.

To improve the transformants selecting rate in the condition without exogenous antibiotic markers and obtain stable phenotype mutants with low citrinin, some approaches were used in the study. First, the REMI transformation method was used, because it can obtain more mutants than other transformation approaches in *Monascus* spp. (26). Second, for high effectiveness of homologous recombination, a fragment length of above 1 kb is recommended in *Monascus* spp. (10), and the sequences used for homologous recombination at both sides of the fusion gene were 1.9 and 1.5 kb separately in this study. Finally, the approaches that the aimed gene replaced by the genomic sequence was related to the positive biological characters of the same strain were performed. Sequences that came from the same genomic DNA and used for gene replacing will be beneficial for genetic stability of mutants, because it has the nearest GC content and base arrangement characters to the target one.
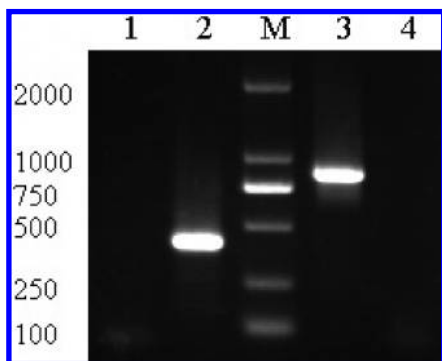

Figure 4. Genotyping analysis of transformants. M, DL2000 marker; lanes 1 and 2, amplification for the joint of *pks1* with ORF3 fragment; lanes 3 and 4, amplification for the *ctnA* gene. Lanes 1 and 4 represented the control, and lanes 2 and 3 represented the mutant, separately.

Table 2. Comparison of Red Pigment Color Values between the Wild Type and Mutants

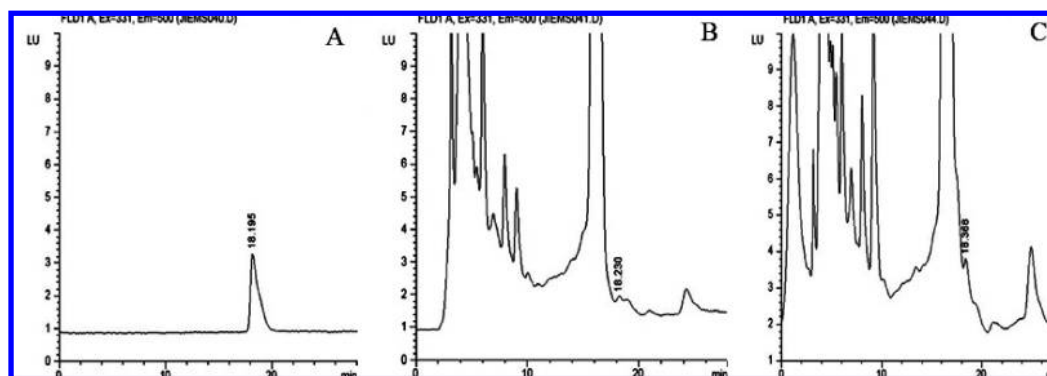| culture time (days)	color value of the wild type (OD$_{505}$ nm)	color value of the mutant (OD$_{505}$ nm)	improved ratio of red pigment (%)
5	0.524	0.599	+14.3
6	0.639	0.774	+21.1
7	0.737	0.939	+27.4
8	0.765	1.021	+33.5
9	0.676	0.905	+33.9

Figure 5. Citrinin analysis of mutants. A, standard citrinin; B, wild-type *M. purpureus* control; C, one of the mutants. The citrinin retention time of the wild type was 18.221 min, and the calculated citrinin content was 3.0 μg/mL. The citrinin retention time of the mutant was 18.360 min with a calculated content of 1.26 μg/mL. It showed that the citrinin content of the mutant was reduced to 42% compared to the wild-type strain.

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