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Original Article

Some Biological Characteristics of *Monascus* Strains Isolated from a Commercial Red Fermented Rice Product

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Abstract: *Monascus purpureus* has been widely used for over 1000 years in Asian countries especially in China and Japan for the production of natural food colorants or red fermented rice. However, *M. purpureus* produces a mycotoxin named citrinin. Therefore, *Monascus* strains used in industry must be controlled for the ability of citrinin production. In Vietnam, red fermented rice products are imported and widely sold on online markets but there are no reports on their safety. From a commercial red fermented rice sold in Vietnam, two strains of *M. purpureus* were successfully isolated, purified, and identified based on morphological characteristics and sequencing of the internal transcribed spacer (ITS) region of rDNA. Additionally, both isolates produce citrinin, a harmful mycotoxin, similar to the reference strains of *M. purpureus*. Effects of nitrogen sources and initial pH values on the production of pigments and citrinin in these *Monascus* strains were also investigated. The results revealed the red fermented rice product containing the citrinin-producing *M. purpureus* strains needed to be managed, evaluated, and monitored more strictly in terms of food safety. Notably, changes in nitrogen sources and initial pH values for fungal cultivation could suppress the toxin production for the quality improvement of red fermented rice products. In addition, two isolates provided in this study are potential strains for pigment production with strict control of fermented conditions.

Keywords: Red fermented rice, *Monascus purpureus*, citrinin, pigment production, food safety.

1. Introduction

Red-fermented rice, the fermentation product of *Monascus* spp., has been used for over 1000 years in China, Japan, and other countries in East and Southeast Asia. This is currently used worldwide as a natural food colorant, fermentation starter, and food additive. Red rice fermented by *Monascus purpureus* has been reported to possess numerous biological properties with hypolipidemic, anti-atherosclerotic, anticancer, neurocytoprotective, hepatoprotective, anti-osteoporotic, antifatigue, anti-diabetic, anti-obesity, immunomodulatory, anti-inflammatory, anti-hypertensive, and anti-microbial activities [1]. The fungus

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M. purpureus can biosynthesize several valuable secondary metabolites such as pigments for natural color and flavoring agents, monacolin K for mitigation of diarrhea and improving blood circulation, gamma-aminobutyric acid (GABA) for producing calming effects [2]. However, a hepatonephrotoxic compound in mammals called citrin can be produced by *Monascus* [2]. The presence of citrinin has raised concerns for the safety of these fermented products and limited the wide application of these filamentous fungi. Because genetically modified strains are hardly permitted for direct use in the food industry, screening of non-citrinin or low-citrinin-producing *Monascus* strains and optimization of culture conditions are of interest. In addition, most *Monascus*-fermented rice follows the traditional process, which is not strictly controlled. As a result, it has a high risk of interference from other microorganisms, especially filamentous fungi [3, 4]. In Vietnam, studies on *Monascus* spp. are limited, and red fermented rice is imported and popularly sold on online markets (Shopee, Lazada, Tiki,...) as functional foods or food coloring ingredients called Hongqu powder. In this study, we have successfully isolated two strains of *M. purpureus* from commercial red fermented rice using the restrictive medium supplemented with lactic acid and ethanol. The isolates showed better sporulation and similar citrinin production to laboratory strains. Moreover, we suggested conditions of the nitrogen source and initial pH for inhibiting citrinin but improving pigment production and provided options for quick detection of citrinin-producing strains of *M. purpureus*.

2. Experimental

2.1. Microbial Strains

M. purpureus NBRC 4478, NBRC 6540, NBRC 30873, and NBRC 32316 were used as the reference strains [5]. *Bacillus subtilis* PY79 [6] was chosen for the examination of citrinin production from different *Monascus* strains.

2.2. Isolation of Monascus Strains from Red-Fermented Rice

A commercial red fermented rice product (whole grain) from Taiwan stored at room temperature was purchased from the Shopee market in Vietnam (https://shopee.vn). An amount of 0.15 g of the pulverized red fermented rice sample was incubated in 50 ml of rice milk medium (1.5% indica rice flour, 0.5% glucose) supplemented with 4.5% lactic acid and 9% ethanol at 30 °C and 200 rpm for 7 days [7]. The culture broth was diluted 50 times and 50 µl of the diluted broth was spread on a PDA (potato dextrose agar) (Himedia, India) supplemented with 1% peptone and incubated for 7 days at 37 °C.

Fungal isolates were purified by single spore isolation and identified by morphological analyses under a microscope [8].

2.3. Preparation of Fungal Spore Suspension and Genomic DNA Extraction

Fungal strains were grown on PDA or PDA supplemented with peptone at 37 °C for 5-7 days for harvesting fungal spores and fungal mycelia. The preparations of fungal spore suspensions and genomic DNA samples were performed as previous study [8]. The spore suspensions were adjusted to 10^6 spores/ml for further experiments.

For examining sporulation ability, a volume of 10 µl (10^6 spores/ml) of 4 laboratory strains and 2 isolates of *Monascus* were incubated on the PDA supplemented with peptone at 37 °C for 5 days to collect spores and count under a microscope using a Thoma counting chamber [8]. This experiment was repeated three times to ensure the reliability of the obtained data. The number of spores was compared with other strains. Statistical analysis was implemented with the Student's *t*-test. The significant difference was considered with $p<0.05$.

2.4. Molecular Identification of Fungal Strains

The DNA samples were used as the templates for PCR amplification of the fungal internal transcribed spacer (ITS) region

of ribosomal DNA (rDNA) with the universal primer pair, including ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [9]. The obtained PCR product was purified with MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, Gyeonggi-do, South Korea) and sequenced by the 1st BASE company (Singapore). The obtained sequences were examined with the BioEdit 7.2 software and comparatively analyzed with the GenBank database using the BLAST tool. The related fungal ITS sequences were extracted from the GenBank database for constructing the phylogenetic tree using the MEGA11 software with the maximum likelihood method and 1000 bootstrap replicates [10].

2.5. Detection of Pigment and Citrinin and Production of Monascus Strains

Different *Monascus* strains were inoculated in the CD medium (3% sucrose; 0.3% $NaNO_3$; 0.1% KH_2PO_4; 0.05% KCl; 0.05% $MgSO_4.7H_2O$; 0.001% $FeSO_4.7H_2O$; 0.005% $CuSO_4.5H_2O$; 0.001% $ZnSO_4.7H_2O$) with different nitrogen sources, including $NaNO_3$, $(NH_4)_2SO_4$, and peptone at 30 °C, 200 rpm for 7 days. Simultaneously, the pH of the culture medium was adjusted to 2.5; 5.5; 8.5. The fermentation broth was used to determine citrinin and pigment production.

A volume of 50 µl of cultured *Bacillus subtilis* PY79 supernatant was spread uniformly on the surface of PDA agar plates. One well with a diameter of 1 cm was made at the center of the agar plate to provide a place for the addition of 50 µl of *Monascus* inoculum culture. The plate was incubated at 30 °C for 2 days. The production of citrinin was detected via the existence of transparent inhibition zones.

The fermentation broth was then filtered through Miracloth (Calbiochem, Germany). The supernatants were observed in the colors and analyzed by a spectrophotometer at different wavelengths of 400 nm (A_{400}), 470 (A_{470}) nm, and 500 nm (A_{500}), corresponding to yellow, orange, and red pigment concentrations [11, 12]. The absorbance represented the kind of pigments produced by *M. purpureus*.

3. Results and Discussion

3.1. Isolation of M. Purpureus Strains from Commercial Products

The *Monascus* in commercial pulverized red fermented rice could successfully grow in rice milk medium supplemented with 4.5% lactic acid and 9% ethanol. Adding lactic acid and ethanol simultaneously could eliminate bacteria and fungi other than *Monascus* [7]. Red fermented rice samples are often stored in different storage conditions, including in wooden drawers without any packaging and stored together with other herbs, in packaging, in the refrigerator, at room temperature, in an air-conditioning system, and in open-air. As a consequence, red fermented rice is usually contaminated with other filamentous fungi, yeasts, and bacteria [13]. The presence of these microorganisms can cause serious disturbance during the preparation of *Monascus* pure culture, especially when conventional mold media such as PDA, Rose Bengal medium, and malt extract agar. Some restrictive media for the enrichment of *Monascus* has been proposed for the enrichment of *Monascus*. Using a restrictive medium based on the synergistic stress of lactic acid and ethanol of *Monascus*, the efficiency of *Monascus* enrichment from red fermented rice was increased [7]. Using the rice milk medium supplemented with lactic acid and ethanol, we obtained colonies with morphological characteristics similar to *Monascus* spp. (Figure 1).

Two red colonies named BG2 and BG5 were selected and separated from enrichment plates. These isolates contained red mycelia related to the secretion of pigments into the medium after 5 days at 37 °C (Fig. 1), the ascomata walls were composed of 2 distinct layers, and the ascomata cavities were filled with unicellular ascospores (Figure 1). These observed results were similar to the morphological characteristics and the sexual structure of *M. purpureus* [14]. Furthermore,

analysis of the ITS region of rDNA from two isolates using the universal primer pair ITS1/ITS4 indicated that the ITS sequences of these fungal strains were identical to the ones of *M. purpureus* deposited in the GenBank database (Figure 2).

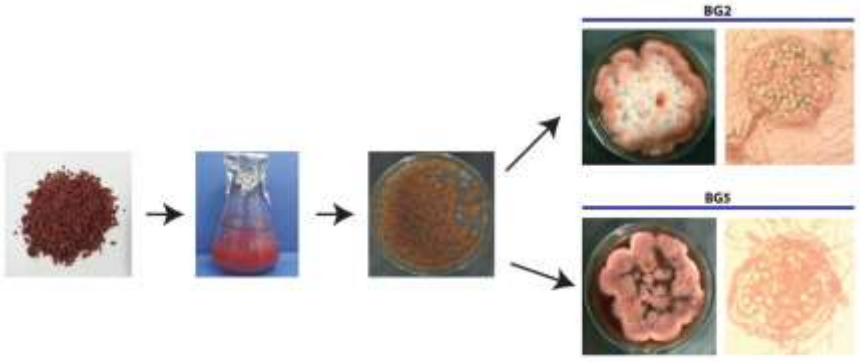

Figure 1. Isolation of *Monascus* spp. from red fermented rice.
The red fermented rice bought from the online market was pulverized and incubated in rice milk medium supplemented with lactic acid and ethanol for 7 days at 30 °C and 200 rpm. The fermentation broth was diluted and spread on a PDA supplemented with peptone. After 7 days of incubation at 37 °C, the colonies with morphologies similar to *Monascus* spp. were selected and observed their ascomata and ascopores under the microscope.

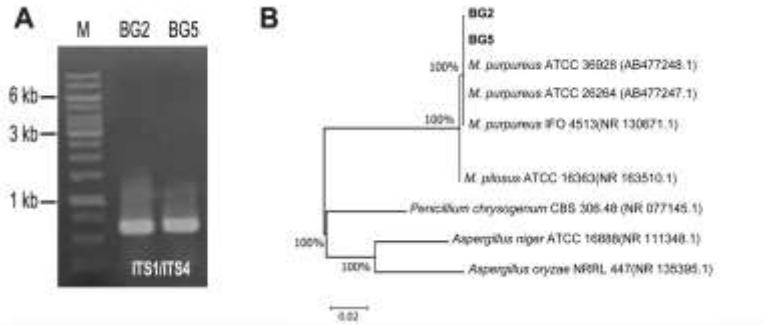

Figure 2. Identification of *Monascus* strains isolated from commercial red fermented rice.
(A) The amplified ITS regions from 2 isolates. M: DNA marker 1 kb (Thermo Fisher Scientific, USA).
(B) The phylogenetic tree based on the ITS region of rDNA.

3.2. Two Isolates Produce more Spores and Pigments Compared with the Laboratory Strains

After 5 days of cultivation, our results revealed that 2 isolates considerably produced more spores than 4 of the laboratory strains (Figure 3A). The BG5 and BG2 strains respectively produced 2.8-3.2 times more spores than *M. purpureus* NBRC 30873 and around 2.6 times compared to *M. purpureus* NBRC 6540 (Figure 3A). These isolates also showed higher pigment accumulation than the laboratory strains after 7 days of inoculation (Figure 3B). The pigments produced by the fungus *Monascus* sp. are of traditional use in the oriental countries as food additives. The strains used for pigment production are usually isolated from Japan, China, Thailand, or Indonesia. The pigment production varies greatly with the species and cultivation

conditions. Besides, strains differ in the amount produced and the tone of the pigments [12]. The OD values of the fermentation broths at wavelengths corresponding to yellow, orange, and red pigments are shown in Table 1.

Table 1. The absorbance of fermentation broths of *M. purpureus* strains at different wavelengths

Strain	A_{400}	A_{470}	A_{500}
NBRC 4478	0.328 ± 0.007	0.361 ± 0.012	0.375 ± 0.009
NBRC 6540	0.214 ± 0.010	0.267 ± 0.014	0.296 ± 0.008
NBRC 30873	0.223 ± 0.005	0.254 ± 0.033	0.239 ± 0.004
NBRC 32316	0.446 ± 0.014	0.515 ± 0.028	0.501 ± 0.013
BG2	**0.708 ± 0.001**	**0.856 ± 0.023**	**0.930 ± 0.001**
BG5	**0.528 ± 0.004**	**0.673 ± 0.019**	**0.680 ± 0.013**

3.3. Establishment of a Cultivation Condition to Reduce Citrinin Production and Enhance the Production of Pigments

In the 1940s, citrinin was characterized as an antibiotic active against most Gram-positive bacteria, including *B. subtilis* [15, 16]. Besides the complex techniques for precise detection of citrinin such as NMR spectroscopy or HPLC with ultraviolet (UV) light and fluorescence (FLD), the activity against *B. subtilis* was selected for quick screening of citrinin production by filamentous fungi [17]. All 6 strains showed antibacterial activities against *B. subtilis* PY79. Notably, the production of citrinin in two isolates from commercial red fermented rice was similar to laboratory strains (Figure 3C). *M. purpureus* NBRC 6540 produces the lowest pigment and the highest citrinin level among 4 laboratory strains [5], so this strain was selected as a reference. In our study, 3 nitrogen sources, including sodium nitrate ($NaNO_3$), ammonium sulfate ($(NH_4)_2SO_4$), and peptone, and 3 initial pH values (2.5, 5.5, 8.5) were examined for their impacts on the citrinin and pigment production of *M. purpureus* (Figure 4). The results showed that at pH 5.5 when peptone was used as the sole nitrogen source, the citrinin production in the culture broth was significantly reduced despite high pigment accumulation (Figure 4).

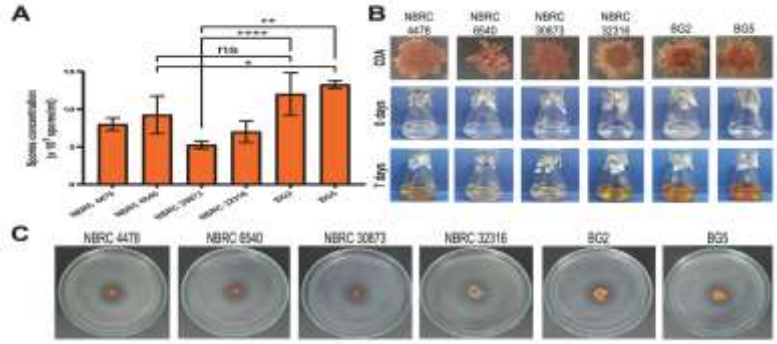

Figure 3. The capacity of sporulation, citrinin, and pigment production of different *M. purpureus* strains. (A) The sporulation ability of 4 laboratory *M. purpureus* strains and 2 *M. purpureus* isolates. Experiments were performed in triplicates. Data showed mean ± SD and significant differences were evaluated with $p<0.05$ using Student's *t*-test. Asterisks indicated a level of statistical significance. No significant difference was indicated with ns ($p>0.05$). (B) The growth of *M. purpureus* strains on CDA, the color of fermentation broths at 0 days and 7 days of incubation. (C) The quick screening of citrinin production in the *M. purpureus* strains via the antibacterial activity assay.

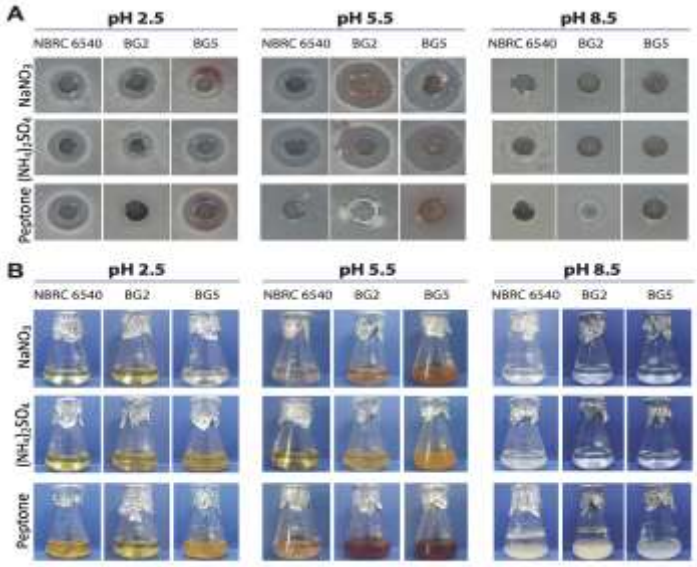

Figure 4. Effects of nitrogen sources and initial pH values on the citrinin and pigment production in *M. purpureus*.
(A) Effects of nitrogen sources and initial pHs on citrinin production. (B) Changes in color of *M. purpureus* culture media with different nitrogen sources and initial pH values after 7 days of shaking incubation.

Interestingly, the presence of peptone as the nitrogen source and the initial pH of 2.5 resulted in the inhibition of citrinin production from the BG2 strain (Figure 4A).

Monascus azaphilone pigments produced by *Monascus* species have been classified into yellow, orange, and red pigment subclasses based on color. The concentration of *Monascus* pigment is often measured using a spectrophotometer at the specific wavelengths of 400 nm, 470 nm, and 500 nm which correspond to the characteristic absorbance of yellow, orange, and red pigments, respectively [11, 12]. According to the results, the pH values of 2.5 and 5.5 were demonstrated to be the suitable pH for the production of pigments in *M. purpureus* (Figure 4B). Besides, sodium nitrate as the nitrogen source and pH 5.5 stimulated orange and red pigment productions, while peptone as the nitrogen source and pH 2.5 enhanced yellow pigment. At pH 5.5 and peptone as the nitrogen source, the pigment production of NBRC 6540 was limited while red pigment was significantly improved in the BG5 strain. The growth and pigment production of all strains were inhibited at pH 8.5 (Figure 4B). The absorbance data are shown in Table 2. To obtain more precise results, we suggest the use of HPLC with suitable extraction methods for citrinin and pigments from *Monascus* spp.

To select strains suitable for added-value fermentation seeds, the production of high pigments but no citrinin was the primary standard. There was no clear correlation between the pigment and citrinin production [5]. The nitrogen source plays an important role in the production of pigments. Inorganic nitrogen, such as ammonium chloride (NH_4Cl), produces higher *Monascus* pigment yield during the stationary phase but suppresses the development of conidial germination and the sexual cycle of *Monascus*. Sodium nitrate ($NaNO_3$) stimulates sporulation and high pigment yield but restricts the growth of *Monascus* [18]. The pigment production of *Monascus* spp. can increase when organic nitrogen such as peptone, yeast extract, and MSG are added as supplements [19, 20].

Table 2. The absorbance values of fermentation broths with different nitrogen sources and initial pH values

Nitrogen source and initial pH	Strain	A_{400}	A_{470}	A_{500}
$NaNO_3$, pH 2.5	NBRC 6540	0.298 ± 0.006	0.125 ± 0.012	0.157 ± 0.002
	BG2	0.467 ± 0.007	0.432 ± 0.008	0.371 ± 0.025
	BG5	0.128 ± 0.005	0.133 ± 0.003	0.091 ± 0.004
$NaNO_3$, pH 5.5	NBRC 6540	0.214 ± 0.010	**0.267 ± 0.014**	**0.296 ± 0.008**
	BG2	0.708 ± 0.001	**0.856 ± 0.023**	**0.930 ± 0.001**
	BG5	0.528 ± 0.004	**0.673 ± 0.019**	**0.680 ± 0.013**
$NaNO_3$, pH 8.5	NBRC 6540	0.012 ± 0.002	0.078 ± 0.007	0.092 ± 0.028
	BG2	0.043 ± 0.011	0.093 ± 0.005	0.106 ± 0.016
	BG5	0.020 ± 0.002	0.099 ± 0.003	0.093 ± 0.006
$(NH_4)_2SO_4$, pH 2.5	NBRC 6540	0.297 ± 0.003	0.256 ± 0.004	0.219 ± 0.005
	BG2	0.315 ± 0.005	0.264 ± 0.017	0.171 ± 0.018
	BG5	0.195 ± 0.004	0.192 ± 0.009	0.204 ± 0.017
$(NH_4)_2SO_4$, pH 5.5	NBRC 6540	0.378 ± 0.010	0.348 ± 0.021	0.321 ± 0.013
	BG2	0.364 ± 0.002	0.282 ± 0.015	0.259 ± 0.005
	BG5	0.374 ± 0.006	0.306 ± 0.009	0.245 ± 0.032
$(NH_4)_2SO_4$, pH 8.5	NBRC 6540	0.004 ± 0.001	0.057 ± 0.004	0.073 ± 0.015
	BG2	0.024 ± 0.003	0.089 ± 0.005	0.081 ± 0.009
	BG5	0.019 ± 0.001	0.083 ± 0.008	0.077 ± 0.011
Peptone, pH 2.5	NBRC 6540	**0.616 ± 0.009**	**0.486 ± 0.009**	**0.424 ± 0.020**
	BG2	**1.153 ± 0.010**	**0.622 ± 0.028**	**0.350 ± 0.100**
	BG5	**1.425 ± 0.017**	**0.821 ± 0.034**	**0.568 ± 0.022**
Peptone, pH 5.5	NBRC 6540	**0.411 ± 0.003**	**0.139 ± 0.006**	**0.112 ± 0.017**
	BG2	**0.521 ± 0.011**	**0.399 ± 0.012**	**0.508 ± 0.022**
	BG5	**0.688 ± 0.005**	**0.731 ± 0.023**	**0.917 ± 0.074**
Peptone, pH 8.5	NBRC 6540	0.123 ± 0.003	0.118 ± 0.007	0.098 ± 0.001
	BG2	0.131 ± 0.003	0.122 ± 0.004	0.076 ± 0.011
	BG5	0.125 ± 0.013	0.109 ± 0.006	0.109 ± 0.011

In this study, we discovered that the sole nitrogen source of peptone and initial culture medium pH at 5.5 could lead to the high accumulation of pigments and the inhibition of citrinin production in both isolates. Some aspects that influence the level of citrinin produced include the *Monascus* species and isolates, amino acids, trace elements, carbon and nitrogen sources, nutritional factors, the ratio of nitrogen to carbon concentration, pH, moisture content, light, oxygen, temperature, and environmental factors. The enhancement of citrinin production might be due to longer cultivation time, red light, and culture medium components [5]. To control the production of citrinin in red fermented rice, many recent studies have focused on the optimization of fermentation conditions and enrichment of nutrients. However, it is very challenging to block citrinin biosynthesis completely in *Monascus* spp. [21]. The *M. purpureus* strains existing in commercial food products still can produce citrinin and even are similar to the strong citrinin-producer of *M. purpureus* NBRC 6540. These strains should be managed, evaluated, and monitored more strictly in terms of food safety. Therefore, the detection and evaluation of citrinin produced by different *M. purpureus* strains are very essential for the safety of food products. In this study, we proposed a quick strategy to inhibit the citrinin production in *Monascus* but still maintain high pigment accumulation with the use of peptone as the nitrogen source and an initial pH of 5.5. In addition, choosing suitable conditions of nitrogen source and initial pH values could help

quickly detect mycotoxin-producing *Monascus* strains. This may be applied for the safety of food with the control of mycotoxin-producing *M. purpureus* isolated from commercial red fermented rice samples.

4. Conclusion

In this study, we have successfully isolated *Monascus* strains from commercial red fermented rice using a restrictive medium. Two strains of *M. purpureus* from commercial red fermented rice were purified and identified based on morphological characteristics and sequencing of the ITS region of rDNA. Two isolates showed better sporulation and pigment production than 4 laboratory strains, but the citrinin production was similar in all 6 strains. We discovered that nitrogen sources and initial pH values had great effects on pigment biosynthesis and citrinin production in 2 isolates of *M. purpureus*. The use of peptone as the sole nitrogen source and the initial pH of 5.5 could result in lower citrinin but higher pigment production. This result can be utilized to develop a fermentation condition to reduce citrinin but still maintain high pigment accumulation, which will be helpful for the control of mycotoxin-producing *M. purpureus* isolates and the enhancement of quality in red fermented rice products.

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