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Citrinin-producing capacity of *Monascus purpureus* in response to low −frequency magnetic fields

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ABSTRACT

Citrinin is a type of mycotoxin that negatively affects *Monascus* products. Application of a low-frequency magnetic field (LF-MF) decreased citrinin production by *M. purpureus* in liquid-state fermentation during shake flask culture. Under 30 °C incubation, six different magnetic field induction intensity (MF-II), four different exposure times and five exposure time periods were tested to discover optimal treatment conditions. The cultures were exposure to a MF-II of 1.6 mT from 0 to 2 d of incubation time. With LF-MF treatment, peak citrinin production decreased by 46.7% while yellow, red, orange pigments and monacolin K production increased by 31.3%, 40.3%, 41.7% and 29.3%, respectively, compared with control groups at 12 d of incubation. Moreover, the relative expression levels of the citrinin biosynthesis genes *pksCT* and *ctnA* were 0.46 and 0.43 times lower, respectively, than the control values relative to the *GAPDH* reference gene. This study provides evidence that LF-MF is a preferable way to alter *M. purpureus* metabolism to reduce citrinin production and to increase pigments and monacolin K production without affecting cell growth. Therefore, LF-MF may be used as a tool to process *Monascus* products to obtain important functional food additive while reducing the adverse effects of citrinin.

1. Introduction

Monascus is a versatile filamentous fungus that can be used to produce various active metabolites, such as pigments, monacolin K, γ-aminobutyric acid (GABA), ergosterol and others [1]. The pigments are widely used as natural food colorants and monacolin K is used as an antihypercholesterolemic agent [2]. GABA is one of the most important inhibitory neurotransmitters in the central nervous system of mammals [3]. Ergosterol is a precursor of liposoluble vitamin D_2, cortisone and the hormone progesterone [4,5]. However, *Monascus* also produces citrinin. Citrinin is a mycotoxin that has nephrotoxic activity in mammals [6], and may have teratogenic, carcinogenic and mutagenic properties [7]. Therefore, many countries have enacted new policies that strictly limit the citrinin content in *Monascus* products. Traditional methods to control citrinin production include optimizing fermentation conditions, selecting strains that produce less citrinin [8,9] and using gene sequencing to construct mutants that do not produce citrinin [10,11]. Such mutants were constructed in the context of biological pathway of citrinin biosynthesis. The citrinin biosynthesis pathway was reported to be a polyketide metabolite pathway, and a gene cluster was identified [12]. The citrinin cluster in *M. purpureus* was deduced to contain six genes, including one polyketide synthase (PKS) gene (*pksCT*), one transcriptional activator (*ctnA*), one membrane transporter gene and three genes for post-PKS-modifying enzymes. *pksCT* and *ctnA* have proven involvement in citrinin biosynthesis [13,14].

On the other hand, numerous studies in many different scientific disciplines have recently found that low-frequency (<300 Hz) magnetic fields (LF-MF) can alter microbial metabolism to enhance the production of desired metabolites [15–18]. At the same time, these studies also confirmed that LF-MF can decrease fermentation production at other magnetic field induction intensities (MF-II) and exposure time. The results thus suggests that LF-MF may be able to decrease the production of harmful metabolites.

In this paper, we optimize LF-MF exposure conditions to decrease citrinin production; we also measure pigments and monacolin K production and the relative expression levels of citrinin biosynthesis gene (*pksCT* and *ctnA*) under LF-MF exposure conditions.

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2. Materials and methods

2.1. Strain and growth conditions

Cultures of *M. purpureus* strain SKY219 were incubated in Czapek yeast extract Agar (CYA) at 30 °C for 10 days. Then, 250 mL Erlenmeyer flasks containing 75 mL of fermentation broth (3 mL of spore suspension inoculated into 72 mL of potato dextrose broth (PDB)) were incubated in a rotary shaker (BS-IE Changzhou Guohua Electric Appliance Co., Ltd, China), at 30 °C for 36 h at 180 rpm. Subsequently, 1 mL of inoculum added to 9 mL of sterile distilled water generated a spore suspension of approximately 105 spores/mL that was then used as an inoculum source. Inocula (1 mL) were added to 99 mL of PDB and mixed. Then 30 mL mixture take in plastic (polyethylene) bottles (diameter, 30 mm; height, 60 mm), which were incubated at 30 °C for 24 h at 180 rpm in a constant-temperature incubator (BS-IE Changzhou Guohua Electric Appliance Co., Ltd, China) and treated as follows. Under 30 °C incubation, six different MF-II (0 (control), 0.5, 0.9, 1.2, 1.6 and 2.0 mT), four different exposure times (0–2, 0–4, 0–6, and 0–8 d of incubation time), and six exposure time periods (0–2, 2–4, 4–6, 6–8, 7–9 and 9–11 d of incubation time) were tested to discover optimal treatment conditions. The control group was not treated with the LF-MF. All cultures were incubated at 30 °C until the 12th day of fermentation.

2.2. Magnetic field

An alternating (frequency, 50 Hz) magnetic field (MF) generator was designed by Yangtze University (Jingzhou, China) with 5 pairs (1 and 1′, 2 and 2′, 3 and 3′, 4 and 4′, 5 and 5′) of cylindrical coils wrapped with magnetic shielding materials and powered by a transformer (Model TDGC2J-2KVA, Wenbo Electric Appliance, Shanghai, China). A detailed description of the MF unit can be found in Zhang et al. [18]. Ten plastic sample bottles containing PDB with *M. purpureus* SKY219 were placed on a nonconductive stand in the center of the coils. The mechanical rotation component of the equipment is composed of a rotating system and a motor operated by a rotation speed controller. The effective current was 0–2.0 mA, corresponding to a MF-II range of 0–2.5 mT at the center of each coil. The sample temperature was maintained at 30 °C by a thermostat (Model KST, Hengdian Thermal Protector Factory, Dongyang, China) during MF treatment. The distribution of MF in the exposure volume corresponded to the specific coil and current. The MF-II of the different coils was measured using a digital Tesla sensor (Model HT 20A, East Star Machinery Industry & Trade, Xiamen, China) with a constant current.

2.3. Determination of biomass

The fermentation broth was withdrawn for centrifugation (22, 400 × *g*, 10 min, 4 °C, Himac CR 21GII, Japan). The cells were collected, washed three times with distilled water, dried in a 60 °C oven to constant weight and then cooled in a desiccator before measuring the dry cell weight. The results were expressed in grams per liter.

2.4. Determination of citrinin and monacolin K content

A volume of fermented liquid was added to an equal volume of toluene-ethyl acetate- formic acid (7: 3: 1, v: v: v) and was powerfully vortexed before being centrifuged at 10, 000 × *g* for 30 min at room temperature. The upper layer was filtered through a 0.20 μm filter and measured by high-performance liquid chromatography (HPLC; Agilent, California, USA). A C_{18} column (5 μm, 25 cm × 4.6 mm) was used for analytical separation. An injector volume of 40 μL was used. The mobile phase contained 65% acetonitrile and 35% nanopure water. The flow rate was set at 1 mL/min and citrinin was detected using a UV detector set at 330 nm. A citrinin standard (Shanghai Yuanye Bio-Technology Co., Ltd, Shanghai, China) was used to construct a standard curve with five points over a range from 0.1 mg/L to 100 mg/L ($y = 8.8053x + 4.4181$, $R^2 = 0.999$).

To estimate the monacolin K concentration by HPLC, The 5 mL fermented liquid was added to 75% methanol 20 mL, then put into 50 °C water bath for 1 h, and oscillated intermittently 3–4 times, eventually constant volume to 25 mL and filtered through a 0.45 μm membrane filter. Chromatographic separation was performed with a C_{18} column (5 μm, 25 cm × 4.6 mm) using a 60: 40 mixture of acetonitrile and phosphate buffer (pH adjusted to 3 with orthophosphoric acid) as the mobile phase. The flow rate was 1 mL/min, and the UV detector was set at 238 nm. A monacolin K standard (Shanghai Yuanye Bio-Technology Co., Ltd, Shanghai, China) was used to construct a standard curve with five points over range from 10 mg/L to 100 mg/L ($y = 0.0513x + 0.0033$, $R^2 = 0.999$).

Table 1
Primers used in this study.

Name	Sequences(5′ → 3′)	Descriptions
ctnA-S	GGTGTAACCTGCGAGGTCAACAG	For qRT-PCR analysis of *ctnA*
ctnA-A	CACCAATTCCGTTATCGGTTCAG	
pksCT –S	CAGAGCCTTCAAAGTTACGTTCG	For qRT-PCR analysis of *pksCT*
pksCT-A	CCAAACCAACATAGGTGGAAACT	
GAPDH-S	GTCTATGCGTGTGCCTACTTCC	For qRT-PCR analysis of *GAPDH*
GAPDH-A	GAGTTGAGGGCGATACCAGC	

2.5. Determination of pigments content

Culture broth was subjected to sonication for 40 min at 120 W and then vacuum-filtered. The absorbance value of the filtrate at 410, 465 and 500 nm was determined on a spectrophotometer (UV1601PC, Shimadzu, Japan) and was multiplied by the dilution factor in distilled water to estimated content of yellow, orange and red pigments; the final value was termed U per milliliter [19].

2.6. Quantitative RT-PCR analysis

The treatment groups of *M. purpureus* were exposed to MF-II at 1.6 mT during days 0–2 of incubation time; after exposure, they were incubated at 30 °C until day 6. Total RNA from the fungal hyphae was extracted as described by Chantasigh et al. [20] and quantified in triplicate using UV absorbance at 260 nm. First-stand cDNA was synthesized using random hexamers and reverse transcriptase (Fermentas, USA) according to the supplier's protocol. Gene expression was monitored by quantitative RT-PCR (qRT-PCR). The obtained first-strand cDNAs served as templates for qRT-PCR. qRT-PCR was performed with the SLAN Fluorescence Detection System (SLAN, Shanghai, China) from Wuhan Goodbio Technology Co., Ltd (Wuhan, Hubei, China). The RNA primers for the citrinin synthesis genes *pksCT* and *ctnA* were designed based on DNA sequences from which the introns had been removed. The specific primers sequences are listed in Table 1. Relative expression was examined in reactions containing 12.5 μL of Toyobo Thunderbird SYBR qRT-PCR Mix (Toyobo, Osaka, Japan), 1.0 μL of 2.5 μM reverse primer, and 2.0 μL of template cDNA. The thermal cycling conditions comprised an initial denaturation at 94 °C for 1 min followed by 40 amplification cycles at 95 °C for 15 s, 58 °C for 20 s, and 72 °C for 20 s, with a final extension step at 72 °C for 5 min *GAPDH* was used as the reference gene.

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