



The relationship between pH, pigments production, and citrinin synthesis by *Monascus purpureus* during red fermented rice fermentation

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ABSTRACT

Red fermented rice (RFR) is consumed as a traditional medicine in many countries. Consumption of food contaminated with citrinin (CIT) has adverse effects on the liver cells and the kidneys. This study aimed to understand the relationship between pH, pigments, and CIT levels during the fermentation of RFR. The rice underwent soaking, steaming, and autoclaving before being inoculated with *Monascus purpureus* isolates (MF1 or MS1) and fermented at 30 °C for 30 days. The difference between these isolates was that MF1 and MS1 required 4 days and 7 days, respectively, to produce light blue fluorescence on Coconut Cream Agar (CCA), an indicator of the presence of CIT. The pH, pigments, and CIT levels were recorded during fermentation using a pH meter, spectrophotometer, and Ultra-High-Performance Liquid Chromatography with a Fluorescence Detector (UHPLC-FLD), respectively. The pH dropped from 6.8 to 5.3 after 9 days of fermentation. The CIT levels increased from day 4 until day 5 and then decreased during the fermentation period. The pigments increased after 5 days of fermentation, suggesting a relationship between pigments production and CIT reduction. Mixing the CIT standards and pigments extracted from MF1 and MS1 (a mixture of yellow, orange, and red pigments) showed that there was a reduction in CIT of 26–68 % and 16–45 %, respectively. It is suggested that future work should determine which pigments are responsible for CIT reduction. The optimization of pigments production with the control of pH at 5.5 may help to control CIT levels during the fermentation of RFR.

1. Introduction

Red fermented rice (RFR) is consumed by more than a billion people worldwide, particularly in the Southeastern and Eastern Asian countries such as China, Korea, Japan, Taiwan, Malaysia, Indonesia, the Philippines, and Thailand (Chen et al., 2015; Fukami et al., 2021; Patel, 2016; Samsudin & Abdullah, 2014; Wu et al., 2020). Rice is a staple food in these countries (Fukagawa & Ziska, 2019), with high temperatures and humidity (Fukami et al., 2021).

RFR is known by various names in different countries, including angkak, anka, Ang-Khan, Anka rice, Anka-Koji, beni-koji, red rice, red koji, red Chinese rice, red mold rice, red rice mold, red yeast rice, red yeast grain, *Monascus*-fermented rice, red leaven, zhitai, hong qu, hung-chu, Hon-Ci, Dan Qu, *tan-giku*, and rotschimmelreid (Chiu et al., 2006; Doughari, 2015; Erdoğan & Azirak, 2004; Farawahida et al., 2022b; Fukami et al., 2021; Patcharee et al., 2007; Poorniammal et al., 2021; Ristiarini et al., 2017).

RFR can be consumed as a food or dietary supplement (Gordon et al., 2010). RFR has been used for centuries to alleviate diarrhoea, improve

blood circulation, and treat indigestion, limb weakness, muscle bruising, and dysentery (Kohama et al., 1987; Zhu et al., 2019). RFR has also been utilized in traditional Chinese medicine with improving health benefits, such as lowering the production of cholesterol in the liver, and serves as a food colouring, flavouring, and preservative (Farawahida et al., 2022b; Fukami et al., 2021; Hong et al., 2011; Shen et al., 2017; Srianta et al., 2014). The product is also sought after as a dietary supplement outside Asia, with American consumers spending \$20 million on RFR dietary supplements in 2008 (Gordon et al., 2010). Since the 1950s, red pigments from RFR have been used in Japan in processed meat products, processed fish paste products, beverages, bread, and confectionaries (Fukami et al., 2021). Instant noodles, Chinese sausages, dairy products, and meat are examples of food applications utilizing pigments extracted from *Monascus* spp. (Meinicke et al., 2012).

RFR is produced by mixing cooked rice with *Monascus* spp. and fermenting the mixture for 2–3 weeks (Chairote et al., 2009; Dogra & Kumar, 2017; Patcharee et al., 2007). Various *Monascus* species such as *M. purpureus*, *M. ruber*, *M. anka*, and *M. pilosus* can be used to produce RFR (Srianta et al., 2014). During fermentation, these *Monascus* spp.

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produce secondary metabolites including six pigments categorized into three colours: yellow, orange, and red (Blanc et al., 1994).

RFR also produces other secondary metabolites such as citrinin (CIT) (Avula et al., 2014; Chen et al., 2016; Liao et al., 2014; Nigović et al., 2013; Twarużek et al., 2021). CIT is a mycotoxin that can contaminate foods such as rice (Twarużek et al., 2021). Prolonged exposure to CIT shows that the kidneys are the primary target organs (Kamle et al., 2022).

There are two prevailing opinions on the biosynthesis pathways of CIT and pigments. Hajjaj et al. (1999) proposed that during the growth of *M. ruber*, CIT and pigments are produced due to the occurrence of a tetraketide serving as a precursor for both secondary metabolites. Conversely, another perspective suggests that all *Monascus* spp. produced CIT independently of their pigments production (Qin et al., 2023).

The substrate's pH is one of the major factor determining metabolic activity in solid-state fermentation (Velmurugan et al., 2011). While many studies have reported the effects of initial pH on pigments production (Orozco & Kilikian, 2008; Patrovsky et al., 2019; Velmurugan et al., 2011), there are limited studies measuring pH changes during the fermentation of RFR.

Orozco and Kilikian (2008) reported that the highest total red pigments production and the lowest CIT concentration were achieved with the growth of *M. purpureus* CCT3802 in submerged culture at pH 5.5, followed by production at pH 8.5. However, maintaining the growth of this isolate at pH 5.5 until the end of incubation resulted in the highest CIT production. In contrast, using ammonium sulphate [(NH₄)₂SO₄] as a nitrogen source led to a low final pH, resulting in no CIT production (Kang et al., 2014). However, both studies were conducted in submerged cultures.

Most published studies have determined CIT levels in commercial RFR products rather than during the fermentation process of RFR. Several countries have established maximum limits for CIT in RFR (Table 1). Most published data report that RFR contains CIT levels exceeding these maximum limits (Chen et al., 2016; Gordon et al., 2010; Ristiarini et al., 2017). Ristiarini et al. (2017) reported that all thirty commercial RFR products from eight different regions in Indonesia exceeded the maximum CIT limits, ranging from 17,940 µg/kg to 124,680 µg/kg. Among 12 commercial RFR products in the United States, 33 % contained CIT levels from 24,000 µg/kg to 189,000 µg/kg, surpassing the maximum limits (Gordon et al., 2010). In Taiwan, 27.2 % of 206 RFR samples contained CIT, with 14 RFR raw materials (42.4 %) and one RFR dietary supplement (1.7 %) exceeding the maximum limits of 5000 µg/kg and 2000 µg/kg, respectively (Chen et al., 2016). Based on these findings, researchers recommended that food manufacturers may choose low-CIT-producing *Monascus* spp., optimize fermentation conditions, and implement self-monitoring practices to reduce CIT levels in RFR products (Chen et al., 2016).

Although it has been reported that RFR contains both pigments and CIT, the relationship between these metabolites during the fermentation of RFR being still unclear. Therefore, this study was conducted to understand the relationship between pH (as an indicator of metabolic products), pigments and CIT levels, during fermentation of RFR.

Table 1
The regulatory limits for citrinin in red fermented rice.

Countries	Maximum permitted citrinin levels (µg/kg)	References
Taiwan	2000	Taiwan-FDA (2020)
China	200	Kamle et al. (2022); Urraca et al. (2016)
Europe	100	EU (2019)
Japan	50	Kamle et al. (2022); Urraca et al. (2016)

2. Materials and methods

2.1. Identification of fungi

Two *M. purpureus* isolates, MF1 and MS1 isolated from RFR (Farawahida et al., 2022a) were used for inoculation. These isolates have been deposited in the International Collection of Micro-organisms from Plants (ICMP) public strain collection at Landcare Research, New Zealand, and are recorded as ICMP 25182 and ICMP 25183, respectively. Based on the previous study (Farawahida et al., 2022a), *M. purpureus* MF1 required 4 days to produced light blue fluorescence on Coconut Cream Agar (CCA) (100 %, 10/10 plates), while *M. purpureus* MS1 required 7 days to produce fluorescence on CCA (30 %, 3/10 plates). CCA is a simple method to detect CIT by the presence of light blue fluorescence. These isolates were stored at -20 °C for a maximum of 4 weeks. For long-term storage, both isolates were cryopreserved with Microbank™ (Cat. No. PL.170, Fisher Scientific, Texas, USA) and stored at -80 °C for a maximum of 3 years.

2.1.1. Morphology observation

Ten microlitres of both isolates were inoculated on Malt Extract Agar (MEA; Cat. No. 1053980500, Merck, Darmstadt, Germany) and incubated at 30 °C for 7 days (Fig. 1). The method from Pitt and Hocking (2009) was used to measure the diameters of the colonies from the reverse side by the transmitted light method (Fig. 2), while a Scotch Tape method was used to observe the fungal structures under a microscope (Model Nikon Eclipse Ni-U, Nikon Corporation, Tokyo, Japan) (Harris, 2000) (Fig. 3). The colony size, conidia shape, conidia size, ascospores shape, and ascospores size of the isolates were recorded (Table 2).

2.1.2. Molecular characterization and phylogenetic analysis

Genomic deoxyribonucleic acid (DNA) of 7-day-old isolates on MEA was purified using the DNeasy Plant Mini Kit (Cat. No. 69104, QIAGEN, Hilden, Germany) based on the manufacturer's instructions. All the primers used were customized from Thermo Fisher Scientific (Auckland, New Zealand). Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region was performed using the primers ITS4 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS5



Fig. 1. *Monascus* spp. on Malt Extract Agar incubated at 30 °C for 7 days.

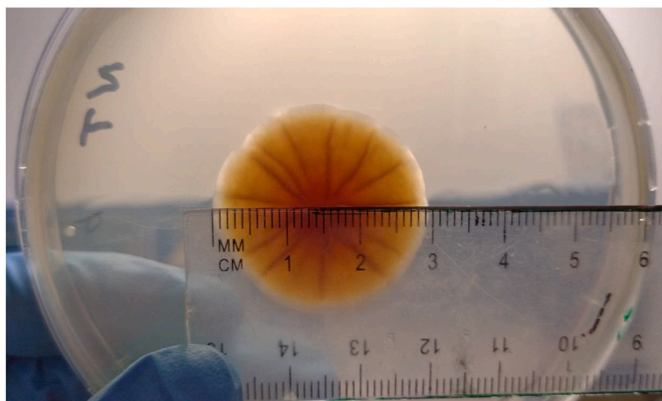


Fig. 2. Measurement of a *Monascus* spp. colony on Malt Extract Agar from the reverse side. The size of the colonies ranged from 23 to 30 mm and was consistent with Pitt and Hocking (2009).

(5'-TCCTCCGCTTATTGATATGC-3'), and the beta-tubulin (β -tubulin) region was amplified using β -tubulin F (5'-CAACTGGGCTAAGGGTCATT-3') and β -tubulin R (5'-GTGAACTCCATCTCGTCCATA-3') primers. To determine the ability of the fungi to produce CIT, polyketide synthase (*pksCT*) and *ctnA* regions were amplified. The primers used for the *pksCT* region were *pksCT* F (5'-TGATGCGACGAAGATGTTAC-3') and

pksCT R (5'-TCTCTATGCTGCGACTGAC-3'). Meanwhile, *ctnA* F (5'-AACGGACAGGAAGAGCGTGC-3') and *ctnA* R (5'-CACACCACCGATGCCATACC-3') primers were used to amplify *ctnA* region.

Fifty microliters of reaction mixtures were prepared by mixing 25 μ L of Platinum™ Green Hot Start PCR 2 \times Master Mix (Cat. No. 13001012, Invitrogen by Thermo Fisher Scientific, Lithuania, Europe), 20 μ L of nuclease-free water, 3 μ L of DNA template, and 1 μ L of each primer (10 μ mol/L). To amplify PCR, the ProFlex 3 \times 32-well PCR System (Cat. No. 4484073, Applied Biosystems by Thermo Fisher Scientific, Singapore) was used with initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, and final extension at 72 °C for 8 min for all primers. The annealing was set for 30 s at 52 °C (ITS), 55 °C (β -tubulin), and 56 °C (*pksCT* and *ctnA*). The setting used for elongation was 72 °C for 45 s (ITS and *pksCT*) and 90 s (β -tubulin and *ctnA*). The PCRs were run for 35 cycles for ITS and β -tubulin, while *pksCT* and *ctnA* were run for 34 cycles.

The PCR products were purified from free deoxynucleotide triphosphates, fluorescent derivatives, and DNA polymerases using DNA Clean & Concentrator-5 [Cat. No. D4013, Zymo Research, The United States of America (USA)] according to the manufacturer's recommendations. The BigDye™ Terminator v3.1 Cycle Sequencing Kit (Cat. No. 4337455, Applied Biosystems by Thermo Fisher Scientific, California, USA) was used to sequence PCR products.

The nucleotide sequences were verified by eyes using the Molecular Evolutionary Genetics Analysis (MEGA) 11 software (Kumar et al.,

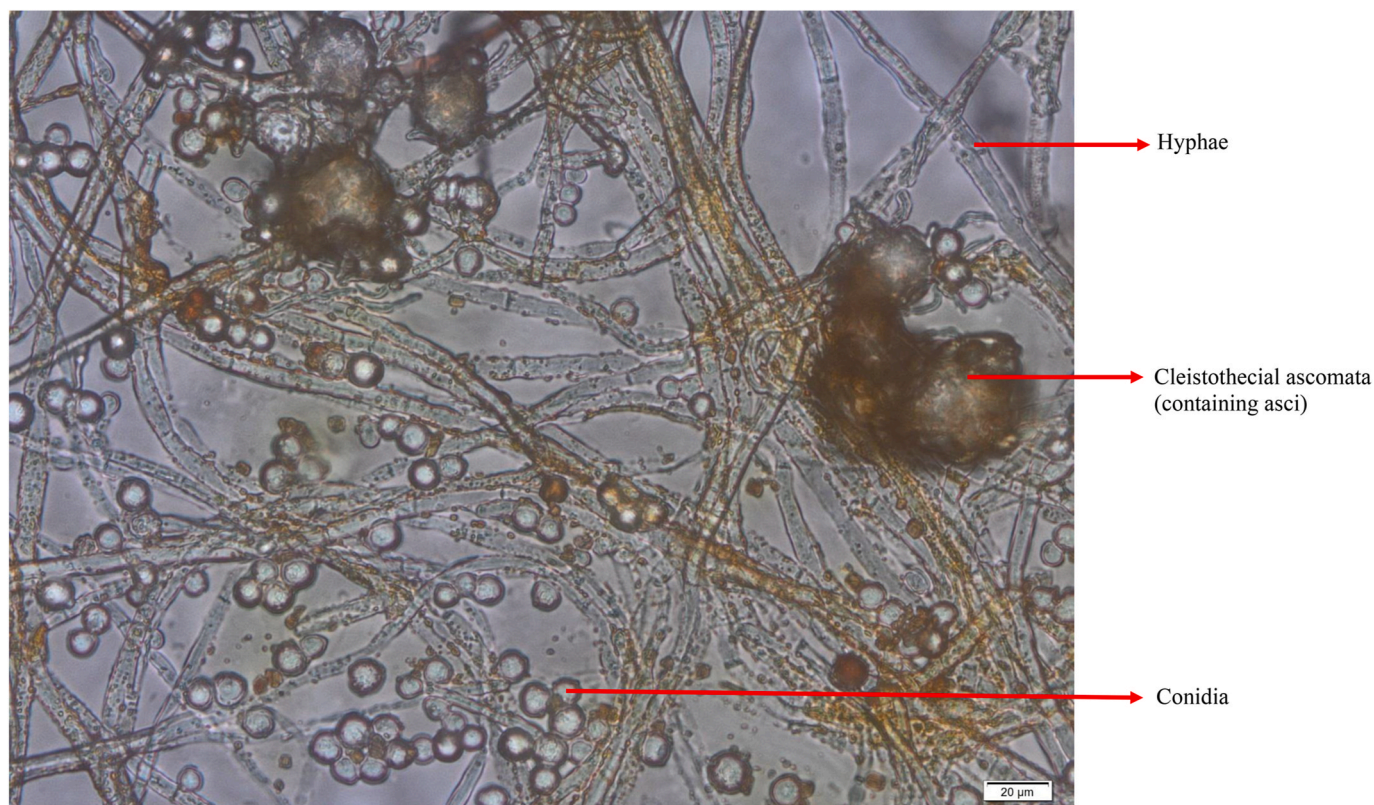


Fig. 3. Microscopic observation of *Monascus* spp. at 40 \times magnification.

Table 2
Morphological characteristics of *Monascus* spp. on Malt Extract Agar (MEA).

Isolates	Colony size (mm) ^a	Conidia shape	Conidia size (μ m) ^a	Ascomata shape	Ascomata size (μ m) ^a	Identified fungi
MF1	23–30	Globular	4–8	Subglobular	13–21	<i>Monascus</i> spp.
MS1	23–30	Globular	4–8	Subglobular	13–21	<i>Monascus</i> spp.

^a The triplicate measurements were conducted for both isolates.

2018). BioEdit 7.7.1 software (Hall, 1999) was used to obtain consensus sequences by assembling forward and reverse sequences. The ITS and *pksCT* sequences for both isolates were deposited into GenBank and BankIt, respectively. To compare the β -tubulin and *ctnA* sequences of these isolates with GenBank databases from the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>), the Basic Local Alignment Search Tool (BLAST) was used. The percentage match of similarity was used to determine the closest matches between the isolates and existing sequences from the BLAST search.

There are nine species of internationally recognized *Monascus* spp.: *M. purpureus*, *M. pilosus*, *M. ruber*, *M. floridanus*, *M. pallens*, *M. lunisporas*, *M. argentinensis*, *M. sanguineus*, and *M. eremophilus* (Abdul-Manan et al., 2017; Chen & Zhang, 2019; Dai et al., 2021). ITS and β -tubulin are the genes used to identify fungi. The accession numbers of ITS and β -tubulin genes for these *Monascus* species were obtained from the National Centre for Biotechnology Information and included in phylogenetic analysis for comparison with MF1 and MS1 isolates (Table 3, Figs. 4 and 5). *Aspergillus* species (*A. flavus*, *A. parasiticus*, and *A. fumigatus*) were used as outgroups because these fungi are closely related to *Monascus* spp. (Li et al., 2020). The accession numbers of ITS and β -tubulin used were listed in Table 3. MEGA 11 software was used for multiple sequence alignment and phylogenetic analysis. The DNA sequences of these species were aligned by MUSCLE. The Neighbor-Joining Tree was used to construct the phylogenetic trees based on the ITS and β -tubulin sequences using the Kimura 2-parameter model. The tree reliability was estimated using a bootstrap method with 1000 replicates.

2.2. Production of RFR

The method of Dogra and Kumar (2017) with slight modification was used to produce RFR. Low-quality white long grain rice available in New Zealand was purchased from local supermarkets. One hundred and 10 g of white rice was weighed in a beaker and 220 mL of distilled water was added to the beaker. After soaking for 2 h, 40 % of distilled water was removed from the beaker. The rice was steamed with a fast slow pressure cooker (Breville Model BPR650 BSSANZ, Breville Pty Ltd, Sydney, Australia) at 100 °C for 20 min. The steamed rice was autoclaved at 121 °C for 15 min. After cooling, 10 mL of MF1 or MS1 spore suspension (10^4 conidia/mL) from a seven-day-old culture on MEA was added as the inoculum to 50 g of steamed rice. The rice was stirred by a spatula. The rice was weighed (1.2 g) and added to a 120 mL polypropylene container with screw cap (Cat. No. 349005, DELTALAB, Bernolsheim, France). This procedure was carried out in an aseptic environment to avoid contamination of the rice. Eighty-four containers were prepared for MF1 and MS1, respectively. All the containers were stored in a box to avoid

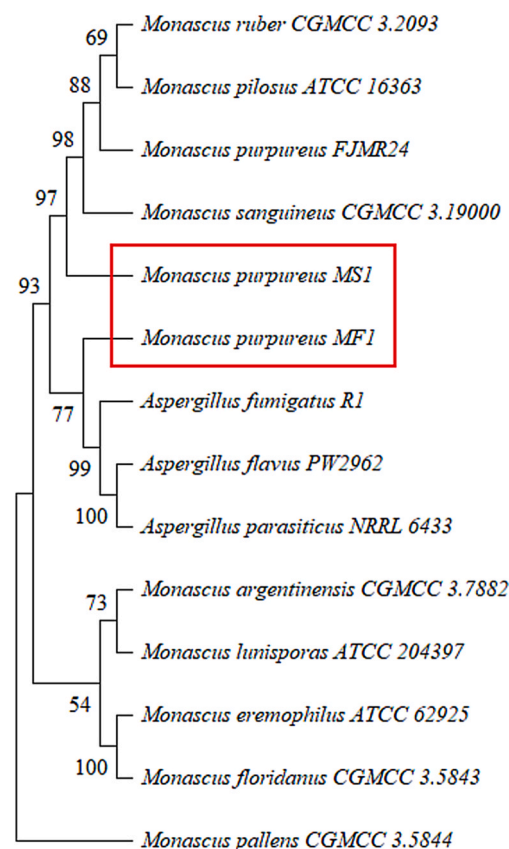


Fig. 4. Neighbor-Joining Tree showing the phylogenetic relationships among *Monascus* and *Aspergillus* spp. based on the internal transcribed spacer sequences using the Kimura 2-parameter model. Values on the branches are bootstrap values.

light exposure at 30 °C for 30 days.

2.3. Determination of pH

Section 2.2 was used to prepare the samples for pH determination. Six containers (three samples for batches A and B, respectively) were removed for analysis at each sampling time (Fig. 6). The sampling was taken daily from day 4 until day 9, followed by three-day intervals until 30 days. Previous studies reported that the minimum requirement to

Table 3

Accession numbers used for the species identification and the phylogenetic analysis.

Isolates	Source	Species	Accession numbers			
			ITS	β -tubulin	<i>pksCT</i>	<i>ctnA</i>
MF1	Red fermented rice	<i>Monascus purpureus</i>	PP060453 ^a	JX221439	PP140672 ^a	AB243687
MS1	Red fermented rice	<i>Monascus purpureus</i>	PP060454 ^a	JX221439	PP140673 ^a	AB243687
<i>M. purpureus</i> FJMR24			MT525241	MT582425	NA	NA
<i>M. ruber</i> CGMCC 3.2093			MN156542	MN229574	NA	NA
<i>M. pilosus</i> ATCC 16363			NR_163510	AY498596	NA	NA
<i>M. argentinensis</i> CGMCC 3.7882			MN156555	MN229606	NA	NA
<i>M. eremophilus</i> ATCC 62925			AY498584	AY498603	NA	NA
<i>M. floridanus</i> CGMCC 3.5843			MN156552	MN229603	NA	NA
<i>M. lunisporas</i> ATCC 204397			AY498583	AY498604	NA	NA
<i>M. sanguineus</i> CGMCC 3.19000			MN156551	MN229602	NA	NA
<i>M. pallens</i> CGMCC 3.5844			MN156553	MN229604	NA	NA
<i>A. flavus</i> PW2962			KF562205	KF562216	NA	NA
<i>A. parasiticus</i> NRRL 6433			EF661568	EF661480	NA	NA
<i>A. fumigatus</i> R1			KJ001801	ON792385	NA	NA

ITS: internal transcribed spacer. β -tubulin: beta-tubulin. *pksCT*: polyketide synthase.

NA: Not applicable.

^a The deoxyribonucleic acid (DNA) sequences for these genes were deposited in the GenBank database.

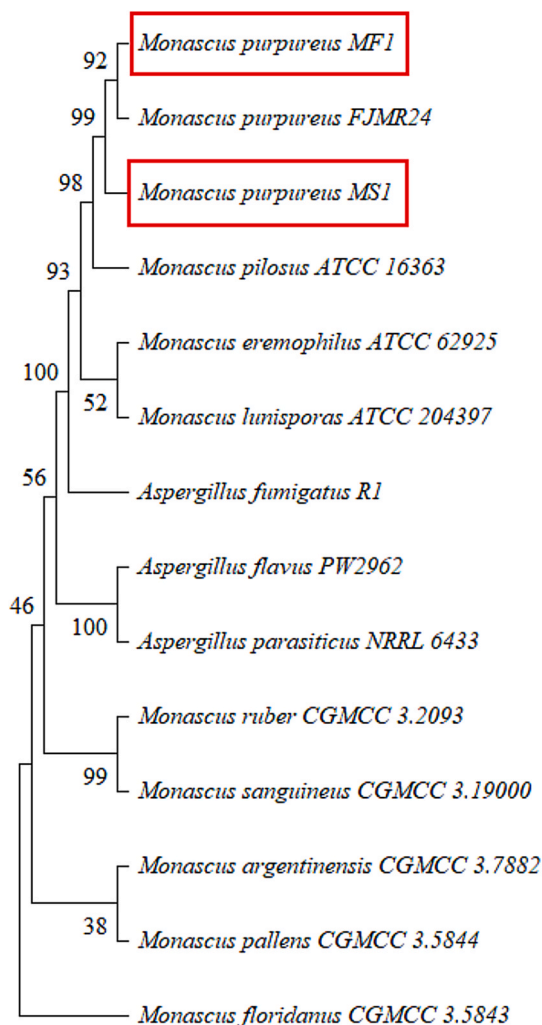


Fig. 5. Neighbor-Joining Tree showing the phylogenetic relationships among *Monascus* and *Aspergillus* spp. based on the beta-tubulin sequences using the Kimura 2-parameter model. Values on the branches are bootstrap values.

produce RFR is several days, and the maximum fermentation time is 30 days (Chairote et al., 2009; Chen et al., 2015; Chen & Hu, 2005; Chiu et al., 2006; Dogra & Kumar, 2017; Li et al., 2003; Patcharee et al., 2007). Therefore, RFR was fermented for 30 days in this study.

There were two batches for each isolate, namely Batches A and B. Batches A and B for both isolates were prepared on the same day under the same conditions to ensure reliability and reproducibility of the results. Then, 10 mL of 60 % ethanol absolute solution (ethanol: distilled water, v/v) (Cat. No. BSPLE975.20, Thermo Fisher Scientific New Zealand Ltd, North Shore City, New Zealand) was added to each container and agitated on an Infors HT Multitron incubator shaker at 25 °C, 200 rpm for 24 h (INFORS HT, Bottmingen, Switzerland). To filter the supernatant, a Minisart® NML surfactant-free cellulose acetate syringe filter (0.2 µm pore size) (Cat. No. S6534—FMOSK, Sartorius, Göttingen, Germany) was used. One millilitre of the filtrate was used to determine the pH of the samples, while 200 µL of the filtrate was used to determine the pigments content. The pH of the filtrate was measured using a pH meter SevenDirect SD20 (Cat. No. 30671550, Mettler-Toledo, Schwerzenbach, Switzerland), which was calibrated prior to the measurements.

2.4. Determination of pigments

To ensure that both pigments and pH analyses were based on the

same extract, 200 µL of the filtrate from section 2.3 was used to determine the pigments. The sampling was taken daily from day 4 until day 9, followed by three days' time interval until 30 days. Batches A and B for both isolates were prepared on the same day with the same condition to produce reliability and reproducibility of the results. The samples were pipetted into Nunc™ MaxiSorp 96-well microplates (Cat. No. 439454, Thermo Fisher Scientific, USA), subjected to a Varioskan LUX Multimode Microplate Reader (Cat. No. VL0000D0, Thermo Fisher Scientific, Vantaa, Finland) and analysed by SkanIt Software 6.0.1.6. (Thermo Fisher Scientific, Vantaa, Finland). The wavelengths used to determine yellow, orange, and red pigments were 400 nm, 460 nm, and 500 nm, respectively (Davoudi Moghadam et al., 2019; Kongruang, 2011). As a control, 60 % of ethanol absolute solution (ethanol: distilled water, v/v) was used. This formulation was used to convert the absorbance into pigments yield, as shown in Equation (1):

$$\text{Pigments yield} = \frac{\text{Optical density (O.D.)} \times \text{dilution} \times \text{volume of extract (mL)}}{\text{Amount of sample (g)}} \quad (1)$$

2.5. Determination of citrinin

2.5.1. Citrinin extraction

Six containers for each isolate (three samples for batches A and B, respectively) were taken at each sampling time to extract CIT from the rice (Fig. 6). The sampling was taken daily from day 4 until day 9, followed by three days' time interval until 30 days. There were two batches for each isolate, namely Batches A and B. Batches A and B for both isolates were prepared on the same day with the same condition to produce reliability and reproducibility of the results. To determine CIT in RFR, 5 mL of 50 % ethanol absolute solution (ethanol: distilled water, v/v) was used as the optimal solid-to-liquid ratio and extraction solvent, respectively (Liu & Xu, 2013). The solvent was added to each container and shaken by a rotary shaker for 1 h at 25 °C at 200 rpm. The mixture was centrifuged by a centrifuge (Sigma® 6-16S, John Morris Scientific, Auckland, New Zealand) for 10 min at 21 °C at 3000×g. To filter the supernatants, a Minisart® NML surfactant-free cellulose acetate syringe filter (0.2 µm pore size) was used. To determine the quantity of CIT levels for each sample, 1 mL of the filtrate was subjected to Ultra-High-Performance Liquid Chromatography with a Fluorescence Detector (UHPLC-FLD) (Thermo Scientific Dionex Ultimate 3000, California, USA).

2.5.2. Preparation of a standard curve for citrinin determination

Citrinin powder from *Penicillium citrinum*, ≥98 % [high-performance liquid chromatography (HPLC)] (Cat. No. C1017, Sigma-Aldrich, Missouri, USA) was used as a CIT standard. Ten millilitres of methanol (HPLC grade) (Cat. No. 34860, Sigma-Aldrich, Missouri, USA) was used as a solvent to dissolve 5 mg of CIT standard to obtain 0.5 mg/mL. Seven concentrations of CIT standards (4, 50, 100, 500, 1000, 2000, and 3000 ng/mL) were prepared and injected to the UHPLC-FLD. A 7-point calibration curve was constructed to determine CIT concentrations in the samples.

2.5.3. Determination of citrinin by Ultra-High-Performance Liquid Chromatography with a Fluorescence Detector (UHPLC-FLD)

The method of Farawahida et al. (2022a) was used to determine the CIT levels in the samples. The presence of CIT was determined by a UHPLC-FLD using a reverse-phase symmetry Kinetex C₁₈ column (250 mm length × 4.6 mm internal diameter, 5 µm psize, 100 Å pore size) (Part No. 00G-4601-E0, Phenomenex, California, USA) as the stationary phase. The column temperature was kept at 30 °C.

All solvents used were of HPLC grade (Sigma-Aldrich, Missouri, USA). Acetonitrile (ACN) (Cat. No. 34851) and 0.1 % trifluoroacetic acid (TFA) (Cat. No. 302031) in purified water were separately filtered through a regenerated cellulose filter (0.20 µm pore size) (Sartorius,

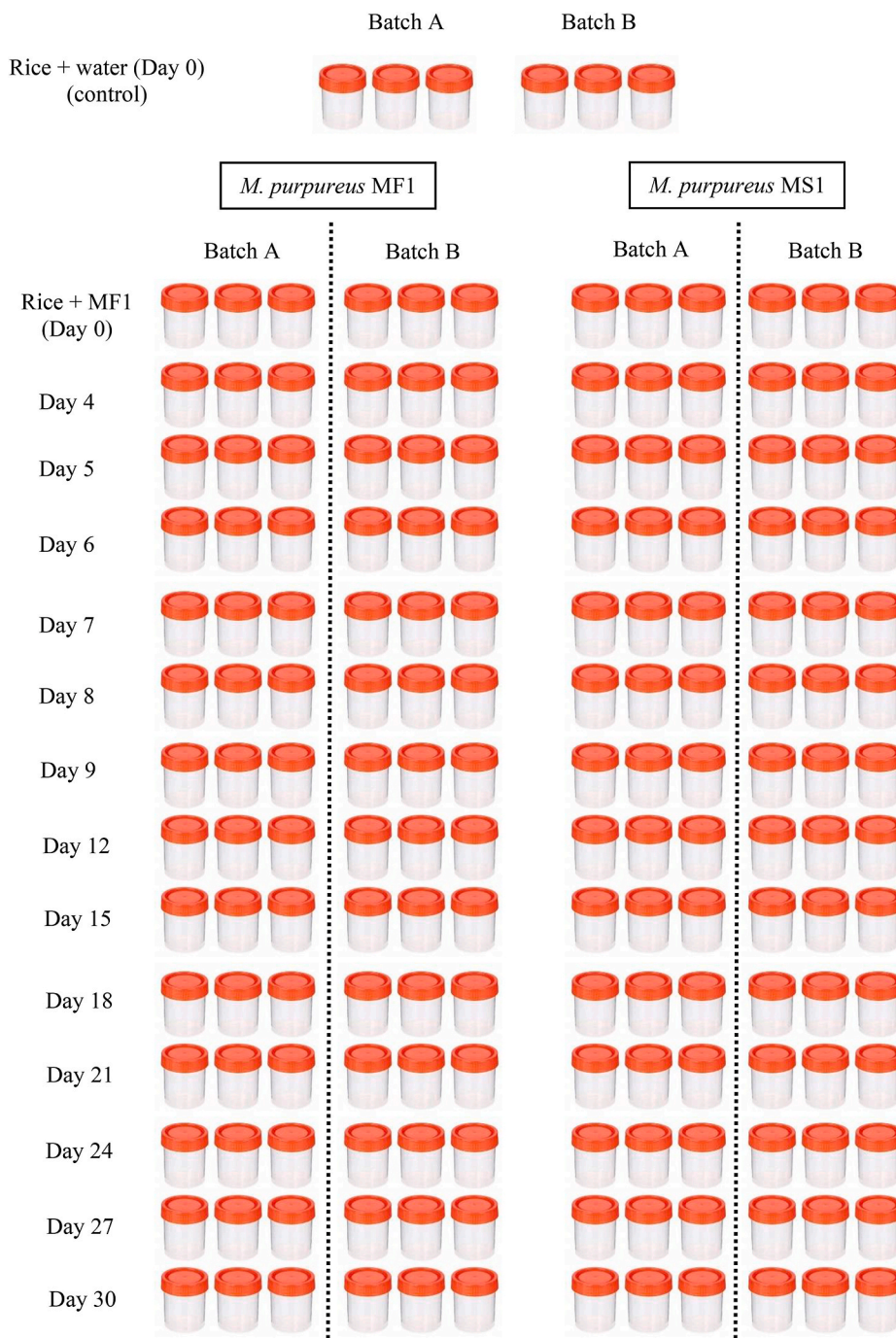
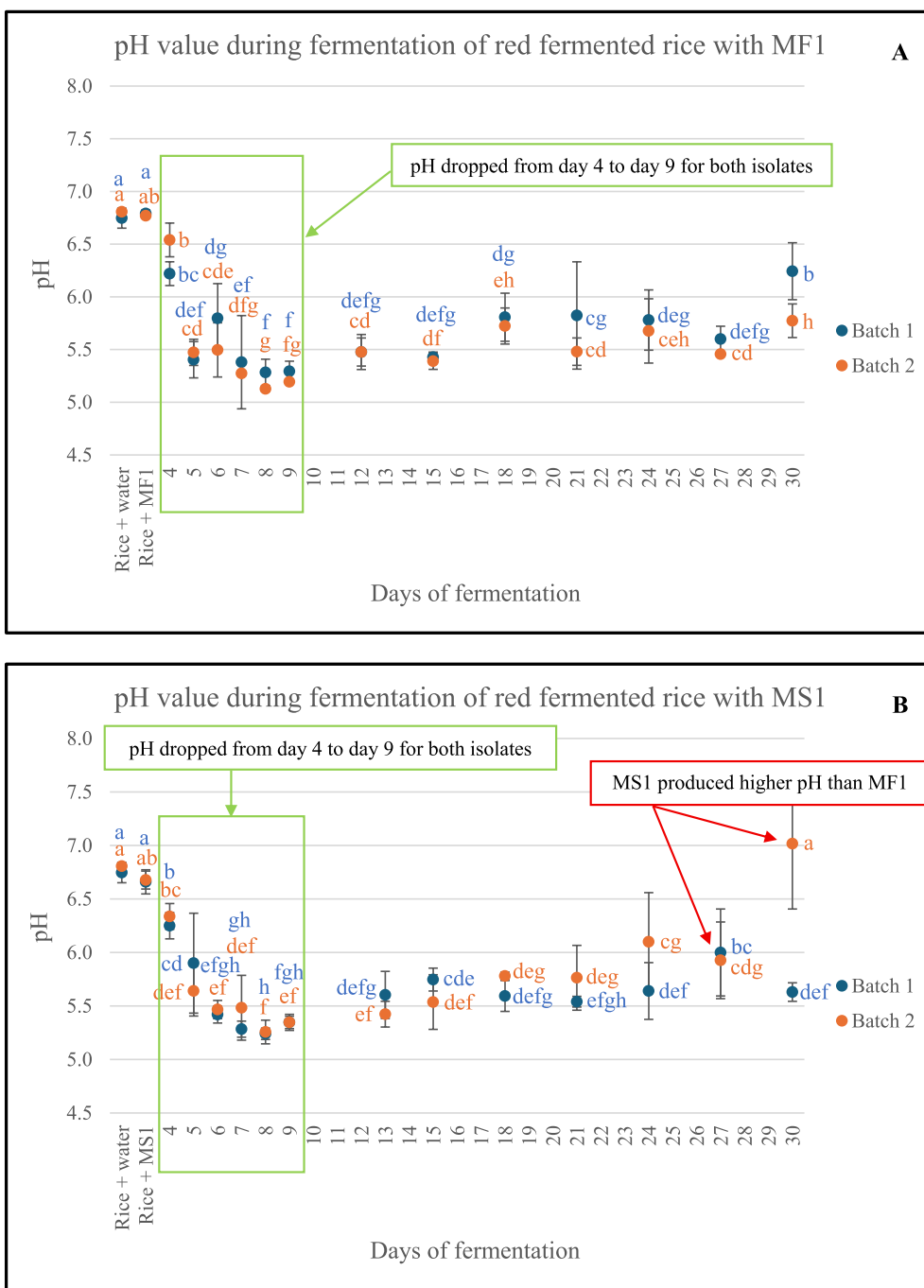


Fig. 6. Schematic diagram for producing red fermented rice using *Monascus purpureus* MF1 and MS1 isolates. Batches A and B were prepared on the same day for both isolates. The experiments were conducted independently to determine (a) pigments and (b) citrinin. All the rice was fermented at 30 °C for 30 days.

Göttingen, Germany). These solvents were used as the mobile phase with a ratio of ACN: 0.1 % TFA (55:45, v/v) at a flow rate of 1.0 mL/min. The data were recorded with a fluorescence (FLD) detector at an excitation wavelength of 330 nm and an emission wavelength of 500 nm. The system was run isocratically for 40 min. Twenty microliters of each sample were injected into the UHPLC–FLD system. All the injections were done in triplicate and to process the data, Chromeleon™ Chromatography Data System 7.0 Software (Thermo Fisher Scientific, California, USA) was used.

2.6. Reduction of citrinin after combining with pigments

MF1 and MS1 were inoculated on CCA and incubated at 30 °C for 8 days. Based on the preliminary study, there was an increase in pigments production and a reduction in CIT when MF1 and MS1 isolates were inoculated on CCA (data not shown). Therefore, 8 days was chosen as the incubation time for this study. *Monascus* mycelium from both isolates secreted a deep red colour on CCA after 8 days of incubation. These mycelia were scraped from CCA, weighed (1 g, 2 g, and 3 g), and 10 mL



* Blue font is for Batch 1 and orange font for Batch 2 for both isolates. Different letters within the same batch for each isolate indicate significant differences ($p < 0.05$).

Fig. 7. The pH values of *Monascus purpureus* isolates (A) MF1 and (B) MS1 after fermentation of red fermented rice at 30 °C for 30 days.

60 % ethanol absolute solution (ethanol: distilled water, v/v) was added to obtain 100 mg/mL, 200 mg/mL, and 300 mg/mL of mycelium. The mixtures were shaken on a rotary shaker at 25 °C, 200 rpm for 24 h. The supernatants were filtered using a Minisart® NML surfactant-free cellulose acetate syringe filter (0.2 µm pore size) to obtain the pigments. The pigments were mixed with 1000 ng/mL CIT and left for 1 h before injection into the UHPLC-FLD. All the samples were done in two batches.

2.7. Statistical analysis

All the samples were measured in triplicate to obtain the mean and standard deviation. The graphs were analysed and plotted using Microsoft Excel. Batches A and B for all parameters were analysed separately to ensure the reliability and reproducibility of the results. The differences between the samples were analysed using the mean and standard deviation and submitted to a one-way analysis of variance (ANOVA) followed by Fisher LSD Method (5 % significance) using Minitab Version 21 software (Minitab, LLC, Pennsylvania, USA).

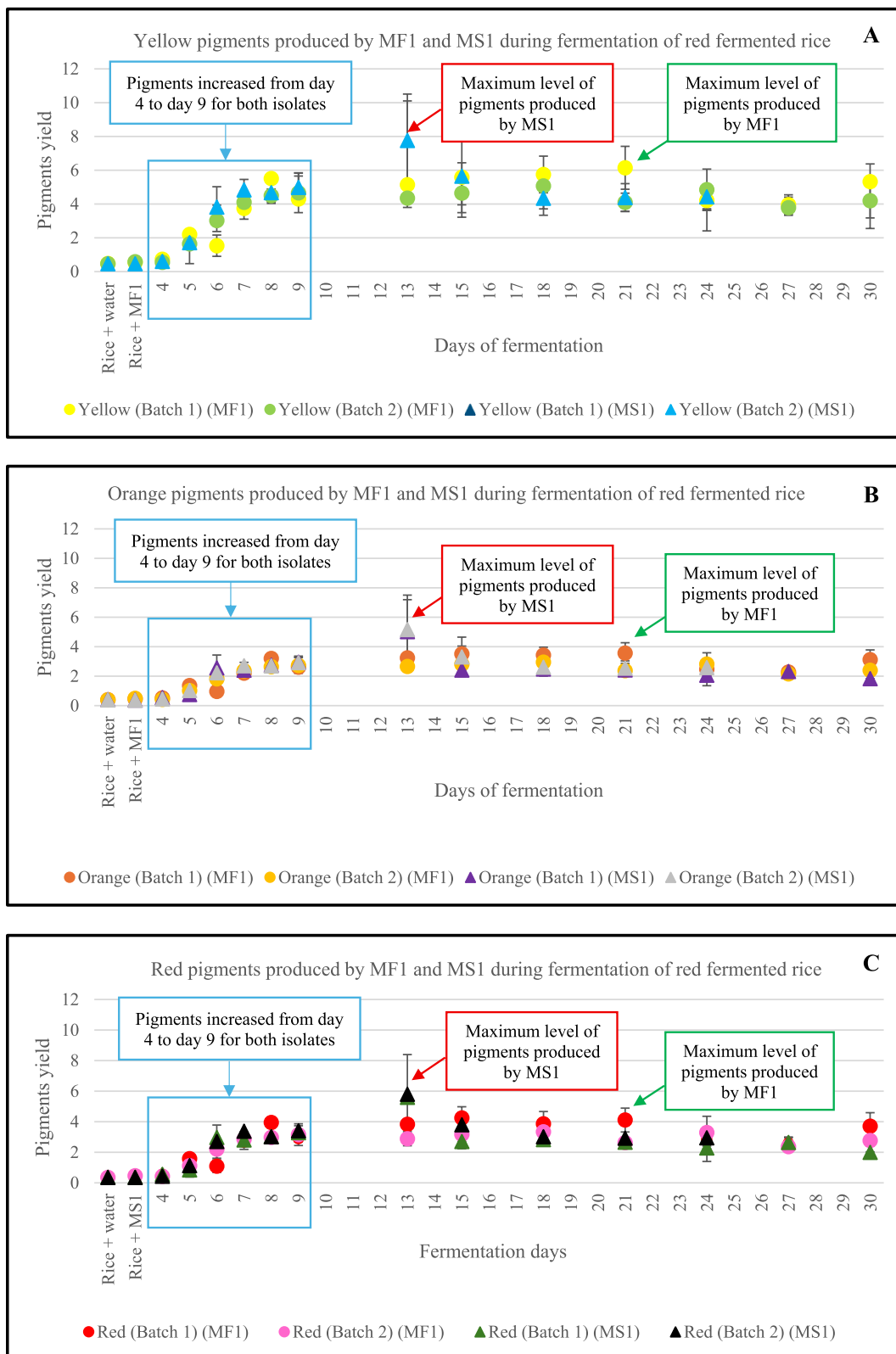


Fig. 8. The yield of (A) yellow, (B) orange, and (C) red pigments produced by *Monascus purpureus* isolates MF1 and MS1 after fermentation of red fermented rice at 30 °C for 30 days.

3. Results

3.1. Identification of fungi

Figs. 1 and 2 show the morphology of fungi on MEA from the upright and reverse sides, respectively. Microscopic observation at 40× magnification revealed the presence of cleistothecium ascomata, hyphae and conidia (Fig. 3).

The Neighbor–Joining Tree was constructed based on the ITS and β -tubulin sequences to illustrate the phylogenetic relationships among *Monascus* spp. and several *Aspergillus* species (Figs. 4 and 5).

3.2. Determination of pH

Fig. 7 shows that the pH of MF1 significantly decreased from pH 6.79 to 5.29 (Batch 1) and from 6.77 to 5.19 (Batch 2) during 9 days of fermentation of RFR. For MS1, the pH also significantly dropped from 6.66 to 5.35 (Batch 1) and from 6.68 to 5.35 (Batch 2) over the same period. However, the pH of RFR from MF1 significantly increased from day 18 (Batch 1) and day 12 (Batch 2) until the end of fermentation. For MS1, the pH significantly increased from day 24 to day 30 (Batch 2).

Both isolates showed similar pH trends from day 4 to day 24. However, MS1 produced a higher pH than MF1 after 27 days of fermentation.

3.3. Determination of pigments

During the fermentation of rice, pigments levels in RFR increased over time (Fig. 8). From day 4 to day 9, pigments production by both MF1 and MS1 increased. However, each isolate produced the maximum pigments levels at different times. MS1 produced maximum levels of yellow, orange, and red pigments after 13 days of fermentation, while MF1 produced the maximum levels of yellow, orange, and red pigments after 21 days.

3.4. Determination of citrinin

3.4.1. Citrinin standard curve

To determine CIT concentration in the RFR, a standard curve was generated using several concentrations of CIT standards (Section 2.5.2.). A good coefficient of determination (R^2) was obtained (0.9999) and the calibration curve further used to determine CIT content in the samples (Fig. 9).

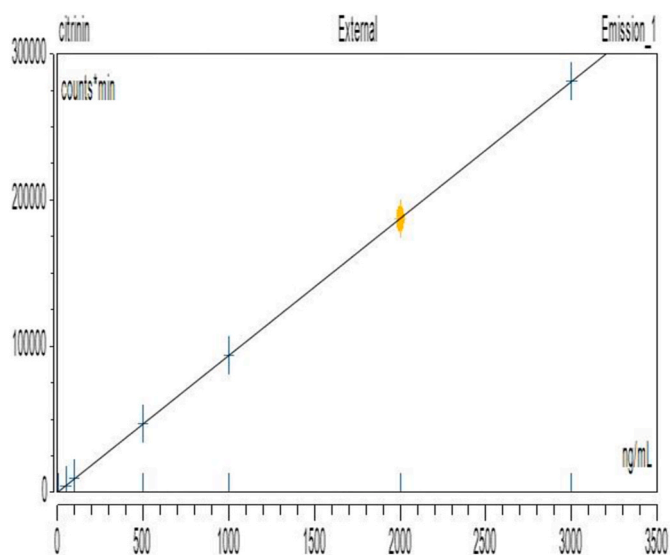


Fig. 9. A citrinin standard curve with seven different concentrations.

3.4.2. Citrinin levels during fermentation of red fermented rice

In this study, CIT was detected in samples after 4 days of RFR fermentation (Fig. 10). For both isolates, CIT levels increased from day 4 to day 5 and then decreased. For MF1 (Batch 1), CIT level increased significantly from 290.65 ng/mL (day 4) to 978.34 ng/mL (day 5), followed by a significant decrease to 701.59 ng/mL (day 6). In Batch 2, the CIT level significantly increased from 333.18 ng/mL (day 4) to 725.17 ng/mL (day 5), then significantly dropped to 389.92 ng/mL after 8 days. A similar trend was observed with MS1. In batch 1, CIT level significantly increased from 438.62 ng/mL (day 4) to 1099.33 ng/mL (day 5), and then significantly dropped to 695.41 ng/mL after 6 days. In batch 2, CIT level significantly increased from 240.15 ng/mL (day 4) to 787.51 ng/mL after 6 days, followed by a significant decrease to 326.36 ng/mL on day 7.

3.5. Reduction of citrinin after combining with pigments

Tables 4 and 5 present the CIT reduction after mixing with pigments extracted from MF1 and MS1, respectively. After combining the CIT standards with pigments from both isolates, CIT levels were significantly reduced by 26–68 % for MF1 and 16–45 % for MS1.

4. Discussion

4.1. Identification of fungi

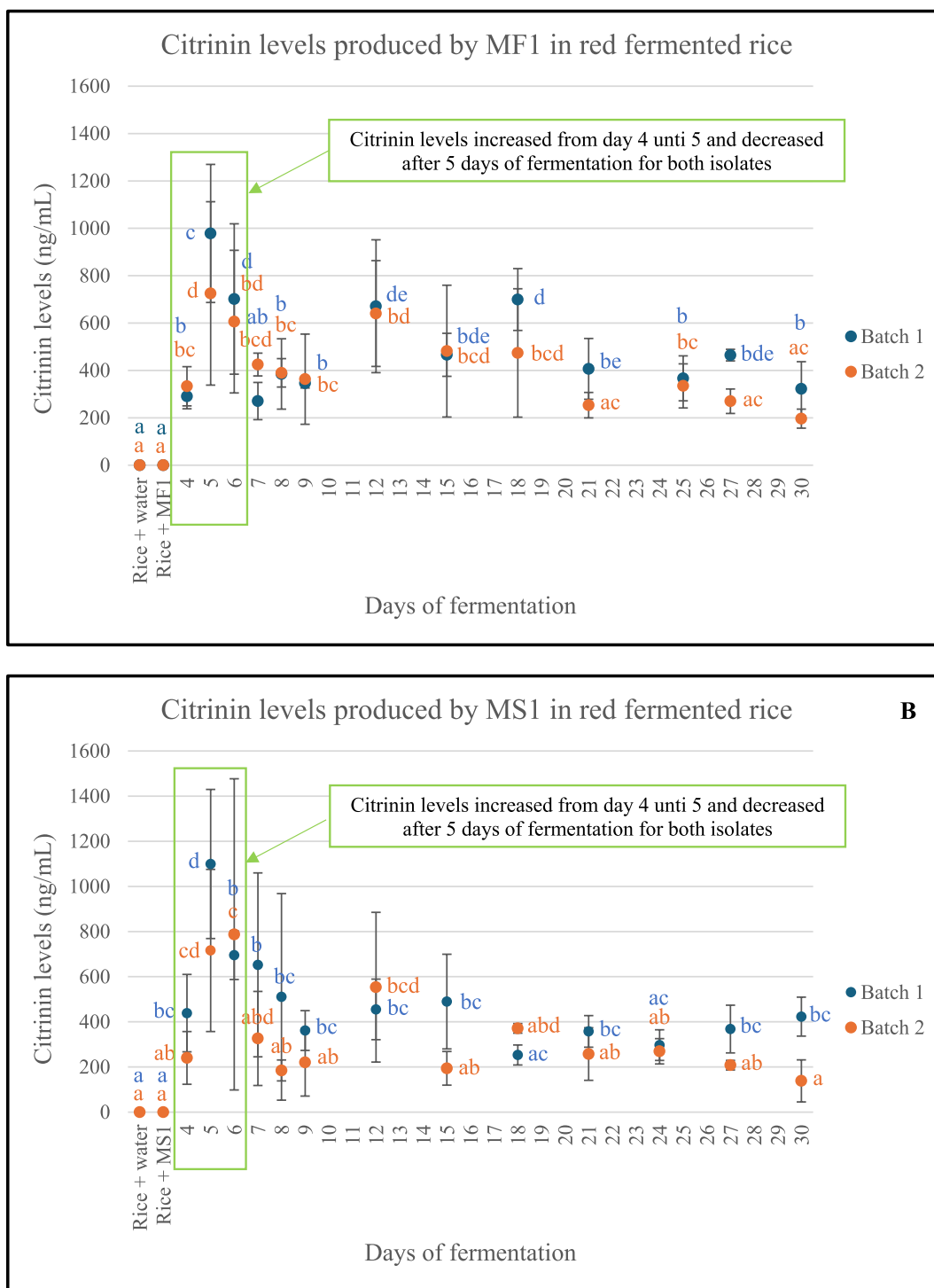
The genus of the isolates was identified based on colony size and fungal structures (Kim et al., 2010; Pitt & Hocking, 2009). The development of cleistothecia is one of the key characteristics of *Monascus* spp. Therefore, MF1 and MS1 were categorized as *Monascus* spp. The conidial size of isolates MF1 and MS1 was similar to that of *M. purpureus* reported by Kim et al. (2010). According to the BLAST database, isolates MF1 and MS1 were classified as *M. purpureus*, possessing CIT biosynthesis genes with 98–100 % similarity.

It was observed that *M. ruber*, *M. pilosus*, *M. sanguineus*, *A. fumigatus*, *A. flavus*, and *A. parasiticus* were grouped in the same clade as *M. purpureus* FJMR24, and *M. purpureus* isolates MF1 and MS1 (Fig. 4). However, these *Monascus* and *Aspergillus* spp. were clearly separated from *M. purpureus* in the analysis (Fig. 5). The reference isolate *M. purpureus* FJMR24 clustered with MF1 and MS1 (Fig. 5). The results showed that the Neighbor–Joining Tree based on the ITS sequence was insufficient to distinguish *Monascus* spp., whereas the Neighbor–Joining Tree based on the β -tubulin sequence provided better separation among *Monascus* species.

4.2. Determination of pH

The pH changes observed in this study were similar to those reported in previous work. Jirasatid et al. (2019) examined the growth and production of pigments, monacolin K, and CIT using rice pasta by-products fermented by *M. purpureus* TISTR 3541 and TISTR 3629. The pH was also monitored in that study. Results showed that during the initial fermentation period (0–810 days), the pH slightly decreased and then increased afterward. Accumulation of organic acids via the tricarboxylic acid cycle caused the pH to drop during the early stage of RFR fermentation. Later, deamination produced ammonium, resulting in an increase in the pH value (Jirasatid et al., 2019).

However, the pH results in this study contrast with findings by Patrovsky et al. (2019), where the pH increased from 5.5 to 7.1 after 14 days of incubation. Patrovsky et al. (2019) also reported that the CIT level increased until day 14 (33 mg/L) and decreased after prolonged incubation. After 24 days, the final pH reached 7.3, and the CIT level declined to 6 mg/L. It was hypothesized that the decrease in CIT levels was linked to degradation into CIT H2 at pH above 7.0 (Patrovsky et al., 2019), likely due to the opening of the CIT ring structure (Bazin et al., 2013). CIT H2 is less toxic and exhibits lower absorbance at 320 nm



* Blue font is for Batch 1 and orange font for Batch 2 for both isolates. Different letters within the same batch for each isolate indicate significant differences ($p < 0.05$).

Fig. 10. Citrinin levels produced by *Monascus purpureus* isolates (A) MF1 and (B) MS1 after fermentation of red fermented rice at 30 °C for 30 days.

compared to CIT (Hirota et al., 2002). Kang et al. (2014) reported that CIT was inhibited at extremely low pH (pH 2 or 2.5). In contrast, the highest total red pigments production and the lowest CIT levels were achieved at pH 5.5, followed by production at pH 8.5 (Orozco & Kili-kian, 2008).

4.3. Determination of pigments

Patrovsky et al. (2019) studied the effects of initial pH and cultivation time on pigments and CIT production by *M. purpureus* in submerged liquid culture. The study found that *M. purpureus* reached maximum growth after 10 days of incubation.

Yellow pigments increased until day 24, while orange pigments

Table 4Reduction of citrinin after combining with pigments extracted from *Monascus purpureus* isolate MF1.

Samples	Reduction of citrinin	
	Batch A	Batch B
100 mg/mL pigments +1000 ng/mL CIT	29.97 ± 8.57 % ^a	26.07 ± 2.88 % ^a
200 mg/mL pigments +1000 ng/mL CIT	55.12 ± 13.77 % ^b	51.60 ± 26.22 % ^{ab}
300 mg/mL pigments +1000 ng/mL CIT	54.33 ± 13.09 % ^b	68.33 ± 7.65 % ^b

* Different letters within the same column indicate significant differences ($p < 0.05$).**Table 5**Reduction of citrinin after combining with pigments extracted from *Monascus purpureus* isolate MS1.

Samples	Reduction of citrinin	
	Batch A	Batch B
100 mg/mL pigments +1000 ng/mL CIT	41.17 ± 12.78 % ^a	16.06 ± 5.98 % ^a
200 mg/mL pigments +1000 ng/mL CIT	32.80 ± 25.10 % ^a	28.67 ± 4.92 % ^a
300 mg/mL pigments +1000 ng/mL CIT	39.60 ± 19.00 % ^a	44.99 ± 7.85 % ^b

* Different letters within the same column indicate significant differences ($p < 0.05$).

increased until day 14 and decreased afterward (Patrovsky et al., 2019). This decline may be due to mycelial damage during later fermentation stages and the reaction of amino-containing substances with orange pigments to form red pigments (Patrovsky et al., 2019). The mycelial damage may have resulted from agitation during fermentation.

Velmurugan et al. (2011) reported complete inhibition of *M. purpureus* growth at initial pH 1 and 2. Maximum red and yellow pigments production was observed at initial pH 5 and 6, respectively. Increasing the initial pH to 7 or 8 led to reduced yellow and red pigments levels. Red pigments production also decreased at initial pH below 5.5 and above 8.5 (Lee et al., 2001). Therefore, Lee et al. (2001) suggested that the optimal initial pH for red pigments production by *M. purpureus* ranges from 5.5 to 8.5, with pH 7 yielding maximum red pigments level.

In this study, yellow, orange, and red pigments produced by MS1 increased from day 4 to day 13, whereas pigments production by MF1 increased from day 4 to day 21. These findings suggest that isolate type and fermentation duration influence pigments levels. A pH between 5 and 6 favors pigments production by *M. purpureus*.

4.4. Determination of citrinin

The secondary metabolite CIT is typically produced during the stationary phase under oxygen-rich and glucose-limited conditions (Hajjaj et al., 2000; Orozco & Kilikian, 2008). Patrovsky et al. (2019) reported that *M. purpureus* DBM 4360 reached maximum CIT production on day 14, followed by a decline until day 24. Bazin et al. (2013) proposed that increases in the pH of RFR above 7.0 can lead to ring opening of CIT and transformation into CIT H2. In contrast, our results show that CIT levels declined after day 5 when the pH was still below 7.0.

CIT and pigments share partial mutual biosynthetic pathways (Mou et al., 2023). The pathway begins with one acetyl coenzyme A (acetyl-CoA) molecule, three malonyl-coenzyme A (malonyl-CoA) molecules, and polyketide synthase, which condense to form a tetraketide*. After this step, the pathways for CIT and pigments diverge. For CIT, another acetyl-CoA and polyketide synthase are added to form an Intermediate, followed by methylation, condensation, reduction, O-alkylation, cleavage between C-1 and C-9 bonding, further reduction, oxidation, and dehydration to produce CIT (Hajjaj et al., 1999, 2000; Suharna et al., 2019). However, Mou et al. (2023) proposed that the CIT is formed by a series of methylation, condensation, reduction, methoxylation, oxidation, and dehydration reactions to the Intermediate.

Pigments are synthesized by the addition of two more malonyl-CoA

molecules to the tetraketide*, followed by oxidation, reduction, hydration, dehydration, decarboxylation, methylation, and glycosylation to form a hexatone chromophore (Mou et al., 2023). Esterification of the hexatone chromophore with fatty acids yields orange pigments. Orange pigments are reduced to form yellow pigments, while amination results in red pigments (Mou et al., 2023).

Li et al. (2021) proposed that there were two hypotheses for the biosynthesis pathways of *Monascus* pigments and CIT. One of the hypotheses is according to the metabolic pathways, which is *Monascus* pigments and CIT shared a common pathway up to a branch point. Another hypothesis is based on genomic analysis, which is *Monascus* pigments and CIT have two different pathways due to the finding of their separate biosynthesis gene clusters.

4.5. Reduction of citrinin after combining with pigments

Monascus pigments contain at least six pigments, which are: (1) ankaflavin and (2) monascin (yellow pigments), (3) monascorubrin and (4) rubropunctatin (orange pigments), and (5) monascorubramine and (6) rubropunctamine (red pigments) (Chai et al., 2020; Egea et al., 2023; Farawahida et al., 2022b; Meinicke et al., 2012). Pigments extracted from MF1 showed greater CIT reduction than those from MS1 when mixed with the CIT standard. This may be due to higher CIT production by MF1.

Fungal pigments are known to reduce mycotoxins. Salama et al. (2021) reported that red pigments from *Penicillium purpurogenum* 2603 reduced aflatoxin B1 and B2 by 9–38 % and 45 %, respectively. For ochratoxin A, reductions of 15 %, 31 %, and 54 % were observed at pigments concentrations of 1, 3, and 5 mg/mL, respectively. Piliu et al. (2011) found that flavonoid pigments accumulation, especially phlobaphenes in maize kernel reduced fumonisin B1. Most carotenes and xanthophylls inhibited aflatoxin production by *A. flavus* and *A. parasiticus*, with greater inhibition observed from alpha-ionone-ring-compounds (alpha-carotene, beta-carotene, or alpha-ionone) than from beta-ionone-ring compounds. *A. parasiticus* was less sensitive to beta-carotenes than *A. flavus* (Norton, 1997).

5. Conclusion

Two *M. purpureus* isolates, MF1 and MS1, were used in this study. Two batches of each isolate were fermented at 30 °C for 30 days to determine the pH, pigments, and CIT levels. While some differences were observed between batches of the same isolate, the trends were consistent. CIT production peaked after 5 days of fermentation, then declined, coinciding with increased pigments production. *M. purpureus* MF1 and MS1 isolates required different fermentation times to achieve maximum pigments levels. The optimal pH range for pigments production was between 5 and 6. The inverse relationship between CIT and pigments levels suggests a possible metabolic link. Future work should identify which specific pigments are responsible for CIT reduction. Optimizing fermentation to enhance pigments production while minimizing CIT contamination in RFR presents a promising avenue for food safety improvement.

CRedit authorship contribution statement

Abdul Halim Farawahida: Writing – original draft, Visualization, Data Curation, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Jon Palmer:** Writing – review & editing, Visualization, Validation, Supervision, Conceptualization. **Steve Flint:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Glossary

β -tubulin	Beta-tubulin
ACN	Acetonitrile
Acetyl-CoA	Acetyl coenzyme A
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
CCA	Coconut Cream Agar
CIT	Citrinin
DNA	Deoxyribonucleic acid
FLD detector	Fluorescence detector
HPLC	High-performance liquid chromatography
ITS	Internal transcribed spacer
Malonyl-CoA	Malonyl-coenzyme A
MEA	Malt Extract Agar
MEGA	Molecular Evolutionary Genetics Analysis
PCR	Polymerase chain reaction
<i>pksCT</i>	Polyketide synthase
R^2	coefficient of determination
RFR	Red fermented rice
TFA	Trifluoroacetic acid
UHPLC-FLD	Ultra-high performance liquid chromatography with a fluorescence detector
USA	The United States of America

Data availability

Data will be made available on request.

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