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CONTROL OF CITRININ AND PIGMENTS PRODUCED BY *Monascus purpureus* DURING THE FERMENTATION OF RED FERMENTED RICE

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ABSTRACT

Monascus spp. is a fungal starter used to produce red fermented rice (RFR). This product has some health benefits and contains pigments that can act as natural colour and flavouring agents. However, *Monascus* spp. can also produce the mycotoxin, citrinin (CIT) which is believed to have adverse effects on human health. CIT in RFR has been reported worldwide by using different methods of detection. In this current research, Coconut Cream Agar (CCA) was developed as a simple and rapid semiquantitative method to screen CIT–producing *Monascus* spp. isolates from RFR. Two *Monascus purpureus* isolates, MF1 and MS1, were isolated. These isolates were identified based on the macroscopic and microscopic observations, and deoxyribonucleic acid (DNA) sequencing [internal transcribed spacer (ITS) and beta–tubulin (β–tubulin) genes]. Further analysis showed that these isolates contained polyketide synthase (*pksCT*) and *ctnA* genes, which are CIT biosynthesis genes. These isolates exhibited fluorescence on CCA and were confirmed as CIT producers by Ultra–high performance liquid chromatography with a fluorescence detector (UHPLC–FLD). A toxicity test was conducted using *Artemia salina* to determine the toxicity of CIT. The results showed that LC_{50} was 66 µg/mL. The fungal growth, CIT, pigments production, and pH of *M. purpureus* isolates were characterized on CCA for 30 days. Decreasing CIT levels were observed after incubation of MF1 and MS1 on CCA for 8 and 7 days, respectively. The pigments increased during this incubation time. There were similar trends for CIT and pigments observed during the production of RFR. CIT increased to a maximum level after 5 days of incubation and pigments increased from 5–9 days. There appears to be a relationship between pigments production and CIT levels during the growth of *M. purpureus*. Mixing CIT and pigments extracted from MF1 and MS1 resulted in a

reduction of CIT by 26–68% and 16–45%, respectively. The results on specific pigments and their effect on CIT showed that ankaflavin, monascin, monascorubrin, rubropunctatin, and monascorubramine significantly reduced CIT, with the highest reduction produced by ankaflavin. The findings of this study suggest pigments production could be optimized to control CIT levels in *Monascus*–fermented products.

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DECLARATION

This thesis is comprised of eight chapters. Chapter 2 and 3 have been published (Appendices A–D). Partial contents of chapter 4, 5, 6, and 7 are structured to be submitted to international journals.

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90–d	90–days
α–carotene	Alpha–carotene
α–ionone	Alpha–ionone
α–tocopherol	Alpha–tocopherol
~	Approximately
β–carotene	Beta–carotene
β–ionone	Beta–ionone
β, ψ–carotene	Beta, psi–carotene
β–tubulin	Beta–tubulin
° C	Degree Celsius
γ–carotene	Gamma–carotene
γ–tocopherol	Gamma–tocopherol
<	Less than
µg/g	Microgram per gram
µg/kg	Microgram per kilogram
µg/mL	Microgram per millilitre
µL	Microliter
µm	Micrometre
µM	Micromolar
×	Multiplication
%	Percentage
±	Plus–minus
:	Ratio
–	To
Å	Angstrom
a_w	Water activity
ACN	Acetonitrile
Acetyl–CoA	Acetyl coenzyme A
AF	Aflatoxin
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFM_1	Aflatoxin M_1
AMD	Age–related macular degeneration
ANOVA	Analysis of variance
BGG–823	Human gastric adenocarcinoma
BLAST	Basic Local Alignment Search Tool
bp	Base pair
Ca	Calcium
$CaCl_2$	Calcium chloride
CAGR	Compound annual growth rate
CCA	Coconut Cream Agar
Cells/mL	Cells per millilitre
CYA	Czapek Yeast Extract Agar
CIT	Citrinin
CIT H2	Citrinin H2
Conidia/mL	Conidia per millilitre

CZE	Capillary zone electrophoresis
CZE–UV	Capillary zone electrophoresis along with an ultraviolet detector
Dihydro–β–ionone	Dihydro–beta–ionone
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DOW	Deep ocean water
DOW–MFD	*Monascus*–fermented *Disoscorea* fermented with deep ocean water
DPPH	2,2–diphenyl–1–picrylhydrazyl
DRBC	Dichloran Rose Bengal Chloramphenicol
EC	European Commission
EFSA	European Food Safety Authority
EIA	Enzyme immunoassays
ELISA	Enzyme–linked immunosorbent assay
ETC	Electron transport chain
EU	The European Union
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FDA	Food and Drug Administration
Fe	Iron
$FeSO_4$	Ferrous sulfate
$FeSO_4 \cdot 7H_2O$	Ferrous sulfate heptahydrate/Iron (II) sulfate heptahydrate
FLD detector	Fluorescence detector
g	Gram
g/mL	Gram per millilitre
g/L	Gram per Liter
G25N	Twenty–five per cent (25%) Glycerol Nitrate Agar
GABA	ɣ-aminobutyric acid
GC–MS	Gas chromatography–mass spectrometry
GLM	Generalized linear model
GOX	Glucose oxidase
h	hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HepG2	Hepatoblastoma/hepatocarcinoma cell line
HEK293	Human embryonic kidney cell line
HFB_1	Hydrolysed fumonisin B_1
HFB_2	Hydrolysed fumonisin B_2
HMG–CoA reductase	Enzyme 3–hydroxy–3–methyl–glutaryl coenzyme A reductase
HPLC	High–performance liquid chromatography
HPLC–FLD	High–performance liquid chromatography with a fluorescence detector
HPTLC–UV/FLD	High–performance thin–layer chromatography with ultraviolet and fluorescence detectors
ic–ELISA	Indirect competitive enzyme–linked immunosorbent assays
ICA	Immunochromatographic assay
ICMP	International Collection of Micro–organism from Plants
ICS	Immunochromatographic strip

ITS	Internal transcribed spacer
K	Potassium
K_2HPO_4	di–potassium hydrogen orthophosphate
Kb	Kilobase
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
LAB	Lactic acid bacteria
LC_{50}	Medium lethal concentration
LC–FLD	Liquid–chromatography with fluorescence detector
LC–MS	Liquid–chromatography coupled with mass spectrometry
LC–MS/MS	Liquid–chromatography and tandem mass spectrometry
LD_{50}	Medium lethal dose
LDL	Low–density lipoprotein
Malonyl–CoA	Malonyl–coenzyme A
MEA	Malt Extract Agar
MeOH	Methanol
MC	Moisture content
MEGA	Molecular Evolutionary Genetics Analysis
Mg	Magnesium
mg/g	Milligram per gram
mg/kg	Milligram per kilogram
mg/L	Milligram per Liter
mg/mL	Milligram per millilitre
$MgSO_4$	Magnesium sulphate
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate/Epsom salt
min	Minute
mL	Millilitre
mL/min	Millilitre per minute
mm	Millimetre
mm^2	Square millimetre
mm/day	Millimetre per day
$MnSO_4 \cdot H_2O$	Manganese (II) sulfate monohydrate
mol/L	the number of moles per Liter
MSG	Monosodium glutamate
n	Sample size
NA	Not applicable
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
NCM–FB_1	*N*–carboxymethyl–fumonisin B_1
NDI	New dietary ingredient
ng/µL	Nanogram per microliter
ng/mL	Nanogram per millilitre
NH_4Cl	Ammonium chloride
NM	Not mentioned
nm	Nanometre
NMR	Nuclear magnetic resonance spectroscopy
NOAEL	No observed adverse effect level
NYDB	Nutrient yeast dextrose broth
OD	Optical density

OTA	Ochratoxin A
p–value	Probability value
PCR	Polymerase chain reaction
PDA agar	Potato Dextrose Agar
PDA detector	Photodiode–array detector
pksCT	Polyketide synthase
psi	Pounds per square inch
ppb	Parts per billion
ppm	Parts per million
R^2	coefficient of determination
rDNA	Ribosomal DNA
RFR	Red fermented rice
RH	Relative humidity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP–HPLC	Reversed–phase high–performance liquid chromatography
rpm	Revolutions per minute
RT	Room temperature
SAMNs	Surface Active Maghemite Nanoparticles
SSF	Solid–state fermentation
SmF	Submerged fermentation
Sec	Second
T	Temperature
TCM	Traditional Chinese medicine
TFA	Trifluoroacetic acid
TLC	Thin–layer chromatography
UHPLC–FLD	Ultra–high performance liquid chromatography with a fluorescence detector
UHPLC–DAD–QTOF–MS	Ultra–high performance liquid chromatography–diode array detector–quadrupole time of flight–mass spectrometry
U/g	Units per gram
U/mL	Units per millilitre
UHT	Ultra–High Temperature processing
USA	The United States of America
USD	United States dollar
USFDA	United States Food and Drug Administration
UV	Ultraviolet
v/v	Volume per volume
w/w	Weight in weight
x g	Earth's surface
XZK	Xuezhikang
YES	Yeast Extract Sucrose
Zn	Zinc
$ZnSO_4.7H_2O$	Zinc Sulphate heptahydrate
ZU	Size of fluorescence region around the colony on the upright side
ZR	Size of fluorescence zone on the reversed side

SCIENTIFIC PARTICIPATION DURING MY STUDY

- Organizing Committee Members of the NZMS Conference 2022.
- Reviewer for peer–reviewed international journals (22 times).

Publications

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Oral presentations

Farawahida, A. H., Palmer, J, & Flint, S. Potential to optimize pigment production to control citrinin levels during fermentation of red fermented rice. New Zealand Microbiology Society (NZMS) Conference, 20-23 November 2023, The University of Auckland, New Zealand.

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Three-minute pitch (3MP) presentations

Farawahida, A. H., Palmer, J, & Flint, S. Changes of citrinin, pigments, and pH during incubation of *Monascus* spp. on Coconut Cream Agar. The New Zealand Institute of Food Science & Technology Inc (NZIFST) Conference, 3-5 July 2023, University of Otago, Dunedin, New Zealand.

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Farawahida, A. H., Palmer, J, & Flint, S. Characterization of fungal growth of *Monascus* spp. isolates on Coconut Cream Agar and citrinin production. NZIFST Conference, 5-7 July 2022, Rotorua, New Zealand.

Farawahida, A. H., Palmer, J., & Manan, M. A., & Flint, S. Simple and rapid method to screen citrinin producers of *Monascus* spp. by using Coconut Cream Agar. 16th Congress of the Federation of Asian and Oceanian Biochemists and Molecular Biologists (FAOBMB) Online Conference, 22-25 November 2021, New Zealand.

Farawahida, A. H., Palmer, J., & Manan, M. A., & Flint, S. Simple and rapid method to screen slow and fast citrinin producer of *Monascus* spp. from red fermented rice by using Coconut Cream Agar. NZIFST Conference, 6-8 July 2021, Palmerston North, New Zealand.

Farawahida, A. H., Palmer, J., & Manan, M. A., & Flint, S. Coconut Cream Agar as a rapid and simple screening method for detection of citrinin producer *Monascus* spp. isolated from red fermented rice. NZMS Online Conference, 26-27 November 2020, New Zealand.

CHAPTER 1

INTRODUCTION

1.1. Research Background

Rice is an important staple food consumed by over half the world's population and crucial for global food security (Phillips et al., 2024), with Asians being the major rice producers, exporters, and consumers in the world (Muthayya et al., 2014; Pratoomchai, 2015). The quality of milled rice can be characterized based on the presence of broken kernels. The higher the percentage of broken rice, the lower the price of milled rice. Broken rice is defined as fragments of rice grains that break during any part of the milling process (Phillips et al., 2024). Whole rice and broken rice are used as sustainable raw materials through fermentation with *Monascus* spp. to produce value-added products (Pratoomchai, 2015). In Western countries, milk, malt, and fruit juice are used for fermentation. In contrast, rice and beans are popular sources to produce fermented products in Eastern and Southeastern Asia. Fungi are widely used in the fermentation process as the humidities and high temperatures of these countries are suitable for the growth of fungi (Fukami et al., 2021).

Monascus spp. are edible filamentous fungi used as a fungal starter to produce red fermented rice (RFR) (Chen et al., 2015). *Monasccus* was named and classified in 1884 by van Tieghem (Vendruscolo et al., 2016). There are more than twenty *Monascus* spp. reported in the literature, but only nine species are internationally recognized as *Monascus* spp. These are *M. purpureus, M. pilosus, M. ruber, M. floridanus, M. pallens, M. lunisporas, M. argentinensis, M. sanguineus,* and *M. eremophilus* (Abdul-Manan et al., 2017b; Chen & Zhang, 2019; Chen et al., 2015; Dai et al., 2021; Samsudin & Abdullah,

2014; Shao et al., 2014; Vendruscolo et al., 2016). Among these species, *M. purpureus*, *M. ruber*, and *M. pilosus* are the most common species in the food industry (Chen & Zhang, 2019; Martínková & Patáková, 1999; Pan & Hsu, 2014; Vendruscolo et al., 2016).

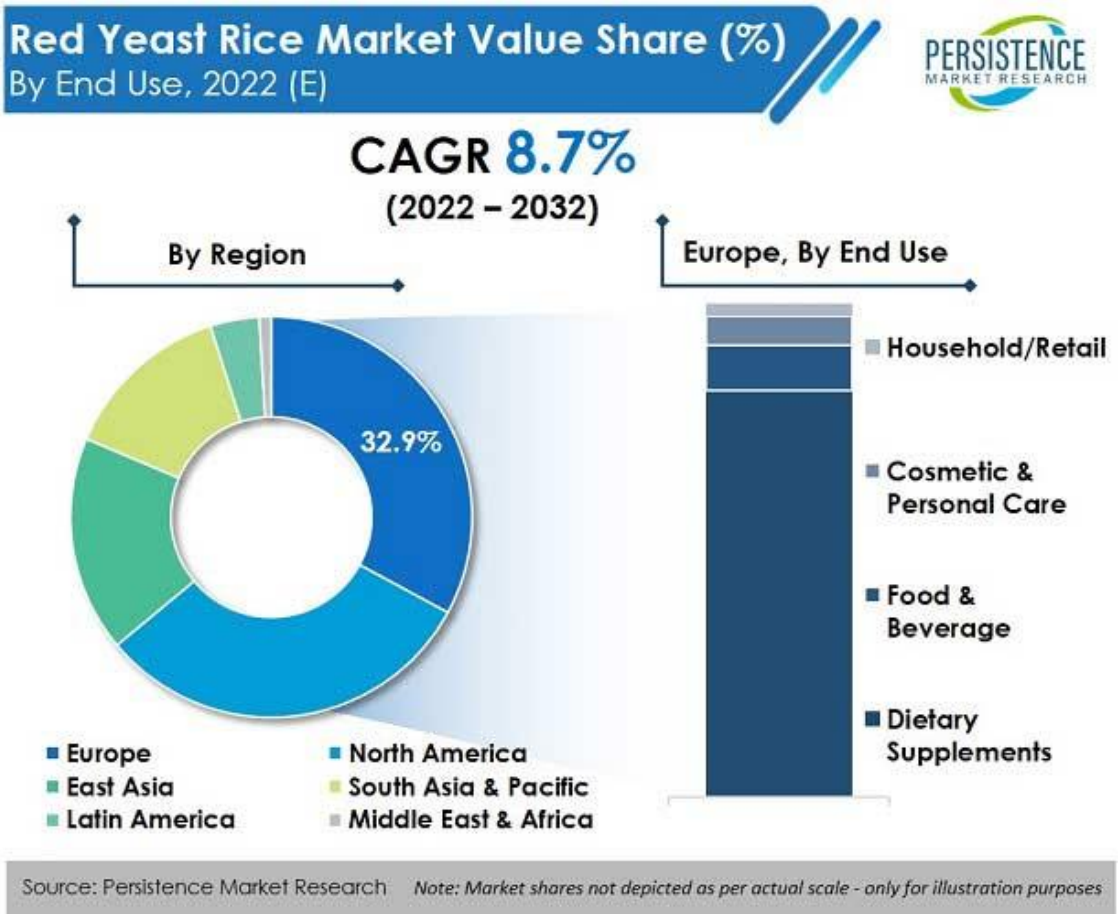
The most common polyketide secondary metabolites produced by *Monascus* spp. are citrinin (CIT), pigments, and monacolin K or lovastatin (Chai et al., 2020). CIT is a mycotoxin, mainly due to its nephrotoxic activity (Touhami et al., 2018). CIT is believed to have adverse effects on human health and animals such as immunosuppression, carcinogenic activity, and could damage the liver cells and kidneys (Blanc et al., 1995b; Jia et al., 2017; Wyatt, 1977; Zhang et al., 2021). *Monascus* spp. can produce yellow, orange, and red pigments. These pigments are categorized as azaphilones and have similar chemical properties and molecular structures (Vendruscolo et al., 2016). Meanwhile, monacolin K is recognised as cholesterol–lowering agents that inhibit HMG–CoA reductase (Aniya et al., 1999; Fukami et al., 2021; Jirasatid et al., 2019; Zhu et al., 2019) since 1979 (Endo, 1979). Monacolin K has some health benefits such as lowering cholesterol, blood pressure, and radical scavenging action to protect liver injury (Aniya et al., 1999; Lee et al., 2010a). Endo (1979) reported that monacolin K reduced plasma cholesterol levels in the New Zealand White Rabbits in the range of 10.1% to 26.2 %.

There are other common names for RFR such as red yeast rice, red mold rice, red rice, red koji, Hongqu, and angkak (Chen & Hu, 2005; Fukami et al., 2021; Sulandari et al., 2021). RFR is available in the market as a consumable food or a source of food supplements (Figure 1.1).

Figure 1. 1. Commercial red fermented rice available in the market as (a) consumable food and (b) food supplements.

RFR has been used as a traditional Chinese medicine (TCM), food supplement, and food additive (Yang et al., 2015b). In Western countries, RFR has been used as a food supplement due to the presence of monacolin K/lovastatin (Le Bloc'h et al., 2015). The product is suitable to be used as a natural colourant for medicine and foods. For example, RFR has been used as a natural food colour, flavour, and preservative in spices, fermented bean curd, ham, sausage, meat, pork stew, Chinese cheese, and fish products such as crab–flavoured–fish cakes (Fukami et al., 2021; Ristiarini et al., 2017; Song et al., 2019; Sulandari et al., 2021; Yang et al., 2015b). RFR is also used as a fermentation starter to produce vinegar in Japan, China and other countries in East and Southeast Asia (Yang et al., 2015b). In Malaysia, the Malaysian Chinese community consumes RFR daily or weekly as porridge, TCM, to remove meaty odour, and to obtain a unique aroma and taste. RFR can be used to extract a natural red colour by sprinkling RFR on vegetables and meat. Those practices are similar to those in China, Taiwan, and Korea (Samsudin & Abdullah, 2014).

RFR and other *Monascus*–fermented products are consumed by more than a billion people worldwide, especially in Asian countries such as China, Japan, Taiwan, Korea, Thailand, and other countries (Li et al., 2012b; Patel, 2016; Yang et al., 2015b). RFR is available in traditional Chinese shops including in small towns, proving that this product has a high demand. Samsudin and Abdullah (2014) reported that Malaysian Chinese shops sold 25–50 kg of RFR per month, with the price being five times more expensive than white rice due to the method of preparation to produce RFR and RFR being an imported product. According to Persistence Market Research, the global sales of RFR were expected to reach US$ 879.7 by 2032, growing at a compound annual growth rate (CAGR) of 8.7 % from 2020–2032 (Figure 1.2).



CAGR: compound annual growth rate.

Figure 1. 2. Global market value for red fermented rice.

Source: Persistence-Market-Research (2022).

1.2. Problem statements

Several authors have reported mycotoxin contamination including CIT in RFR and RFR products in various countries such as China, Taiwan, USA, Malaysia, and Indonesia (Avula et al., 2014; Liao et al., 2014; Marley et al., 2016; Ristiarini et al., 2017; Samsudin & Abdullah, 2013).

Samsudin and Abdullah (2013) found that *Monascus* spp. in 50 RFR samples, confirming that *Monascus* spp. has been used as a starter fungus to produce RFR. *Monascus* spp. identified in this study were *M. purpureus* and *M. pilous.* However, the study reported that there was a serious contamination of mycotoxigenic fungi and

mycotoxins in the RFR from Selangor, Malaysia. Out of 50 samples, 62 %, 54 %, and 44 % of the samples were contaminated with *Penicillium chrysogenum*, *Aspergillus niger*, and *A. flavus*, respectively. The studies found that 100 % of RFR were contaminated with CIT (230–20,649 µg/kg), 92 % with aflatoxin (AF) (0.61–77 µg/kg), and 100% with Ochratoxin A (OTA) (0.23–2.48 µg/kg). The maximum allowable limits of CIT are 50, 100, 200, and 2000 µg/kg in Taiwan, China, Europe, and Japan, respectively (EU, 2019; Kamle et al., 2022; Taiwan-FDA, 2020; Urraca et al., 2016). Meanwhile, 5 and 10 µg/kg are the maximum permitted levels of total AF in rice before human consumption in Malaysia and Europe, respectively (EU, 2010; Food-Regulation, 1985). There is no maximum limit of OTA established specifically for rice. Improper production, handling, transportation, and storage practices might contribute to the levels of contamination observed (Samsudin, 2011). Therefore, it is important to investigate the *Monascus* spp. contamination and control the CIT production during the fermentation of RFR.

Coconut Cream Agar (CCA) has been used to detect mycotoxins (Dyer & McCammon, 1994; Heenan et al., 1998). Mohamed et al. (2013) used CCA to determine the mycotoxin production by the fungi isolated from smoked and dried tuna fish. A further study by a thin–layer chromatography (TLC) confirmed that fluorescence was a CIT produced by *P. citrinum.*

It is important to determine the toxicity of CIT in animals. Lee et al. (2010a) studied oral administration of RFR diets containing different levels of CIT in male Wistar rats for 90 days. The results showed that there was no toxic effects as determined by food consumption, body weight, organ weight, and histopathology in the 90–d Wistar rats, even though the highest CIT level in RFR for the diet was 200 ppm. No cytotoxicity was observed on hepatoblastoma cell line (HepG2) and human embryonic kidney cell line

(HEK293) after treating the Wistar rats with 20 µM (5 ppm) CIT after 72 h. Lee et al. (2010a) recommended 2 ppm as the safe level of CIT in *Monascus*–fermented products.

Another study was conducted to evaluate acute and chronic toxicity, and genetic toxicity of RFR extract, Xuezhikang (XZK) (Gao et al., 2020). Interestingly, there was no acute toxicity even though the Sprague Dawley rats were treated with XZK at maximum doses of 10g/kg. Further study to determine chronic toxicity for 26–weeks showed that there were no differences in weight, food intake, organ weight, organ to weight ratio, microscopic and macroscopic examination of organs, urinalysis, and hematological parameters. Furthermore, there was no genotoxicity markers at any dose of XZK. Gao et al. (2020) suggested that 2000 mg/kg has no observed adverse effect level (NOAEL) for XZK, based on the 26–weeks toxicity study.

A comparative study to determine the toxicity of plant extracts by using mice and brine shrimp (*in vivo* and *in vitro*, respectively) showed that these methods have a good correlation (Parra et al., 2001). Brine shrimp assays have been used to determine the toxicity of medicinal plants, brown seaweed, and mycotoxins (Abbas et al., 2018; Alim et al., 2022; Nguyen et al., 2022). Therefore, brine shrimp assays can be used to determine the toxicity of CIT.

Monascus spp. produces many metabolites, but the relationship between these metabolites is still unclear. Salama et al. (2021) reported that red pigment extracted from *P. purpurogenum* reduced aflatoxin B1 (AFB_1), aflatoxin B2 (AFB_2), and OTA.

1.3. Hypotheses

1) CCA can be used as a simple and rapid screening method for the detection of *Monascus* spp. isolates that can produce CIT, and can be verified by using macroscopic and microscopic observation, deoxyribonucleic acid

(DNA) sequencing, and an Ultra–high performance liquid chromatography with a fluorescence detector (UHPLC–FLD).

2) Toxicity of CIT can be determined using a brine shrimp test.
3) There is a relationship between the growth, CIT levels, pigments, and pH of *M. purpureus* isolates on CCA. These parameters might have a similar trend when the *M. purpureus* isolates are grown on rice.
4) CIT levels can be reduced after prolonged incubation or fermentation days due to pigments production.
5) CIT levels can be controlled by optimizing pigments production during fermentation of RFR.

1.4. Objectives

The objectives of this research are:

1) To identify a simple and rapid method for screening CIT–producing *Monascus* spp. isolated from RFR.
2) To determine the toxicity of CIT using the mortality of brine shrimp bioassay.
3) To determine the fungal growth, CIT, pigments, and pH of *M. purpureus* on CCA for a prolonged incubation period.
4) To understand the relationship between pH, pigments, and CIT levels during fermentation of RFR.
5) To determine the relationship between *Monascus* pigments and CIT.

1.5. Overview of the thesis

This thesis comprises eight chapters (Figure 1.2). The current chapter (Chapter 1) is a general introduction to the study, introducing the research background, problem

statements, research questions, hypotheses, and objectives of this study. This chapter includes the thesis outline to illustrate the overview of this thesis. The relevant literature for this study has been compiled in Chapter 2, and this review has been published in the International Journal of Food Microbiology (Appendices A and B). In Chapter 3, a simple and rapid screening method was developed using CCA to identify CIT producers of *Monascus* spp. isolated from commercial RFR. The reliability of this screening method has been verified by morphology (macroscopic and microscopic observation), DNA sequencing, and UHPLC–FLD. This chapter has been published in the Journal of Microbiological Methods (Appendices C and D).

There are few studies to determine the toxicity of CIT by using simple methods. Therefore, a toxicity test was conducted and discussed in Chapter 4 using brine shrimp to determine if CIT is toxic to animals. There is an unclear relationship between secondary metabolites produced by *M. purpureus*, therefore a study was conducted to determine the relationship between *M. purpureus* growth, CIT, pigments, and pH on CCA. The findings from this study are detailed in Chapter 5. It has been questioned whether these parameters might have a similar trend when the *M. purpureus* isolates were grown on rice. Therefore, another study was conducted monitoring the growth of *M. purpureus* on rice, and the findings were reported in Chapter 6.

In Chapters 5 and 6, there was a reduction of CIT levels on CCA and rice after certain days of incubation and fermentation, respectively. The reason for CIT reduction might be due to the production of pigments. Chapter 7 supports this idea by using five different pigments to determine their contribution to CIT reduction. Chapter 8 is the summary of this study including the conclusions, limitations, and recommendations for future research.

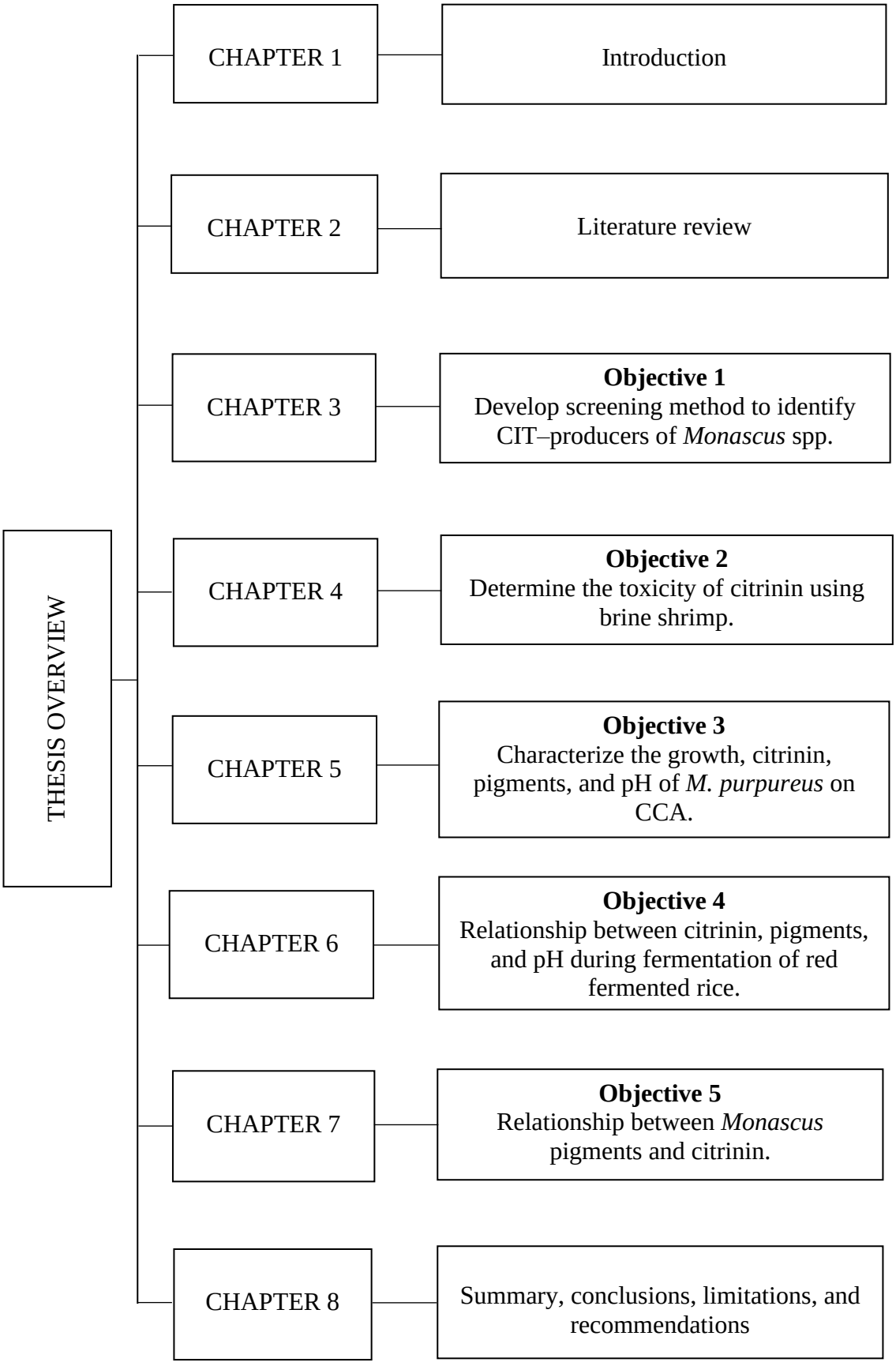


Figure 1. 3. Overview of the thesis.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

RFR is a fermented product consumed in East Asia for centuries and is particularly popular in Chinese dishes. RFR is also known as red rice, red rice mold, *Monascus*–fermented rice, red leaven, zhitai, Chiqu, Fuqu, hong–qu, angkak, and hung–chu among the Chinese while the Japanese call the product red koji, beni–koji and akakoji (Doughari, 2015; Erdoğrul & Azirak, 2004; Pratoomchai, 2015; Song et al., 2019; Sulandari et al., 2021). Other names for RFR are rotscimmelreid (Europe), hong–gug (Korea), Hon–Ci, Dan Qu, anka, ang–kak, angkhak, Ang–Khan, Ankak rice, angquac, Anka–Koji, red Chinese rice, red mould rice, red yeast rice, and red yeast grain (Chiu et al., 2006; Doughari, 2015; Fukami et al., 2021; Patcharee et al., 2007; Poorniammal et al., 2021; Pratoomchai, 2015; Ristiarini et al., 2017; Yuan et al., 2023). RFR is widely used as a therapy for hyperlipemia. RFR is available in the market in dried and powder forms (Abdul-Manan et al., 2017b). RFR has been used to give colour and flavour, and act as a preservative in East Asian foods and cuisine, especially in China, Korea, and Japan. RFR is also consumed as a TCM (Doughari, 2015; Nguyen et al., 2017) and dietary supplement in Western countries (Zhu et al., 2019). Table 2.1 summarizes the benefits of RFR to improve the quality of human health.

Table 2. 1. The benefits of red fermented rice.

Benefits	References
Improve the quality of eggs for human consumption: (a) Decreasing the level of serum and egg yolk cholesterol (b) Enhance the egg quality in laying hens without disablement of laying production	(Pengnoi et al., 2018; Sun et al., 2015; Zhu et al., 2019)
Anticarcinogenic compounds against liver, colon, breast, and prostate cancer	(Chiu et al., 2013; Klingelhöfer & Morlock, 2019)
Inhibitors of cholesterol biosynthesis	(Man et al., 2002)
Lipid and cholesterol–lowering properties	(Becker et al., 2009; Bogsrud et al., 2010; Heber et al., 1999; Ma et al., 2009; Wei et al., 2003; Yang et al., 2021; Zhou et al., 2019)
Prevent the build–up of fats, cholesterol, and other substances in and on the artery walls	(Lin et al., 2008; Lin et al., 2011; Liu et al., 2017; Shen et al., 2017; Wu et al., 2017)
Neurocytoprotective activity	(Lee et al., 2009; Lee et al., 2007b; Lee et al., 2010c; Lin et al., 2015)
Preventing osteoporosis	(Cho et al., 2010; Gutierrez et al., 2006; Wang et al., 2015; Wong & Rabie, 2008)
Anti–obesity activity	(Chen et al., 2008)
Anti–fatigue effects	(Wang et al., 2006; Xue et al., 2017)
Anti–diabetic benefits	(Chen & Liu, 2006; Chen et al., 2008)
Regulatory effects on the immune system	(Patakova, 2013)
Anti–microbial activities	(Ferdeş et al., 2009; Milanda et al., 2021)
Anti–inflammatory effects	(Ding et al., 2014; Hsu et al., 2010)
Anti–hypertensive benefits	(Wang et al., 2010)
Anti–cancer activity	(Hong et al., 2011; Hsu et al., 2010; Lee et al., 2013; Lin et al., 2007; Xue et al., 2017)
Prevent damage to the liver	(Cheng & Pan, 2011; Hong et al., 2007; Lee et al., 2012)

During fermentation of the RFR, secondary metabolites such as pigments, lovastatin/monacolin K, polysaccharide, ɣ–aminobutyric acid (GABA), ergosterol, dimerumic acid, and CIT are produced (Srianta et al., 2014). Monacolin K, also known as lovastatin in lactone form (Younes et al., 2018), is a natural statin, acting as an inhibitor of the enzyme 3–hydroxy–3–methyl–glutaryl coenzyme A reductase (HMG–CoA reductase) that prevents the formation of mevalonate from HMG–CoA during cholesterol biosynthesis (Suraiya et al., 2018). The United States Food and Drug Administration

(USFDA) has approved the use of statins to treat hyperlipidaemia (abnormally high level of fats/lipids in the blood including cholesterol and triglycerides) (Gregory et al., 2012). Statins can be used either as a single–ingredient such as Lipitor (atorvastatin), Lescol (fluvastatin), Mevacor (lovastatin), Altoprev (lovastatin extended–release), Livalo (pitavastatin), Pravachol (pravastatin), Crestor (rosuvastatin), and Zocor (simvastatin); or in combination with other products including Advicor (lovastatin/niacin extended–release), Simcor (simvastatin/niacin extended–release), and Vytorin (simvastatin/ezetimibe) (USFDA, 2016). The average amount for the prescription of lovastatin (Monacolin K) is 10–80 mg/day (Gregory et al., 2012). However, consumption of RFR containing CIT could lead to adverse effects on the liver, and musculoskeletal system including a breakdown of muscle tissue. This muscle tissue breakdown releases a damaging protein (myoglobin) into the blood and can damage the kidneys (Younes et al., 2018). Consequently, the European Food Safety Authority (EFSA) Panel cannot recommend a safe dietary intake of monacolins from RFR (Younes et al., 2018). CIT, a mycotoxin of food safety concern, can be produced by some of *Monascus* spp. such as *M. purpureus* during fermentation of RFR (Silva et al., 2021).

This literature will focus on *Monascus* spp. as the starter fungi for RFR, secondary metabolites produced during the fermentation of RFR including beneficial compounds such as pigments and monacolin K (Table 2.1), methods used including different ways to ferment RFR, factors affecting the growth, pigments production by *Monascus* spp. and CIT production during fermentation of RFR, and the occurrence, detection and reduction of CIT in RFR. This study will help the food industries, researchers, and consumers to understand food safety risks associated with the consumption of RFR, and the possibility of producing RFR with low or no CIT.

2.2. Methods used to ferment RFR

There are variations in the methods used for the fermentation of rice to produce RFR, but in general the processes are the same. All rice types can be used as a substrate for the RFR, but non–glutinous rice is preferable to avoid the agglomeration of rice during the fermentation process (Wen et al., 2020). A variety of substrates and nutritional broths are used in solid–state fermentation (SSF) or liquid/submerged fermentation (SmF) respectively, for improving RFR quality in terms of yield, pigments, and monacolin K. Most of the SSF processes require several weeks to produce RFR (Chairote et al., 2009; Chen & Hu, 2005; Dogra & Kumar, 2017; Li et al., 2003; Patcharee et al., 2007). The type of inoculum, inoculum concentration and size (the ratio of the inoculum to the substrate, v/w) are important to produce RFR. Selection of *Monascus* spp. isolates, medium formulation and optimization of fermentation conditions help to improve RFR (and other *Monascus* fermented products), enhancing the pigments and monacolin K production, and possibly novel unknown compounds that might be produced during the fermentation of RFR. Comprehensive research and development are needed to identify additional bioactive compounds. Table 2.2 shows the methods that have been used in the literature to ferment RFR.

Table 2. 2. Methods used to ferment red fermented rice.

Country	Fermentation type	Type of rice	Culture media	Inoculum	Amount of inoculum	Red fermented rice process										Reference
						Soaking time	Steaming time	Cooling time	Weight of steamed rice	Sterilization condition	Cooling temperature	Incubation temperature	Incubation time	Drying information	Final product	
India	SSF	Basmathi white rice	Rose Bengal Agar (30 °C, 7–8 days)	Pre–culture *M. purpureus* (1 week old)	5 %	2 h	20 min	Yes (NM)	20 g	15 psi, 121 °C, 15 min	NA	30 °C	2–3 weeks	65 °C, 2 h (Oven)	Dried RFR	(Dogra & Kumar, 2017)
Thailand	SSF	Polished rice	PDA (30 °C, 10 days)	*M. purpureus/ M. ruber*	NA	NA	NA	Yes (RT)	NA	121 °C, 15 min	RT	RT	14 days	55 °C, 3 days (NM)	Powder RFR	(Patcharee et al., 2007)
Thailand	SSF	Non–glutinous rice and glutinous rice	NA	*M. purpureus* (1 week old)	NA	6 h	20 min	Yes (NM)	50 g	15 psi, 121 °C, 15 min	NA	30 °C	2–3 weeks	65 °C, 6 h (Oven)	-Dried RFR -Dried RFR + [a]1 mL of 0.25 g/mL soybean milk solution	(Chairote et al., 2009)
China	SSF	NM	[b]GBP medium slant	*Monascus*	5 ml spore solution (1 x 10^6/mL)	2 h (Soaked in water at 30 °C)	100 g rice, 1 g wheat, 70 mL water/ 50 mL water + 20 ml [c]nutritional broth were mixed well			121 °C, 30 min	NA	30 °C	Several days	50 °C (until constant weight)	Dried RFR	(Chen & Hu, 2005)
China	SSF (Industrial, using Modified Nagata type koji maker)	Indica rice	NA	*M. purpureus*	0.7–4 %	0–25 min	Yes (100 °C, 30 min)	From 100 °C to 30 °C (5 min)	NA	NA	NA	SSF: 37–38 °C 86th hour until the end of fermentation: 26, 30, 34 °C		NA	Dried RFR	(Chiu et al., 2006)
China	SSF (Traditional)	NM	NA	*Monascus* koji	NA	6-8 hours	Yes (NM)	Yes (40 °C)	NA	1st day: Inoculated with *Monascus* koji (33–35 °C) 2nd day: Stirring and mixing of koji (34 °C) 3rd day: 1st water soaking of koji for 30 min (moisture: 50 %) 4th day: 2nd water soaking of koji (moisture: 47 %) 5th day: last time water soaking of koji (moisture: 48 %) 6th day: Post maturing, stirring every 10 h (30 °C)				45 °C, 22 h	Dried RFR	(Chiu et al., 2006)
China	SSF (Traditional)	Long–shaped non–glutinous rice	NA	*Monascus* starter culture	NA	1 day	Yes (steamed in [d]ZENG with vapour)	NA	NA	NA	NA	25–28 °C	2–4 weeks	NA	Powder RFR	(Chen et al., 2015)

China	SSF	Long–grained non–glutinous rice	NA	SJS1–SJS35	NA	24 h (soaked in acidified water (pH 4.5)	60 g of soaked rice were assigned to a 500 mL flask. After autoclaved at 121 °C for 20 min, 6 mL seed cultures[e] were inoculated and incubated. Triplicate flasks were inoculated with each strain.	121 °C, 20 min	NA	30 °C, 3 weeks ([f]RH 70 %) 1–3 days: All flasks were left standing and hereafter shaken daily to reduce clumping. To maintain MC, autoclave water (pH 4.5) was added during the later stage of incubation		NA	Dried RFR	(Li et al., 2003)
China	SmF	NA	NA	*Monascus* single–spore culture (10–days old)	NA	300 mL flasks containing 50 mL of submerged culture (g/L): Glucose (20), monosodium glutamate (5), KH_2PO_4 (5), K_2HPO_4 (5), MgSO4.7H_2O (0.5), CaCl2 (0.1), $FeSO_4.7H_2O$ (0.01), $ZnSO_4.7H_2O$ (0.01) and $MnSO_4.H_2O$ (0.03). Triplicate flasks were inoculated with each isolate	121 °C, 20 min	NA	30 °C	3 weeks	NA	SmF broth	(Li et al., 2003)

NM: not mentioned. NA: not applicable. RT: room temperature. MC: moisture content.

[a] To study the effect of adding nitrogen-containing nutrients.

[b] GBP medium slant: 10 g glucose, 5 g beef extract, 10 g protease peptone, 5 g NaCl, 15 g agar, 1000 mL distilled water, pH 5.0, sterilized for 30 min at 121 °C.

[c] Nutritional broth: 40 g glucose, 4 g sodium glutamate 3 g protease peptone, 3 g NH_4NO_3, 1000 mL distilled water.

[d] ZENG: a kind of wooden rice steamer.

[e] Seed cultures: Transferring a small piece of 10-day-old *Monascus* single-spore culture from an MEA slant into a 500 mL flask containing 50 mL autoclaved yeast extract. sucrose medium (by mixing 10 g yeast extract and 100 g sucrose in 1 L distilled water). Cultures were incubated in the shaker at 30 °C for 3 days.

[f] RH: Relative humidity.

2.3. Production of RFR

RFR can be produced by traditional (Figures 2.1 and 2.2) and industrial methods. In the traditional process (Figure 2.1) (Chiu et al., 2006), cooked rice is inoculated with *Monascus* spp., and put in a round shallow bamboo tray (about 5–6 cm in depth) to control aeration and temperature. The trays are stored on shelves in a fermentation room at 33–35 °C. Hands are used to turn over the rice koji (rice inoculated with *Monascus* spp. culture) to remove the heat of fermentation (the frequency unknown and probably varies). To maintain the appropriate moisture content of the koji, each tray is taken out at least three times from the room and soaked in water during the fermentation process. Some of the traditional methods to ferment RFR involve hand stirring and water soaking for several days (Figure 2.1). Another method that does not involve hand stirring and water soaking, adjusts the moisture content and temperature of incubation of RFR in a temperature–controlled (the temperature was not mentioned) chamber with regularly stirring and moistening (Figure 2.2) (Chen et al., 2015).

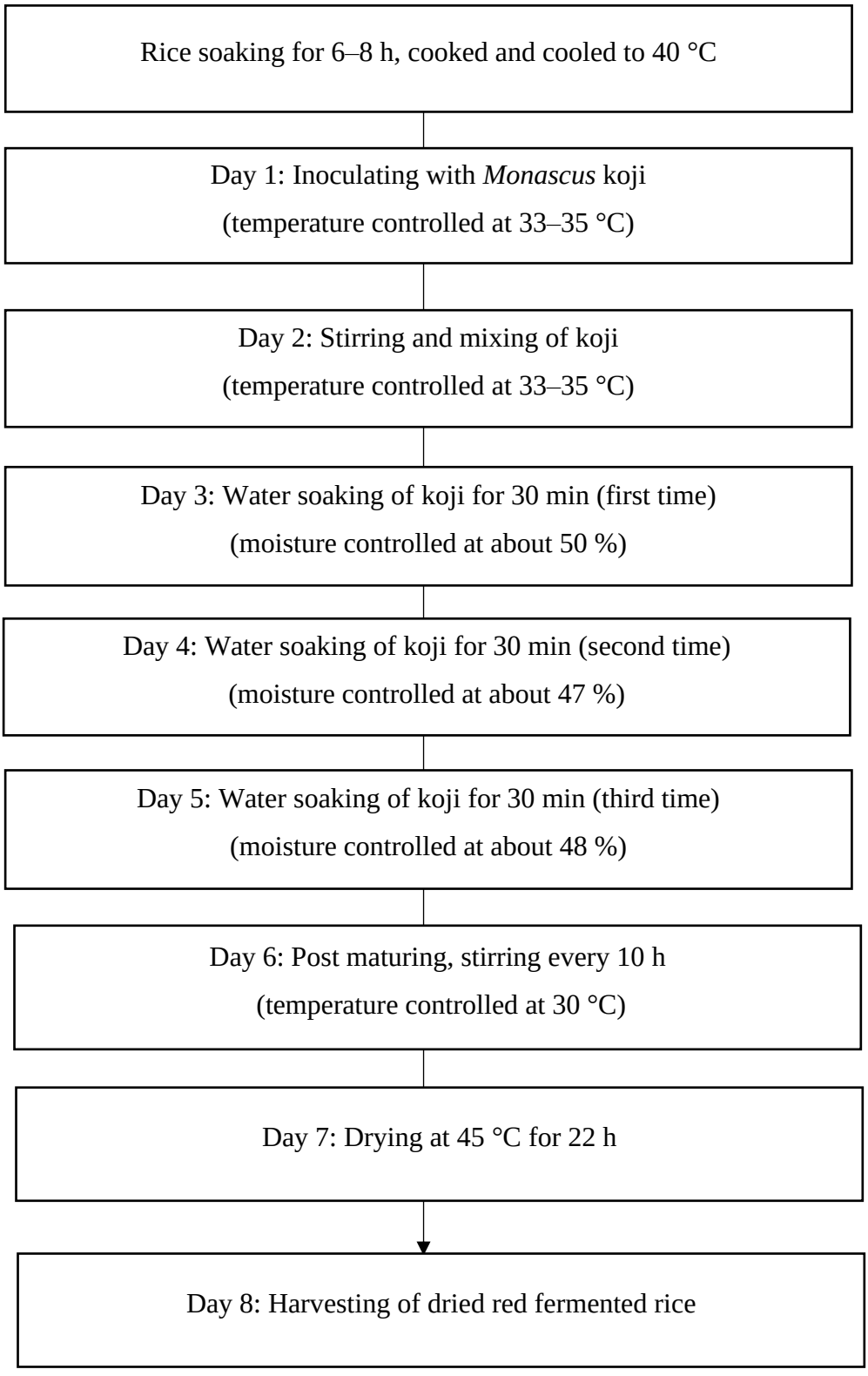


Figure 2. 1. Red fermented rice production by the traditional process.

Source: Chiu et al. (2006).

Figure 2. 2. The traditional process of red fermented rice production.

Source: Chen et al. (2015).

During fermentation, the rice may be contaminated by the environment and hand-turning. High labour costs are involved in stirring and water soaking. Fermentation in a closed environment (anaerobic) produces an unsatisfactory product (Chiu et al., 2006) with a very low monacolin K level (9 µg/g) compared to aerobic fermentation (157 µg/g) under the same fermentation conditions (Tsukahara et al., 2009).

There are two different ways of producing RFR, either by SSF or SmF. Both methods are used in commercial production (Fukami et al., 2021). SSF involves the addition of starter fungi into the solid medium (rice) whereas SmF provides nutrients for fungal growth in a liquid medium. For both fermentations, the temperature is very important as it affects microbial growth, spore formation and germination, and pigments and monacolin K production (Darwesh et al., 2020; Tsukahara et al., 2009). A low moisture content (20–70 %) is needed for fungal growth, whereas bacteria need a moisture content higher than 70 % (Babitha et al., 2007). At high initial moisture content, the rice will agglomerate and this limits the supply of oxygen for fungal growth, resulting in low pigments production (Gautam, 2002). A low moisture level will reduce the risk of contamination with bacteria and yeast, resulting in high productivity during SSF (Kraboun et al., 2019).

SSF also provides ideal conditions for fungal hyphae to grow on the surface of the rice and penetrate the rice substrate generating high pigments production. One of the advantages of SSF is the low cost of production because a variety of carbon sources such as jackfruit seed powder, sesame oil cake, coconut oil cake, wheat bran, palm kernel cake, grape waste, rice bran, cassava powder, spent brewing grain, and tamarind seed powder can be used as substrates (Babitha et al., 2007). Zhang et al. (2013) showed that the type of fermentation (SSF or SmF) influenced the production of CIT

and pigments, with SSF producing 1000 times lower CIT than SmF (0.018 ug/g and 19.02 ug/g respectively).

Fungi in SmF culture are often grown in pellets, which are the form of compact spherical masses of mycelium (Pirt, 1966). There are two types of fungal pellet formations, which are coagulative and non–coagulative (Veiter et al., 2018). In the coagulative agglomeration type, spores agglomerate fast and subsequently germinate involving hyphal tip growth (Zhang & Zhang, 2016). Finally, a great number of spores of the coagulative type form pellets. Meanwhile, for the non–coagulative agglomeration type, spores germinate to hyphae. Branched hyphal elements subsequently agglomerate to form a pellet. In theory, one spore can form a pellet (Veiter et al., 2018). Fungi in SmF can also grow in the filamentous form, featuring homogeneously dispersed hyphae (Veiter et al., 2018).

The drawback of SmF is that the growth of fungi during the fermentation results in an increase in the viscosity of the broth. After 8–9 days of fermentation, pigments productivity decreases due to the lack of oxygen (Agboyibor et al., 2018). The formation of pellets might be disturbed due to the high shear force at high agitation speed (Wang et al., 2014), leading to low pigments concentration and high CIT. Non-coagulative pellet formation is connected with agitation and aeration (Pazouki & Panda, 2000).

Yang et al. (2015a) studied the effect of oxygen supply on the pigments and CIT production by *M. ruber* HS.4000 in a shake-flask and fermenter. High agitation speeds lead to a high oxygen supply. The results showed that an agitation speed of 300 rpm produced lower CIT compared to 600 rpm. In contrast to earlier findings by Pereira et al. (2008), the optimum agitation speed was 600 rpm with a dissolved oxygen concentration of 60 %. Yang et al. (2015a) suggested ending the fermentation

once the optimum pigments concentration was produced before most of the CIT is produced.

The high viscosity of the broth was observed when *Monascus* sp. J101 was fermented at 30 °C, resulting in poor oxygen transfer and low pigments yield. Lower viscosity was achieved when the culture was incubated at 25 °C due to a reduced fungal growth rate, resulting in 10 times higher pigments yield than when incubated at 30 °C (Ahn et al., 2006). Low RFR yields occur when *Monascus* mycelia readily attach to the stirring paddle and fermentation tank wall (Srianta et al., 2014). Another problem with SmF is low pigments production. Due to the presence of free water in SmF, the pigments are not associated with the fungal cells (intracellular) but in the surrounding medium (Hamano et al., 2005). Intracellular pigments are insoluble in water, while extracellular pigments are water-soluble and can be affected by the nitrogen source and pH of the liquid medium. The intracellular pigments accumulate mainly in the mycelium and is retained within the fungal cells resulting in a lower pigments yield (Hamano et al., 2005). In contrast, Mukherjee and Singh (2011) stated that high pigments yield can be achieved by SmF, and the pigments yield is affected by fermentation conditions such as pH, medium composition and agitation (Hamdi et al., 1996; Mukherjee & Singh, 2011). In the food industry, the production of RFR by SmF remains confidential to many companies. Therefore, there is limited technical information published in the literature (Srianta et al., 2014).

SmF has many advantages over SSF such as a smaller surface area, less labour, and a short fermentation time. Less labour also reduces the chance of contamination (Agboyibor et al., 2018; Srianta et al., 2014). Therefore, the purification of RFR products from SmF is easier compared to SSF (Subramaniyam & Vimala, 2012). It is

also easier to control the production of secondary metabolites of RFR in SmF such as pigments, CIT, and monacolin K (Patakova, 2013).

2.4. Fungal growth identified in RFR

RFR is usually produced by traditional methods in most Asian countries. However, there is usually no monitoring procedure during production, storage and transportation to ensure the safety of RFR (Samsudin & Abdullah, 2013). Fungal contamination and growth by genera such as *Aspergillus, Fusarium* and *Penicillium* may result in mycotoxins such as CIT, AF, and OTA (Pisareva et al., 2005; Samsudin & Abdullah, 2013). Most of the fungal growth in RFR is from *Monascus* spp. (as a fungal starter), but RFR can be contaminated with other species such as *Aspergillus* spp. and *Penicillium* spp. Table 2.3 shows the fungi that have been isolated from RFR.

Table 2. 3. Fungal isolates from red fermented rice.

Fungal isolates	References
M. purpureus	(Abdul-Manan et al., 2017b; Chen et al., 2015; Samsudin & Abdullah, 2014)
M. pilosus	(Abdul-Manan et al., 2017b; Chen et al., 2015; Dai et al., 2021; Samsudin & Abdullah, 2014)
M. ruber	(Abdul-Manan et al., 2017b; Chen et al., 2015; Samsudin & Abdullah, 2014)
M. floridanus	(Barnard & Cannon, 1987; Chen et al., 2015)
M. pallens	(Abdul-Manan et al., 2017b; Chen et al., 2015; Shao et al., 2014)
M. lunisporas	(Abdul-Manan et al., 2017b; Chen et al., 2015; Shao et al., 2014)
M. argentinensis	(Abdul-Manan et al., 2017b; Chen et al., 2015; Shao et al., 2014)
M. sanguineus	(Abdul-Manan et al., 2017b; Chen et al., 2015; Shao et al., 2014)
M. eremophilus	(Abdul-Manan et al., 2017b; Chen et al., 2015; Shao et al., 2014)
P. chrysogenum	(Samsudin & Abdullah, 2013)
A. niger	(Samsudin & Abdullah, 2013)
A. flavus	(Samsudin & Abdullah, 2013)

2.5. Occurrence of mycotoxins in RFR and RFR products

Table 2.4 shows the occurrence of mycotoxins in RFR in different countries, especially in Asia. Samsudin and Abdullah (2013) found that RFR is often contaminated with CIT and other mycotoxins, such as AF and OTA. Meanwhile, other researchers only found CIT in RFR. This may be because RFR was stored in different storage conditions: (1) in wooden drawers without any packaging and stored together with other herbs, (2) in packaging, (3) in the refrigerator, (4) at room temperature, (5) in an air-conditioning system, and (6) in open-air (Samsudin & Abdullah, 2013). The study found that RFR was contaminated with *A. flavu*s and *A. niger*, which are AFs and OTA producers, respectively (Samsudin & Abdullah, 2013).

Avula et al. (2014) found that there was no detectable CIT in authentic RFR samples (obtained from Beijing Peking University WBL Biotech Co., Ltd., China) and dietary supplements (labelled as 600 or 1200 mg of RFR was purchased online from supplement retailers in the USA), but 19 % of commercial RFR were contaminated with CIT (10,000–80,000 µg/kg). This was thought to be due to variations in storage conditions in the RFR shops that may affect fungal contamination of RFR, leading to CIT production (Samsudin & Abdullah, 2013).

Most of the studies on RFR focus on the occurrence of CIT in the final products. However, other studies have determined CIT, pigments or monacolin K during fermentation of RFR (Table 2.5). Li et al. (2003) compared the CIT level and pigments of RFR produced from SSF and SmF. The results found that SSF produced RFR with higher CIT and pigments value compared to SmF. The researchers also mentioned that long-grained non-glutinous rice was the preferable medium for CIT production.

Table 2. 4. Occurrence of citrinin, aflatoxin, ochratoxin A, and monacolin K in red fermented rice and red fermented rice products.

Samples	Country	Methods of detection	Total of tested samples	Percentage of positive and mycotoxins level (μg/kg)			Monacolin K (mg/g)	References
				CIT	AF	OTA		
RFR[a]	China	[c]LC–FLD	9	<3–3200	NA	NA	NA	(Marley et al., 2016)
RFR	Malaysia	[d]ELISA	50	100 % (230–20,650)	92 % (0.61–77.33)	100 % (0.23–2.48)	NA	(Samsudin & Abdullah, 2013)
RFR (SSF)	China	[e]HPLC, [f]LC–MS	35	100 % (280–2,460,000)	NA	NA	NA	(Li et al., 2003)
RFR (SmF)	China	[e]HPLC, [f]LC–MS	30	86 % (90–56,000)	NA	NA	NA	(Li et al., 2003)
RFR (raw material)	Taiwan	[g]HPLC–FLD	84	84 % (400–93,500)	NA	NA	NA	(Liao et al., 2014)
RFR (supplements)	Taiwan	[g]HPLC–FLD	77	35 % (100–15,200)	NA	NA	NA	(Liao et al., 2014)
RFR (processed products)[b]	Taiwan	[g]HPLC–FLD	141	6 % (100–1300)	NA	NA	NA	(Liao et al., 2014)
RFR (raw material)	Taiwan	[h]LC–MS/MS, HPLC–FLD	33	64 % (1450–63,400)	NA	NA	NA	(Chen et al., 2016b)
RFR (dietary supplements)	Taiwan	[h]LC-MS/MS, HPLC–FLD	58	24 % (70–4900)	NA	NA	NA	(Chen et al., 2016b)
RFR (processed products)[a]	Taiwan	[h]LC–MS/MS, HPLC–FLD	115	18 % (70–1290)	NA	NA	NA	(Chen et al., 2016b)
RFR	China	[e]HPLC	10	50 % (2.13–11.97)	NA	NA	NA	(Xu et al., 2003)
RFR food additives	China	[g]HPLC–FLD	11	73 % (127–4960)	NA	NA	NA	(Li et al., 2012b)
RFR medicinal materials	China	[g]HPLC–FLD	19	100 % (18.2–5253)	NA	NA	NA	(Li et al., 2012b)

RFR functional food and medicine products	China	[g]HPLC–FLD	29	14 % (16.6–62.5)	NA	NA	NA	(Li et al., 2012b)
Authentic RFR samples	China	[i]UHPLC–DAD–QTOF–MS	3	0 %	NA	NA	100 % (1.97–2.33)	(Avula et al., 2014)
Commercial RFR materials	China	[i]UHPLC–DAD–QTOF–MS	31	19 % (10,000–80,000)	NA	NA	71 % (0.54–24.27)	(Avula et al., 2014)
RFR dietary supplements (600 or 1200 mg)	USA	[i]UHPLC–DAD–QTOF–MS	14	0 %	NA	NA	100 % (0.03–2.62)	(Avula et al., 2014)

RFR: red fermented rice. CIT: citrinin. AF: aflatoxin. OTA: ochratoxin A. NA: not applicable.
[a] Granules – sold loose, powder – sold loose and packet, capsules, tablets.
[b] Including RFR sauce, crackers, oatmeal, soy sauce and wine.
[c] LC–FLD: liquid–chromatography with fluorescence detector.
[d] ELISA: enzyme–linked immunosorbent assay.
[e] HPLC: high–performance liquid chromatography.
[f] LC–MS: liquid–chromatography coupled with mass spectrometry.
[g] HPLC–FLD: high–performance liquid chromatography with a fluorescence detector.
[h] LC–MS/MS: liquid–chromatography and tandem mass spectrometry.
[i] UHPLC–DAD–QTOF–MS: ultra–high performance liquid chromatography–diode array detector–quadrupole time of flight–mass spectrometry.

Table 2. 5. Citrinin, pigments or monacolin K production during the fermentation of red fermented rice by traditional and industrial methods.

Samples/ media	Country	Type of fermentation	Fermentation approach	Methods of detection	CIT	Pigments/ Monacolin K	References
1. YES medium (SmF) 2. Wet rice medium (SSF) 3. Synthetic medium	France	SSF and SmF	Laboratory scale	[a]UV spectrometer, spectrophotodensitometer, [b]NMR spectroscopy, [c]TLC, [d]HPLC, and mass spectrometer	1. [e]YES medium -*M. ruber*: 370 mg/L -*M. purpureus*: 240 mg/L 2. [f]Wet rice medium -*M. ruber*: 300 mg/kg dried fermented rice powder -*M. purpureus*: 100 mg/kg dried fermented rice powder 3. [g]Synthetic medium: No CIT produced	No pigments are produced in synthetic medium	(Blanc et al., 1995a)
RFR	Taiwan	SSF	Industrial (modified Nagata type koji maker)	NM	331 – 617 ppb	-Red pigments:0.75–0.91 [h]$OD_{500\ nm}$ -Monacolin K: 47–54 ppm	(Chiu et al., 2006)
Rice waste	Taiwan	SmF	Laboratory scale	[d]HPLC	From 30.36 ppb (control) reduced to 0.057 ppb (forecast optimal conditions) and 0.055	The best yield of pigments: - Yellow: 4.132 ppm - Red: 8.480 ppm - Orange: 4.573 ppm	(Chung et al., 2009)

					ppb (verification experiment).		
RFR	China	SSF and SmF	Laboratory scale (autoclaved at 121°C for 20 min)	HPLC	SSF: 0.28–2460 mg/kg SmF: 0.09–56 mg/L	Pigments: SSF: 26–1134 U/g SmF: 0.11–35.33 U/mL	(Li et al., 2003)
RFR	Korea, USA, Taiwan, China	SSF	Traditional (culturing *M. ruber* on steamed rice)	HPLC	0–11.97 µg/g (samples were cultured in the lab)	NA	(Xu et al., 2003)

RFR: red fermented rice. CIT: citrinin. SSF: solid–state fermentation. SmF: submerged fermentation. NM: not mention. NA: not applicable.
[a] UV spectrometer: ultraviolet spectrometer.
[b] NMR spectroscopy: nuclear magnetic resonance spectroscopy.
[c] TLC: thin–layer chromatography.
[d] HPLC: high–performance liquid chromatography.
[e] Yeast extract sucrose (YES) medium: composed of yeast extract (40 g) and sucrose 160 g/L of deionized tap water. This medium was incubated at 27 °C without agitation for 2 weeks.
[f] Wet rice medium (50% of water w/w): incubated at 27 °C for 2 weeks.
[g] Synthetic medium: composed of monosodium glutamate (MSG) (5g), di-potassium hydrogen orthophosphate (K_2HPO_4) (5 g), potassium dihydrogen phosphate (KH_2PO_4) (5 g), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (0.5 g), calcium chloride ($CaCl_2$) (0.5 g), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) (0.5 g), zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) (0.01 g), magnesium sulfate heptahydrate($MnSO_4.H_2O$) (0.03 g), and ethanol or glucose (20 g/L deionized tap water). The initial pH of the medium was adjusted to 6.5 with ammonium hydroxide. This medium was incubated with or without agitation at 27 °C until exhaustion of ethanol.
[h] OD: optical density.

2.6. Factors affecting growth and pigments production of *Monascus* spp.

Red mould species such as *Monascus* spp. are xerophilic fungi (Silbir & Goksungur, 2019) and are commonly used as fungal starters in RFR (Dogra & Kumar, 2017). RFR is produced by inoculating rice (preferably white rice) with a fungal starter consisting of one or more *Monascus* spp. There are nine species of *Monascus* spp. identified globally, these are *M. purpureus, M. pilosus, M. ruber, M. pallens, M. lunisporas, M. argentinensis, M. sanguineus, M. floridanus,* and *M. eremophilus* (Chen et al., 2015; Shao et al., 2014). However, the main fungal starters for RFR production are *M. purpureus*, *M. pilosus*, and *M. ruber* (Chen et al., 2015; Samsudin & Abdullah, 2014). The taxonomy of *Monascus* is kingdom *Fungi*, phylum *Ascomycota*, class *Ascomycetes*, order *Eurotiales*, family *Monascaceae*, and genus *Monascus* (Pan & Hsu, 2014). These fungal strains can secrete various secondary metabolites of polyketide structure. Polyketides are rich sources of pharmaceuticals, including antibiotics, anticancer drugs, cholesterol–lowering drugs, and immune suppressant (Chakravarti & Sahai, 2004; Jůzlová et al., 1996).

The growth of *M. purpureus* starts with whitish-coloured mycelium, followed by rich orange and then a clear rich red colour. The colour change happens because of the increase in acidity of the medium resulting in the production of red-orange hyphae. As the culture ages, *M. purpureus* will change to a deep crimson (Abdul-Manan et al., 2017b; Blanc et al., 1994). There are several factors affecting the growth and colour production (pigments) of *Monascus* spp. These include *Monascus* isolates, cultivation conditions, the substrate, nutrient supplement, inorganic nitrogen, organic nitrogen, amino acid, carbon source, pH, temperature, initial moisture content, inoculum size, sample size, air rate, respiration rate, and hyphal morphology (Chen & Johns, 1993; Doughari, 2015; Kraboun et al., 2019; Lee et al., 2002; Liu & Chen, 2019; Yin et al., 2022).

Monascus spp. can produce three types of pigments, which are yellow (monascin and ankaflavin), orange (rubropunctatin and monascorubrin), and red (rubropunctamine and monascorubramine) pigments (Figure 2.3). The maximum absorbance wavelengths for the yellow, orange and red pigments are 330–450 nm, 460–480 nm, and 490–530 nm, respectively (Liu & Chen, 2019). The maximum wavelengths of pigments produced by *Monascus* vary among species. For example, 370 nm for *M. kaoliang*, 420 nm for *M. anka*, and 500 nm for *M. purpureus* and *M. barkeri* (Kraboun et al., 2019). These pigments have benefited human health.

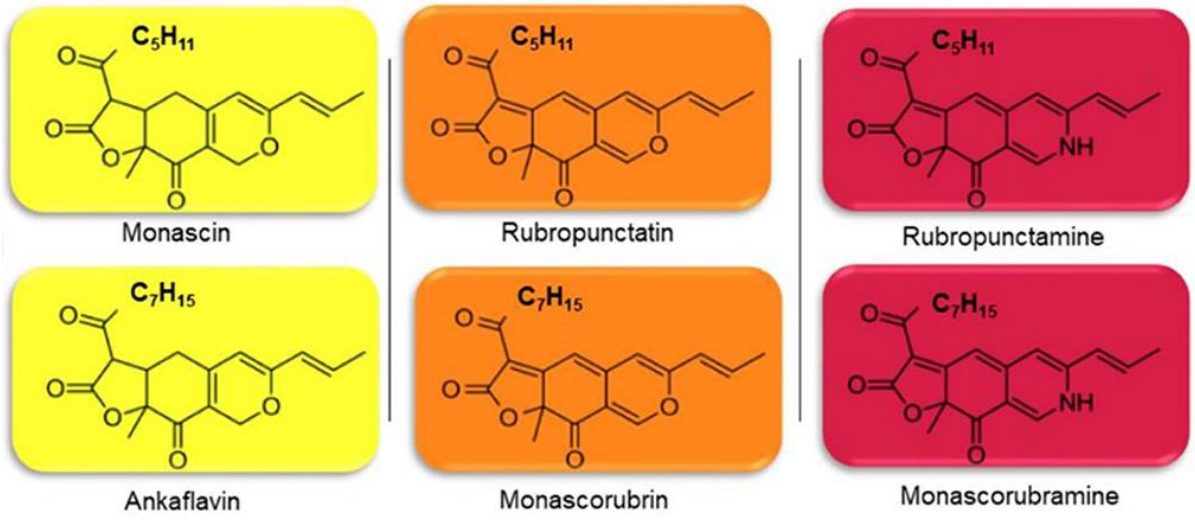


Figure 2. 3. Structures of *Monascus* pigments.
Source: Egea et al. (2023).

Yellow *Monascus* pigments has anti-obesity activity and obesity–related diseases such as hyperlipidemia, steatohepatitis, and hyperglycemia (Hsu & Pan, 2014). ANKASCIN 568–R, is a patented RFR, free of monacolin K, high level of yellow pigments, and have been verified by animal and clinical studies. The product has been accepted by USFDA as a new dietary ingredient (NDI) (Liu et al., 2018). Rubropunctatin, an orange *Monascus* pigment, has an anti-proliferative effect on BGG–823 (human gastric adenocarcinoma) cells and has the potential to be developed as a new natural anti–cancer agent (Zheng et al., 2010). The red

pigments produced by *Monascus* spp. is important for food additives, food colourants and condiments (He et al., 2020; Lagashetti et al., 2019). Red pigments can also induce cellular senescence and reduce viability in the hepatocarcinoma cell line (HepG2). Wei and Popovich (2013) suggested that this capability may halt the progression of carcinomatous cells to invasive malignancy and provide an alternative strategy for cancer prevention.

The substrate is important for the growth of *Monascus* spp. and the production of RFR. Chairote et al. (2008) found that RFR produced from non–glutinous rice with the addition of soybean milk and fermented for 3 weeks had a softer texture, a pleasant sweet odour and a dark red colour compared with RFR from non–glutinous rice without the soybean milk.

Inorganic nitrogen, such as ammonium chloride (NH_4Cl), produces higher *Monascus* pigment yield during the stationary phase even though the development of conidial germination and the sexual cycle of *Monascus* is suppressed (Chen & Johns, 1994). Sodium nitrate ($NaNO_3$) stimulates sporulation and high pigments yield, but the growth of *Monascus* is restricted (Chen & Johns, 1994).

To increase the pigments production of *Monascus* spp., organic nitrogen such as peptone, yeast extract, and monosodium glutamate (MSG) can be added as supplements (Dufossé et al., 2005; Lin & Demain, 1991; Silveira et al., 2008; Subsaendee et al., 2014; Vidyalakshmi et al., 2009). The carbon source also affects the production of *Monascus* spp. pigments. Ghada and Walid (2017) studied the factors affecting pigments production by *M. purpureus*. The results showed that the optimal growth and pigments production was achieved when corn starch was used as a carbon source, yeast extract was used as a nitrogen source, initial pH was adjusted to pH 6, and incubated at 30 °C for 12 days with shaking speed at 150 rpm.

The use of glucose as a carbon source increased *M. purpureus* growth. In contrast, cell mass was inhibited by the use of sucrose (Ajdari et al., 2011). Li et al. (2017) studied the effect

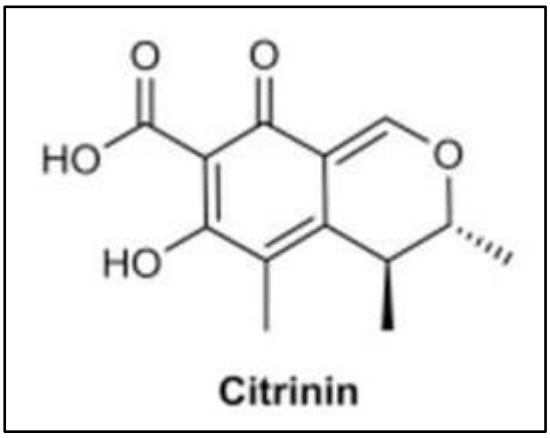
of different carbon sources on CIT production by *P. citrinum* and used transcriptome analysis to study the mechanism at the molecular level. CIT produced by glucose–cultured *P. citrinum* was 49 % higher than sucrose–cultured *P. citrinum.* The glucose–cultured *P. citrinum* changed its primary metabolic pathways, with more carbon passing through acetyl–CoA and malonyl–CoA, resulting in increased levels of precursors for polyketide synthesis. The polyketide synthase involved in secondary metabolism and CIT biosynthesis increased (up–regulated) in the glucose–cultured *P. citrinum*, resulting in higher CIT production. As a carbon source, glucose suppresses *P. citrinum* to produce energy, activates the electron transport chain (ETC) process, forms reactive oxygen species (ROS), and produces higher hydrogen peroxide (H_2O_2) content due to the up–regulation of glucose oxidase (GOX*).* In response to oxidative stress, *P. citrinum* might produce higher CIT concentrations by altering expression levels of signalling pathway genes, antioxidant enzymes and others via transcriptional regulation. Ajdari et al. (2011) suggested that glucose and sucrose are a good combination of carbon sources to enhance sporulation and cell mass of *Monascus* spp. The effects of these combinations on the CIT produced by *Monascus* spp. and understanding the mechanism for CIT biosynthesis at the molecular level are interesting areas to be discovered.

According to Wang et al. (2004), the pH required for *Monascus* pigments production is between pH 2.5 to 10.0. The production of yellow and orange pigments is higher at pH 2.5 than more alkaline pH (Babitha et al., 2006). Another main factor involved in the hydrolysis of *Monascus* spp. is temperature. Temperatures between 35 °C and 37 °C encourage growth and glucoamylase production, while temperatures in the range of 30–40 °C produce pigments (Babitha et al., 2007; Kraboun et al., 2019). The highest pigments intensity occurs when the initial moisture content is between 30 % to 50 % (Kongbangkerd et al., 2014). For high pigments intensity, Kraboun et al. (2019) suggested the moisture content of the rice must be less than 30%. Moisture content higher than 50 % reduces oxygen transfer, heat exchange and

ventilation, leading to carbon dioxide accumulation. This is unsuitable for the formation of secondary metabolites, resulting in lower pigments intensity (Kongbangkerd et al., 2014).

2.7. Factors affecting CIT production in RFR

It is critical to design a safe process for RFR production, as it can be contaminated with mycotoxins such as CIT, AF, and OTA (Samsudin & Abdullah, 2013). The most reported mycotoxin in RFR is CIT (Figure 2.4) (Avula et al., 2014; Chen et al., 2016a; Chen et al., 2016b; Li et al., 2003; Li et al., 2012b; Liao et al., 2014; Marley et al., 2016; Xu et al., 2003). However, other mycotoxins such as AF and OTA are reported (Samsudin & Abdullah, 2013) but appear to be associated with *P. chrysogenum*, *A. niger*, and *A. flavus* contamination in RFR from contaminated air. This review will focus on CIT production by *Monascus* during the production of RFR (Blanc et al., 1995a).



$C_{13}H_{14}O_5$

Figure 2. 4. Structure of citrinin.
Source: Kamle et al. (2022).

Some aspects that influence the level of CIT produced include the *Monascus* species and strains, medium–chain fatty acids, amino acids, trace elements, carbon and nitrogen source, nutritional factors, the ratio of nitrogen to carbon concentration, pH, moisture content, humidity, light, oxygen, temperature, environmental factors, respiration rate, and hyphal

morphology (Blanc et al., 1995a; Comerio et al., 1998; Doughari, 2015; Hajjaj et al., 2012; Hajjaj et al., 2000b; Kang et al., 2014; Marič et al., 2019; Ostry et al., 2013; Patakova, 2013; Wong & Koehler, 1981; Yang et al., 2019; Yang et al., 2015a).

Monasus species and isolates can produce different levels of CIT. Blanc et al. (1995a) studied the CIT concentration produced by *M. ruber* and *M. purpureus* in Yeast Extract Sucrose (YES) medium (SmF), wet rice (SSF), and synthetic media (Table 2.5). YES medium is used to produce fungal toxins, wet rice is traditionally used to produce RFR, and synthetic medium is used to produce red pigments. The results showed that *M. ruber* produced higher CIT levels than *M. purpureus* on the YES and wet rice medium. In contrast to later findings, five of the *M. ruber* isolates produced no CIT, four of the *M. ruber* isolates produced CIT in the range of 59–137 ng/g, and two of the *M. purpureus* produced higher CIT concentrations (8470–11,064 ng/g) (Li et al., 2020). Selecting a *Monascus* spp. that does not produce CIT or produced minimal CIT would help in ensuring a safe product of RFR.

Different fungal species required different optimal temperatures for optimal growth and CIT production. The optimum temperature for *P. viridicatum* and *P. citrinum* to produce CIT is 30 °C, but CIT can be produced over a wide temperature range between 15 °C to 37 °C (Montani et al., 1988; Silva et al., 2021; Wu et al., 1974). The optimum temperatures for *M. purpureus* to produce CIT in SSF and SmF are 35 °C and 32 °C, respectively (Zhang et al., 2013). However, 30 °C was used as the fermentation temperature after inoculating rice and *Monascus* spp. inoculum (Table 2.2). In contrast, the study by Camardo Leggieri et al. (2016) found that the highest amount of CIT produced by *P. citrinum* occurs when incubated at 35 °C and 0.99 water activity (a_w). Another important finding was CIT production decreased rapidly when a_w decreased from 0.99 to 0.96 (T = 20 °C) and stopped production at 0.93 a_w. This result differs from Comerio et al. (1998) who reported that the minimum a_w for *P. citrinum* growth on MEA (30 °C) and CIT accumulation in the substrate was 0.775 and 0.810, respectively.

The humidity required for the growth of CIT producing fungi such as *Monascus* spp. is between 16.5–19.5 % (Doughari, 2015). High red pigments and low CIT are achieved when RFR is fermented at pH 5.5, followed by the addition of an alkaline medium (the medium added was not mentioned) to adjust the pH to 8.5 (Orozco & Kilikian, 2008).

2.8. Detection methods of CIT

There are several methods used to detect CIT in RFR: (1) Colorimetric techniques such as fluorometer, ultraviolet (UV) spectrometer and luminescence material (eg: carbon dot) as a fluorescence probe for CIT detection in the picomole range; (2) Spectrophotodensitometer; (3) nuclear magnetic resonance (NMR) spectroscopy; (4) enzyme immunoassays (EIA) such as enzyme–linked immunosorbent assay (ELISA) and indirect competitive enzyme–linked immunosorbent assays (ic–ELISA); (5) immunochromatographic assay (ICA); (6) immunochromatographic strip (ICS); (7) capillary zone electrophoresis (CZE), including CZE along with an ultraviolet detector (CZE–UV); (8) micellar electrokinetic capillary chromatography; (9) TLC; (10) mass spectrometer; (11) high–performance liquid chromatography (HPLC) with UV light, fluorescence (FLD), or photodiode–array (PDA) detector; (12) liquid–chromatography coupled with mass spectrometry (LC–MS); (13) liquid–chromatography and tandem mass spectrometry (LC–MS/MS); (14) liquid–chromatography with fluorescence detector (LC–FLD); (15) ultra–high performance liquid chromatography–diode array detector–quadrupole time of flight–mass spectrometry (UHPLC–DAD–QTOF–MS); and (16) gas chromatography–mass spectrometry (GC–MS) (Avula et al., 2014; Blanc et al., 1995a; Chen et al., 2016b; Cheng et al., 2018; Kamle et al., 2022; Marley et al., 2016; Nigović et al., 2013; Xu et al., 2006; Zhang et al., 2021). Some of them were listed in Tables 2.4 and 2.5.

HPLC is used to determine 80 % of the world's organic compounds due to providing accurate results and is the most frequently used method to detect CIT in RFR (Ji et al., 2015; Singh & Mehta, 2020). The limitations of using HPLC are the practical issues on the choice of calibration, sample preparation, sample type, and matrice effects (Singh & Mehta, 2020). Even though GC–MS provides high specificity and sensitivity, this method has drawbacks such as requiring samples volatilization, the nonlinearity of calibration curves, reminiscence properties from earlier samples, weak fluorescent and absorption groups, drifting responses, column blockage, and the risk of contamination compared to HPLC (Perez et al., 2016; Pettersson & Langseth, 2002).

Some of the mycotoxins such as CIT and AF have natural fluorescence properties, and HPLC–FLD is used to identify analytes based on the occurrence of the chromophore in the particles (Singh & Mehta, 2020; Vazquez et al., 1997). Ji et al. (2015) compared the use of HPLC–FLD and LC–MS/MS for the quantification of CIT in RFR. The results showed that LC–MS/MS offers better sensitivity, accuracy, and reproducibility than HPLC–FLD. However, these instrument-based methods have disadvantages such as the instruments need maintenance, the use of large amounts of organic solvents, cost, time, complex pre-treatments, and require a good technique and training for troubleshooting, method development, system testing, analysing chromatograms and data analysis (Haidar Ahmad, 2017; Kamle et al., 2022; Singh & Mehta, 2020). Meanwhile, the drawbacks of biological methods such as TLC and ELISA are lack of sensitivity, difficult reproducibility, and the possibility of false-positive results (Kamle et al., 2022; Shekhar et al., 2017). Therefore, it is important to find a simple and rapid method for screening fungi that can produce CIT.

CCA is a white culture medium consisting of coconut cream (50 %) and agar (1.5%) (Dyer & McCammon, 1994). CCA has been used as a screening method for the detection of other mycotoxins such as AF (Dyer & McCammon, 1994) and OTA (Heenan et al., 1998).

Studies from Mohamed et al. (2013) found that CCA can be used to detect CIT from *P. citrinum* isolated from Maldives fish. This method has been verified by Farawahida et al. (2022a) to screen CIT–producing *Monascus* spp. isolates isolated from RFR. This method can be adopted to select *Monascus* spp. isolates without or with low CIT to produce RFR.

2.9. Toxicity of CIT

AF, CIT, and OTA can be produced during the fermentation of RFR (Blanc et al., 1995a; Dogra & Kumar, 2017; Samsudin & Abdullah, 2013). *Monascus* pyridines are toxic metabolites that can also be present in RFR (Blanc et al., 1995a). CIT can be produced by *Aspergillus*, *Fusarium* and *Penicillium* species across the temperature range of 15–37 °C, but the optimum temperature is 30 °C (Silva et al., 2021). Zhang et al. (2013) reported the optimum temperatures for CIT production by *M. purpureus* in SSF and SmF are 35 °C and 32 °C, respectively. CIT production could be minimised by incubation of *M. purpureus* for SSF and SmF at 28 °C. CIT has antibacterial properties against Gram–positive bacteria. However, due to its high mammalian nephrotoxicity, it is not allowed to be used as a drug (Flajs & Peraica, 2009).

CIT exposure has been tested on human cells and animals such as guinea pigs, mice, rats, rabbits, bovines, hamsters, and zebrafishes (de Oliveira Filho et al., 2017). CIT has detrimental health consequences for humans and animals including nephrotoxicity and hepatoxicity, but the level of acute toxicity varies in different species (Kumar et al., 2010). CIT is embryocidal and fetotoxic in mice, meanwhile, rats exposed to high doses of CIT cause renal tumours, and teratogenic effects and induce the enlargement of tubular necrosis of the kidney (Mayura et al., 1984). The major target organ of CIT is the kidney (Kamle et al., 2022). In cereals and grains, CIT and OTA are the two mycotoxins frequently occurring together as co–contaminants and cause toxicity in the kidneys and reduce ribonucleic acid (RNA) synthesis in

murine kidneys (Knecht et al., 2005; Sansing et al., 1976). Consumption of foods contaminated with these mycotoxins increased the toxicity due to the addition or combination effect of these mycotoxins, leading to kidney diseases in humans and animals (Bouslimi et al., 2008; Vrabcheva et al., 2000). Exposure of CIT to male F344 rats for 60 and 80 weeks leads to tumour formation in their kidneys (Arai & Hibino, 1983). Vero cells (kidney cell culture) exposed to CIT for 24 h produce DNA damage, and a combination of OTA and CIT exposure simultaneously causes renal diseases due to enhanced oxidative stress (Bouslimi et al., 2008).

CIT also targets other organs such as the liver, heart, immune and reproductive system. The toxic effects of CIT have been associated with CIT–mediated oxidative stress and mitochondrial dysfunction in biological systems (de Oliveira Filho et al., 2017). Kumar et al. (2010) studied the immunotoxicity of New Zealand White rabbits. The combination of CIT and OTA caused several humoral immune deficiencies in the rabbits.

In 1979, monacolin K was isolated from *M. ruber* and *A. terreus* (Endo, 2004). Then, Merck, Sharp, and Dohme commercialized monacolin K as a drug, to lower cholesterol (lovastatin) in 1987 after obtaining validation from the Food and Drug Administration (FDA) (Endo, 2004; Le Bloc'h et al., 2015). As RFR produces a drug (monacolin K) and mycotoxin (CIT), the USFDA does not approve the RFR as a dietary supplement (Gordon et al., 2010). However, some researchers have shown that RFR or *Monascus* fermented products pose no threat to human or animal health, and this may be due to the low levels of CIT in RFR or other *Monascus*–fermented products (Lee et al., 2006a; Mohan Kumari et al., 2009; Venero et al., 2010). Due to inadequate exposure data, risk assessment of CIT in food was estimated based on the CIT concentrations in grains and grain–based products, resulting in an exposure equal to the level of no concern for nephrotoxicity (0.2 µg/kg body weight per day) (EFSA, 2012). However, most researchers consider that some action should be taken to control CIT concentration in RFR (Chen & Hu, 2005).

The European Commission (EC) has established the maximum permissible level of CIT in food supplements based on rice fermented with *M. purpureus* is 100 µg/kg (EU, 2019). Meanwhile, the maximum permitted levels of CIT in RFR products are 50 µg/kg in China (Srianta et al., 2014), 200 µg/kg in Japan (Srianta et al., 2014), and 2000 µg/kg in Taiwan (Taiwan-FDA, 2020). The regulations vary among the countries due to different dietary patterns, the risk analysis of toxicological data available, sampling and analytical capabilities, information on susceptible commodities, the effect on the availability of an adequate food supply, environmental conditions, and national practices (FSANZ, 2019; Stoloff et al., 1991).

2.10. Control and reduction of CIT

CIT problems can be minimised by: (1) preventing contamination, (2) removing contaminated material from the RFR, (3) reducing CIT in RFR, and (4) treating exposed individuals (Karlovsky et al., 2016). The CIT production during the fermentation of RFR is difficult to avoid since *Monascus* spp. produce CIT. However, it may be possible to select isolates that can produce high levels of desirable pigments and monacolin K but no or minimal CIT (Blanc et al., 1995a; Li et al., 2020). Another approach is to reduce the amount of CIT produced by manipulating the fermentation conditions (Yang et al., 2015a). Three other approaches to prevent CIT production are (1) generating mutant *Monascus* spp. isolates free of the gene *pksCT* to obtain an isolate that does not produce CIT (Dikshit & Tallapragada, 2018; Jia et al., 2010; Li et al., 2020), (2) optimizing the culture media for SmF (Chen et al., 2016a), and (3) genetic engineering such as disrupting the *pksCT* gene (Jia et al., 2010).

Treatments to reduce CIT concentration in CIT standard, RFR and other samples have been reported (Table 2.6). These treatments can be categorized as physical, chemical, natural substances, and microbiological.

Table 2. 6. Methods to control and reduce citrinin.

Category	Control of CIT during fermentation	Fungal species	Media/ Substrates	Treatment	Reduction of CIT	References
Physical	Initial $a_{w=}$ 0.800	*P. citrinum*	Wheat	NA	NA	(Comerio et al., 1998)
Physical	pH = 5.5, then adjust the pH to 8.5	*M. purpureus*	[a]Bioreactor cultures	NA	NA	(Orozco & Kilikian, 2008)
Physical	NA	*P. citrinum*	[b]YES broth	Heating at 140 ºC – 160 ºC	20 %	(Hirota et al., 2002; Trivedi et al., 1993)
	NA	*M. purpureus, M. ruber*	[c]Rice medium	SAMNs	70 %	(Magro et al., 2016)
Chemical	NA	*M. purpureus*	RFR	Phosphate-ethanol extraction	92 % CIT was reduced, and 80 % monacolin K has remained	(Lee et al., 2007a)
Natural substance	NA	*M. aurantiacus*	[d]SmF	Genistein (Flavonoid)	80 %	(Wang et al., 2020)
Microbiology	NA	NA	Nutrient yeast dextrose broth (NYDB)	*Cryptococcus podzolicus* Y3	94 %	(Zhang et al., 2017)
	NA	*M. purpureus*	[e]YES medium	*Saccharomyces cerevisiae*	98 %	(Davoudi Moghadam et al., 2019)
	NA	NA	[f]Mineral broth	*Klebsiella pneumoniae*	100 %	(Chen et al., 2011)

CIT: citrinin. NA: not applicable. RFR: red fermented rice. SmF: submerged fermentation. SAMNs: Surface Active Maghemite Nanoparticles.

[a] Bioreactor cultures (g/L): [di–potassium hydrogen orthophosphate (KH_2PO_4) (1.5 g), potassium dihydrogen phosphate (K_2HPO_4) (1.5 g); zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (0.01 g), monosodium glutamate (MSG) (7.6 g), sodium chloride (NaCl) (0.4 g), ferrous sulfate ($FeSO_4$) (0.01 g), and yeast extract (1.0 g) (pH 5.5)] with addition of glucose solution (g/L) [glucose (20.0 g); magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (4.8 g)].
[b] Yeast Extract Sucrose (YES) broth: 2 % yeast extract and 15 % sucrose.
[c] Rice medium: (20 g/L, 5 g/L glycine and 20 g/L agar-agar).
[d] Submerged fermentation (SmF): (2.0 % rice powder, 0.2 % $NaNO_3$, 0.05 % KH_2PO_4, 0.1 % K_2HPO_4, 0.1 % $MgSO_4 \cdot 7H_2O$), with addition of rice powder inorganic salt medium (20.0 g/L) and 2.0 g/L of genistein.
[e] YES medium: yeast extract 40 g and sucrose 160 g/L in distilled water.
[f] Mineral broth (1 L of deionised water): [potassium chloride (KCl) (0.7 g), KH_2PO_4 (2 g); disodium hydrogen phosphate (Na_2HPO_4) (3 g), $MgSO4 \cdot 7H_2O$ (0.7 g); and calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) (0.02 g)] containing 10 ppm of CIT, 1.2 % glucose, 0.3 % peptone, and 100 ppm of vitamin C.

Physical methods include heating at 140 °C–160 °C which reduced 20 % of CIT and converted it to CIT H2 (non–cytotoxic to human cervical cancer, HeLa cells) (Chen et al., 2013; Hirota et al., 2002). Surface Active Maghemite Nanoparticles (SAMNs) removed 70 % CIT by adsorption and binding iron (III) in solution (Magro et al., 2016). This study suggested that the addition of SAMNs can also reduce other mycotoxins such as dihydrocitrinone and OTA due to the presence of a strong iron–chelating agent on the toxin molecule, namely the keto–enol group. However, the effects of SAMNs on other compounds such as pigments and monacolin K have not been reported. Cold plasma can reduce fungi and mycotoxins contamination in roasted coffee and may be able to be applied to RFR. *A. westerdijikiae, A. steynii, A. versicolor,* and *A. niger* were unable to produce CIT after the exposure to cold plasma for 6 min, while exposure for 30 min reduced 33–61 % of OTA produced by these fungi (Casas-Junco et al., 2019).

Treatment with chemicals such as phosphate during extraction (phosphate–ethanol extraction) of RFR was sufficient to reduce CIT and retain the monacolin K content. CIT was reduced up to 92 % and 80 % monacolin K was retained under optimized conditions (45 % ethanol, 1.5 % phosphate, and extraction for 70 min) (Lee et al., 2007a).

Wang et al. (2020) studied the effect of adding nine flavonoids (natural substances) to SmF medium, incubated in a shaking incubator (180 rpm) for 12 days at 30 °C. The mycelium was measured after obtaining a constant weight. The results showed that by the addition of 20 g/L rice powder and 2 g/L genistein, pigments and biomass were increased by 20 % and 80 % of CIT was reduced.

Cryptococcus podzolicus Y3 can be used to reduce CIT by intracellular enzymes (Zhang et al., 2017). CIT was reduced by 94 % using *C. podzolicus* Y3 at 1×10^8 cells/mL incubated in nutrient yeast dextrose broth (NYDB) at 28 ºC for 42 h. CIT can be reduced by 98 % when 20 µg/mL of CIT is incubated at pH 4.0.

Davoudi Moghadam et al. (2019) studied the effects of adding heat–treated *Saccharomyces cerevisiae* at different temperatures and yeast concentrations on CIT and pigments produced during SmF using *Monascus* spp. When *S. cerevisiae* (10^5 cells/mL) was heated at 121 °C, CIT was reduced by 98 % through binding to the yeast. However, this treatment also significantly reduced extracellular pigments (Davoudi Moghadam et al., 2019).

Chen et al. (2011) isolated CIT–degrading strains in 24 soil samples (>50 g each) (the species were not characterized). CIT (1 ppm) was added to suspensions of the soil samples and incubated in a shaking incubator at 150 rpm and 30 °C for 2–3 days. The microbial growth was monitored using optimal density (OD) at 660 nm. When the OD unit of the suspension had an OD value greater than 2, the CIT level in the suspension was measured by HPLC. If there was a reduction in CIT, the suspension was transferred to a new broth containing 2 ppm of CIT, and the same process was repeated at a higher CIT concentration (4 ppm). After the CIT was fully degraded, samples were diluted and inoculated onto mineral agar plates containing 10 ppm of CIT and incubated at 30 °C for 1 day. Over 300 colonies grown on the CIT–containing mineral agar plates were picked and re–suspended in the fresh mineral broth added with 10 ppm CIT and incubated in a shaking incubator at 30 °C for 2–3 days. From the screening process, 10 strains were isolated and characterized as *Klebsiella pneumoniae* based on 16S ribosomal DNA (rDNA) and these bacteria degraded CIT, with the most effective strain NPUST–B11. The addition of *K. pneumoniae*, resulted in a 2 % reduction in CIT when incubated at 37 °C at 200 rpm for 1 h. After 5 h, 91 % of CIT was degraded, and CIT was completely degraded after 10 h of incubation (Chen et al., 2011).

Lactic acid bacteria (LAB) can reduce other mycotoxins such as AF, OTA, aflatoxin M_1 (AFM_1), patulin, deoxynivalenol, zearalenone, fumonisin B_1 (FB_1), and fumonisin B_2 (FB_2) (Muhialdin et al., 2020). There is a possibility that LAB may also be able to degrade CIT.

To control CIT production in RFR, recent studies focused on optimization of fermentation conditions, enrichment nutrients used in SmF, and post–harvest degradation by physical, chemical, natural substances, and microorganisms. Optimization of fermentation and enrichment nutrients can be used to control CIT production, but it is difficult to block CIT biosynthesis completely in *Monascus* spp. (Li et al., 2020).

Variation in the CIT production and concentration could be due to different extrinsic factors during fermentation. Comerio et al. (1998) studied the influence of a_w on *P. citrinum* growth and CIT production in wheat. The finding showed that CIT in wheat was not detected at a_w 0.800, even though there was the development of *P. citrinum* mycelium and sporulation after 6 days and 12 days of incubation, respectively. Ristiarini et al. (2017) reported that RFR in Indonesia has a_w between 0.75 to 0.80. However, there is no information available on the effect of a_w on CIT produced by *Monascus* spp. (Silva et al., 2021).

Different *Monascus* species and isolates also can affect CIT production. Li et al. (2020) showed that *M. purpureus* R9 produced higher CIT than *M. purpureus* R3. However, some *M. ruber* isolates (R4, R15, R16, R17) produce CIT while other *M. ruber* isolates (R1, R2, R19, R20, R21) do not produce any CIT. The results contradict the results obtained by Blanc et al. (1995a), who reported *M. ruber* produced higher CIT than *M. purpureus* in SSF and SmF. Another study found that from eight isolates of *M. purpureus*, two of the isolates (isolates IFRPD 4044 and IFRPD 4046) do not produce any CIT after fermentation for 14 days at 30 °C (Saithong et al., 2019).

Therefore, it is important to select *Monascus* spp. isolates as the inoculum for RFR production. CCA has been successfully used to screen other mycotoxins such as AF and OTA, and CIT from Maldives fish (Mohamed et al., 2013). This method has been used to screen *Monascus* spp. isolates from RFR and reported in Chapter 3 in this thesis.

2.11. Conclusion

RFR is consumed as a traditional Chinese medicine producing a variety of metabolites, in particular, monacolin K, beneficial to human health for cholesterol reduction, and red pigments to add flavour and colour to food. However, CIT is produced during the fermentation of RFR and this is a concern for human health. To produce safe RFR, CIT production must be reduced. Several methods can reduce CIT in the final product, but many of these methods are not fully understood or are impractical for routine use. The ideal approach is to prevent CIT production. One of the methods is to select *Monascus* spp. isolates that produce no CIT, while still producing the desirable bioactive properties valued in RFR. There is another scope to explore which is applying different fermentation conditions, such as a_w, oxygen levels, and temperature to prevent CIT production. Rapid screening tests would be useful to help select *Monascus* spp. isolates that do not produce CIT. The kinetics of CIT production during RFR fermentation does not appear to have been studied and is important to optimise the fermentation to minimise CIT levels. Alternative methods for CIT reduction could be investigated to produce safe RFR.

CHAPTER 3

COCONUT CREAM AGAR AS A SIMPLE AND RAPID SEMIQUANTITATIVE METHOD TO SCREEN CITRININ–PRODUCING *MONASCUS* SPP. ISOLATES ISOLATED FROM RED FERMENTED RICE

3.1. Introduction

RFR is a fermented product that is widely used in East Asian countries, especially in Chinese dishes. RFR is produced by fermenting rice with *Monascus* spp. as a fungal starter. RFR is also known as red rice, red leaven, zhitai, hong qu, hung–chu (Chinese), beni-koji (Japanese), rotscimmelreid (Europe), angkak, Anka, Ang–Khan, Anka–Koji, red mould rice, and red yeast rice (Chiu et al., 2006; Erdoğrul & Azirak, 2004; Patcharee et al., 2007; Ristiarini et al., 2017).

RFR can be consumed as porridge, or used as TCM, food flavour, food colourant, food preservative, to remove meaty odour, and for fermentation of traditional Chinese red yeast rice wine/ red fermented rice wine (Samsudin & Abdullah, 2014). Red yeast rice wine is produced by mixing cooked white glutinous rice with RFR and incubating for a month. RFR has health benefits such as improved blood circulation, as an anti–hypercholesterolemic agent (Cicero et al., 2019; Cicero et al., 2021), produces hypolipidemic effects (Zhou et al., 2019), has anti–fatigue and anti–cancer (Xue et al., 2017) benefits, and anti–inflammatory activity (Hsu et al., 2010). RFR is available in traditional Chinese shops and is in high demand (Samsudin & Abdullah, 2014). More than a billion people consume RFR and *Monascus*–fermented products worldwide (Yang et al., 2015b).

However, RFR might be contaminated with CIT as a secondary metabolite produced by some *Monascus* spp. (Liao et al., 2014). CIT is a known hepatonephrotoxin, which changes

the metabolism of the liver, and impairs the structure and function of the kidneys (Silva et al., 2021). Based on a CIT toxicokinetic study in humans, the absorption of CIT in humans >40 % based on the 40 % of CIT eliminated in the urine (Degen et al., 2018). Due to its high mammalian nephrotoxicity, it is not allowed to be used as a drug (Flajs & Peraica, 2009) or as a dietary supplement (Gordon et al., 2010). Nevertheless, some researchers claim that RFR and *Monascus*–fermented products represent no threat to the health of animals or humans possibly due to the low amount of CIT in RFR or other *Monascus* fermented products (Lee et al., 2006a; Mohan Kumari et al., 2009; Venero et al., 2010). However, most researchers consider that some action should be taken to control CIT levels in RFR (Chen & Hu, 2005). For CIT in RFR fermented with *M. purpureus*, Commission Regulation of the European Union (EU) has set a 100 µg/kg maximum permitted limit (EU, 2019).

Different methods can be used to detect CIT in RFR, including chromatographic techniques such as LC–MS/MS, UHPLC–DAD–QTOF–MS, HPLC, and high–performance thin–layer chromatography with ultraviolet and fluorescence detectors (HPTLC–UV/FLD) (Avula et al., 2014; Chen et al., 2016a; Chen et al., 2016b; Li et al., 2003; Li et al., 2012b; Liao et al., 2014; Marley et al., 2016; Xu et al., 2003). Biological methods such as ELISA can also be used (Samsudin, 2011; Samsudin & Abdullah, 2013). Even though UHPLC–DAD-QTOF–MS, HPLC and LC–MS/MS can provide accurate results, using these instruments requires skill and they are expensive. These instruments can determine CIT in samples but are unable to screen the *Monascus* spp. strains that produce CIT. Studies from Mohamed et al. (2013) found that CCA can be used to detect CIT from *P. citrinum* isolated from Maldives fish, and this method has also been used for the detection of other mycotoxins such as AF (Dyer & McCammon, 1994) and OTA (Heenan et al., 1998). This study aimed to identify a simple and rapid method by using CCA to screen the *Monascus* spp. isolates that can produce CIT. To the

best of the author's knowledge, this is the first study using CCA as a screening method to identify CIT–producers of *Monascus* spp. isolated from RFR.

3.2. Materials and methods

3.2.1. Source of samples

Two samples of RFR (100 g each) from Malaysia were exported to New Zealand with importation permits approved by Ministry for Primary Industries in February 2020. All the samples were packed in air–tight polyethylene bags, labelled, and stored at 4 °C before analysis.

3.2.2. Materials and microorganisms

All solvents used were HPLC grade. CIT standards were supplied by Sigma–Aldrich (Missouri, USA). Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) were purchased from Merck (Darmstadt, Germany) and Czapek Yeast Extract Agar (CYA) was supplied by HiMedia (Mumbai, India). Twenty-five per cent (25%) Glycerol Nitrate Agar (G25N) was prepared with this formulation: di-potassium hydrogen orthophosphate, K_2HPO_4 (0.75 g), Czapek concentrate (7.5 mL), yeast extract granulated (3.7 g), glycerol (250 g), microbiology agar-agar (12 g) and distilled water (750 mL). CCA was prepared by using 1000 g of Ultra High–Temperature processing (UHT) coconut cream (Kara, Riau, Indonesia), 1000 g of distilled water, and 30 g of microbiology agar–agar (Merck, Darmstadt, Germany). One litre of YES agar was prepared by using yeast extract (4 g), sucrose (20 g), potassium dihydrogen phosphate (KH_2PO_4) (1 g), magnesium sulphate ($MgSO_4$) (0.5 g), microbiology agar–agar (15 g) and distilled water (1 L). All the media used included 1 mL of chloramphenicol (0.05 g/L) added to inhibit the growth of bacteria during the incubation period. All the primers used were supplied by Thermo Fisher Scientific (Auckland, New Zealand). *M. ruber* Tiegh ICMP 15220,

P. glabrum (Wehmer) Westling ICMP 5686, and *P. expansum* ICMP 1178 were supplied by the International Collection of Micro-organisms from Plants (ICMP) (Landcare Research Centre, New Zealand).

3.2.3. Identification of fungi in RFR

Macroscopic and microscopic (40 × magnification) observations were used to identify the fungi in RFR using the method from Pitt and Hocking (2009) with minor modification. Firstly, RFR was ground using a coffee grinder. Then, 1 g of the ground sample was added to 90 mL of 0.1 % peptone water and homogenized using a peristaltic blender for 120 sec. Serial 10–fold dilutions from 10^{-2} to 10^{-6} were prepared in 0.1 % peptone water, and spread plated on CYA, MEA, and G25N in triplicate and incubated for 7 days. CYA plates were incubated at three temperatures (5 °C, 25 °C, and 37 °C), while MEA and G25N agars were incubated at 25 °C. The diameters of the colonies were measured from the reverse side by transmitted light (Pitt & Hocking, 2009). A Scotch Tape method was used to prepare fungal slides for microscopic observation (Harris, 2000).

3.2.4. Screening of CIT–producers of *Monascus* spp. isolates in RFR

The method from Mohamed et al. (2013) was used with a slight modification to screen CIT–producers of *Monascus* spp. isolates. The RFR dilutions were prepared as in 3.2.3. and spread plated on CCA, MEA, CYA, and YES. All the plates were incubated at 30 °C for 7 days. Colonies were observed under long–wavelength UV light (365 nm) using UVP Chromato–Vue® Cabinet C–10 and UVP UVGL–58 (Analytik Jena, California, USA). Daily observations were made for 7 days and the growth of *Monascus* spp. isolates were recorded by the photography. By using a 1–point inoculation method (inoculation needle was placed into the fungal colony and transferred to the centre of a new medium), each *Monascus* spp. isolates

on CCA were transferred to 10 new CCA plates and incubated at 30 °C and observed under long–wavelength UV light. Finally, 10 mol/L hydrochloric acid (HCl) (about 10 mL) was sprayed with a spray bottle onto the colonies on CCA plates and the enhancement of the colour change around the colonies in CCA under UV light was recorded by photography as an indication of CIT. For positive controls, 50 000 ng/mL of CIT standard (5 mL), *M. ruber* Tiegh ICMP 15220 and *P. expansum* ICMP 1178 were used and *P. glabrum* (Wehmer) Westling ICMP 5686 was used as a negative control.

To prepare the stock culture *for M. ruber, P. expansum and P. glabrum*, 10 µL of each isolate was inoculated onto PDA and incubated at 30 °C for 7 days. Ten mL of 0.1 % Tween 80 was added onto PDA and scraped with a sterile microscope glass slide. The mixture was poured into a beaker containing 20 g of glass beads. To break up the mycelia, the mixture was shaken vigorously by hand for 15 s followed by a vortex mixer for 15 s. A sterile tea strainer was used to filter the mixture. The filtrate was transferred to 1.5 microcentrifuge tubes and stored at -20 °C for later use.

3.2.5. Preparation of inoculum and stock culture

Based on the screening results on CCA, the inoculum and stock cultures of the two of *Monascus* spp. isolates were prepared. Each of the *Monascus* spp. isolates were isolated onto new MEA agar plates by the 1–point inoculation method and incubated at 30 °C for 7 days. Pure cultures for each *Monascus* spp. isolates were removed from MEA agar plates using 10 mL of 0.1 % Tween 80, and scraping with a sterile microscope glass slide and the mixture was collected in a beaker containing 20 g of glass beads. The mixture was shaken vigorously by hand for 15 s followed by a vortex mixer for 15 s to break up the mycelia, filtered by using a sterile tea strainer and the filtrate was collected as the inoculum.

To increase the volume of inoculum, 200 µL of inoculum was inoculated into bottles containing 10 mL of MEA agar slants and incubated at 30 °C for 7 days. The same process was used to obtain the inoculum stock culture by pouring 5 mL of 0.1 % Tween 80 onto the MEA agar slants and scraping with a sterile spatula. The conidia of the inoculum were observed with a microscope at 40 × magnification, and the concentration of the inoculum was measured by using a haemocytometer. Two samples were observed to obtain the inoculum data. Then, 1 mL of the inoculum was transferred to 1.5 microcentrifuge tubes and stored at -20 °C and -80 °C for later use.

3.2.6. Genomic DNA extraction of Monascus spp. isolates

The stock culture for both *Monascus* spp. isolates were thawed for further extraction process. For purification of DNA, DNeasy Plant Minikit (QIAGEN, Hilden, Germany) was used following the manufacturer's instructions and kept at -20 °C as a DNA template.

3.2.7. Polymerase chain reaction (PCR) amplification and sequencing of internal transcribed spacer (ITS), beta–tubulin (β–tubulin), polyketide synthase (*pksCT*), and *ctnA* genes

M. ruber ICMP 15220 was used as a positive control from the *Monascus* spp., *P. expansum* ICMP 1178 was used as a positive control from other fungal species that potentially can produce CIT, and *P. glabrum* (Wehmer) Westling ICMP 5686 was used as a negative control. The method from Li et al. (2020) was used with minor modification to amplify the ITS, β–tubulin, *pksCT*, and *ctnA* genes from the purified DNA extracted from Section 3.2.6. The primers that were used in this study are listed in Table 3.1. Platinum™ Green Hot Start PCR 2 × Master Mix (Thermo Fisher Scientific, Lithuania) (25 µL) and 20 µL of nuclease–free water were mixed, followed by adding 3 µL of DNA template (~100 ng) and 1 µL of each

primer (10 µM) (Thermo Fisher Scientific, Lithuania) to obtain 50 µL reaction mixtures. The ProFlex^{TM} Base (Applied Biosystems®by Life Technologies, Singapore) was used to amplify PCR. The settings for the thermal cycler for PCR amplification are listed in Table 3.2.

Table 3. 1. The primers of internal transcribed spacer, beta–tubulin, polyketide synthase, and *ctnA* used for the polymerase chain reaction amplification.

Target genes	Primers	Primer sequences
ITS	ITS4 (forward)	5'–GGAAGTAAAAGTCGTAACAAGG–3'
	ITS5 (reverse)	5'–TCCTCCGCTTATTGATATGC–3'
β-tubulin	β–tubulin F (forward)	5'–CAACTGGGCTAAGGGTCATT–3'
	β–tubulin R (reverse)	5'–GTGAACTCCATCTCGTCCATA–3'
pksCT	*pksCT* F (forward)	5'–TGATGCGACGAAGATGTTAC–3'
	pksCT R (reverse)	5'–TCTCTATGCTGCGACTGAC–3'
ctnA	*ctnA* F (forward)	5'–AACGGACAGGAAGAGCGTGC–3'
	ctnA R (reverse)	5'–CACACCACCGATGCCATACC–3'

ITS: internal transcribed spacer. β–tubulin: beta–tubulin. *pksCT*: polyketide synthase.
The primer sequences were adopted from Li et al. (2020).

Table 3. 2. The conditions for polymerase chain reaction amplification.

Region	Steps	Temperature	Time	Cycles
ITS	Initial denaturation	94°C	3 min	35
	Denaturation	94°C	30 s	
	Annealing	52°C	30 s	
	Elongation	72°C	45 s	
	Final extension	72°C	8 min	
β-tubulin	Initial denaturation	94°C	3 min	35
	Denaturation	94°C	30 s	
	Annealing	55°C	30 s	
	Elongation	72°C	90 s	
	Final extension	72°C	8 min	
pksCT	Initial denaturation	94°C	3 min	34
	Denaturation	94°C	30 s	
	Annealing	56°C	30 s	
	Elongation	72°C	45 s	
	Final extension	72°C	8 min	
ctnA	Initial denaturation	94°C	3 min	34
	Denaturation	94°C	30 s	
	Annealing	56°C	30 s	
	Elongation	72°C	90 s	
	Final extension	72°C	8 min	

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

3.2.8. Gel electrophoresis

Gel electrophoresis was used to separate the PCR amplification products using the 2 % E-gel®EX Agarose Gels (Thermo Fisher Scientific). To estimate the size of the PCR products, 20 µL of PCR products were loaded into the well and compared with Trackit 1 Kb Plus DNA ladder (Thermo Fisher Scientific, Lithuania). Nucleus–free water was used as a negative control. The gel electrophoresis was analysed using the E–Gel® Safe Imager™ Real–time Transilluminator (Life Technologies) for 12 min.

3.2.9. Purification of PCR products and suspension preparation before sequencing

The PCR products obtained from Section 3.2.8 were cleaned by DNA Clean & Concentrator–5 (Zymo Research, USA) following the manufacturer's instructions to remove

any DNA polymerases, free deoxynucleotide triphosphates (dNTPs), and fluorescent derivatives. The concentration of ultra–pure DNA products was measured based on nucleic acid concentration by using the Colibri Spectrometer (Titertek Berthold, Germany). The ultra–pure DNA products (1 µL) were measured and diluted into a concentration range of 25–50 ng/µL.

The suspensions for sequencing were prepared by mixing 15 µL of nuclease–free water, 4 µL of forward/ reverse primer (1 µM), and 1 µL of ultra–pure DNA products (25–50 ng/µL). Two suspensions (forward and reverse primers) were prepared per sample. All the samples were sent to the Massey Genome Service, Palmerston North for Sanger sequencing using Applied Biosystems™ 3730 and 3500xL Genetic Analyzer. Dye–terminator DNA sequencing PCR reaction was run on Thermofisher Veriti ThermalCycler following manufactory's guideline for The BigDye Terminator v3.1 Cycle Sequencing Kit.

3.2.10. Sequence alignment and species identification

The Molecular Evolutionary Genetics Analysis (MEGA X) 11 software (Kumar et al., 2018) was used and the sequences were edited based on verification by eye. To assemble forward and reverse sequences and to obtain consensus sequences, BioEdit 7.7.1 software was used. The ITS and *pksCT* genes for both isolates were deposited into GenBank and BankIt, respectively. The Basic Local Alignment Search Tool (BLAST) was used to compare the sequences of β–tubulin and ctnA genes with existing sequences in the GenBank database from http://www.ncbi.nlm.nih.gov (Norlia et al., 2019). The closest matches between the query and existing sequence from the BLAST search were reported as the percentage match of similarity (per identical), identifying the isolates.

3.2.11. Phylogenetic analysis

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., 2017b; Chen & Zhang, 2019; Dai et al., 2021). ITS and B–tubulin are the genes used to identify fungi. The nucleotide sequences of ITS and B–tubulin genes for these *Monascus* species were obtained from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and included in phylogenetic analysis for comparison with MF1 and MS1 isolates. *Aspergillus* species (*A. flavus*, *A. parasiticus,* and *A. fumigatus*) were used as outgroups because this fungi was closely related to *Monascus* spp. (Li et al., 2020). The accession numbers of ITS and B–tubulin used were listed in Table 3.4. 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.

3.2.12. Preparation of samples for CIT determination

Negative control [*P. glabrum* (Wehmer) Westling ICMP 5686], positive controls from other fungi (*P. expansum* ICMP 1178) and *Monascus* spp. isolates isolated from RFR were used. Ten µL of each isolate was inoculated onto CCA and incubated at 30 °C for 7 days. Then, all the isolates on CCA were observed under UV light, and the presence of fluorescence was noted. 10 mol/L HCl (about 10 mL) was sprayed onto the isolates and the enhancement of the UV light reaction was recorded by the photography. A sterile cork borer (wrapped with an aluminium foil and sterilized at 121 °C for 15 min,) was used to remove some area of the CCA agar, and 50,000 ng/mL of CIT standard (1 mL) was put on that area on CCA and incubated at

room temperature. CIT standard was added to CCA as a positive control to confirm that the fluorescence is from CIT and not from other mycotoxins or *Monascus* spp. metabolites. To confirm that the fluorescence is CIT, the area with CIT standard on CCA was sprayed with HCl, and the colour changes were recorded by the photography.

3.2.13. CIT extraction

The method from Marič et al. (2019) was used for CIT extraction with minor modification. The *Monascus* spp. isolates were removed from CCA by a sterile spatula. One gram of the fungal mycelium was weighed and transferred to a 120 mL container, and 50 % ethanol solution (ethanol: water, v/v) (5 mL) was added to the container containing mycelium. Then, the mixture was extracted with a rotary shaker (200 rpm, 25 °C, 1 h), followed by centrifugation at 3000 x g for 10 min. The supernatant liquid was filtered through a Minisart® NML syringe filter (0.2 µm pore size, Sartorius, Göttingen, Germany), and then 1 mL of the filtrate was subjected to the Ultra–high performance liquid chromatography with a fluorescence detector (UHPLC–FLD) (Dionex Ultimate 3000) for CIT determination.

3.2.14. Preparation of a standard curve for CIT determination

CIT standard (5×10^5 ng/mL) was prepared by mixing 5 mg of CIT standard (Sigma–Aldrich) with 10 mL of methanol (MeOH) (0.5 mg/mL = 5×10^5 ng/mL). Seven concentrations of CIT standard were prepared: 4, 50, 100, 500, 1000, 2000, and 3000 ng/mL.

3.2.15. UHPLC–FLD condition

The method from Lee et al. (2006b) was adopted with minor modification to determine CIT in the samples by using UHPLC–FLD (Dionex, California, USA). An HPLC system equipped with a reverse–phase symmetry Kinetex C_{18} column (250 mm length × 4.6 mm

internal diameter, 5 µm particle size, 100Å pore size) was used together with an FLD detector at an excitation wavelength of 330 nm and an emission wavelength of 500 nm. The column temperature was kept at 30 °C. Acetonitrile (ACN) (Sigma–Aldrich, Missouri, USA) and 0.1 % trifluoroacetic acid (TFA) (Fisher Scientific, Loughborough, UK) in water were separately filtered through a regenerated cellulose filter (0.20 µm pore size, Sartorius, Göttingen, Germany). The mobile phase used was ACN: 0.1 % TFA (55:45, v/v) with a flow rate of 1.0 mL/min, running isocratically for 40 min. Twenty microliters (20 µL) was used as the sample injection volume. CIT quantifications were done in triplicate and processed with Chromeleon 7.0 software (Thermo Fisher Scientific).

3.2.16. Statistical analysis

Triplicate measurements were performed to obtain means and standard deviations. The significance probability for each independent variable was shown by the *p*–value to be considered as significant ($p < 0.05$). All data were analysed by univariate one–way analysis of variance (ANOVA) using Minitab (Version 18, PA., State College, USA).

3.3. Results and discussion

3.3.1. Identification of *Monascus* spp.

Based on visual observations, the fungi started to produce white mycelium after 3 days of incubation. From the original dilution plating, all the colonies produced orange colonies on MEA. Two colonies were isolated for macroscopic and microscopic observation. According to the guidelines introduced by Pitt and Hocking (2009), these isolates are considered *Monascus* spp. This was in agreement with the statement by Abdul-Manan et al. (2017b) which mentions that *M. purpureus* produce white mycelium in the early stages of incubation. Due to the development of red–orange hyphae and an increase in the acidity of the agar, the colour of

mycelium changes rapidly from rich orange to a rich red colour. Prolonged incubation also changes the substratum (e.g. rice) to a deep crimson colour (Abdul-Manan et al., 2017b). The size of colonies after 7 days of incubation ranged from 23 to 30 mm in diameter (Figures 3.1 and 3.2).

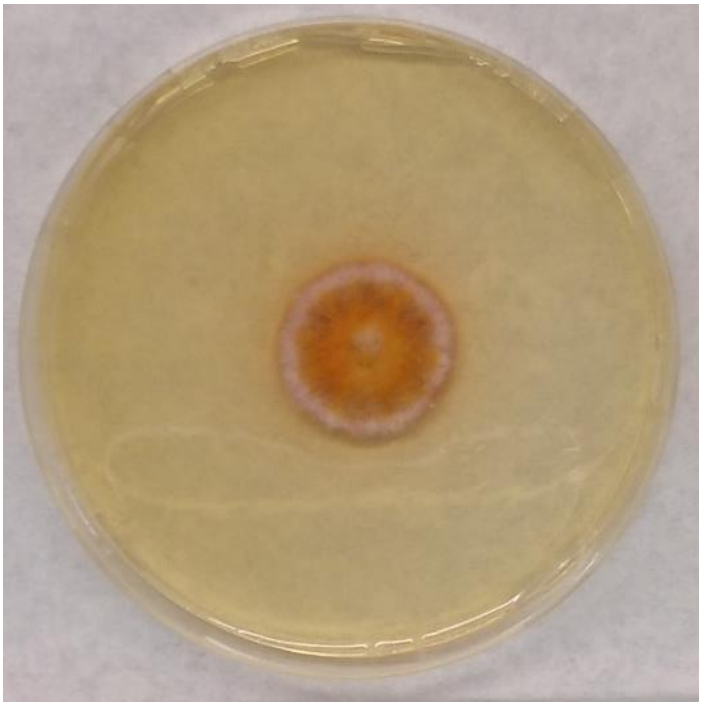


Figure 3. 1. *Monascus* spp. on Malt Extract Agar incubated at 30 °C for 7 days.

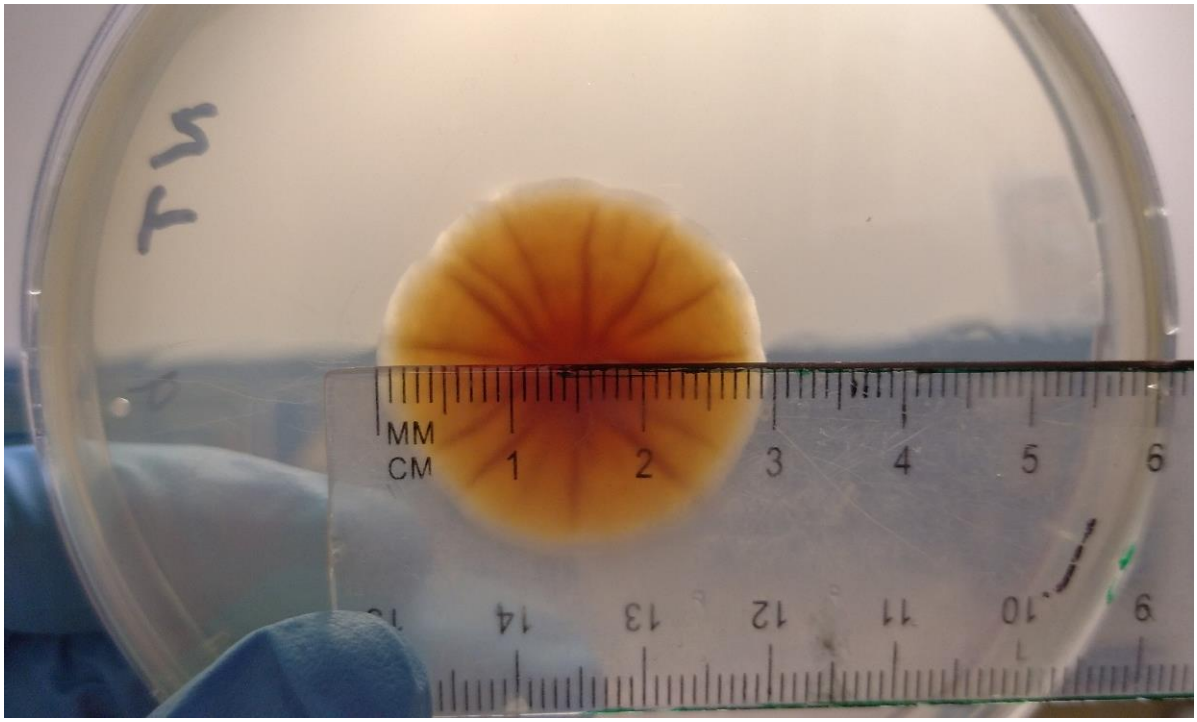


Figure 3. 2. Measurement of a *Monascus* spp. colony on Malt Extract Agar from the reversed side. The size of the colonies was in the range of 23–30 mm and in line with Pitt and Hocking (2009).

The microscopic results at 40 × magnification showed hyphae, conidia and cleistothecium ascomata (Figure 3.3). Cleistothecium ascomata were completely closed fruiting bodies with asci not regularly arranged (Samsudin, 2011). Two isolates from RFR that met these criteria were therefore regarded as *Monascus* spp.

3.3.2. Screening of CIT–producers of *Monascus* spp. in RFR

Any *Monascus* spp. that produced light blue fluorescence or a halo on CCA were categorized as CIT producers. Then, CIT–producers of *Monascus* spp. were sprayed with HCl to give an acidic environment to the media to enhance fluorescent change. CIT–producers of *Monascus* spp. detected by the presence of light blue fluorescence on CCA, was not observed on other media (MEA, CYA, and YES). However, *M. ruber* Tiegh ICMP 15220 (positive control) on MEA secreted a deep red pigment seen on the reverse side of the agar plate, while *P. glabrum* (Wehmer) Westling ICMP 5686 produced blue–grey colonies (negative control).

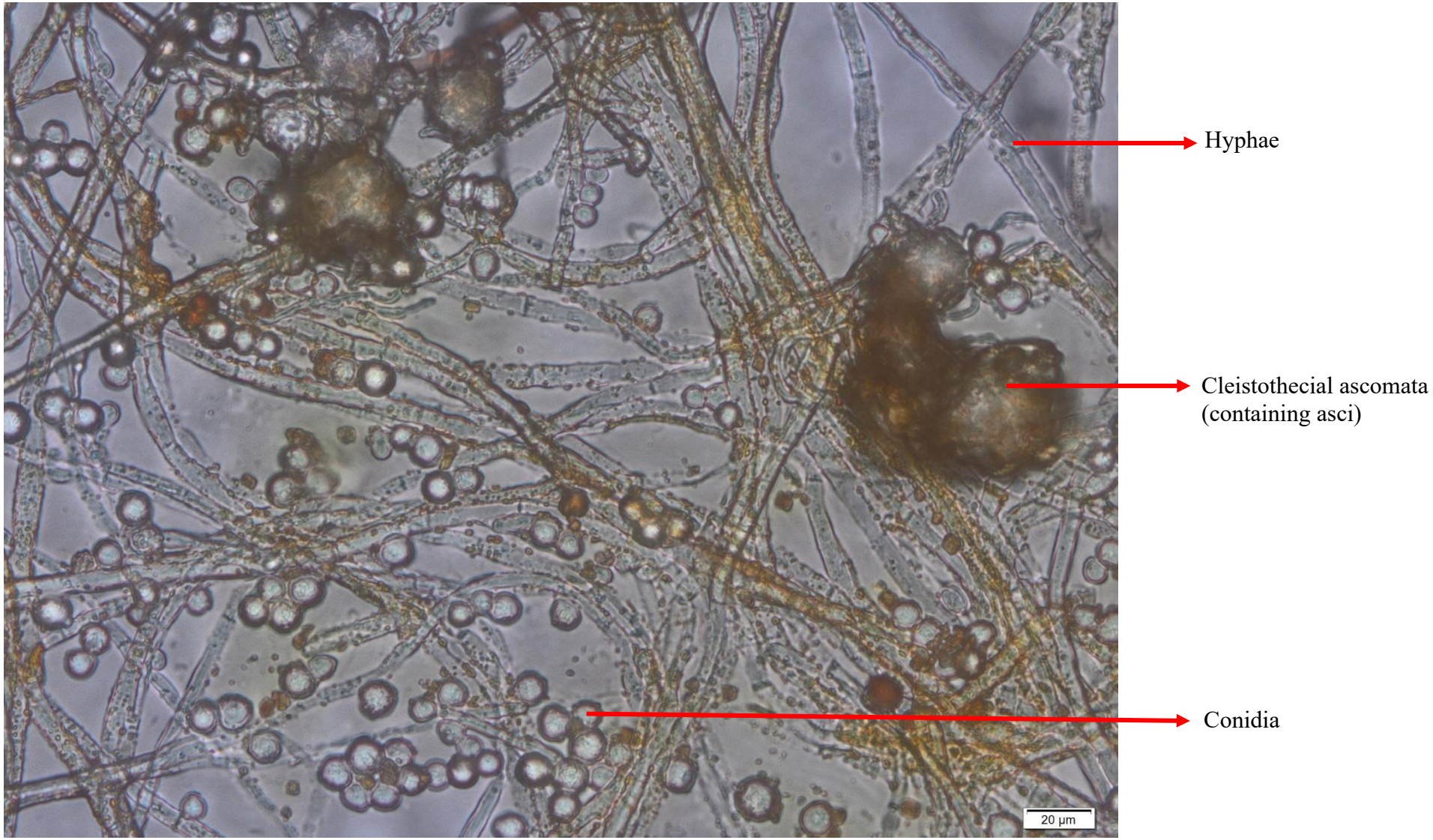
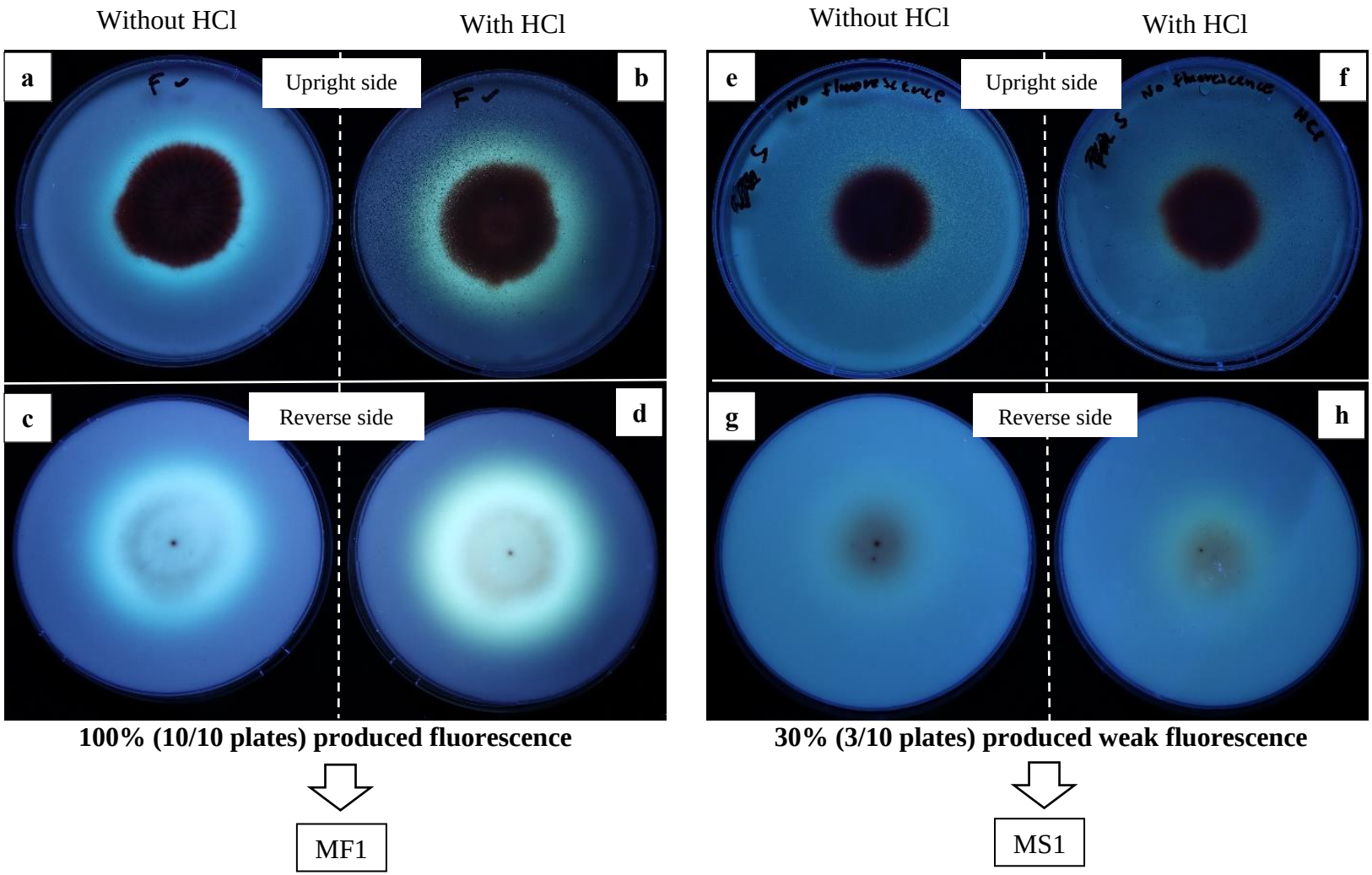


Figure 3. 3. Microscopic observation of *Monascus* spp. with 40 × magnification.

The *Monascus* spp. isolates were observed by the naked eye and under UV light from day 3 to day 5 of incubation. The *Monascus* spp. began to grow with a white colony that turned red on CCA on day 3, but fluorescence was only seen from the upright side of the agar. The optimum incubation time for the *Monascus* spp. was on day 4 when fluorescence was seen from both sides. On day 4, the *Monascus* spp. colonies from the upright side exhibited yellow fluorescence while light blue fluorescence was observed from the reverse side. On day 5, the red colour of the *Monascus* spp. became darker, and some of the colonies started to fade. Mohamed et al. (2013) observed that the intensity of the *P. citrinum* colonies on CCA and the size of the fluorescence ring around the colony increased until 7 days of incubation.

By using CCA and observing the colonies under UV light, three different isolates of *Monascus* spp. were isolated from RFR, namely strains MF1, MS1 and MW1, based on the colour of the colony and development of fluorescence on CCA. All the *Monascus* spp. isolates were CIT–producers. Both isolates MF1 and MS1 were red colonies, typical of *Monascus* spp. All (10/10) of the MF1 colonies produced light blue fluorescence on CCA faster than isolate MS1 after incubation for 4 days. Thirty percent of the MS1 colonies produced weak fluorescence on CCA plates (3/10) after incubation for 7 days (Figure 3.4). Isolate MW1 was a white colony and produced light blue fluorescence after incubation for 4 days. Since MW1 was a white colony (presumptive non–pigment producer) and produced light blue fluorescence (presumptive CIT producer), this isolate was excluded for further analysis. Other mycotoxins such as AF produce a different pastel blue fluorescence on CCA (Dyer & McCammon, 1994). The testing of *Monascus* spp. for CIT production on CCA has not been previously reported.



HCl: hydrochloric acid.

Figure 3. 4. Isolates of *Monascus spp.* on Coconut Cream Agar were observed under ultraviolet light after incubation at 30 °C for 7 days: (a, e) from the upright side; (b, f) from the upright side after spraying with hydrochloric acid; (c, g) from the reverse side; and (d, h) from the reverse side after spraying with hydrochloric acid. The isolates were selected and named MF1 and MS1.

The presence of CIT on CCA was confirmed by spraying HCl (10 mol/L) and observing the change in fluorescence colour and intensity (Mohamed et al., 2013). The results show the absence or presence of fluorescence of CIT standard (Figure 3.5), *M. ruber* Tiegh ICMP 15220 (Figure 3.6) (positive control), and isolates MF1 and MS1 (Figure 3.7).

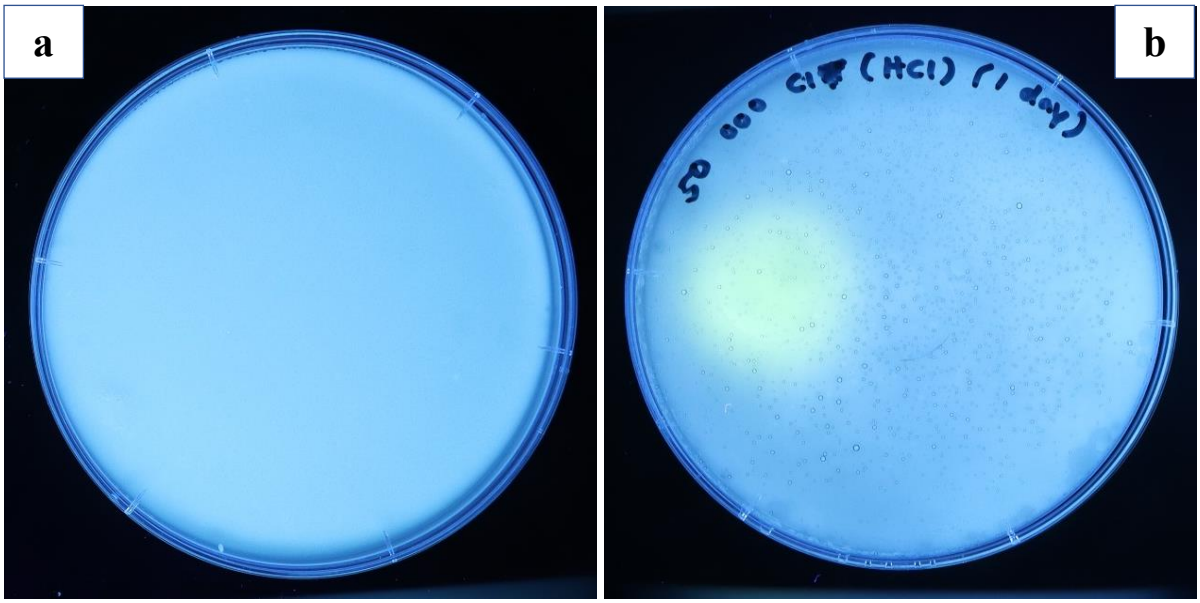


Figure 3. 5. (a) Negative control, (b) Yellow fluorescence from the upright side after 50, 000 ng/mL of citrinin standard was sprayed with hydrochloric acid.

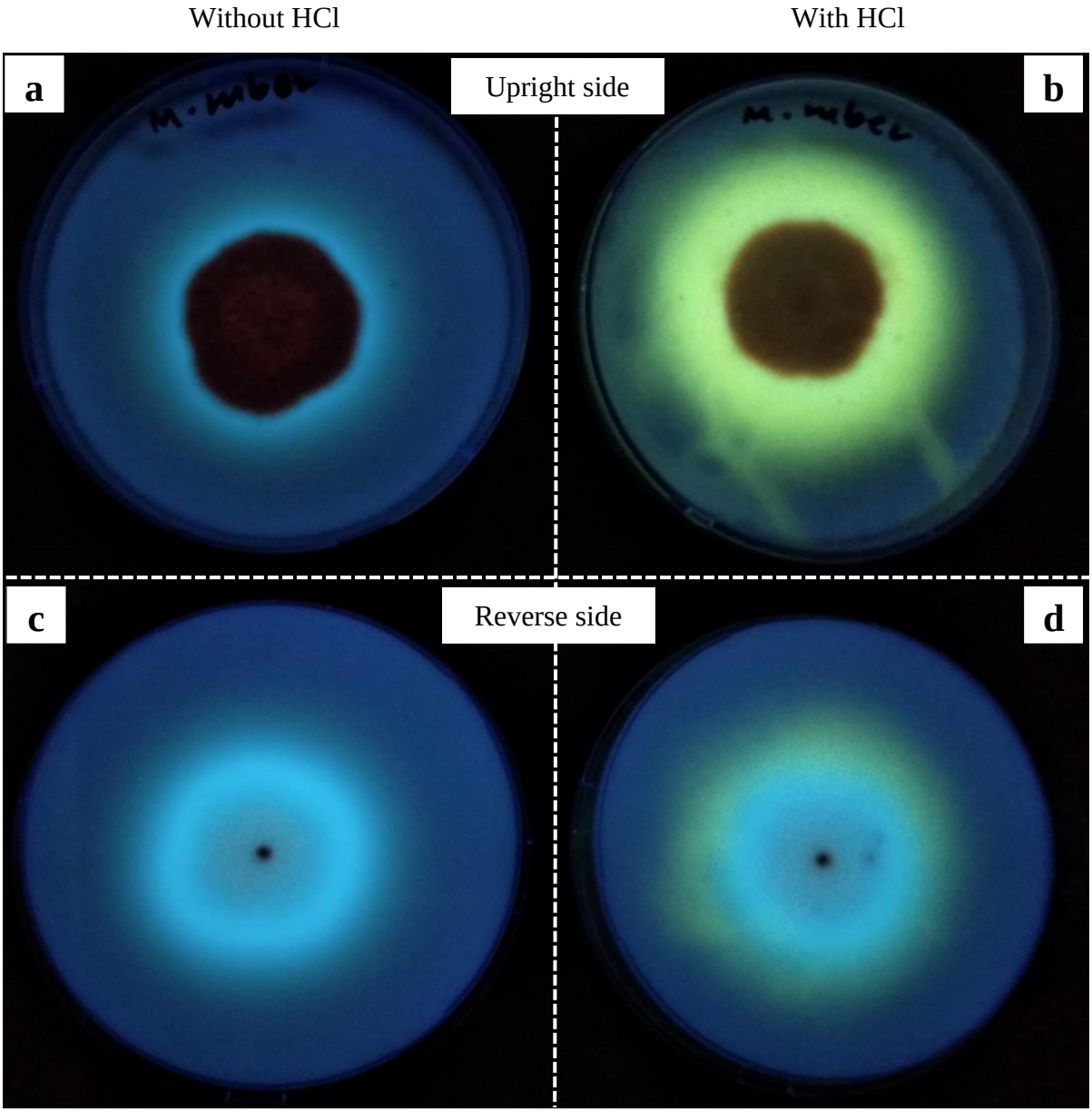


Figure 3. 6. ***Monascus ruber*** **ICMP 15220 (positive control) on Coconut Cream Agar observed under ultraviolet light after incubation at 30 °C for 7 days: (a) light blue fluorescence from the upright side; (b) larger yellow fluorescence from the upright side after spraying with hydrochloric acid; (c) light blue fluorescence from the reverse side; and (d) yellow–blue fluorescence from the reverse side after spraying with hydrochloric acid.**

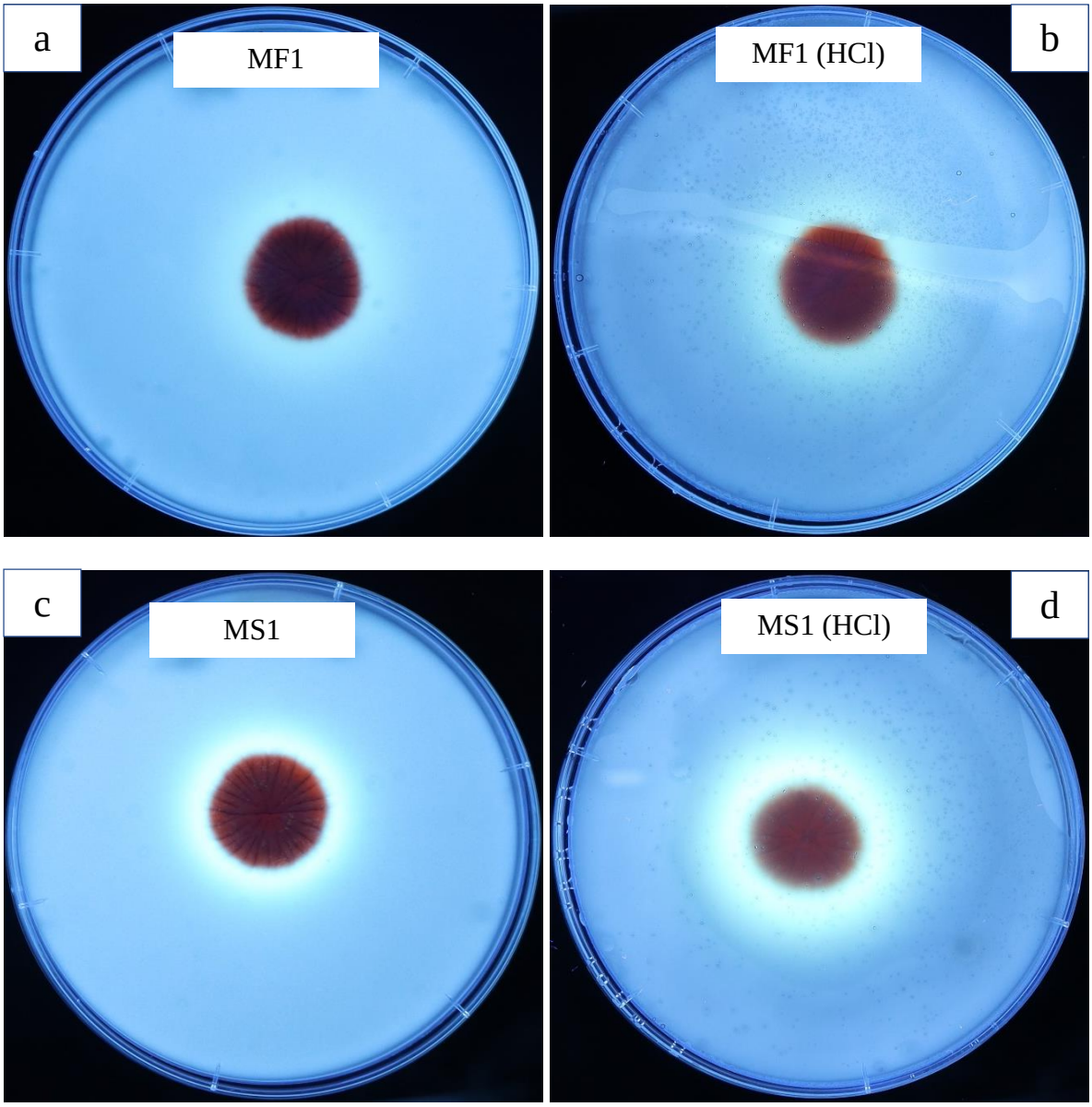


Figure 3. 7. Isolates of MF1 and MS1 from red fermented rice on Coconut Cream Agar were observed under ultraviolet light from the upright side after incubation at 30 °C for 7 days; (a) Weak light blue fluorescence around MF1; (b) The light blue fluorescence around MF1 changed to yellow fluorescence when sprayed with hydrochloric acid; (c) Strong light blue fluorescence from MS1; and (d) The fluorescence zone around the MS1 colony changed from light blue to yellow colour, as the size and intensity of the fluorescence increased.

CIT is an acidic lemon–yellow crystalline solution and changes colour from lemon–yellow at pH 4.6 to cherry red at pH 9.9 (Silva et al., 2021). CIT also has a conjugated (under neutral pH) and planar (under acidic pH) structure, that exhibits a natural fluorescence, but it is weak compared to other toxins. The non–ionized (planar) form of CIT is detected in the acidic environment, which is at its maximum at pH 2.5. The non–ionized form exhibits fluorescence because of the intermolecular hydrogen bond linking the phenol and keto functional group of the CIT, producing an increase in planarity (Vazquez et al., 1997).

Based on the red colony, the speed of growth (growth rate) and development of fluorescence on CCA, MF1 and MS1 were selected as the potential isolates to be used as the inoculum for the managed fermentation of rice to produce RFR. The growth rate of *Monascus* spp. is one of the important criteria for the preparation of an inoculum because it will affect the fermentation time of RFR. Generally, the fermentation of RFR requires several days to four weeks (Chen & Hu, 2005; Chen et al., 2015). Therefore, the stock cultures for these isolates were prepared.

3.3.3. Stock culture concentration

The concentration of MF1 and MS1 stock cultures were 10^4 conidia/mL determined by counting in a haemocytometer.

3.3.4. PCR amplification and gel electrophoresis of ITS, β–tubulin, *pksCT* and *ctnA* genes

The *Monascus* spp. is mainly identified by morphological and physiological features, but culture pigments, size and shape of the spores and colonies can be affected by the culture media and other factors. Therefore, it is important to confirm the *Monascus* spp. by using a molecular approach such as PCR.

PCR, gel electrophoresis, and nucleotide sequences were used to identify the MF1 and MS1 isolates and compare them with the positives (*P. expansum* ICMP 1178 and *M. ruber* Tiegh ICMP 15220) and negative control [*P. glabrum* (Wehmer) Westling ICMP 5686]. Table 3.3 shows the absence or presence of the bands in the gel electrophoresis as the indicators of the existence of the nucleotide sequences for specific genes. Jia et al. (2017) mentioned that it was insufficient to use 18S rRNA gene sequences and ITS–5.8S rRNA gene sequences to identify the species of *Monascus* spp. The β–tubulin gene sequences showed more accurate results with 100 % similarity when compared with public databases (Jia et al., 2017). Therefore, ITS and β–tubulin genes were used to identify the species of the fungi (Jia et al., 2017; Li et al., 2020; Park et al., 2004). Meanwhile, the *pksCT* and *ctnA* (a major activator for CIT biosynthesis) genes in *Monascus* spp., involved in the biosynthesis of CIT, can be used to identify the ability of the *Monascus* spp. to produce CIT (Li et al., 2020; Touhami et al., 2018; Xu et al., 2009). By gel electrophoresis, all the primer pairs for the isolates showed the presence of a single band except β–tubulin and *ctnA* of *P. glabrum* (Wehmer) Westling ICMP 5686.

Table 3. 3. Gel electrophoresis of the primers for the isolates.

Primers	**Isolates**				
	***M. ruber* ICMP 15220 (positive control)**	***P. expansum* ICMP 1178 (positive control)**	***P. glabrum* (Wehmer) Westling ICMP 5686 (negative control)**	**MF1**	**MS1**
ITS (gene for fungi)	+	+	+	+	+
β–tubulin (gene for fungi)	+	+	-	+	+
pksCT (biosynthesis–related genes)	+	+	+	+	+
ctnA (biosynthesis–related genes)	+	+	-	+	+

ITS: internal transcribed spacer. β–tubulin: beta–tubulin. *pksCT*: polyketide synthase.
+ indicates the presence of the band on the gel electrophoresis.
- indicates the absence of the band on the gel electrophoresis.

Fungi such as *Monascus* spp. can be identified using the ITS region. Both MF1 and MS1 isolates shared similar ITS regions (650 bp), and their sizes were larger than the positive and negative controls. Based on the ITS sequence, these isolates matched 100 % with those of *Monascus* spp. The ITS sequences from different types of *Monascus* spp. can be adjusted by the insertion or deletion of the bases in the genome of the fungi. The identification of the species based on the ITS sequence only is not totally reliable and difficult to differentiate from other species. Therefore, β–tubulin, which is a protein–coding gene, was also used as a secondary indicator for *Monascus* spp. (Li et al., 2020). *Monascus* spp. and *P. expansum* (positive control) had similar sized β–tubulin (1000–1500 bp), but the *P. glabrum* (negative control did not produce a band. When β–tubulin genes were used, the MF1 and MS1 were easily identified as *Monascus purpureus* with high sequence similarity. The sizes of ITS and

β–tubulin in this study were similar to Li et al. (2020), who reported the sizes of ITS to be between 500 and 700 bp, and β–tubulin in the range of 900–1200 bp.

For *pksCT* and *ctnA* genes, both MF1 and MS1 gave positive results. The sizes of PCR products of *pksCT* and *ctnA* were 500 bp and 1500–2000 bp, respectively. Li et al. (2020) reported that the *pksCT* gene was present in CIT–producers of *Monascus* spp., and the *ctnA* gene was absent in non–CIT producers. It was suggested that the if *pksCT* is successfully amplified and is functional by sequence analysis in *Monascus* isolates, then the same technique can be carried out to determine the presence of the *ctnA* gene. But if the amplification of *pksCT* is negative or *pksCT* becomes non–functional, then the amplification of *ctnA* is not necessary (Li et al., 2020). Shimizu et al. (2007) proved that *ctnA* is a major activator of CIT biosynthesis. The studies showed that disruption of *ctnA* significantly decreased the transcription of *pksCT* and *orf*, followed by reduction of CIT production. Li et al. (2020) reported that *M. ruber* varies in the presence of *pksCT* and *ctnA* genes. Out of nine *M. ruber* isolates, the *ctnA* gene was absence in five of isolates and these could not synthesis CIT efficiently. However, two *M. purpureus* isolates showed positive results for ITS, β–tubulin, *pksCT*, and *ctnA* genes, and both of the isolates can produce CIT (Li et al., 2020). The current study is in line with the findings of Li et al. (2020).

3.3.5. Sequence alignment and species identification

The BLAST search of the sequences of the PCR products from ITS, β–tubulin, *pksCT* and *ctnA* from MF1 and MS1 confirmed these isolates as *M. purpureus* with having CIT biosynthesis genes, with 98.04 to 100 % similarity (Table 3.4).

Table 3. 4. Accession numbers used for species identification and phylogenetic analysis.

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

ITS: internal transcribed spacer. β–tubulin: beta–tubulin. *pksCT*: polyketide synthase.
NA: Not applicable.
* The DNA sequences for these genes were deposited to GenBank databases.

3.3.6. Phylogenetic analysis

The Neighbor–Joining Tree was created based on the ITS and β–tubulin sequences to provide details of the phylogenetic relationships among *Monascus* spp. and several species of *Aspergillus* (Figures 3.8 and 3.9). It was observed that *M. ruber, M. pilosus, M. sanguineus, A. fumigatus, A. flavus,* and *A. parasiticus* were in the same clade with *M. purpureus* FJMR24, and *M. purpureus* isolates MF1 and MS1 (Figure 3.8). However, these *Monascus* and *Aspergillus* spp. were clearly separated with *M. purpureus* (Figure 3.9). The reference of *M. purpureus* (*M. purpureus* FJMR24) was grouped together with *M. purpureus* isolates MF1 and MS1 (Figure 3.9). The results showed that the Neighbor–Joining Tree based on the ITS sequence is insufficient to distinguish the *Monascus* spp. However, the Neighbor–Joining Tree based on the β–tubulin sequence provides better separation among *Monascus* spp.

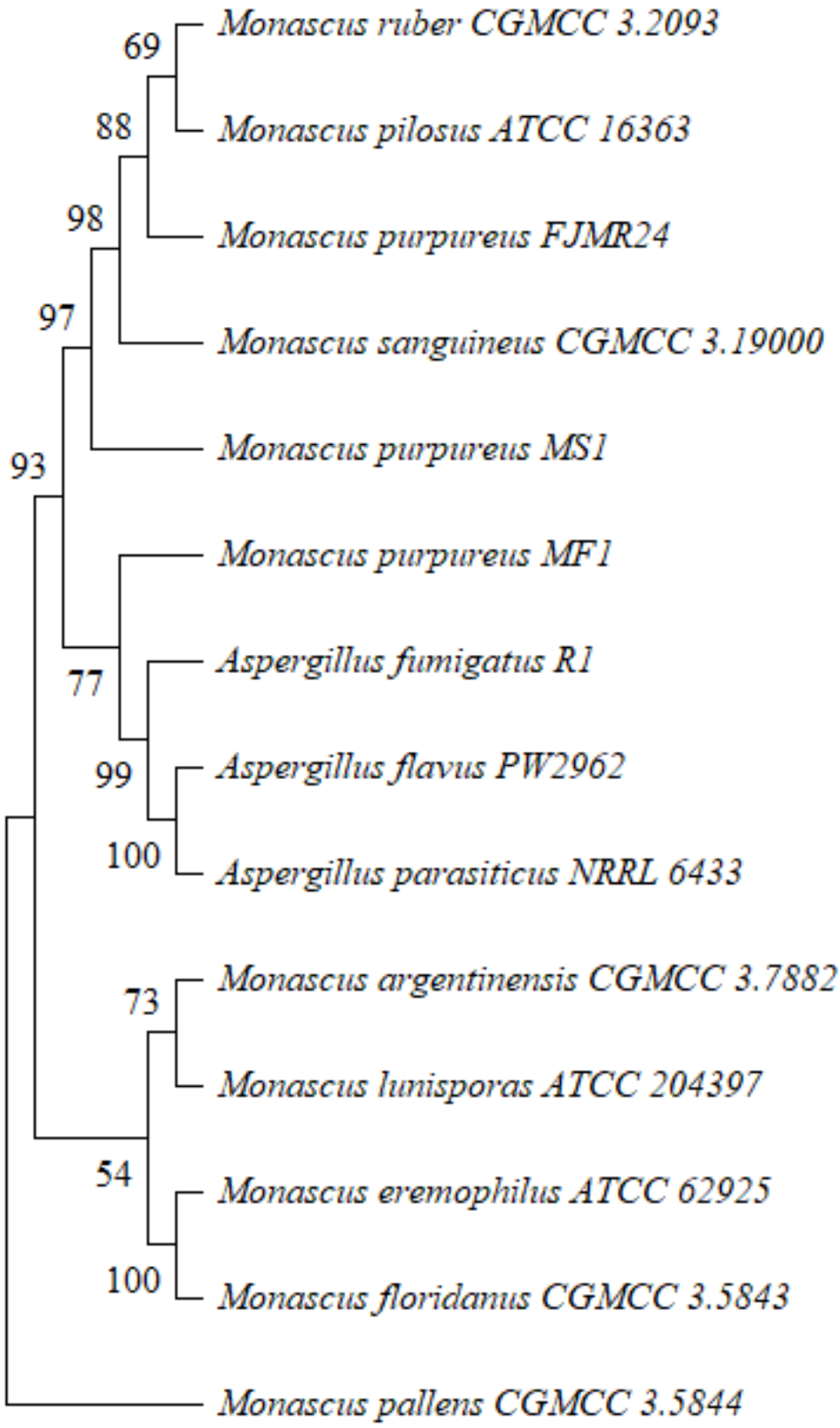


Figure 3. 8. Neighbor–Joining Tree showing the phylogenetic relationships among the *Monascus* and *Aspergillus* spp. based on the internal transcribed spacer sequences using the Kimura 2–parameter model. Values on branches are the bootstrap values.

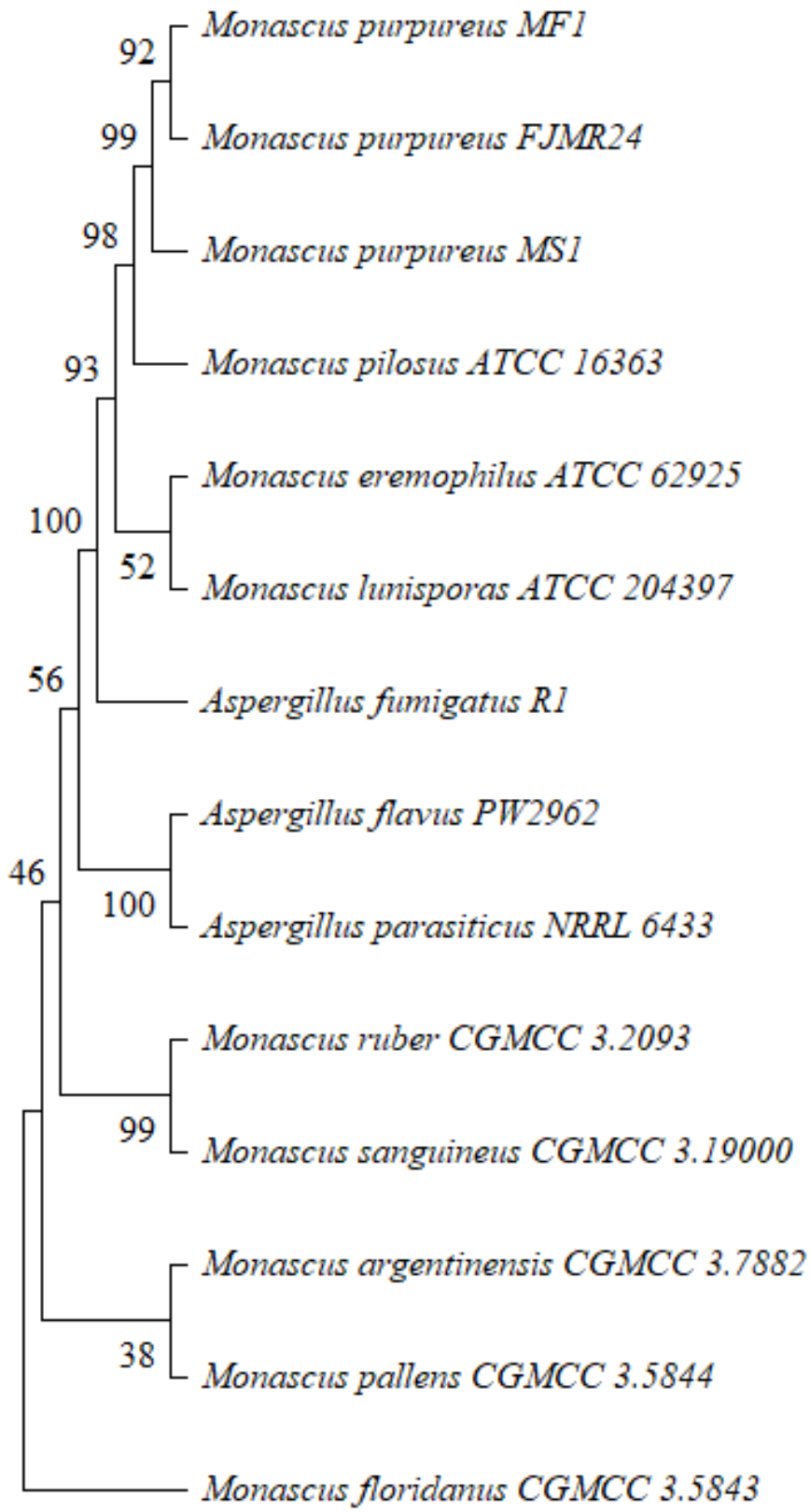


Figure 3. 9. Neighbor–Joining Tree showing the phylogenetic relationships among the *Monascus* and *Aspergillus* spp. based on the beta–tubulin sequences using the Kimura 2–parameter model. Values on branches are the bootstrap values.

3.3.7. CIT standard curve

Different concentrations of CIT standards were used to develop a standard curve for further quantification of CIT in the samples. The retention time was around 5.07 min for CIT standards. The calibration curve for CIT was good and can be used for quantification as the coefficient of determination (R^2) was 0.9999.

3.3.8. Confirmation of fluorescence from CIT by UHPLC–FLD

Table 3.5 shows the relationship between the presence or absence of the fluorescence on CCA with the CIT level analysed by HPLC. *P. glabrum* (Wehmer) Westling ICMP 5686 as negative control did not produce any fluorescence after incubating at 30 °C for 7 days, did not have the *ctnA* gene (one of the biosynthesis genes) and did not produce any peak using HPLC. Meanwhile, *M. ruber* Tiegh ICMP 15220 and *P. expansum* ICMP 1178 (positive controls), MF1 and MS1 produced light blue fluorescence on CCA and changed to yellow after spraying with HCl. Further analysis with the HPLC confirmed that MF1 and MS1 isolates produced 1.35 ± 0.37 and 1.25 ± 0.68 µg/mL of CIT, respectively, after incubation at 30 °C for 7 days. *M. purpureus* MF1 and MS1 isolates produced lower CIT than *M. ruber* Tiegh ICMP 15220.

The production of CIT is strain–dependent. Blanc et al. (1995b) reported that wild *M. ruber* produced higher CIT than wild *M. purpureus* in YES medium (SmF) and rice powder (SSF). Li et al. (2020) analysed the CIT produced by nine isolates of *M. ruber* and two isolates of *M. purpureus* by HPLC. The results showed that five of the *M. ruber* isolates produced no CIT while the other four isolates produced CIT in the range of 59–137 ng/g. However, *M. purpureus* produced higher CIT (8470–11, 064 ng/g) than *M. ruber*. Another study conducted by Pisareva et al. (2005) found that there was no CIT produced by three isolates of *M. purpureus*, while eight of the *M. purpureus* produced CIT between 59–74 µg/mL in broth culture. Three *M. ruber* isolates produced CIT within a similar range. One of the two *M. pilosus*

isolates do not produce CIT, while another *M. pilosus* isolate produced CIT with the concentration of 63 µg/mL.

Table 3. 5. Fluorescence on Coconut Cream Agar and citrinin levels analysed by Ultra–performance liquid chromatography with a fluorescence detector.

Isolates	Fluorescence on CCA (7 days)	CIT level (µg/mL)
M. ruber Tiegh ICMP 15220 (positive control)	+	153.25 ± 7.83^{a}
P. expansum ICMP 1178 (positive control)	+	35.12 ± 2.00^{b}
P. glabrum (Wehmer) Westling ICMP 5686 (negative control)	-	0^{c}
MF1	+	1.35 ± 0.43^{c}
MS1	+	1.25 ± 0.79^{c}

CCA: Coconut Cream Agar. CIT: citrinin. Number of samples, $n = 3$.
+ indicates the presence of fluorescence on Coconut Cream Agar.
- indicates the absence of fluorescence on Coconut Cream Agar.
* Different letters within the same column indicate significant differences ($p < 0.05$).

3.4. Conclusion

The present study identified two *Monascus* spp. isolates from RFR, MF1 and MS1, based on the development of fluorescence in CCA. Further analysis with macroscopic and microscopic observations, gel electrophoresis, and DNA sequences of PCR products confirmed both isolates of *M. purpureus* with *pksCT* and *ctnA* genes, indicating that these isolates can produce CIT. MF1 and MS1 produced fluorescence on CCA and UHPLC–FLD analysis confirmed CIT production. CCA can be used as a simple and rapid method to screen the *Monascus* spp. isolated from RFR for CIT production. This method may be used to select *Monascus* spp. that do not produce CIT for production of RFR.

CHAPTER 4

TOXICITY TEST OF CITRININ USING THE BRINE SHRIMP (*Artemia salina*) BIOASSAY

4.1. Introduction

CIT is one of the mycotoxins that can contaminate foods. CIT is produced by several fungi including *Aspergillus, Penicillium* and *Monascus* spp. (Flajs & Peraica, 2009; Lee et al., 2010a). CIT can contaminate a wide variety of foods including white rice, paddy rice, parboiled rice, brown rice, RFR, wheat, maize, fermented maize, cereals, rye, grain–based products, barley, oat, *ogi* (pudding made from maize–based fermented gruel), *Tom bran* (pudding formulated from several whole grains including maize, peanuts, wheat, soybean and millets), infant formula, industrially–processed complementary foods, black olives, apples, lager bees, tomato, tomato juice, pomaceous fruits, fruit and vegetable juices, bean, roasted nuts (pistachio nuts, peanuts, almonds, hazelnuts), herbs, spices (fennel, black pepper, turmeric, coriander, cumin, and cardamom), oilseeds (sunflower), fermented meat and dry meat products, cheese, sausages, *sufu* (fermented soybean products), cooked foods, ham, and snacks (Abramson et al., 2009; Abramson et al., 1996; Avula et al., 2014; Chen et al., 2016b; Flajs & Peraica, 2009; Kamle et al., 2022; Kpodo et al., 1996; Li et al., 2003; Li et al., 2012a; Liao et al., 2014; Marley et al., 2016; Ojuri et al., 2019; Samsudin & Abdullah, 2013; Silva et al., 2021; Tölgyesi et al., 2015; Xu et al., 2003).

CIT is believed to be nephrotoxic and fetotoxic, and is embryocidal (Flajs & Peraica, 2009). Lee et al. (2010a) conducted a study by subchronic administration in the diet at levels up to 100 mg/kg body weight per day RFR with 200 ppm CIT for 90 consecutive days in male Wistar rats. Interestingly, the results showed no toxic response in body weight, organ weight, clinical chemistry parameters, and histopathology of Wistar rats. Based on this finding, it is

suggested that *Monascus*–fermented products containing 2 ppm CIT are safe to consume (Lee et al., 2010a). The International Agency for Research on Cancer has categorized CIT as a group 3 carcinogen (not classifiable) due to the lack of data on carcinogenesis in humans and animals (Hou et al., 2021; IARC, 1987; Ostry et al., 2017).

Even though *in vivo* assays on laboratory animals have been widely used for toxicity tests (Hamidi et al., 2014), this method is time–consuming and expensive. Alternative biological assays for preliminary toxicity tests use *Artemia salina, A. urmiana, A. franciscana, A. sinica,* and *Thamnocephalus platyurus* (Carballo et al., 2002; El-Magsodi et al., 2016; Hipsher et al., 2021; Kim et al., 2009; Lu & Yu, 2020; Manfra et al., 2012; Mayorga et al., 2010; Mirzaei & Mirzaei, 2013; Ruiz-González et al., 2023; Shaukat et al., 2014; Veni & Pushpanathan, 2014). *Artemia* species such as *A. salina, A. urmiana, A. franciscana, and A. sinica* are commonly referred to as brine shrimp (Azra et al., 2022; Ntungwe N et al., 2020), meanwhile *T. platyurus* is freshwater fairy shrimp (Mayorga et al., 2010). Due to the sensitivity of brine shrimp to a variety of substances, the Brine Shrimp Assay has been widely used for the toxicity test for the past 40 years and more than 90 % of the studies use *A. salina* compared to other *Artemia* species (Hamidi et al., 2014; Ntungwe N et al., 2020; Parra et al., 2001).

A. salina is a zooplanktonic crustacean found in lakes and oceans. It is one of the popular food sources for many fish and aquatic invertebrates. *A. salina* is a popular model organism for toxicological tests as it is cheap, safe, simple, rapid, robust, has a short–life cycle, easy to culture, has commercially available cysts, high offspring production, year–round availability, feeding is not required during the assay, requires a small amount of sample for testing, has good repeatability, is efficient, and convenient (Banti & Hadjikakou, 2021; Hamidi et al., 2014; Naidu et al., 2014; Solis et al., 1993; Trompeta et al., 2019).

A. salina larvae (nauplii) is more sensitive to toxic agents compared to the adult *Artemia* (Banti & Hadjikakou, 2021; Trompeta et al., 2019). The toxicological tests using *A.*

salina can be conducted using many aspects such as during hatching, mortality, swimming, morphology, and biomarkers (Ates et al., 2013; Banti & Hadjikakou, 2021; Zhu et al., 2018; Živković et al., 2016).

A. salina nauplii has been used to detect a variety of toxic substances such as nanoparticles, natural extracts, plant extracts, metal complexes, heavy metals, pesticides, metal ions, cyanobacteria, algae, dental materials, marine natural products, bioactive molecules, and mycotoxins (Ates et al., 2013; Banti & Hadjikakou, 2021; Carballo et al., 2002; da Silveira Carvalho et al., 2017; Hamidi et al., 2014; Hartl & Humpf, 2000; Hlywka et al., 1997; Parra et al., 2001; Sharma et al., 2013; Solis et al., 1993; Zhu et al., 2018).

This study aimed to determine the toxicity of CIT using the mortality of brine shrimp.

4.2. Materials and methods

4.2.1. Materials and chemicals

The CIT standard and sea salts were purchased from Sigma–Aldrich. (MO, USA). A bottle of MeOH used was HPLC grade and supplied by Fisher Scientific (Belgium). The 6–well tissue culture plates with flat bottoms and low evaporation lids were obtained from Falcon (Durham, USA). A bottle of JBL Artemio Pur (Neuhofen, Germany) was purchased to provide top–quality *A. salina* eggs with a maximum hatching rate.

4.2.2. Preparation of CIT standards

One millilitre of methanol was added to the 5 mg of CIT standard to obtain a stock solution (5000 µg/mL). Five concentrations of CIT standards were prepared from this stock culture, which were 60, 90, 100, 110, and 150 µg/mL.

4.2.3. Toxicity of CIT by using brine shrimps

CIT toxicity of brine shrimp used a method developed by Nguyen et al. (2022) with minor modifications. Four percent of artificial seawater (pH: 6.70 ± 0.01) was prepared by mixing 40 g of sea salts in 1 L of distilled water. The 100 mL of 4 % seawater was prepared in three 250 mL conical flasks, and 100 mg of *A. salina* eggs were added to each conical flask. The conical flasks were shaken on a rotary shaker (200 rpm speed, 30 °C, 24 h) in the presence of lateral light to hatch the *Artemia* eggs.

On the same day, 1 mL of different concentrations of CIT standards were pipetted into each well of the culture plate (n = 9 for each CIT concentration). All the 6–well tissue culture plates containing CIT concentrations were dried overnight at 30 °C to remove MeOH from the solution. After 24 h (200 rpm speed, 30 °C), *A. salina* nauplii were hatched. A Pasteur pipette was used to transfer some of the solution containing *A. salina* nauplii to a small petri dish and separated manually from the eggs by pipetting *A. salina* nauplii to another small petri dish. One mL of distilled water and 1 mL of 4 % seawater were added to each well to dissolve CIT standards (final concentration: 30, 45, 50, 55, and 75 µg/mL of CIT standards in 2 % seawater). Wells containing 2 % seawater (n = 9) without any CIT were used as a control.

Ten *A. salina a* nauplii were added to each well and incubated again at 30 °C under fluorescence white bulb light for 24 h. The 6–well tissue culture plates need to be sealed with masking tape to avoid evaporation of the solution. After incubation for 24 h, the plates were observed under an aCOLade 2 colony counter (Symbiosis, Cambridge, UK) and the numbers of dead *A. salina* nauplii in each well were counted. The following equation was used to calculate the percentage of *A. salina* nauplii mortality rate:

$$\% \text{ Mortality} = \frac{\text{Total number of dead nauplii}}{\text{Total number of nauplii test}} \times 100\%$$

4.2.4. Statistical analysis

Nine measurements for every control and different CIT concentrations ($n = 3 \times 3$) were conducted in this experiment to obtain the mortality of brine shrimp. The medium lethal concentration (LC_{50}) of brine shrimp was estimated by Microsoft Excel.

4.3. Results and discussion

One of the important conditions for hatching the brine shrimp eggs is the pH of the seawater. Hamidi et al. (2014) suggested the optimal pH value of the seawater to hatch the brine shrimp eggs is 8.0 ± 0.5. In contrast to this study, the pH of 6.70 ± 0.01 was used to hatch the brine shrimp. In this study, the fluorescence of white bulb light was very important to keep the brine shrimp alive. The mortality of brine shrimp after being exposed to five different concentrations of CIT for 24 h is shown in Figure 4.1.

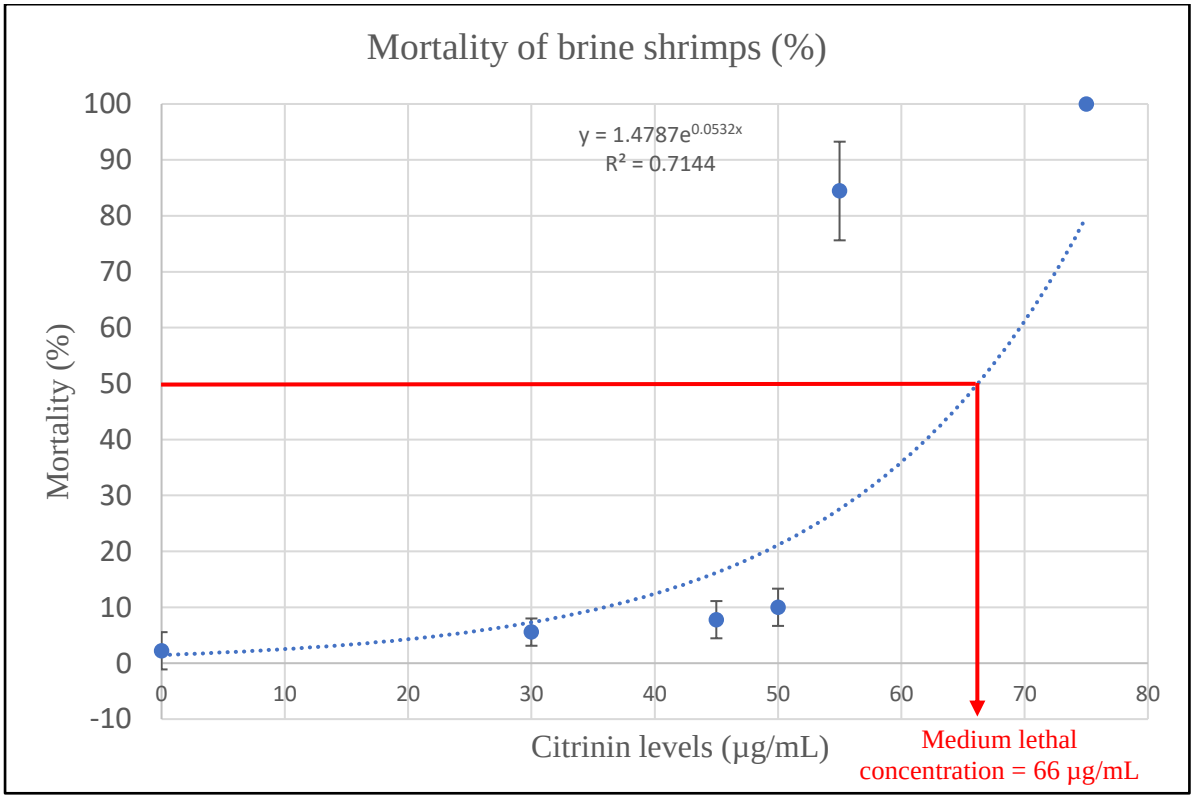


Figure 4. 1. Mortality of brine shrimp after exposure to citrinin standards and estimation of medium lethal concentration.

Based on Figure 4.1, the higher the concentration of CIT, the higher the percentage of mortality of brine shrimp. The LC_{50} of brine shrimp after being treated with CIT standards for 24 h is estimated at 66 µg/mL. Based on this result, the toxicity of CIT can be classified according to Meyer's and Clarkson's toxicity index (Clarkson et al., 2004; Meyer et al., 1982). Based on Meyer's toxicity index, the compounds with LC_{50} less than 1000 µg/mL are considered toxic, while compounds with LC_{50} more than 1000 µg/mL are considered non–toxic. Meanwhile, Clarkson has a broader range of toxicity index of the compound, which are: (1) LC_{50} from 0 to 100 µg/mL is highly toxic, (2) LC_{50} from 100 to 500 µg/mL is medium toxic, (3) LC_{50} from 500 to 1000 µg/mL is low toxic, and (4) LC_{50} higher than 1000 µg/mL is non–toxic (Alim et al., 2022; Clarkson et al., 2004; Hamidi et al., 2014). Therefore, the CIT toxicity test using brine shrimp in this study was categorized as toxic and highly toxic according to Meyer's and Clarkson's toxicity index, respectively.

Several studies have been conducted to compare the toxicity test by using brine shrimp and other methods. Parra et al. (2001) reported that there was a good correlation between the results of the medium lethal dose of mice (LD_{50}) and LC_{50} of brine shrimp (*in vivo* vs *in vitro*) to determine the toxicity of medicinal plants of the Cuban flora, which were (1) *Aloe vera* (L.) Burm. F. (Aloeaceae), (2) *Artemisia absinthium* L. (Asteraceae), (3) *Citrus aurantium* L. (Rutaceae), (4) *Cymbopogon citratus* (DC. Ex Nees) Stapf (Poaceae), (5) *Datura stramonium* L. (Solanaceae), (6) *Justicia pectoralis* Jacq. (Acanthaceae), (7) *Musa x paradisiaca* L. (Musaceae), (8) *Ocimum basilicum* L., (9) *O. gratissimum* L., (10) *O. tenuiflorum* L. (Lamiaceae), (11) *Orthosiphon aristatus* (Blume) Miq. (12) *Pimenta dioica* (L.) Merr. (Myrtaceae), (13) *Piper auritum* Kunth (Piperaceae), (14) *Plantago major* L. (Plantaginaceae), (15) *Plectranthus amboinicus* (Lour.) Spreng. (Lamiaceae), (16) *P. amboinicus* (Lour.) Spreng. (Lamiaceae) (aqueous extract), (17) *Ruta graveolens* L. (Rutaceae), (18) *Senna alata* (L.) Roxb. (Fabaceae), (19) *Stachytarpheta jamaicensis* (L.) Vahl (Verbenaceae), and (20) *Thuja*

occidentalis L. (Cupressaceae). Naidu et al. (2014) conducted a study to determine the toxicity of *Mentha spicata* (Lamiaceae) extracts by acute oral toxicity of rats and the brine shrimp lethality test showed that the *Mentha spicata* extract was non–toxic by applying both approaches. *Mentha spicata* is a garden mint known as "pudina", a perennial herb with a characteristic spearmint odour (Naidu et al., 2014). The results by Parra et al. (2001) and Naidu et al. (2014) showed that there was no significant difference in toxicity test by mice or rats and brine shrimp, and there was a good correlation between both toxicity tests.

The toxicity of other mycotoxins such as FB_1 has been studied using brine shrimp. Hlywka et al. (1997) used the chicken embryos and brine shrimp nauplii to determine the toxicity of FB_1, and the results showed that the chicken embryo was similar to the brine shrimp bioassay because both bioassays were sensitive to FB_1. However, the chicken embryo bioassay has limitations due to the high dose of FB_1 needed per egg. The toxicity of *Fusarium* mycotoxins such as FB_1, FB_2, hydrolysed fumonisin B_1 (HFB_1), hydrolysed fumonisin B_2 (HFB_2), *N*–palmitoyl–HFB_1, and *N*–carboxymethyl–fumonisin B_1 (NCM–FB_1) were conducted by the brine shrimp bioassay test and the results showed that FB_1 is the most toxic whereas NCM–FB_1 is the least toxic (Hartl & Humpf, 2000).

Abdel-Mallek et al. (1993) conducted research to determine the toxicity and mycotoxins production by different isolates of the *Penicillium, Fusarium,* and *Aspergillus* genera. Sixty–three isolates of *Penicillium, Fusarium,* and *Aspergillus* were isolated from sunflower seeds and corn grains. These isolates were screened qualitatively for mycotoxins production by TLC, and brine shrimp were used for toxicity tests. The level of toxicity was categorized as: (1) high toxicity, when the mortality of brine shrimp larvae is more than 75 %, (2) moderate toxicity, when the mortality of brine shrimp larvae is between 50–75 %, (3) low toxicity, when the mortality of brine shrimp larvae is between 25–49 %, and (4) non–toxic, when the mortality of brine shrimp larvae is less than 25 % were observed. Based on the results,

one isolate of each *A. candidus*, *A. terreus*, and *A. ustus* proved to be a CIT–producer. *A. candidus* has lower toxicity, but both *A. terreus* and *A. ustus* have moderate toxicity. Most *Penicillium* species produced CIT and had low toxicity. These included *P. jenseni*, *P. kapuscinskii*, *P. chrysogenum*, *P. citrinum*, and *P. steckii*. Only *P. corylophilum* had moderate toxicity.

4.4. Conclusion

Brine shrimp can be used to determine the toxicity of CIT. LC_{50} values of brine shrimps after being treated with CIT standards for 24 h was 66 µg/mL. Based on Meyer's toxicity index, LC_{50} less than 1000 µg/mL is considered toxic, meanwhile Clarkson categorised LC_{50} from 0–100 µg/mL as highly toxic (Clarkson et al., 2004; Meyer et al., 1982). Therefore, CIT is considered toxic and highly toxic according to Meyer's and Clarkson's toxicity indices, respectively. For future research, it is recommended to determine the toxicity of CIT extracted from *Monascus* spp. and RFR, respectively. However, the CIT needs to be purified by using an immunoaffinity column.

CHAPTER 5

RELATIONSHIP BETWEEN *Monascus purpureus* GROWTH, CITRININ, PIGMENT, AND pH ON COCONUT CREAM AGAR

5.1. Introduction

Monascus spp. is one of the filamentous and xerophilic fungi (Chen et al., 2015; Silbir & Goksungur, 2019). This fungus is classified in the family *Monascaceae*, the order *Eurotiales*, the subclass *Eurotiomycetidae*, and the class *Eurotiomycetes* (Chen et al., 2015). Twenty–nine *Monascus* species have been identified worldwide including *M. purpureus, M. ruber, M. pilosus, M. argentinensis, M. eremophilus, M. floridanus, M. lunisporas, M., sanguineus,* and *M. pallens* (Chiu et al., 2006; Shao et al., 2014). The most common *Monascus* spp. for the food and pharmaceutical industry are *M. pilosus, M. purpureus,* and *M. ruber* (Chen et al., 2015). *M. pilosus* and *M. purpureus* are usually used to produce pigments, while *M. ruber* is used to decompose several foods (de Carvalho et al., 2005).

Pigments are usually used as food colourants and can be classified as synthetic and natural pigments. One of the sources of natural pigments is microbial fermentation using *Monascus* spp. (Abdul-Manan et al., 2017a). Pigments are secondary metabolites produced by *Monascus* spp. through SSF or SmF (Farawahida et al., 2022b; Srianta et al., 2014). *Monascus* spp. have been used to produce RFR, *Monascus*–fermented soybean, *Monascus*–fermented dioscorea, Chinese cheese, wine, sake, bagoong, tofu, miso, and sausage (Srianta et al., 2014).

There are six molecules of natural pigments produced by *Monascus* spp., which are yellow (ankaflavin and monascin), orange (monascorubrin and rubropunctatin), and red (monascorubramine and rubropunctamin) pigments. These pigments are categorized as azaphilones. Other fungal genera that can produce azaphilones pigments are *Aspergillus, Penicillium, Pleosporales, Chaetomium, Hypoxylon, Talaromyces, Muycopron,* and

Phomopsis. However, *Monascus* spp. are prolific pigment producers (Afroz Toma et al., 2023). Among these pigments, red pigments are the most interesting in the food industry due to their natural colour in food such as fish, ketchup, meat, and liquor (Hamano et al., 2005).

However, *Monascus* spp. also can produce CIT, a mycotoxin that is hepatonephrotoxic (Blanc et al., 1995a; Li et al., 2020). The International Agency for Research on Cancer has categorized CIT as a group 3 carcinogen (not classifiable) due to the limited data that CIT can causes cancer in humans and animals (Hou et al., 2021; IARC, 1987). Other fungi that can produce CIT are *Aspergillus* spp. and *Penicillium* spp. (Doughari, 2015).

The most common culture media used for *Monascus* spp. are Rose Bengal Agar, PDA, Dichloran Rose Bengal Chloramphenicol (DRBC) agar, and MEA (de Carvalho et al., 2005; Dogra & Kumar, 2017; Samsudin & Abdullah, 2013; Samsudin & Abdullah, 2014). These media are incubated either at room temperature, or 29 °C to 35 °C for 7–10 days to grow *Monascus* spp. that are used as an inoculum to produce RFR (Chairote et al., 2009; Chairote et al., 2008; Dogra & Kumar, 2017; Patcharee et al., 2007). RFR is usually fermented at 25–30 °C for 2–4 weeks (Chen et al., 2015; Dogra & Kumar, 2017; Li et al., 2003).

According to Arseculeratne et al. (1969), coconut is an excellent substrate for AF production due to its fat content. This study reported that *A. flavus*, one of the filamentous fungi hydrolysed fat in the coconut to glycerol. Glycerol is an important carbon source for growth and AF production by *A. parasiticus* (Davis & Diener, 1968). Dyer and McCammon (1994) introduced a new medium, CCA, which is easily prepared with coconut cream. Coconut cream is a concentrated cream extract from the freshly grated kernel of the mature coconut. CCA is easily prepared by mixing 50 % coconut cream, distilled water, and 1.5 % microbiology agar before being sterilized at 121 °C for 15 min. The advantage of using CCA is CCA has a white background which promotes visualization of blue or blue–green fluorescence in the agar under UV light (Lin & Dianese, 1976).

CCA has been used as a screening method to detect mycotoxins such as AF, OTA, and CIT (Dyer & McCammon, 1994; Farawahida et al., 2022a; Heenan et al., 1998; Mohamed et al., 2013). The mycotoxins produced by these fungi were detected by the presence of fluorescence on CCA. The presence or absence of fluorescence on CCA surrounding the growing fungi was determined by observing the CCA under UV light. CCA has been used to detect AF production by *Aspergillus* spp. *A. flavus* produced pastel blue fluorescence while *A. parasiticus* and *A. nomius* exhibited bluish–white fluorescence. OTA produced by *A. carbonarius* and *A. niger* can be detected by using this medium (Heenan et al., 1998). This method has also been used to detect CIT produced by *Monascus* spp. (Farawahida et al., 2022a) and *P. citrinum* (Mohamed et al., 2013). AF, OTA, and CIT are mycotoxins commonly produced by *Aspergillus*, *Penicillium*, and *Monascus* spp. These fungi are filamentous fungi and contain mycelium (Abraham et al., 2022; Doughari, 2015; Kamle et al., 2022). Camardo Leggieri et al. (2020) reported 90 % of the mycotoxins produced on grana cheese is available in the mycelium of *Penicillum* spp. such as *P. crustosum*, *P. nordicum,* and *P. roqueforti.*

pH is one of the factors affecting the fungal growth and pigments production of *Monascus* spp. (Farawahida et al., 2022b). However, there is no information available in the literature about the relationship between the growth of *Monascus* spp., CIT, pigments, and pH after prolonged incubation. Thus, this study was conducted to characterize the growth, CIT, pigments, and pH of *M. purpureus* on CCA as a rapid method for indicating CIT production. Based on the authors' knowledge, this is the first study to measure the fungal growth, CIT, pigments, and pH of *M. purpureus* on CCA for a prolonged incubation.

5.2. Materials and methods

5.2.1. Source of samples

Refer to Section 3.2.1 for the source of samples.

5.2.2. Materials and microorganisms

The CIT standard was purchased from Sigma–Aldrich. All solvents used were HPLC grade. Solvents were supplied by Merck (Darmstadt, Germany). The formulation to prepare CCA was by using one litre of distilled water and UHT coconut cream, respectively, and this mixture was added to 30 g of Microbiology Agar–Agar prior to autoclaving. To prevent the growth of bacteria, 1 mL of chloramphenicol with the concentration of 0.05 g/L was added to the media. MF1 and MS1 were isolated from RFR according to the method of Farawahida et al. (2022a). These isolates have been deposited to the ICMP, Landcare Research, New Zealand for public strain collection and are labelled as ICMP 25182 and ICMP 25183, respectively. Macroscopic and microscopic examination, PCR, gel electrophoresis, and DNA sequences were conducted as described by Farawahida et al. (2022a) and the isolates were recognized as *M. purpureus Went.* These isolates were used for the current study.

5.2.3. Screening of CIT–producers of *Monascus* spp. isolates in RFR

Refer to Section 3.2.4. for screening *Monascus* spp. isolates from RFR that can produce CIT. Two *Monascus* spp. were isolated, namely MF1 and MS1.

5.2.4. Preparation of inoculum and stock culture

Refer to Section 3.2.5 for the preparation of inoculum and stock culture. The concentration of the inoculum for both isolates was diluted to 10^4 conidia/mL.

5.2.5. Fungal growth of *M. purpureus* isolates

MF1 and MS1 isolates (10 µl) were inoculated on the centre of CCA plates with two batches for each isolate (six CCA plates per respective day for each isolate) (Figure 5.1). All the CCA were sealed in zip lock plastic bags, put in large paper boxes, and incubated at 30 °C for 30 days. The CCA plates were observed daily to determine if visible growth has started. The CCA plates were examined visually for colony morphology and pigmentation. The sizes of colonies, sizes of fluorescence region around the colony on the upright side (ZU), sizes of fluorescence on the reversed side (ZR), and the mass of fungi were recorded. The long wavelength UV light at 365 nm in a dark cabinet was used to observe the size of ZU and ZR as an indicator of CIT production. The sizes of colonies, ZU and ZR were measured with a ruler by diameter measurements along two perpendicular directions (Figure 5.2). The intensity of fluorescence produced by both *M. purpureus* isolates was estimated by the number of "+" signs. The mass of fungi was measured by scraping mycelium from the CCA plates by a spatula and weighing using a balance. All the measurements were carried out for 30 days.

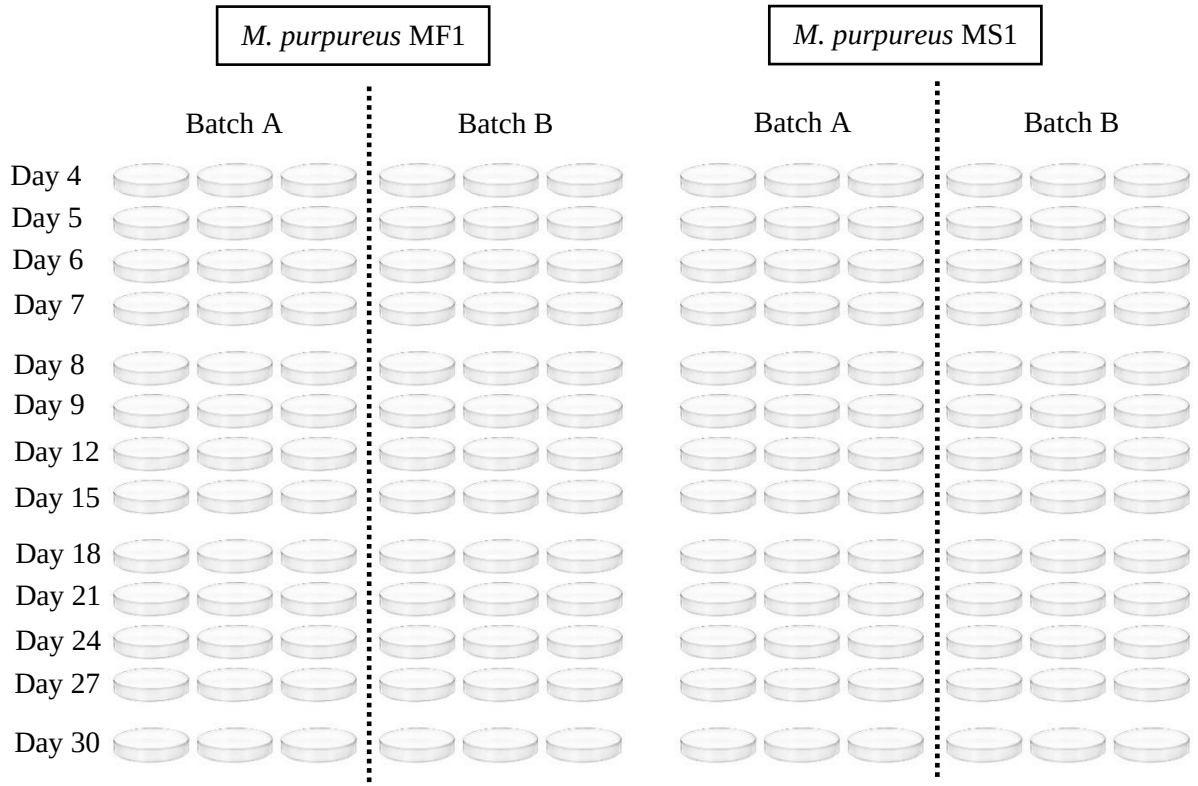
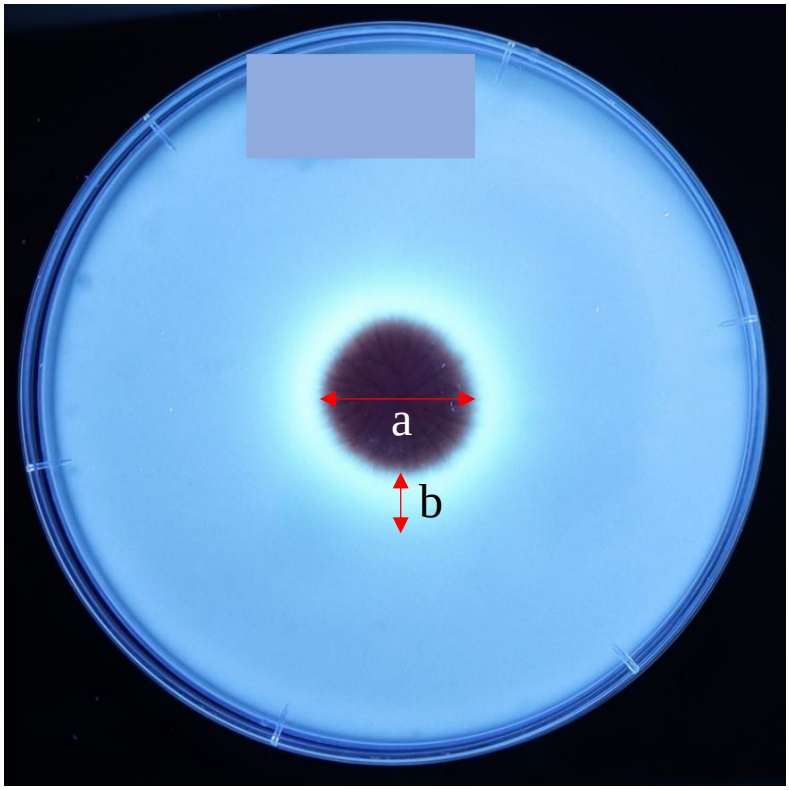
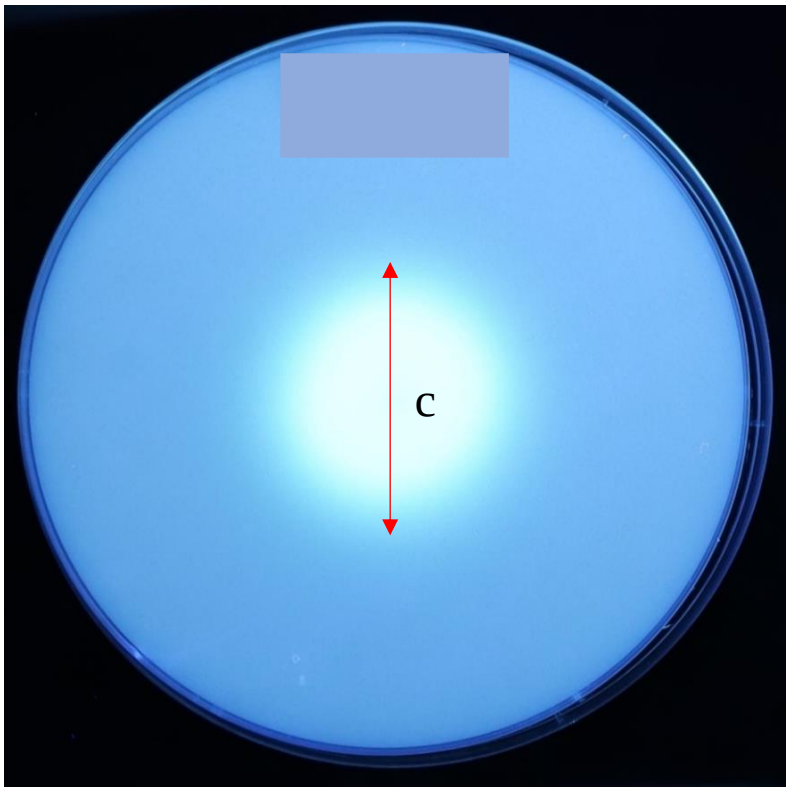


Figure 5. 1. The schematic diagram of the experiments with *Monascus purpureus* MF1 and MS1 isolates on Coconut Cream Agar. These experiments were conducted independently: (a) measurement of the fungal growth, (b) determine citrinin levels, and (c) determine pigments. All the Coconut Cream Agar plates were incubated at 30 °C for 30 days.



Upright side



Reversed side

Figure 5. 2. The fungal growth of *Monascus* spp. isolate on CCA was measured, (a) the size of the colony from the upright side, (b) the size of fluorescence region around the colony on the upright side (ZU), and (c) the size of fluorescence on the reversed side (ZR).

5.2.6. CIT extraction

Ten millilitres of MF1 and MS1 isolates were inoculated onto CCA plates with two batches for each isolate (six CCA plates per respective day for each isolate) (Figure 5.1). All the CCA were sealed in zip lock plastic bags to avoid the CCA plates drying. All the plates were put in the boxes to avoid the light and incubated at 30 °C for 30 days.

To extract the CIT from the isolates, the method from Farawahida et al. (2022a) was applied. A sterile spatula was used to scrape the mycelia of MF1 and MS1 and weighted individually (1 g). After transferring the mycelia to a 120 mL container, 5 mL of 50 % ethanol (ethanol: water, v/v) was added and the mixture was agitated on a rotary shaker for 1 h at 25 °C with the speed 200 rpm. The mixture was centrifuged for 10 min at the speed 3000 x *g*. A Minisart® NML syringe filter with the pore size 0.2 µm was used to filter the supernatants before detection by UHPLC–FLD (Dionex Ultimate 3000).

5.2.7. Preparation of a standard curve for CIT determination

Five milligrams of standard (Sigma–Aldrich) was dissolved in 10 mL of MeOH to obtain 0.5 mg/mL. These concentrations were prepared and injected to the UHPLC–FLD: 4, 50, 100, 500, 1000, 2000, and 3000 ng/mL. A 7–point calibration curve was constructed to quantify CIT in the samples.

5.2.8. Determination of CIT by UHPLC–FLD

The UHPLC–FLD was used to determine CIT in the MF1 and MS1 isolates (Farawahida et al., 2022a). A Kinetex C_{18} column (4.6 mm × 250 mm, i.d., 5µm) was used as a stationary phase. The column temperature was kept at 30 °C. A mixture of ACN and 0.1 % TFA in water with the ratio of 55:45 (v/v) was used as the mobile phase, and the flow rate was 1.0 mL/min. Fluorescence detection was performed at 330 nm excitation wavelength and 500

nm emission wavelength. The injection volume was 20 µL, and the column temperature was set at 30 °C. Triplicate measurements were conducted and Chromeleon 7.0 software was used to process the data.

5.2.9. Determination of pigments

Both *M. purpureus* isolates were inoculated (10 µl) on CCA plates with two batches for each isolate (six CCA plates per respective day for each isolate) (Figure 5.1). The CCA plates were put in the zip lock plastic bags and sealed. Then, the plates were put in the box and incubated in an incubator at 30 °C for a month. The pigments were determined daily from day 4 until 9, followed by three days intervals until the end of incubation of CCA plates (30 days).

The method from Marič et al. (2019) with minor modifications was used to estimate pigment production produced by *M. purpureus* isolates on CCA. By using a sterile spatula for each sample, the fungal mycelium was scraped from the centre of the CCA plates. One gram of the mycelium for each sample was transferred to a 120 mL container and mixed with 10 mL 60 % ethanol solution (ethanol: water, v/v). The extraction was carried out on a shaker at 25 °C for 24 h at 200 rpm speed. The mycelium was removed and a Minisart® NML syringe filter (0.2 µm pore size, Sartorius, Göttingen, Germany) was used to filter the supernatant liquid. Two hundred microlitres of the filtrate for each sample was pipetted into NUNC MaxiSorp 96–well microplate (Thermo Fisher Scientific, USA), subjected to a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Vantaa, Finland) and analysed by SkanIt Software 6.0.1.6. The wavelengths were set at 400 nm, 460 nm, and 500 nm for yellow, orange, and red pigments, respectively (Davoudi Moghadam et al., 2019; Kongruang, 2011). The 60 % of ethanol solution (ethanol, water, v/v) was used as a control. This formula was used to convert the absorbances into pigments yield:

$$\text{Pigments yield} = \frac{\text{Optical density (OD)} \times \text{dilution} \times \text{volume of extract (mL)}}{\text{Amount of sample (g)}}$$

5.2.10. Determination of pH

One millimetre of the filtrate from section 5.2.9 was used to determine the pH of the samples. A pH meter (Mettler–Toledo, Schwerzenbach, Switzerland) was used to measure the pH of each sample.

5.2.11. Statistical analysis

Mean and standard deviation of the data were calculated by triplicate measurements. All data and graphs were analysed and plotted with Microsoft Excel. The results for the differentiation between the samples were analyzed using mean and standard deviation and submitted to one–way ANOVA followed by Fisher LSD Method (5 % significant) using MINITAB (Version 21).

5.3. Results

5.3.1. Fungal growth of *M. purpureus* isolates

After inoculating 10 µl each of MF1 and MS1 on CCA, the fungal growth of *M. purpureus* isolates was observed visually for 30 days. The visible growth of *M. purpureus* for both isolates was noticed by the appearance of a white colony on CCA after 3 days of incubation. The colonies changed to orange colonies on day 4, therefore the photos of colonial morphologies and the fungal growth of *M. purpureus* for both isolates were recorded from day 4. Figure 5.3 shows the colonial morphologies of *M. purpureus* isolates on CCA starting from day 4 to 30 days, while Table 5.1 shows the fluorescence intensity observed on CCA by MF1 and MS1 isolates.

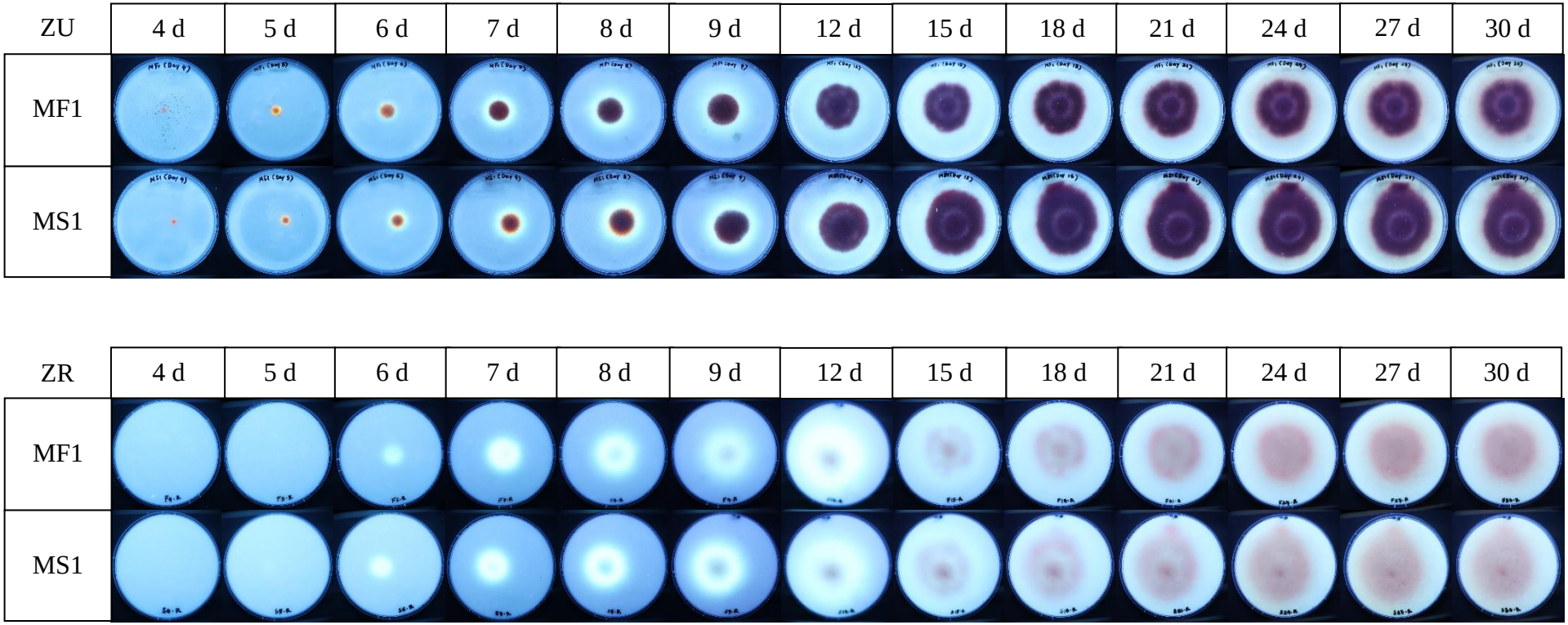


Figure 5. 3. Colonial morphologies of *Monascus purpureus* isolates MF1 and MS1 on Coconut Cream Agar over 30 days incubation at 30 °C.

Table 5. 1. Fluorescence intensity of *Monascus purpureus* isolates MF1 and MS1 on Coconut Cream Agar at 30 °C during 30 days of incubation.

Days	MF1		MS1	
	ZU	ZR	ZU	ZR
4	-	-	-	-
5	-	-	-	-
6	++	++	+++	+++
7	+++	+++	+++	+++
8	+++	+++	+++	+++
9	+	++	+++	+++
12	-	++	+	++
15	-	-	-	-
18	-	-	-	-
21	-	-	-	-
24	-	-	-	-
27	-	-	-	-
30	-	-	-	-

ZU: the size of fluorescence region around the colony on the upright side.
ZR: the size of fluorescence on the reversed side.
-: no fluorescence.
+: weak fluorescence.
++: medium fluorescence.
+++: strong fluorescence.

5.3.2. Colour and size of colonies of *M. purpureus* isolates

The colony diameters for MF1 and MS1 were measured from day 4 to 30, and a graph was plotted against incubation time (Figure 5.4). In this study, the size of *M. purpureus* colonies increased over the incubation period. On day 3, MF1 and MS1 on CCA started to grow by producing mycelium in white colour. The mycelium for both *M. purpureus* isolates changes to orange colour from day 4 until 6. After 4 days of incubation, MF1 grew faster than MS1 based on the colony diameter.

In the present study, on day 7, the mycelium of MF1 completely change to dark red, meanwhile, the mycelium of MS1 changed to orange red from days 7 and 8, followed by dark red on day 9. However, the size of the MS1 colony (2.70 cm) was larger than MF1 (2.67 cm) after 8 days of incubation.

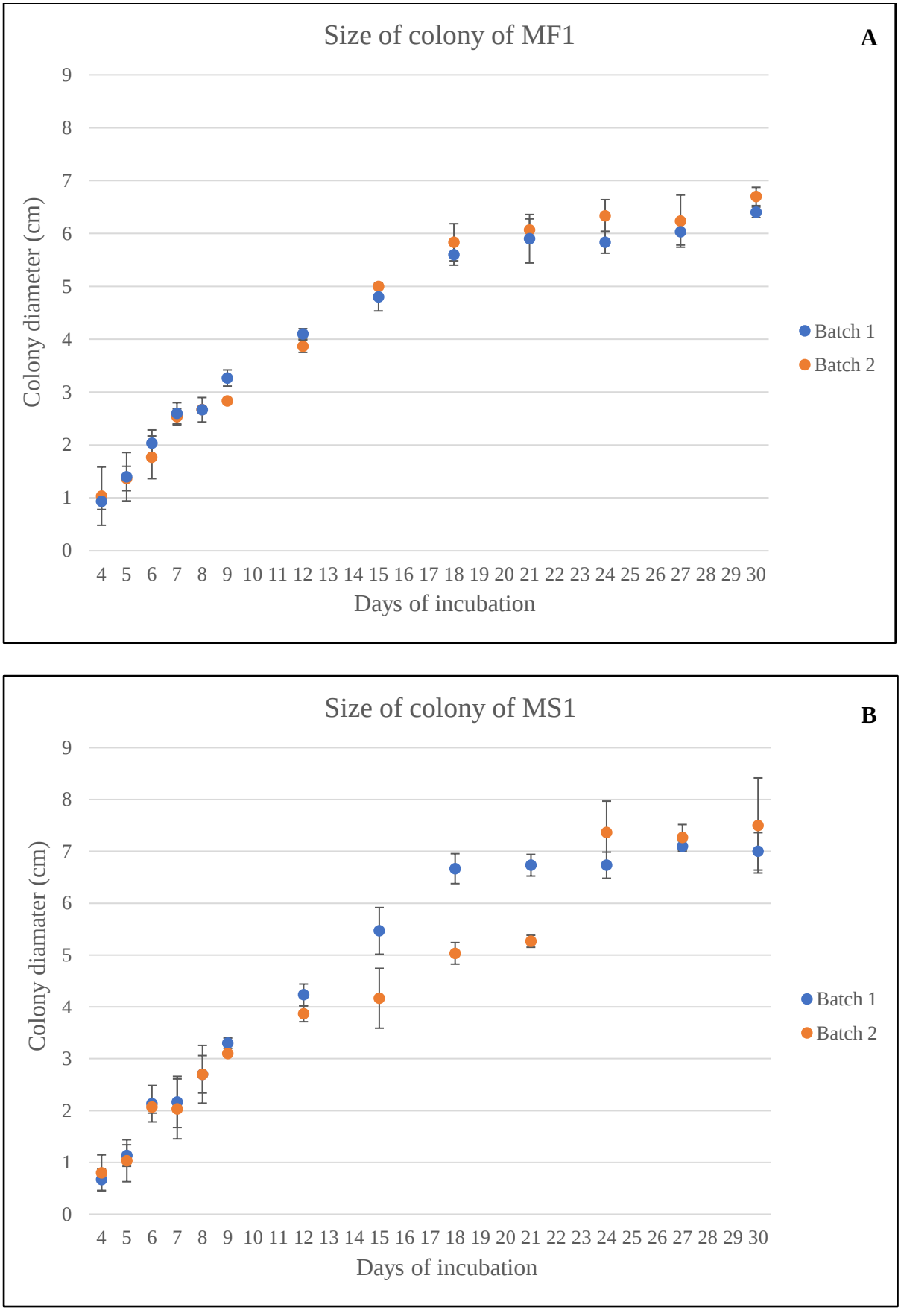
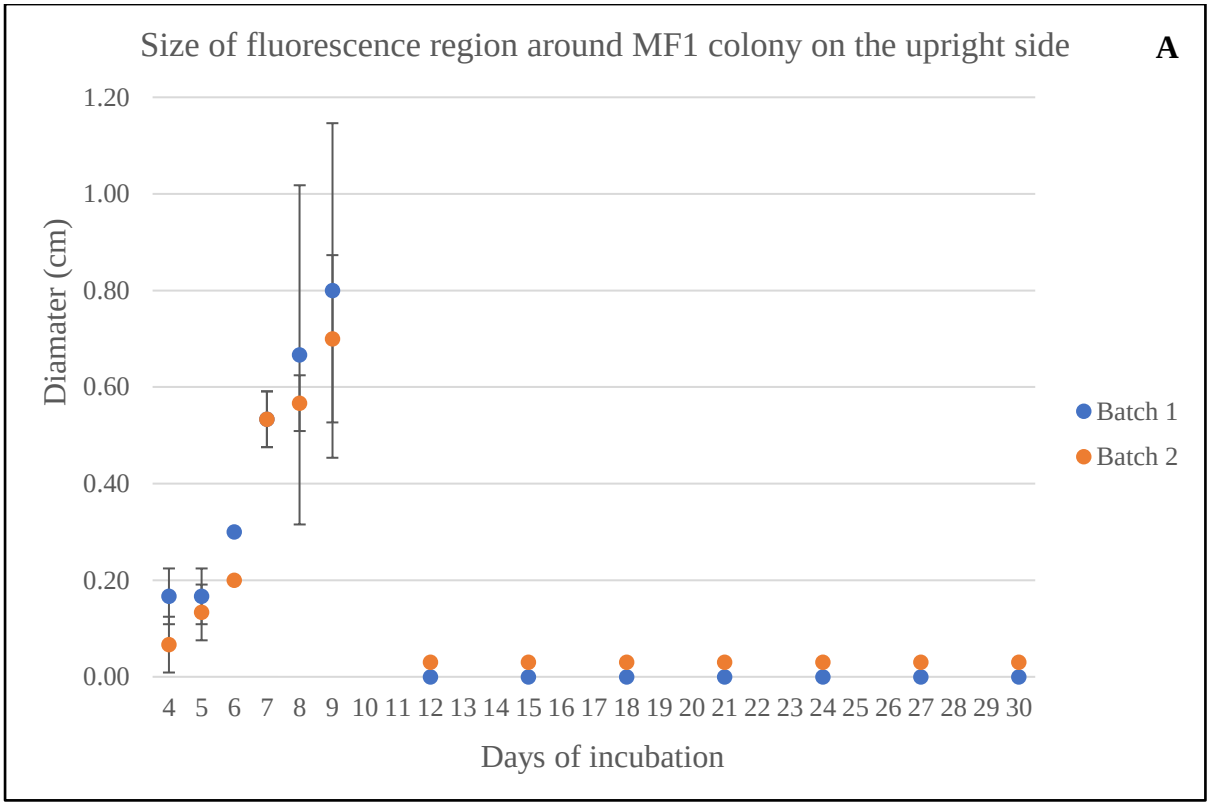


Figure 5. 4. Size of colonies of *Monascus purpureus* isolates (A) MF1 and (B) MS1 after incubation of Coconut Cream Agar at 30 °C for 30 days.

5.3.3. Size of fluorescence region around the colony on the upright side (ZU)

For the present study, daily observation of CCA agar under UV light for MF1 and MS1 showed a fluorescence region around the colony on the upright side after 6 days of incubation. Figure 5.5 shows the ZU for both isolates. The size increased from day 4 reaching a maximum size on day 9. However, MF1 and MS1 produced strong fluorescence on days 7–8 and 6–9, respectively (Table 5.1). In the present study, prolonged incubation showed that no fluorescence was observed on MF1 (from day 12) and MS1 (from day 15) until 30 days of incubation.



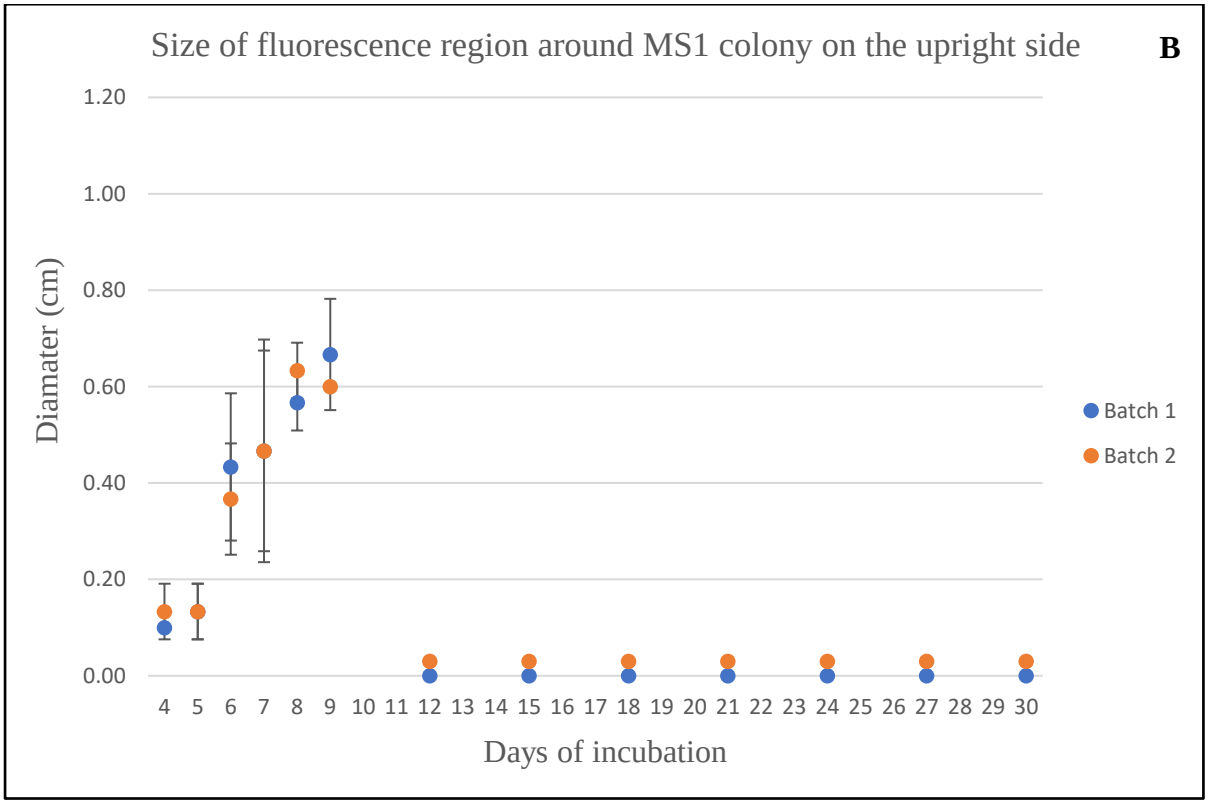


Figure 5. 5. Size of fluorescence region around *Monascus purpureus* isolates (A) MF1 and (B) MS1 on the upright side after incubation of Coconut Cream Agar at 30 °C for 30 days.

5.3.4. Size of fluorescence zone on the reversed side (ZR)

In the present trial, MF1 and MS1 started producing fluorescence on the reversed side after 6 days of incubation (Figure 5.6). The ZR increased until days 9 and 12 for MF1 and MS1, respectively. However, MF1 produced strong fluorescence on the reversed side after 7 and 8 days of incubation, while MS1 produced strong fluorescence on the reversed side after days 6 to 9.

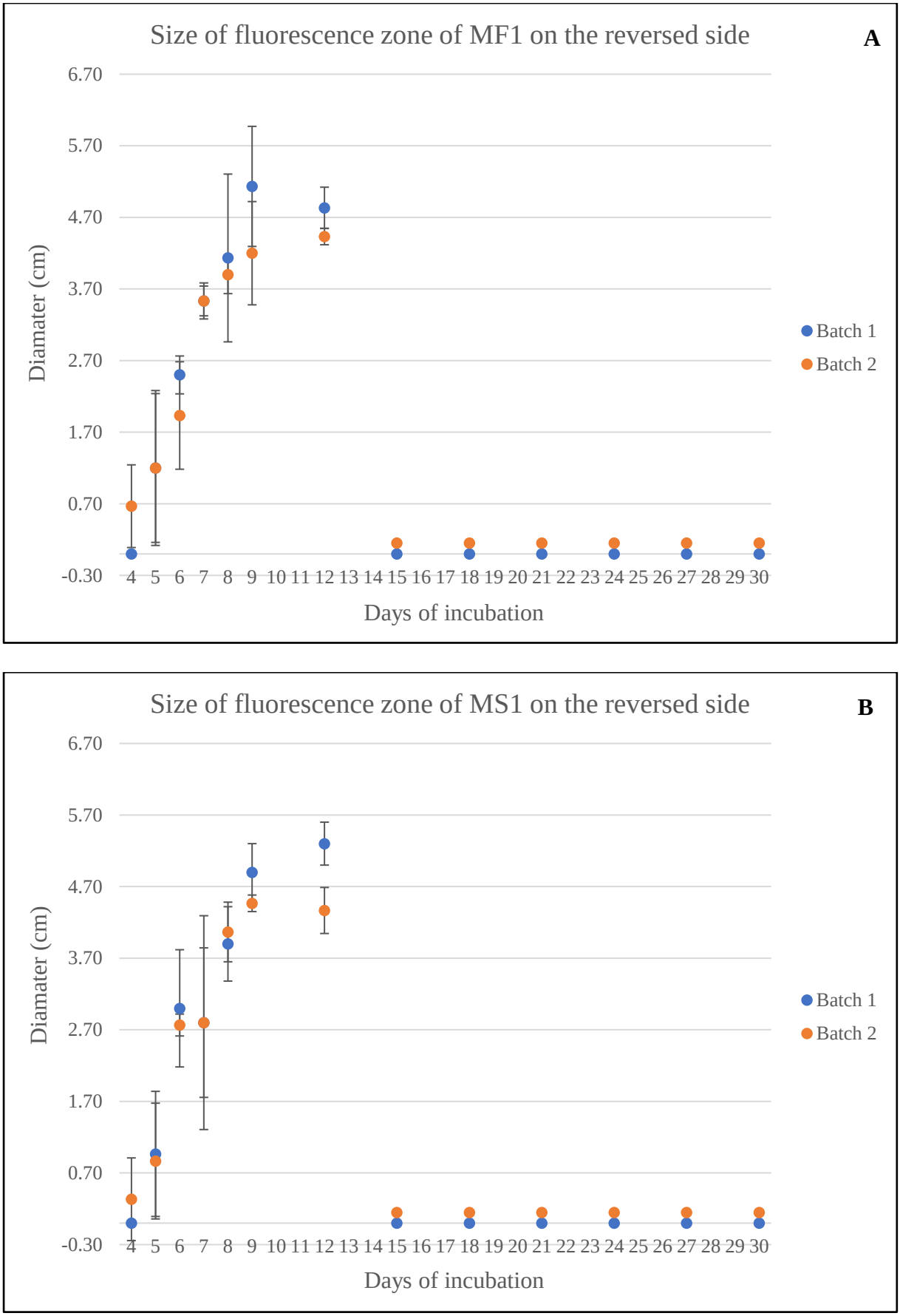


Figure 5. 6. Size of fluorescence zone of *Monascus purpureus* isolates (A) MF1 and (B) MS1 on the reversed side after incubation of Coconut Cream Agar at 30 °C for 30 days.

5.3.5. Mass of fungi of *M. purpureus* isolates

Figure 5.7 shows that the mass of fungi for both isolates increased with incubation time. Early in the incubation, MF1 had more mass of fungi than MS1. However, MS1 produced a greater mass of fungi than MF1 at the end of incubation.

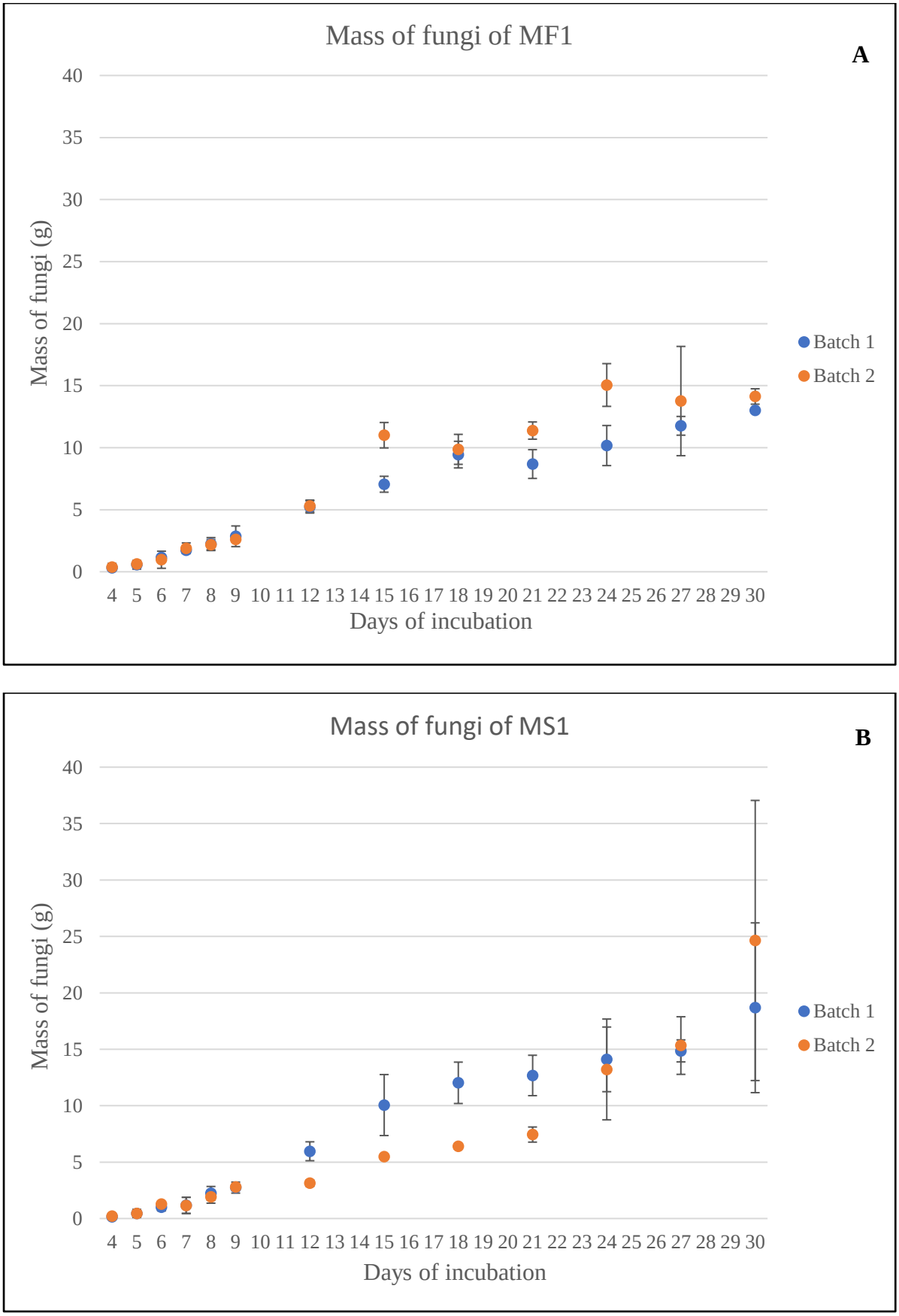
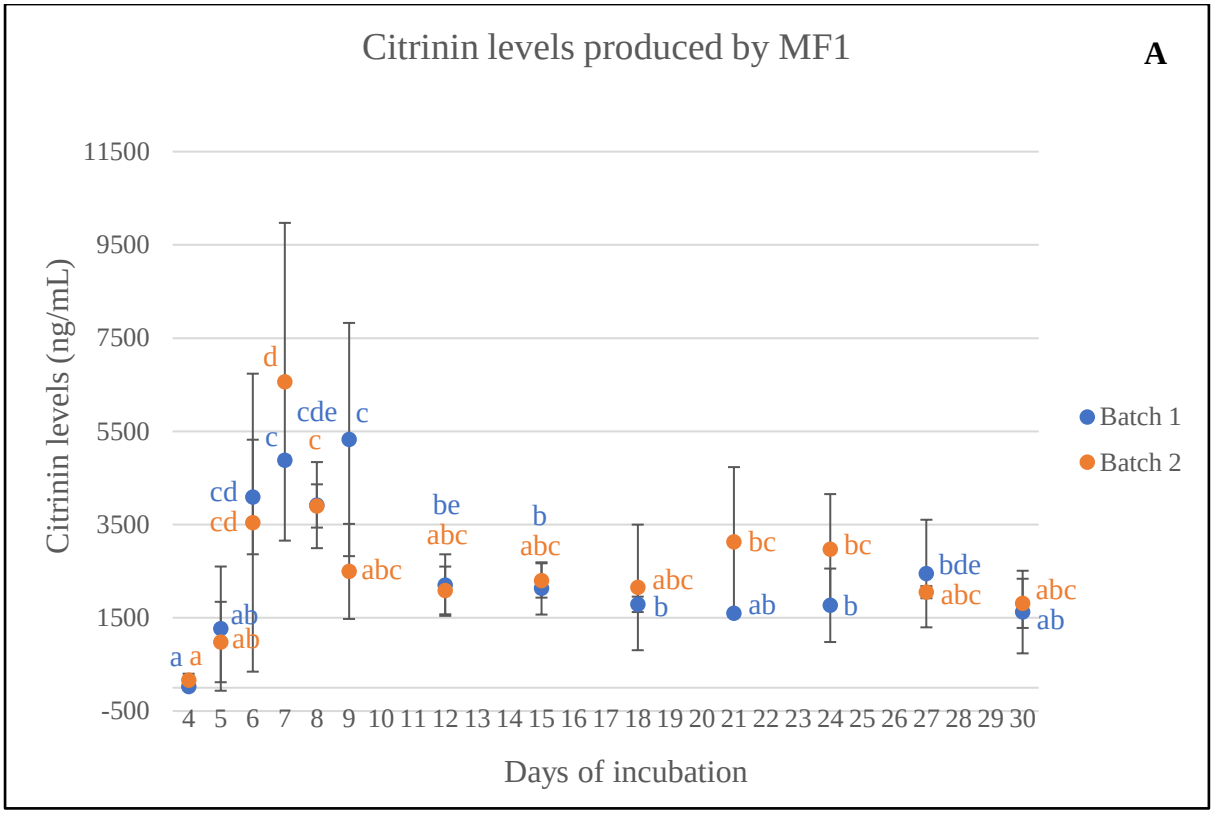


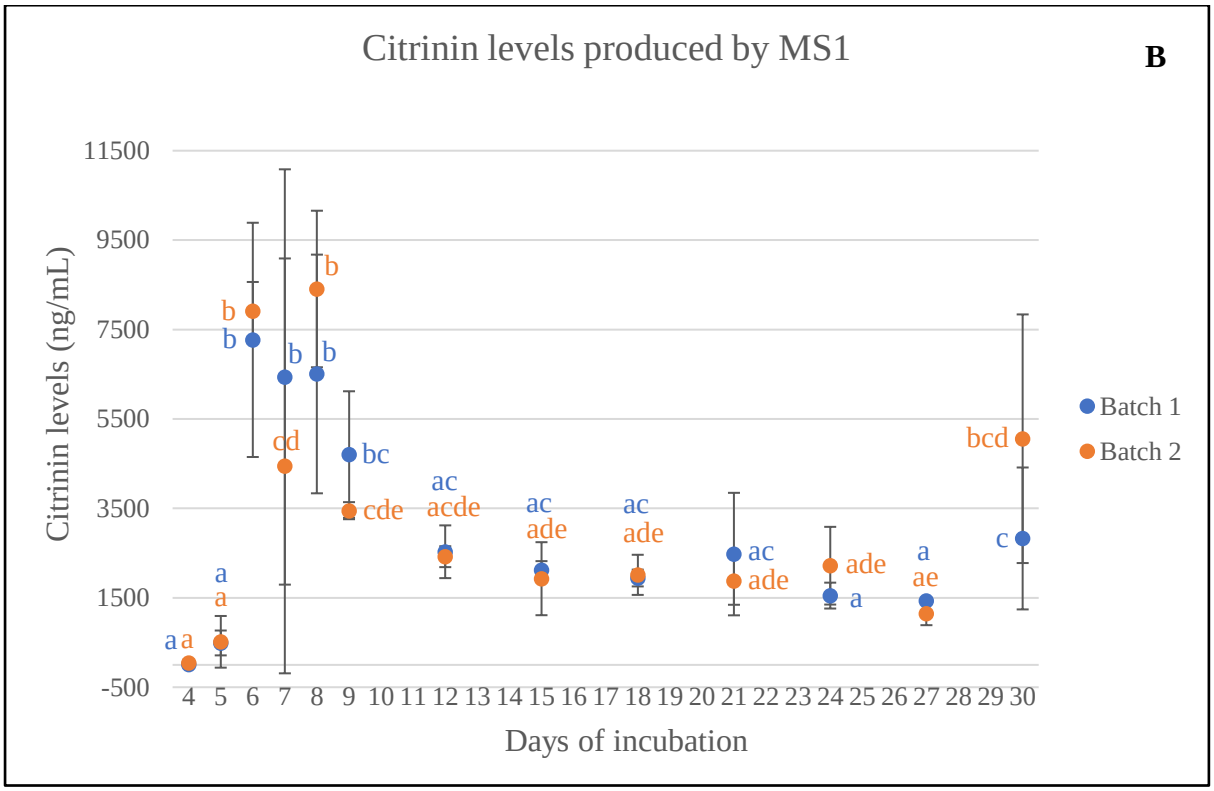
Figure 5. 7. Mass of fungi of *Monascus purpureus* isolates (A) MF1 and (B) MS1 after incubation of Coconut Cream Agar at 30 °C for 30 days.

5.3.6. CIT production of *M. purpureus* isolates

Figure 5.8 shows the results of CIT levels after incubation of MF1 and MS1 on CCA for a month and detected by UHPLC–FLD. CIT significantly increased from day 4 until 7 for MF1, and from day 4 until 8 for MS1. The maximum CIT levels for MF1 and MS1 were achieved after 7 and 8 days of incubation, respectively. Based on the colonial morphologies (Figure 5.3), MF1 produced a dark red colony on the upright side and strong fluorescence zones on the upright and reversed side after 7 days of incubation. Meanwhile, on day 8, MS1 produced an orange–red colony and strong fluorescence zones on the upright and reversed sides. Incubation of MF1 and MS1 on CCA after 12 days showed that there was no fluorescence on the upright and reversed sides which coincided with decreasing CIT levels produced by these isolates.

MF1 and MS1 changed from yellow orange to the orange–red colony, and the intensity of fluorescence and CIT levels increased with incubation. When *M. purpureus* isolates started producing red colonies, the fluorescence intensity and CIT levels decreased. Prolonged incubation for both isolates until 30 days was proportional to the increment in the size of colonies and mass of fungi. However, CIT levels significantly decreased after 8– and 9–days incubation of MF1 and MS1, respectively on CCA. It is hypothesized that there was a correlation between CIT levels and pigments production by these isolates.





* 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 ($p < 0.05$).

Figure 5. 8. Citrinin levels produced by *Monascus purpureus* isolates (A) MF1 and (B) MS1 after incubation on Coconut Cream Agar at 30 °C for 30 days.

5.3.7. Pigments production of *M. purpureus* isolates

Yellow, orange, and red pigments have different wavelengths which were 330–450 nm, 460–480 nm, and 490–530 nm, respectively (Liu & Chen, 2019). Based on daily monitoring, white mycelium was observed on CCA after 3 days of incubation, followed by the change of colony colour to orange (Figure 5.3). Figure 5.9 shows the pigments yield of MF1 and MS1 on CCA. Generally, the pigments increased from day 4 until 21. The maximum pigments yield for yellow, orange, and red pigments produced by MF1 on CCA were on days 18 and 21, 15 and 21, and 15 and 21 for each batch, respectively. Meanwhile, MS1 produced highest yellow, orange, and red pigments after 24 and 30 days of incubation.

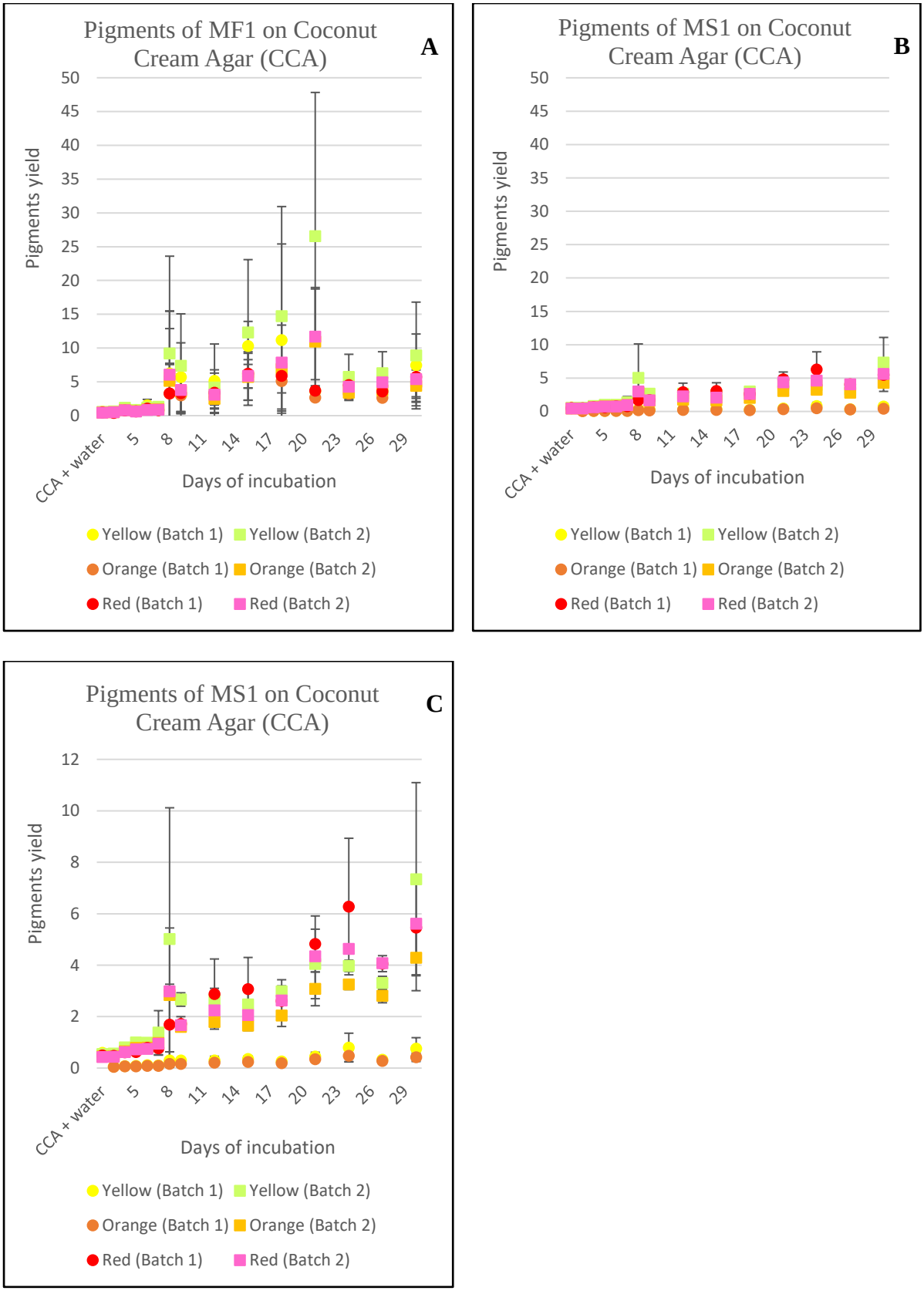
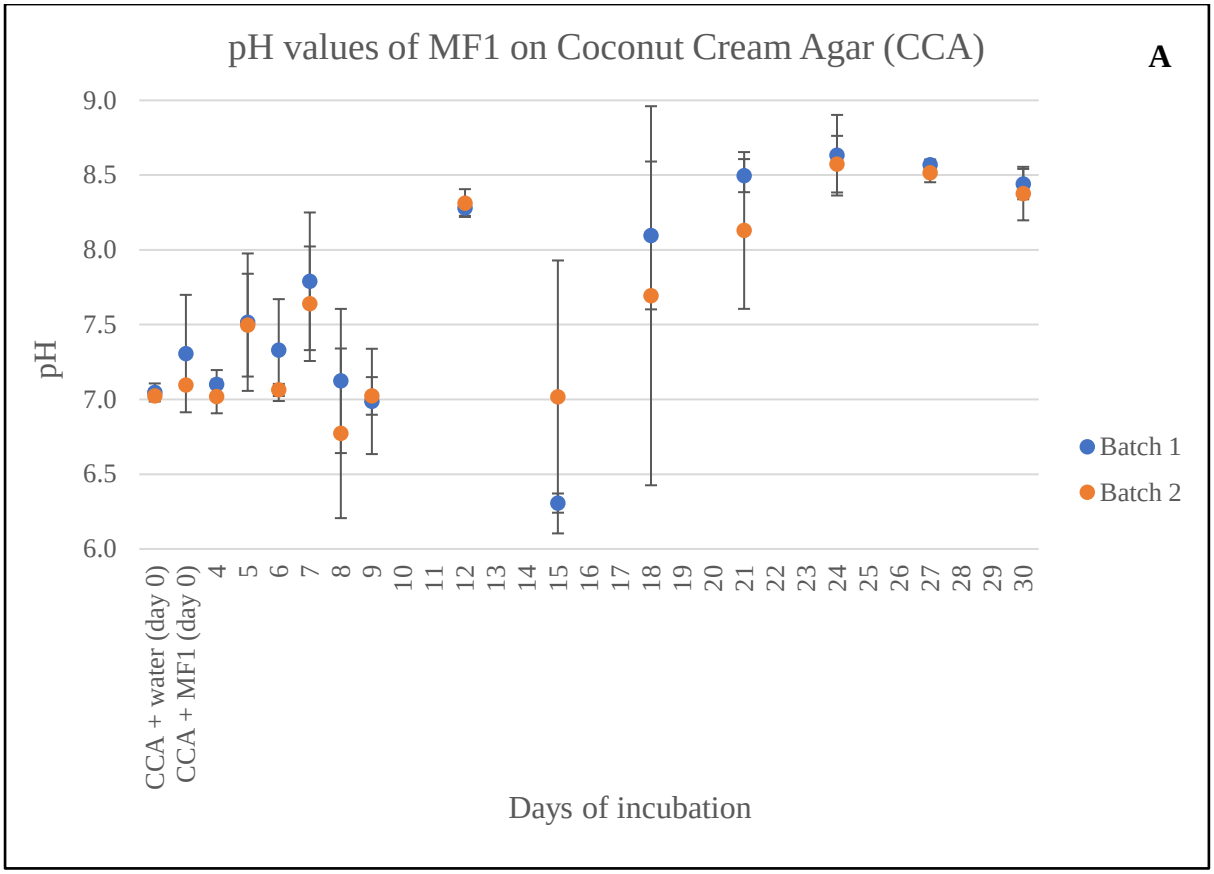


Figure 5. 9. The yield of pigments produced by *Monascus purpureus* isolates (A) MF1 (with a scale of 0–50 pigments), (B) MS1 (with a scale of 0–50 pigments), and (C) MS1 (with a scale of 0–12 pigments) after incubation of Coconut Cream Agar at 30 °C for 30 days.

5.3.8. pH of *M. purpureus* isolates

The pH of the MF1 and MS1 on CCA was measured for 30 days. In this study, the pH for both isolates were not controlled and increased from approximately 7.0 to 8.6 (Figure 5.10).



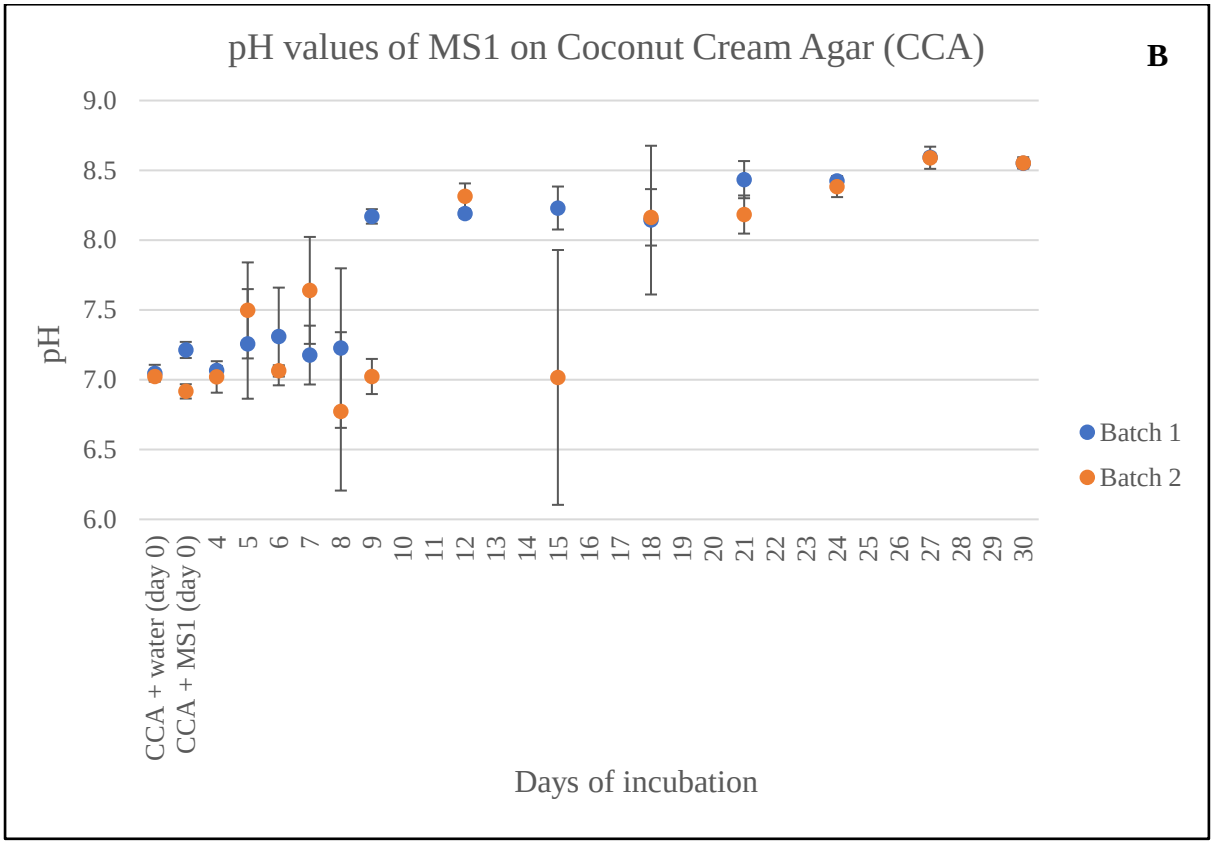


Figure 5. 10. The pH values of *Monascus purpureus* isolates (A) MF1 and (B) MS1 after incubation of Coconut Cream Agar at 30 °C for 30 days.

5.4. Discussion

5.4.1. Colour and size of colonies of *M. purpureus* isolates

The fungal growth based on the colony diameters was usually measured within the lag period of fungal growth (1–7 days) (Baert et al., 2007; Tannous et al., 2016). Hawksworth and Pitt (1983) reported that the colony diameter of *M. purpureus* should be less than 2.8 cm after incubation for 7 days, and has a floccose hyphal texture and deep red colony colour. This is in line with our findings with MF1 having a 2.53–2.60 cm diameter and MS1 a 2.03–2.17 cm diameter after 7 days of incubation. Samsudin and Abdullah (2014) reported that after a week, *M. purpureus* on MEA produced smaller colonies (0.8–2.2 cm) with little aerial growth. The *M. purpureus* growth started with white or reddish mycelium, followed by strong orange or red shade pigments. As the colonies matured, the colour changed to reddish–orange and the pigment diffused into the agar.

The study by Kim et al. (2010) found that *M. purpureus* grew very slowly and produced the smallest colony compared with other *Monascus* spp., which were 1.30 ± 0.00 cm. Possible explanations for this difference in results may be different inoculation methods, media and incubation temperatures. *M. purpureus* had an orange–to–red colony from the upright side and secreted deep red pigments. These fungi had large red subglobular ascomata (30–60 µm diameter) but small globular conidia (3–8 µm diameter). *M. purpureus* can be differentiated from *M. pilosus* by having slow growth, secretion of deep red pigments, abundant ascomata, and less production of conidia (Kim et al., 2010).

According to Afroz Toma et al. (2023), orange pigments are biosynthesized first, and then synthesized to yellow and red pigments, depending on culture conditions. Yellow pigments are the result of the depletion of orange pigments, while Schiff base formation of orange pigments produces red pigments (Figure 5.11).

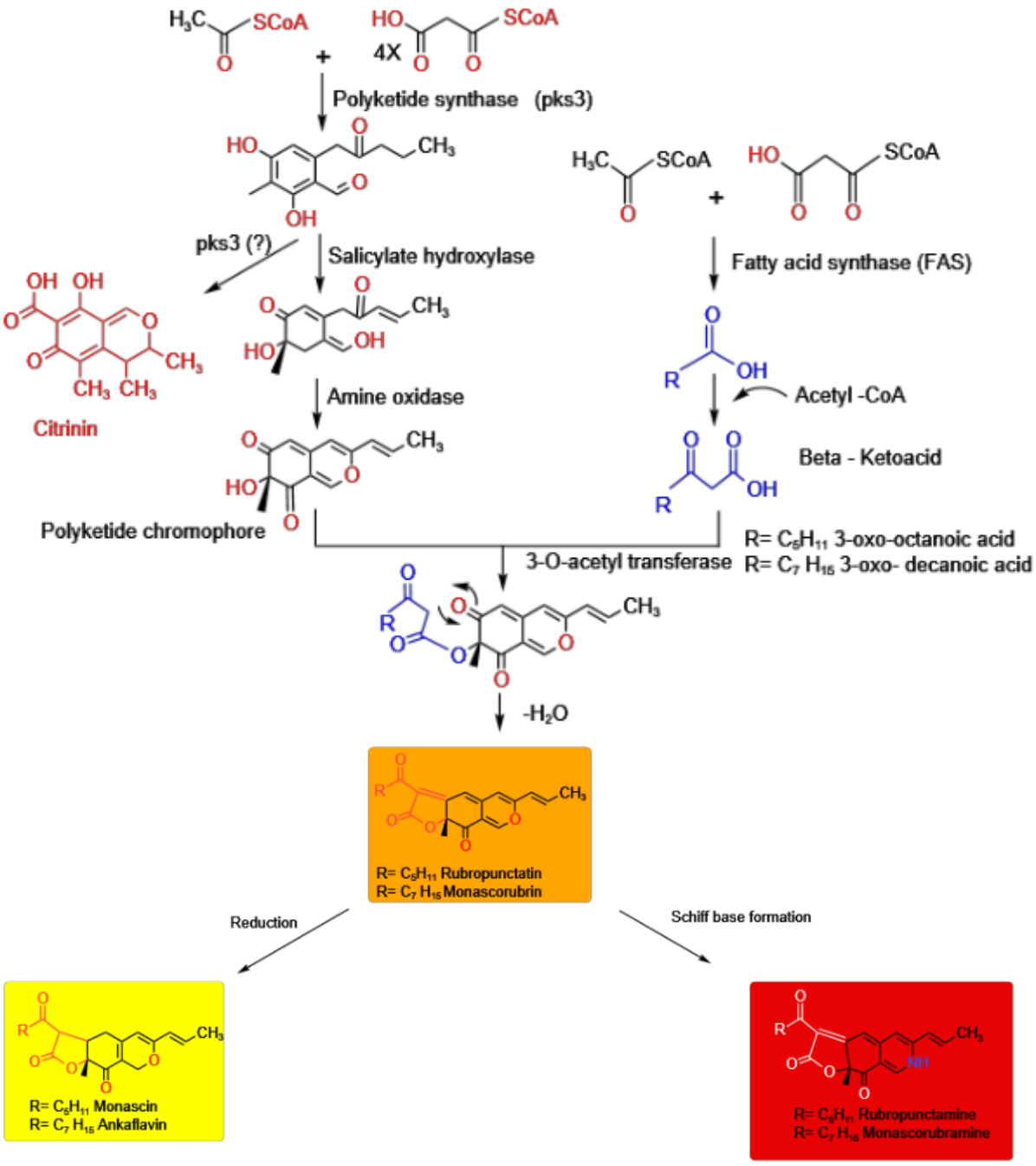


Figure 5. 11. The hypothetical pathway for citrinin and *Monascus* pigments biosynthesis. Source: Afroz Toma et al. (2023).

According to Ajdari et al. (2011), the fungal growth of *M. purpureus* were determined by radial growth measurements. Six different media (PDA, MEA, Improved Medium, Power Medium, Hiroi, and Hiroi–PDA) were used to grow the *M. purpureus*, and the growth rate was reported between 1.67 to 3.03 mm/day after 12 days incubation. These *M. purpureus* isolates had a larger radial growth rate due to different methods used to grow the fungi. In current study,

10 µL of stock culture was inoculated in the centre of CCA plates, but the previous study inoculated 2 mm^2 vegetative cells in the centre of different media.

In the present trial, at the end of incubation, the colony sizes for MF1 and MS1 were 6.40–6.70 cm and 7.00–7.50 cm in diameter, respectively. The fungal growth study has some limitations due to the Petri plates' dimensions. After reaching approximately 7 cm as the limiting diameter, the growth curves lose their linear appearance (Tannous et al., 2016).

5.4.2. Size of fluorescence region around the colony on the upright side (ZU)

For other mycotoxins such as AF, the AF–positive isolates produce blue or blue–green fluorescence. After transferring the mycelia plug with 3 mm in diameter of *Aspergillus* spp. from *Aspergillus*–Differential Medium (ADM) onto CCA and incubating at 20, 24, 28, and 32 °C, fluorescence was observed after 32 hours and 3 days for strong and weak AF producers, respectively (Lin & Dianese, 1976).

5.4.3. Size of fluorescence zone on the reversed side (ZR)

Figure 5.6 shows the ZR for MF1 and MS1 isolates on CCA when observed under UV light which has been reported as a qualitative method to detect AF (Dyer & McCammon, 1994; Sultan & Magan, 2010), OTA (Sultan & Magan, 2010), and CIT (Mohamed et al., 2013). These authors reported the detection of these mycotoxins by observing the fluorescence of the CCA, but none of them reported ZU and ZR over incubation time.

The light blue fluorescence converted to yellow fluorescence after spraying with HCl, and the yellow fluorescence is an indicator that CIT is produced by *Monascus* spp. isolates (Farawahida et al., 2022a). Vazquez et al. (1997) explained that CIT has a conjugated and planar structure, providing the natural fluorescence. However, the fluorescence was weaker than other toxins. In the acidic environment until a maximum pH 2.5, the keto and phenol

functional groups were linked to the carboxyl group of the CIT nucleus by intramolecular hydrogen bonds, resulting increasement of planarity. The non–ionized CIT was formed and producing fluorescence.

The addition of HCl in the culture medium produces fluorescence as an indicator of the presence of CIT (Vazquez et al., 1997). The lower CIT produced by *P. citrinum* and *P. expansum* requires a higher volume of HCl to exhibit yellow fluorescence. The study mentioned that after the addition of HCl to the culture medium, five days are necessary to show the presence of CIT.

Mohamed et al. (2013) reported that after incubation of *P. citrinum* on CCA at 25 °C for four days, the isolates secreted a strong yellow–green halo when the reversed side of CCA media was observed under long UV light. The fluorescence grew larger and intensity on the reversed side increased until 7 days of incubation. For several isolates, prolonged incubation after 7 days showed that the fluorescence started to disappear. These results were in line with the present study, where there was no fluorescence produced on the reversed side of CCA on day 15.

5.4.4. Mass of fungi of *M. purpureus* isolates

There are five visible growth stages for filamentous fungi; (1) lag phase, (2) first transition phase, (3) log phase, (4) second transition phase, and (5) stationary phase (Meletiadis et al., 2001). Said et al. (2014) reported that there were four phases of fungal growth, which were lag, exponential, stationary, and decline phase. The highest biomass production of *M. ruber* was achieved at 96 h even though after 72 h of incubation, almost all glucose sources had been used. Decreasing glucose decelerated the growth of *M. ruber* until the growth ended (Said et al., 2014). However, those studies were conducted in SmF, which may explain the difference from the present study that used SSF.

Ajdari et al. (2011) found that the growth rate based on cell mass for *M. purpureus* was between 0.04 to 0.16 after 12 days of incubation on different media. The addition of fluconazole, an azole–based antifungal agent, to the SmF medium affected ergosterol, biomass, and red pigments of *M. purpureus* (Koli et al., 2017). Ergosterol is a sterol that resides on the cells membranes of fungi, and the pigments binds with the membrane due to having both hydrophobic and hydrophilic structures (amphiphilic). Increment of fluconazole causes reduction of ergosterol levels and the total biomass of *M. purpureus*. Reduction of ergosterol levels affected the development of fungal cell membranes, resulting to a rising of extracellular pigments production. This study suggested that extracellular pigments production can be increased by changing the composition and structure of the cell membrane of *Monascus* spp., by adding 30 µg/mL of fluconazole (Koli et al., 2017).

5.4.5. CIT standard curve

A standard curve was developed using different concentrations of CIT standards. The R^2 for the calibration curve was 0.9999, indicating that the calibration curve is good. The calibration curve was used to quantify CIT in the samples extracted from section 5.2.6.

5.4.6. CIT production of *M. purpureus* isolates

CIT is a toxic fermentation by–product of *Monascus* isolates, and this mycotoxin can alter liver metabolism, and cause structural and functional kidney damage (Nigović et al., 2013). Even though it has been reported that CIT is produced by *Monascus, Penicillium* and *Aspergillus* genus, it is still unclear when these fungi start producing this secondary metabolite. Therefore, the present study examined CIT levels produced by *M. purpureus* isolates on CCA.

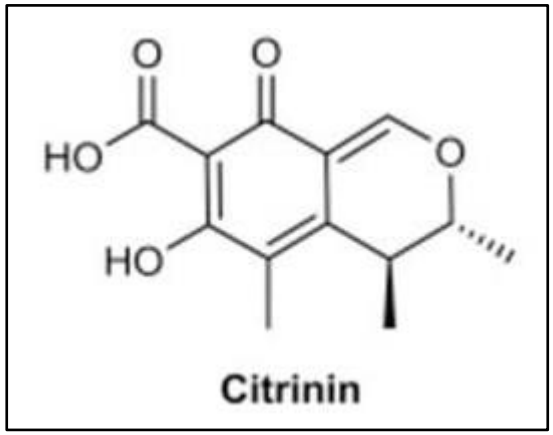
Mohamed et al. (2013) reported that another fungus, *P. citrinum*, produced yellow–green fluorescence when the reversed side of CCA plates was observed under long UV light,

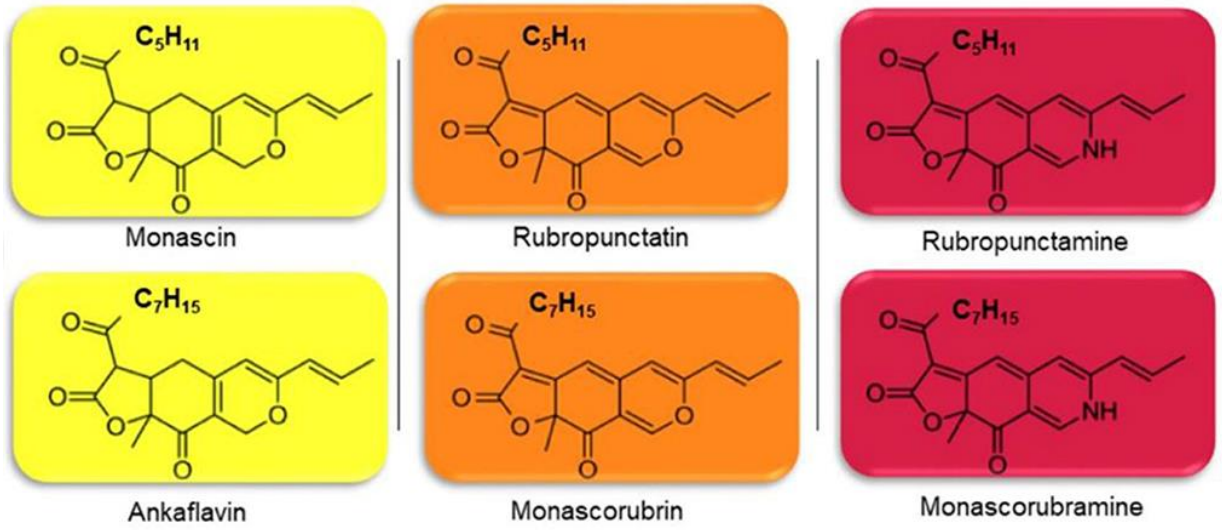
and the maximum detection was observed after 4–5 days of incubation at 25 °C. The isolates were obtained from dried tuna and smoked fish. The fluorescence produced on CCA was detected as CIT by TLC. The addition of HCl to yeast extract broth with 20 % sucrose (YES broth) and incubation for 21 days at 25 °C showed that there was yellow fluorescence produced, which was confirmed as CIT by reversed–phase high–performance liquid chromatography (RP–HPLC) (Franco et al., 1996; Vazquez et al., 1997).

AF and OTA have been successfully detected by using selective media such as CCA (Dyer & McCammon, 1994; Heenan et al., 1998). *A. nomius, A. flavus,* and *A. parasiticus* showed fluorescence after incubating of this medium at 30 °C for a week. A further study by TLC plates showed that the fluorescence was due to AF and there was a high correlation between CCA and TLC (Dyer & McCammon, 1994). An early study by Lin and Dianese (1976) found that *Aspergillus* spp. showed a blue or blue–green fluorescence on CCA and was confirmed as AF by the TLC of culture broths. The authors also reported that an orange–yellow pigmentation on CCA was consistently connected with the exhibition of fluorescence and suggested that the pigmentation can be used to identify *Aspergillus* spp. isolates that can produce AF without applying the UV light.

Figure 5.12 shows the chemical structures of CIT and pigments. Monascin and ankaflavin are yellow pigments, rubropunctatin and monascorubrin are orange pigments, while rubropunctamine and monascorubramine are red pigments (Egea et al., 2023). There is a probability that there was interaction between CIT and pigments, leading to the reduction of CIT.

$C_{13}H_{14}O_5$





Monascus pigments

Figure 5. 12. Structures of citrinin and *Monascus* pigments.

Source: Kamle et al. (2022) and Egea et al. (2023).

5.4.7. Pigments production of *M. purpureus* isolates

The pigments production during SmF have been studied by other researchers (Patrovsky et al., 2019; Said et al., 2014). The red pigments are favourable compared to yellow and orange pigments because red is often a preferred colour in food and it is not easy to obtain red pigments from other sources (Said et al., 2014). However, red pigments produced by *Monascus* spp. is not formed biosynthetically (Patrovsky et al., 2019). The red and orange pigments produced in SmF are linked with the growth of *M. ruber*, but not yellow pigments (Said et al., 2014). At a suitable pH, there is a chemical reaction between the red pigments and other compounds that have primary amino groups such as aminos and nucleotides, and these reactions continue during extraction (Shi et al., 2015).

5.4.8. pH of *M. purpureus* isolates

These results agree with the findings of studies by Said et al. (2014), in which pH was changed from ~ 6.5 to 7.0 after cultivation of *M. ruber* in SmF media for 120–140 h. The changes in pH were due to the chemical conversion of orange pigments to red pigments (Mak et al., 1990).

In the present study, the highest red pigments production was achieved after prolonged incubation, and the mycelium changed from neutral to alkaline. In contrast to the earlier findings, the change in *M. purpureus* colony colour was due to the increasing acidity of the medium (Abdul-Manan et al., 2017b). Orozco and Kilikian (2008) suggested that an alkaline medium is suitable for red pigments production by *M. purpureus* CCT3802. The total red pigments in SmF were achieved at the growth phase (with glucose) at pH 5.5 followed by the production phase (without glucose) at pH 8.5 (Orozco & Kilikian, 2008). The study reported that the alkaline medium can inhibit CIT synthesis, which did not happen in this study. In this

study, the CIT levels reduced after 7–8 days of incubation and were not associated with the pH of the *M. purpureus* on CCA media.

5.5. Conclusion

In summary, the fungal growth, CIT, pigments, and pH of *M. purpureus* isolates can be characterized after incubation of MF1 and MS1 isolates on CCA for a prolonged period. The *M. purpureus* growth was determined based on the size of colonies, ZU, ZR, and the mass of fungi. The size of colonies increased over time, ZU increased from day 4–9, and no fluorescence was observed around the colony on the upright side of CCA after that. A similar trend was also observed for ZR. ZR for both isolates increased from day 4 until day 9–12, and there was no fluorescence on the reversed side after the prolonged incubation. Decreasing CIT levels were observed after incubation of MF1 and MS1 on CCA for 8 and 9 days, respectively. The pigments increased during these incubation days. There appears to be a relationship between pigments and CIT produced by *M. purpureus* isolates. This knowledge is beneficial for the food industry to control the fermentation conditions to produce safe food using *M. purpureus*.

CHAPTER 6

THE KINETICS OF pH, CITRININ, AND PIGMENT PRODUCTION DURING THE FERMENTATION OF RED FERMENTED RICE

6.1. Introduction

RFR is consumed by more than a billion people worldwide, especially in Southeastern and Eastern Asia 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 humidities (Fukami et al., 2021).

RFR is called by other names in different countries such as angkak, anka, Ang–Khan, Ankak rice, Anka–Koji, beni–koji, red rice, red koji, red Chinese rice, red mould 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 rotscimmelreid (Chiu et al., 2006; Doughari, 2015; Erdoğrul & 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 have been used for centuries to mitigate diarrhoea, improve blood circulation, treat indigestion, limb weakness, bruising of muscles, and dysentery (Kohama et al., 1987; Zhu et al., 2019). RFR has been used as a TCM with improving health benefits such as lowering the production of cholesterol in the liver, and acts 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). Red pigments from RFR have been used since the 1950s 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 of 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). Many *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. produce secondary metabolites including six pigments divided into three colours, yellow, orange, and red (Blanc et al., 1994). RFR produces other secondary metabolites such as CIT (Avula et al., 2014; Chen et al., 2016b; 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 kidneys are the major target organs (Kamle et al., 2022).

Substrate pH is one of the major factors to determine the metabolic activity in SSF (Velmurugan et al., 2011). Many studies reported the effects of initial pH on pigments (Orozco & Kilikian, 2008; Patrovsky et al., 2019; Velmurugan et al., 2011), but there are limited studies on measuring the pH during the fermentation of RFR.

Even though it has been reported that RFR contains pigments and CIT, the kinetics of the production of these metabolites during the fermentation of RFR is 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.

6.2. Materials and methods

6.2.1. Microorganisms

Two isolates MF1 and MS1 isolated from RFR (Farawahida et al. (2022a) were used for inoculation. Both isolates were *M. purpureus* based on macroscopic and microscopic

observations, gel electrophoresis, and DNA sequencing. These isolates were stored at -20 °C for further use.

6.2.2. 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. A hundred and ten grams of white rice was weighed in a beaker and 220 mL of water was added to the beaker. After soaking for two hours, 40 % of water was removed from the beaker. The rice was steamed with a steamer for 20 min. The steamed rice was autoclaved at 121 °C for 15 min. After cooling, 10 mL of MF1 or MS1 was added as the inoculum to 50 g of rice. The rice was stirred by a spatula. The rice was weighed (1.2 g) and added to a 120 mL container. 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 light exposure at 30 °C for 30 days.

6.2.3. CIT 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 (Figure 6.1). To determine CIT in RFR, 5 mL of 50 % ethanol solution (ethanol: 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 for 10 min at 3000 x g. To filter the supernatants, a Minisart® NML syringe filter (0.2 µm pore size, Sartorious) was used. To determine the quantity of CIT levels for each sample, I mL of the filtrate was subjected to UHPLC–FLD (Dionex Ultimate 3000, California, USA).

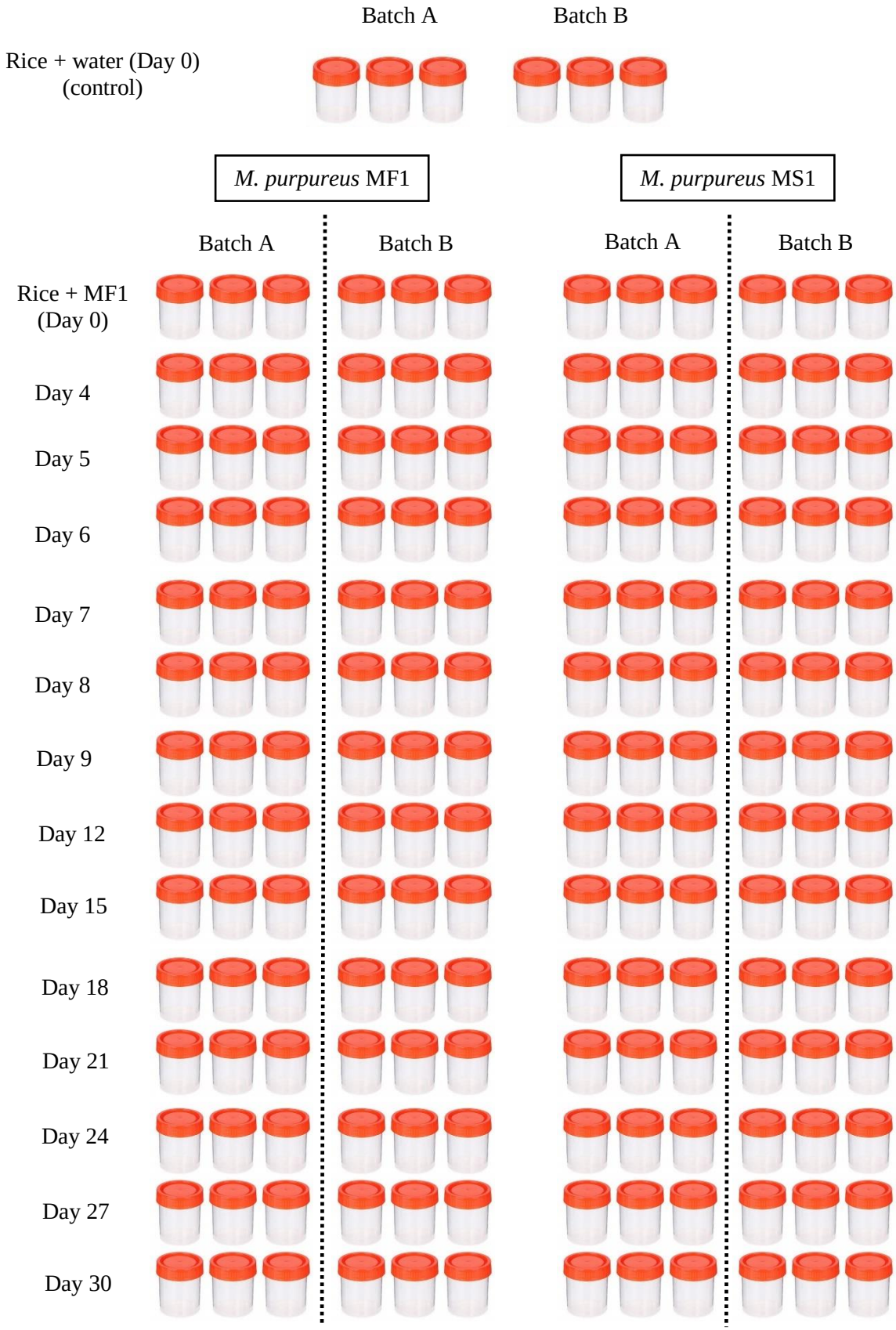


Figure 6. 1. The schematic diagram to produce red fermented rice using *Monascus purpureus* MF1 and MS1 isolates. The experiments were conducted independently to determine (a) citrinin and (b) pigments. All the rice were fermented at 30 °C for 30 days.

6.2.4. Preparation of a standard curve for CIT determination

CIT standard was supplied from Sigma–Aldrich. Ten millilitres of MEOH 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 in the samples.

6.2.5. Determination of CIT by UHPLC–FLD

The method of Farawahida et al. (2022a) was used to determine CIT levels in the sample. The presence of CIT was determined by a UHPLC–FLD using a reverse–phase symmetry Kinetex C_{18} column (250 mm length × 4.6 mm internal diameter, 5 µm particle size, 100 Å pore size) was used as a stationary phase. The column temperature was kept at 30 °C. All solvents used were HPLC grade, and the CIT standard was supplied by Sigma–Aldrich.

ACN (Sigma–Aldrich, Missouri, USA) and 0.1 % TFA in water were separately filtered through a regenerated cellulose filter (0.20 Ø µm; Sartorius, Göttingen, Germany). These solvents were used as a mobile phase with the ratio ACN: 0.1 % TFA (55:45, v/v) with a flow rate of 1.0 mL/min. The data was recorded with an FLD detector at an excitation wavelength of 330 nm and an emission wavelength of 500 nm. The system was running isocratically for 40 min. A 20 µL for each sample was injected into the UHPLC–FLD system. All the injections were done in triplicate and to process the data, Chromeleon 7.0 software (Thermo Fisher Scientific) was used.

6.2.6. Determination of pigments

Section 6.2.2 was used to prepare the samples for pigments determination. Six containers (three samples for batches A and B, respectively) were removed for analysis at each sampling time (Figure 6.1). Then, 10 mL of 60 % ethanol solution (ethanol: water, v/v) was

added to each container and agitated on a rotary shaker at 25 °C with 200 rpm speed for 24 h. To filter the supernatant, a Minisart® NML syringe filter (0.2 µm pore size, Sartorius, Göttingen, Germany) was used. Each sample (200 µL) was pipetted into NUNC MaxiSorp 96–well microplates (Thermo Fisher Scientific, USA), subjected to a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Vantaa, Finland) and analysed by SkanIt Software 6.0.1.6. 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 solution (ethanol, water, v/v) was used. This formulation was used to convert the absorbances into pigments yield:

$$\text{Pigments yield} = \frac{\text{OD} \times \text{dilution} \times \text{volume of extract (mL)}}{\text{Amount of sample (g)}}$$

6.2.7. Determination of pH

To determine the pH of the sample, 1 mL of the filtrate from section 6.2.6 was tested using a pH meter (Mettler–Toledo, Schwerzenbach, Switzerland).

6.2.8. Reduction of citrinin after combining with pigments

MF1 and MS1 were inoculated on CCA and incubated at 30 °C for 8 days. *Monascus* mycelium for both isolates were scaped from CCA, weighed (1 g, 2 g, and 3 g), and 10 mL 60 % ethanol added to obtain 100 mg/mL, 200 mg/mL, and 300 mg/mL of pigments. The mixtures were shaken on a rotary shaker at 25 °C, 200 rpm for 24 h. The supernatants were filtered by syringe filter to obtain pigments. The pigments were mixed with 1000 ng/mL CIT and left for 1 h before injecting into the UHPLC–FLD. All the samples were done in two batches.

6.2.9. Statistical analysis

All the samples were measured in triplicate to obtain the mean and standard deviation. The graphs were analysed and plotted with Microsoft Excel. The results for the differentiation between the samples were analyzed using mean and standard deviation and submitted to one–way ANOVA followed by Fisher LSD Method (5 % significant) using MINITAB (Version 21).

6.3. Results and discussion

6.3.1. CIT standard curve

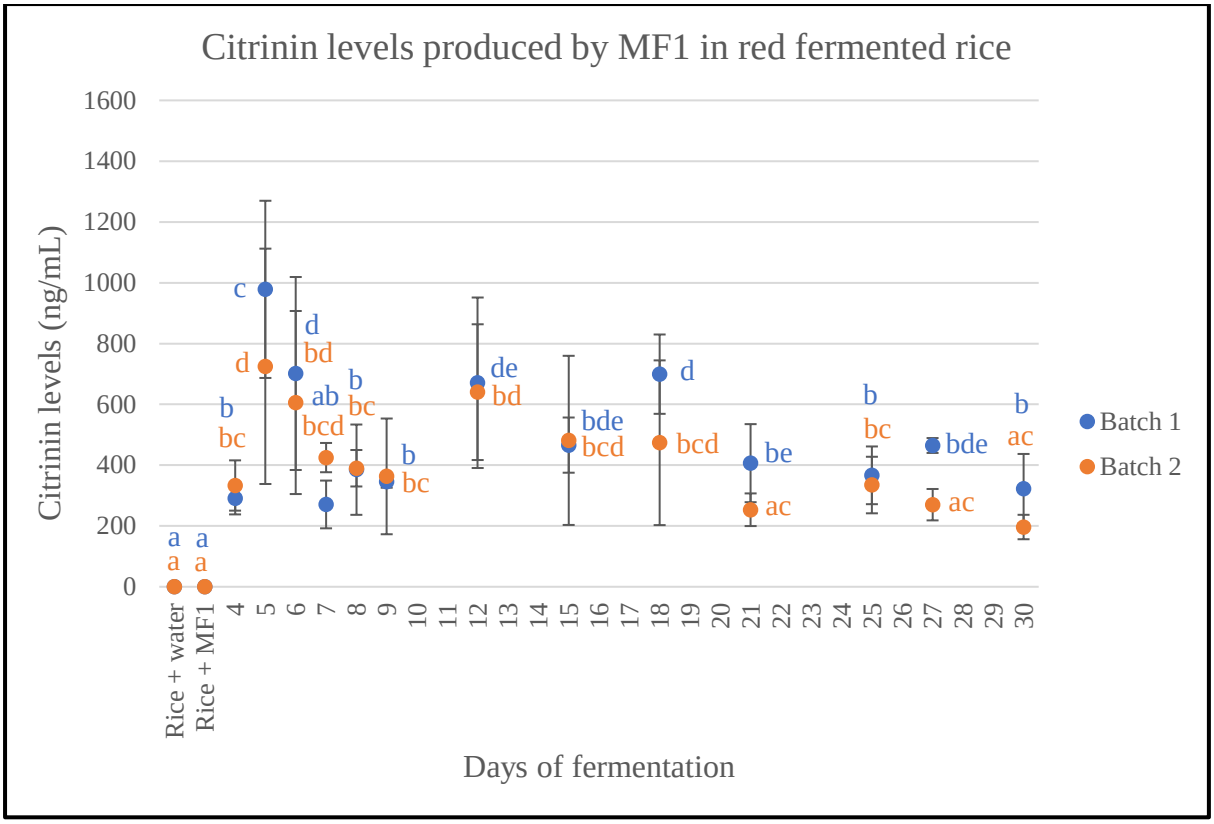
To determine CIT in the RFR, a standard curve was constructed by using several concentrations of CIT standards. A good R^2 was obtained (0.9999) and the calibration curve further used to determine CIT in the samples.

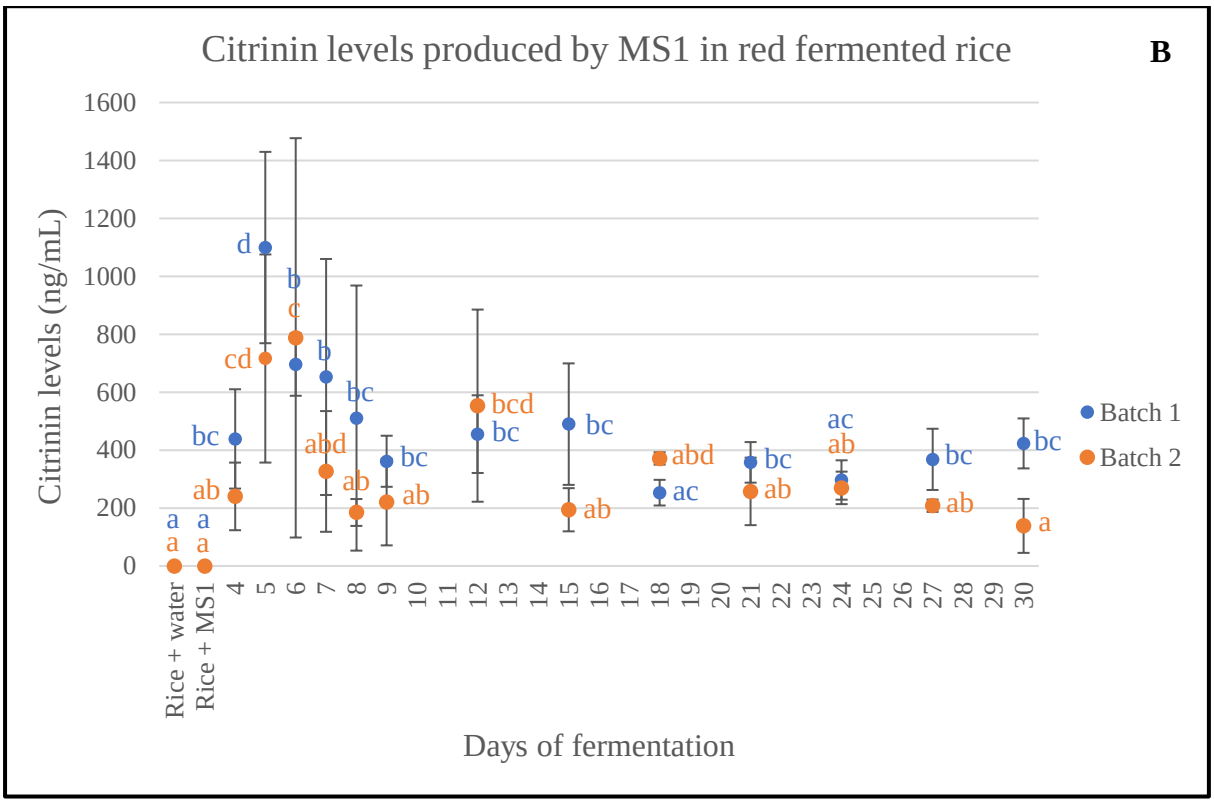
6.3.2. Determination of CIT

Most published studies determined CIT levels of commercial RFR products not the CIT levels during the fermentation of RFR. Most of published data report that RFR contains CIT above the maximum limits (Chen et al., 2016b; Gordon et al., 2010; Ristiarini et al., 2017). The maximum limits for CIT in China, Europe, Japan and Taiwan are 50, 100, 200, and 2000 µg/kg (ppb), respectively (EU, 2019; Srianta et al., 2014; Taiwan-FDA, 2020). Ristiarini et al. (2017) reported that all thirty RFR commercial products from eight different regions in Indonesia exceeded the maximum CIT limits, varying from 17.94 ppm to 124.68 ppm. From 12 commercial RFR products in the United States, 33 % of the samples contained CIT from 24 to 189 ppm, which was higher than the maximum limits (Gordon et al., 2010). In Taiwan, 27.2 % of the 206 RFR samples had CIT, with 14 RFR raw materials and one RFR supplement exceeding the maximum limits of 5 ppm and 2 ppm, respectively (Chen et al., 2016b). Based

on the findings, the researchers suggested that food manufacturers choose low–CIT–producing *Monascus* spp., optimize fermentation conditions, and implement self–management to reduce CIT levels in the RFR products (Chen et al., 2016b).

In this study, during fermentation of RFR, the samples contained CIT after 4 days of fermentation (Figure 6.2). For both isolates, CIT levels increased from day 4 until 5 and decreased after 5 days of fermentation. During fermentation of RFR using MF1 isolate, the CIT level significantly increased from 290.65 ng/mL (day 4) to 978.34 ng/mL (day 5) and significantly decreased to 701.59 ng/mL (day 6) for batch 1. For batch 2, the CIT level was 333.18 ng/mL after 4 days of fermentation. The CIT level significantly elevated to 725.17 ng/mL (day 5) and significantly reduced to 389.92 ng/mL after 8 days of fermentation. A similar trend was observed for the fermentation of RFR using MS1 isolate. For batch 1, the CIT level was 438.62 ng/mL on day 4. The CIT level significantly elevated to 1099.33 ng/mL (day 5) and significantly dropped to 695.41 ng/mL after 6 days of fermentation. For batch 2, the CIT level started from 240.15 ng/mL (day 4) and significantly increased to 787.51 ng/mL after 6 days of fermentation. It was observed that on the following day (day 7), the CIT level significantly decreased to 326.36 ng/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 ($p < 0.05$).

Figure 6. 2. Citrinin levels produced by *Monascus purpureus* isolates (A) MF1 and (B) MS1 after fermentation of red fermented rice at 30 °C for 30 days.

Patrovsky et al. (2019) reported that *M. purpureus* DBM 4360 produced maximum CIT levels on day 14, then the CIT levels decreased until 24 days of incubation. Bazin et al. (2013) suggested that an increase in the pH of the RFR above pH 7.0 could cause the ring of the CIT structure to open and transform the CIT into CIT H2. This differs from the findings presented here in that the CIT levels decreased after 5 days of fermentation when the pH was below 7.0.

CIT and pigments share partial mutual biosynthesis pathways and are produced by polyketide synthase (Mou et al., 2023) (Figure 6.3). The addition of one acetyl–CoA molecule and three malonyl–CoA molecules are condensed into a tetraketide, followed by methylation, condensation, reduction, O–alkylation, cleavage between C–1 and C–2 bonding, oxidation, and dehydration resulting in CIT formation (Hajjaj et al., 2000a; Hajjaj et al., 1999b; 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. Meanwhile, pigments are produced when two malonyl–CoA molecules are added to tetraketide followed by esterification (Hajjaj et al., 2000a; Hajjaj et al., 1999b; Mou et al., 2023; Suharna, 2015). 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 to a certain branch. Another hypothesis is based on the analysis of the whole genome sequence, which is *Monascus* pigments and CIT have two different pathways due to the finding of their separate biosynthesis gene clusters.

Citrinin

Figure 6. 3. Citrinin biosynthesis pathway.
Source: Mou et al. (2023).

6.3.3. Determination of pigments

During the fermentation of RFR, the pigments of RFR increased over time (Figure 6.4). MF1 produced maximum levels of pigments after 21 days fermentation. However, isolate MS1 produced the maximum levels of pigments after 13 days fermentation. Patrovsky et al. (2019) studied the effects of different initial pH and cultivation times of *M. purpureus* in submerged liquid culture on the production of pigments and CIT. The studies reported that *M. purpureus* had maximum fungal growth after 10 days of incubation. Yellow pigments increased until day

24, meanwhile, orange pigments increased until day 14 and decreased after that. This may be due to mycelial damage in the later days of cultivation and an easier reaction of amino–containing substances with orange pigments to form red pigments (Patrovsky et al., 2019). However, the findings from the current study have different trends (Patrovsky et al., 2019). The yellow, orange, and red pigments produced by MF1 increased from day 4 until 21, meanwhile, the production of pigments by MS1 increased from day 4 until 13. This finding suggested that different isolates and fermentation days can affect pigments levels.

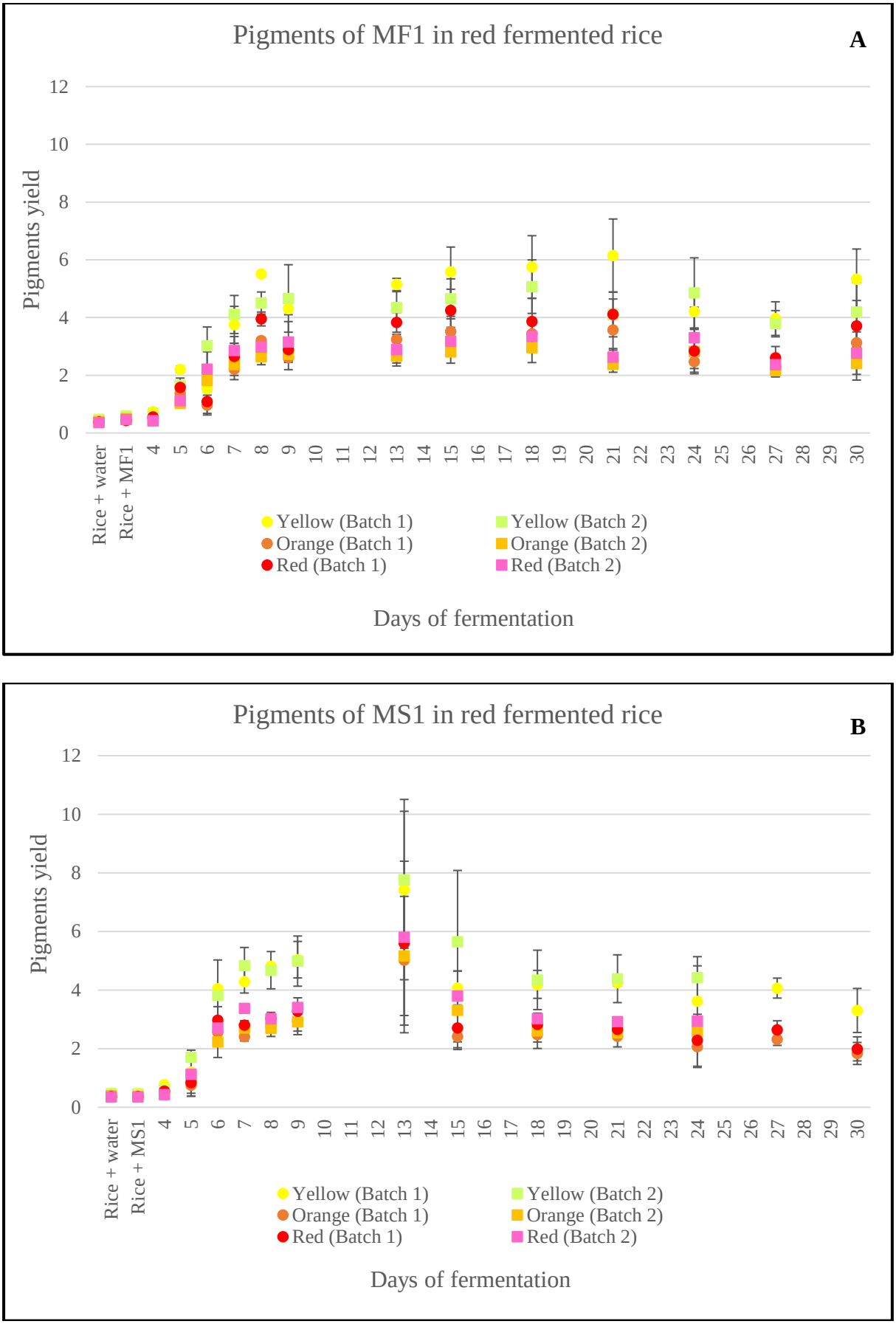
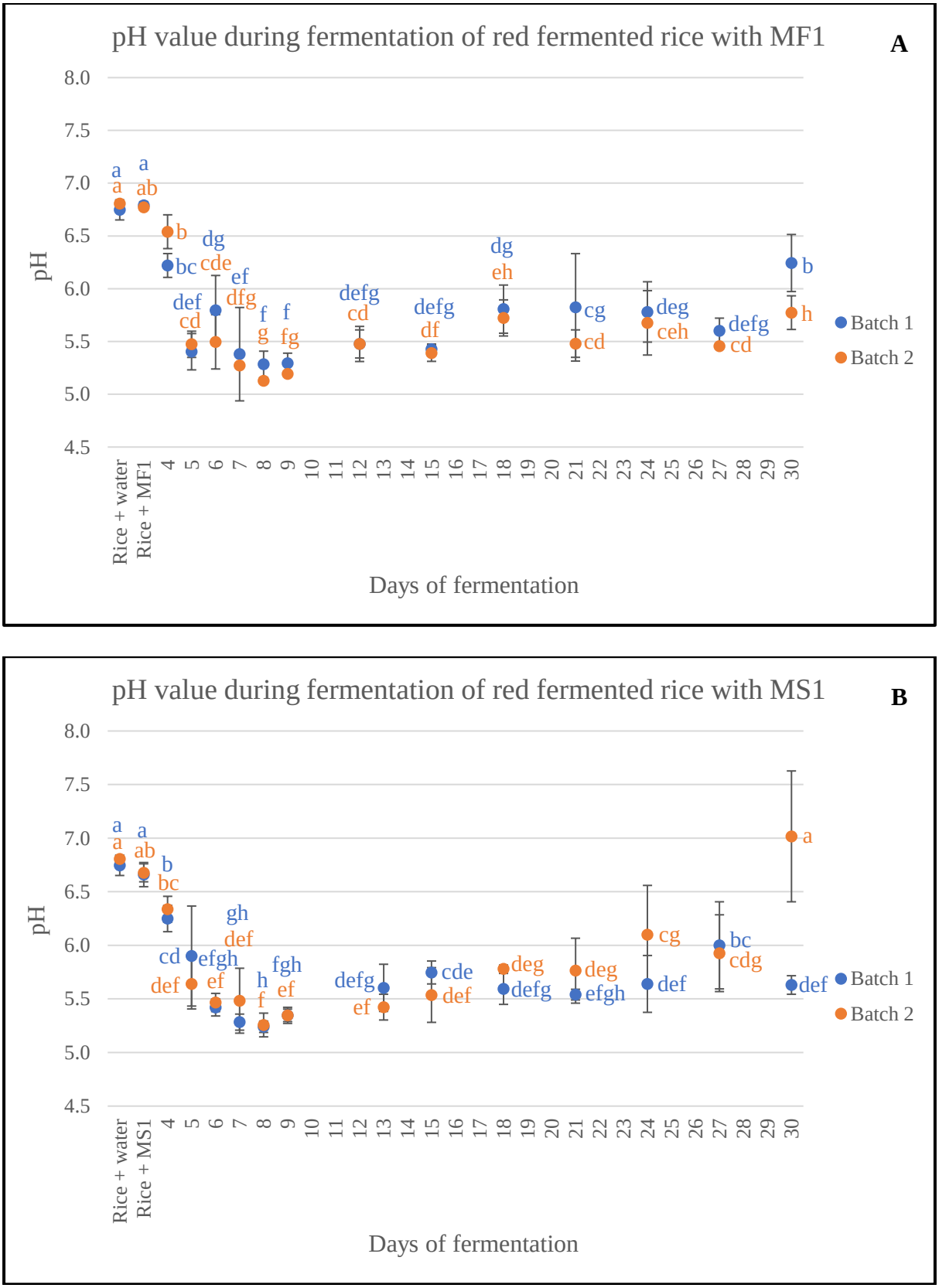


Figure 6. 4. The yield of pigments produced by *Monascus purpureus* isolates (A) MF1 and (B) MS1 after fermentation of red fermented rice at 30 °C for 30 days.

6.3.4. Determination of pH

Figure 6.5 shows the pH from MF1 significantly dropped from pH 6.79 to 5.29 (Batch 1) and 6.77 to 5.19 (Batch 2) during fermentation of RFR for 9 days. After 9 days of fermentation using MS1 isolate, the pH significantly decreased from 6.66 to 5.35 (Batch 1) and 6.68 to 5.35 (Batch 2). However, the pH of RFR from MF1 isolate significantly increased from day 18 (Batch 1) and day 12 (Batch 2) until end of fermentation. For MS1 isolate, the pH significantly increased from day 24 until day 30 (Batch 2).

This is in contrast to findings by Patrovsky et al. (2019), where the pH increased from 5.5 to 7.3 after 24 days of incubation. Patrovsky et al. (2019) also reported the CIT level increased until day 14 and decreased after a prolonged incubation period. It has been hypothesized that the decreasing CIT level is linked to the degradation of CIT to CIT H2 at pH above 7.0 (Patrovsky et al., 2019), due to the opening of the CIT ring structure (Bazin et al., 2013). However, the present study shows contradictory results of CIT levels when compared to Patrovsky et al. (2019). A possible explanation for the CIT reduction after five days of fermentation of RFR is there is an effect of the increasing level of pigments. It has been reported that the deletion of one of the *Monascus* pigments, *mrpigH,* results in an increase in other pigments and a reduction of CIT (Li et al., 2021).



* 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 ($p < 0.05$).

Figure 6. 5. The pH values of *Monascus purpureus* isolates (A) MF1 and (B) MS1 after fermentation of red fermented rice at 30 °C for 30 days.

6.3.5. Reduction of CIT after combining with pigments

Tables 6.1 and 6.2 show the results of CIT reduction after combining with pigments extracted from MF1 and MS1, respectively. After mixing the CIT standard and pigments from both isolates, CIT significantly reduced by 26–68 % and 16–45 % for MF1 and MS1, respectively. The pigments extracted from MF1 had a higher reduction of CIT compared to MS1 after mixing the pigments and CIT standard. This result may be explained by the fact that MF1 produced higher CIT compared to MS1.

Table 6. 1. Reduction 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 6. 2. 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 ($p < 0.05$).

Pigments from fungi can reduce mycotoxins. A study by Salama et al. (2021) found that red pigments from *P. purpurogenum* 2603 reduced AFB_1 and AFB_2 by 9–38 % and 45 %, respectively. For OTA, the reduction was recorded at 15 %, 31 %, and 54 % for the pigments concentrations of 1, 3, and 5 mg/mL in media, respectively. Pilu et al. (2011) reported that accumulation of flavonoid pigments especially phlobaphenes in maize kernel can reduce FB_1.

Most of the carotenes and xanthophylls pigments showed inhibition of AF produced by *A. flavus* and *A. parasiticus,* with inhibition greater for compounds with an α–ionone–type ring (α–carotene, β–carotene, or α–ionone) compared with compounds with a β–ionone ring. The results showed that *A. parasiticus* was less sensitive to β–carotenes than *A. flavus* (Norton, 1997).

6.4. Conclusion

CIT production peaked after 5 days of growth of both *M. purpureus* MF1 and MS1 isolates on rice. However, after 5 days of fermentation, CIT levels dropped which coincides with an increase in pigments production. Consequently, there appears to be a relationship between pigments and CIT produced by *M. purpureus* with an increase in pigments levels corresponding to a decrease in CIT. It is recommended to optimize fermentation to maximize pigments production of RFR and reduce CIT in food.

CHAPTER 7

EFFECTS OF *Monascus* PIGMENTS ON CITRININ REDUCTION

7.1. Introduction

Colour plays an important role in food production and processing, especially in enhancing first impressions and sensory characteristics (Aberoumand, 2011; Sen et al., 2019). In the food industry, food colourants increase the market value of the product because they indicate that the food is safe, fresh, delicious, and has nutritional and aesthetic value (Aberoumand, 2011; Downham & Collins, 2000; Manikprabhu & Lingappa, 2013; Narsing Rao et al., 2017). Food colourants can be divided into four categories: (1) natural colours, (2) nature–identical colours, (3) synthetic colours, and (4) inorganic colours (Aberoumand, 2011). Natural colours are pigments such as canthaxanthin, riboflavin, and carotene produced by living organisms while nature–identical colours are artificial pigments. Synthetic colours are artificial colours that are non–existent in the environment. Examples of inorganic colours are gold, silver, and titanium dioxide (Aberoumand, 2011).

Many synthetic colourants have been prohibited due to food safety and health issues such as toxicity, cancer, allergenicity, carcinogenicity, and hyperactivity in sensitive children (Dey & Nagababu, 2022; McCann et al., 2007; Potera, 2010; Sen et al., 2019). Therefore, there is a high demand for natural colours, and this is estimated to increase by 7 % every year (Pailliè-Jiménez et al., 2020; Sen et al., 2019; Tuli et al., 2015). The market for natural food colours and flavours was estimated to be USD 5.01 billion in 2020 and it is projected to reach USD 6.83 billion by 2025, growing at a CAGR of 5.4 % (Markets-and-Markets, 2020). Natural colour has many advantages such as being renewable, safe, biodegradable, with no risk to the environment, non–carcinogenic, non–allergic, and non–toxic (Pailliè-Jiménez et al., 2020; Sen et al., 2019).

Natural pigments have many benefits, such as intensifying colour, functional food ingredients, antioxidants, additives, antimicrobial properties (actibacterial, antifungal, and antiviral activity), antifouling, anti–algicidal, anti–insecticidal, anti–herbicidal, antiparasitic, antiprotozoal, antileishmanial, antitrypanosomal, antinematodal, ichthy deterrent, anti–biofilm, and use as protein markers for gel electrophoresis (Lin & Xu, 2020; Narsing Rao et al., 2017; Ramesh et al., 2019). Many of the natural pigments have bioactive compounds and have health benefits such as anti–inflammatory, antimetastatic, immunosuppressive, antitumor, anti–Alzheimer's, antiatherosclerosis, antihypertensive, anticancer, photosensitizers, antineoplastic, anti–Tuberculosis, antiulcerogenic, antilipoperoxidant, anti–HIV, anti–hypertriglyceridemia, anti–Atherosclerotic, cholesterol–lowering, anti–Proliferation, anti–aging, anti–obesity, anti–diabetic, antiadipogenic, are used for conjugated antibodies, prevent cardiovascular diseases, improve cognitive function, and avoid age–related macular degeneration (AMD) (Lin & Xu, 2020; Ramesh et al., 2019; Wang et al., 2013a).

There are many sources of natural pigments such as minerals, seeds/ roots, fruits, plants, insects, and microorganisms (Egea et al., 2023; Gmoser et al., 2017; Sen et al., 2019). The use of microbial fermentation to produce natural pigments is preferable due to scalability, availability, stability, easy and fast growth, ease of extraction and production, cost–effective production using low–cost substrates from a wide range of agro–industries waste, low labour costs, high yields, no seasonal dependence and variations, easy downstream processing, and strain improvement techniques to increase natural pigments (Akilandeswari & Pradeep, 2016; Chaudhary et al., 2022; Panesar et al., 2015; Sen et al., 2019). Fungi are preferable among many microorganisms because they can produce larger amounts of pigments as the fungi are readily be cultured in the laboratory (Afroz Toma et al., 2023; Kalra et al., 2020).

Filamentous and xerophilic fungi such as *Monascus* spp. are well known for the production of many secondary metabolites such as natural pigments, monacolins such as

monacolin K/lovastatin/mevinolin, GABA, dimerumic acid, polysaccharide, ergosterol, unsaturated fatty acids, condensed tannins, terametylpyrazine, and CIT (Chaudhary et al., 2022; Farawahida et al., 2022b; Feng et al., 2016; Gmoser et al., 2017; Hong et al., 2012; Jia et al., 2017; Liu et al., 2005; Mahmoud et al., 2021; Patel, 2016; Srianta et al., 2014). *Monascus* is a homothallic fungus and is classified into the class *Ascomycetes* and the family *Monascaceae* (Egea et al., 2023; Mahmoud et al., 2021). There are seven *Monascus* species internationally recognized, but the most relevant species in the food industries are *M. purpureus, M. pilous,* and *M. ruber* (Agboyibor et al., 2018; de Carvalho et al., 2005; Egea et al., 2023).

In many Asian countries such as China, Japan, and South East Asia, *M. purpureus* has been used for more than twenty centuries to produce RFR and natural pigments (Jia et al., 2019; Mahmoud et al., 2021; Woo et al., 2014). The yearly production of *Monacus* pigments, usually in the form of RFR, is almost 20,000 tons in China. It is estimated that more than one billion people consume products containing *Monascus* pigments during their daily lives (Jia et al., 2019). At least six pigments are produced by *Monascus* spp.: ankaflavin and monascin (yellow pigments), monascorubrin and rubropunctatin (orange pigments), and monascorubramine and rubropunctamine (red pigments) (Chai et al., 2020; Egea et al., 2023; Farawahida et al., 2022b; Meinicke et al., 2012). There are more than 50 patents from The United States of America (USA), France, Federal Republic of Germany, and Japan to use *Monascus* spp. in the food (Lin et al., 1992), indicating that there is considerable interest to use natural pigments extracted from *Monascus* spp. (Magro et al., 2016). The red pigments have been widely applied in food such as candy, pastry, meat, sausage, ham, “koji”, soybean cheese, tofu, red wine, and fruit juice (Hajjaj et al., 1999a; Wang et al., 2013b; Woo et al., 2014). However, the use of *Monacus* spp. to produce pigments and *Monascus*–fermented products has some limitations in the food industry due to the production of CIT (Frisvad et al., 2013; Yang et al., 2019). There is a

restriction on the importation of *Monascus*–fermented products from China to Japan, USA, and European countries due to CIT contamination (Liang et al., 2018).

Taiwan has recommended a limit of 2000 µg/kg for RFR products (Taiwan-FDA, 2020). Japan and China have set a maximum limit of 50 µg/kg and 200 in RFR products, respectively (Kamle et al., 2022; Urraca et al., 2016). Meanwhile, European authorities established a maximum level of 100 µg/kg CIT in food supplements based on rice fermented with *M. purpureus* (EU, 2019) (Table 7.1).

Table 7. 1. Regulatory limits of 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)

It is important to reduce CIT levels in RFR. The findings from previous chapters (Chapters 5 and 6) showed that there was a relationship between CIT reduction and pigments production during incubation and fermentation of *M. purpureus* isolates MF1 and MS1 on CCA and RFR, respectively. Therefore, this study aimed to determine the relationship between *Monascus* pigment and CIT. There is a possibility that specific pigments or all pigments contributed to CIT reduction.

7. 2. Materials and methods

7.2.1. Materials

The CIT standard was supplied by Sigma–Aldrich. Ankaflavin, monascin, monascorubrin, rubropunctatin, and monascorubramine (*Monascus* pigment standards) were purchased from Biopurify Phytochemicals Ltd. (Sichuan, China).

7.2.2. Preparation of a standard curve for CIT determination

The CIT standard was purchased from Sigma–Aldrich. To obtain 0.5 mg/mL CIT, 5 mg of CIT standard was mixed with 10 mL of MeOH. Different CIT concentrations were prepared from 0.5 mg/mL CIT, which were 4, 50, 100, 500, 1000, 2000, and 3000 ng/mL. These CIT standards were injected to the UHPLC–FLD. Seven concentrations of CIT were used to build a calibration curve and this calibration curve was used to quantify CIT in the samples.

7.2.3. Mixing of pigments and CIT standard

Three different concentrations (10, 100, and 1000 µg/mL) of ankaflavin, monascin, monascorubrin, rubropunctatin, and monascrobramine were prepared separately by adding MeOH to fully dissolve. All the prepared pigments were mixed with 1000 ng/mL of CIT (1:1 v/v) and the mixture were left for 1 h. A mixture of water and 1000 ng/mL of CIT (1:1 v/v) was used as a control. The samples were injected into UHPLC–FLD and the CIT levels were compared with the control to determine reduction.

7.2.4. Determination of CIT content

UHPLC-FLD (Dionex Ultimate 3000, California, USA) was used to determine the CIT. The mobile phase used was (1) ACN (Sigma-Aldrich, Missouri, USA) and (2) 0.1 % TFA in water, and all the solvents used were HPLC grade. These solvents were separately filtered through a regenerated cellulose filter (0.20 µm pore size; Sartorius, Göttingen, Germany) before being used. The ratio of the mobile phase used in the system was ACN: 0.1 % TFA (55:45, v/v) with a flow rate of 1.0 mL/min. For the stationary phase, a reverse–phase symmetry Kinetex C_{18} column (250 mm length × 4.6 mm internal diameter, 5 µm particle size, 100 Å pore size) was used. The column temperature was set at 30 °C. The excitation and emission

wavelengths for the FLD detector were 330 nm and 500 nm, respectively. The injection volume was 20 µL, and the system was running isocratically for 40 min. All the injections were done in triplicate and Chromeleon 7.0 software (Thermo Fisher Scientific) was used to process the data.

7.2.5. Statistical analysis

Samples were conducted in triplicate, and the data obtained were subjected to univariate analysis. All the data obtained were subjected to ANOVA using the generalized linear model (GLM) procedure and significant differences between means ($p < 0.05$) were confirmed using the Tukey Pairwise comparison. All the variables and their interactions were tested as independent variables. Minitab software (version 21) was used to analyse the data.

7.3. Results and discussion

Seven different CIT concentrations were used to develop a standard curve. The results showed that the calibration curve is good ($R^2 = 0.9999$). The calibration curve was further used to determine the CIT levels in the samples.

This study examined, for the first time, the effects of *Monascus* pigments on CIT reduction. The results showed that only pigments and combinations of pigments and their levels significantly reduced CIT ($p < 0.05$). Table 7.2 shows the effects of pigments on the reduction of CIT. Combinations of pigments and their levels on the reduction of CIT are reported in Table 7.3 and Figure 7.1.

Table 7. 2. The effects of *Monascus* pigments on the reduction of citrinin.

Groups of pigments	Pigments	Mean of citrinin reduction (%)
Yellow pigments	Ankaflavin	34.62 ± 8.18^{a}
	Monascin	33.50 ± 5.08^{a}
Orange pigments	Monascorubrin	17.07 ± 3.01^{c}
	Rubropunctatin	25.70 ± 7.39^{b}
Red pigments	Monascorubramine	22.05 ± 3.73^{bc}

* Concentrations of pigments used were 10, 100, and 1000 µg/mL (n = 9 for each pigment) to obtain mean. Mean values with different superscripts within same column are significantly different ($p < 0.05$).

Table 7. 3. The effects of *Monascus* pigments and their levels on the reduction of citrinin.

Group of pigments	Pigments	Mean of citrinin reduction (%)		
		Levels of pigments		
		10 µg/mL	100 µg/mL	1000 µg/mL
Yellow pigments	Ankaflavin	41.47 ± 7.83^{a}	32.62 ± 8.92^{abc}	29.76 ± 3.86^{abcde}
	Monascin	37.00 ± 7.61^{ab}	32.51 ± 3.22^{abc}	30.99 ± 2.32^{abcd}
Orange pigments	Monascorubrin	15.20 ± 4.18^{e}	16.91 ± 2.37^{de}	19.11 ± 1.27^{cde}
	Rubropunctatin	18.67 ± 8.33^{cde}	32.34 ± 2.39^{abc}	26.09 ± 1.68^{bcde}
Red pigments	Monascorubramine	21.40 ± 4.08^{cde}	22.96 ± 4.18^{bcde}	21.80 ± 4.43^{bcde}

* Mean values (n = 3 for each level) with different superscripts are significantly different ($p < 0.05$).

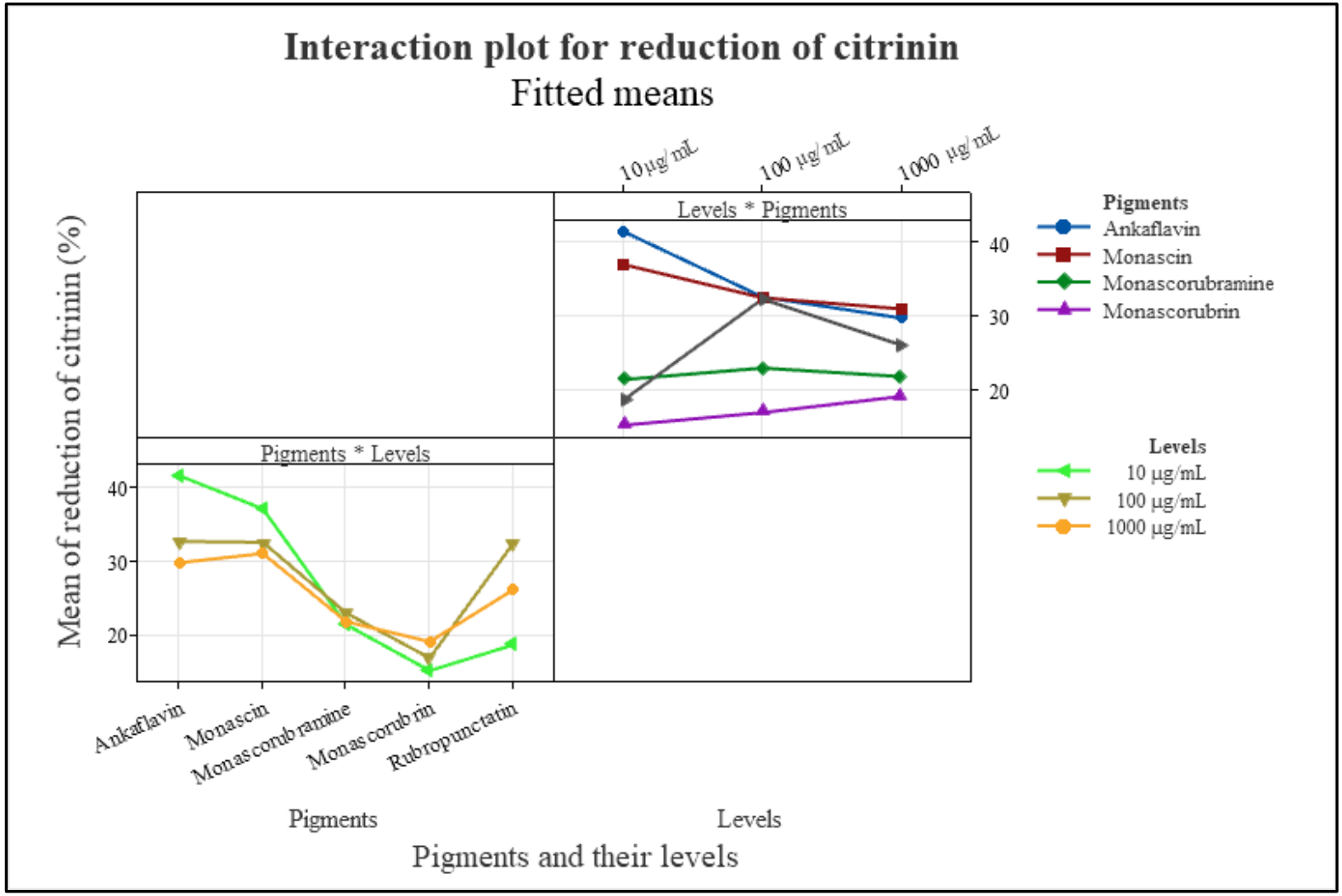


Figure 7. 1. The illustration of the effects of a combination of pigments and their levels on the reduction of CIT. The maximum error bar is 8.92 with *n* = 3 for each level.

Ankaflavin and monascin (yellow pigments) produce a higher CIT reduction compared to the orange and red pigments (Table 7.2), with the reduction of CIT after mixing with these pigments at 30–41 % and 31–37 %, respectively (Table 7.3). From a previous study, Lung et al. (2016) demonstrated that the *Monascus*–fermented *Disoscorea* (a traditional Chinese herb) fermented by using deep ocean water (DOW) (ocean water from a depth of more than 200 m) (DOW–MFD) significantly increased ($p < 0.05$) monascin and ankaflavin production, while CIT levels were significantly reduced ($p < 0.05$) due to presence of magnesium (Mg), calcium (Ca), potassium (K), zinc (Zn), and iron (Fe). Monascin and ankaflavin have other benefits such as decreasing the levels of total cholesterol, triglycerides, and low-density lipoprotein (LDL) cholesterol contents in the blood (Lee, 2015; Lee et al., 2010b). In this study, monascorubrin and rubropunctatin reduced CIT by 15–19 % and 19–32 %, respectively. Meanwhile, monascorubramine reduced CIT from 21 % to 23 % after mixing the pigment and CIT standard. Table 7.4 shows the effects of factors on the reduction of CIT. Pigments and combinations of pigments and their levels significantly reduced CIT.

Table 7. 4. The effects of different factors on the reduction of citrinin.

Factors	***p*–value**
Pigments	0.000
Levels of pigments	0.584
Pigments × levels of pigments	0.022

A strong relationship between pigments and mycotoxins has been reported in the literature. Recent studies found that blue and black–grained wheat were more contaminated with mycotoxins compared to red and purple varieties (Gozzi et al., 2023). Another similar finding showed that black barley was more resistant to deoxynivalenol accumulation than yellow barley (Choo et al., 2015). Landoni et al. (2020) conducted a study to determine the amount of fumonisins in the maize flour extracted from coloured lines (which contain

phlobaphenes pigment) and non–coloured lines of maize kernels for three years. The study found that the fumonisins levels were lower in the colour flour compared to the colourless flour, with average fumonisins reduction of 19 % and 39.2 %, respectively. The study showed a strong relationship between pigmentation and fumonisins reduction. This result was consistent with an earlier study that phlobaphene pigments could be associated with a reduced level of fumonisin B_1 in maize kernels (Pilu et al., 2011).

Yellow maize naturally contains carotenoids and xanthophylls, and most of these carotenoids are found in the hard endosperm of the kernel (Muzhingi et al., 2008). Carotenoids are natural pigments and can be categorized into two groups based on their chemical composition, which are carotenes and xanthopylls (Doukani et al., 2022; Maoka, 2020), Examples of carotenes are α–carotene, β–carotene, β,ψ–carotene (γ–carotene), phytofluene, lycopene, and phytoene, meanwhile compounds such as lutein, astaxanthin, zeaxanthin, β-cryptoxanthin, spirilloxanthin, antheraxanthin, fucoxanthin, peridinin, and echinenone are xanthophylls (Doukani et al., 2022; Maoka, 2020). AF produced by *A. flavus* and *A. parasiticus* was inhibited by most carotenes and xanthophylls occurring in maize endosperm, without affecting their growth (Norton, 1997). However, *A. parasiticus* was less sensitive than *A. flavus* (Norton, 1997).

β–carotene can be converted into β–ionone by carotenoid cleavage dioxygenase 1 (Paparella et al., 2021). α–ionone and dihydro–β–ionone are two analogs of β–ionone that act as plant volatiles and are important in the interaction between plants and herbivores. The difference between α–ionone and β–ionone is the position of endocyclic double bond, which is located at C–2 and C–1 position of the ring in α–ionone and β–ionone, respectively (Li et al., 2019). The presence of an α–ionone ring is almost 25 times more inhibitory of AF production than β–ionone ring (Norton, 1997). In contrast, tocopherols (α– and γ–tocopherol) increased approximately 20 % of AF production when 2 mg/mL of α– and γ–tocopherol were added,

respectively (Norton, 1997). Tocopherols occur primarily in the germ of corn. This compound has a similar structure to carotenoids, and both of these compounds has similar anti–oxidant properties (Norton, 1997). However, carotenoids and tocopherols have contradictory affects to AF production. The difference between these compounds is carotenoids have conjugated double bonds in the tail or central portion of the compound, allowing them to integrate into membranes. Norton (1997) suggested there are two possibilities for the inhibition AFB_1 production: (1) the structure or activity of carotenoids can alter the cell membranes and indirectly affect the polyketide synthase, which is in the cytosol (Dutton, 1988), (2) carotenoids connected with hydrophobic domains of the synthase or AF pathway enzymes and, thereby, affect synthesis. Further study showed that AF synthesis appears to be inhibited before the formation of norsolorinic acid, either indirectly by affecting general cell metabolism or directly by affecting the polyketide synthase steps (Norton, 1997).

It has been reported that the red pigments extracted from *P. purpurogenum* has antibacterial, antifungal, and antimycotoxigenic properties (Salama et al., 2021). Further studies showed that there was a reduction of AFB_1 between 9 % and 38 %, for 1 mg/mL and 5 mg/mL red pigments, respectively. For AFB_2, the reduction was 45 % for 5 mg/mL. OTA had the highest reduction, which was 15 %, 31 %, and 5 % for 1, 3, and 5 mg/mL red pigments (Salama et al., 2021). Rubropunctatin, orange pigment extracted from *Monascus* spp. has antifungal activity by disruption of cell membrane of *P. expansum* MTCC 4900, *Rhizopus stolonifer* MTCC 10595, and *A. niger* MTCC 8652 (Majhi et al., 2023).

7.4. Conclusion

There is a high demand for natural colours in the food industry. Natural colours can be extracted from many sources including fungi, such as *Monascus* spp. However, there are some limitations to utilising *Monascus* spp. due to the ability of this fungus to produce CIT, a

carcinogenic toxin, as a secondary metabolite. This study was conducted to determine the effects of *Monascus* pigments on the reduction of CIT. The results showed that ankaflavin, monascin, monascorubrin, rubropunctatin, and monascorubramine significantly ($p < 0.05$) reduced CIT, with the highest reduction seen from ankaflavin. A possible explanation for yellow pigments (ankaflavin and monascin) producing a higher reduction of CIT compared to other pigments is ankaflavin and monascin have fewer double bonds (Figure 7.2). The possible reason is fewer double bonds means that the pigments are more saturated and have a less reactive structure. This condition can avoid any unwanted side reactions happened, resulting more effective interaction between pigments and CIT, leading to higher CIT reduction. The finding of this study demonstrates the possibility of optimizing pigments production to control CIT levels in the *Monascus*–fermented products.

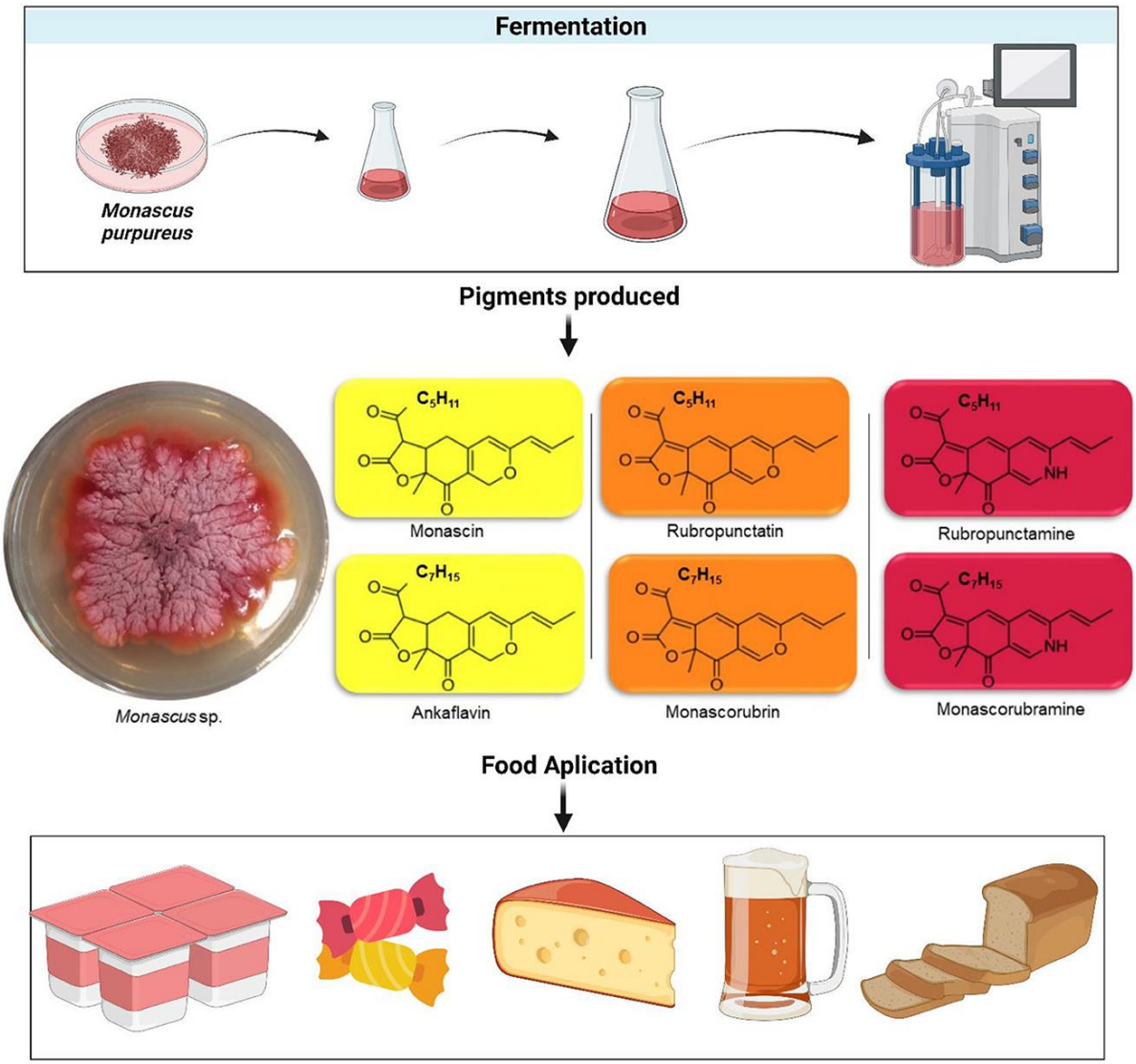


Figure 7. 2. Production of yellow (monascin and ankaflavin), orange (rubropunctatin and monascorubrin) and red (rubropunctamine and monascorubramine) pigments from *Monascus* spp. and their food applications. Monascin and ankaflavin have fewer double bonds than other pigments.

Source: Egea et al. (2023).

CHAPTER 8

SUMMARY, CONCLUSIONS, LIMITATIONS, AND RECOMMENDATIONS FOR FUTURE RESEARCH

8.1. Summary

RFR is rice fermented using *Monascus* spp. as a fungal starter. This product contains secondary metabolites produced by *Monascus* spp. such as pigments. Pigments are used as natural colour and flavouring agents. However, *Monascus* spp. can also produce the mycotoxin, CIT which is believed to have adverse effects on human health and animals. RFR can be produced by SSF and SmF. Therefore, Chapter 2 of the thesis is a review on *Monascus* spp. as the starter fungi for RFR production, secondary metabolites produced during the fermentation of RFR including beneficial compounds such as pigments and monacolin K, the production of RFR by SSF and SmF, factors affecting the growth and pigments production of *Monascus* spp., factors affecting CIT production during fermentation of RFR, toxicity of CIT, and the occurrence, detection, and reduction of CIT in RFR.

CIT in RFR has been reported worldwide by using different methods of detection. However, those methods required high skills and do not screen *Monascus* spp. isolates that can produce CIT. In Chapter 3, CCA was developed as a simple and rapid screening method for the detection of *Monascus* spp. isolates that can produce CIT. RFR was spread onto CCA and other media (MEA, PDA, CYA, G25N, and YES) and incubated at 30 °C for 7 days. All the media were observed daily under UV light and any *Monascus* spp. colony that produced light blue fluorescence was recorded as a CIT–producer. Two different isolates (MF1 and MS1) isolated from RFR on CCA were selected for further analysis. All CCA plates (100 %; 10/10) inoculated with MF1 produced light blue fluorescence after incubation for 4 days, meanwhile,

30 % (3/10 plates) of MS1 produced weak fluorescence on CCA after incubation for 7 days. The presence of CIT on CCA was indicated by the change of fluorescence from light blue to yellow colour. Presumptive *Monascus* spp isolated from RFR were observed macroscopically and microscopically for preliminary identification. The isolates were extracted and purified for DNA sequencing. Four primers (ITS, β–tubulin, *pksCT*, and *ctnA*) were used to confirm identification. ITS and β–tubulin were used to identify the species of the fungi, , while *pksCT*, and *ctnA* are the specific genes for CIT production (Li et al., 2020; Touhami et al., 2018; Xu et al., 2009). MF1 and MS1 isolates have been deposited in ICMP, Landcare Research, New Zealand for public strain collection and are labelled as ICMP 25182 and ICMP 25183, respectively. These isolates were identified as *M. purpureus* Went. Both isolates can produce CIT using *pksCT* and *ctnA* genes. CIT was quantified by UHPLC–FLD. CCA has been identified as a simple and rapid method for screening CIT–producing *Monascus* isolates (Objective 1). This method may be used to select *Monascus* spp. that do not produce CIT for production of RFR.

There was a lack of studies to determine the toxicity of CIT by using a simple method. In Chapter 4, different concentrations of CIT standards were tested for toxicity using *A. salina* (brine shrimp) showing an LC_{50} of 66 µg/mL. CIT is considered toxic and highly toxic according to Meyer's and Clarkson's toxicity index, respectively. Brine shrimp bioassay can be used to determine the toxicity of CIT by estimation of CIT concentration that can kill 50 % of the brine shrimp after exposure to CIT for 24 h (Objective 2). This was a simple method to determine the toxicity of CIT.

There are secondary metabolites produced by *Monascus* spp. such as pigments and CIT. In Chapter 5, a study was designed to characterize the growth, pigments, CIT, and pH of *M. purpureus* isolates on CCA for prolonged incubation period. MF1 and MS1 isolated from RFR were inoculated onto CCA. The CCA plates were incubated for 30 days at 30 °C. The fungal

growth of both isolates was determined based on the size of colonies, ZU, ZR, and the mass of fungi. During the incubation period, pigments, CIT, and pH of the extracts were measured using a microplate reader, UHPLC–FLD, and a pH meter, respectively. From day 4 until days 6–8, the CIT levels increased and then decreased during further incubation. The pigments produced by *M. purpureus* increased after 8 days of incubation indicating an inverse relationship between CIT reduction and pigments production. The pH of these isolates on CCA increased from 7 to 8.6. The fungal growth, CIT, pigments, and pH of *M. purpureus* can be determined by inoculating *M. purpureus* isolates onto CCA and incubating the CCA plates at 30 °C for 30 days (Objective 3). These results provide fundamental knowledge of the relationship between the pigments, CIT, and pH levels during the incubation of *M. purpureus* isolates on CCA.

It is unclear whether secondary metabolites produced during the fermentation of RFR follow a similar trend or not. Therefore, Chapter 6 uses a similar approach by applying MF1 and MS1 on rice to produce RFR. In this study, RFR is produced by SSF. The rice underwent soaking, steaming, and autoclaving before inoculating with one of two *M. purpureus* isolates (MF1 and MS1) and was fermented at 30 °C for 30 days. CIT and pigments produced during the fermentation of RFR followed a similar trend to the incubation of these isolates on CCA. However, the pH followed a different trend. 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 produced by *M. purpureus* 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 showed that there was a reduction in CIT by 26–68 % and 16–45 %, respectively. There was a relationship between CIT reduction and pigments production during fermentation of RFR (Objective 4). This is the

first study reported that there was an inverse relationship between CIT reduction and pigments production during incubation of *M. purpureus* on CCA and fermentation of RFR, respectively.

The effects of *Monascus* pigment on the reduction of CIT is discussed in Chapter 7. Different *Monascus* pigments with different concentrations were mixed with CIT standards. The results showed that ankaflavin, monascin, monascorubrin, rubropunctatin, and monascorubramine significantly ($p < 0.05$) reduced CIT, with the highest reduction observed from ankaflavin. There was an inverse relationship between *Monascus* pigments (ankaflavin, monascin, monascorubrin, rubropunctatin, and monascorubramine) and CIT (Objective 5). The findings of this study suggest the possibility of optimizing pigments production to control CIT levels in *Monascus*–fermented products, resulting in improved and safer products.

8.2. Conclusion

CCA can be used as a semiquantitative method to detect CIT–producers of *Monascus* spp. Based on this method, two isolates from RFR, MF1 and MS1 were selected for this study. These isolates are *M. purpureus* with the ability to produce CIT based on macroscopic and microscopic observation, PCR, DNA sequencing, and UHPLC–FLD. The toxicity test with brine shrimp showed that CIT is toxic. Incubation and fermentation of *M. purpureus* on CCA and rice, respectively, produced a similar trend for CIT and pigments levels. There was an increase in CIT levels to a certain point of incubation and fermentation followed by a reduction. The pigments increased during the reduction of CIT, suggesting that there was a relationship between CIT and pigments. Mixing of pigments and CIT standards showed that there was a reduction in CIT. Further study using different *Monascus* pigments showed that ankaflavin, monascin, monascorubrin, rubropunctatin, and monascorubramine reduce CIT, with the highest reduction observed from ankaflavin. The finding of this study suggests the possibility of optimizing pigments production to control CIT levels during the production of

RFR. This study is important because it can help the RFR industry to produce high–quality and safe RFR. The authorities can set specific regulations for controlling CIT in RFR to protect consumers' health based on the findings of this study.

8.3. Limitation

RFR was imported from Teluk Intan, Perak, Malaysia to Massey University, Palmerston North, New Zealand with a permit, due to the unavailability of RFR in New Zealand. From the food industry perspective, it is better to use broken rice to produce RFR because that is considered the lowest quality of rice. However, there is no broken rice in New Zealand. Therefore, low–quality white long–grain rice from supermarkets was used in this study to produce RFR.

8.4. Recommendations for further research

There was a limited number of isolates used in this study due to limited RFR imported into New Zealand. For further research, it is recommended that RFR from different places be imported to obtain more *Monascus* spp. isolates from different species. There is a possibility to isolate *Monascus* spp. that cannot produce CIT. Furthermore, DNA sequencing for pigments and monacolin K biosynthesis genes can be included in the study.

In this study, secondary metabolites such as CIT, pigment, and pH were measured during the incubation and fermentation of *M. purpureus* on CCA and rice. Other important metabolites such as monacolin K, GABA, and dimerumic acid may be produced during incubation. This project provides new information towards understanding the relationship between secondary metabolites produced by *M. purpureus* on CCA and rice.

Rice used in this study is the common substrate used for the fermentation of *Monasscus* spp. to produce RFR. However, *Monacus* spp. can be used to ferment other substrates or agro–

industrial wastes to produce new *Monacus*–fermented products with associated health benefits. Additional monacolin K, GABA, and dimerumic acid content information during the fermentation of *Monascus*–fermented products are strongly recommended. Furthermore, the total phenolic content and antioxidant activity of RFR can be measured based on the 2,2–diphenyl–1–picrylhydrazyl (DPPH) scavenging activities.

Several *Monascus* spp. such as *M. pilosus* MS–1 and *M. ruber* (isolates R1, R2, R19, R20, R21) have been reported that do not produce CIT. (Blanc et al., 1995b; Li et al., 2020). However, the production of pigments by these isolates have not mentioned. Therefore, there is a limitation to use these isolates to produce RFR and *Monascus*–fermented products. There is a possibility to optimize pigments production to control CIT levels during fermentation of RFR by controlling various factors. There are many factors affecting pigments production such as incubation conditions, *Monascus* spp. isolates, substrate, carbon source, organic nitrogen, inorganic nitrogen, temperature, pH, and moisture content (Kraboun et al., 2019). Water activity and concentration of the inoculum used might affect pigments production. It is recommended to manipulate variable fermentation conditions to optimize pigments production during production of RFR. A predictive model should be developed to elucidate the relationship between CIT and pigments production in RFR and help designing the best combination of conditions for fermentation.

Identifying the mechanism between pigments production and reduction of CIT, either *in vitro* or over time during fermentation of RFR would be an important and interesting area for further study. Some differences were observed between *M. purpureus* isolates MF1 and MS1 in terms of their pigments and CIT production. These differences can be explored further by whole genome sequencing and detection of genes responsible for pigments, monacolin K, and CIT biosynthesis genes (other than *pksCT* and *ctnA* genes).

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APPENDICES

Appendix A: Statement of Contribution (Chapter 2)

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Appendix B

First page of publication (Chapter 2)

International Journal of Food Microbiology 379 (2022) 109829

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Review

Monascus spp. and citrinin: Identification, selection of *Monascus* spp. isolates, occurrence, detection and reduction of citrinin during the fermentation of red fermented rice

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ARTICLE INFO

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ABSTRACT

Red fermented rice (RFR) is rice fermented using *Monascus* spp. This product contains monacolin K, providing health benefits including mitigation of diarrhoea and improving blood circulation. RFR can produce pigments that can act as natural colour and flavouring agents. However, *Monascus* spp. (a fungal starter to ferment RFR) can also produce the mycotoxin, citrinin (CIT) which is believed to have adverse effects on human health. CIT in RFR has been reported worldwide by using different methods of detection. This review focuses on the production of RFR by solid-state fermentation (SSF) and submerged fermentation (SmF), the occurrence of CIT in RFR, CIT quantification, the factors affecting the growth of *Monascus* spp., pigments and CIT production in RFR, and possible methods to reduce CIT in RFR. This review will help the food industries, researchers, and consumers understand the risk of consuming RFR, and the possibility of controlling CIT in RFR.

1. Introduction

Red fermented rice (RFR) is a fermented product consumed in East Asia for centuries and is particularly popular in Chinese dishes. RFR is also known as red rice, red leaven, zhitai, hong qu, angkak, and hung-chu among the Chinese while the Japanese call the product beni-koji (Erdoğrul and Azirak, 2004). Other names for RFR are rotscimmelreid (Europe), Anka, Ang-Khan, Anka-Koji, red mould rice and red yeast rice (Chiu et al., 2006; Patcharee et al., 2007; Ristiarini et al., 2017). RFR is widely used as a therapy for hyperlipemia. RFR is available in the market in dried and powder forms (Abdul-Manan et al., 2017). RFR has been used to give colour and flavour, and act as a preservative in East Asian foods and cuisine, especially in China, Korea, and Japan. RFR is also consumed as a traditional Chinese medicine (Nguyen et al., 2017) and dietary supplement in Western countries (Zhu et al., 2019). Table 1 summarizes the benefits of RFR to improve the quality of human health.

During fermentation of the RFR, secondary metabolites such as pigments, lovastatin/monacolin K, polysaccharide, γ-aminobutyric acid (GABA), ergosterol, and CIT are produced (Srianta et al., 2014). Monacolin K, also known as lovastatin in lactone form (Younes et al., 2018), is a natural statin, acting as an inhibitor of the enzyme 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG-CoA reductase) that prevents the formation of mevalonate from HMG-CoA during cholesterol biosynthesis (Suraiya et al., 2018). The United States Food and Drug Administration (USFDA) has approved the use of statins to treat hyperlipidaemia (abnormally high level of fats/lipids in the blood including cholesterol and triglycerides) (Gregory et al., 2012). Statins can be used either as a single-ingredient such as Lipitor (atorvastatin), Lescol (fluvastatin), Mevacor (lovastatin), Altoprev (lovastatin extended-release), Livalo (pitavastatin), Pravachol (pravastatin), Crestor (rosuvastatin) and Zocor (simvastatin); or in combination with other products including Advicor (lovastatin/niacin extended-release), Simcor (simvastatin/niacin extended-release), and Vytorin (simvastatin/ezetimibe) (USFDA, 2016). The average amount of prescription lovastatin (Monacolin K) is 10–80 mg/day (Gregory et al., 2012). Consumption of RFR containing RFR could lead to adverse effects on the liver, and musculoskeletal system including a breakdown of muscle tissue. This muscle tissue breakdown releases a damaging protein (myoglobin) into the blood and can damage the kidneys (Younes et al., 2018). However, the European Food Safety Authority (EFSA) Panel is unable to recommend a safe dietary intake of monacolins from RFR due to several uncertainties (Younes et al., 2018). Another metabolite such as citrinin (CIT), a mycotoxin of food safety concern, can be produced by some of *Monascus* spp. such as *M. purpureus* during fermentation of RFR

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Appendix C

Statement of Contribution (Chapter 3)

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Farawahida, A. H., Palmer, J., & Flint, S. (2022). Coconut Cream Agar as a simple and rapid semiquantitative method to screen citrinin-producing Monascus spp. isolates isolated from red fermented rice. Journal of Microbiological Methods, 199, 106523. https://doi.org/https://doi.org/10.1016/j.mimet.2022.106523

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Appendix D

First page of publication (Chapter 3)

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Coconut Cream Agar as a simple and rapid semiquantitative method to screen citrinin-producing *Monascus* spp. isolates isolated from red fermented rice

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ABSTRACT

Red fermented rice (RFR) is produced using *Monascus* spp. This product has some health benefits. However, RFR can also contain the mycotoxin, citrinin (CIT) and that has adverse effects on human health. The objective of the study was to develop a simple and rapid screening method for the detection of *Monascus* spp. isolates that can produce CIT by using Coconut Cream Agar (CCA). RFR was spread onto CCA and other media and incubated at 30 °C for 7 days. All the media were observed daily under ultraviolet (UV) light and any *Monascus* spp. colony that produced light blue fluorescence was recorded as a CIT-producer. Two different isolates (MF1 and MS1) isolated from CCA were selected for further analysis. All (100%; 10/10 plates) of CCA inoculated with MF1 produced light blue fluorescence after incubation for 4 days, meanwhile 30% (3/10 plates) of MS1 produced weak fluorescence on CCA after incubation for 7 days. Isolates MF1 and MS1 were identified as *M. purpureus* with the ability to produce CIT by having polyketide synthase (*pksCT*) and transcriptional regulator (*ctnA*) genes. CIT was quantified by high-performance liquid chromatography (HPLC). CCA is a simple and rapid method to detect CIT-producers of *Monascus* spp.

1. Introduction

Red fermented rice (RFR) is a fermented product that is widely used in East Asian countries, especially in Chinese dishes. RFR is produced by fermenting rice with *Monascus* spp. as a fungal starter. RFR is also known as red rice, red leaven, zhitai, hong qu, hung-chu (Chinese), beni-koji (Japanese), rotscimmelreid (Europe), angkak, Anka, Ang-Khan, Anka-Koji, red mould rice and red yeast rice (Chiu et al., 2006; Erdoğrul and Azirak, 2004; Patcharee et al., 2007; Ristiarini et al., 2017).

RFR can be consumed as porridge, or used as traditional Chinese medicine (TCM), food flavour, food colourant, food preservative, remove meaty odour, and fermentation of traditional Chinese red yeast rice wine/red fermented rice wine (Samsudin and Abdullah, 2014). Red yeast rice wine is produced by mixing cooked white glutinous rice with RFR and incubating for a month. RFR has health benefits such as improved blood circulation, as an anti-hypercholesterolemic agent (Cicero et al., 2019), produces hypolipidemic effects (Zhou et al., 2019), has anti-fatigue and anti-cancer (Xue et al., 2017) benefits, and anti-inflammatory activity (Hsu et al., 2010). RFR is available in traditional Chinese shops and is in high demand (Samsudin and Abdullah, 2014). More than a billion people consume RFR and *Monascus*-fermented products worldwide (Yang et al., 2015).

However, RFR might be contaminated with citrinin (CIT) as a secondary metabolite produced by some *Monascus* spp. (Liao et al., 2014). CIT is a known hepatonephrotoxin, which changes the metabolism of the liver, and impairs the structure and function of the kidneys (Silva et al., 2021). Based on a CIT toxicokinetic study in humans, the absorption of CIT in humans was >40% based on the 40% of CIT eliminated in the urine (Degen et al., 2018). Due to its high mammalian nephrotoxicity, it is not allowed to be used as a drug (Flajs and Peraica, 2009) or as a dietary supplement (Gordon et al., 2010). Nevertheless, some researchers claim that RFR and *Monascus* fermented products represent no threat to the health of animals or humans possibly due to the low amount of CIT in RFR or other *Monascus* fermented products (Lee et al., 2006a; Mohan Kumari et al., 2009; Venero et al., 2010). However, most researchers consider that some action should be taken to control CIT levels in RFR (Chen and Hu, 2005). For CIT in RFR fermented with *Monascus purpureus*, Commission Regulation (EU) has set a 100 μg/kg maximum permitted limit (EU, 2019).

Different methods can be used to detect CIT in RFR, including

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Appendix E

Statement of Contribution (Chapters 4 and 7)

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Appendix G

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