

IMPACT OF THE FERMENTATION CONDITIONS ON PIGMENT, MONACOLIN K, AND CITRININ PRODUCTION OF *Monascus purpureus* Went

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ABSTRACT

Red fermented rice, commonly used as a natural food colorant, contains monacolin K (MK), a bioactive compound known for its cholesterol-lowering properties. However, concerns have emerged regarding its safety due to the potential production of citrinin (CT), a toxic byproduct synthesized during fermentation. To promote safer utilization, this study aimed to determine a fermentation protocol for producing *Monascus*-biopigment that yields high MK content while minimizing CT concentrations. The study investigated the impact of various parameters over a 10-day fermentation period, including substrate type, initial pH, 0.5% ethanol supplementation, and incubation temperature. *Monascus*-biopigments were extracted using a 70% (v/v) ethanol solution, and concentrations of both MK and CT were quantified using HPLC-UV-Vis analysis. Among the tested conditions, the control setup, utilizing rice as the fermentation substrate, no pH modification, and incubation at 30°C, resulted in the highest biopigment concentration. Meanwhile, the C3 treatment, which utilized corn as a substrate, a pH adjusted to 2.5, with 0.5% ethanol supplementation, and fermentation temperature of 26°C, yielded the highest MK-to-CT ratio. Although using corn without altering pH enhanced pigment, MK, and CT, lowering the pH to 2.5 significantly suppressed CT concentration. Moreover, the combination of ethanol supplementation and lower temperature further enhanced MK production. In conclusion, the findings provide valuable insights into optimizing fermentation conditions for safer and more potent *Monascus*-biopigment, supporting its potential application in the food and nutraceutical industries.

Keywords: *Monascus purpureus* Went, monacolin K, citrinin, biopigment, HPLC

INTRODUCTION

Elevated blood cholesterol is a significant risk factor in the development of cardiovascular diseases (CVDs), which continue to be among the primary causes of death worldwide (WHO, 2021). Despite global health initiatives to reduce the burden of CVDs, the prevalence remained high in most countries. This alarming trend urges the need for the health sector to meet the target set by the United Nations' Sustainable Development Goals, specifically in reducing premature deaths from lifestyle diseases such as CVDs, chronic respiratory conditions, diabetes, and cancer (Roth, et al., 2020; DOST-FNRI, 2015). As public awareness grows, people have become more mindful of their diets and turning to functional foods and nutraceuticals. Several products have been explored and developed to help reduce the risk of CVDs, one of which is *angkak*, or red fermented rice (RFR).

RFR is a traditional *Monascus*-based product from Asia, commonly used for its natural coloring, flavoring, and preservative properties in various food products such as cheese, bread, beverages, fish, and meat (Bule et al., 2019; Chen and Hu, 2005). In the Philippines, RFR is used to color *bagoong* and other fermented fish products (Pattanagul et al., 2007b; Fukami et al. 2021). In addition to its function as a food additive, RFR is valued as a dietary supplement in China and other Asian countries due to the medicinal properties of MK.

MK, a secondary metabolite byproduct of *M. purpureus* showed to lower serum cholesterol levels by inhibiting HMG-CoA reductase, the key enzyme in cholesterol biosynthesis (Le Bloc'h et al., 2015; Pengnoi et al., 2017). Numerous research studies have also demonstrated the efficacy of RFR containing MK in treating hyperlipidemia, reducing total cholesterol, low-density lipoprotein, and triglycerides (Mazza et al. 2018; Liasi et al. 2024). Some clinical research linked its use to decrease mortality from coronary heart disease (Le Bloc'h et al., 2015; Pengnoi et al., 2017). However, despite its benefits, regulatory standards vary among countries, including France, Finland, and Estonia which limits the daily MK intake to 10 mg or less per day, while other countries, including Belgium and Denmark, impose no restrictions (Le Bloc'h et al., 2015).

A major challenge with RFR utilization is the potential production of CT. CT is a mycotoxin associated with hepatorenal injury in mammals (Lin et al., 2008; Pengnoi et al., 2017). To manage this risk, the European Commission reduced the allowable CT level in RFR from 2 mg L⁻¹ to 0.1 mg L⁻¹ to mitigate potential genotoxicity and carcinogenicity, given the limited toxicological data available.

The fermentation parameters highly influenced the biosynthesis of pigment, MK, and CT. RFR is typically produced through solid-state fermentation using *Monascus purpureus* Went, with rice as a substrate (Ajdari, et al., 2011). This substrate supports microbial metabolism by supplying carbon and nutrients. During the early stage of fermentation, primary metabolites such as carbon dioxide and energy are generated, which subsequently contribute to the synthesis of secondary metabolites, including pigment, MK, and CT. The growth and pigmentation of *M. purpureus* are good indicators of metabolite production. Initially, the mycelium is white, but after 10 days or more, it gradually transforms into an intense red hue (Pattanagul et al., 2007a). Several studies have explored how various substrates and their nutrient compositions, such as carbon, nitrogen, and fatty acids affect MK and CT levels (Wang et al., 2003; Pattanagul et al., 2007a; Saithong et al., 2019; Chen and Hu, 2005). Other factors such as ethanol supplementation, pH regulation (Lee et al., 2007a; Lee et al., 2007b), and incubation temperature (Tsukara et al., 2009) have also been found to significantly influence metabolite production.

To maximize the potential benefits of MK while minimizing the risks posed by CT, it is essential to determine the ideal fermentation conditions to obtain high *Monascus*-biopigment with MK-to-CT ratio. A thorough assessment of variables such as substrate type, pH level, ethanol supplementation, and incubation temperature is therefore necessary to enhance the yield of MK and pigment while reducing or eliminating CT production.

MATERIALS AND METHODS

Acquisition of Materials and Chemicals

The pure culture of *M. purpureus* Went was obtained from the Institute of Food Science and Technology, College of Agriculture and Food Science, University of the Philippines Los Baños. The fermentation substrates used were ordinary polished rice (C4 variety), fresh grated corn (yellow variety), and steamed ground peanut. These were purchased from the local market in Los Baños, Laguna, Philippines. Analytical-grade reagents, including CT and MK standards, and other chemicals such as acetonitrile, methanol, acetic acid, and ethanol, were procured from Sigma-Aldrich Ltd. (St. Louis, MO, USA).

Preparation of Seed Culture

Monascus purpureus Went was prepared following the method of **Dizon (1983)** and **Reginio Jr et al. (2016)**, with modifications. The stock culture was transferred onto PDA slants and incubated at 30°C for 7 days. After incubation, spore suspensions were prepared by adding 5 mL sterile distilled water to each slant. The growth of the mold from the PDA was gently scraped from the surface and thoroughly mixed to ensure even spore distribution. The spore suspension was then poured into 250-mL Erlenmeyer flasks containing the respective substrates for fermentation.

Preliminary Screening of Substrates

A preliminary experiment was done to identify substrates, pH, and moisture levels suitable for growth of *M. purpureus* Went over a 10-day fermentation period. Among the substrates tested, steamed rice, steamed ground peanut, and fresh grated corn with moisture content ranges from 35% to 65% showed favorable growth support. Furthermore, *M. purpureus* Went also demonstrated growth on substrates supplemented with 0.5% (v/v) ethanol and adjusted pH levels of 2.5 and 5.7, based on the methodology adapted from **Lee et al. (2007a)**.

Solid-State Fermentation

A total of 10 different fermentation conditions were evaluated in the experiment (Figure 1). The control setup was based on the protocol of **Dizon (1983)** and **Reginio Jr et al. (2016)**, with minor modifications. In the control condition, 50 g of steamed rice was transferred into a 250-mL Erlenmeyer flask, covered with cotton plug and aluminum foil, and sterilized at 121°C for 15 minutes. Once cooled, the sterilized substrate was inoculated with spore suspension of *M. purpureus* Went and incubated at 30°C for 10 days. The remaining nine treatments utilized three different substrates such as steamed rice (R), dehulled steamed peanut (P), and fresh grated corn (C). Each substrate type was divided into three portions of 50 g and adjusted to one of the following conditions: pH levels: 1= no pH adjustment, 2= pH adjusted to 5.7, and 3= pH adjusted to 2.5. The pH adjustments were made by adding a food-grade citric acid solution directly into the fermentation flasks. Each treatment was prepared into a 250-mL Erlenmeyer flask, sealed with cotton and foil, and sterilized at 121°C for 15 minutes. After cooling, 0.5% (v/v) ethanol was added to each flask, followed by inoculation with 5 mL of *M. purpureus* Went inoculum. The fermentation was conducted at 26°C for 10 days.

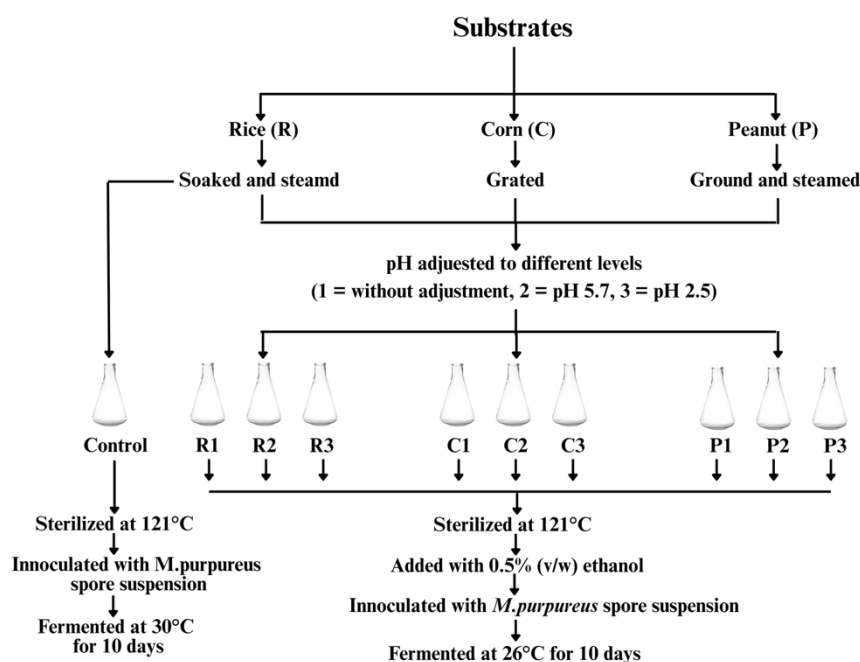


Figure 1 Experimental setup for solid-state fermentation using different fermentation conditions.

Note: Control refers to the condition using rice as the substrate, with no pH adjustment, no ethanol supplementation, and incubation at 30°C. Treatments include variations in substrate (R = rice, C = corn, and P = peanut) and pH levels (1 = no pH adjustment, 2 = pH 5.7, and 3 = pH 2.5). All treatments were supplemented with 0.5% (v/v) ethanol and incubated at 26°C.

Extraction of *Monascus*-biopigment

Biopigment extraction was performed using a method adapted from **Mamucod (2011)**, with slight modifications. The *Monascus*-fermented substrates were first dried in an oven at 55°C for 24 hours. The dried samples were then pulverized with a blender (Shimono, Japan) and passed through a No. 40-mesh sieve to ensure uniform particle size. Approximately 1.0 g of each sample was placed in a 250-mL Erlenmeyer flask, followed by the addition of 10 mL of 70% (v/v) ethanol solution. This mixture was shaken for 1 hour, then underwent centrifugation at 400 rpm for 30 minutes using a centrifuge (Allegra 25R, Beckman Coulter, California, USA). The supernatant was decanted and concentrated using a rotary evaporator (Shimadzu Corp., Japan) at 50°C until the ethanol was evaporated. The crude pigment extract was stored at -20°C until further analysis.

Pigment Quantification

The concentration of biopigment was determined following the method of **Nimnoi and Lumyong (2011)**, with slight modifications. Each *Monascus*-biopigment extract was transferred into cuvettes and measured using a spectrophotometer (Spectronic 20, Mettler Toledo, USA). Absorbance readings were taken at 400, 470, and 500 nm to quantify the yellow, orange, and red pigments, respectively. The blank used was 70% ethanol. Appropriate dilutions were made to ensure accurate readings. The optical density (OD) was calculated using the equation.

$$\text{Optical density (OD)} = \frac{\text{Absorbance (nm)} \times \text{dilution factor}}{\text{weight of sample}} \quad (\text{Eq. 1})$$

Monocolin K Quantification

The concentration of MK was determined using high-performance liquid chromatography (HPLC) (Sigma-Aldrich, USA), following the method of **Lee et al. (2006)** with slight modifications. Standard solutions were prepared by dissolving powdered MK with acetonitrile to obtain concentrations of 100, 200, 300, 400, and 500 mg mL⁻¹. Both standards and sample extracts were filtered through 0.45 µm syringe filters prior to injection. HPLC analysis was carried out using a UV-Vis detector set at 237 nm. Chromatographic separation was achieved on a Nova-Pak C18 column (3.9 mm x 150 mm, 5 µm) with a mobile phase consisting of 55% acetonitrile, 45% ultra-pure water, and 0.05% trifluoroacetic acid, run at a flow rate of 0.7 mL min⁻¹. The MK content in the samples was quantified using a linear regression calibration curve, and results were reported in mg MK per liter of extract (mg L⁻¹).

Citrinin Quantification

The CT content was analyzed using a procedure similar to MK. CT standards were diluted in acetonitrile and passed through a 0.45 µm syringe filter. HPLC analysis employed the same chromatographic conditions and mobile phase composition, but detection was carried out at 325 nm to target CT. The values were expressed in mg CT per liter of extract (mg L⁻¹).

Statistical Analysis

All analyses were done in triplicate and subjected to analysis of variance (ANOVA) to determine significant differences among treatments. Post-hoc analyses were conducted using Tukey’s Honest Significant Difference, with a confidence level of 95%. Statistical computations were done using R software version 4.1.3.

RESULTS

Effect of Various Fermentation Parameters on Biopigment, MK and CT Production of *Monascus purpureus*

The production of *Monascus*-biopigment, MK, and CT were significantly affected by variations in fermentation conditions over a 10-day incubation period. The study identified the ideal fermentation conditions that enhanced the synthesis of *Monascus*-biopigment and MK, while simultaneously reducing CT levels. Key fermentation parameters evaluated included substrate type, pH adjustments, 0.5% ethanol supplementation, and incubation temperature. The combined effects of these variables on the metabolic output of *M. purpureus* Went are summarized in Table 1.

Table 1 Concentrations of *Monascus*-biopigments, MK, CT, and MK-to-CT ratio produced under various fermentation conditions.

Treatment	Optical Density			Monacolin K (mg L ⁻¹)	Citrinin (mg L ⁻¹)	MK-to-CT Ratio
	Yellow	Orange	Red			
Control	58.25 ± 0.5 ^a	61.50 ± 1.9 ^a	58.00 ± 1.0 ^a	1.26 ± 0.1 ^f	3.95 ± 0.1 ^c	0.32 ± 0.0 ⁱ
R1	34.25 ± 1.0 ^c	37.75 ± 1.2 ^c	38.50 ± 0.5 ^c	13.99 ± 0.2 ^b	2.72 ± 0.2 ^{de}	5.13 ± 0.0 ^c
R2	32.00 ± 1.4 ^c	30.00 ± 0.5 ^c	32.50 ± 0.5 ^{de}	8.70 ± 0.0 ^d	1.62 ± 0.3 ^{gf}	5.39 ± 0.2 ^d
R3	19.25 ± 0.2 ^e	16.10 ± 1.9 ^e	17.75 ± 0.7 ^b	13.48 ± 0.3 ^b	1.66 ± 0.1 ^f	8.32 ± 0.1 ^c
C1	52.25 ± 0.2 ^b	46.50 ± 0.5 ^b	46.00 ± 0.4 ^b	15.22 ± 0.1 ^a	18.17 ± 0.3 ^b	0.84 ± 0.0 ^h
C2	37.00 ± 1.0 ^{cd}	29.40 ± 0.7 ^c	27.50 ± 0.5 ^f	8.88 ± 0.1 ^d	22.95 ± 0.1 ^a	0.38 ± 0.0 ⁱ
C3	16.50 ± 0.4 ^h	11.50 ± 0.5 ^h	11.25 ± 0.1 ⁱ	12.45 ± 0.5 ^c	1.14 ± 0.1 ^{hg}	11.17 ± 0.2 ^a
P1	38.75 ± 0.4 ^c	11.50 ± 0.6 ^d	33.50 ± 0.0 ^{de}	8.48 ± 0.2 ^d	2.31 ± 0.1 ^c	3.57 ± 0.0 ^f
P2	34.50 ± 1.5 ^{de}	11.50 ± 0.7 ^{de}	31.25 ± 0.9 ^e	6.47 ± 0.5 ^e	3.04 ± 0.0 ^d	2.04 ± 0.0 ^g
P3	26.25 ± 0.9 ^f	11.50 ± 0.8 ^f	24.00 ± 0.2 ^e	8.80 ± 0.1 ^d	0.96 ± 0.0 ^h	8.61 ± 0.1 ^b

Means within the same column followed by the same letter are not significantly different at $p < 0.05$. **Note:** Control refers to the condition using rice as the substrate, with no pH adjustments, no ethanol supplementation, and incubation at 30°C. Treatments include variations in substrate (R = rice, C = corn, and P = peanut) and pH levels (1 = no pH adjustment, 2 = pH 5.7, and 3 = pH 2.5). All treatments were supplemented with 0.5% (v/v) ethanol and incubated at 26°C.

***Monascus*-biopigment Concentration**

The production of *Monascus*-biopigment varied significantly across various fermentation conditions. The most intense pigment development was observed under control conditions, which used rice as a substrate, without pH adjustments or ethanol supplementation, and incubated at 30°C. Among the treatments, corn (C1) produced the most vibrant coloration, followed by rice (R1) and peanut (P1) under their respective unmodified pH conditions. However, lowering the pH to 2.5 significantly reduced pigment intensity in all substrates. The reduction was

noticeable in corn, where the color shifted from a deep red to a more yellow-orange hue. Furthermore, the combined effects of 0.5% ethanol supplementation and reduced incubation temperature to 26°C further suppressed pigment formation. This was evident in the decreased color intensity observed in R1, compared to the control. These findings suggest that the optimal condition for maximum biopigment production is the use of rice as a substrate, with no pH alteration, no ethanol addition, and fermentation at 30°C. Figure 2 shows *Monascus*-fermented products produced under the different fermentation conditions.

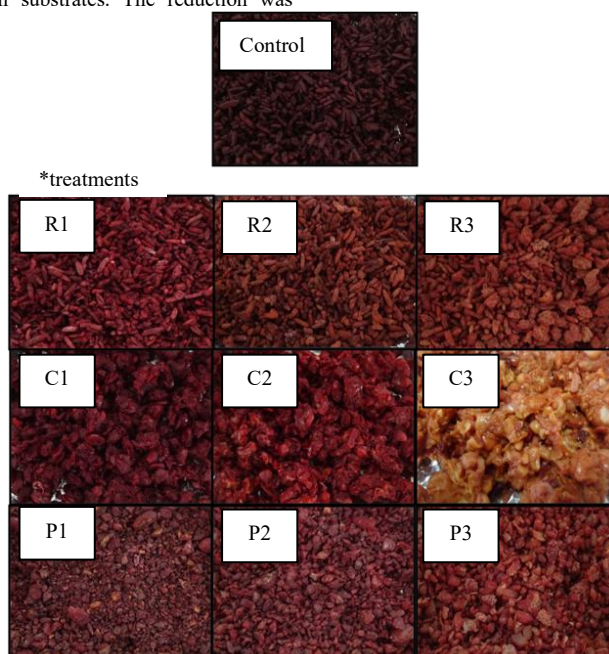


Figure 2 Photographs of *Monascus*-fermented products under different fermentation conditions.

Note: Control refers to the condition using rice as the substrate, with no pH adjustments, no ethanol supplementation, and incubation at 30°C. *Treatments include variations in substrate (R = rice, C = corn, and P = peanut) and pH levels (1 = no pH adjustment, 2 = pH 5.7, and 3 = pH 2.5). All treatments were supplemented with 0.5% (v/v) ethanol and incubated at 26°C.

Monacolin K Concentration

The concentration of MK varied among the different treatments. The highest MK concentration was observed in C1 treatment, followed by R3, while the lowest concentration was recorded in the control condition. When comparing substrates

under identical treatment conditions, corn (C1) consistently produced significantly higher MK than rice (R1) or peanut (P1). Adjustment of pH to 2.5 pH did not significantly affect MK levels in rice and peanut, as evidenced by the close values in R1 and R3, and P1 and P3. However, the combined application of 0.5% ethanol supplementation and reduced fermentation temperature of 26°C in rice as a

substrate significantly increased MK production, elevating it from 1.26 mg L⁻¹ (control) to 13.99 mg L⁻¹ (R1). A similar trend was observed in corn, where the same conditions improved MK from 1.26 mg L⁻¹ (control) to 15.22 mg L⁻¹ (C1).

Citrinin Concentration

The concentration of CT also showed substantial variation across treatments. The highest CT concentration was obtained in C2, followed by C1, while the lowest CT concentration was found in P3. Among the three substrates, corn (C2) exhibited the highest CT levels, followed by rice (control) and peanut (P2). Acidifying the substrates to pH 2.5 significantly reduced CT synthesis across all substrates. In corn, CT levels dropped from 18.17 mg L⁻¹ (C1) to 1.14 mg L⁻¹ (C3), and in peanut, from 2.31 mg L⁻¹ (P1) to 0.96 mg L⁻¹ (P3). Similarly, the application of 0.5% ethanol and incubation at 26°C significantly lowered CT concentration in rice, from 3.95 mg L⁻¹ (control) to 2.72 mg L⁻¹ (R1), though this still exceeded the EU's safety threshold of 0.1 to 2 mg L⁻¹. Notably, samples from treatments R2, R3, C3, and P3 obtained CT levels below 2 mg L⁻¹.

Monacolin K to Citrinin Ratio

The MK-to-CT ratio which serves as an indicator of product safety and efficacy was obtained highest in C3, followed by P3, while the control exhibited the lowest ratio. Among the substrates, corn exhibited the highest MK-to-CT ratio, followed by peanut and rice. Lowering the pH to 2.5 significantly improved the MK-to-CT ratio in all substrates. As observed in corn, the ratio increased from 0.84 (C1) to 11.17 (C3). Ethanol supplementation and reduced temperature also had a strong synergistic effect when applied to rice, enhancing the MK-to-CT ratio from 0.32 in the control to 5.13 (R1). This observation suggests that adjusting fermentation conditions, particularly the substrate type, pH, and temperature can significantly improve the functional quality and safety of *Monascus*-fermented products.

DISCUSSION

The fermentation factors, namely substrate type, pH level, ethanol supplementation, and incubation temperature significantly influenced the production of biopigments and MK-to-CT ratio in *Monascus*-fermented products after 10 days of solid-state fermentation under varying fermentation conditions. Each factor played a significant role in the metabolic activity of *M. purpureus*, emphasizing the importance of optimizing fermentation conditions for producing safer and functionally potent *Monascus*-based products.

Among the substrates tested, rice emerged to be the most suitable for biopigment production, particularly under control conditions with no pH adjustment or ethanol supplementation, and fermentation at 30°C. These findings align with previous research showing that rice is the ideal source of nutrients for biopigment production of *M. purpureus* (Rosenblitt et al., 2000; Teng and Feldheim, 2001; Dikshit and Tallapragada, 2011; Srianta et al., 2016). This efficiency may be attributed to rice's rich carbohydrate content and availability of micronutrients such as iron, zinc, and calcium, and low fat levels, which create favorable conditions for biopigment biosynthesis (DOST-FNRI, 2022).

On the other hand, corn yielded the highest MK and CT concentrations, which may be attributed to its elevated levels of sugars, fat-soluble vitamins, and essential macronutrients such as potassium, phosphorus, magnesium, and sodium (DOST-FNRI, 2022). Peanut, despite having relatively low carbohydrate content, resulted in the lowest CT concentrations. This may imply that higher protein and lipid content may divert metabolic pathways toward the production of other secondary metabolites. The observed trend strengthened the idea that the nutritional profile of substrates can influence fungal metabolism, as confirmed in the findings of Ajdari, et al. (2011) and Wen et al. (2020), who emphasized the importance of carbon-to-nitrogen ratio, with limited nitrogen condition enhances the synthesis of MK.

The addition of 0.5% (v/v) ethanol in the substrate as a supplemental carbon source had a significant positive impact towards enhancing MK-to-CT ratio. It significantly boosted MK production while suppressing CT synthesis. This aligns with the study of Wang et al. (2003) which showed that ethanol can enhance MK biosynthesis. These findings further support the hypothesis that optimizing carbon source not only enhances MK yield but also modulates CT concentrations.

The pH level also played a significant effect in the synthesis of *Monascus* metabolites. *M. purpureus* Went is capable of growing across a broad range of pH; however, results showed that biopigment production was optimal under near-neutral pH conditions, while acidic environments (pH 2.5) led to a substantial decline in both biopigment and CT levels. This result aligns with the study of Lee et al. (2007a) and Orozco et al. (2008), who demonstrated that biopigment synthesis is favored within the pH range of 5.5 to 6.5. Importantly, acidic conditions inhibited CT biosynthesis, similar to the study of Patrovsky et al. (2019), who observed negligible CT production at pH 2.5, and Silva et al. (2020) who observed sensitivity of CT production to pH changes such as structural instability at lower pH levels. In terms of MK production, acidic pH did not hinder yields in rice and peanut substrates. These findings were comparable with the investigation of Lee et al. (2007), who showed that MK biosynthesis remains active in low-pH environments. Furthermore, Pattanagall et al. (2007)

emphasized the importance of the beta-hydroxy acid to lactone ratio in MK, with higher ratios indicating better bioactivity. Kraboun et al. (2019) also found that MK production was suppressed at pH levels above 5, highlighting the importance of in-depth investigations in optimizing fermentation parameters to achieve a high MK-to-CT ratio in *Monascus*-fermented products compliant in the regulatory standards.

CONCLUSION

The study demonstrated that key fermentation parameters, including substrate type, pH adjustment, ethanol supplementation, and incubation temperature, significantly influenced the synthesis of biopigments, MK, and CT by *Monascus purpureus* Went. Among the tested conditions, rice as a substrate with no pH modification and incubated at 30°C yielded the highest biopigment levels, confirming its effectiveness for maximizing pigment synthesis. On the other hand, corn substrate combined with a pH of 2.5, 0.5% ethanol supplementation, and a reduced temperature of 26°C produced the most favorable MK-to-CT ratio, highlighting an effective fermentation condition for reducing CT levels while enhancing MK production. These findings emphasize the importance of optimizing fermentation conditions to balance functional efficacy with product safety of *Monascus*-fermented products. The findings also offer valuable insights for the development of safer, high-value *Monascus*-fermented products with potential applications in the food and nutraceutical industries. Further investigation should explore additional fermentation variables and alternative nutrient sources to further improve pigment quality and MK-to-CT ratio while ensuring compliance with international safety standards.

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