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Research Article

SCREENING OF *Monascus purpureus* STRAIN WITH MONACOLIN K ACTIVITY AND CITRININ-FREE CHARACTERISTICS FOR RED YEAST RICE PRODUCTION BY LC-MS/MS ANALYSIS BY LC-MS/MS ANALYSIS

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ABSTRACT

Red yeast rice is produced from rice using Monascus purpureus fungus, which results in the red colour of the fermented rice. Red yeast rice has been used for a thousand years as a food preservative and in traditional medicine to support vascular and digestive health. The compound known as monacolin K is the main active component that helps decrease blood cholesterol. However, the byproduct of fermentation is citrinin, which causes hepato-nephrotoxic mycotoxin. This study used Liquid Chromatography-Mass Spectrometry (LC-MS/MS) to concurrently analyse citrinin and monacolin K produced by M. purpureus strain. The pigmentations were varied when cultured on various media such as PDA, PGA, MGA, MCM, and SDAY media. MGA (61.12 – 1,996.20 AU/g) and PGA (8.78 – 507.26 AU/g) displayed the highest levels of red pigment, while SDAY (10.55–31.79 AU/g) showed the lowest levels. Morphological examination revealed typical features of Monascus sp., including spherical or oval shapes and spore chains of two to four spores. Qualitative analysis of chromatographic plates TLC revealed consistent bands of Monacolin K standard across most strains, indicating their potential for producing this compound, with exceptions like strain BS3-GLTT. Notably, strains C3.12, C5.17, C4.1, C1.15, and BS3-GLTT exhibited either faint or absent spots corresponding to citrinin, suggesting their potential as citrinin-free strains. Furthermore, the C5.17 strain identified as Monascus purpureus, under specific conditions, yielded a monacolin K concentration of 292,32 ppm, with no detectable citrinin by LC-MS/MS analysis, highlighting its suitability for monacolin K production.

Keywords: citrinin; monacolin K; *Monascus purpureus*; LC-MS/MS; Red yeast rice

1. Introduction

Monascus sp. belongs to the Monascaceae family, genus Monascus, which is one of the species capable of producing natural bioactive pigments (Mussalbakri et al., 2017).

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Pigments are formed through the polyketide biosynthetic pathway, in which polyketide synthesis and fatty acid synthesis play essential roles (Rasheva et al., 1998). One of the processed rice that brings economic and health value is red yeast rice (RYR) (Seenivasan et al., 2008; Song et al., 2019). RYR is the product after fermenting rice with *Monascus purpureus* fungus, which has many beneficial biological effects such as improving cardiovascular circulation, reducing cholesterol to prevent metabolic disorders, and in cosmetics for skin rejuvenation and digestive support (Fukami et al., 2021). Currently, RYR is recognized as the leading natural therapy for high cholesterol individuals. The total cholesterol level of the body in general, including bad LDL cholesterol and triglycerides, is significantly reduced compared to the control group with patients using RYR (Cicero et al., 2019). The reason cholesterol has been extensively researched is that the leading cause of death worldwide is cardiovascular disease, accounting for 32.3%. There is an alarming situation of lipid disorders worldwide, with 33.6 million people in the US having high lipid levels, accounting for 15% of the adult population; in the UK, two-thirds of the population have higher cholesterol levels than recommended; in Vietnam, 29% of adults have high lipid levels, with the urban population accounting for 44.3%. Many complications occur, such as 48% of cases of stroke, 56% of cases of myocardial ischemia, and myocardial infarction due to complications of atherosclerosis from lipid disorders. Monacolin K activity in *Monascus* sp. is similar to statin drugs used in lipid treatment (Heber et al., 1999). Natural monacolins, especially monacolin K, have anti-osteoporosis effects and inhibit cholesterol synthesis by HMG-CoA enzyme. In 2007, Lê Đức Mạnh investigated the pigment-producing and lovastatin-producing capabilities of *Monascus purpureus* MD and *Monascus purpureus* 3403 strains, revealing the latter's ability to synthesise lovastatin hydroxy acid at 60 µg/g in dried samples. Despite low citrinin production, its presence in both strains raised concerns for their application in colouring agents or health food production.

2. Material and method

2.1. Microorganism and inoculum

The *M. purpureus* strain used in this study was obtained from rice from the HCM Biotech collection. The cultured strain will be inoculated onto a PDA medium and then incubated at room temperature (24-30°C) for 14 days. Spore suspension will be prepared by adding distilled water to the PDA culture medium to obtain a spore density of 10^7 spores/mL.

2.2. Morphological identification

We perform a dilution series ranging from 10^{-2} to 10^{-5} using 1 gram of untreated and fermented rice samples in physiological saline solution, followed by vortexing to homogenise the samples. Then, pipette 100 ul onto Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar with Yeast Extract (SDAY) plates for screening strains with red pigment. To observe the morphological characteristics of fungal colonies, inoculate spots onto Petri dishes containing PDA, Potato Glucose Agar (PGA), Malt Glucose Agar (MGA),

Malt Extract Agar (MEA), and SDAY media, and incubate at room temperature. Successfully cultured strains will produce fungal hyphae. Use a moist chamber method for microscopic observation. The growth of fungal colonies is observed under an automated microscope (Darwesh et al., 2020; Carvalho et al., 2005).

2.3. Method for determining the content of red pigment

Fungal biomass is obtained and dried for 24 hours at 50°C, then finely ground. Consequently, 70% ethanol is added to the sample at a ratio of 1:20, and the mixture is agitated at 150 rpm for 2 hours at room temperature. Afterwards, the sample is centrifuged at 7000 rpm for 10 minutes to collect the supernatant, then diluted with 70% ethanol. The absorption at 505 nm (red pigment) is measured with the sample, using 70% ethanol as the blank control. The total absorbed pigment (AU/g) is calculated using the formula: AU/g = Abs x (10/0.5) x df, where Abs represents the sample absorbance and df denotes the dilution factor (Passos et al., 2019).

2.4. Selection of Monascus strains capable of producing monacolin K using the Thin Layer Chromatography (TLC) method

The TLC method was employed for the rapid screening of citrinin and monacolin K using thin-layer chromatography (TLC), with silica gel thin-layer chromatography plates as the stationary phase and chloroform: methanol (90:10, v/v) as the mobile phase. Standard citrinin and monacolin K were simultaneously analysed. Monascus strains were identified according to the method of Babitha et al. (2007). Fungal biomass obtained after freeze-drying for 24 hours at 50°C was finely ground. 70% ethanol was added to the sample at a ratio of 1:20, and the mixture was shaken at 150 rpm at room temperature for 2 hours. Subsequently, the mixture was centrifuged at 7000 rpm for 10 minutes to collect the supernatant, which was then diluted with 70% ethanol. Absorbance at 505 nm (red pigment) was measured with 70% ethanol as the negative control (Seenivasana et al., 2015).

2.5. Mass spectrometry condition LC-MS/MS

Weigh 0.5 grams of dry sample into a 50 mL falcon tube. *Monascus* fungal spores are extracted with 50 mL of Acetonitrile (ACN) in an ultrasonic bath at 30°C for 30 minutes. The resulting mixture is then heated in a water bath at 60°C for 1 hour and centrifuged at 3500 rpm for 10 minutes. Add 0.01g of adsorbent material (MgSO4: Na-Acetate, adjusted) to 1 mL of the upper phase, followed by centrifugation at 10000 rpm for 5 minutes. The supernatant is then filtered through a 0.2 µm filter membrane. A volume of 5µL of the filtered solution is injected into the LC-MS/MS system. Transfer the sample into a vial and analyse it using LC-MS/MS Waters. Standards are determined with known concentrations and peak areas. The chromatographic conditions include a C18 column, a 0.3 mL/minute flow rate, and a sample injection volume of 5 µL. Mobile phase A consists of 10 mM ammonium formate in H2O (0.1% HCOOH); B1: MeOH (0.1% HCOOH) for citrinin and monacolin K (Svoboda et al., 2017; Fogarty et al., 2018). Monacolin K, ESI +, primary ion

405 (m/z), daughter ion 303 and 199 (m/z). Citrinin, ESI -, primary 281 (m/z), daughter ion 205 and 249 (m/z).

2.6. Method for DNA sequencing and identification

Total DNA is extracted by adding fungal fibres into a 1.5 mL Eppendorf tube containing 500 µL of a phenol-chloroform-isoamyl alcohol and STES buffer mixture. The mixture is centrifuged at 10000 rpm for 5 minutes to collect the supernatant. The supernatant is transferred to a new 1.5 mL Eppendorf tube containing 300 µL of isopropanol and CH_3COONa. After centrifugation at 10000 rpm for 5 minutes, the supernatant is discarded, and the precipitate is collected. The precipitate is washed with ethanol and dried. 50 µL of TE buffer is added to dissolve the DNA. For the PCR reaction, the reaction mixture contains 2.5 µL of 10X.

Add 2.5 µL of dNTPs, 0.5 µL of 10µM ITS5 forward primer, 0.5 µL of 10µM reverse primer LR7, 0.25 µL of Taq DNA polymerase, 2 µL of DNA template, and ddH2O to a total volume of 25 µL. The PCR thermal cycling conditions are as follows: 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 56°C for 45 seconds, 72°C for 1 minute, and a final extension step at 72°C for 7 minutes. The PCR products are then electrophoresed on a 1.5% agarose gel for 20 minutes and purified using the GeneJET PCR Purification Kit. For PCR sequencing, the reaction mixture includes 1 µL of BigDye™ Terminator 3.1 Ready Reaction Mix, 1 µL of primer, 10 - 100 ng of purified PCR product, and ddH2O to a total volume of 10 µL. The sequencing products are cleaned using Sephadex G-50, sequenced with an ABI 3500 Genetic Analyzer, and analysed using the BLAST program from NCBI. Phylogenetic trees are constructed using MEGA-10 software, and species identification is performed using the Neighbor-Joining method with a bootstrap index of 1000 (Joaquin et al., 2016; Suharna et al., 2005).

3. Results and discussion

3.1. Morphological identification

To observe the morphological characteristics of fungal colonies, inoculate spots onto Petri dishes containing PDA, Potato Glucose Agar (PGA), Malt Glucose Agar (MGA), Malt Extract Agar (MEA), and SDAY media, and incubate at room temperature. A description of fungal strains is presented in Figure 1. After 21 days of cultivation on different media, including PDA, PGA, MGA, MCM, and SDAY, the isolated fungal strains generally exhibited robust growth, with most mycelia densely covering the entire Petri dish surface.

Pigmentation was observed on the agar plates' upper and lower surfaces, accompanied by distinct morphologies. The strains exhibited characteristic pigments of *Monascus* sp., predominantly red, pink, orange, yellow, and white. Specifically, fungal colonies grown on PDA, MCM, and SDAY media mainly displayed pink pigmentation, while those on PGA and MGA media showed deeper shades of pink, red, or orange. The morphology of fungal colonies

appeared round, with mycelia arranged in a flat or slightly raised manner at the centre, often forming concentric rings with smooth or undulating edges.

The identified fungal strains were characterised morphologically and described in detail in Figure 1. Morphological characteristics, including spore capsule size, morphology of asexual spores, and the number of spores in chains, were examined. Overall, the strains exhibited spherical or oval shapes, occurring singly or in chains of 2 to 4 spores, indicating characteristics typical of *Monascus* sp. In the five different cultivation media, dextrose was the carbon source in PDA, MCM, and SDAY, while glucose was used in PGA and MGA.

Therefore, it can be concluded that the carbon source in the cultivation media influences pigment variation and growth in the examined strains. Studies by Kujumdzieva et al. (1998) and Lee et al. (2001) also concluded that glucose is preferred as a substrate for pigment and biomass production in *Monascus* sp. over other carbon sources.

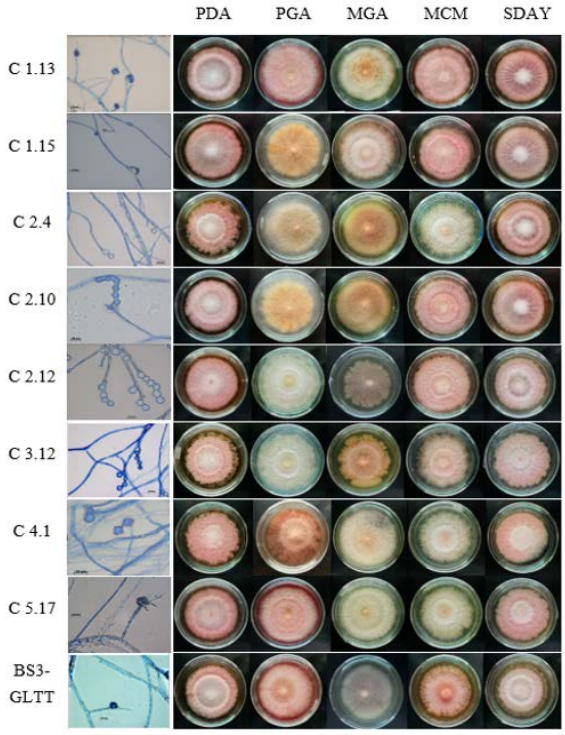

Figure 1. *Morphological characteristics of Monascus sp. on different media, including PDA, PGA, MGA, MCM, and SDAY*

3.2. Method for determining the content of red pigment

From Table 1, it can be observed that there are differences in the red pigment content among the cultured strains and between different media within the same strain. When cultured on various media such as PDA, MCM, MGA, SDAY and PGA media, the pigmentations were varied PDA (11.37-41.49 AU/g), MCM (14.51-129.99 AU/g), MGA (61.12-1,996.20 AU/g), SDAY (10.55-31.79 AU/g) and PGA (8.78-507.26 AU/g).

Specifically, the C2.12 strain had high red pigment levels of 1,996.20 AU/g in the MGA medium, 54.58 AU/g in the MCM medium, 41.49 AU/g in the PDA medium, 18.58 AU/g in the SDAY medium, and had the lowest red pigment levels 8.78 AU/g in PGA medium. However, the C2.10 strain had high red pigment levels of 420.77 AU/g in the PGA medium, 389.35 AU/g in the MGA medium, 73.82 AU/g in the MCM medium, 38.80 AU/g in the PDA medium, and had the lowest red pigment levels 29.56 AU/g in SDAY medium.

Table 1. *Average pigment data on five media after 21 days measured at a wavelength of 505 nm*

STT	Strains	The content of red pigment on mediums (AU/g)				
		PDA	**MCM**	**MGA**	**SDAY**	**PGA**
1	C 1.13	34.57	98.13	366.33	31.79	30.79
2	C 1.15	33.60	88.11	433.33	22.11	113.98
3	C 2.4	19.25	17.63	415.15	16.10	52.48
4	C 2.10	33.80	73.82	389.35	29.56	420.77
5	C 2.12	41.49	54.58	1,996.20	18.58	8.78
6	C 3.12	16.07	20.12	209.58	27.06	12.55
7	C 4.1	13.96	19.78	61.12	17.39	507.26
8	C 5.17	20.07	14.51	45.27	18.23	24.73
9	BS3-GLTT	11.37	129.99	485.77	10.55	20.39

3.3. Results of selecting Monascus strains capable of producing monacolin K using thin layer chromatography (TLC)

The qualitative results showed that most of the isolated strains, as well as the control strain *M. purpureus*, were separated into similar spots on the chromatographic plate, with corresponding bands of Monacolin K standard appearing prominently, except for the strain BS3-GLTT, which exhibited faint spots. The qualitative results showed that most of the isolated strains, as well as the control strain *M. purpureus*, were separated into similar spots on the chromatographic plate, with corresponding bands of Monacolin K standard appearing prominently, except for the strain BS3-GLTT, which exhibited faint spots.,

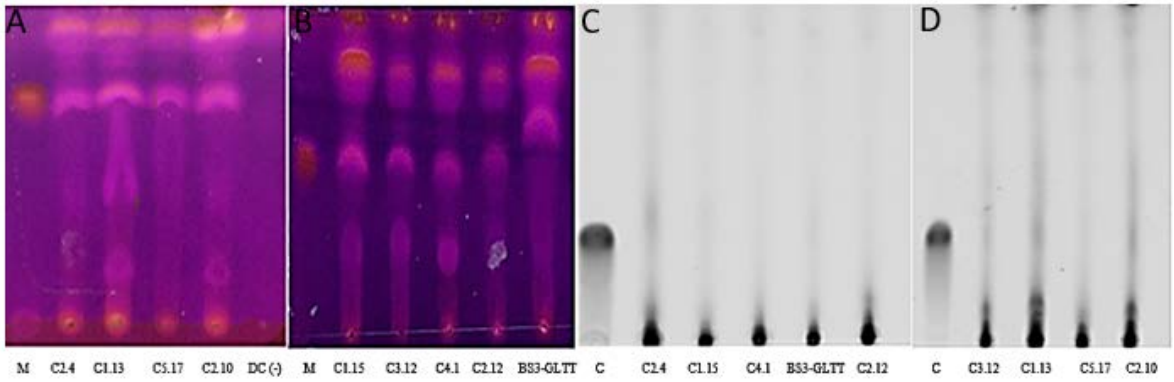

Figure 2. *Qualitative results of monacolin K determination using TLC method in isolated Monascus sp. strains (where A-B: M - Monacolin K; C-D: C - Citrinin; (-) DC - PGA medium)*

Figure 2 exhibited the qualitative results of monacolin K (A-B) and citrinin (C-D) determination using the TLC method in nine isolated strains. Based on the spots corresponding to the standard citrinin spots in the figure, it can be inferred that most strains produce monacolin K and citrinin at varying levels. The Rf values differ with each TLC run, so all comparisons are made relative to the standard when performing a specific version of TLC. Among the strains isolated from natural samples, strains that did not appear or showed faint spots corresponding to citrinin are considered potential strains, including C3.12, C5.17, C4.1, C1.15, and BS3-GLTT.

Table 3. *Summary of qualitative determination of Monacolin K and citrinin in the surveyed strains Note: (+) positive; (-) negative, Rf retention factor*

No	Strains	Monacolin K			Cinitrin		
		Positive	***Negative***	***Rf***	***Positive***	***Negative***	***Rf***
1	C 1.13	++		0.67	+		0.33
2	C 1.15	++		0.58		-	-
3	C 2.4	++		0.67	+		0.30
4	C 2.10	++		0.67	+		0.33
5	C 2.12	++		0.58	+		0.33
6	C 3.12	++		0.58		-	-
7	C 4.1	++		0.58		-	-
8	C 5.17	++		0.67		-	-
9	BS3-GLTT		-	-		-	-

Table 3 shows that most of the strains could produce monacolin K except BS3-GLTT strains; the four potential strains, including C3.12, C5.17, C4.1, C1.15, which either produced high amounts of monacolin K and produced little citrinin or do not detect citrinin, were selected for further investigations.

3.4. Analysis of monacolin K and citrinin in selected strains by LC-MS/MS

After conducting the TLC method, strains that exhibit no citrinin streaks will be chosen for citrinin content analysis using the LC-MS/MS method. The strain selected will ideally have either the lowest citrinin concentration or no citrinin, enabling it to grow on rice without producing the bioactive compound citrinin.

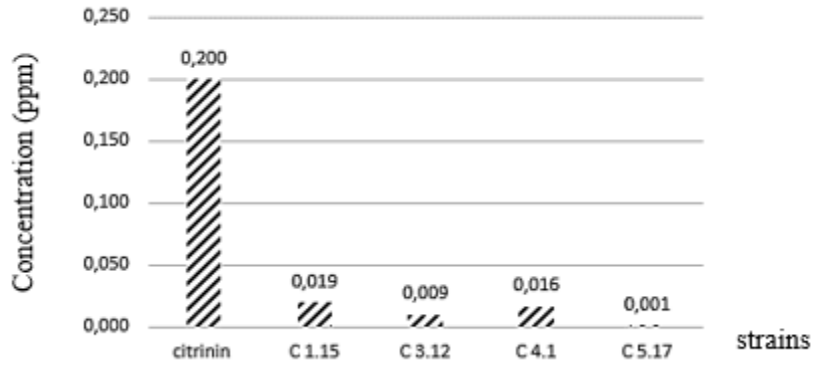

Figure 3. *Results of citrinin analysis in strains by LC-MS/MS method*

The concentration of citrinin results in strains C1.15, C3.12, C4.1, and C5.17 are shown in Figure 3. The analysis results indicated that the citrinin concentration of all strains was below 0.2 ppm. Among them, strain C5.17 has the lowest level of 0.001 ppm. Therefore, the monacolin K produced by this strain was further measured. The results are shown in Figure 4.

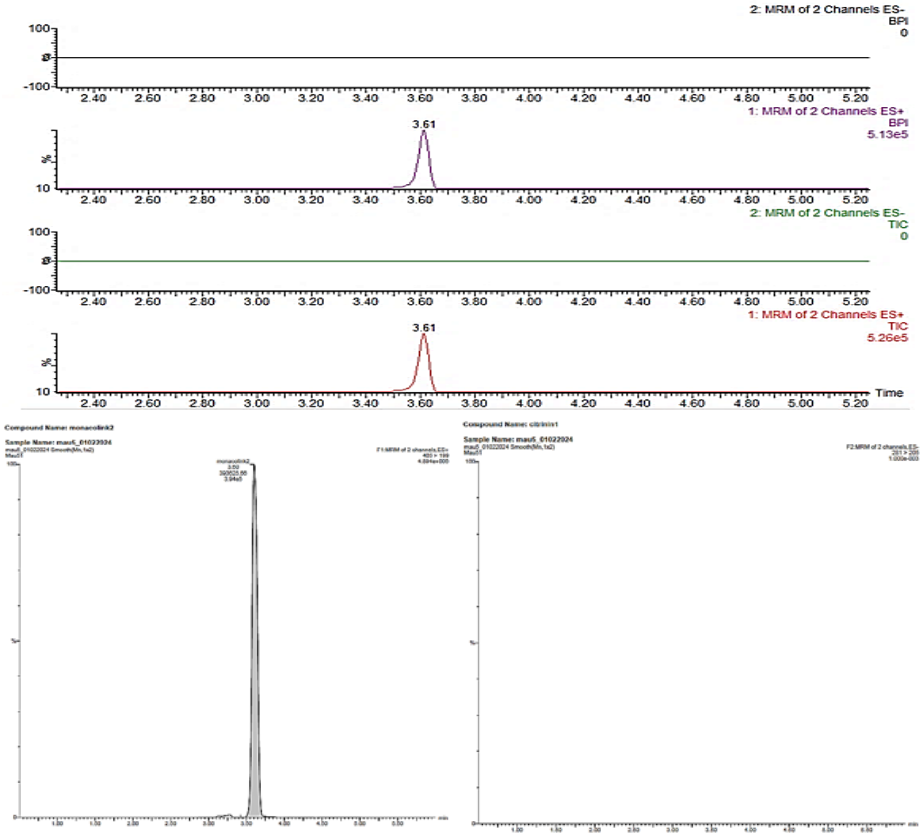

Figure 4. *Result of monacolin K and citrinin content in samples by LC-MS/MS*

When cultivating the strain on red yeast rice under the conditions of cultivation for 12 days with 8% inoculum at a temperature of 24°C, the concentration of monacolin K was found to be 292,32 ppm (Y=134.305 * X + 1030.09 with r^2= 0.99). The results show that when simultaneously analysing monacolin K and citrinin, Figure 4 indicates that the sample only contains monacolin K, and no citrinin was detected.

3.5. Method for DNA sequencing and identification

The PCR results using the primer pair ITS5 and LR7 yielded an electrophoresis band at approximately 2000 bp. A phylogenetic tree was constructed based on the highest sequence similarities of the ITS5-ITS (Internal transcribed spacer) and LR7-LSU (Large subunit) regions of fungal strains using MEGA XI software. The results indicated that the strains in the screening set belonged to the evolutionary branch of *Monascus purpureus*. Additionally, the fungal species formed a distinct branch separate from all species within the genus *Monascus* sp.

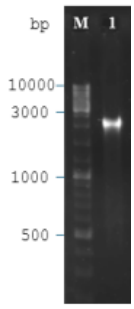

Figure 5. *The PCR results using the primer pair ITS5 and LR7 yielded an electrophoresis*

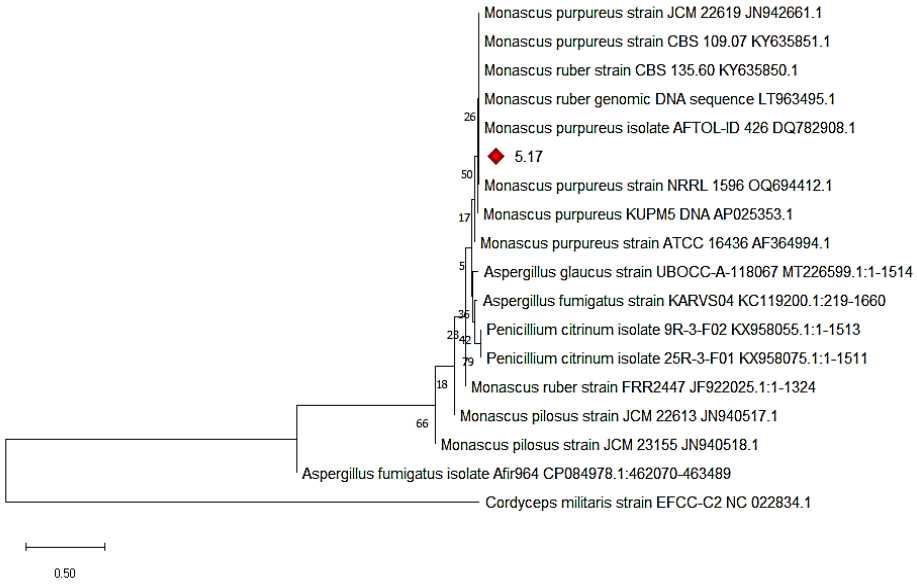

Figure 6. *Phylogenetic tree of the ITS5 and LR7 regions. The phylogenetic tree was constructed using the Neighbor-joining method with a bootstrap value of 1000 in MEGA. The bar (0.50) represents the genetic distance.*

4. Conclusions

Using Liquid Chromatography-Mass Spectrometry (LC-MS/MS), this study simultaneously analysed citrinin and monacolin K in *M. purpureus* strains. Various media cultures displayed diverse pigmentations, while TLC analysis confirmed consistent Monacolin K presence across most strains, except for BS3-GLTT. Strain C5.17, identified as *Monascus purpureus*, exhibited a notable monacolin K concentration of 292.32 ppm without detectable citrinin, suggesting its potential for red yeast rice production with high monacolin K.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

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SÀNG LỌC CHỦNG *Monascus purpureus* SINH MONACOLIN K, KHÔNG SINH CITRININ TRONG GẠO LÊN MEN ĐỎ BẰNG PHƯƠNG PHÁP LC-MS/MS

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TÓM TẮT

Gạo lên men đỏ được sản xuất từ gạo bằng cách lên men vi nấm Monascus purpureus có sinh sắc tố đỏ. Gạo lên men đã được sử dụng trong hàng nghìn năm nay như một chất bảo quản thực phẩm và trong y học cổ truyền để hỗ trợ sức khỏe mạch máu và tiêu hóa. Hợp chất được biết đến với tên monacolin K là thành phần hoạt động giúp giảm cholesterol trong máu. Tuy nhiên, sản phẩm phụ của quá trình lên men là citrinin, gây ra một loại nấm độc hại cho gan và thận. Nghiên cứu này sử dụng kĩ thuật Liquid Chromatography-Mass Spectrometry (LC-MS/MS) để phân tích đồng thời citrinin và monacolin K được sản xuất bởi chủng M. purpureus. Khảo sát sự phát triển của các chủng trên các môi trường PDA, PGA, MGA, MCM và SDAY, kết quả cho thấy màu sắc và hàm lượng sắc tố khác nhau, MGA (61.12-1,996.20 AU/g) và PGA (8.78-507.26 AU/g) thể hiện mức độ màu đỏ cao nhất, trong khi SDAY (10.55-31.79 AU/g) cho thấy mức độ thấp nhất. Quan sát hình thái học đã phát hiện tính năng đặc trưng của Monascus sp., bao gồm hình dạng tròn hoặc hình bầu dục và chuỗi bào tử từ 2 đến 4 bào tử. Phân tích các chất bằng TLC đã phát hiện ra các dải Monacolin K trên hầu hết các chủng, chỉ ra tiềm năng của chúng để sản xuất hợp chất này, ngoại trừ BS3-GLTT. Đáng chú ý, các chủng C3.12, C5.17, C4.1, C1.15 và BS3-GLTT đã thể hiện dấu vết mờ hoặc không có tương ứng với citrini. Hơn nữa, chủng C5.17 đã được xác định là Monascus purpureus, được nuôi cấy trên gạo cho ra nồng độ monacolin K là 292,32 ppm, mà không phát hiện citrinin qua phân tích LC-MS/MS, nhấn mạnh tính phù hợp của nó cho việc sản xuất monacolin K, không có độc tố citrinin.

Từ khóa: citrinin; cholesterol; monacolin K; *Monascus purpureus*; LC-MS/MS; Gạo lên men đỏ