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Original Article

Solid-state fermentation parameters for kojic acid production by marine *Aspergillus flavus* DIKSI C35 and cytotoxicity against B16F10

[Parámetros de fermentación en estado sólido para la producción de ácido kójico por *Aspergillus flavus* marino DIKSI C35 y citotoxicidad contra B16F10]

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Abstract

Context: Kojic acid (KA) is an organic compound generated through the fermentation process involving various species of *Aspergillus* fungi. It has attracted considerable attention within the cosmetic industry and serves a vital role in pharmaceuticals. Remains a paucity of research focused on the production of KA from marine fungi, presenting a significant opportunity for further investigation.

Aims: To optimize KA production from the marine coral-associated fungus by adjusting factors such as incubation time, medium pH, temperature, and fermentation type.

Methods: Coral material was collected from Nipah, Lombok, Indonesia, ground into small pieces, and incubated in PDA medium with seawater at 28°C. Purified fungi were identified through DNA sequencing. Fermentation was performed on solid media, and extracts were obtained using ethyl acetate. KA crystals were formed by storing the liquid extract at 4°C. Optimization of KA production on fermentation parameters, including incubation time, medium pH, temperature, and fermentation type. KA levels were assessed by HPLC, and toxicity was assessed in B16F10 cells.

Results: Marine coral-associated fungi produced significant amounts of KA, identified as *Aspergillus flavus* DIKSI C35 through BLAST DNA analysis. The optimal yield of KA was obtained at pH 3, 25°C, and after 15 days of incubation. The crude extracts demonstrated excellent non-cytotoxicity at 125 µg/mL.

Conclusions: Marine fungi have demonstrated the capacity to produce valuable compounds, including KA. Through the optimization of fermentation parameters, yields can be substantially enhanced, and a toxicity assay is conducted to ensure the safety of the extracts.

Keywords: *Aspergillus flavus*; B16F10 melanoma; cell viability; kojic acid; marine fungi.

Resumen

Contexto: El ácido kójico (KA) es un compuesto orgánico producido mediante el proceso de fermentación por diversas especies de hongos de la familia *Aspergillus*. Ha atraído considerable atención en la industria cosmética y desempeña un papel vital en la farmacéutica. Aún existe escasez de investigación centrada en la producción de KA a partir de hongos marinos, lo que representa una importante oportunidad para futuras investigaciones.

Objetivos: Optimizar la producción de KA a partir de hongos asociados a corales marinos mediante el ajuste de factores como el tiempo de incubación, el pH del medio, la temperatura y el tipo de fermentación.

Métodos: Se recolectó material de coral en Nipah, Lombok, Indonesia, se trituró en pequeños trozos y se incubó en medio PDA con agua de mar a 28°C. Los hongos purificados se identificaron mediante secuenciación de ADN. La fermentación se realizó en medios sólidos y se obtuvieron extractos con acetato de etilo. Los cristales de KA se formaron al almacenar el extracto líquido a 4°C. Optimización de la producción de KA en función de los parámetros de fermentación, incluidos el tiempo de incubación, el pH del medio, la temperatura y el tipo de fermentación. Los niveles de KA se determinaron mediante HPLC y la toxicidad se evaluó en células B16F10.

Resultados: Los hongos marinos asociados a corales produjeron cantidades significativas de KA, identificado como *Aspergillus flavus* DIKSI C35 mediante análisis de ADN BLAST. El rendimiento óptimo de KA se obtuvo a pH 3, 25°C y tras 15 días de incubación. Los extractos crudos demostraron una excelente no citotoxicidad a 125 µg/mL.

Conclusiones: Los hongos marinos han demostrado la capacidad de producir compuestos valiosos, entre ellos KA. Mediante la optimización de los parámetros de fermentación, se puede mejorar sustancialmente el rendimiento y se realiza un ensayo de toxicidad para garantizar la seguridad de los extractos.

Palabras Clave: ácido kójico; *Aspergillus flavus*; hongos marinos; melanoma B16F10; viabilidad celular.

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Abbreviations: B16F10: A type of mouse melanoma cell line, particularly derived from the B16 melanoma; BLAST DNA: Basic local alignment search tool deoxyribonucleic acid crystals conversion; ddH2O: Distilled deionized water; DIKSI 35: The isolation of fugus *Aspergillus flavus* from the marine coral; DMEM: Dulbecco's modified eagle medium; DMSO: Dimethyl sulfoxide; DNA: Deoxyribonucleic acid; FBS: Fetal bovine serum; HPLC: High performance liquid chromatography; IC_{50}: Half maximal inhibitory concentration; ITS: Internal transcribed spacer; KA: Kojic acid; LD_{50}: Median lethal dose; MEA: Malt extract agar; MeOH: Methanol; MNA: Malt nutrient agar; MTT assay: A colorimetric assay that measures cell mitochondrial metabolic activity based on MTT into formazan; NMR: Nuclear Magnetic Resonance; PDA: Potato dextrose agar; rRNA: Ribosomal RNA; TLC: Thin layer chromatography.

INTRODUCTION

Kojic acid (KA; structure can be seen in Fig. 1E) is a chelating organic acid belonging to the pyrone derivative (Happi et al., 2015) family, predominantly produced by microorganisms, particularly from the *Aspergillus* genus, including *A. oryzae* (Liu et al., 2016), *A. tamari* (Gould, 1938), *A. parasiticus* (Coupland & Niehaus Jr, 1987), and *A. flavus* (Rosfarizan & Ariff, 2000). The crystalline form of kojic acid was first identified in 1907 by Saito, who isolated it from the mycelia of *A. oryzae* cultured on steamed rice (Beélik, 1956). The chemical structure, represented by the formula $C_6H_6O_4$, was established in 1924. Kojic acid has become prominent in skincare formulations due to its effectiveness as a melanogenesis inhibitor, with significant potential to address skin hyperpigmentation (Saeedi et al., 2019). Its reputation extends beyond cosmetics; it also possesses antibacterial properties (Wu et al., 2018), and it is an essential component in various products. In both the cosmetic and food industries, KA serves as a precursor to taste enhancers and helps preserve the vibrant red colors of fruits and seafood (El-Kady et al., 2014). The potential applications of kojic acid are extensive, attributed to its remarkable range of biological activities. These include anti-inflammatory, antibacterial, antifungal, antiviral, antitumor, antidiabetic, anticancer, antileukemic, antiparasitic, anti-toxoplasmic, and antinematodal properties, as well as exhibiting pesticidal, insecticidal, larvicidal, and analgesic effects (Felipe et al., 2023).

Research has convincingly established KA's toxicity, which can vary depending on species, route of administration, and the specific enzyme being evaluated. The IC_{50} values range from 20 to 100 mM, while the IC_{50} for human tyrosinase is significantly higher at 500 mM. The LD_{50} values indicate oral toxicity levels of 2 g/kg in rats and 5.1 g/kg in mice. Notably, assessments of thyroid tumor promotion in both mice and rats have shown no adverse effects at a dose of 15.5 mg/kg/day. Additionally, a 21-day feeding study in chickens at concentrations up to 146 mg/kg also showed no adverse effects. Furthermore, topical human sensitization tests conducted at doses of up to 2% have demonstrated no harmful effects. In general, KA found in food does not raise safety concerns. In Japan, there is a strong belief that meal products containing KA promote beauty and health, leading to their widespread popularity in the market (Phasha et al., 2022; Saeedi et al., 2019).

Numerous fungal species have been identified as producers of KA, including species from the genera *Chaetomium*, *Cunninghamella*, *Fusarium*, *Mucor*, *Pleospora*, *Trichoderma*, and *Penicillium*. Nonetheless, the genus *Aspergillus* is acknowledged as the most prolific producer (Felipe et al., 2023). Within this genus, *A. oryzae*, *A. flavus*, *A. tamarii*, and *A. parasiticus* are distinguished by their superior capacities for KA production. Numerous studies have effectively investigated methods to enhance KA yields in *Aspergillus*, especially through the use of genetically modified strains and monosporic cultures (Chib et al., 2019). Several critical factors influence KA production, including the composition of the culture medium, pH levels, temperature, agitation, incubation duration, and dissolved oxygen tension. Each of these variables has undergone meticulous examination to optimize productivity (Chib et al., 2019; Coupland & Niehaus Jr, 1987; Liu et al., 2016; Rosfarizan et al., 2002). Various solvents, such as ethanol, ethyl acetate, acetone, chloroform, and methanol, are effectively employed for KA extraction (Chib et al., 2019; Felipe et al., 2023; Futamura et al., 2001). The purification process generally involves recrystallization, with a preliminary treatment with activated charcoal crucial when the isolate exhibits coloration. The purest form of KA is identified by its yellowish prismatic needles. An alternative crystallization method entails cooling the supernatant to approximately 0–5°C to induce crystal formation (Al-Saleem et al., 2022; El-Kady et al., 2014). Rapid detection of KA can be achieved by thin-layer chromatography (TLC), in which the sample reacts with ferric chloride solution, yielding a distinctive reddish color. High-performance liquid chromatography (HPLC) is used to precisely quantify KA in fermentation samples (Felipe et al., 2023).

This investigation aimed to enhance KA production from the marine coral-associated fungus *Aspergillus flavus* DIKSI C35 by optimizing incubation time, medium pH, temperature, and fermentation type. While several studies have successfully improved KA production from marine-associated fungi, some have specifically isolated KA from marine-associated fungus *Aspergillus* (Hamed et al., 2019; Sun et al., 2014; Yang et al., 2011), including research focused on marine coral-associated fungus *Aspergillus* (Al-Saleem et al., 2022).

MATERIAL AND METHODS

Experimental materials

The hard coral material was collected in May 2023 off the coast of Nipah (8°26'05.6"S 116°02'35.6"E), Lombok, West Nusa Tenggara, Indonesia, at a depth of 1.5 m. The morphological characteristics of *Seriatopora hystrix* were identified by the authors, including colony form, individual branches, the tips, corallites, and size. The materials were placed in clean, dry plastic bags and transported to the laboratory under freezing conditions. To disinfect the surface, it was treated with 70% ethanol for 30 seconds, followed by a 0.01% sodium hypochlorite solution, and then rinsed twice with distilled deionized water (ddH_2O). After cleaning, the coral was ground into small pieces and incubated in PDA medium with seawater at 28°C. The fungal strain isolated from the coral sample was subsequently separated and purified. The mycelium grown on the medium was carefully cut, lyophilized, and ground, and then DNA was extracted using the Quick-DNA Magbead Plus Kit (ZYMO Research, USA). The primer ITS1 was employed to amplify the ITS rRNA region. The resulting PCR products were analyzed at Genetika Science Co., Ltd. (Banten, Indonesia). Through detailed BLAST analysis and phylogenetic evaluation of the gene sequences, the fungus was identified as *Aspergillus flavus*. This strain has been deposited in GenBank under accession number PQ198489 with the strain code DIKSI C35 and is currently housed in the Department of Pharmacy, Faculty of Medicine, Mataram University, Mataram, Indonesia.

Fermentation, metabolite extraction, and isolation of the compound

The *A. flavus* strain DIKSI C35 was initially grown on agar plates and subsequently inoculated into 250 mL flasks, each containing a medium composed of 50 g of rice, 1 g/L of malt extract, 25 g/L of sea salt, and 25 mL of water. The flasks were incubated at 25°C for 15 days. Following incubation, the fermentation broth and mycelia were combined with 25 mL of distilled water to form a suspension. This suspension underwent three thorough extractions with equal volumes of ethyl acetate, which were then evaporated under reduced pressure to yield crystalline compounds that adhered to the walls of the rotary flask. The crystals were purified by rinsing the flask walls with ethyl acetate until the yellow color disappeared. The resulting crystals were collected using methanol (MeOH). Finally, the crystalline compound was subjected to NMR spectroscopy to obtain proton, carbon, and 2D-NMR data.

Optimization of media

Optimization of growing media was performed using three types: Malt Extract Agar (MEA), Malt Nutrient Agar (MNA), and Potato Dextrose Agar (PDA). Each medium was supplemented with 25 g/L sea salt and incubated for 10 days at room temperature, with pH maintained at 6.0. After incubation, the biomass was collected, dried at 70°C until constant weight, and the results were measured in mg/mL. Based on the findings, MNA media was selected for further optimization.

Optimization of incubation times, temperatures, and pH

The DIKSI C35 strain was initially inoculated using MNA medium for 10 days, after which it was transferred to 250 mL flasks containing rice (50 g), malt extract (1 g/L), sea salt (25 g/L), and 25 mL of water. These flasks were incubated at 25°C for durations of 10, 15, 20, 25, and 30 days. The metabolite was extracted to obtain a crude extract, and the KA content in the extract was analyzed by HPLC. Based on the KA content, a 15-day incubation period was selected for further optimization. Temperature optimization was performed with the same media, incubating for 15 days at 25, 30, and 40°C. The results indicated that 25°C was the optimal temperature for KA production and was subsequently selected. Furthermore, pH was optimized at 3.0, 4.0, 5.0, and 6.0, with each condition incubated for 15 days at 25°C.

KA analysis by HPLC

A total of 2 mg of crude extract obtained from various optimizations was dissolved in 1 mL of methanol (MeOH), and then 20 µL of this solution was injected into the HPLC system. The analysis used a Waters C18 column (5 µm, 150 × 4.6 mm) at a flow rate of 0.5 mL/min, with UV detection at 269 nm, and 100% MeOH as the mobile phase. KA was used as the standard, with the analytical concentration ranging from 0.2 to 1 mg (Ola et al., 2019).

Unspecific cytotoxicity by MTT assay

The nonspecific cytotoxicity of the crude extract from DIKSI C35 against melanoma was thoroughly examined in vitro using the B16F10 cellular model via the MTT assay. This method effectively measures cellular metabolic activity by quantifying the reduction of a yellow tetrazolium salt to insoluble purple formazan crystals, which are generated by viable cells. It is important to note, however, that while the MTT assay provides valuable insights, it has limitations and cannot accurately distinguish between metabolically inactive cells and completely dead cells (Lajis et al., 2012).

B16F10 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Wako) supplemented with 10% FBS and penicillin/streptomycin (100 IU/50 µg/mL). The cells were maintained in a 5% CO_2 environment at 37°C. Approximately 1 × 10^4 cells were seeded in 96-well plates containing 200 µl of DMEM and 10% FBS and incubated at 37°C with 5% CO_2 for 24 hours. After this incubation, the medium was replaced with fresh medium containing various concentrations of the crude extract, and the mixture was incubated for an additional 24 hours. Subsequently, the medium was replaced with a fresh medium containing 0.01 mg/mL MTT, and the cells were incubated for 2 hours at 37°C. Finally, the liquid medium was carefully removed, and 200 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formed formazan crystals. The absorbance was then measured at 570 nm using an Agilent BioTek Epoch microplate spectrophotometer. Each concentration was assayed three times, and we confidently included six additional controls (cells in medium) in each test. The data are robustly presented as IC_{50} values, representing the concentration of the compound required to achieve a 50% reduction in cell viability (Lajis et al., 2012).

Statistical analysis

Comparison of the groups (mean ± SD) by One-Way Analysis of Variance (ANOVA) with Duncan's. Significant as *p<0.05 and **p<0.01 by the GraphPad Prism (version 9.0, San Diego, CA, USA).

RESULTS AND DISCUSSION

This research demonstrates the successful fermentation of fungi isolated from the coral species *Seriatopora hystrix* using semi-solid media as the substrate. The fermentation products were extracted with ethyl acetate, and half of the solvent volume was efficiently evaporated. Following this, the extract was stored at 4°C for 12 hours, during which distinct yellow needle-like crystals formed. These crystals were meticulously washed and effectively separated from the filtrate, preparing them for thorough analysis. For detailed characterization, we utilized NMR spectroscopy. The analysis, conducted with both ^1H (Fig. 1A) and ^{13}C (Fig. 1B) NMR at a frequency of 500 MHz in MeOD, revealed three distinct proton signals at δ_H 7.90 (1H), 6.50 (1H), and 4.40 (2H) Hz, as well as six carbon signals at δ_C: 170.4, 110.8, 176.9, 147.4, 141.0, and 61.2 ppm. The 2D-HSQC (Fig. 1C) spectrum clearly illustrated correlations between the proton signal at δ_H 7.90 Hz and the carbon signal at δ_C 141.0 Hz, as well as between the signals at δ_H 6.50 Hz and δ_C 110.8 Hz, confirming the presence of a duo -CH-aromatic structure. Furthermore, the correlation of the proton signal at δ_H 4.40 (2H) Hz with δ_C 61.20 Hz strongly indicates a methylene group. The ^{13}C NMR spectrum unequivocally confirms the presence of three oxygenated carbons at δ_C 170.40, 147.4, and 141.0 Hz, while the signal at δ_C 176.90 Hz clearly denotes a carbon-lactone structure. The ^1H-NMR spectrum further reinforces the presence of aromatic protons at δ_H 7.90 and 6.50. The 2D-HMBC spectrum (Fig. 1D) distinctly confirmed a correlation between the proton signal at δ_H 4.40 and the carbon signals at 110.8 and 170.4 Hz. Additionally, the signal at δH 6.50 exhibited correlations with the carbon signals at 61.2, 110.8, 147.4, 170.4, and 176.9 Hz. Furthermore, the proton signal at δH 7.90 displayed strong correlations with the four carbon signals at 141.0, 147.4, 170.4, and 176.9 Hz. These NMR results robustly support the characterization of the kojic acid compound, as depicted in Fig. 1E.

The nucleotide sequence of the fungi responsible for KA production has been precisely determined at the Internal Transcribed Spacer (ITS) using 16S rRNA primers. A BLAST search confirmed an exact match to *Aspergillus flavus*, achieving 100% query coverage. The strain DIKSI C35, associated with GenBank accession number PQ198489, was utilized in this study. The morphological characteristics of DIKSI C35 on agar medium, along with the phylogenetic tree presented in Fig. 2, were clearly defined. Research by Chang (2023) convincingly demonstrated that nearly all parts of *A. flavus* harbor gene clusters responsible for the complete biosynthesis of KA. This finding highlights the significant genetic potential of this fungus for efficient KA biosynthesis, establishing it as a valuable resource for the pharmaceutical and cosmetic industries. Such insights are poised to propel future research aimed at optimizing production processes and enhancing the yields of this important compound. The result of the fermentation period's influence on KA production levels by DIKSI C35 reveals that significant KA production begins on the 15th day, steadily increasing until the 20th day, with concentrations reaching between 23.5 and 25.8 g/L (Fig. 3A). These yields are consistent with the findings of prior research (Quiterio-Gutiérrez et al., 2023; Rosfarizan & Ariff, 2000; Wan et al., 2005). The observed decline in KA production after the optimal fermentation period is likely due to its conversion into other secondary metabolites.

Recent research has established that several *Aspergillus* species are highly effective in producing KA. In our study, we thoroughly examined the impact of varying pH levels on KA production during solid fermentation with sea salt. Notably, strain DIKSI C35 achieved a maximum KA yield of 31.2 g/L at pH 3 (Fig. 3B). As the pH of the fermentation medium increased, we observed a distinct reduction in KA production, reinforcing the conclusion that pH 3 is optimal for maximizing yields.

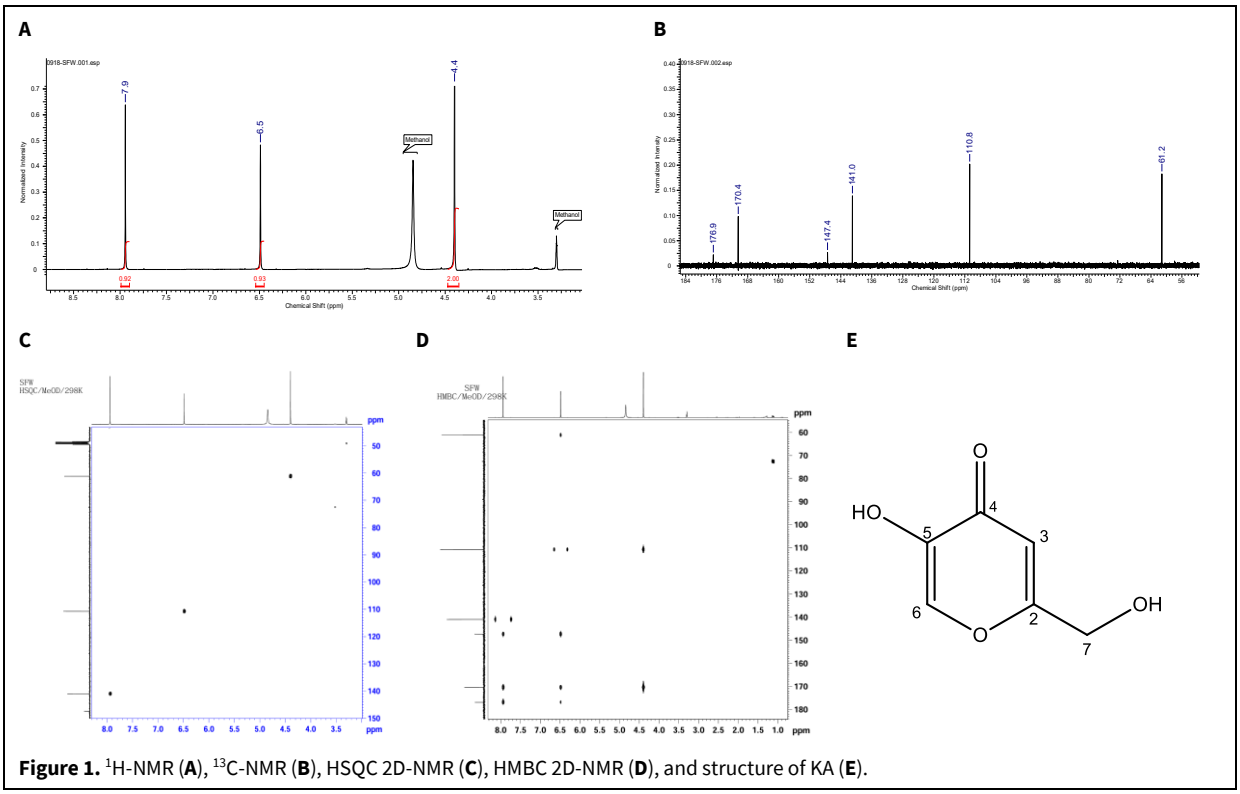

Figure 1. ^{1}H-NMR (**A**), ^{13}C-NMR (**B**), HSQC 2D-NMR (**C**), HMBC 2D-NMR (**D**), and structure of KA (**E**).

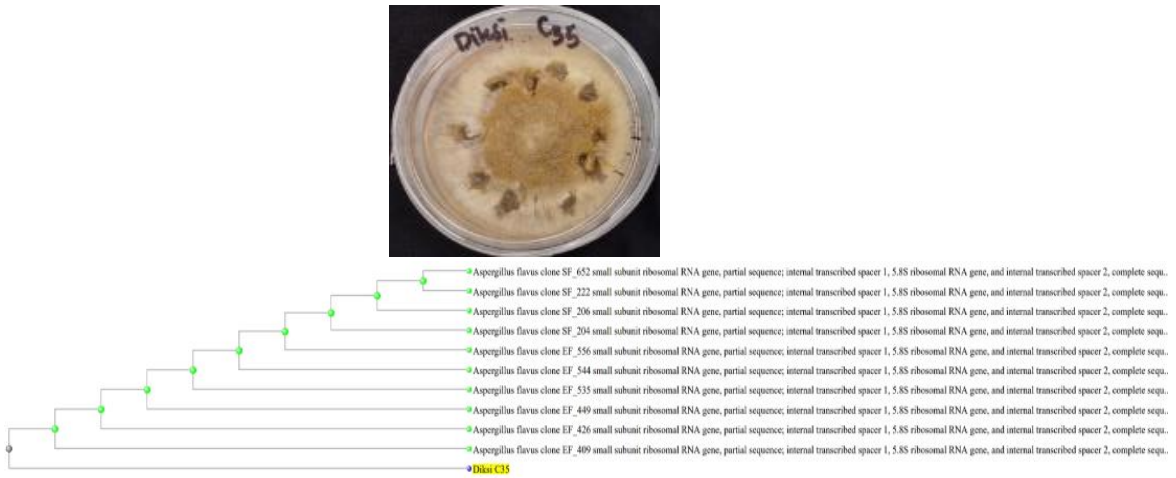

Figure 2. Morphology (**A**) and phylogenetic tree (**B**) of DIKSI C35.

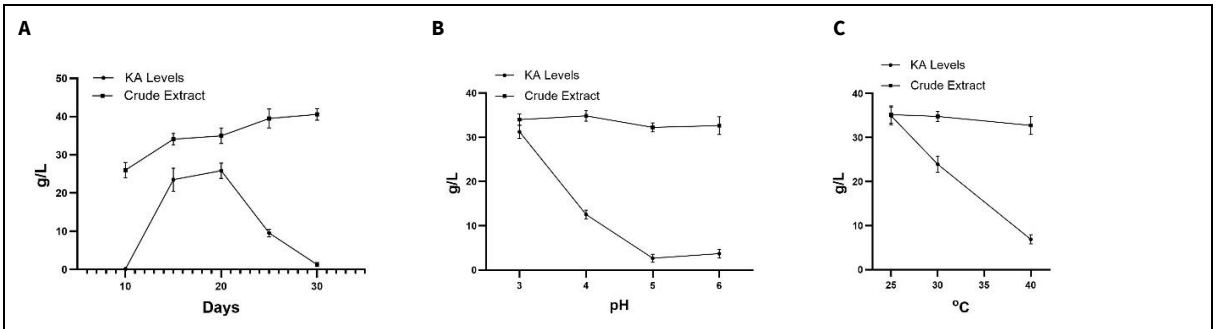

Figure 3. Effect of time (**A**), pH (**B**), and temperature (**C**) on KA production by strain Diksi C35 (n = 3).

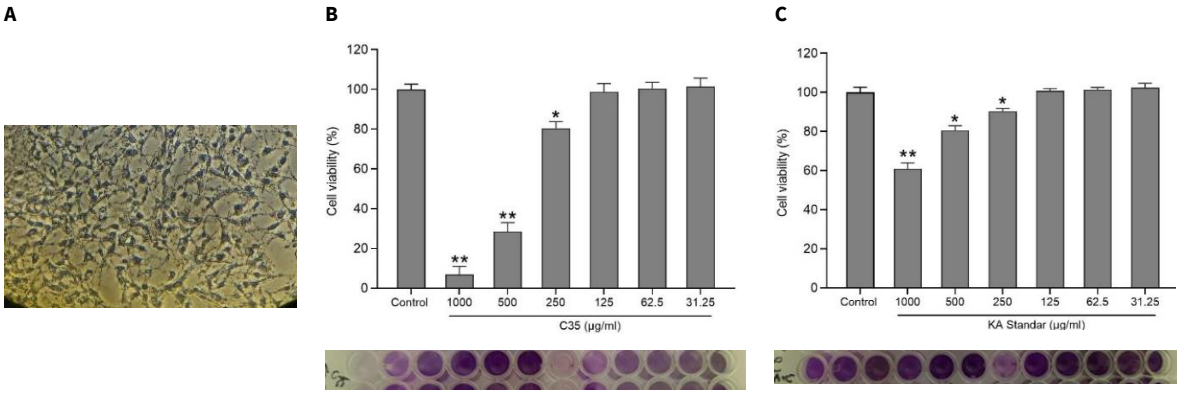

Figure 4. Morphology of B16F10 treatment as 31.25 µg/mL of C35 for 24 h in cell culture conditions (**A**). Percent cell viability of cells treated by C35 (**B**) and KA standard (**C**) for 24 h. **p<0.001, *p<0.05 compared to control groups.

These findings align well with earlier studies conducted by Rosfarizan et al. (2002), which reported similar trends under varying pH conditions. Specifically, increasing the pH of the solid fermentation medium containing sea salt resulted in a significant decrease in KA levels. This shift indicates that, at higher pH levels, the fungus alters its metabolic pathways, favoring the production of alternative compounds and thereby limiting KA synthesis (Rosfarizan et al., 2002). In conclusion, strain DIKSI C35 demonstrated its highest KA production at pH 3, while higher pH levels effectively ceased production. pH is a critical factor influencing KA yields in *Aspergillus* strains, with optimal results consistently observed at acidic pH values of 2–3 (Rosfarizan et al., 2002). Maintaining strict pH control throughout the fermentation process is essential, as it significantly enhances KA production compared to processes that lack such regulation.

The production levels of KA by strain Diksi C35 were observed to depend significantly on the culture temperatures after a 15-day fermentation period. Temperature plays a critical role in influencing both the levels of KA and the growth of *A. oryzae*. Notably, the highest growth rates for strain C35 occurred at 25°C, where KA levels were remarkably elevated. In contrast, at 30°C, KA levels were considerably lower (Fig. 3C). On day 15, the measured KA concentrations were 34.95 g/L at 25°C, 23.95 g/L at 30°C, and 6.88 g/L at 40°C, highlighting a clear trend of declining KA levels with increasing temperature. Moreover, it is established that 28°C is the optimal temperature for *A. oryzae* (Rasmey & Abdel-Kareem, 2021). This finding is consistent with reports from Ishak et al. (2018) and Rosfarizan & Ariff (2000), which also underscore the significant effects of temperature on KA production levels.

The toxicity of both crude extract and standard KA against B16F10 melanoma cells was rigorously examined using MTT assays following a 24-hour incubation at concentrations of 1000, 500, 250, 125, 62.5, and 31.25 µg/mL (Fig. 4). Our findings indicate a clear dose-dependent reduction in cell viability of B16F10 cells with both the crude extract and standard KA. Notably, KA has been confirmed to be nontoxic at doses as low as 100 µg/mL, with toxicity directly linked to higher concentrations. Previous studies support the assertion that KA poses no toxic risks in reproductive, chronic, genotoxicity, and acute assessments related to human skin. In our research, we observed that crude extracts with high concentrations of KA demonstrate toxicity against B16F10 cells at elevated doses ranging from 250 to 1000 mg/mL, while concentrations of 150 µg/mL and below exhibited no toxic effects. A similar pattern was observed for the KA standard, which showed toxic effects at doses exceeding 250 mg/mL against B16F10 melanoma cells (Lajis et al., 2012).

CONCLUSION

Kojic acid, produced by the marine coral-associated fungus *Aspergillus flavus* strain DIKSI C35, can be optimized by adjusting fermentation duration, pH, and culture temperature, yielding 34.95 g/L after 15 days at pH 3 and 25 °C. Importantly, the high concentration of kojic acid in the crude extract from strain DIKSI C35 demonstrated no toxicity at a dosage of 125 against B16F10 melanoma cells.

CONFLICT OF INTEREST

The research was conducted entirely free from any involvement in commercial, financial, or personal matters that might be perceived as a possible conflict of interest or that could have influenced the outcomes or interpretation of the study.

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GENERATIVE ARTIFICIAL INTELLIGENCE (AI)

The authors declare that no generative artificial intelligence (AI) or AI-assisted technologies were used in the preparation, writing, or editing of this manuscript. All content was developed solely by the authors, who take full responsibility for its accuracy and integrity.

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Contribution	Safwan S	Aini SR	Ridwan S	Pradiningsih A	Pratiwi ET	Wahid AR
Concepts or ideas	x					
Design		x				
Definition of intellectual content			x			
Literature search	x					
Experimental studies	x				x	
Data acquisition						x
Data analysis	x					x
Statistical analysis			x			
Manuscript preparation	x		x	x		
Manuscript editing		x			x	x
Manuscript review	x	x	x	x	x	x

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