Cyanide-Degrading Indigenous *Aspergillus* Spp. Isolated From *Kabuto*, A Southeast Sulawesi Fermented Food

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Abstract.

Kabuto is a traditional fermented food from Muna, Indonesia, produced by the solid-state fermentation of cassava roots. This process involves coating the cassava with naturally occurring fungal colonies. This study aimed to investigate the fermentation of cassava roots using defined Aspergillus spp. strains to enhance its nutritional profile, with a specific focus on reducing cyanogenic compounds. Cassava samples were subjected to solid-state fermentation by various Aspergillus spp. isolates for six days at approximately 28°C. The resulting products were analyzed for moisture content (%), total protein (mg), and cyanide detoxification (µg g⁻¹). Isolates K03, K23, K1c, and K31 demonstrated significant antimicrobial activity and produced hydrolytic enzymes, including amylase and cellulase. These same isolates also effectively inhibited the growth of Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 35218. Based on phenotypic characterization, isolates K03, K23, and K1c were identified as Aspergillus niger, while isolate K31 was identified as Aspergillus terreus. A notable reduction in the moisture content of Kabuto commenced on the fourth day of fermentation. Upon completion, isolates K03, K23, K31, and K1c yielded final moisture contents of 7.1%, 6.74%, 8.69%, and 7.07%, respectively. The highest protein content was achieved through fermentation with Aspergillus terreus K31, which yielded 346 mg of protein and concurrently reduced the cyanide concentration to 0.48 µg g⁻¹. The optimal duration for Kabuto fermentation was determined to be six days. In conclusion, this study demonstrates that controlled fermentation using specific Aspergillus strains, particularly A. terreus K31, significantly improves the nutritional quality and safety of Kabuto by enhancing its protein content and effectively detoxifying cyanide.

Keywords: *Aspergillus; Cassava; Cyanide; Fermented food and HCN.*

I. INTRODUCTION

Kabuto is an indigenous fermented food from the Muna community of Southeast Sulawesi, Indonesia, produced by the spontaneous fermentation of cassava tubers (*Manihot esculenta* Crantz) and consumed as a staple accompaniment. This traditional product is characterized by dried cassava roots that undergo fermentation until becoming fully enveloped in fungal colonies. During this process, the proliferating fungi assimilate the root's nutrients, leading to significant modifications in the product's texture and nutritional profile [1]. Cassava is notably rich in carbohydrates but inherently deficient in proteins and lipids. On a dry matter basis, its carbohydrate content ranges from 70 to 82%, comprised predominantly of starch with varying ratios of amylopectin and amylose [2,3]. A major concern, however, is the tuber's content of anti-nutritional compounds, specifically cyanogenic glycosides which release hydrogen cyanide (HCN). Reported concentrations can reach 1090–1550 mg HCN/kg, levels that pose a significant risk to human health [4,5].

Chronic dietary exposure to high cyanogen levels can cause '*konzo*', a permanent upper motor neuron disease. Populations reliant on cassava-based diets, particularly children and the elderly, are therefore at heightened risk of cyanide poisoning and require monitoring [6]. The fermentation process in kabuto production is initiated by ambient atmospheric inoculum. The abundant carbohydrates in cassava serve as the primary nutrient source for the growth of these indigenous fungi. A key objective of this bioprocess is to enhance the functionality and beneficial properties of the final fermented product [7,8]. In related fermented cassava foods, applied fungal inoculants frequently belong to the genus Aspergillus, with species such as *A. fumigatus*, *A. niger*, *A. oryzae*, and *A. terreus* being commonly reported [9-12]. This study aimed to isolate and identify the indigenous fungi, with a focus on *Aspergillus* species, responsible for the spontaneous fermentation of kabuto. Selected fungal strains will subsequently be applied as a defined starter culture to standardize the fermentation process and evaluate the efficacy of these strains in reducing the cassava's cyanide content.

II. METHODS

Sampling Procedures

Kabuto is made by locals of Southeast Sulawesi, Indonesia. The process of *Kabuto* begins with the natural drying of sunlight around the community housing. Natural fermentation was carried out after the cassava roots dried (Fig. 1A). Fermentation occurred in sacks for 9 days until the cassava roots were colonized by the mold (Fig. 1B). The temperature of fermentation was recorded at 28 °C, and the humidity at 61%. These environmental factors are highly determining the presence of microorganisms, especially the colony of mold that needs a moist environment.

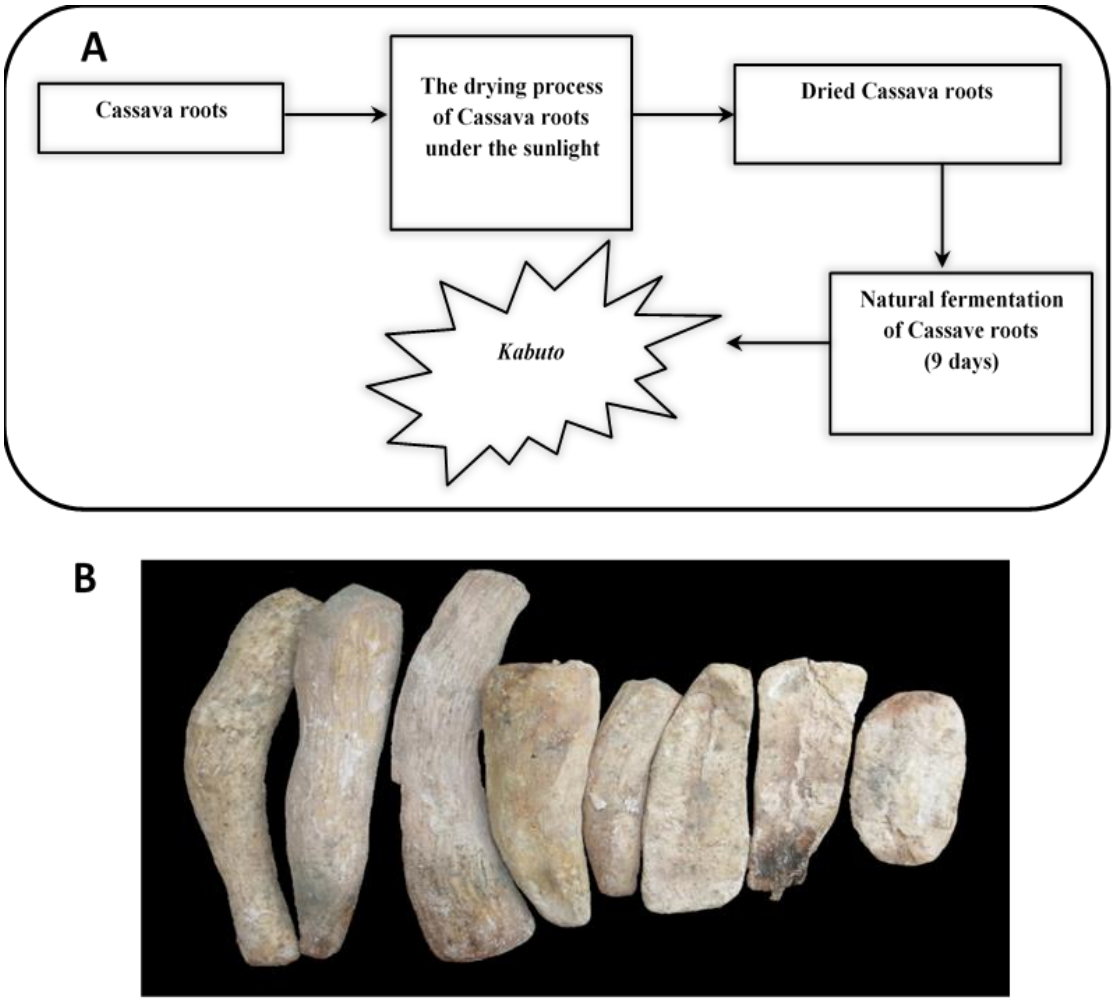

Fig 1. *Kabuto*, the traditional fermented food of Munanese, Southeast Sulawesi: A) Schemmatic diagram on the process of preparing *kabuto*; B) Appearance of final product of fermented cassava, *kabuto*.

Isolation and Enumeration of Fungal Colonies

Fungal isolation was performed using the pour plate method with a Potato Dextrose Agar (PDA) medium. A 10-gram sample of Kabuto powder was added to a dilution bottle containing 90 mL of sterile distilled water and suspended until a homogeneous mixture was achieved. Subsequently, a 1 mL aliquot of this suspension was transferred to the first dilution bottle containing 9 mL of sterile distilled water, yielding a 10^{-2} dilution. This serial dilution was repeated to achieve a final dilution of 10^{-7}. Aliquots of 1 mL from the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were then pipetted into separate, empty Petri dishes. Molten PDA medium, cooled to approximately 45°C, was then poured into the plates. To ensure an even distribution of fungal cells, the plates were gently swirled in a figure-eight motion on the bench surface. After the agar solidified, the plates were incubated in an inverted position at ambient temperature for 24-48 hours. The resulting fungal colonies were enumerated, and the microbial load was calculated as colony-forming units per gram (CFU/g) using the standard plate count formula: CFU/g = (Number of colonies) × (1 / Dilution Factor). Individual mold colonies were purified using the point inoculation method on PDA slants and incubated at 26–28 °C for 24–48 hours. To prepare pure stock cultures, these purified isolates were similarly transferred to PDA slants via point inoculation, incubated under the same conditions, and then stored in a refrigerator.

Antibacterial Test

The bacterial strains, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 35218 were acquired from the Food and Nutrition Culture Collection (FNCC) at Gajah Mada University. For the assays, 1 mL of each pure bacterial isolate was transferred into 5 mL of nutrient broth. The cultures were adjusted to a cell density of 10^8 CFU/mL, standardized according to the McFarland turbidity standard [13], and incubated at 37 °C for 24 hours. The antibacterial activity of the *Aspergillus* isolates was evaluated using the well diffusion assay. The base layer, consisting of penassay base agar, was poured into Petri dishes and allowed to solidify. A sterile spacer was placed aseptically on the solidified base. Subsequently, a soft agar medium seeded with 1 mL of the test bacterial suspension was poured over it. After the overlay solidified, the spacer was removed, creating uniform wells in the medium. A 50 µL aliquot of the mold supernatant was dispensed into each well. Distilled water and penicillin were used as negative and positive controls, respectively, to benchmark the clear zones produced by the mold isolates. The plates were refrigerated for 60 minutes to facilitate the diffusion of antimicrobial compounds into the medium, followed by incubation at 37 °C for 24 hours. The formation of a clear zone around a well indicated the presence of antibacterial substances.

Amylolytic and Cellulolytic Activity

Amylolytic activity was assessed on a starch minimum medium, while cellulolytic activity was tested on a cellulose agar medium. The starch minimum medium was prepared by dissolving 2 g of PDA and 0.3 g of soluble starch in 100 mL of distilled water. The mixture was heated on a hot plate with a magnetic stirrer until homogeneous and then sterilized by autoclaving. The cellulose agar medium was composed of the following: 0.2 g yeast extract, 0.1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g carboxymethyl cellulose (CMC), and 2 g agar [14]. These components were dissolved in 100 mL of distilled water, heated with constant stirring using a magnetic stirrer to achieve a homogeneous solution, and subsequently sterilized by autoclaving. Following sterilization and cooling to approximately 45-50°C, the media were poured aseptically into sterile Petri dishes and allowed to solidify. Fungal colonies from pure, actively-growing cultures were then spot-inoculated onto the center of the respective agar plates. After the incubation periode, the plates were developed to visualize enzymatic activity. The starch agar plates were flooded with Gram's iodine solution. A clear, colorless zone (halo) surrounding the fungal colony against a dark blue background of the starch-iodine complex indicated the hydrolysis of starch. The cellulose agar plates were flooded with an aqueous 1% (w/v) Congo Red solution for 15-20 minutes. The dye was then poured off, and the plates were destained with 1 M NaCl for another 15-20 minutes. A clear zone around the fungal growth against a red background indicated the degradation of carboxymethyl cellulose.

Characterization of Fungal Isolates

The characterization of fungal isolates was conducted after the initial selection of promising strains based on their antimicrobial activity and hydrolytic enzyme production. The Kabuto isolate was characterized by its hyphal morphology, the characteristics of its sexual and asexual spores (including shape and color), rhizoid formation, and sporangiophore structure. Macroscopic colony observation involved assessing color, surface topography, margin morphology, texture, the presence of exudate droplets, sporulation patterns, zonation, radial furrowing, growth density, and the appearance of the reverse side. Microscopic morphological analysis included the identification of sexual and asexual spores, their type, morphology, and dimensions; the structure of the respective spore-producing bodies; hyphal type, pigmentation, and width; along with other distinctive features.

Fungal Inoculation and Kabuto Fermentation

A modified Potato Dextrose Broth (PDB) was used as an inoculum medium for subsequent inoculation onto cassava roots. The modified PDB medium was composed of 13.25 g cassava flour, 13.25 g commercial PDB powder, and 1000 mL distilled water. The medium was prepared by mixing the cassava flour and PDB powder, followed by heating and homogenization using a magnetic stirrer. The modified medium was then sterilized by autoclaving. A 96-hour-old fungal culture grown on modified Potato Dextrose Agar (PDA) was suspended in 4 mL of the modified PDB within a test tube. Spores from this suspension were then aseptically transferred to a slant of PDA using an inoculation loop and subsequently incubated in a 100 mL Erlenmeyer flask. A 10 mL aliquot of this fungal isolate from the Erlenmeyer flask was transferred into a sterile glass bottle (385 x 290 x 40 mm). The spore concentration was determined using a hemocytometer to standardize the inoculum to a density of 10^6 spores/mL [15]. Ten mL of inoculum solution, was aseptically inoculated onto sterilized cassava tubers. The inoculum was mixed thoroughly to ensure even distribution across the substrate. The inoculated cassava was then fermented in an incubation room maintained at approximately 28°C. Fermentation was carried out for six days in small plastic jars covered with sterile fabric to allow for aeration. Samples were analyzed at 48-hour intervals, starting from day 0.

Determination of Water Content

The water content of the Kabuto samples was monitored throughout the fermentation process to track moisture loss. The analysis was performed by weighing approximately 1 g of sample (initial weight), drying it in an oven at 105 °C for 4 hours, and then re-weighing the sample (final weight). The percentage water content was calculated: % Water Content = [(Initial Weight - Final Weight) / Initial Weight] × 100%.

Determination of Total Protein

The Biuret reagent was prepared by dissolving 3 g of $CuSO_4 \cdot 5H_2O$ and 9 g of sodium potassium tartrate in 500 mL of 0.2 N NaOH. Subsequently, 5 g of potassium iodide (KI) was added, and the solution was diluted to a final volume of 1000 mL with 0.2 N NaOH. A standard curve was constructed using Bovine Serum Albumin (BSA) with a concentration of 5 mg/mL. Aliquots of the BSA standard solution (0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mL) were pipetted into test tubes. Distilled water was added to each tube to bring the total volume to 4 mL. Then, 6 mL of Biuret reagent was added to each tube. The tubes were incubated at 37 °C for 10 minutes, and the absorbance was measured at 645 nm using a UV-Vis spectrophotometer. Two grams sample of fermented cassava flour was suspended in 10 mL of distilled water. The suspension was centrifuged at 3000 rpm for 10 minutes to precipitate denatured proteins. The supernatant was decanted and discarded. The resulting pellet was resuspended in 4 mL of distilled water, and 6 mL of Biuret reagent was added. The alkaline reagent dissolved the residual protein precipitate, and the solution was homogenized using a vortex mixer. The absorbance of the sample solution was measured at 645 nm. The protein concentration was calculated based on the regression equation ($Y = ax + b$, where Y is absorbance and x is protein concentration) derived from the standard curve.

Quantification of Cyanide (HCN) using Argentometric Titration

A 10 g sample of Kabuto powder was mixed with 100 mL of distilled water in a Kjeldahl flask and macerated for 2 hours. An additional 100 mL of distilled water was added, and the mixture was subjected to steam distillation. The distillate was collected in an Erlenmeyer flask containing 20 mL of 2.5% (w/v)

sodium hydroxide (NaOH) solution. Distillation was halted once 100 mL of distillate had been collected. The distillate was then treated with 8 mL of ammonium hydroxide (NH_4OH) and 5 mL of 5% (w/v) potassium iodide (KI) solution. This mixture was titrated with a standardized 0.02 N silver nitrate ($AgNO_3$) solution until the first appearance of persistent turbidity, indicating the formation of a silver cyanide complex and a white silver iodide precipitate. The volume of $AgNO_3$ titrant consumed was used to calculate the cyanide acid content according to the stoichiometry of the reaction as follows:

$$HCN = \frac{Volume\ (mL)\ of\ AgNO_3 \times 0.01\ N\ AgNO_3 \times 54}{100} \times 1000\ mg/kg$$

Extraction, qualitative and quantitative analysis of fungal genomic DNA

DNA isolation from fungal isolates was performed by first growing the isolated fungi in sterilized PDB containing ampicillin (30mg/L), and incubation was done in a shaking incubator at 150rpm, 30 ºC for 5 days. For five days, 1100 mg of mycelia were frozen in liquid nitrogen and crushed to a fine powder using a mortar and pestle in order to expose the content of the nucleus, facilitate the DNA extraction process, and were put in 1.5 mL microcentrifuge tubes. The DNA extraction was conducted using Geneaid plant DNA extraction kits as per the manufacturer's protocol. The quality and quantity of isolated genomic DNA were analyzed for DNA size and purity, as well as to check the success of DNA isolation. The analysis of genomic DNA was performed using a Biophotometer (Thomas Scientific USA) at a 260/280 ratio of DNA concentration measured. The quality and quantity of DNA were also analyzed using 0.8% (w/v) Agarose gel electrophoresis; the formed bands were visualized using a gel documentation system, and the visualized bands were documented.

Polymerase chain reaction (PCR) amplification of the ITS regions and sequencing

The fungal ITS2 gene sequence from the total fungal community and genomic DNA was amplified using the ITS2 primers (ITS86F – GTGAATCATCGAATCTTTGAA and ITS4 – TCCTCCGCTTATTGATATGC). Amplification of ITS regions was performed using My Taq HS red mix (Bioline, Germany) using Verity 96-well Thermal cycler (Thermo Fisher Scientific, USA). The reaction was performed in 50µl reaction mixtures, which contained 25µl PCR mix (Bioline, Germany), 2.5µl DNA sample, 2.25 µl of each primer, and it was made to 50µl by adding 17.5µl of ddH2O. PCR was carried out at an initial denaturation step at 94°C for 2 min, followed by 30 cycles at 94°C for 30 sec, 45°C for 30 sec, and 72°C for 2 min, and a final extension step at 72°C for 5 min. The obtained PCR products were subjected to agarose gel electrophoresis (Bio-Rad) at 85V for 60 minutes using 1.5% (W/V) agarose gel electrophoresis in 1x TAE (TrisAcetate-EDTA) buffer. The forming bands were stained using Ethidium bromide (EtBr) for 15 minutes and were washed in distilled water for 10 minutes. The formed DNA bands were visualized using a gel documentation system and were recorded. The obtained PCR products were sequenced both in forward and reverse directions at a 1st base Laboratory in Singapore.

Data analysis

The data for each treatment were subjected to a two-factorial analysis of variance (ANOVA). This model was employed to evaluate the effects of the two independent variables and their interaction. For parameters where the treatment demonstrated a significant effect (indicated by an F-statistic exceeding the critical F-value), the analysis was followed by Duncan's Multiple Range Test. This post-hoc test was applied to discern specific differences between treatment means at a 95% confidence level ($\alpha = 0.05$). All statistical computations were performed using SAS software (Statistical Analysis System).

III. RESULT AND DISCUSSION

Extrinsic and Intrinsic Characteristics of Spontaneous Kabuto Fermentation

Measurements of key environmental parameters, i.e. temperature, humidity, and moisture content at the source, demonstrated that the fermentation process significantly altered the conditions within the Kabuto (Fig. 2). The correlations between these parameters and mold growth over the 9-day fermentation period are detailed in Table 1. Table 1 presents the correlation matrix for the measured characteristics of Kabuto: internal temperature (TK), ambient temperature (T), internal humidity (RHK), ambient humidity (RH),

moisture content (ω), and total fungal count (TFC) as a measure of mold growth. A correlation coefficient approaching +1 indicates a strong positive relationship, while a value approaching -1 signifies a strong negative (inverse) relationship. The analysis revealed a strong positive correlation (r = 0.754) between the internal temperature of the Kabuto (TK) and mold growth (TFC). However, the only statistically significant correlation (*) was the strong negative relationship between ambient temperature and the internal humidity of the Kabuto (r = -0.998).

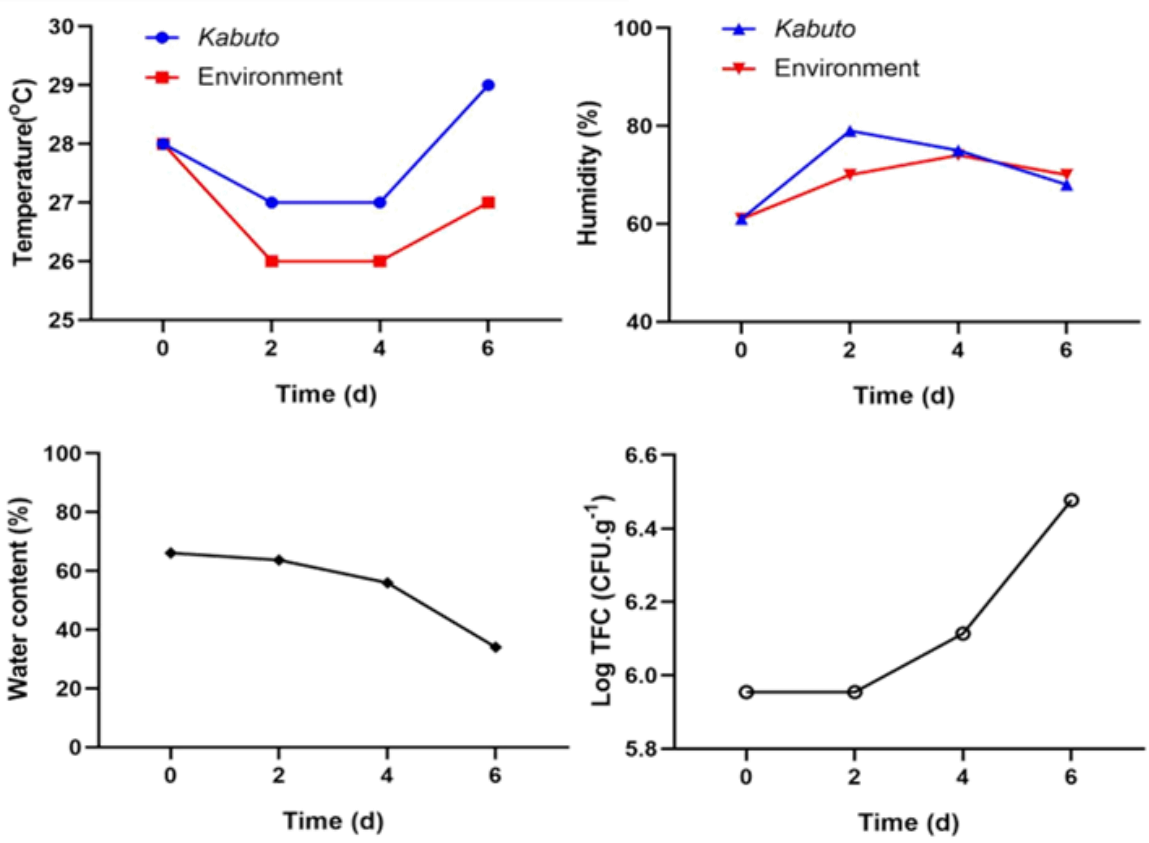

Fig 2. Characteristics of *kabuto* within 9-days of the fermentation period

Table 1. Correlation (*r*) among extrinsic and intrinsic characteristics of *kabuto*

Parameters	TK (°C)	T (°C)	RHK (%)	RH (%)	ω (%)	TFC (CFU·g⁻¹)
TK (°C)	1	0.636	−0.669	−0.332	−0.731	0.754
T (°C)	0.636	1	−0.977*	0.902	0.034	0.015
RHK (%)	−0.669	−0.977*	1	0.800	0.072	−0.129
RH (%)	−0.332	0.902	0.800	1	−0.401	0.367
ω (%)	−0.731	0.034	0.072	−0.401	1	−0.998*
TFC (CFU·g⁻¹)	0.754	0.015	−0.129	0.367	−0.998*	1

Values represent Pearson's correlation coefficient (r). * denotes a statistically significant correlation (P < 0.05). TFC: Total Fungal Count.

Antibacterial and Enzymatic Activity of Fungal Isolates from Kabuto

Eleven fungal isolates from *kabuto* were evaluated for antibacterial activity and the production of hydrolytic enzymes. As summarized in Table 2, seven isolates showed antibacterial activity against *Staphylococcus aureus* ATCC 25923, while all eleven isolates were active against *Escherichia coli* ATCC 35218. Furthermore, four isolates (K03, K23, K31, and K1c) demonstrated the ability to produce both amylase and cellulase. This enzymatic activity was confirmed by the formation of a clear hydrolysis zone around the fungal colonies following a four-day incubation period (Fig. 3). The high carbohydrate content of the *kabuto* substrate likely facilitated the induction of these substrate-specific enzymes in the mold isolates.

Table 2. Antibacterial and enzymatic profiles of *kabuto*-associated fungal isolates

Isolate	Inhibition zone (*Staphylococcus aureus* ATCC 25923)	Inhibition zone (*Escherichia coli* ATCC 35218)	Amylase (clear zone)	Cellulase (clear zone)
K01	+	+	–	–
K32	+	+	–	–
K21	+	+	–	–
Ksr2	–	+	–	–
K51	–	+	–	–
K04	–	+	–	–
K03	+	+	+	+
K23	+	+	+	+
K31	+	+	+	+
K1c	+	+	+	+
K1d	–	+	–	–

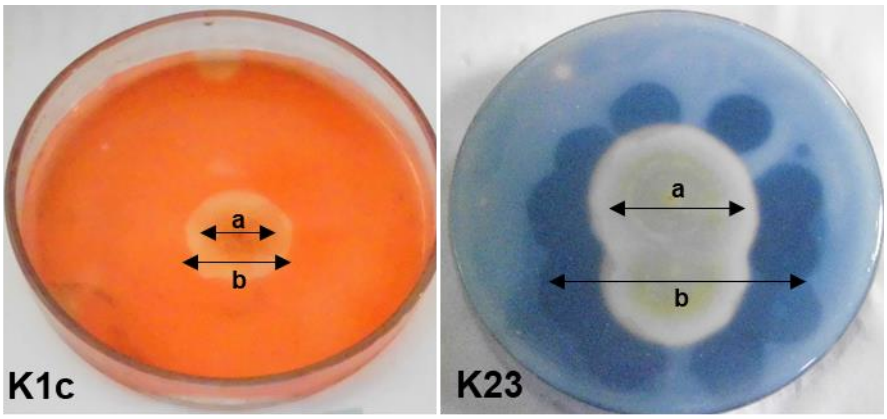

Fig 3. Extracellular cellulase by K1c and amylase by K23 as visualized using plate-based assays.
Note: a) Fungal colonies, b) Clear zones indicating positive activities

Morphological Characteristics of Fungal Isolates

The morphological characteristics of the four fungal isolates were assessed through both macroscopic and microscopic examination. Macroscopic evaluation was conducted on colonies grown on PDA, focusing on traits such as surface and reverse coloration, sporulation, presence of radial furrows, and distinct growth zones. Microscopic analysis, performed using the slide culture technique, identified key features including asexual spore morphology, rhizoid development, whether the hyphae were septate or aseptate, and mycelial pigmentation. The consolidated morphological profiles of the four isolates, based on these PDA culture observations, are presented in Table 3 and Fig. 4.

Table 3. Morphological characteristics of *Aspergillus* spp. isolated from *kabuto*

Characteristics	Fungal Isolates			
	K03	K23	K1c	K31
Color (surface)	Black	Black	Black	Brown
Color (reverse)	White	White	Yellow	Brown
Texture (colony)				
Spore color	Black	Black	Black	Brown
Concentric ring (+/-)	+	+	+	+
Radial line (+/-)	-	-	+	-
Rhizoid cell (+/-)	+	+	+	+
Conidiospore (+/-)	+	+	+	+
Hyphae				
Septate	+	+	+	+
Color	Hyaline	Hyaline	Hyaline	Hyaline
Conidiophore				

Texture	Smooth	Smooth	Smooth	Smooth
Length	Long	Short	Long	Long
Vesicle (+/-)	+	+	+	+
Phialide(s)	Biserriate	Biserriate	Biserriate	Biserriate

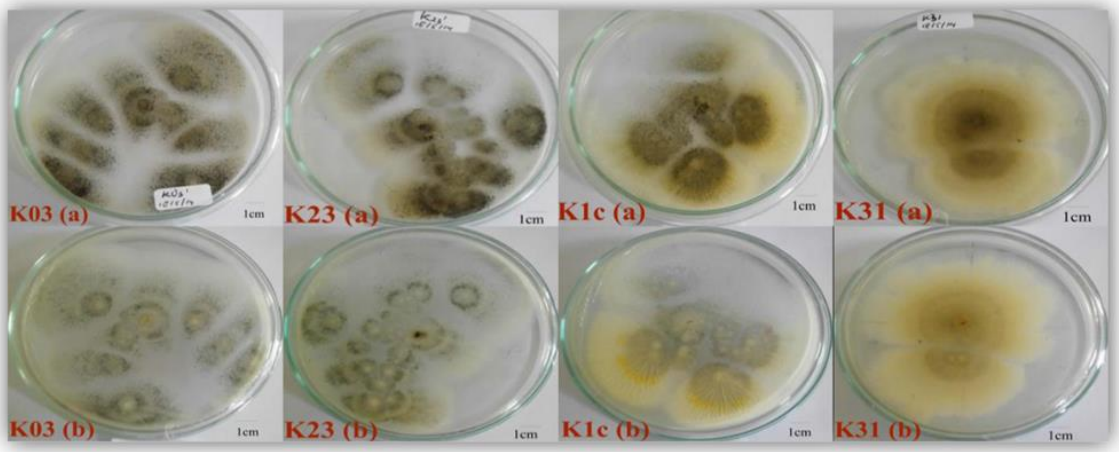

Fig 4. Colony images of *Aspergillus* spp. isolated from *kabuto* showing surface (a) and reverse colony (b)

Identification of Fungal Isolates based on ITS-rDNA Sequence

The three isolates obtained in this study were identified as *Aspergillus niger,* while the fourth was identified as *Aspergillus terreus*. Figure 5 presents a maximum likelihood phylogenetic tree, illustrating the evolutionary relationships among the studied isolates and reference strains. The isolates from this work (denoted K03, K23, K1c, and K31) form distinct clades. Although they share relatively high similarity with known sequences, the phylogenetic analysis reveals that they are not closely related to strains currently deposited in GenBank, as indicated by a degree of homology of less than 99% with the reference strains [16].

Growth Profiles of Selected Fungal Isolates for Experimental Kabuto

Mold growth was quantified by enumerating colonies on Potato Dextrose Agar (PDA) using the cup count method. Figure 6 illustrates the correlation between fungal proliferation and the moisture content of *kabuto* for each treatment throughout the fermentation period. The data indicate a direct relationship between fermentation duration and mold biomass. Conversely, the water content exhibited a declining trend. This reduction in moisture commenced on the fourth day post-inoculation. After four days of fermentation, the final water content values for the respective isolates were as follows: K03 (7.1%), K23 (6.74%), K31 (8.69%), and K1C (7.07%).

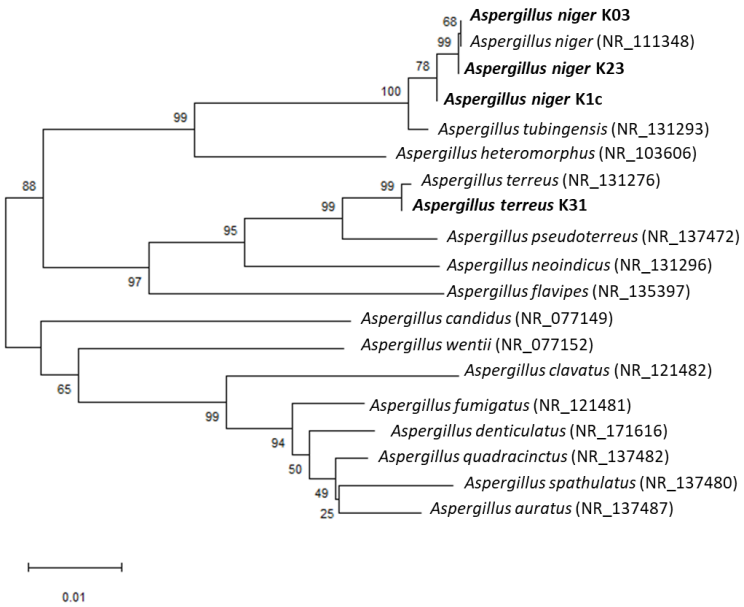

Fig 5. The maximum likelihood phylogenetic tree of the sequenced isolates and other reference strains retrieved from GenBank (*strains ending with K03, K23, K1c and K31 are isolates from the present study)

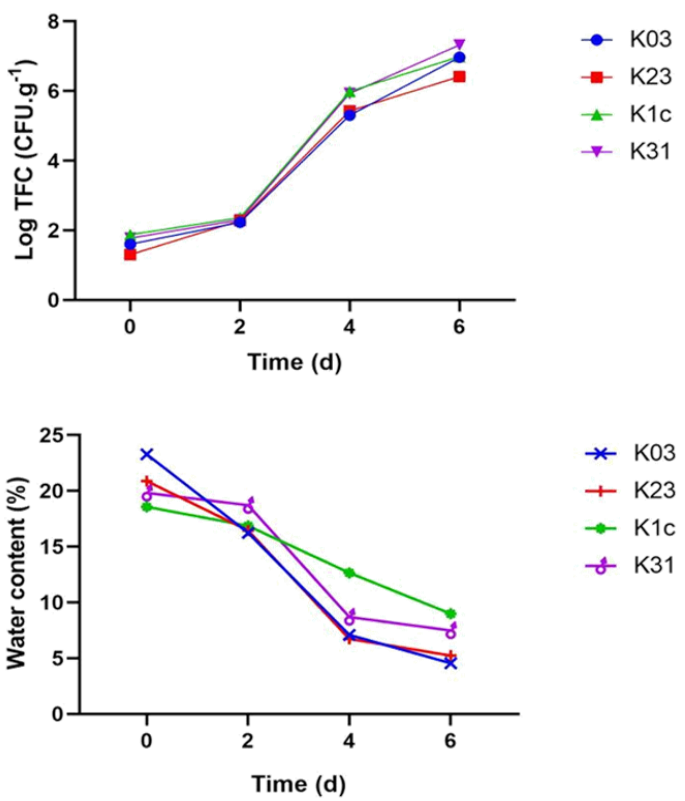

Fig 6. Characteristics of experimental kabuto based on the high correlation between log TFC and water content.

Protein Content of Experimental Kabuto

The analysis revealed that both the application of the four fungal isolates and the fermentation duration significantly affected the protein content of Kabuto compared to the untreated control (Fig. 7). A significant interaction was observed, with the K31 isolate producing the highest protein concentration on the sixth day of fermentation. These changes in protein content were correlated with fungal growth, indicating a positive contribution of the molds to the protein enrichment of the substrate. Fermentation with the fungal isolates led to a substantial increase in protein levels beginning on the fourth day. The K03 and K23 isolates increased protein content by 187 mg and 182 mg, respectively. In contrast, isolates K1c and K31 demonstrated a more pronounced effect, elevating protein levels by 226 mg and 208 mg over the same period. By the sixth day, all isolates had further increased the protein content, with the K31 treatment yielding the most substantial increase of 346 mg.

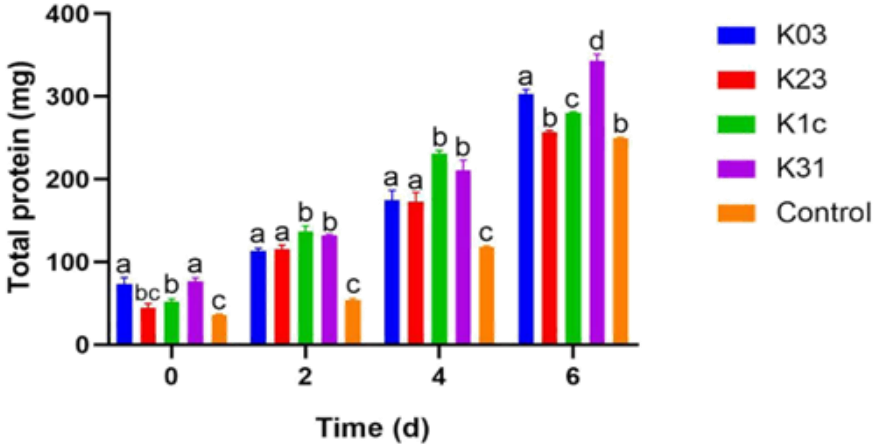

Fig 7. Profile of total protein in *kabuto* fermented by fungal isolates analyzed using ANOVA test. Bars that share the same letters are not significantly different (Tukey test, $P< 0.05$) within a similar time period.

Cyanide (HCN) Content in Experimental Kabuto

Cassava tubers undergo processing prior to consumption to reduce toxicity, particularly through the detoxification of cyanogenic compounds such as hydrogen cyanide (HCN). The HCN levels in Kabuto were measured to evaluate the efficacy of the fungal isolates in degrading these compounds during fermentation. Fig. 8 shows that the results of the *Kabuto* fermentation using isolate K03 experienced a decrease in HCN levels on the fourth day, namely 17.88 $g.g^{-1}$,and on the sixth day was only 0.97 $g.g^{-1}$. Additionally, the results of *Kabuto* fermentation using K23 isolate decreased HCN levels on the fourth day from 7.56 $g.g^{-1}$ to 0.48 $g.g^{-1}$ on the sixth day. *Kabuto* fermentation using K31 isolate also decreased HCN levels on the fourth day, namely 6.48 $g.g^{-1}$, and on the sixth day, HCN levels decreased to 0.97 $g.g^{-1}$. *Kabuto* fermentation using K1c isolate undertook a reduction of HCN levels on the fourth day(7.02 mg/kg) and on the sixth day (0.54 $g.g^{-1}$).

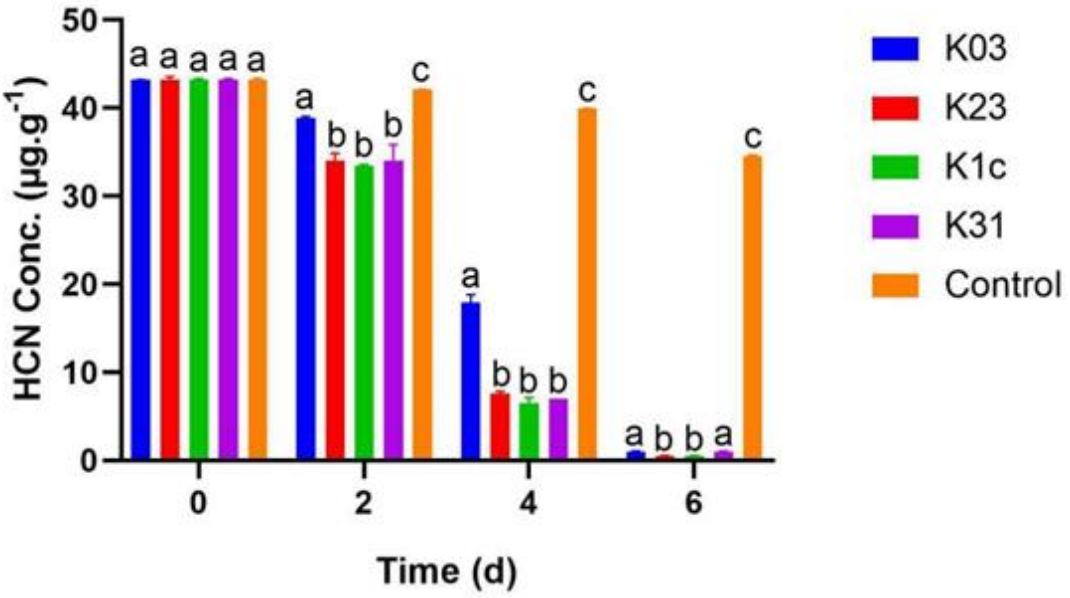

Fig 8. Profile of cyanide detoxification in *kabuto* fermented by fungal isolates analyzed using ANOVA test. Bars that share the same letters are not significantly different (Tukey test, $P< 0.05$) within a similar time period

Discussion

Research on *kabuto*, a traditional food from Southeast Sulawesi, remains limited. This study provides preliminary data on the application of *Aspergillus* spp. molds to reduce cyanide content in Kabuto. We also report complementary data on antimicrobial activity, hydrolytic enzyme production (amylase and cellulase), microbial growth, and protein content during fermentation. The investigation utilized both locally produced Kabuto and laboratory-prepared samples. Abiotic parameters were monitored as supporting factors for mold growth during a nine-day natural fermentation process conducted by local producers. The data indicate that fermentation altered the temperature, humidity, and moisture content of the *kabuto* substrate. At a 95% confidence level, ambient temperature showed a positive but statistically insignificant correlation with *kabuto* temperature. Conversely, the humidity of the *kabuto*, ambient humidity, and substrate moisture content were negatively, though not significantly, correlated with *kabuto* temperature. Overall, these four parameters did not exert a significant influence on temperature fluctuations within the substrate. However, *kabuto* temperature exhibited a strong, near-significant correlation ($r \approx 0.754$) with total fungal count (TFC), as detailed in Table 1.Recorded fermentation temperatures ranged from 25–30°C (data not shown), which falls within the optimal range for mold growth. A logarithmic growth phase was observed commencing on the fourth day (Fig. 2). Ambient temperature demonstrated a significant effect on Kabuto humidity, indicating that higher fermentation temperatures corresponded with lower substrate moisture levels (Table 1). A significant negative correlation was also observed between substrate moisture content and TFC, meaning that higher moisture levels were associated with suppressed mold growth.

Natural (Spontaneous) *kabuto* fermentation induced a transition in water content; the substrate moisture decreased progressively with extended fermentation time (Fig. 2). This phenomenon aligns with the fermentation of cassava flour, where moisture content is reduced. Fermentation exceeding 24 hours typically elevates temperature due to microbial metabolic activity [17]. The concomitant decrease in water content is attributed to hydrolysis, a chemical reaction that cleaves water molecules (H_2O) into hydrogen cations (H^+) and hydroxide anions (OH^-). This process is driven by starch-degrading enzymes secreted by microorganisms. The increasing enzymatic activity over time efficiently degrades the starch present in Kabuto. Starch degradation releases bound water, resulting in a softer, more porous texture and facilitating evaporation during subsequent drying. Mold isolates, distinguished by morphological differences, were screened for antimicrobial activity against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 35218, as well as for hydrolytic enzyme production (amylase, cellulase), to assess their potential for improving Kabuto quality. Of the 11 isolates tested, four (K03, K23, K1c, and K31) exhibited both antimicrobial properties and positive hydrolytic enzyme activity (Table 1). Generally, antimicrobial compounds function by disrupting cell wall synthesis, compromising membrane integrity, leading to increased permeability, inactivating essential enzymes, or damaging genetic material [18,19].

Isolates K03, K23, K1c, and K31 produced antimicrobial compounds capable of inhibiting the transpeptidase enzyme. This inhibition disrupts the cross-linking of peptidoglycan in the final stage of cell wall synthesis in both Gram-positive and Gram-negative bacteria, resulting in cell lysis [18]. Furthermore, these four isolates potentially produce antimicrobial peptides (AMPs). AMPs are oligopeptides comprising a variable number of amino acid chains (from five to over a hundred) [20] and have been historically classified under various mechanisms, including cationic host defense peptides [21], anionic antimicrobial peptides/proteins [22], cationic amphipathic peptides [23], and host defense peptides [25].Amylolytic testing of isolates K03, K1c, K23, and K31 revealed clear zones around the colonies, confirming their ability to produce extracellular amylase enzymes. These enzymes hydrolyze starch into simpler reducing sugars that cannot complex with iodine [18]. The same isolates also demonstrated cellulolytic activity, evidenced by clear zones on carboxymethyl cellulose (CMC) agar after Congo red staining and NaCl washing. These zones indicate that the isolates consumed the cellulose in the media for growth [27]. The starch and cellulose degradation capabilities of K03, K1c, K23, and K31 are inherent traits likely influenced by their original environment. Cassava roots, being carbohydrate-rich, select for microorganisms that produce amylase and cellulase to utilize these carbohydrates for growth. Macroscopic morphological analysis of isolates K03, K23, K1c, and K31 revealed both similarities and differences in characteristics such as surface and reverse colony color, growth zones, radial lines, growth rate, and sporulation.

All four isolates formed concentric growth zones. Isolates K03 and K23 (Fig. 4) initially displayed white colonies on both surfaces, with the surface darkening to black due to sporulation as they aged. Isolate K1c also had a white surface that turned black, while its reverse side transitioned from white to yellow. Isolate K31 was distinct, with both surface and reverse colors shifting from white to yellowish-brown and finally to dark brown with age. All four isolates reproduce asexually via conidia formation. Microscopic observation revealed that K03, K23, K1c, and K31 possess septate hyphae and rhizoids. Their non-branching conidiophores are characteristic of the genus *Aspergillus*. Key features of the class Ascomycetes, genus *Aspergillus*, include septate hyphae, asexual spore formation via conidia, and sexual spore formation in asci [28]. Molecular identification confirmed that isolates K03, K23, and K1c are similar to *Aspergillus niger*, while K31 is identical to *Aspergillus terreus*.Controlled fermentation with these four isolates showed optimal growth occurring after six days. The total growth was 9.3×10^6 CFU/g for K03, 2.6×10^6 CFU/g for K23, 9.7×10^6 CFU/g for K1c, and 2.1×10^7 CFU/g for K31. The fermentation duration directly influences mold density, with growth continuing as long as nutrients are available. This is consistent with findings that mold counts in attieke increased from 3×10^3 CFU/g pre-fermentation to 2.5×10^7 CFU/g by the third day [29]. Starch serves as an excellent carbon source for amylase production in *A. niger* [30], with glucose and fructose being optimal for supporting mold growth and enzyme activity [31].

The decrease in Kabuto moisture content during fermentation is linked to water consumption during the mold growth phase and the metabolic process of starch hydrolysis by fungal α-amylase [32]. Post-fermentation growth of the four isolates enhanced the nutritional profile of Kabuto. A key finding was the concurrent decrease in cyanide levels and increase in protein content (Fig. 7-8). All four isolates significantly reduced cyanide concentration after six days of fermentation. This reduction was strongly influenced by mold growth on the Kabuto substrate, a phenomenon supported by Tivana *et al.* [33], who reported that a four-day cassava root fermentation reduced cyanide levels (16.86 mg/kg). The decrease in HCN in all treatments was strongly correlated with fermentation time and the concomitant increase in mold cell count. This aligns with the findings of Zubaidah & Irawati [34], where HCN in MOCAF fermented with *Aspergillus niger* decreased from 33.60 ppm at 38 hours to 30 ppm at 48 hours. The enzymatic degradation of linamarin into less harmful compounds during fermentation reduces these plant toxins (cyanogenic glycosides). As HCN levels decreased during fermentation time, the ability of enzymes to degrade linamarin into compounds was not as harmful as it was. This means that fermentation causes degradation of plant toxins (e.g. cyanogenic glycosides), as well as decrease the risks of various illnesses and diseases including cardiovascular disease, arthritic disease, type 2 diabetes, periodontitis, respiratory problems, bladder disorders, bone problems, liver problems, and skin problems [35-43]. In contrast to the results of the analysis of protein content (Fig. 7), after six days of fermentation, there was an increase in all *Aspergillus* spp. isolate.

The longer the fermentation time, the more likely the *Kabuto* protein will increase. This is because the *Aspergillus* genus can increase the protein content in the *Kabuto* material. Due to the long fermentation time, *Aspergillus* has the opportunity to grow and develop, so it improves protein-rich cell mass. Kompiang *et al.* [44] reported an increase in protein content of sliced cassava roots from 3% to 18-42% through SSF with *Aspergillus niger*. The increase in the protein content was followed by the number of molds because the main contributor to protein content was the biomass of mold cells. It has implications for changes in the nutritional content of fermented products where the protein content is representative of microbial biomass amount [45]. According to Sharma *et al.* [46], on the fifth-day, fermentation could increase *A. niger* biomass after observing the OD (optical density) value on a UV spectrophotometer [47]. The dense fermentation of cassava pulp using *Aspergillus terreus* can increase protein starting on the second day by 4.5%, and on the fourth day by 7.6%. It was asserted by Kurniati *et al.* [48], the development of the population of the combination of *Aspergillus niger*, *Rhizopus oryzae* and the length of time during fermentation will boost the nutritional quality of organic substrates (especially crude protein) which are used as substrates for mold growth as long as fermentation, molds grow and develop on suitable organic substrates.

IV. CONCLUSION

Several isolated fungal strains from *kabuto*, specifically isolates K03, K23, K1c, and K31, demonstrated potential for antimicrobial production and exhibited enzymatic activity for amylase and cellulase. These isolates formed inhibition zones against both *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 35218. Phenotypic characterization indicated that all four fungal isolates belong to the genus *Aspergillus*. Isolates K03, K23, and K1c displayed macroscopic and microscopic characteristics consistent with *Aspergillus niger*, notably black colony surfaces and the presence of black spores. In contrast, isolate K31 shared morphological traits with *Aspergillus terreus*, featuring brown colony surfaces and reverses, along with dark brown spores. In subsequent fermentation trials, the application of isolate K31 proved most effective in enhancing the protein content of *kabuto*. Conversely, isolates K23 and K1c yielded the most significant reduction in HCN content. A fermentation period of six days was established as optimal for simultaneously maximizing protein enrichment and minimizing HCN concentration.

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