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Molecular Identification and Antifungal Activity of *Candida chrysomelidarum* and *Rhodotorula toruloides* Isolated from Indonesian Indigenous Fruit *Flacourtia inermis* Roxb. (Lobi-lobi) against Pathogenic Mold *Aspergillus flavus*

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Abstract

Aspergillus flavus is one of the main contaminating mold species in most feed products, significantly impacting human and animal health. This study aims to isolate the epiphytic yeasts from the fruit *Flacourtia inermis* Roxb and test their potential ability to act as biocontrol agents for *A. flavus*. The antagonistic test was performed using a dual culture method, and the potential yeast isolates were then identified using a combination of morphology characterization and molecular sequence analysis. From 42 yeast isolated from *F. inermis* fruit, 2 yeast isolates (LCA 75 and LCA 102) demonstrated the highest inhibition growth against *A. flavus* (32.32 and 32.30 %, respectively). The isolates LCA 75 and LCA 102 were identified as *Candida chrysomelidarum* and *Rhodotorula toruloides*, respectively. *Rhodotorula toruloides*, identified as one of the yeast isolates (LCA 102), is an unconventional red yeast demonstrating notable antagonistic effects against *A. flavus*, inhibiting its growth by 32.30 %. This yeast, also known as *Rhodosporidium toruloides*, *R. rubescens*, *R. glutinis*, or *R. gracilis*, belongs to the subphylum Pucciniomycotina within the division

Basidiomycota. The significant inhibition of mycelium growth and sporulation observed in our study highlights the potential of *R. toruloides*, along with *C. chrysomelidarum* (LCA 75), as effective biocontrol agents to reduce mycotoxin contamination in chicken feed by *A. flavus*. This study represents a significant advancement in the management of *A. flavus* contamination in animal feed. The findings not only underscore the viability of yeast-based biocontrol strategies but also contribute to a safer, more sustainable approach to preventing mycotoxin proliferation in feedstocks.

Keywords: Molecular identification, Antifungal activity, *Candida chrysomelidarum*, *Rhodotorula toruloides*, *Flacourtia inermis* fruit, *Aspergillus flavus*

Introduction

The contamination of animal feed by *A. flavus* poses a significant threat due to its ability to produce mycotoxins, particularly aflatoxins, which can cause severe health issues in both humans and animals. *Aspergillus* spp. are notorious for their ability to thrive on food and feed, leading to substantial losses in agriculture and endangering public health and the economy [1,2]. *Aspergillus flavus*, in particular, is a concerning species due to its wide temperature tolerance (12 - 48 °C) and its pathogenicity towards various crops including tomatoes, cereals, peanuts, corn, grains, pistachio trees and nuts [3-6]. This mold can inflict damage at 3 crucial stages of crop development: Pre-harvest, during harvesting and post-harvest [7]. Consumption of feed contaminated with *A. flavus* can result in adverse health effects due to the presence of aflatoxins, which are potent secondary metabolites [8,9]. Globally, aflatoxins are highly dangerous due to their mutagenic properties, including teratogenic and carcinogenic effects [10]. These toxins are primarily produced during fungal growth on various crops and agricultural commodities [11,12]. Efforts to control *A. flavus* contamination have included the exploration of biological agents sourced from nature [13]. However, despite these efforts, effective and sustainable biocontrol methods remain limited, highlighting the ongoing challenge of mitigating the risks associated with Aspergillus contamination in food and feed.

Recent studies have emphasised the potential of employing biological agents to combat fungal contaminants like *A. flavus.* However, many of these agents either originate from common, often ineffective sources or require synthetic enhancement for optimal efficacy. Notably, beneficial yeasts isolated from plants have emerged as promising biocontrol agents, demonstrating effectiveness against fungal pathogens [14]. These beneficial yeasts employ various mechanisms to combat fungal pathogens, including antagonism [15-17]. They can inhibit the mycelium growth and sporulation formation of *Aspergillus* spp. [18,19], compete nutritionally with fungal pathogens and produce a range of antifungal compounds, including volatile ones [20-22]. These volatile compounds have been found to degrade cell wall integrity, thus inhibiting the growth of plant pathogens such as *A. flavus* [23].

The competition for nutrition represents a particularly fascinating mechanism affecting the growth of plant fungal pathogens both *in vitro* and *in vivo* [24]. Yeasts have demonstrated the ability to control plant pathogens like *A. flavus* [3], proving effective as a biocontrol treatment during both pre-harvest and post-harvest stages [25]. Previous research has showcased the efficacy of yeast isolates in managing pre-harvest mango fruit rot caused by fungal pathogens such as *Neofusicoccum parvum* and *Lasiodiplodia theobromae*, with 4 isolates showing significant control [26]. Jaibangyang *et al.* [27] reported that various yeasts can mitigate the activity of *A. flavus* by inhibiting mycelium growth, reducing conidia germination and decreasing aflatoxin production. Furthermore, studies have demonstrated that yeasts can suppress *A. flavus* growth in dual culture assays and reduce conidia germination by producing antifungal compounds [28]. Building on this foundation, the current study seeks to isolate yeasts from the local fruits with the goal of identifying new biocontrol agents capable of combating the mycotoxin-producing *A. flavus.*

An intriguing prospect in this regard is *F. inermis* Roxb., a local Indonesian fruit known by various names such as Lobi-lobi (Java, Lampung), Tombi-tombi (South Halmahera), Tomi-tomi (Manado, Maluku), Lubi-lubi (Batak, Minangkabau and Sumatera), Batoko, Louvi and Thornless Rukam [29]. This fruit is classified as a Salicaceae group member, which grows in tropical regions at altitudes up to 1,300 m above sea level. *F. inermis* are native plants from the Maluku region with 3 - 15 m in height and have spread to the Malesia region and Papua New Guinea [29]. *F. inermis* plant has round fruit with a diameter of 1.3 - 2.5 cm with a green colour and becomes shiny red when ripe. Each fruit contains about 10 seeds nestled within 5 stipules. *F. inermis* fruit is noted for producing various chemical compounds [30], including polyphenols [31], as well as exhibiting antibacterial activity, notably through the presence of 2,3-Dihidrobenzoic acid compounds [32] and demonstrating antifungal properties [33]. Harnessing the bioactive compounds present in *F. inermis* holds promise for developing natural, effective strategies to combat fungal contaminants in food and feed, potentially offering a sustainable solution to the persistent challenge of Aspergillus contamination. Further research into the specific mechanisms of action and application methods of these compounds is warranted to fully exploit their potential in mitigating fungal risks.

This research, therefore, focuses on the epiphytic yeasts isolated from *F. inermis* Roxb. (Lobi-lobi), a less explored source that may offer novel biocontrol properties. *F. inermis*, an indigenous fruit to Indonesia, hosts a unique microbial community which has not been extensively studied for its antagonistic potential against pathogenic fungi. The urgency of this research lies in its exploration of these unique yeast isolates as a natural, potent alternative to chemical fungicides. Isolating and identifying high-antagonistic yeasts from *F. inermis* provides a critical understanding of their inherent properties and effectiveness, potentially leading to innovative strategies for managing *A. flavus* in feed products. Furthermore, this study addresses a significant gap by systematically evaluating the antagonistic activity of these yeasts, offering insights into their practical applications and benefits over existing biocontrol methods.

Material and methods

Preparation of mold pathogen

This study used 1 isolate of *A. flavus* P8 from the Microbiology Laboratory, Universitas Negeri Jakarta Culture Collection (UNJCC), Indonesia. The *A. flavus* isolate was further maintained on PDA (Potato Dextrose Agar) slants.

Isolation of epiphytic yeasts

Twelve ripe and healthy *F. inermis* Roxb. fruits were collected from 2 trees in Kridaloka Park, Senayan. The yeast was isolated using the direct washing method [34] with modifications aimed to suit the specific requirements of the study. A total of 5 g of pulp was collected and mashed aseptically. Samples were put into 50 mL of yeast malt broth (YMB) medium at pH 3.0 in duplicate, followed by vortex homogenization and incubation for 48 h at 28 °C. A total of 1 mL solution was transferred into 9 mL sterile distilled water was used to prepare 10^1 serial dilutions. One mL of the dilution 10^3 to 10^5 was added to the yeast malt agar (YMA) medium with pH 3.0 in duplicate by spread plate method. Samples were flattened using the Drigalski spatula and incubated for 48 h at 28 °C based on Chanchaichaovivat *et al.* [35]. The growing yeast colony was transferred into a YMA medium (pH 3.0) using sterile toothpicks to make a colony library. The representative yeast from the colony library was purified on a YMA medium (pH 3.0) using a quadrant streak based on Cappuccino and Sherman [36]. The single colony obtained was then made into a stock and working culture.

Screening of yeast isolates against *A. flavus*

The screening of antagonistic assay was carried out using the quadrant streak method to select the best isolates with inhibition activity against *A. flavus* [37]. A total of 42 yeast isolates were collected and tested against *A. flavus* in this experiment. To prepare *A. flavus* for testing, the fungus was cultivated on coconut agar (CA) medium for 4 days. Subsequently, a spore suspension was meticulously prepared and then appropriately diluted with 0.1 mL of Tween 20 for further experimentation.

An amount of 0.2 µL of 5-day-old *A. flavus* cell suspension with 10^7 CFU/mL cell density was used here. The spore suspension of all yeast isolates in 10 µL with a cell density of 10^6 CFU/ml was used here. The spore suspension of all yeast isolates (48 h old) was streaked. The spore suspension of *A. flavus* was inoculated into the middle part of the CA medium on the other side at 1 cm from the yeasts. Observations were carried out for 5 days at 28 °C. The observation on the plates was based on a visual assay and grouped the score into 2 levels, as follows [38]:

1) "+" is the estimated as positive by showing clear zone of *A. flavus*.

2) "-" is no showing clear zone for *A. flavus*.

Antagonistic assay against *A. flavus*

The dual culture method determined the antagonistic assay for 16 isolates and was carried out on 90 mm Petri dishes with potato dextrose agar (PDA) medium. A spore suspension of *A. flavus* was inoculated at the centre of PDA with a distance of 1.5 cm of the spore suspension of yeasts. Observations were performed for 5 days at 28 °C. The observation includes 1) The diameter of treatment and control fungi, 2) The growth spacing of yeast treatment isolates, 3) The growth of treatment and control mycelium fungi and 4) The amount of sporulation of treatment and control. The percentage of growth inhibition was calculated as follows:

$$Inhibition\ Percentage\ (IP) = \frac{A_p - A_{t+p}}{A_p} \times 100\ \% \quad (1)$$

where IP is an inhibition percentage, A_p is the growth rate of pathogenic *A. flavus* in control (cm) and A_{t+p} is the growth rate of pathogenic *A. flavus* in treatment with biocontrol agent yeasts (cm).

Morphological characterization of antagonistic yeasts

Two yeast isolates were selected depending on their high inhibition activity against *A. flavus*. The morphology of these 2 isolates was identified based on macroscopic and microscopic characteristics. Colony morphology was observed, such as texture, colour, surface, profile and the colony's edge [39]. Microscopic observation of the colonies was done using a phase contrast microscope (Olympus) at ×400 magnification to determine their budding type and cell shape.

Molecular identification of antagonistic yeasts

Molecular identification to confirm the species of the 2 antagonistic yeasts was performed based on the D1/D2 region of 26S rDNA. A forward primer of NL1 (5-'GCATATCAATAAGCGGAGAAAG-3') and a reverse primer of NL4 (5'-GGTCCGTGTTTCAAGACGG-3') were used for DNA multiplication. The preparation for molecular identification was conducted as follows: The colony of each yeast isolate was inoculated into YMA medium and incubated overnight at 37 °C. Yeast was obtained by harvesting a culture that had been incubated on an oblique YMA (Yeast Malt Agar) medium for 48 h at 28 °C. The cultivated yeast was gathered by transferring approximately 3 - 4 loops of yeast culture into a 1.5 mL

Eppendorf tube containing 250 µL of ultrapure distilled water. This solution was then homogenized for 1 min using a vortex and was used for DNA isolation using a boiling method based on [40].

The tube was put into a beaker glass filled with water and incubated for 25 min at 100 °C. The tube was lifted and centrifuged for 15 min at 13,000 rpm. The supernatant was collected and used for PCR reaction as a DNA template. Three µL of DNA template added with nuclease-free water (8.5 µL), KAPA2G Robust HotStart Readymix (12.5 µL), NL1 forward primer (0.5 µL), NL4 reverse primer (0.5 µL) for the PCR reaction. PCR conditions set for 35 cycles as follows; 2 min pre-denaturation at 95 °C, 30 s denaturation at 95 °C, 30 s annealing at 60 °C, 1-minute elongation at 72 °C, 10 min final elongation at 72 °C and 10 min cooling at 4 °C. The results of PCR products were visualized using electrophoresis with 1.7 % agarose gel and TEA 1X buffer (Tris Acetate EDTA). A total of 3 µL PCR products and 1 Kb DNA marker were put into agarose gel wells, 0.5 µL loading dye was added and then electrophoresis (Mupid EXU) with 110-volt voltage for 25 min was performed. Electrophoresis results were stained using ETBR 1 % (ethidium bromide) for 30 min.

The DNA fragments produced were visualized under ultraviolet light using a UV transilluminator. DNA fragment band samples were compared with DNA ladder band fragments for analysis. PCR products were purified and sent to Macrogen DNA sequencing services to obtain the sequence reads. Sequence data result was then edited using the ChromasPro version 1.7.7 application program and analyzed using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov) to get the closest homologous species with the yeast isolates. The phylogenetic tree was constructed using the MEGA X application program with 1,000 times bootstrap with a neighbour-joining method [41,42].

Statistical analysis

The treatments *in vitro* are organized in a completely randomized design. The data *in vitro* IP variables were subjected to ANOVA, and a comparison of the means by LSD at $p < 0.05$ was conducted. All analyses were performed in SPSS for Windows version SPSS 16.

Results and discussion

Yeast isolates obtained from *F. inermis* Roxb. fruits

A total of 42 yeast isolates with different morphological characteristics were successfully obtained as shown in **Table 1**. The yeast isolates were dominated with cream colour colonies (14), followed by white clear (8). It is noteworthy that yeasts have the capacity to produce various pigments, including carotenoids. These pigments are synthesised through a biochemical pathway that initiates with acetyl-CoA and progresses through several enzymatic steps, leading to the formation of compounds such as lycopene, β-carotene and astaxanthin [43]. These pigments contribute to the vibrant colours of the organisms and play a crucial role in protecting them from the damaging effects of UV radiation by absorbing and dissipating the energy as heat [44]. This information sheds light on the diverse metabolic capabilities of yeasts and underscores the potential significance of these microorganisms, not only in terms of their biocontrol properties against fungal pathogens like *A. flavus* but also in their capacity to produce valuable compounds with applications in various fields, including biocontrol and health.

Table 1 Morphological characteristic of yeast isolates from *F. inermis* fruit incubated on YMA medium at 28 °C for 48 h.

Tree	Number of isolates	Cream	White clear	Orange	Pink
1	23	14	8	0	1
2	19	9	8	2	0
Total	42	23	16	2	1

The isolation results revealed that 55 % of the yeast isolates from fruit exhibited a creamy white pigment. It is worth noting that white yeast isolates typically indicate a lack of pigment production. Yeasts thrive on fruit as it serves as a rich source of nutrients essential for their growth and proliferation. Moreover, several yeasts isolated from fruits exhibit antagonistic properties against other fungi. For instance, Sukmawati *et al.* [34] highlighted the antagonistic abilities of yeasts isolated from the fresh parts of *Broussonetia papyrifera.*

Interestingly, yeasts isolated from UV-exposed environments, such as the phylloplane, are often dominated by pigmented species [45,46]. While white to creamy yeast isolates (lacking pigmentation) are commonly found in ripe fruit [47,48], pigmented yeasts, such as those exhibiting orange or pink hues, typically contain pigments in the form of carotenoids [49,50]. For instance, *Rhodotorula mucilaginosa* produces carotenoids as a protective measure against UV-B radiation [51]. Additionally, black-pigmented yeasts indicate the presence of melanin pigments [49,52].

Yeasts are relatively scarce during the fruit development phase and in immature fruit. However, their numbers tend to increase significantly as the fruit ripens [53]. This condition is likely due to the higher carbohydrate content present in ripe fruit compared to unripe fruit, providing a more favourable environment for yeast growth. Carbohydrates serve as a crucial nutrient substrate for yeast growth. Swami *et al.* [54] found that ripe jackfruit contains more carbohydrates than unripe jackfruit. Various types of yeasts have been identified in ripe fruit, including *Saccharomyces cerevisiae*, R. *mucilaginosa* and *Pichia* sp. [47,53]. These findings underscore the complex interplay between yeast ecology and fruit maturation, highlighting the importance of understanding the dynamics of yeast populations in fruit environments.

Screening of yeast isolates against *A. flavus*

Out of the 42 yeast isolates tested, 16 showed antagonistic activity against *A. flavus* (**Table 2**). The antagonistic activity was characterized by the formation of clear inhibitory zones around the yeast colonies, indicating the suppression of *A. flavus* growth. Additionally, the growth spacing between yeasts and fungi was observed, suggesting competition for space and nutrients. Stunted mycelium growth and inhibited sporulation further confirmed the antagonistic effects of the yeast isolates (**Figure 1**).

Table 2 Inhibition percentage of *A. flavus* by yeast isolates incubated at CA medium at 28 °C for 5 days.

Yeast isolates	Inhibition distance (mm)	Inhibition code
LCB 79	4.91^{a}	++
LCC 80	4.60^{ab}	+
LCA102	4.53^{ab}	++
LCA 53	3.79^{abc}	+
LBA 90	3.64^{abcd}	+
LCC 84	3.61^{abcd}	+

Yeast isolates	Inhibition distance (mm)	Inhibition code
LBB 97	3.59^{abcd}	+
LBA 89	3.30^{bcde}	+
LCB 109	3.04^{cde}	+
LCC 127	2.96^{cde}	+
LBB 45	2.73^{cde}	+
LCA 73	2.38^{de}	+
LCA 56	1.95^{ef}	+
LBC 98	0.88^{f}	+
LBB 28	0.74^{f}	+
LCA 75	0.72^{f}	+

Note: Numbers followed by the same letter are a significant level ($p < 0.05$). "+" = Low clear zone; "++" = high clear zone.

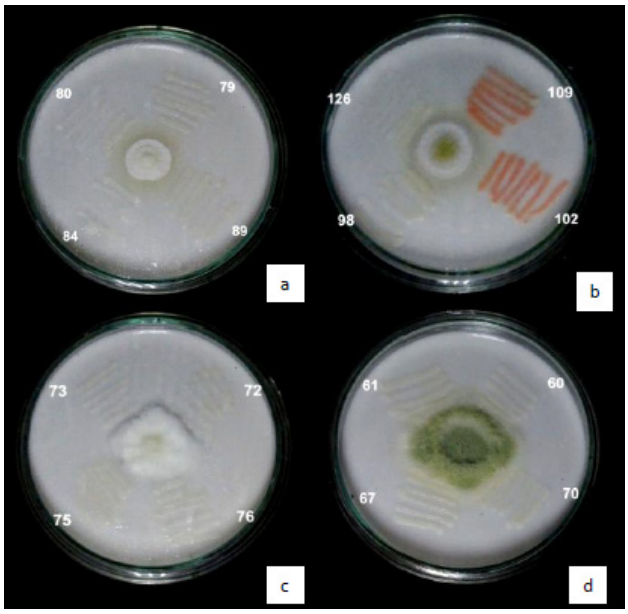

Figure 1 Screening result of antagonistic test against *A. flavus* in coconut medium for 5 days incubation at temperature of 28 °C: (a) inhibiting the growth of spores and mycelium, (b) inhibiting mycelium growth and spore growth, (c) inhibiting spore growth and (d) not antagonistic.

Isolates LCA 75 and LCA 102 stood out for their particularly high inhibitory zones against *A. flavus*, indicating strong antagonistic potential. These isolates may possess unique mechanisms for inhibiting *A. flavus* growth, which warrant further investigation. The antagonistic activity of yeast isolates against *A. flavus* holds promise for the development of biocontrol agents to manage aflatoxin contamination in agricultural products. Understanding the mechanisms underlying the antagonistic effects of yeasts can help in the selection of effective biocontrol agents for commercial applications.

Antagonistic assay against *A. flavus*

The antagonistic assay involving 16 yeast isolates had a significant impact on inhibiting the growth of *A. flavus* ($p < 0.05$). Several yeast isolates demonstrated strong inhibitory effects on *A. flavus* growth.

These yeast isolates effectively restricted the expansion of the *A. flavus* colony, exhibiting inhibition ranging from 0.72 to 4.91 mm, in comparison to the negative control (*A. flavus* only). Notably, 3 isolates, namely LCA 75 (4.91 mm), LCA 80 (4.60 mm) and LCA 102 (4.53 mm), exhibited particularly a higher antagonistic activity, while 3 other isolates, LBC 98 (0.88 mm), LBB 28 (0.74 mm) and LCA 75 (0.72 mm), demonstrated relatively lower levels of inhibition (**Figure 2**).

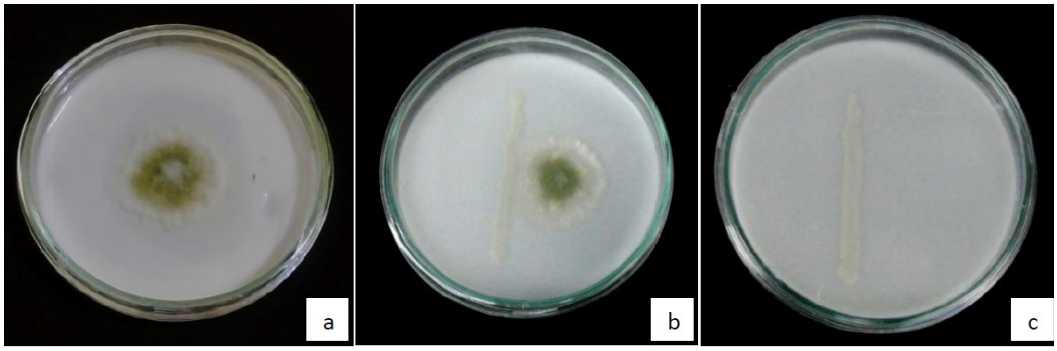

Figure 2 Antagonistic test result against *A. flavus* (a) positive control, (b) isolate LCA 75 and (c) negative control.

The presence of clear zones between *A. flavus* and yeast isolates in the screening test of antagonistic activity suggests the secretion of antifungal compounds against *A. flavus* [55]. These yeast isolates possess the capability to produce various wall-degrading enzymes, including protease, exo-beta-1,3-glucanase and chitinase, as mentioned by Bar-Shimon *et al.* [56]. Additionally, volatile compounds, siderophores and killer toxins secreted by these yeast isolates contribute to inhibiting pathogenic growth [57-59]. Yeast isolated from tropical fruits have various molecular mechanisms in inhibiting the growth of fungal pathogens. For instance, *Pichia membranifaciens* strain FY-101 has been found to secrete the enzyme β-1,3 glucanase as an antagonistic mechanism in inhibiting the growth of *Botrytis cinerea* [60]. The β-1,3 glucanase enzyme secreted by such yeast can cause a degradation of the fungal cell walls into simpler compounds, which yeast can then utilize as a source of nutrients. Additionally, *P. membranifaciens* produces killer toxins that can inhibit the growth of *B. cinerea* [61]. Another study conducted by Marquina *et al.* [62] also mentioned several types of yeast, such as *Saccharomyces cerevisiae*, produced some killer toxins K1, K2 and KT28. A killer toxin was a mechanism to change formation in ion channels, increase cell membrane permeability, endocytosis and inhibit the cell cycle.

The presence of an inhibition zone and reduced mycelium growth of *A. flavus* during a confrontation with yeast isolates are possible incidences of competition for nutrients and space. The competition mechanism of the nutrient in yeast caused an inhibition growth of fungal mycelium [63] and the germination of spores [64,65]. Li *et al.* [66] found that the mechanism of competition on nutrition and space could inhibit the mycelium growth of *Rhodotorula glutinis* and the germination of *B. cinerea* spores. *In vitro* research conducted by Mekbib *et al.* [67] proved that healthy competition in the medium could inhibit the germination of *Penicillium digitatum* spores for 24 h by *Cryptococcus laurentii* and *Candida sake*. A mode of action as competition on the nutrient that possibly occurs between yeast isolates and *A. flavus* is the competition for carbohydrate intake. These carbohydrates are essential macronutrients needed for mould growth.

Some studies used a CA medium for growth and detecting aflatoxin content in *A. flavus* [68,69] because coconut is rich in carbohydrates, proteins, fats and moisture [70]. Additionally, research by Moor and Landecker [71] in Hafsari and Salamah [72] suggests that the growth of mycelium and asexual

reproduction of fungi are influenced by a decrease in the availability of carbohydrates (monosaccharides, disaccharides and polysaccharides), nitrogen, vitamins and minerals in the medium. Furthermore, specific sugars such as D-glucose, D-xylose and D-mannose have been shown to trigger the germination of conidia in *A. niger* [73], while D-galactose in the medium could induce the germination of *A. nidulans* [74].

Research on the antagonistic activity of the yeast of *C. chrysomelidarum* and *R. toluroides* is still limited, but species such as *Candida* sp. and *Rhodotorula* sp. are reported to have antagonistic abilities against fungi. Gholamnejad *et al.* [75] demonstrated that 2 species, *C. membranifaciens* and *R. mucilaginosa*, have antagonistic abilities against *Penicillium expansum*. Afsah-Hejri [3] also reported that *R. fragaria* and *R. hinula* could inhibit the growth of *A. flavus*, with *R. fragaria* exhibiting the ability to inhibit the spore production by 90.2 spores/mL, decrease *A. flavus* biomass by 18.14 mg and inhibit the production of aflatoxin B1 of 1.18 mg/mL. Meanwhile, *R. hinula* could inhibit the production of 89.7 spores/mL, 27.61 mg in reducing biomass and 1.17 mg/mL in inhibiting the production of aflatoxin B1 [3].

Moreover, beneficial fungi exhibit another mode of action by producing antifungals. The inhibition zones detected in our study indicate the effective role of 2 yeast isolates in restricting the growth of *A. flavus* by appearing in empty areas around the plant pathogen. Beneficial fungi utilize several mechanisms, including the production of antifungals. For example, *Trichoderma* spp. has shown inhibition zones against *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (FocTR4) by producing secondary metabolites with potential antifungal activity [76]. The antifungal activity observed by Mohammed *et al.* [77] affected the sporulation and mycelium growth of FocTR4. Additionally, the linear mycelium growth of *A. niger* was decreased by antifungals [78]. Parafati *et al.* [79] also mentioned the ability of yeast isolates to act as biocontrol agents against *A. flavus*. Therefore, it is plausible that the 2 isolates of LCA75 and LCA 102 are likely to produce antifungal compounds that affect the growth of *A. flavus* and reduce conidia production.

Identification of antagonistic yeasts

The 2 yeast isolates exhibiting the highest inhibitory activity, namely LCA 75 and LCA 102, were chosen for identification, employing a combination of morphological characterization and molecular analysis. In terms of macroscopic characteristics, isolate LCA 75 displayed creamy white colonies with smooth surfaces, a thick butter-like texture, mountainous colony profiles and flat colony edges. Microscopic examination revealed that isolate LCA 75 exhibited cells with dimensions ranging from 2 to 4 µm in width and 2.25 to 4 µm in length, featuring both round and oval shapes, with no observed budding. On the other hand, macroscopic analysis of isolate LCA 102 revealed colonies with an orange hue, characterized by a smooth and shiny surface, a thick texture and a mountainous profile with flat colony edges. Microscopic analysis revealed that isolate LCA 102 exhibited both round and oval cells, with dimensions ranging from 1.5 to 4.75 µm in length and 1.5 to 3.25 µm in width. and no budding structures were detected (**Figure 3**).

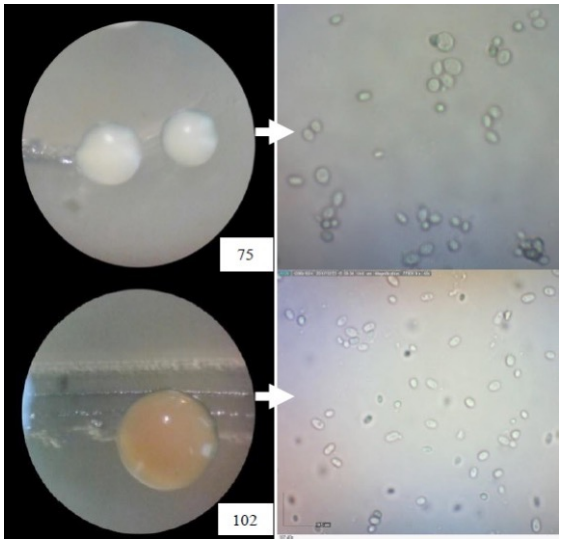

Figure 3 Macroscopic and microscopic morphology of isolate LCA 75 and LCA 102 on YMA medium incubated for 48 h at 28 °C.

The molecular identification based on the D1/D2 region using NL1 and NL4 primers on isolates LCA 75 and LCA 102 yielded nucleotide base sequences of 568 and 585 bp, respectively. Although the DNA bands of each isolate LCA 75 and LCA 102 were less than 600 bp, the amplified regions were sufficient for analysis, as suggested by [40,80]. The sequences were then compared with the NCBI gene bank database using the BLAST program to determine species closely related to the 2 yeast isolates. BLAST results showed that isolate LCA 75 has the closest homology to *C. chrysomelidarum* and *Metschnikowia reukaufii*, with a similarity identity of 99 and 90 %, respectively. BLAST results for isolate LCA 102 showed sequence similarity of 90 % with *Rhodotorula toruloides* and 90 % with *Rhodosporidium toruloides* (**Table 3**).

Table 3 BLAST result for yeast isolates LCA 75 and LCA 102 based on D1/D2 regional sequencing analysis.

Isolate codes	BLAST results	Max score	Query cover	*E*-value	Accession	Identity	Gaps
LCA 75	*C. chrysomelidarum*	910	92 %	0.0	NG055373	99 %	3/525 (0 %)
	M. reukaufii	733	98 %	0.0	FJ455114	90 %	11/557 (1 %)
LCA 102	*R. toruloides*	623	84 %	2e-174	KY109171	90 %	13/499 (2 %)
	Rh. toruloides	623	84 %	2e-174	AF070426	90 %	13/499 (2 %)

The results of sequence alignment using MEGA 6 software indicated the presence of gaps. Gaps or the insertion of deletions (indels) play an essential role in making phylogenetic trees and the evolution of a genome [81-83]. The gaps in the BLAST result (**Table 3**) show the differences in the nucleotide sequences between the isolates and the reference strains, while the comparison underscores the homology between the nucleotide bases. The isolate LCA 75 has gaps with a value of 0 % in the nucleotide sequence of *C. chrysomelidarum*, while *M. reukaufii* has gaps of 1 %. In addition, LCA 102 isolate has 2 % gaps with *R. toruloides* and *Rh. toruloides*. The absence of gaps in the BLAST result for isolate LCA 75 indicates a complete alignment with the nucleotide sequence of *C. chrysomelidarum*, suggesting a high degree of similarity between them.

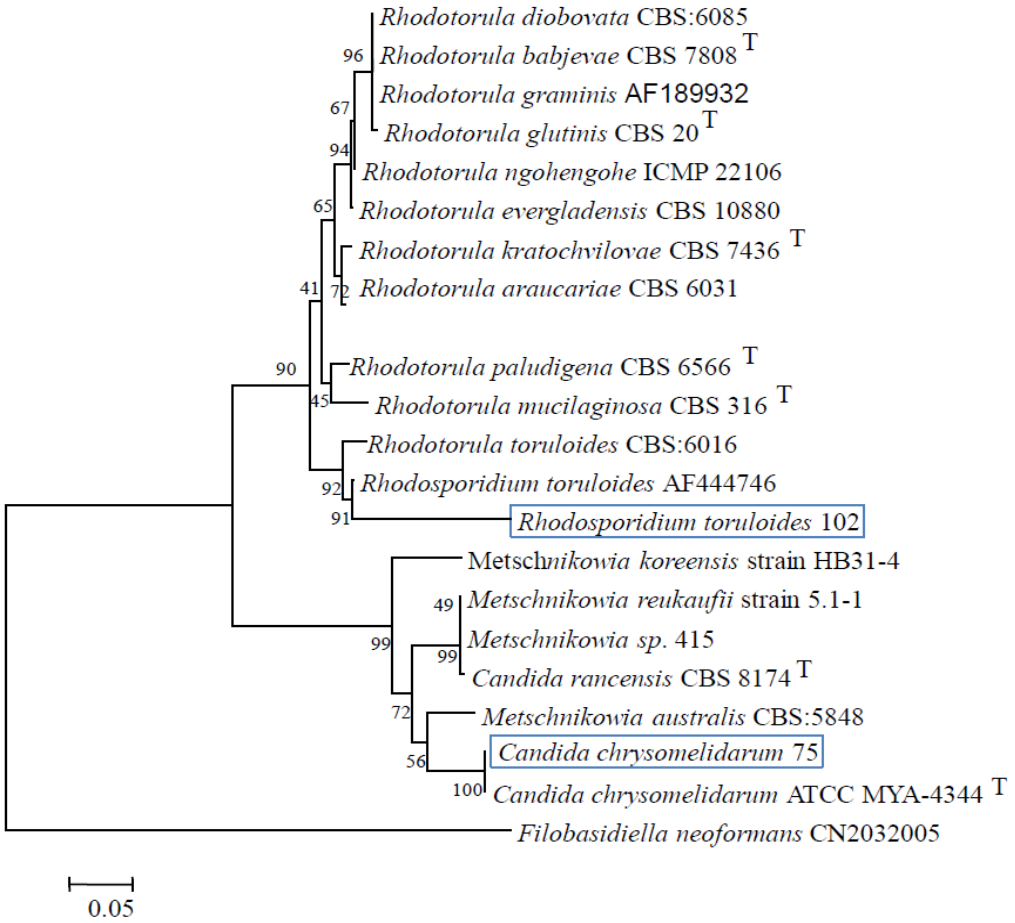

Figure 4 The phylogenetic tree of potential antagonist yeast isolates from *F. inermis* Roxb. was reconstructed by a maximum likelihood algorithm based on the distance calculated by Kimura's 2-parameter model from sequences of D1/D2 regions of rDNA. Bootstrap values greater than 50 % from 1,000 replicate bootstrap resamplings. *Filobasidiella neoformans* was used as an outgroup.

Conversely, the presence of a 1 % gap in the alignment with *M. reukaufii* suggests a slight divergence or variation in the nucleotide sequence between the isolate and this reference strain. In essence, the absence of gaps implies a close resemblance and homology between the nucleotide sequences of isolate LCA 75 and *C. chrysomelidarum*, while the minimal gap observed with *M. reukaufii* suggests a slightly lower level of similarity, likely due to minor differences or mutations in the nucleotide sequence. This analysis is further supported by the results of the phylogenetic tree reconstruction based on the D1/D2 rDNA region sequence (**Figure 4**), which places isolate LCA 75 in a monophyletic clade with *C. chrysomelidarum*, with a bootstrap value of 100 %. This indicates that the characteristics of isolate LCA 75 closely resemble those of *C. chrysomelidarum*. The isolate LCA 102, on the other hand, is located in a monophyletic clade with *Rh. toruloides* with a bootstrap value of 91 %. Although there are differences between the BLAST results and the resulting branch length, it might be concluded that isolate LCA 102 has the same characteristics as *Rh. toruloides.* Based on Dharmayanti [84], differences in the length of these branches indicate the presence of polymorphisms in nucleotide bases. These differences may also occur due to the inaccurate cutting process in the MEGA X program. However, a bootstrap value of 70 - 100 % can be a basis for determining a high confidence level in phylogenic tree construction [85]. Lastly, the use of *Filobasidiaella neoformans* as an

outgroup in phylogenetic tree reconstruction is based on the research of Staton [86]. Outgroups are important in the phylogenic tree reconstruction process [87], it defines as a taxa that have a close kinship with the group of species to be studied but are not part of the group.

Antifungal activity of *C. chrysomelidarum* and *R. toruloides* against *A. flavus*

The antifungal activity of microbial species against pathogenic fungi is of significant interest in various fields, including agriculture, medicine and food safety. In particular, the efficacy of certain yeasts, such as *C. chrysomelidarum* and *Rhodotorula toruloides*, in inhibiting the growth of fungal pathogens like *A. flavus* has garnered attention due to its potential applications in biocontrol strategies and the management of fungal contamination. Understanding the antagonistic effects of these yeasts on *A. flavus* can provide valuable insights into their potential as biocontrol agents and contribute to the development of sustainable methods for controlling fungal contamination in agricultural settings and food production facilities.

This study found that *C. chrysomelidarum* and *Rhodotorula toruloides* isolated from the Indonesian indigenous fruit *F. inermis* Roxb. demonstrated antifungal activities against *A. flavus.* Nguyen *et al.* [88] reported that the phylogenetic analysis of the D1/D2 loop sequences placed *C. chrysomelidarum* as a sister taxon of *Candida rancensis*. A recent study conducted by Dhami *et al.* [89] found that *Candida rancensis* is the 2nd most dominant yeast species after *M. reukaufii* found in *M. aurantiacus* nectar. Both *M. reukaufii* and *C. chrysomelidarum* have white colonies, butyrous textured, vegetative reproduction (by germination) and pseudohyphae [90]. They can hydrolyse sucrose and generate a mixture of oligosaccharides [91], as well as produce volatile compounds that can attract pollinators [92]. Wang *et al.* [93] also reported that nectar yeasts like *C. chrysomelidarum* demonstrated antimicrobial activity and prebiotic activity by producing mannoproteins.

The demonstration of the antifungal ability of *R. toruloides* in this study showed the potential of this yeast species to be used as a biocontrol agent for *A. flavus*. the phylogenetic tree indicates that the isolate LCA 102 is located in 1 clade with *R. toruloides* with a bootstrap value of 95 %. Wang *et al.* [94] reported that *Rh. toruloides* is the old name of *R. toruloides.* Barnett *et al.* [90] reported that *Rh. toluroides* have pink to red colonies, have mucoid to butyrous textures and do not represent hypnotic hyphae. *R. toluroides* is closely related to *R. mucilaginosa* [95]. In this study, *R. mucilaginosa* is used as comparative data with the closest kinship with isolate LCA 102 to determine the characteristics of the yeast morphology. *Rhodotorula mucilaginosa* exhibits a round to oval cell shape, with dimensions ranging from 2 to 8 µm in width and 2 to 12 µm in length, having a single cell arrangement in pairs or short chains on 5 % malt extract medium, 3 days incubation at 25 °C [96]. Recent studies found that *Rhodosporidium toruloides* is a potential red yeast for producing lipids [97,98] and it is an ideal microbial cell factory to produce oleochemicals, carotenoids and other products [99].

Conclusions

This study found that 42 epiphytic yeasts have been successfully isolated from *F. inermis* Roxb. fruit originating from Indonesia. Among all isolates, 2 isolates of LCA 75 and LCA 102 exhibited the highest inhibition against *A. flavus*, with inhibition rates of 32.32 and 32.30 %, respectively. Molecular analysis identified isolate LCA 75 as *C. chrysomelidarum*, or known as Candida rancensis, and isolate LCA 102 as *Rhodotorula toruloides*. Notably, *Rhodotorula toruloides* (LCA 102) demonstrated significant antagonistic effects against *A. flavus*, inhibiting its growth by 32.30 %. This unconventional red yeast, belonging to the subphylum Pucciniomycotina within Basidiomycota, is commonly found on various plant surfaces, suggesting its adaptability and potential utility in biocontrol applications. *C. chrysomelidarum* is classified as nectar yeast as it is present in nectar flowers in certain plants, such as *M. aurantiacus*. *C.*

chrysomelidarum has white colonies, butyrous textured, vegetative reproduction (by germination) and pseudohyphae. The substantial inhibition of *A. flavus* mycelium growth and sporulation by these yeasts underscores their efficacy as biocontrol agents for reducing mycotoxin contamination in chicken feed. This study represents a significant advancement in *A. flavus* contamination management in animal feed, highlighting the viability of yeast-based biocontrol strategies.

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