



Isolation and Characterization of Endophytic Fungi Producing L-Asparaginase Enzyme from Kencur Plant (*Kaempferia galanga* L.)

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ABSTRACT

L-Asparaginase is an enzyme that catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia. This enzyme has important benefits in the medical field, particularly as a biological agent in the therapy of acute lymphoblastic leukemia, due to its ability to inhibit the growth of asparagine-dependent cancer cells. This study aims to isolate and characterize endophytic fungi derived from the rhizome of the aromatic ginger (*Kaempferia galanga* L.) plant, and to test the ability of these isolates to produce the L-asparaginase enzyme. The research method used is an experimental method conducted in a pharmaceutical microbiology laboratory. Endophytic fungi were isolated using surface sterilization techniques and culturing on PDA media. Enzyme production was performed using the submerged fermentation method, while enzyme purification used the ammonium sulfate fractionation method with concentration ranges of 0-20%, 20-40%, 40-60%, 60-80%, and 80-100%. The L-asparaginase enzyme activity test was conducted using the Nesslerization method, with *Asparagus* as an inducer. The results showed that several endophytic fungal isolates were capable of producing L-asparaginase, as evidenced by a brownish color in the Nessler test. The purified fractions showed varying enzyme activity at each level of ammonium sulfate saturation. Thus, endophytic fungi from the aromatic ginger rhizome have potential as an alternative source for producing the L-asparaginase enzyme, which can be further developed in the fields of pharmacy and biotechnology.

INTRODUCTION

L-asparaginase is an enzyme that converts L-asparagine into L-aspartate and ammonia. Leukemia cancer cells are known to utilize L-asparagine as one of their nutritional sources. L-asparaginase has potential as a biological agent in cancer therapy because this enzyme specifically inhibits the availability of nutrients required by cancer cells (Alrumman et al., 2019). According to Jia et al. (2021), the administration of L-asparaginase is crucial in disrupting cancer cell nutrition and can inhibit their growth.

L-asparaginase is widely distributed in both eukaryotic and prokaryotic organisms. According to Jia et al. (2021), this enzyme can be produced by various microorganisms such as *Escherichia coli*, *Erwinia chrysanthemi*, *Aspergillus oryzae*, *Aspergillus niger*, *Penicillium crustosum*, and *Candida*. However, the enzyme activity produced may vary depending on the type of microorganism.

Microorganisms that have the potential to serve as sources of L-asparaginase include endophytic fungi. Endophytic fungi are microorganisms that grow within plant tissues without causing negative effects and often form mutualistic symbiosis with their host plants. These fungi can be isolated from roots, seeds, stems, leaves, flowers, and fruits, and are capable of producing bioactive compounds that can be developed as pharmaceutical raw materials. Endophytic microorganisms enter plant tissues through lenticels, stomata, or wounds in lateral roots and germinating roots (Purwaningsih, 2015). Based on research by Dion et al. (2021), rhizomes harbor endophytic fungi belonging to the genera *Trichoderma*, *Aspergillus*, and *Fusarium*.

Rhizome plants, such as kencur (*Kaempferia galanga* L.), possess various medicinal properties due to their active compounds, including flavonoids, phenolics, terpenoids, polyphenols (curcumin), and essential oils. These compounds exhibit anti-inflammatory, antimicrobial, antidiarrheal, antiviral, and anticancer activities. These bioactive compounds can also influence the composition and activity of endophytic microorganisms living within

the plant. Endophytic fungi from rhizome plants have the potential to produce new isolates capable of producing L-asparaginase (Krishnapura et al., 2016). According to Jia et al. (2021), rhizome plants such as white turmeric and ginger have potential as sources of L-asparaginase enzymes, both from plant tissues and associated endophytic microorganisms.

This study aims to isolate and characterize endophytic fungi from kencur (*Kaempferia galanga* L.) that have the potential to produce L-asparaginase enzyme, which may serve as an alternative source for biotechnological enzyme production.

RESEARCH METHODS

This study employed an experimental laboratory method to isolate and characterize endophytic fungi producing L-asparaginase enzyme from kencur (*Kaempferia galanga* L.).

Sterilization of Equipment

All equipment used in this study was sterilized prior to use. Glassware such as Petri dishes and test tubes were sterilized in an oven at 170°C for 1 hour. Inoculating loops and forceps were sterilized by direct flaming over a Bunsen burner. Media sterilization was carried out using moist heat in an autoclave at 121°C under 1 atm pressure for 15 minutes (Pakaya et al., 2024).

Media Preparation

Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) media were prepared according to standard procedures. PDA medium was prepared by weighing PDA powder and chloramphenicol, while PDB medium was prepared according to preparation instructions and dissolved in distilled water. The media were mixed until homogeneous, covered with cotton and aluminum foil at the mouth of the Erlenmeyer flask, and sterilized in an autoclave at 121°C for 15 minutes under 1 atm pressure (Prihanto et al., 2017).

Isolation of Kencur Plant (*Kaempferia galanga* L.)

Fresh rhizomes of kencur were used as samples. The samples were washed under running water, cut into 1–2 cm pieces, and separated according to plant parts. The samples were surface sterilized by immersion in 70% alcohol for 1 minute, 1% sodium hypochlorite for 5 minutes, followed by 70% alcohol for 30 seconds, and rinsed with sterile distilled water. Sterilized samples were placed on PDA medium containing chloramphenicol and incubated at 25–28°C for 3–7 days (Pakaya, 2025).

Purification of Endophytic Fungi

Potato Dextrose Agar (PDA) medium was used for fungal purification. Fungal isolates grown on PDA were transferred to fresh media using the streak method until single isolates were obtained. The isolates were incubated for 5–7 days. Pure cultures were maintained on two media: one stored at 4°C as stock culture and the other used as a working culture at 37°C (Effendi, 2019; Mohan et al., 2016; Pakaya, 2022).

Characterization of Endophytic Fungi

Macroscopic Observation

Macroscopic identification was based on colony color, surface texture, radial lines from the center to the edge, concentric rings, and reverse colony color (Rusli et al., 2016).

Microscopic Observation

Microscopic identification was performed by aseptically taking fungal isolates using an inoculating needle, placing them on a glass slide, adding methylene blue stain, and observing under a microscope. Observations included hyphal structure and other microscopic features (Rusli et al., 2016).

Production of L-Asparaginase Enzyme

Fungal isolates and asparagus inducer were inoculated into a 250 mL Erlenmeyer flask containing 100 mL of sterile production medium. The culture was incubated in a shaker incubator at $28 \pm 2^\circ\text{C}$ with a speed of 150 rpm for 7 days. The culture was then transferred into centrifuge tubes and centrifuged at 3000 rpm at 4°C for 10–15 minutes. The obtained cell-free supernatant was filtered and used for qualitative enzyme testing as crude L-asparaginase (Pratiwi & Ardiansyah, 2022; Septiani et al., 2019).

Purification of L-Asparaginase Enzyme

The crude L-asparaginase-containing supernatant was purified using ammonium sulfate precipitation at saturation levels of 0–20%, 20–40%, 40–60%, 60–80%, and 80–100%. After adding ammonium sulfate, the solution was stirred until dissolved, centrifuged at 3000 rpm for 10 minutes, and filtered. The results were stored overnight at 4°C and then dissolved in Tris-hydroxymethyl-aminomethane buffer (pH 8.6) (Suryani et al., 2017).

L-Asparaginase Enzyme Assay

Six test tubes were prepared, each containing 0.1 mL of suspended precipitate (EK, F1, F2, F3, F4, F5) and 0.4 mL of 0.2 M Tris-hydroxymethyl-aminomethane buffer (pH 8.5). The samples were incubated at 37°C for 30 minutes, followed by the addition of 1 mL of 1.5 M TCA solution. The samples were then centrifuged at 3000 rpm for 15 minutes. A total of 0.25 mL of filtrate was taken and mixed with 4.25 mL distilled water and 0.5 mL Nessler reagent. A positive result was indicated by a color change to yellowish-brown in each reaction (Martina et al., 2021).

RESULTS

Isolation Results of Kencur Plant (*Kaempferia galanga* L.)

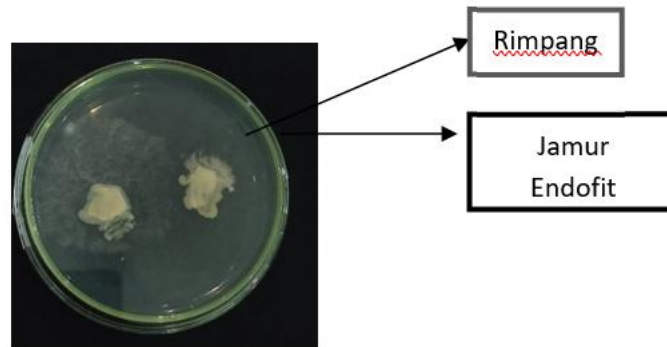


Figure 1. Isolation Results of Kencur Plant (*Kaempferia galanga* L.)

Based on Figure 1, the isolation results of the kencur plant (*Kaempferia galanga* L.) showed ivory-white coloration, circular colony shape, irregular colony margins, and flat colony elevation.

Results of Endophytic Fungi Characterization Macroscopic Observation

Table 1. Macroscopic Characterization Results of Endophytic Fungi from Kencur (*Kaempferia galanga* L.)

Endophytic Fungi	Surface Color	Colony Shape	Colony Margin	Colony Elevation
<i>Candida</i>	Ivory white	Irregular	Irregular	Flat

Based on Table 1, the macroscopic characterization results show that the isolated endophytic fungi from kencur (*Kaempferia galanga* L.) exhibited an ivory-white surface color. The colony texture was non-cottony, with an irregular shape, irregular colony margins, and flat colony elevation. The results of the macroscopic characterization of the endophytic fungi can be seen in Figure 2 below.

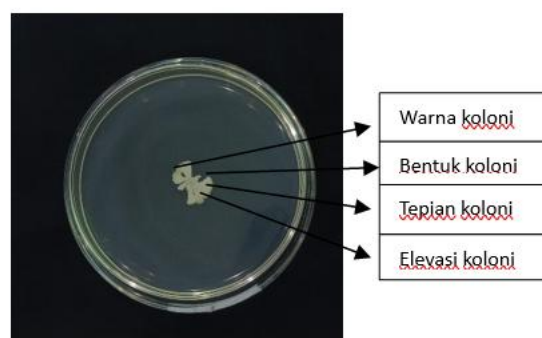


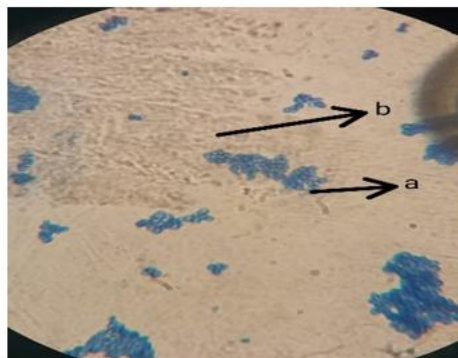
Figure 2. Macroscopic Characterization Results of Endophytic Fungi from Kencur Plant (*Kaempferia galanga* L.)

Microscopic Observation

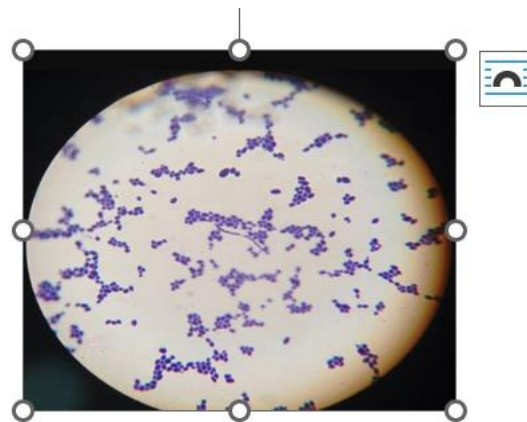
Table 2. Microscopic Characterization Results of Endophytic Fungi from Kencur (*Kaempferia galanga* L.)

Endophytic Fungi	Conidia	Hyphae
<i>Candida</i>	Blastoconidia (yeast cells)	Chlamydospore

Based on Table 3, the results of microscopic observations using Gram staining showed that the kencur plant (*Kaempferia galanga* L.) contains yeast-shaped cells. The endophytic fungi produce chlamydospores, which are spores formed from hyphae, characterized by a rounded shape and thickened cell walls. These characteristics indicate that the endophytic fungi isolated from kencur (*Kaempferia galanga* L.) belong to the genus *Candida* (Sri Indrayati, 2018). The microscopic results can be seen in Figure 3 below.



(Magnification 100×)



(Sri Indrayati, 2018) (Magnification 100×)

Figure 3. Microscopic Characterization of Endophytic Fungi *Candida*

Note:

a = Conidia (spore shape)

b = Hyphae (fine filamentous structures)

Results of L-Asparaginase Enzyme Production



Table 3. (a) Results of L-Asparaginase Enzyme Production, (b) Cell-Free Supernatant Based on Figure 4.4, the supernatant of *Candida* endophytic fungi incubated for 3–7 days using a shaker incubator changed color to a yellowish turbid appearance. After centrifugation at 3000 rpm, it produced a cell-free supernatant in liquid form with a clear yellow color.

Results of L-Asparaginase Enzyme Purification

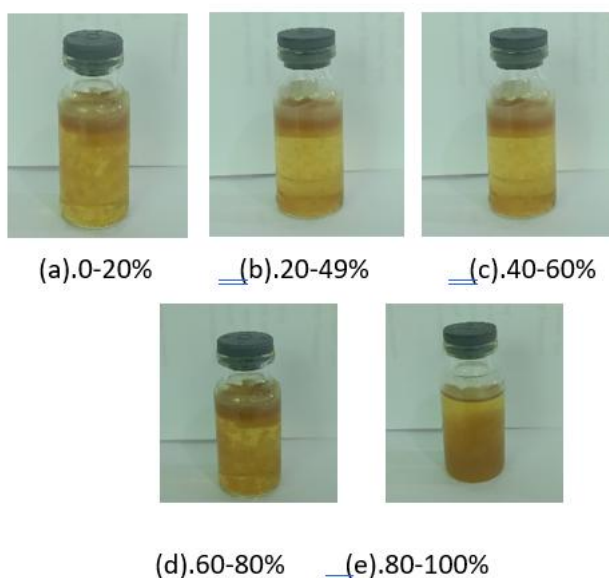
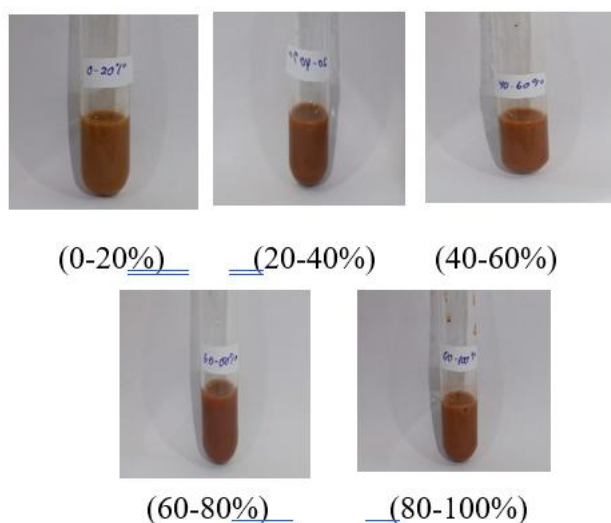


Figure 4. Purification of L-Asparaginase Enzyme from Endophytic Fungi of Kencur Plant (*Kaempferia galanga* L.) Using Ammonium Sulfate at Different Saturation Levels

Based on Figure 4, the fractionation of L-asparaginase enzyme from *Candida* endophytic fungi using ammonium sulfate produced five fractions. The first fraction had a saturation level of 0–20%, the second 20–40%, the third 40–60%, the fourth 60–80%, and the fifth 80–100%. The resulting fractions were filtered to obtain protein precipitates produced from the enzyme protein precipitation process, in order to yield higher enzyme activity.

Results of L-Asparaginase Enzyme Assay



DISCUSSION

Isolation Results of Kencur Plant (*Kaempferia galanga* L.)

The results of endophytic fungal isolates obtained from kencur rhizomes (*Kaempferia galanga* L.) in this study showed ivory-white colonies with a circular shape and smooth colony surface. These morphological characteristics are commonly found in endophytic fungi during the early stages of growth, particularly before the formation of pigments or reproductive structures.

Based on Figure 4.1, these results are consistent with previous studies reporting that endophytic fungi from kencur rhizomes and plants of the Zingiberaceae family generally exhibit white to cream-colored colonies with relatively uniform shapes. Waraswati et al. (2025) reported isolates suspected to belong to the genera *Aspergillus*, *Cladosporium*, *Fusarium*, and *Chlamyospora*. Differences in colony characteristics between studies may be influenced by variations in culture media, incubation time, and environmental conditions.

Purification of Endophytes

The purification results showed that the endophytic fungal isolate had ivory-white colony color, smooth surface texture, and irregular colony growth patterns. These characteristics indicate that the isolate was in a pure condition and relatively free from contamination by other microorganisms, making it suitable for further identification and biological activity testing.

These findings are consistent with Waraswati et al. (2025), who reported that endophytic fungal isolates from rhizomes generally exhibit white colonies with smooth textures after purification. In addition, Gary Strobel and Bryce Daisy (2003) stated that macroscopic morphological observations such as colony color, surface texture, and growth patterns are important initial parameters in confirming the purity of endophytic fungal isolates.

Characterization Results

Macroscopic

Macroscopic observations of endophytic fungi, as reported by Purwanto and Ukhradiya (2014), include surface color, reverse color, and colony texture. Based on Figure 4.3, the macroscopic characterization of the endophytic fungal isolate showed that both the surface and reverse colony colors were ivory white (non-pigmented), with a circular colony shape and non-cottony texture. According to Rahmayanti et al. (2022), these characteristics are consistent with those of endophytic yeast commonly found in plant rhizomes.

Microscopic

Microscopic observations of the endophytic fungi isolated from kencur rhizomes (*Kaempferia galanga* L.) showed that the isolates consisted of oval to round cells resembling yeast, capable of forming blastoconidia, possessing septa, and lacking branched conidiophores typically found in filamentous fungi. These characteristics are consistent with the morphological features of fungi belonging to the genus *Candida*, a group of yeast fungi that reproduce asexually through blastoconidia formation and generally possess pseudohyphae without complex conidiophore structures.

These findings are in agreement with the morphological description of *Candida* reported by Charles P. Kurtzman et al. (2011) and other studies on endophytic fungi from medicinal plants, which also reported the presence of *Candida* with similar microscopic characteristics (Kharwar et al., 2011). Furthermore, several previous studies have shown that the genus *Candida* has the potential to produce L-asparaginase, which plays an important role in biomedical applications, particularly as an anticancer therapeutic agent.

Production of L-Asparaginase Enzyme Using Asparagus Inducer

The supernatant obtained from the culture of *Candida* endophytic fungi incubated for three days using a shaker incubator showed a change in color to a yellowish turbid appearance. This change indicates active fungal growth and the release of extracellular metabolites and proteins into the medium, including L-asparaginase produced during the fermentation process.

El-Gendy et al. (2022) reported that L-asparaginase from endophytic fungi is generally produced extracellularly, increasing the concentration of dissolved proteins in the medium and affecting the color and clarity of the supernatant. Additionally, amino acid metabolism during incubation can produce nitrogenous compounds that contribute to the yellowish coloration of the culture medium (Kumar et al., 2020).

Purification of L-Asparaginase Enzyme

Purification of L-asparaginase from the cell-free supernatant of *Candida* endophytic fungi induced by asparagus was carried out through stepwise precipitation using ammonium sulfate at saturation levels of 0–20%, 20–40%, 40–60%, 60–80%, and 80–100%. This technique is widely used as an initial step in protein purification because it is relatively simple, efficient, and capable of separating proteins based on differences in solubility at various salt concentrations.

The addition of ammonium sulfate reduces protein solubility through a salting-out mechanism, causing the target protein, including L-asparaginase, to precipitate at specific saturation ranges (Kumar et al., 2020).

L-Asparaginase Enzyme Assay

Based on observations in Figure 4.5, the L-asparaginase enzyme assay showed that the cell-free supernatant and all fractions obtained from ammonium sulfate precipitation (0–20%, 20–40%, 40–60%, 60–80%, and 80–100%) indicated positive results for the presence of L-asparaginase enzyme. This was evidenced by a color change to brownish after the addition of the test reagent, indicating the formation of ammonia as a result of the

hydrolysis of L-asparagine into L-aspartate by enzyme activity.

Studies by El-Gendy et al. (2022) and Rahman et al. (2023) explain that the color change from yellow to brown in qualitative L-asparaginase assays is caused by the reaction of ammonia produced during catalysis with Nessler's reagent, making this color change an initial indicator of enzyme activity. Furthermore, Kumar et al. (2021) reported that the appearance of brownish color in both the supernatant and purified fractions indicates that L-asparaginase remains distributed across multiple precipitation fractions, although its activity levels may vary.

CONCLUSION

Endophytic fungi were successfully isolated from kencur rhizomes (*Kaempferia galanga* L.) using Potato Dextrose Agar (PDA) medium following surface sterilization, resulting in one isolate. Characterization results showed that macroscopically, the colonies were ivory white, irregular in shape with uneven margins and flat elevation. Microscopically, the isolate exhibited yeast-like cells, producing blastoconidia and thick-walled chlamydoconidia, and was identified as belonging to the genus *Candida*.

The isolate was also capable of producing L-asparaginase enzyme when cultured in media with an asparagus inducer, indicated by the formation of a clear yellow cell-free supernatant after incubation and centrifugation. Furthermore, qualitative testing using the Nesslerization method showed a color change to yellowish-brown across various ammonium sulfate purification fractions (0–100%), indicating the presence of L-asparaginase enzyme activity.

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