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Original research article

Endophytic Fungi Associated With Turmeric (*Curcuma longa* L.) Can Inhibit Histamine-Forming Bacteria in Fish

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ABSTRACT

Turmeric (*Curcuma longa* L.) is a medicinal plant that is commonly used as spice and preservative. Many types of endophytic fungi have been reported as being associated with medicinal plants and able to synthesize secondary metabolites. In this study, endophytic fungi were isolated from all plant parts of turmeric plants, including the root, rhizome, stem, inflorescence, flower, and leaf. Identification of the endophytic fungi was done using morphological characteristics and sequencing of the ITS region of ribosomal DNA. The dual culture method was used for screening antibacterial activity of the endophytic fungi against *Morganella morganii*, a common histamine-producing bacteria. The disc diffusion method was used to test the ability of water fractions of selected endophytic fungi to inhibit *M. morganii* growth. Two-dimensional thin layer chromatography was used to determine the fungal extract inhibition activity on histamine formation. In total, 11 endophytic fungi were successfully isolated from the various parts of turmeric plant and identified as *Arthrobotrys foliicola*, *Cochliobolus kusanoi*, *Daldinia eschscholzii*, *Fusarium oxysporum*, *Fusarium proliferatum*, *Fusarium solani*, *Fusarium verticillioides*, *Phanerochaete chrysosporium*, and *Phaeosphaeria ammophilae*. Five isolates, *A. foliicola*, *C. kusanoi*, *F. proliferatum*, *F. verticillioides*, and *P. ammophilae* showed inhibition activity against *M. morganii* in the dual culture tests. Based on the disc diffusion assay, *A. foliicola* and *F. verticillioides* inhibited the growth of *M. morganii* as a histamine-producing bacteria, as well as inhibiting histamine formation in fish. The best effects in inhibiting growth of the histamine-producing bacteria (1.10^2 cfu/g) and histamine formation inhibition in fish (3.12 ppm) were produced with *F. verticillioides* water fraction at 0°C incubation.
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1. Introduction

Turmeric (*Curcuma longa* L.) is a zingiberacean plant that grows in the Southeast and South Asia. In Indonesian society, turmeric is used for cooking, natural food dyes, and as a remedy for various diseases. Either on its own or combined with salt, it is also used as a natural preservative for fresh fish to extend shelf life (Akter *et al.* 2013). It has been reported that medicinal plants are potential sources of endophytic fungi that have the ability to produce bioactive compounds (Ginting *et al.* 2013). Turmeric as a medicinal plant, could be host to various endophytic fungi, which potentially have either the same or different biological activities as their host (Zhao *et al.* 2011). Endophytic fungi are fungi that live inside plant tissues and perform a beneficial function for their host, through production of bioactive compounds that protect the host from biotic and abiotic stress (Dai *et al.* 2008). In return, the host provides a unique ecological niche for growth and development of the endophytic fungi (Barrow *et al.* 2008).

Histamine is a chemical compound normally produced by the decarboxylation of free histidine by the L-histidine decarboxylase enzyme, which is synthesized by histamine-producing bacteria. These bacteria are often found in improperly preserved scombroid fish (Bjornsdottir *et al.* 2009). Under normal conditions, eating fish containing small quantities of histamine will have little negative effect on the health. However, in larger amounts, consumption may cause histamine poisoning, whose effect could include diarrhea, headache, hypotension, pruritus, and flushes (Wohrl *et al.* 2004). Therefore, histamine levels need to be controlled to reduce cases of histamine poisoning, especially after consuming fish containing histamine.

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Several studies have been performed on turmeric extract as a growth inhibitor of histamine-producing bacteria. Paramasivam *et al.* (2007) reported that a 5% concentration of turmeric extract inhibited the growth of histamine-producing bacteria, *Morganella morganii* also inhibited the formation of histamine. Previous studies on growth inhibition of histamine-producing bacteria in fresh fish in Indonesia have been carried out only in respect of the effect of bioactive compounds derived from turmeric plant. There has been no report on the uses of endophytic fungi isolated from turmeric plant, specifically, to inhibit the growth of histamine-producing bacteria and thus inhibit histamine formation in fish. Studies on the application of endophytic fungi associated with the turmeric plant should provide information on the potential of these fungi as an alternative natural product for reducing histamine levels in fish. Therefore, this research was carried out to study the endophytic fungi associated with the turmeric plant, and to determine their inhibition effects on the growth of the histamine-producing bacteria, *M. morganii*, and on the formation of histamine in fresh fish.

2. Materials and Methods

2.1. Isolation and identification of endophytic fungi

Turmeric plants were obtained from Tanah Sareal District, Bogor, West Java. The plant organs used for isolation were the root, rhizome, stem, leaf, inflorescence, and flower. Isolation of endophytic fungi was carried out using the same process described by Hallmann *et al.* (2006). The samples were washed with water to remove dirt, and then dried at room temperature using sterile filter paper. Each piece of the plant organ was cut into 2 cm long segments for the roots, rhizomes, and stems, or 2 × 2 cm square pieces for leaves, inflorescences, and flowers.

Surface sterilization of each sample was performed by soaking the samples in 70% ethanol for 1 minute, 5.3% sodium hypochlorite for 5 minutes, 70% ethanol for a further 30 seconds and then rinsed three times using sterile distillated water. All samples were dried for 6 hours on sterile filter paper in a safety cabinet. Samples were then further cut into 1 cm long segments for the roots, rhizomes, and stems, whereas leaves, inflorescences, and flowers were further cut into 1 × 1 cm square pieces. Three pieces or segments of each organ were placed in Petri dishes on low carbon agar (LCA) medium containing rose bengal (25 mg/L) as a fungistatic agent and chloramphenicol (250 mg/L) as a suppressor of bacterial growth. Three replicates were prepared for each organ. As a negative control, 1 mL of sterile water from the final rinse was plated on LCA medium and then incubated at the ambient temperature. The hyphae from the fungal colonies were transferred to a fresh LCA medium without rose bengal and chloramphenicol, and incubated at the ambient temperature for 7 days.

Isolated endophytic fungi were identified by morphological characteristics (Barnett and Hunter 1998), and by molecular analysis using ITS1, ITS2, and 5.8 region sequences, using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) as a forward primer and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as a reverse primer (White *et al.* 1990). For DNA extractions, fungal mycelium was grown in a 50-mL flask containing Potato Dextrose Broth, incubated in 120 rpm shaker at 29°C for 7 days. Mycelia were harvested by filtration with sterile filter paper then washed by sterile distillated water, then ground in a sterile pestle and mortar with the addition of liquid nitrogen. DNA extraction was performed using the CTAB method (Sambrook and Russell 2001).

Polymerase chain reaction (PCR) amplification was performed on a total volume of 30 μL containing 10.5 μL sterile free base water, 15 μL 2× PCR Master Mix (Promega), 0.75 μL 10 pmol of each of ITS1 and ITS4 primers, and 3 μL (~250 ng/μL) of the DNA template. Amplification reaction was performed in 35 cycles as follows: predenaturation at 95°C for 1.5 minutes, denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1.5 minutes, final extension at 72°C for 5 minutes, and then stored at 25°C for 10 minutes. An amount of 5 μL PCR product was analyzed using gel electrophoresis (250 volt; 75 watt) containing 1% agarose for 30 minutes, stained with ethidium bromide for 25 minutes and observed under UV light.

Purification and sequencing of PCR products using the same primers were carried out by a service provider, First BASE (Malaysia). The sequences were analyzed using the ChromasPro program and determined using available DNA fungal sequences, via searches of the MycoBank database (http://www.mycobank.org) and the BLAST (http://www.blast.ncbi.nlm.nih.gov/blast). Phylogenetic analyses were conducted using the neighbor-joining (N-J) method in MEGA5 (Tamura *et al.* 2011), and an N-J tree was constructed using the Kimura-2 parameter model. All characters were equally weighted and unordered. Gaps and missing data were treated as partial deletions. Support for specific nodes on the N-J tree was estimated using 1000 bootstrapping replications.

2.2. Screening of antibacterial activity by endophytic fungi

Histamine-producing bacteria, *M. morganii* FNCC 0122 for microbial testing were obtained from the Food and Nutrition Culture Collection, PAU Food and Nutrition Gadjah Mada University, Yogyakarta, Indonesia. The bacteria were grown firstly on a modified Niven agar medium to confirm that the bacteria produced histamine. The dual culture method (Zhang *et al.* 2009) was used to screen antibacterial activity. Each pure isolate of 7-day-old endophytic fungi in Petri dishes subsequently were perforated with a hole punch (6 mm in diameter), and placed on a nutrient agar medium containing a 24-hour-old bacterial test culture. The Petri dishes were incubated at the ambient temperature for 24 hours. Clear zone diameters produced around the pieces of endophytic fungi were observed.

Endophytic fungal isolates that produced positive inhibition zones were selected for further assay, to determine their antibacterial activity. The isolates were cultured in 2 L of Potato Dextrose Broth medium in an Erlenmeyer flask, shaken at 120 rpm for 15 days at the ambient temperature. Three replicates were prepared for each isolate. The mycelia were harvested using vacuum filtration to separate culture filtrate and mycelia. The filtrate was extracted using ethyl acetate and partitioned with water to obtain water fraction, whereas the mycelial biomass was extracted using water. The Kirby-Bauer disc diffusion method was used to test the extracts against *M. morganii* FNCC 0122. Each extract with serial concentrations of 10,000, 20,000, and 40,000 ppm was tested against *M. morganii* FNCC 0122 in a Müller-Hinton agar medium. Each treatment was carried out using three replicates. The diameter of the clear zone around the disc after incubation for 24 hours at ambient temperature was measured and recorded. The isolates that produced the highest activity were selected for testing inhibition of histamine formation and of histamine-producing bacterial growth in fresh fish.

2.3. Inhibition test of histamine and histamine-producing bacterial growth in fresh fish

Tests were carried out on tuna fish, which was obtained from fish auctions at traditional markets. The fresh fish was washed with sterile distilled water, and the entrails and skin were cleaned to produce 10 grams pieces of fish fillet. The fillets were divided into two groups, the 1st group was immersed for 30 minutes in the filtrate water fraction of selected endophytic fungi, and the 2nd group in sterile distilled water. The concentration of the filtrate

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