Isolation and Identification of Endophytic Fungus, *Curvularia* and its Antibacterial Activity on *Staphylococcus aureus*

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Abstract

The endophytic fungus (KTL-07) from *Aquilaria crassna* Lam. belonging to Thymelaeaceae grown in Pathein showed the best antibacterial activity (29.90 mm of inhibitory zone) on *Staphylococcus aureus* among 25 endophytic fungi. This fungus was identified as *Curvularia* sp. according to the macroscopical and microscopical characters. For the kinetic growth of KTL-07, the highest packed cell volume was 0.44 g at 96 hrs. The inoculum period was 24 hrs as the best age (25.93 mm of inhibitory zone) and 30% of inoculum was the best size (26.59 mm of inhibitory zone) on *Staphylococcus aureus* for fermentation. In the substitution of medium sources, sucrose and malt extract were the best antibacterial activity (11.00 mm and 16.21 mm of inhibitory zones) on *Staphylococcus aureus* and also growth conditions as carbon and nitrogen sources. In the fermentation conditions on the different pH levels (pH-4 to 7), pH-5.5 showed the highest antibacterial activity (30.26 mm of inhibitory zone) on *Staphylococcus aureus.* On the effects of different temperatures (20℃ to 35℃), 30℃ was the best (30.92 mm of inhibitory zone) for the production of antibacterial metabolite in the fermentation condition on *Staphylococcus aureus*. Then, the shaking condition was suitable for fermentation as 22.55 mm of inhibitory zone on *Staphylococcus aureus*.

Keywords: endophytic fungus, antibacterial activity, antibacterial metabolite

Introduction

Cancers and various infectious diseases were threatening worldwide human health. Endophytes are microorganisms that live in the intercellular spaces of healthy host tissues without causing obvious symptoms. Bioprospecting studies of endophytic microorganisms for pharmaceutical and biotechnological purposes are fundamental for the discovery of new substances for human therapeutics including antibiotics, anti-malarias, and anticancer. Microorganisms are important sources of bioactive natural products with enormous potential for the discovery of new molecules for drug discovery, industrial and agricultural applications. In comparison to other natural sources like plants, microorganisms are highly diversed but narrowly explored.

Studies based on the microbial populations have revealed that only about 1% of bacteria and 5% of fungi have been characterized and the rest remain unexplored for their contribution to the human welfare. In addition, more than 60% of the anticancer and 70% of the antimicrobial drugs currently in clinic are natural products or natural product derivatives. A study of the World Health Organization (WHO) revealed that 90% of the bacteria strains are resistant to drugs. Drug resistance in bacteria has become a global concern and the urgently search for new antibacterial agents. In Recent years, the isolation of endophytic fungi and screening of antimicrobial activity have gained more attention (Nwakanma *et al.*, 2016).

Agarwood formation is widely used by the Buddhism for cultural and rituals activity (TRAFFIC, 2004). Its market values also focus on novel antibiotics and perfumes for daily used. Due to its high market value, the demand for agarwood

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increases and *Aquilaria* trees are heavily harvested for commercial. This cause the available *Aquilaria* tree to be decreased aggressively and being listed as "endangered" species in both CITES and IUCN Red list (TRAFFIC, 2004: IUCN, 2014).

Therefore, the selected endophytic fungus or *Curvularia* sp. from *Aquilaria crassna* Lam. was investigated the production of antibacterial metabolite and identification. The aim and objectives of this research paper were to isolate and identify the endophytic fungus according to its macroscopical and microscopical characters and to investigate the fermentation condition for the production of antibacterial metabolite.

Materials and Methods

Isolation of endophytic fungi and their antimicrobial activity

The leaves, barks and roots of *Aquilaria crassna* Lam. (Thit-hmwe) belonging to Thymelaeaceae grown in Pathein were collected for the isolation of endophytic fungi. The plant parts were washed in running tap water for 10 min. Plant parts were cut into small pieces. The surface of cut plant pieces were sterilized by socked in 95% alcohol for 15 seconds. Next, the surface of cut plant pieces into smaller pieces. The samples were dried on sterilized tissue paper. Then, the samples were cut pieces were incubated on nutrient agar plate for 2 days to 1 week at room temperature (Glucose 1g, Polypeptone 0.3 g, Agar 1.8 g and DW 100 mL) (Suto, 1999).

The isolated endophytic fungi were incubated in seed medium (Glucose 1 g, Polypeptone 0.3 g, $CaCO_3$ 0.01 g and DW 100 mL) for 3 days and transferred into the fermentation medium (Glycerol 1.0 mL, Yeast extract 0.3 g, $CaCO_3$ 0.01 g and DW 100 mL) and then carried out for 3-12 days. Then, the fermentation broth was used to check the antimicrobial activity on 8 different test organisms by the agar well diffusion assay (glucose 1.0 g, peptone 0.3 g, agar 1.8 g, distilled water 100 mL at pH 7.0) method (Ando, *et al.*, 2004).

Table 1. Different test organisms used for antimicrobial activity

No	Test organisms	Sources	Infections
1	*Escherichia coli*	AHU 5436	Diarrhea
2	*Micrococcus luteus*	NITE 83297	Skin disease
3	*Candida albicans*	NITE 09542	Candidiasis
4	*Bacillus subtilis*	IFO 90571	DNA topoisomerase I
5	*Staphylococcus aureus*	AHU 8465	Food poisoning, Methicillin Resistance
6	*Pseudomonas fluorescens*	IFO 94307	Rice disease
7	*Malasseia furfur*	AVU 0255	Dandruff, Seborrhea dermatitis
8	*Bacillus pumilus*	IFO 12092	Wound and burn infection, Fever

Identification of the selected endophytic fungus (KTL-07)

The macroscopical and microscopical characters of the selected endophytic fungus (KTL-07) were examined under light microscope for 7 days old culture on the

nutrient agar medium. According to Barnett and hunter (1972), the selected endophytic fungus (KTL-07) was keyed out by its macroscopical and microscopical characters.

The kinetic growth, age and sizes of KTL-07

For the investigation of the kinetic growth of KTL-07, the dry weight of packed cell volume was measured the time course from 24 hrs to 144 hrs.

The seed culture of KTL-07 was undertaken for fermentation from 12 hra to 72 hrs at room temperature as the age of inoculum optimization.

In the sizes of inoculum optimization, 24 hrs of seed culture was utilized as 5 % to 40 % for fermentation. Each fermentation was carried out 7 days and tested the antibacterial activity by agar well diffusion assay on *Staphylococcus aureus*.

The fermentation medium composition as carbon and nitrogen sources of KTL-07

For the growth conditions and the antibacterial metabolite production of KTL-07, 7 different carbon (rice powder, glucose, sucrose, potato powder, glycerol, wheat power, honey) and nitrogen (malt extract, KNO_3, gram pea powder, yeast extract, peptone, fish extract, beef extract) media sources were utilized for 7 days as the growth conditions and tested the antibacterial activity for 5 days by agar well diffusion assay on *Staphylococcus aureus* at room temperature.

The different pH levels and temperatures for fermentation of KTL-07

For the antibacterial metabolite production of KTL-07, pH 4 to 7 and temperature 20ºC, 25ºC, 30ºC, 35ºC were utilized at 5 days of fermentation for the antibacterial activity by agar well diffusion assay on *Staphylococcus aureus*.

The shaking and static conditions for fermentation of KTL-07

The shaking and static conditions of KTL-07 were inoculated for 5 days of fermentation at 100 spm and tested the antibacterial activity by agar well diffusion assay on *Staphylococcus aureus* at room temperature.

Results

A total of 25 endophytic fungi including 8 from leaves, 10 from Barks and 7 from roots were isolated from *Aquilaria crassna* Lam. (Thit-hmwe) belonging to Thymelaeaceae grown in Pathein Township.

Outstanding Characters of *Aquilaria crassna* Lam. (Thit-hmwe) (Thymelaeaceae)

An evergreen tree, 6 to 20 m tall. The smooth bark is grayish to dark gray, and the wood is white to yellowish. Its leaves are alternate, leathery, obovate to elliptic, generally 5 to 11 cm long and 2 to 4 cm wide, with 15 to 20 pairs of inconspicuous and nearly parallel lateral vein which is a helpful diagnostic feature in the field. Its flowers are yellowish green, fragrant, in a terminal or axillary umbel. Perianth campanulate: lobe 5 broad, spreading; scales above the stamens 5, hairy, connate at the base. Stamens 10: anthers subsessile, oblong; connective broad. Disk 0. Ovary subsessile, villous, perfectly or imperfectly 2-cell; stigma large, subsessile. Capsule

compressed, oblanceolate or ovulate, loculicidal; pericarp coriaceous or hard and woody.

Figure 1. Habit and parts of *Aquilaria crassna* Lam. (Thit-hmwe)

Table 2. No. of isolated endophytic fungi

Plants Parts	Designated isolated	Isolated fungi
Aquilaria crassna Lam. (Leaves)	KTL-01,02,03,04,05,06,07,08	8
Aquilaria crassna Lam. (Barks)	KTB-01,02,03,04,05,06,07,08,09,10	10
Aquilaria crassna Lam. (Roots)	KTR-01,02,03,04,05,06,07	7
	Total	25

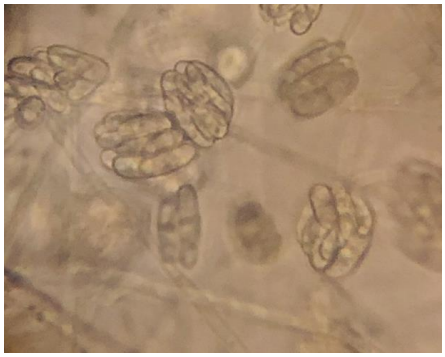
According to the antimicrobial activity of 25 isolated endophytic fungi, KTL-06 and KTL-07 from the leaves, KTB-03, KTB-07 and KTB-09 from the barks and KTR-01 and KTR-03 from the roots showed the antibacterial activity on *Staphylococcus aureus*. Among them, KTL-07 had the best antibacterial activity (29.90 mm of inhibitory zone) on *Staphylococcus aureus* for 3 days of fermentation.

Front view Reverse view

Figure 2. Isolated endophytic fungus (7 days old culture of KTL-07) from *Aquilaria crassna* Lam.

The macroscopical and microscopical characters of KTL-07

Dark mycelium, conidiophores simple and sympodial. The conidia are thick walled with septa and ellipsoidal, often curved due to an enlarged middle cell, rounded at the ends or sometimes tapering slightly towards the base, pale brown.



Micrographs of *Curvularia* (KTL-07) (X400)

Figure 3. Microscopical characters of *Curvularia* (KTL-07)

Key for Identification of KTL-07

1a. Conidia often thin walled ---------- 2
1b. Conidia often thick walled ---------- 91
91a. Conidia with cross and oblique septa ---------- 92
91b. Conidia with transverse septa only ---------- 94
94a. Conidiophores with conidia catenulate ---------- *Dendryphion*
94b. Conidiophores branched, conidia not catenulate ---------- 95
94c. Conidiophores simple, conidia not catenulate --------- 96
96a. Conidia in chain ---------- *Torula*
96b. Conidia not in chains ---------- 97
97a. Conidia bent by enlargement ---------- *Curvularia*
97b. Conidia not bent by enlargement ---------- 100

Classification of KTL-07

The isolated endophytic fungus (KTL-07) from *Aquilaria crassna* Lam. (Thit-hmwe) belonging to Thymelaeaceae can be classified the genus as *Curvularia* belonging to family Pleosporaceae, order Pleosporales, class Dothideomycetes, division Ascomycota and kingdom Fungi.

The effects of the kinetic growth, age and sizes of KTL-07

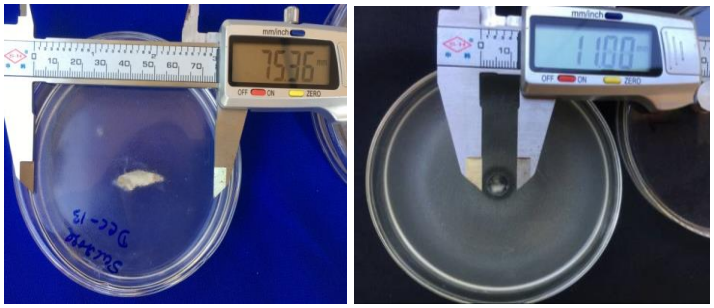
In the investigation of the kinetic growth of KTL-07, 0.44 g with dry weight was the best at 96 hrs. Twenty-four hrs of inoculum was the best age (25.93 mm of inhibitory zone) on *Staphylococcus aureus* for 3 days of fermentation at room temperature. For the production of antifungal metabolite, 30% of seed culture (24 hrs age) was the best size (26.59 mm of inhibitory zone) among 5% to 40% for 3 days of fermentation on *Staphylococcus aureus* at room temperature.

The effects of carbon and nitrogen sources of KTL-07

The growth condition and the antibacterial activity of the sucrose were the excellent (75.36 mm in colony diameter and 11.00 mm of inhibitory zone) among 7 different carbon sources and that of the malt extract (70.19 mm of colony diameter and 16.21 mm of inhibitory zone) on *Staphylococcus aureus* at 3 days of fermentation for the production of antibacterial metabolite.

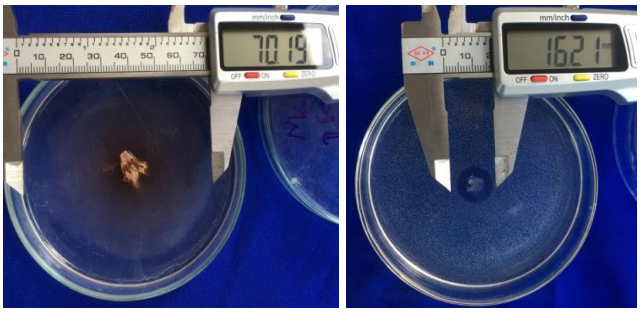
3 days of fermentation **24 hrs** **30%**

Figure 4. Antibacterial activity of KTL-07 on *Staphylococcus aureus* by age and size



growth condition on sucrose antibacterial activity of **sucrose** on *Staphylococcus aureus*

Figure 5. Growth condition and antibacterial activity of KTL-07 as carbon source



growth condition on malt extract antibacterial activity of malt extract on *Staphylococcus aureus*

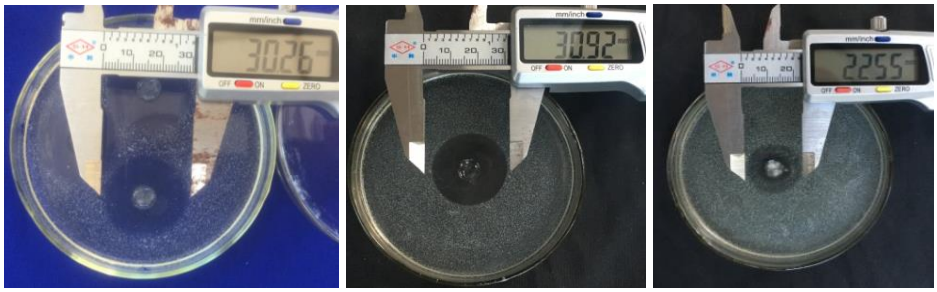
Figure 6. Growth condition and antibacterial activity of KTL-07 as nitrogen source

The effects of different pH levels and temperatures of KTL-07

In the different pH levels and temperatures for fermentation of KTL-07, pH 5.5 and 30ºC had the highest antifungal activity (30.26 mm and 30.92 mm of inhibitory zones) at 3 days on *Staphylococcus aureus* for the production of antibacterial metabolite.

The effects of shaking and static conditions of KTL-07

The isolated endophytic fungus (KTL-07**)** exhibited the more antibacterial activity on the shaking condition (22.55 mm of inhibitory zone) than on the static condition (22.06 mm of inhibitory zone) on *Staphylococcus aureus* at 3 days of fermentation for the antibacterial metabolite production.



pH 5.5 **30°C** **shaking condition**

Figure 7. Antibacterial activity of KTL-07 on *Staphylococcus aureus* by pH, temperature and shaking condition

Discussion and Conclusion

Fungi are commonly noticed as the main microbial component for agarwood formation. Agarwood can be used as a carminative, stimulant for heart palpitation, tonic during pregnancy, remedy during the post-natal recovery period and cure for the disease of the female genital part. The bark of agarwood is believed to heal jaundice and body pain. Moreover, agarwood helps to relieve body pain, warm the abdomen, relieve asthma, treat coughs and acroparalysis as antihistamine, analgesic and anti-inflammatory. In Chinese traditional medicine, it is helpful as a sedative to relieve gastric problems, rheumatism and high fever. Agarwood properties were able to fight cancer cells. Current findings proved that artificial agarwood may produce quality equal to natural agarwood and may not be affected by fungi interacting with the tree which can be used as pharmaceutical application in the treatment of various diseases (Ramli, *et al.*, 2022).

Many filamentous fungi are known to produce several secondary metabolites or bioactive compounds during their growth and reproduction with biological activities. Genus *Curvularia* (Pleosporaceae) is a dematiaceous filamentous fungus that exhibits endophytic lifestyle. Genus *Curvularia* contains approximately 213 species. Apart from their negative effects, there are some beneficial implications like production of enzymes of industrial value, bioherbicides and source of nanoparticles (Mehta, *et al.*, 2022).

The total 25 endophytic fungi were isolated from the leaves, barks and roots of *Aquilaria crassna* Lam. (Thit-hmwe) belonging to Thymelaeaceae. According to the antimicrobial activity, the selected endophytic fungus was identified as *Curvularia* sp. by the macroscopical and microscopical characters. For the production of antibacterial metabolite, the selected endophytic fungus was at 24 hrs age, 30% size in 3 days of fermentation condition with pH 5.5 and 30 ºC on *Staphylococcus aureus*.

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References

Ando K., M. Suto and S. Inaba 2004: **Sampling and isolation methods of fungi**, Workshop at University of Pathein.

Barnet, H. L and B. B. Hunter, 1972: **Illustrated genera of imperfect fungi**, West Virginia University, United states of America.

IUCN, 2014, 2014: **Annual Report of the Species Survival Commission and The Globel Species Programme,** CH-1196, Gland, Switzerland.

Mehta T., M. Meena and A. Nagda, 2022: **Bioactive compounds of *Curvularia* species as a source of various biological activities and biotechnological applications,** Frontiers Microbiology, Mohantal Sukhadia University, India.

Nwakanma C, Njoku EN2 and Pharamat, 2016: **Antimicrobial Activity of Secondary Metabolites of Fungi Isolated fromLeaves of Bush Mango,** T31Department of Environmental Management and Toxicology, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria2Department ofBiological Sciences, Godfrey Okoye University, Enugu State, Nigeria.

Ramli A. N. M., S. Yusof, P. Bhuyar, A. W. Aminan and S. N. Tajuddin, 2022: **Fungi mediated agarwood (*A. malaccensis*) production and their pharmaceutical applications,** International Journal of Plant based Pharmaceuticals, University of Malaysia Pahang, Malaysia.

Suto, M., 1999: **Isolation of endophytes from plants, in Molecular tools on isolation and screening of microbes for useful materials**, Workshop in Malaysia.

TRAFFIC, 2004: **Summaries of the ICUN/FRAFFIC analyses of the proposal to amend the CITES appendices at the 13 the meeting of the Conference of the Parties**, Bangkok, Thailand.