ISOLATION OF ENDOPHYTIC FUNGI FROM THE LEAVES OF *STEPHANOTIS VOLUBILIS* (L.F.) S. RUESS, LIEDE & MEVE AND THE STUDIES OF ANTIMICROBIAL ACTIVITIES & SOME FERMENTATION PARAMETERS

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Abstract

Endophytes are microbes (mostly bacteria and fungi) present in plants asymptomatically. Endophytic fungi are known to produce a wide range of metabolites of pharmaceutical importance. This research paper deals with the endophytic fungi isolated from the leaves of *Stephanotis volubilis* (L.f.) S.Ruess, Liede & Meve. It is a well-recognized medicinal plant in traditional medicine. A total of 31 fungal species were isolated from the leaves. The morphology of endophytic fungi was studied and antimicrobial activities of these fungi were carried out by using paper disc diffusion method on six test organisms. All isolated fungi showed antimicrobial activities on *Bacillus pumilus*, *Bacillus subtilis*, *Candida albicans*, *Micrococcus luteus*, *Pseudomonas* sp., *Staphylococcus aureus*. According to the results, MM-25 showed the best antimicrobial activity against six test organisms. As a result of morphological and microscopical characters, this strain may be *Rhizoctonia* sp. In the study of fermentation parameters, the age of seed culture and the size of inoculum were investigated under two different states such as static and shaking. It was resulted that three-day old cultures provided the highest antimicrobial potentials on six test organisms. Moreover, 25% size of inoculum in fermentation was also observed the best antimicrobial activity on *Staphylococcus aureus* in both static and shaking states.

Keywords: Antimicrobial activity, *Rhizoctonia* sp.

Introduction

Endophytes are ubiquitous that spend their entire or a significant portion of their life cycle within the living tissues of their host plant without causing negative or overt symptoms (Petrini, 1991; Bacon and White, 2000; Kusari and Spiteller, 2012; Meshram *et al*., 2016). During the alliance, neither of the interacting partners is harmed and the benefits obtained are dependent on the interacting partners. Endophytes can provide benefits to their host plants by mediating abiotic and biotic stress tolerance, defending from pests and microbial infections. Thus, endophytes play a significant role in plant symbiosis, protecting their host from pathogens, pests and abiotic stresses (Kusari *et al*., 2014; Meshram *et al*., 2016; Hodkinson and Murphy, 2019).

Endophytes are proving to be a novel source of metabolites for the pharmaceutical and biochemical industries providing biologically active compounds such as antibiotics, antioxidants, anticancer agents, immunosuppressive compounds, insecticides, plant growth-promoting (PG) agents and volatile antimicrobial agents representing a wide range of organic molecules including terpenoids, peptides, carbohydrates, aromatics and hydrocarbons (Strobel, 2018; McNees *et al*., 2019).

The use of herbal medicine for the treatment of diseases and infections is a safe and traditional therapy. Hence, medicinal plants have been receiving great attention worldwide by the researchers because of their safe utility. The plant *Stephanotis volubilis* (L.f.) S.Ruess, Liede & Meve is belonging to the family Apocynaceae and is native to North-East Pakistan to South China and West Malesia. It is a climber and grows primarily in the wet tropical biome. It is also found

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to be growing in high rainfall as well as in low rainfall regions. It is a well-recognized medicinal plant in folklore and traditional medicines like Ayurveda, Siddha and Unani (Najafi, 2011). Traditionally, leaves are used as a treatment to boils and abscesses. Different plant parts can be used in cold, eye diseases and snake bites (Nadkarni, 2009).

In the present work, the existence of endophytic fungi in the leaf tissues of *Stephanotis volubilis* (L.f.) S.Ruess, Liede & Meve is detected and their potentials of antimicrobial activity were surveyed. Moreover, a strain which showed the highest antimicrobial activity was selected for the fermentation parameters such as age of culture and size of inoculum were studied.

Materials and Methods

Collection of plant sample

Endophytic fungal strains were screened from the fresh leaves of *Stephanotis volubilis* (L.f.) S.Ruess, Liede & Meve. The plant samples were collected from the Botanical Garden, Department of Botany, University of Yangon, Kamaryut Township, Yangon Region.

Isolation of endophytic fungi (Suto, 1999)

Isolation of endophytic fungal strains was carried out by the following procedure. The plants were washed in running tap water for about three minutes.

1. The plant part (leaves) was cut into about 1cm pieces.
2. The parts were surface sterilized by soaking in 75% ethanol for 15 seconds.
3. The plant pieces were cut into smaller pieces.
4. These parts were dried on sterilized paper and then placed on agar plates containing nutrient agar medium (NA medium), sucrose yeast extract medium (SY medium), potato glucose agar medium (PGA medium) and glucose yeast extract medium (GY medium and supplemented with chloramphenicol (0.25 mg / 100 ml) to inhibit bacterial growth.
5. These plates were incubated at room temperature for 3-7 days and transferred to pure culture plates.
6. Isolated fungal strains were then transferred into slant culture of each test tube containing respective medium.

Media used to isolate endophytic fungi (Compositions gram per liter)

Nutrient agar medium	
Nutrient agar	28 g
pH	7.4 ± 0.2

Sucrose-Yeast extract (SY) medium (Strobel and Sullivan, 1999)	
Sucrose	10 g
Yeast extract	3.0 g
NaCl	0.5 g
$CaCO_3$	0.1 g
Agar	18.0 g
pH	6.8 ± 0.2

Potato Glucose Agar Medium (PGA) (Atlas,1993)	
Potato	200 g
Peptone	3 g
Glucose	20 g
Agar	20 g
pH	5.6 ± 0.2

Glucose Yeast Extract Medium (GY)	
Glucose	10.0 g
Yeast Extract	3.0 g
NaCl	0.5 g
$CaCO_3$	0.1 g
Agar	18.0 g
pH	6.8 ± 0.2

Chloramphenicol (0.25 mg/ 100 ml) was added after autoclaving.

Antimicrobial activities of isolated fungal strains (Phay, 1997)

The isolated endophytic fungi were grown on different media for 5 days. The fungal isolates were inoculated into seed medium and incubated at room temperature for 3 days. Ten ml of seed culture was transferred into the fermentation medium. The fermentation was carried out for 7 days. The fermented broth was used to check the antimicrobial activity against test organisms by paper disc diffusion assay.

After autoclaving, the conical flasks containing nutrient agar medium were cooled down to 30-35 ºC, 0.3 ml of test organisms were also added into the flasks and shaken and poured into each sterilized Petridishes. After solidification, paper disc impregnated with samples (fermented broth) were applied on the agar plates and the plates were incubated for 24-36 hours at room temperature.

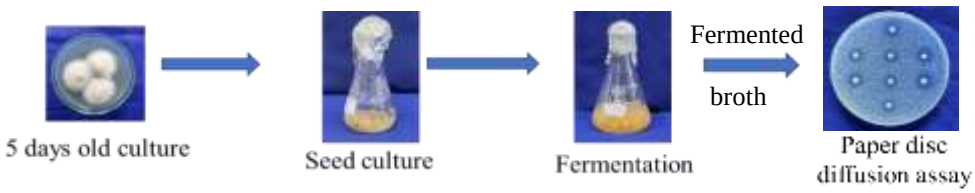


Figure.1. Steps in antimicrobial test of isolated fungi

Morphological and microscopical characters of isolated fungal strains (Barnett, 1998)

Isolated fungi grown on stock culture were transferred on to the plates containing the respective media. Then the plates were incubated at room temperature for 5-7 days. Margins and surface and reverse pigments of isolated fungi were studied for morphological characters and they were identified according to the references of Barnett 1998. The microscopical characters were studied at Microbiology Lab, Department of Botany, University of Yangon.

Fermentation studies

Effects of ages of culture on the fermentation by isolated fungi MM-25

The seed culture of MM-25 was incubated at room temperature. And then about 10 ml of seed culture was separately transferred into fermentation medium at one day intervals. Ages of culture with (1-day, 2-day, 3-day, 4-day, 5-day and 6-day) were utilized for fermentation 6 days. The antimicrobial activity was carried out paper disc diffusion method by using six test organisms (Strobel and Sullivan, 1999). (Figure-2).

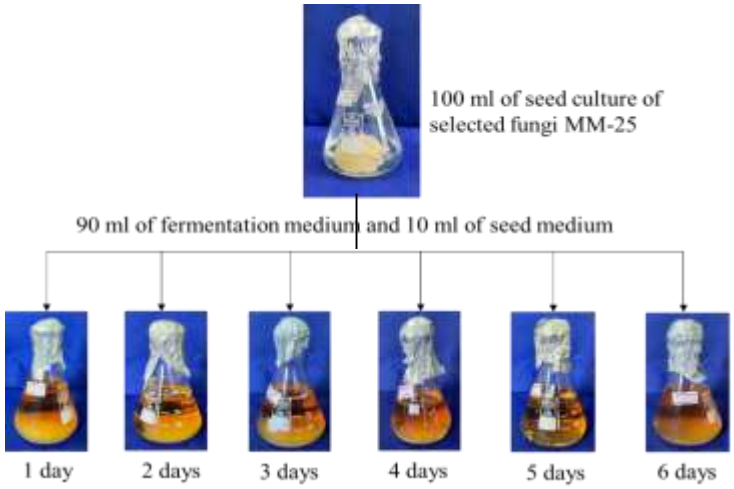


Figure 2. Seed culture and fermentation for age of inoculum

Effect of sizes of inoculum for the fermentation medium of isolated fungi MM-25

The proper cultivation and transfer (size of inoculum) are essential for the production of bioactive metabolites. A piece from fungal plate culture of strain MM-25 was inoculated into 125 ml conical flasks containing 100 ml of Glucose Yeast Extract (GY) medium. The flasks were incubated at room temperature for three days. After three days, the exact culture of the seed cultures was transferred into six conical flasks containing fermentation medium as shown in Figure 5. The fermentation was carried out for six days at room temperature in static state as well as shaking state. After the fermentation period, the fermented broth sample were subjected in the antimicrobial activity test.

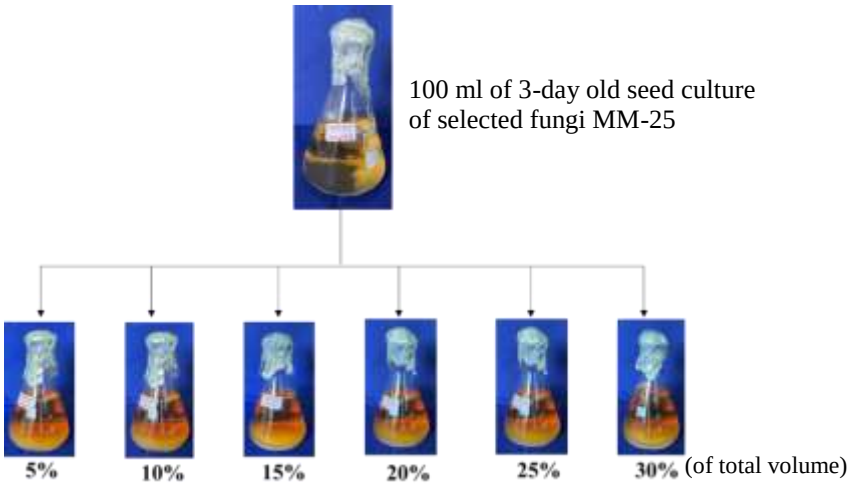


Figure 5. Seed Culture and fermentation for size of inoculum

Conditions of fermentation studies

The seed culture inoculated flask were examined under two fermentation conditions at room temperature. The first is static and the second is shaking condition (reciprocal condition by 180 stroke per minute).

Result

Figure 3. Habit of *Stephanotis volubilis* (L.f.) S.Ruess, Liede & Meve

Scientific Name	-*Stephanotis volubilis* (L.f.) S.Ruess, Liede & Meve
Synonyms	-*Dregea volubilis* (L.f.) Benth. Ex Hook.f. in Fl.Brit. India 4:46 (1883)
	-*Wattakaka volubilis* (L.f.) Stapf in Bot. Mag. 148: t. 8976 (1923)
Habit	-Climber
Distribution	-Wide
Common Name	-Gwedauk-nwe
Family	-Apocynaceae

Outstanding Characters

Lianas to 12 m. Branches pale gray, lenticellate, branchlets green, smooth. Leaves ovate, sparsely pubescent on the veins; Petiole 2.5-6 cm; leaf blade broadly ovate or suborbicular, 7-18 × 4-17 cm, glabrous or soft pubescent, base shallowly cordate, apex acute or short acuminate; lateral veins ca. 4 pairs. Inflorescences pendent, many flowered; peduncle 2-6 cm, slender, puberulent. Pedicel 2-2.5 cm; flowers green or yellowish green, fragrant. (Figure-8)

Sepals ovate-oblong, 2.5-3 mm, pubescent, ciliate. Corolla glabrous; lobes broadly ovate, 6-12 × 5-12 mm, obtuse, ciliate. Corona yellowish green, 4-4.5 mm in diam. Anther appendages white; pollinia oblong. Ovaries pilose. Follicles narrowly ovoid, 10-15 × 3-4 cm, longitudinally wrinkled-striate or irregularly ribbed. Seeds ovate, ca. 1.2 cm × 6 mm, flattened, marginate.

Flowering period: April-September

Fruiting period: July-December

Isolation of Endophytic Fungi

In the course of investigation of fungi, thirty one fungi were isolated from the leaves of *Stephanotis volubilis* (L.f.) S.Ruess, Liede & Meve (Gwedauk-nwe). These fungi were designated as MM-01 to MM-31. MM-01 to MM-03 were isolated from NA (Nutrient Agar) medium, MM-04 to MM-10 were isolated from PGA (Potato Glucose Agar) medium, MM-11 to MM-17 were isolated from SY (Sucrose/ Yeast Extract) medium and MM-18 to MM-31 were isolated from GY (Glucose/ Yeast extract) medium.

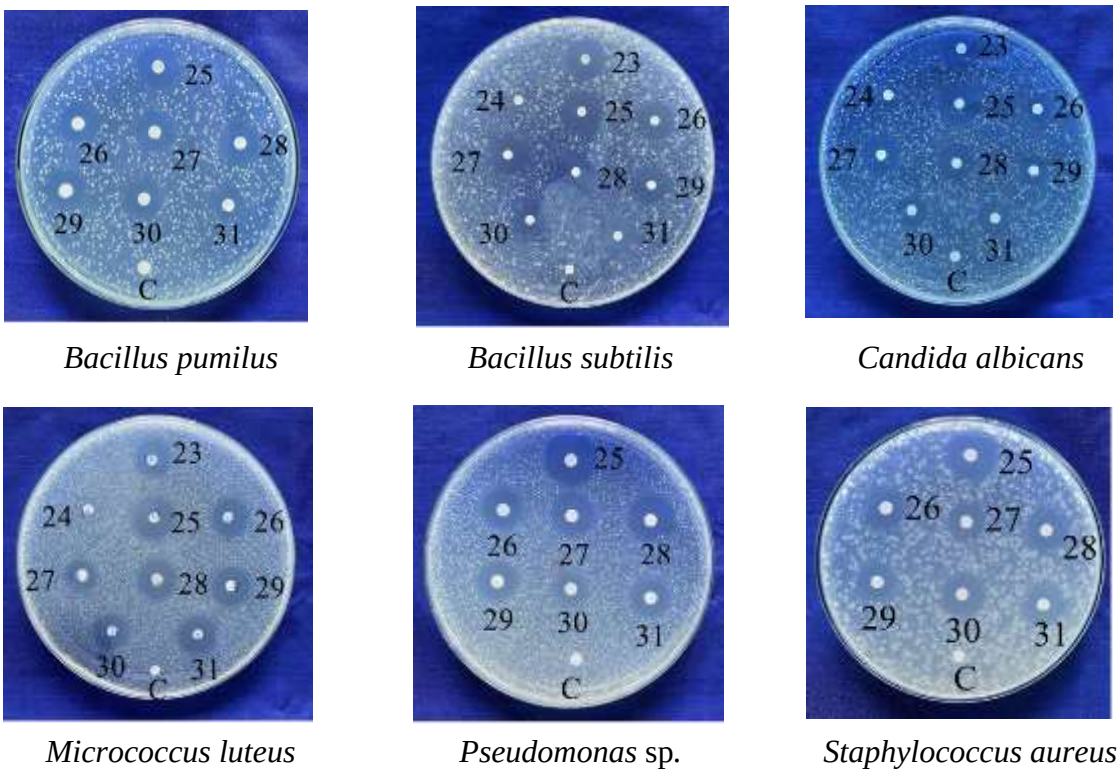
Antimicrobial activity of isolated endophytic fungi

To test the antimicrobial activity, all the endophytic fungi were cultured in respective broth for 2-6 days and their activities were examined against six test organisms. In this study, all strains (MM-01 to MM-31) showed the activities against *Bacillus pumilus, Bacillus subtilis, Candida albicans, Micrococcus luteus, Pseudomonas* sp. and *Staphylococcus aureus.* Among these strains, comparative analysis provided that MM-25 was the most efficient in producing antimicrobial potential as shown in Table 1 and Figure 4.

Table 1. Antimicrobial activities of isolated fungi with 4 days after fermentation by paper disc diffusion method (the numbers indicated the size of clear zones in mm)

	Bacillus pumilus	***Bacillus subtilis***	***Candida albicans***	***Micrococcus luteus***	***Pseudomonas sp.***	***Staphylococcus aureus***
MM-01	20	28	20	22	20	22
MM-02	20	26	21	20	20	21
MM-03	18	21	16	14	19	19
MM-04	19	23	18	18	18	19
MM-05	20	24	19	20	18	23
MM-06	10	22	17	13	15	18
MM-07	20	24	27	15	19	18
MM-08	16	22	15	15	13	14
MM-09	13	15	18	18	17	19
MM-10	12	17	17	12	15	13
MM-11	18	18	15	15	14	12
MM-12	18	21	15	17	14	15
MM-13	20	17	18	13	19	20
MM-14	19	23	20	20	15	20
MM-15	19	22	18	18	17	17
MM-16	17	21	20	20	16	17
MM-17	19	24	18	18	15	19
MM-18	20	18	20	21	19	16
MM-19	15	19	19	15	17	19
MM-20	14	22	19	14	15	18
MM-21	19	23	17	21	13	14
MM-22	17	21	20	20	17	18
MM-23	17	23	19	19	15	15
MM-24	-	-	-	-	-	-
MM-25	22	26	21	24	25	26
MM-26	20	24	14	20	19	21
MM-27	20	25	20	17	19	17
MM-28	20	25	20	21	22	20
MM-29	19	23	15	20	18	16
MM-30	19	25	16	17	15	20
MM-31	17	25	18	21	17	20

Antimicrobial activities of MM-25 with 4 days after fermentation by paper disc diffusion method



Bacillus pumilus *Bacillus subtilis* *Candida albicans*

Micrococcus luteus *Pseudomonas* sp. *Staphylococcus aureus*

Figure. 4. Inhibitory zones of isolated strains against six test organisms

Morphological and microscopical characters of strain MM-25

Colonies of MM-25 were circular to irregular; periphery was whitish filamentous. The inner zone was thicker, raised and white. The center was like a yellow brown doom. The creamy white color can be seen in reverse view of the colony and irregular filamentous edges were prominent. Under the microscope (400X) hyphae were septate, hyaline and multinucleate, branches that are produced at right angle.

Studies on fermentation parameters (age of culture and size of inoculum)

In the studies of fermentation parameters under static and shaking conditions up to 6 days, the observed antimicrobial activities were checked and comparatively shown in Table 2 to 5.

Effects of ages of seed culture on the fermentation by isolated soil fungi MM-25

In the age of inoculum, seed culture one day old, two-day old, three-day old, four-day old, five-day old and six-day old culture were used for the fermentation. According to the result, it was observed that three-day old culture of seed culture was suitable for the fermentation. (Table 2 & 3 and Figure 12).

Table 2. Effect of age of inoculum on antimicrobial activity (inhibitory zone in mm) against six test organisms (Static state)

Test organisms	One-day old culture	Two-day old culture	Three-day old culture	Four-day old culture	Five-day old culture	Six-day old culture
Bacillus pumilus	13	18.5	17.7	17	11	10.4
Bacilus subtilis	13	17.4	16.2	16.2	9.3	7.3
Candida albicans	12.5	15.8	17.4	15.3	14.2	8.8
Micrococcus luteus	9.5	16.5	20.8	19.1	13.7	9.3
Pseudomonas sp.	10	16.1	17.2	15.6	10.7	8.4
Staphylococcus aureus	12.8	18.9	22.9	18	10.2	9.3

Table 3. Effect of age of inoculum on antimicrobial activity (inhibitory zone in mm) against six test organisms (Shaking state)

Test organisms	One-day old culture	Two-day old culture	Three-day old culture	Four-day old culture	Five-day old culture	Six-day old culture
Bacillus pumilus	11.9	19.7	18.5	16.7	12.3	12.1
Bacilus subtilis	13.4	20.7	21.1	17.4	17	15.2
Candida albicans	11	17.1	18.4	17	14	13.2
Micrococcus luteus	10.7	18.6	20.9	16.5	16.4	9.6
Pseudomonas sp.	12.8	18.7	18.5	18.5	14	13.2
Staphylococcus aureus	10.7	19.7	22.1	21.5	14.7	13.9

Static state Shaking state

Figure 12. Inhibitory zones of three-day old seed culture of strain MM-25 against *Staphylococcus aureus*

Effect of sizes of inoculum for the fermentation medium of isolated fungi MM-25

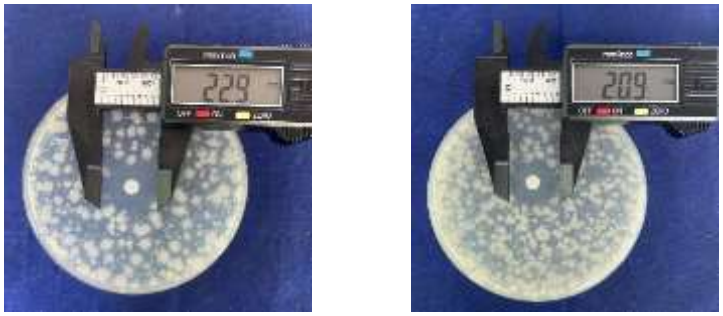
In the study of size of inoculum optimization, different size of inoculum (5%, 10%, 15%, 20%, 25% and 30%) were tested, 25% of the size of inoculum concentration showed the largest clear zone among other concentrations. Therefore, 25% of the seed culture was suitable for the fermentation to produce the bioactive metabolites (Table 4,5 and Figure 13).

Table 4. Effect of size of inoculum on antimicrobial activity (inhibitory zone in mm) against *Staphylococcus aureus* (Static state)

Days / Size of inoculum	1st day	2nd day	3rd day	4th day	5th day	6th day
5%	15.1	9.4	-	-	-	-
10%	18.3	17.2	16.8	16.2	13	-
15%	18	16.9	16.8	16.3	14.9	14.6
20%	22.9	22.3	18	16.9	16.4	14.6
25%	22.9	22.8	19.7	18.1	17	16.2
30%	20.7	19.6	18.7	15	14.7	-

Table 5. Effect of size of inoculum on antimicrobial activity (inhibitory zone in mm) against *Staphylococcus aureus* (Shaking state)

Days / Size of inoculum	1st day	2nd day	3rd day	4th day	5th day	6th day
5%	18	17.1	15.7	13.9	13.5	-
10%	18	17.8	17.6	16.3	16	15.8
15%	20.5	19	17.6	16.7	16.2	15.5
20%	18.1	17.5	17	16.4	15.9	14.9
25%	20.9	20.7	19.9	19.5	18.1	11.3
30%	15.9	15.6	15	14.7	-	-



Static state Shaking state

Figure 13. Inhibitory zones of size of inoculum of strain MM-25 against *Staphylococcus aureus*

Discussion and conclusion

In this present study, thirty one endophytic fungal strains were isolated from the leaves of *Stephanotis volubilis* (L.f.) S.Reuss, Liede & Meve (Gwedauk-nwe). Among thirty-one endophytic fungal strains, MM-01 to MM-03 were isolated from NA (Nutrient Agar) medium, MM-04 to MM-10 were isolated from PGA (Potato Glucose Agar) medium, MM-11 to MM-17 were isolated from SY (Sucrose/ Yeast Extract) medium and MM-18 to MM-31 were isolated from GY (Glucose/ Yeast extract) medium. According to the results of antimicrobial activity, endophytic fungi MM-25 showed the highest activity on different test organisms and it may be *Rhizoctonia* sp.

In Myanmar, Aye Pe (2001), Ni Ni Win (2011), Kyawt Kyawt Aung (2014), Kyi Kyi Khine (2014), Phoo Wint Yee Thaw (2015), Soe Soe Yu Hnin (2018), Hnin Wit Mhon (2018), Kay Thwe Lwin (2021) and Wint Yati (2022) have isolated many endophytic fungal (including *Rhizoctonia* sp.) and bacterial strains from different plant species to isolate the bioactive compounds and had good antimicrobial activity on *Bacillus pumilus, Bacillus subtilis, Candida albicans, Escherichia coli, Malassezia furfur, Micrococcus luteus, Pseudomonas* sp. *Salmonella typhi* and *Staphylococcus aureus.*

In the study of fermentation, seed culture (three-day old) of the age of inoculum was the best for fermentation. In the 1st day, the seed culture didn't show the high activity(< 13.4 mm) on the test organisms. It showed the high activity on *Bacillus pumilus* (18.5 mm) and *Bacillus subtilis* (16.2) on the 2nd day. It also showed the result with highest inhibition rate at the 3rd day on *Candida albicans* (17.4 mm), *Micrococcus luteus* (20.8 mm), *Pseudomonas* sp. (17.2 mm) and *Staphylococcus aureus* (22.9 mm) in the static state. In the shaking state, it showed the high activity on *Bacillus subtilis* and *Pseudomonas* sp. (18.5 mm) at 2nd day. It also showed the highest activity on *Bacillus pumilus* (18.5 mm), *Candida albicans* (18.4 mm), *Micrococcus luteus* (20.9 mm) and *Staphylococcus aureus* (22.1 mm) in 3rd day. In the size of inoculum optimization, fermentation (25%) showed the best antimicrobial activity on the test organism, *Staphylococcus aureus* (22.9 mm in static state and 20.9 mm in shaking condition) at 1st day of fermentation in both static and shaking state.

In conclusion, present study obtained thirty-one endophytic fungi which showed antimicrobial activities and were found to inhibit harmful diseases and infection causing agents such as *Bacillus pumilus, Bacillus subtilis, Candida albicans, Micrococcus luteus, Pseudomonas* sp., *Staphylococcus aureus*. Strain MM-25 may be *Rhizoctonia* sp. according to Barnett (1998) and it indicated the highest antimicrobial activity on tested pathogenic organisms.

In 2021, Sonawane *et al*., had reported that an endophytic fungi *Rhizoctonia* acted as facultative plant pathogen. But it may become a valuable medically important fungi because of the presence of 33 bioactive metabolites in *Rhizoctonia* were found to show active antimicrobial activity on gram positive *Staphylococcus aureus* as well as gram negative *Escherichia coli* bacteria. It is very interested that the extract of fermented broth of *Rhizoctonia* can give anticancer potentials on antileukemic activity, cardioprotective and antifungal activity. Therefore, these fungi may become one of the top targets to do research in the future.

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