Isolation and Effect of Ages and Sizes of Endophytic Fungus TFO-07 on *Agrobacterium tumefaciens*

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Abstract

In the present investigation, the isolation and effect of ages and sizes of endophytic fungus TFO-07 has been conducted. This fungus isolated from the petiole of *Vigna mungo* (L.) Hepper in Thae Phyu Village, Hinthada Township, Ayeyawady Region during June in 2020. The screening of endophytic fungus was carried out by surface sterilization method. In the study of effect of ages of inoculums (36 hrs, 48 hrs, 60 hrs, 72 hrs, 84 hrs, 96 hrs and 108 hrs) and 72 hrs seed culture showed the best activity on *Agrobacterium tumefaciens* than other seed culture. In the study effect of sizes of inoculum (5%, 10%, 15%, 20%, 25%, 30%) were used and it was determined that 25% size of inoculum gave the best activity *Agrobacterium tumefaciens*. The highest antibacterial activity reached at 6 days fermentation and antibacterial activity was tested by agar well diffusion assay method. This study reveals that the endophytic fungus serves as a potential source for the production of an effective bioactive compound.

Keywords: Isolation, age of inoculum, size of inoculum, antibacterial activity

INTRODUCTION

Fungi are the largest group of living organism present on the earth that has economic importance to human being, whether involved in production of various diseases in animals and plants as pathogen or as a source of various therapeutic compounds for the treatment of various diseases. A large number of compounds are produced by various genera of fungi that are used in day to day life besides serving as food. Endophytic fungi are also one such group which is emerging as a new and unexplored field for the isolation and production of medicinally important compounds (Tan and Zou, 2001).

Endophytes are group of microbes which are associated with plants and play very important role in defense mechanism and production of secondary metabolites. This group generally contains bacteria and fungi. Fungal endophytes are more important than bacterial once because of their larger role in plant metabolism. They have been isolated from large number of plant families all over the world. Reports are available in the literatures related to the production of large number of useful compounds in plants by endophytic fungi. Some researchers have even pointed out that the some medicinal chemicals produced by plants are infects produced by the fungal endophytes of that plant. They also play important role in plant defense mechanism by producing some toxic substance which hampers the growth of pathogens (Sharma, 2016).

Recently, legumes are gaining interest because they are excellent sources of bioactive compounds, which play a significant role as a nutraceuticals, pharmaceuticals, pesticides and industrial products. The genus *Vigna* belong to family fabaceae and more than 200 species comes under the genus *Vigna* that are of considerable economic importance in many developing countries. Annual worldwide production of the different *Vigna* species are 20 million hectares and major production is contributed by developing countries. These species are grown successfully in extreme environment conditions such as high temperatures, low rain fall and poor soils, with few economic inputs (Fery, 2002).

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Endophytic fungi are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural and industrial areas (Sowparthani and Kathiravan, 2011). Therefore, this study was devoted to investigation of endophytic fungus derived from *Vigna mungo* (L.) Hepper (Mat Pe) plant and aims at finding the antibacterial activity by isolated endophytic fungus TFO-07.

MATERIALS AND METHODS

Sample Collection

Vigna mungo (L.) Hepper (Mat Pe) plant was collected from Thae Phyu Village, (Lat 17º 40.274´ N and Long 95º 16.16´ E), Hinthada Township, Ayeyawady Region. Healthy plant sample was collected in plastic bags and pressed, labeled with date and site of collection until isolation procedure was completed. The collected plant sample was recorded by photographs for taxonomic description and identified the specimen according to the available literature such as Darwin, R., D. Hooker and D. Jackson, 1895.

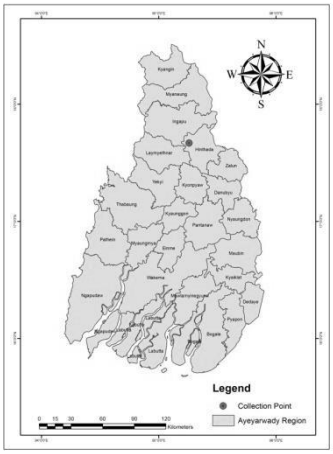


Figure 1. Map of Hinthada Township

(Source: Department of Geography, Pathein University)

Isolation of Endophytic Fungus TFO-07

The collected plant sample was carried out by surface sterilization methods (NITE, 2004). Plant sample was washed thoroughly in running tap water to remove adhering soil particles and microbial surface epiphytes and air dried before it was processed. Plant samples were cut into small fragments (approximately 5 × 5 mm) with a sterile sharp blade. To minimize the risk of isolating epiphytic fungi during sampling, the plant parts were surface sterilized by immersing them sequentially in 70% ethanol for 1 minute and then, also immerse 10% sodium hypochloride for 1 minute and rinsed thoroughly with sterile distilled water under aseptic conditions. Then, with a sterile scalpel, outer tissues were removed and the inner tissues of 0.5 cm size were carefully dissected and placed on petri-disc containing Czapek–Doz Agar (CZA) medium. The medium was supplemented with chloramphenicol to suppress bacterial growth. The Petri-disc was incubated at room temperature for three to seven days until fungal growth appeared.

Effect of Ages of Inoculum on the Fermentation

The selected fungus TFO-07 was grown on CZA medium at room temperature for 4 days and then was transferred into seed medium. Seed cultures of 36 hrs, 48 hrs, 60 hrs, 72 hrs,

84 hrs, 96 hrs and 108 hrs were inoculated into the flasks containing fermentation medium. The fermented broth (20 µL) was carefully added into the wells and incubated at room temperature for twenty four hours. The diameter of inhibitory zone around each well was measured and recorded after twenty four hours for antibacterial activity against *Agrobacterium tumefaciens*.

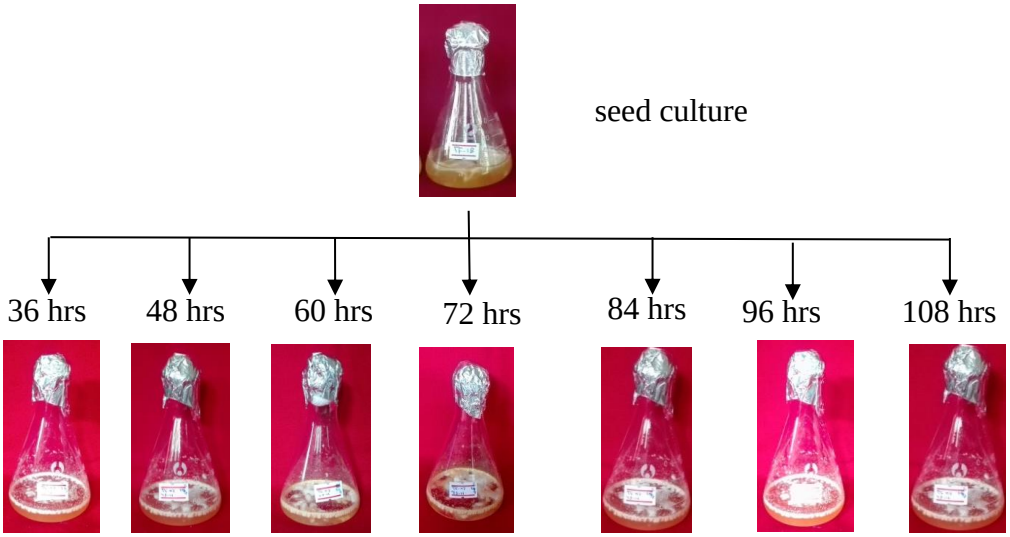


Figure 2. Procedure for the study of ages of inoculum

Effect of Sizes of Inoculum on Fermentation

In the sizes of inoculum, 5%, 10%, 15%, 20%, 25%, 30% of TFO - 07 seed cultures were utilized for the fermentation condition against *Agrobacterium tumefaciens*. Antimicrobial activity was tested by agar well diffusion assay method for four to ten days against *Agrobacterium tumefaciens.*

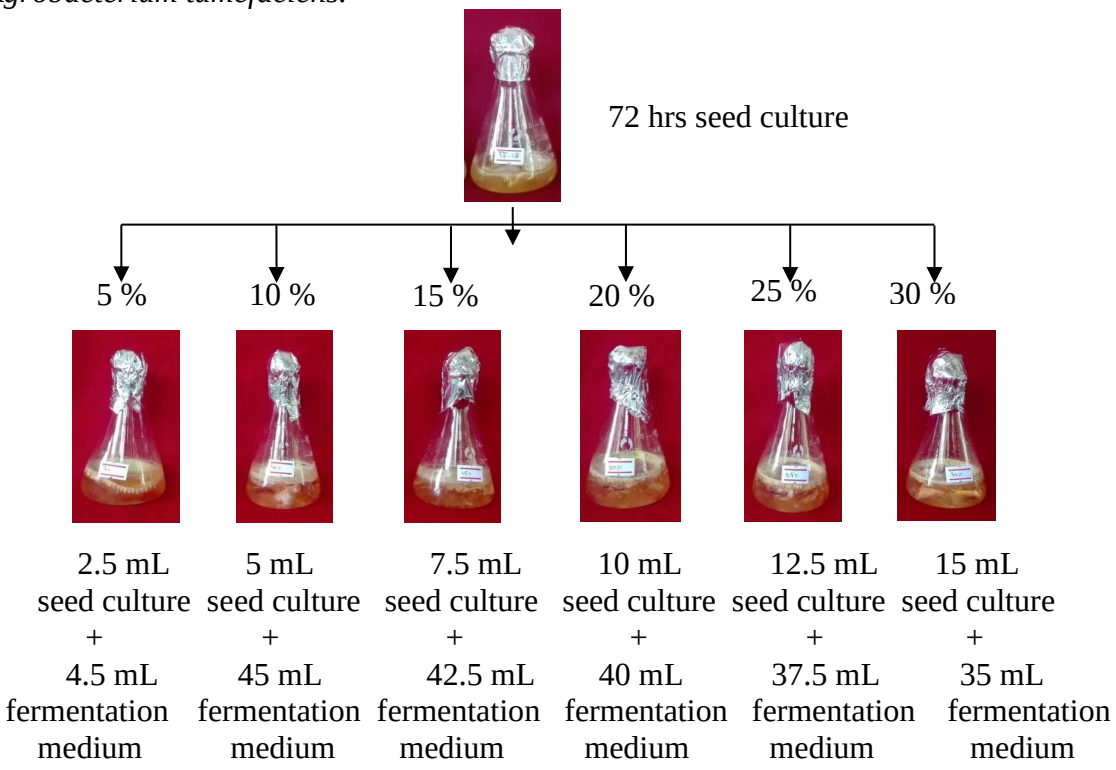


Figure 3. Procedure for the study of sizes of inoculum

Relationship between Cultivation Time and Dry Cell Weight

Fungal dry cell weight was measured by using digital balance on fermentation period one to ten days. The fungus mycelium from the fermentation medium was filtrated by using Whatman No. 1 filter paper for one night, and then fungus mycelium was measured for dry cell weight of the fungus (Ooijkaas *et al* ., 1998).

The dry cell weight of the fungus mycelium was calculated by using the following formula:

Dry weight = (weight of filter paper + mycelium) – (weight of filter paper)

The standard curve was prepared using the collective data.

Time course of Fermentation for the Production of Antibacterial Metabolites

Fermentation was undertaken using with suitable conditions of 72 hrs ages and 25 % sizes of inoculums. Antibacterial activity was tested by ager well diffusion assay method for 3 days to 10 days against *Agrobacterium tumefaciens*.

RESULTS

Sample Collection

The plant sample was collected from Thae Phyu Village in Hinthada Township, Ayeyawady Region. It is located at N 17º 40.274´ and E 95º 16.16´. Scientific name of this plant is *Vigna mungo* (L.) Hepper, which belonging to the Family Fabaceae. This plant is commonly known in Myanmar as Mat Pe.

Isolation of Endophytic Fungus TFO-07

In this research work, endophytic fungus was isolated from petiole of *Vigna mungo* (L.) Hepper. The endophytic fungus was isolated by surface sterilization method. The surface view is pale yellow in the center and edge white colour and reverse view is pale orange colour . Microscopic characters of fungus TFO-07 is septate mycelium, conidiosphore long, conidia elongate shape, cluster conidia Figure 4.

Figure 4. Colony morphology and photomicrograph of fungus TFO-07

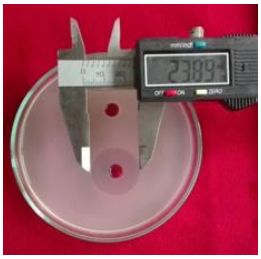
Effect of Ages of Inoculum on the Fermentation

In the study for the effect of ages of inoculum, it was found that 72 hours age give the highest activity (Figure.5 and Table 1).

36 hrs

48 hrs

60 hours



72 hours

84 hours

96 hours

108 hours

Figure 5. Effect of ages of Inoculum on fermentation TFO - 07 against *Agrobacterium tumefaciens*

Table1. Effect of Ages of Inoculum on Fermentation TFO - 07 against ***Agrobacterium tumefaciens***

Ages (hours)	**Inhibitory zone (mm)**
36	17.09
48	19.52
60	20.39
72	23.89
84	20.60
96	18.82
108	18.15

Agar well – 8 mm

Effect of Sizes of Inoculum on Fermentation

In this study, it was observed that 25 % size of inoculum is the best for the fermentation (Figure-6 and Table- 2).

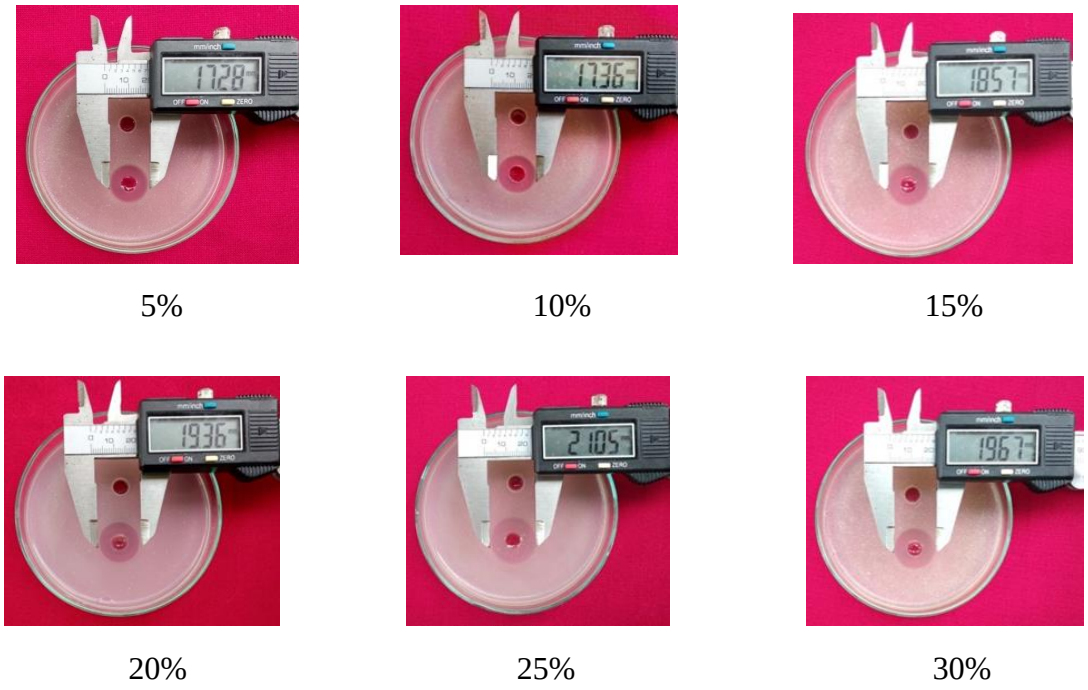


Figure 6. Effect of sizes of inoculum on fermentation TFO-07 against *Agrobacterium tumefaciens*

Table2. Effect of Sizes of Inoculum on Fermentation TFO-07 against ***Agrobacterium tumefaciens***

Sizes of inoculums (%)	**Activity (Clear zones, mm)**
5	17.28
10	17.36
15	18.57
20	19.36
25	21.05
30	19.67

Agar well – 8 mm

Relationship between Fermentation Time and Dry Cell Weight

In the study of relationship between fermentation time and dry cell weight, the highest dry cell weight was six days fermentation (Figure-7).

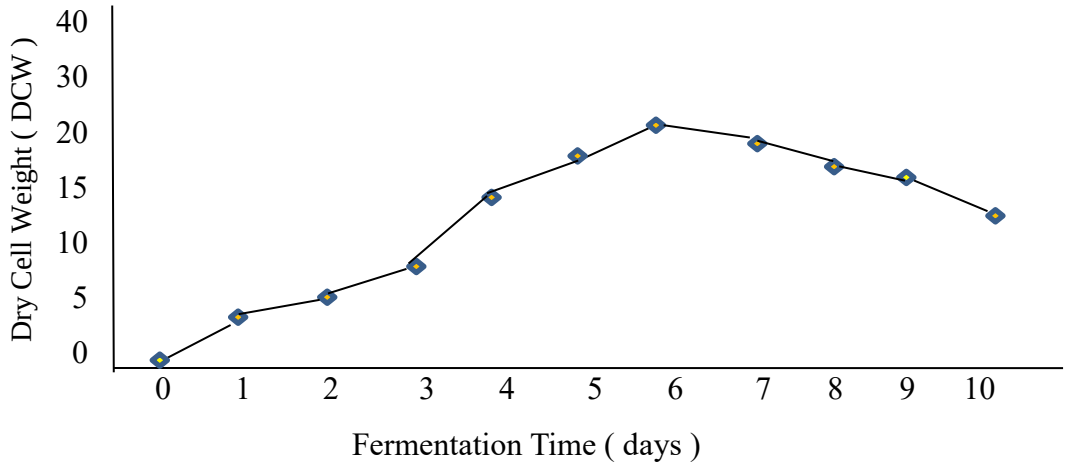


Figure 7. Relationship between fermentation time and dry cell weight

Time course of Fermentation for the Production of Antibacterial Metabolites

Time course of fermentation such as relationship between fermentation time and antibacterial activity, and relationship between fermentation time and pH, 6 days of fermentation period is the maximum activity (22.58 mm) at pH 5.7 (Figure-8 and Table-3).

3 day fermentation period

4 day fermentation period

5 day fermentation period

6 day fermentation period

7 day fermentation period

8 day fermentation period

9 day fermentation period

10 day fermentation period

Figure 8. Time course of fermentation

Table 3. Time course of Fermentation for the Production of Antibacterial Metabolites

Fermentation Time (days)	**Inhibitory Zone (mm)**	**DCW (%)**	**pH**
1	-	3.0	6.0
2	-	6.0	5.9
3	12.35	9.5	5.9
4	15.98	15.1	5.8
5	19.05	18.0	5.7
6	22.58	20.0	5.7
7	20.35	19.1	5.6
8	19.52	17.2	5.5
9	18.84	16.0	5.5
10	17.72	14.1	5.4

(Agar well – 8 mm)

DISCUSSION AND CONCLUSION

Xiao *et al*., 2014 stated that endophytic fungi are considered valuable sources of metabolites production having pharmaceutical importance. Many of commercially potential and bioactive metabolites including those against various cancer cell lines are produced by endophytic fungi.

Proper cultivation age and size are also crucial for the production of primary and secondary metabolites (Omura,1985 and Crueger,1989). In this investigation seven different hours of 36 hrs, 48 hrs, 60 hrs, 72 hrs, 84 hrs, 96 hrs and 108 hrs were consumed (Figure-5 and Table -1). For the size of inoculum 5%, 10%, 15%, 20%, 25%, 30% were used respectively (Figure-6 and Table -2). According to the results from this study, it is considered that the optimum age of inoculum is 72 hrs (23.89 mm) and optimum size is 25% (21.05 mm).

Biomass is a fundamental parameter in the characterization of microbial growth (Suraini *et al*., 2008). Time course of fermentation such as relationship between fermentation time and antibacterial activity, and relationship between fermentation time and pH, 6 days of fermentation period is the maximum activity (22.58 mm) at pH 5.7 (Figure –8 and Table – 3).

Verna *et al*., 2014 said that the culture filtrate recovered after 6 days of incubation was acidified in the pH range (1-6). The period of 6 days fermentation, biomass was constantly increased up (20.0 % dry wt/100 mL followed by a decline (Figure-7 and Table – 3).

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