STUDY ON THE ISOLATION OF SOIL FUNGI PRODUCING ANTIMICROBIAL METABOLITE FROM TU MYAUNG VILLAGE IN LABUTTA TOWNSHIP

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Abstract

In this research paper, fifteen soil fungi were isolated from four different soil samples of Tu Myaung village in Labutta Township of Ayeyarwady Region. A total of fungi were isolated on Low Carbon Agar (LCA) medium and Potato Glucose Agar (PGA) medium by using feeding and chemical treatment dilution methods. These isolated fungi were tested on seven test organisms by using paper disc diffusion assay method for the screening of antimicrobial activities. In the biological properties of isolated fungi, HY-08 showed highest antibacterial activity of clear zone (27.03 mm) against *Bacillus pumilus.* Therefore, this fungus (HY-08) was selected for further investigation. This fungus was studied for the age of culture and size of inoculum. According to the result, 60 hrs of ages and 20 % of sizes of inoculum were suitable for the best bioactive metabolite production of selected fungus.

Keywords: Feeding method, Chemical Treatment Dilution method, Paper disc diffusion assay method

Introduction

Soil microbiology is the study of organism in soil, their function and how they affected soil properties. Soil microorganisms can be classified as bacteria, actinomycetes, fungi, algae and protozoa. Each of these groups has characteristics that define them and their function in soil (Omura, 1985).

Continuous use of chemical fertilizers over a long period may cause imbalance in soil mycoflora and thereby indirectly affect biological properties of soil leading to soil degradation. Fungi are an important component of the soil micro biota. Micro fungi play a focal role in nutrient cycling by regulating soil biological activity (Vinay *et al.*, 2015).

Distribution of soil fungi depend upon the nature of the organic content, climatic condition, surface vegetation and soil texture. Direct relationship is observed between the soil texture and moisture content. Silt and clay soil holds the highest moisture content that's why there is increased population of fungi is observed (Marchner *et al.*, 2003).

Antibiotics (metabolites) may be more useful than synthetic chemicals in the treatment and control of diseases. These metabolites are produced from microorganisms such as fungi, bacteria and actinomycetes (Kurtzman, 1992).

Microorganisms are the important sources of bioactive compounds with enormous potential to be developed as new molecules for drug discovery. Microorganisms grow in unique and extreme habitats that provide them the capability to produce unique and unusual metabolites. The antimicrobial properties of secondary metabolites from various groups of fungi are widely reported. Several fungal species produce bioactive compounds, secondary metabolites and

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chemical models having pharmaceutical importance. Antibiotics can be classified according to their mode of actions (Lambert, 1977).

Microbial growth kinetics is necessary to understand before fermentation to produce the metabolites. Proper cultivation ages and sizes are also crucial for the production of primary and secondary metabolites (Omura, 1985).

The composition of the fermentation medium must include the nutrient essential to support the growth of the microbial strain and the formation of the desired product. Production of secondary metabolites from microbial sources is greatly influenced by cultural and nutritional conditions used in the fermentation process (Sanchez *et al*., 2010).

Therefore, the aim and objectives of this research are to find out the various fungi in different soil samples, to observe the morphological characters, to study the antimicrobial activity and to know the proper cultivation ages and sizes of these isolated fungi.

Materials and methods

I. Area of Study

The soil sample was collected at Tu Myaung Village of Labutta Township in Ayeyarwady Region, July 2020. Tu Myaung village located coordinates in degrees, minutes and seconds (DMS) of 16° 21′ 00.9″N and 94° 41′ 38.9″E.

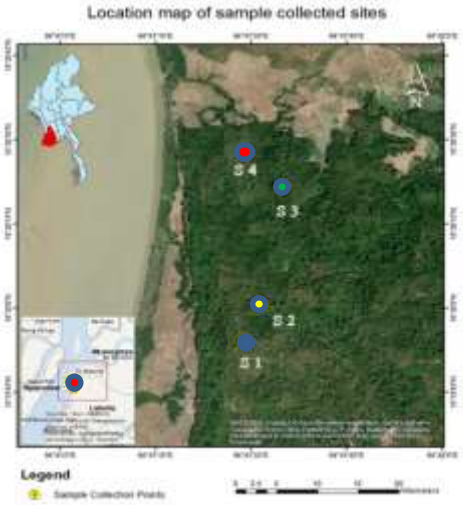


Figure.1. Tu Myaung village in Labutta township

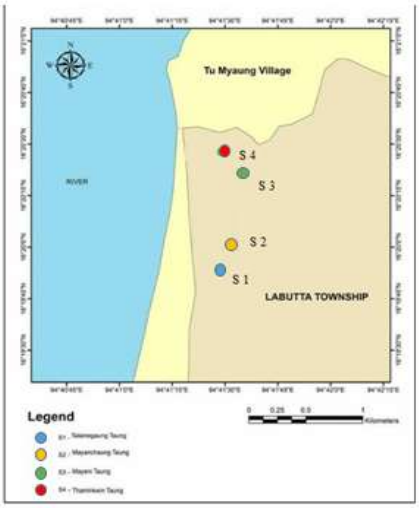


Figure.2. Tu Myaung village sites of soil samples

II. Collection of Soil Samples

Soil samples were collected from different forest fields (15 cm depth) with the help of spatula, in sterile plastic bags. The samples were properly labeled and brought to the Microbiology Laboratory, Department of Botany, Pathein University for further studies. The collected soil samples were transported to Department of Agriculture (Land Use) Yangon Township for tested the analytical texture and pH.

Table 1. Four different samples collected from four different places

Soil sample No.	Collected place	Soil type	pH	Location	Collected date
1.	Talainegaung taung	Clay	5.16	N 16˚19′ 43.897 ″ E 94˚41′ 19.478 ″	17.7.2020
2.	Mayanchaung taung	Clay	5.37	N 16˚20′ 52.012 ″ E 94˚41′ 40.527 ″	17.7.2020
3.	Mayeni taung	Clay loam	5.59	N 16˚20 ′ 34.362 ″ E 94˚41 ′ 30.584 ″	18.7.2020
4.	Thaminkwin taung	Clay	5.09	N 16˚20 ′ 26.463 ″ E 94˚41 ′ 32.486 ″	18.7.2020

III. Feeding Method (NITE, 2004)

Soil sample (1.0g) was poured into 100 mL SLN culture medium (Glucose 0.2g, sucrose 0.2g, K_2HPO_4 0.1g, $MgSO_4.7H_2O$ 0.05g, KNO_3 0.1g, KCl 0.05g, DW 100mL) and it was incubated overnight. Next day, 0.1mL soil suspension was transferred into 5mL of sterile water tube and shaked for minutes. And then, 0.5mL suspension was transferred into 4.5mL sterile water tube. Then 1.0 mL suspension was also transferred into 4.0mL sterile water tube. After shaking for minutes, 30 µL samples were cultured on low carbon agar medium (LCA medium) plates (Glucose 0.2g, Sucrose 0.2g, K_2HPO_4 0.1, KNO_3 0.1g, KCL 0.05g, Agar 1.8g, DW 100mL) and incubated for 5 - 10 days.

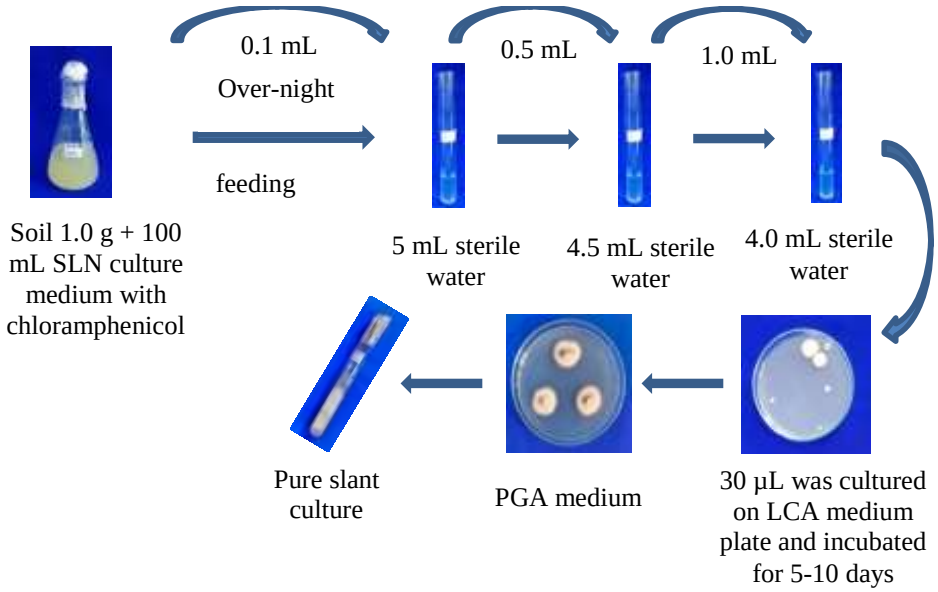


Figure.3. Procedure of feeding method for isolation soil fungi

IV. Chemical Treatment Dilution Method (Harayama and Kobayashi, 2005)

The collected soil samples were air-dried at room temperature, grounded and sieved. And then 2 g of soil samples was added into the 4 mL of sterile water tube. After 6 hours, 14 mL of 70% ethanol were added into this tube and then settle for 1 minute. 0.1 mL soil suspension from this tube was transferred into the 5 mL of sterile water tube, and then 0.5 mL soil suspension was transferred into the 4.5 mL of sterile water tube. After that, 1.0 mL soil suspension was transferred

into the 4.0 mL of sterile water tube and 30 µL soil suspension of this tube was placed on LCA medium plate. The plate was incubated for 3-5 days. After the colonies of fungi were observed on medium surface, isolated on PGA medium and stored as the slant cultures.

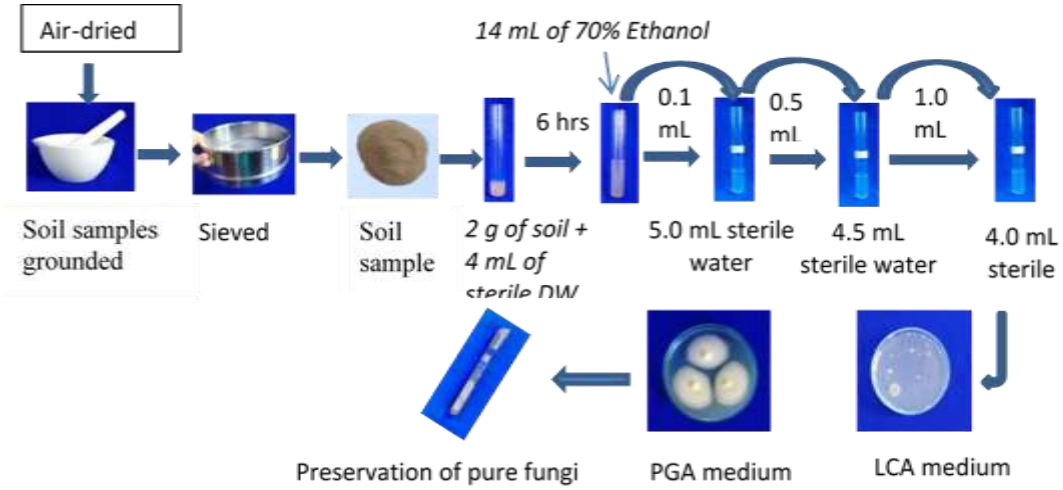


Figure.4. Procedure of chemical treatment dilution method for soil fungi

V. Preliminary Antimicrobial Activity by Paper Disc Diffusion Assay Method (Tomita, 1988)

The isolated fungi were incubated on PGA medium at room temperature for 3 days. Twenty mL of seed culture was transferred into the fermentation medium and incubated at room temperature for 5 days. 20 µL of fermented broth was put on the paper disc and placed on assay plate containing test organisms.

Table 2. Test organisms used in antimicrobial activities

No.	Test organisms	Diseases
1	*Agrobacterium tumefaciens*	Plant disease
2	*Bacillus pumilus*	Fever
3	*Candida albicans*	Candidosis
4	*Escherichia coli*	Diarrhoea
5	*Micrococcus luteus*	Skin disease
6	*Pseudomonas fluorescens*	Rice disease
7	*Staphylococcus aureus*	Boils and Food poisoning

Study on the Microbial Growth Kinetics of Fungus HY-08 (Omura, 1985; Crueger and Crueger, 1989)

The fungus HY-08 was inoculated into 100 mL medium (Glucose 1.0 g, Yeast extract 0.2 g, NZ amine type 0.3 g, pH 6.5) and incubated for 108 hrs at 100 rpm rotary shaker. The culture sample (5 mL) was checked in 12 hrs intervals for the growth. The sample (5 mL) was centrifuged at 2000 rpm for 30 mins and PCV% (Packed Cell Volume) was calculated.

Study on the Effects of Ages of Inoculum for the Fermentation (Omura, 1985; Crueger and Crueger, 1989)

A pure culture of selected fungus was transferred into the seed medium. According to the growth kinetic results, seed cultures were transferred into the fermentation medium at the incubation time (36 hrs, 48 hrs, 60 hrs, 72 hrs and 84 hrs), and tested antibacterial activities by using paper disc diffusion assay method for 7 days.

Study on the Effects of Sizes of Inoculum for the Fermentation (Omura, 1985; Crueger and Crueger, 1989)

A pure culture of selected fungus was transferred into the seed medium. 5%, 10%, 15%, 20%, 25%, 30% and 35% of seed culture (60 hrs) were transferred into the fermentation medium, and tested antibacterial activities by using paper disc diffusion assay method for 7 days.

Seed medium		**Fermentation medium**	
Glucose	2.0 g	Glucose	2.0 g
Peptone	0.3 g	Yeast extract	0.8 g
KNO_3	0.1 g	K_2HPO_4	0.01 g
K_2HPO_4	0.1 g	$MgSO_4$	0.01 g
DW	100 mL	$CaCO_3$	0.1 g
pH	6.5	DW	100 mL

Results

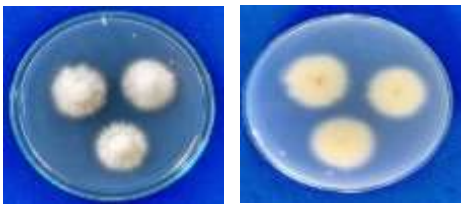
The results revealed the colony morphological characters of fungi from soil samples of Tu Myaung village. A total number of 15 fungi were isolated by chemical treatment dilution and feeding methods.

Table 3. Isolation of soil fungi from four different soil places

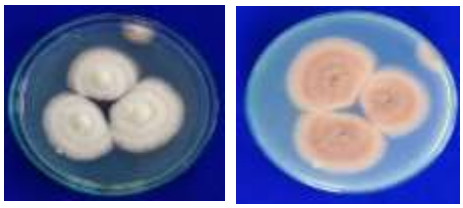
Soil samples	**Soil types**	**Isolated fungi by two methods**	
		Chemical treatment dilution method	**Feeding method**
S-1	Clay	HY-01 to HY-02	HY-03 to HY-04
S-2	Clay	HY-05 to HY-07	HY-08 to HY-09
S-3	Clay loam	HY-10	HY-11 to HY-12
S-4	Clay	HY-13 to HY-14	HY-15
	Total	**8 fungi**	**7 fungi**

Table 4. Morphological colours of isolated fungi

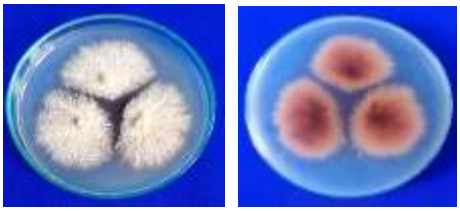
No.	Isolated Fungi	Morphological Color	
		Front Color	**Reverse Color**
1	HY - 01	White	White
2	HY - 02	White	Brown
3	HY - 03	Whitening pink	Reddish
4	HY - 04	Yellow	Yellowish
5	HY - 05	Yellow	Golden yellow
6	HY - 06	Centre yellow, white at margin	Yellow
7	HY - 07	Centre green, white at margin	Greenish yellow
8	HY - 08	White	Cream
9	HY - 09	Purple	Yellowish
10	HY - 10	Dark brown	Yellowish brown
11	HY - 11	Yellow	Yellowish
12	HY - 12	Cream	Yellowish
13	HY - 13	White	White
14	HY - 14	Gray	Grayish
15	HY - 15	Center green, white at margin	Pale green



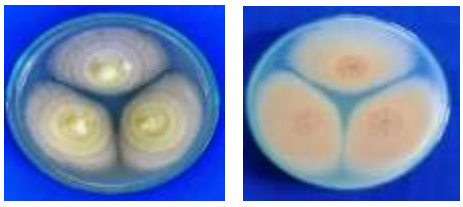
(Front and reverse view of HY-01)



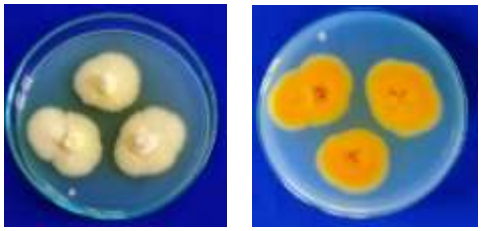
(Front and reverse view of HY-02)



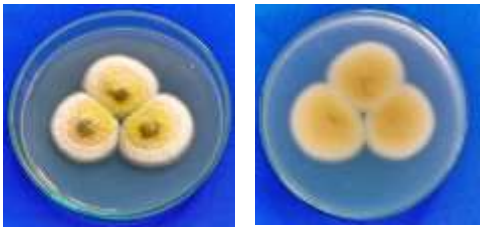
(Front and reverse view of HY-03)



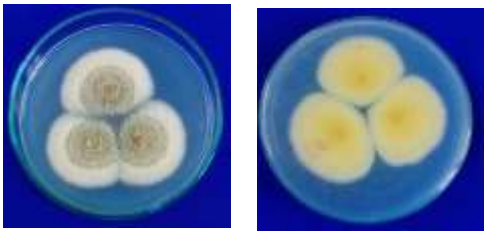
(Front and reverse view of HY-04)



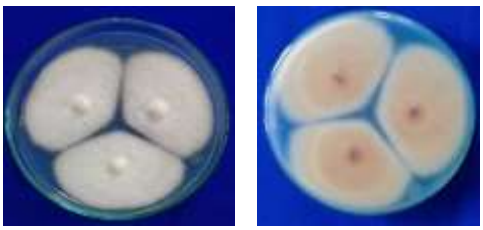
(Front and reverse view of HY-05)



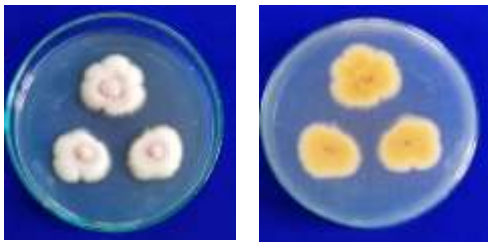
(Front and reverse view of HY-06)



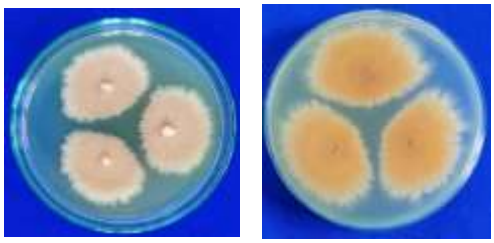
(Front and reverse view of HY-07)



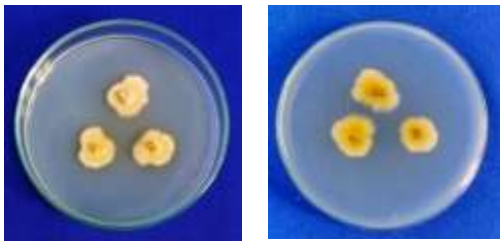
(Front and reverse view of HY-08)



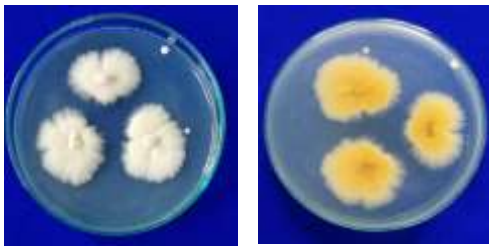
(Front and reverse view of HY-09)



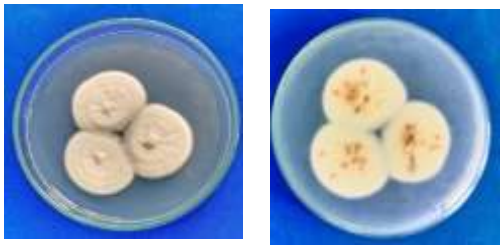
(Front and reverse view of HY-10)



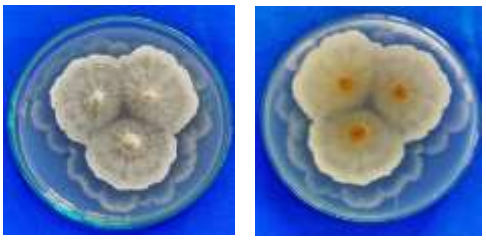
(Front and reverse view of HY-11)



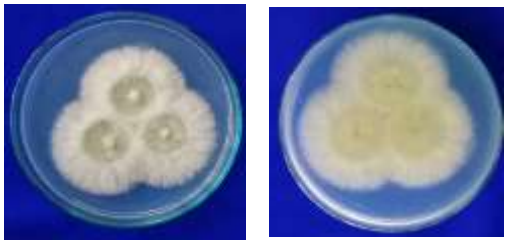
(Front and reverse view of HY-12)



(Front and reverse view of HY-13)



(Front and reverse view of HY-14)



(Front and reverse view of HY-15)

Figure 5. Morphological characters of isolated fungi (7 days old culture)

Table 5. Preliminary of antimicrobial activities on seven test organisms

Fungi No.	Antimicrobial activities on test organisms (inhibitory zone, mm)						
	A. tumefaciens	***B. pumilus***	***C. albicans***	***E. coli***	***M. luteus***	***P. fluorescens***	***S. aureus***
HY-01	-	20.48	20.16	20.07	-	14.76	-
HY-02	19.00	21.53	21.53	-	18.32	15.77	10.00
HY-03	-	20.85	21.34	19.95	-	14.56	17.80
HY-04	-	22.13	24.28	-	14.74	19.34	-
HY-05	-	24.19	21.04	-	-	12.32	-
HY-06	12.56	25.22	24.13	22.50	14.32	14.62	-
HY-07	-	25.40	19.40	-	15.86	14.96	18.12
HY-08	12.24	27.03	26.79	26.22	23.25	-	-
HY-09	-	25.82	25.21	-	18.97	13.20	-
HY-10	-	23.85	25.00	-	22.75	10.76	15.70
HY-11	-	20.77	25.35	21.71	16.46	-	12.28
HY-12	-	21.00	-	24.42	14.34	12.43	-
HY-13	-	18.31	20.02	-	-	-	-
HY-14	-	24.17	-	-	-	-	14.36
HY-15	-	17.34	21.22	-	15.02	-	-

(-) = No activity Paper disc size = 8 mm

Bacillus pumilus *Candida albicans* *Escherichia coli* *Micrococcus luteus* *Pseudomonas fluorescens*

Figure 6. Antimicrobial activities of isolated fungi on test organisms

The Effect of Ages of Inoculum on Selected Fungus HY-08 against *Bacillus pumilus*

According to the growth kinetic results, the selected fungus HY-08 was studied at the incubation times (36 hrs, 48 hrs, 60 hrs, 72 hrs and 84 hrs) for the age of seed culture.

Table 6. The effects of ages of inoculum HY-08 antibacterial activity against *Bacillus pumilus*

No.	Culture Times (Ages of culture)	Antibacterial Activity (Clear zone, mm)
1.	36 hrs	24.15
2.	48 hrs	25.92
3.	60 hrs	30.21
4.	72 hrs	26.58
5.	84 hrs	25.46

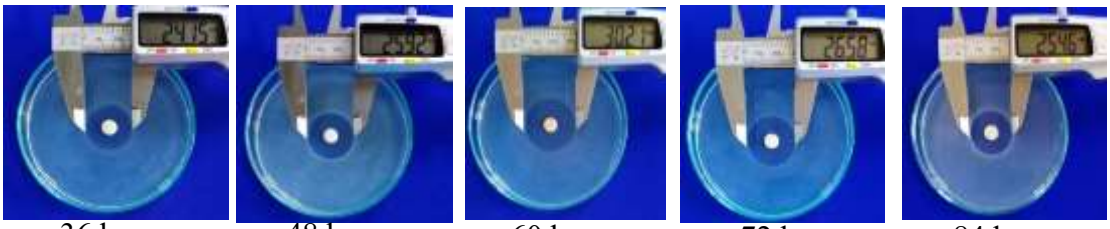


Fig.7. Antibacterial activity of ages of inoculum on *B. pumilus*

The Effect of Sizes of Inoculum on Selected Fungus HY-08 against *Bacillus pumilus*

In this study, 5%, 10%, 15%, 20%, 25%, 30% and 35% of seed cultures were utilized for the antibacterial activity. Incubated seed cultures were transferred as the above sizes into the fermentation conical flasks. Antibacterial activities were tested for seven days. The highest activity showed at 20% sizes of inoculum.

Table 7. The effects of sizes of inoculum HY-08 antibacterial activity against *Bacillus pumilus*

No.	Culture Times (Sizes of culture)	Antibacterial Activity (Clear zone, mm)
1.	5 %	21.00
2.	10 %	22.18
3.	15 %	23.12
4.	20 %	32.19
5.	25 %	26.72
6.	30 %	28.03
7.	35 %	25.35

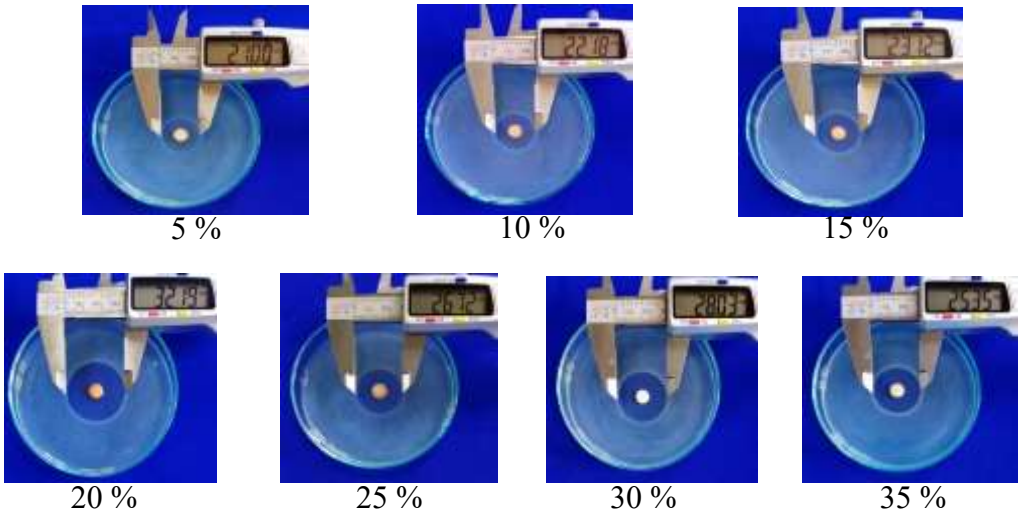


Figure.8. Antibacterial activity of sizes of inoculum on *B. pumilus*

Discussion and conclusion

Soil microorganisms have continually been screened for their useful biological active metabolites, such as antibiotics since long ago. Antibiotics have an important role in human health. Their necessity emerged from the spread of various diseases. As a result, scientists are trying to produce and discover more antibiotics. To help this purpose in some way, this research study was firstly started to discover antibiotics from four different soil samples from Tu Myaung village. The purification of the culture was done either by single spore isolation or by culturing of the hyphal tips on the LCA medium and then was transferred to fresh agar plates of PGA medium. Gaddeyya *et al.* (2012) reported that PGA or PDA medium is the most commonly used culture media and it was stated to be the best media for mycelia growth due to its simple formulation and potential to support wide range of fungal growth.

Two fungi from soil 1, two fungi from soil 2, two fungi from soil 3 and one fungus from soil 4 were also cultured by feeding method. In addition, two fungi from soil 1, three fungi from soil 2, one fungus from soil 3 and two fungi from soil 4 were isolated respectively by using chemical treatment dilution method. The pure isolated fungi were preserved by slants culturing.

In the report of Roberts (1998), antimicrobial agents play the most important role in the treatment of microbial infections and wide spread efforts have been carried out by many scientists in order to screen for novel antibiotic producing microbes. The emergence of new diseases and reemergence of multiple-antibiotic resistance pathogens that render the effectiveness of existence clinically used antibiotics have spurred the needs for the discovery of new antibiotics. According to this aim, the isolated fungi were tested with seven test organisms; *Agrobacterium tumefaciens, Bacillus pumilus, Candida albicans, Escherichia coli, Micrococcus luteus, Pseudomonas fluorescens and Staphylococcus aureus,* by using paper disc diffusion assay method. Exceptively *Agrobacterium tumefaciens* and *Staphylococcus aureus,* nearly all isolated fungi showed more antimicrobial activities on *B. pumilus* and *C. albicans*. Among these fungi, HY-08 was against more on *B. pumilus* than the other isolated fungi. So, this soil fungus (HY-08) was selected for further studies.

Borsa reported that *Bacillus pumilus* is a bacterium, though rarely, as the causative agent of various infections such as sepsis, endocarditis, skin infections and food poisoning in human. So, the study was continued against on *B. pumilus*.

Growth media and incubation conditions have a very strong influence of secondary metabolite production. Therefore, the growth kinetic was studied for the optimization of inoculum age. The selected fungus HY-08 was cultured at the incubation times (36 hrs, 48 hrs, 60 hrs, 72 hrs and 84 hrs) and tested the antibacterial activities. The highest antibacterial activity was found at 60 hrs (30.21 mm). For the proper size of inoculum, 5%, 10%, 15%, 20%, 25%, 30% and 35% of seed culture were inoculated and tested the antibacterial activities. The highest activity showed at 20% (32.19 mm) size of inoculum.

Variations in the fermentation environment often result in an alteration of antibiotic production. The alteration involves changes both in yields and in the composition of the substances. Therefore, the selected fungus HY-08 will be cultured base on these above results for further studies such as identification, extraction and purification of antibacterial compounds. This study may also contribute in providing information on the antibiotic producing microorganisms in soil.

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