ISOLATION AND FERMENTATION CONDITIONS OF SELECTED SOIL FUNGUS TT-27 BETWEEN YENANCHAUNG AND MAGWAY TOWNSHIP, MAGWAY REGION

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Abstract

Six soil samples were collected from six different places of Magway and Yenanchaung Township, Magway Region. Twenty-nine fungi were isolated from these soil samples. Isolation of soil fungi were carried out in Biotechnology Development Center (BDC) from Pathein University. Isolations of fungi were undertaken by the serial dilution method, and cultured on Blakeslee's Malt Extract Agar (BMEA Medium), Czapek-Dox Agar (CZA Medium) and Malt Extract Agar (MEA Medium). The isolated fungi were given as TT-1 to TT-29. In the preliminary study of antimicrobial activity, all fungi were evaluated by agar well diffusion method with seven test organisms. Among them, four fungi TT-22, 23, 24 and 27 exhibited the highest antimicrobial activities. Especially, TT-27 showed the highest antimicrobial activity (39.10mm) on *Escherichia coli*. Therefore TT-27 was selected and the fermentation conditions of TT-27 were carried out by the study of fermentation period, proper age, size, different carbon and nitrogen sources, fermentation medium (FM), effects of pH, temperature, static and shaking culture.

Keywords: soil fungi, antimicrobial activity, fermentation

Introduction

Soil fungi play an important role as major decomposers in the soil ecosystem. Fungi are an important component of the soil microbiota typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions. (Hawksworth *et al*., 1995). Fungi are considered as a good natural source for a production of bioactive secondary metabolites that contain different bioactive agents including antibiotics, antitumor and antioxidants (Elaasser *et al.*, 2011). The secondary metabolite is obtained by fermentation process. During fermentation, the organisms produce the antibiotic material, which can then be isolated for used as a drug (Fenical, 1993).

Fermentation is a metabolic process that produces chemical changes in organic substrates through the action of enzymes. The media used in fermentation process can either be synthetic or complex. Complex media are often used in enzyme and antibiotic production (Chistic, 1999). The composition of the fermentation medium must include the nutrient essential to support the growth of the microbial strains and the formation of the desired products. The fungi have been widely studied for their bioactive metabolites and sources of noval anticancer, antibacterial and anti-viral agents. Therefore, the present study was carried out the isolation and fermentation studies for antibacterial compounds from selected fungus. The aim and objectives of this study were to isolate the soil fungi from six different places, to observe the antimicrobial activities of selected fungi on seven test organisms and to know the optimal fermentation conditions of fungus TT-27.

Materials and Methods

Study Area and Collection of Soil Samples

Soil samples were collected from six different places between Yenanchaung and Magway Township, Magway Region from July, 2017 to August, 2017. These samples were isolated by using three media (MEA, BMEA and CZA).

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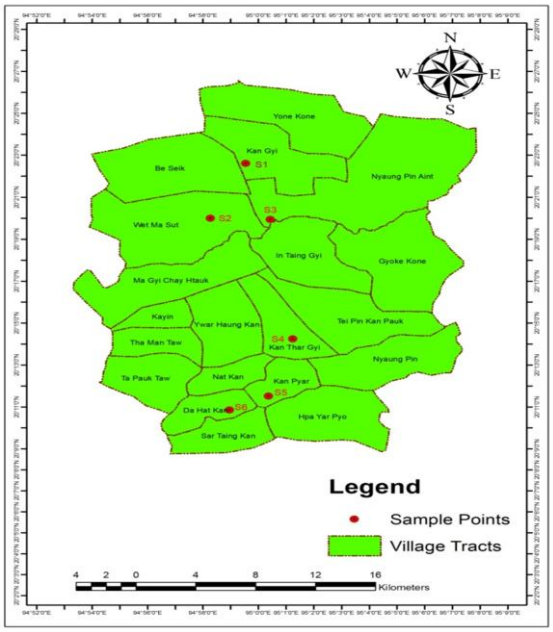


Source from Geography Dept. Pathein University

Figure 1 Location map between Yeanchaung and Magway Township in Magway Region

Table 1 Six different soil samples collected between Yenanchaung and Magway Township

Samples Collected areas	pH	Soil type	Location
Gway gone	6.83	Sandy loam	N20º 22.613′ E 94º 59546′
Pay taw	6.94	Sandy loam	N20º 20.251′ E 94º 59.77′
Nyaung bin aing	6.98	Loamy Sand	N20º 19.944′ E 95º 0.433′
Kanthar gyi	6.84	Sandy loam	N 20º 14.249′ E 95º 1.247′
West of Kanbya	7.09	Sand	N 20º 11.514′ E 95º0.367′
Kan hla	4.6	Sand	N 20º 10.856′ E 54º 58.956′

Isolation of soil fungi by using serial dilution Method

1g of soil sample was introduced into a conical flask containing 99mL of distilled water. The flask was then shaken for about 30 minutes in order to make the soil particles free from each other. This solution was then serial diluted from 10^{-3} to10^{-7} dilution in separate test tubes and 0.5 mL each of the above dilution was separately transferred into sterile petridishes under aseptic condition. Chloramphenicol was added to the sterilized medium for preventing bacterial growth before pouring in to petri plate. The sterilized medium in conical flask was cooled down to about 45º and separately poured into each of the petridish containing the respective soil dilutions. The inoculated plates were shaken clock-wise and anticlockwise direction for about 5 minutes so as to make uniform distribution of the fungi inoculums. When the agar was solidified, the inoculated plates were inverted and incubated at 27º -30º C for 3 -6 days.

Screening for antimicrobial activities (NITE 2005)

The isolated fungi were grown on BMEA medium at room temperature for 5 days. After incubation period, these fungi inoculated into the seed medium (glucose 0.5 g, peptone 0.3 g, yeast extract 0.3 g, $K_2HPO_4$0.01 g, $CaCO_3$0.01 g, DW 100 mL at pH 6.5) for 3 days at room temperature. After three days, the seed medium (2%) was transferred into the fermentation medium (glucose 1.0 g, peptone 0.5 g, yeast extract 0.5 g, $K_2HPO_4$0.01 g, $CaCO_3$0.01 g, DW 100 mL at pH 6.5) and carried out for 3-10days and evaluated the antimicrobial activity by agar well diffusion method.

Screening of antimicrobial activity by agar well method (Collins, 1965)

1 day old culture test broth (0.2 mL) was added to 25 mL warm assay medium (glucose 1.0 g, peptone 0.3 g, KNO_3 0.1 g, DW 100 mL, agar 1.8 g) and thoroughly mixed and poured into plate. After solidification, the agar was left to set. Cork borer was used to make the wells (8 mm in diameter). And then, the fermented broth (20 µL) was carefully added into the well and incubated at room temperature for 24-48 hours. The diameter of the zones of inhibition around each well measured and recorded after 24-48 hours incubation.

Test organisms

Candida albicans NITE 09542, *Bacillus subtilis* IFO 905771, *Bacillus pumilus* IFO 905771, *Escherichia coli* AHU 5436, *Pseudomonas fluorescens* IFO 94307, *Agrobacterium tumefaciens* NITE 09678, *Staphylococcus aureus* AHU 8465, were obtained from NITE (National Institute of Technology and Evaluation, Kisarazu, Japan).

Study on the effect of Fermentation Period

The fermentation period of isolated fungi was studied by 3 days to 10 days and 5 mL of seed culture medium was added to 25 mL of fermentation medium. The flasks were incubated at room temperature and the fermentation medium was assayed for antimicrobial activity by using agar well diffusion method.

The effect of ages of inoculum

In this study, incubation time (48, 60, 72, 84, 96, 108, 120, 132, 144 hrs) were used for the production of antimicrobial metabolites and the procedure of seed culture medium was also used as the previous method. And then, seed culture was transferred to 100 mL conical flask containing of fermentation medium and incubated at room temperature. Fermentations were worked from 48 to 144 hrs and antibacterial activity was tested by agar well diffusion method.

The effect of sizes of inoculum

The inoculum level (5%, 10%, 15%, 20%, 25%, 30%, 35%) were used for the production of antimicrobial metabolite. In the investigation of size of inoculum, well sporulated selected strain was taken and added into 100 mL of seed culture medium and incubated for 3 days at room temperature. After that 3days old seed cultures were transferred to 100 mL conical flasks containing 25 mL of fermentation medium respectively. The flasks were incubated for 5 days at room temperature and the fermentation medium was assayed for antimicrobial activity by using agar well diffusion method.

Effects of carbon and nitrogen sources for growth

The different carbon sources such as potato, corn, carrot, oat, fructose, tapioca powder, lactose, soluble starch powder, mannitol, maltose, sucrose, glucose, glycerol, molasses and sweet potato were used for growth morphology.And then the different nitrogen sources such as NH_4Cl, NH_4SO_4, NH_4NO_3, $NaNO_3$, KNO_3, soybean, peanut, meat extract, peptone, gelatin, urea, yeast extract, asparagine, casein and fish cake were also used.

The effects of pH

Optimum pH was studied by varying the pH as 4, 5, 6,7, 8, 9 and 10. The different pH of seed medium was adjusted by using HCl and NaOH. The fermentation medium was assayed for antibacterial activity.

The effects of temperature

The selected fungus was inoculated and incubated at five different temperature by using 20°C, 25°C, 30°C, 35°C and 40°C.The fermentation medium was carried out 5 days and antibacterial activity was studied by agar well diffusion method.

Effects of carbon and nitrogen utilization

There were variations in the level of antibacterial activity when the different carbon potato, corn, carrot, oat, fructose, tapioca powder, lactose, soluble starch, mannitol, maltose, sucrose, glucose, glycerol, molasses and sweet potato were used. The different nitrogen sources such as NH_4Cl, NH_4SO_4, NH_4NO_3, $NaNO_3$, KNO_3, soybean, peanut, meat extract, peptone, gelatin, urea, yeast extract, asparagine, casein and fish cake were also used for fermentation.

Effects of fermentation medium

Fermentation media were undertaken with suitable conditions of 25% sizes and 108 hrs ages of inoculum with twenty three different media. Fermentation media was carried out for 5 days and antibacterial activity test was carried out every 24 hrs.

Comparison of static and shaking culture

100 mL conical flask containing 50 mL of the best fermentation medium was incubated on the rotary shaker (100 rpm) for 5 days. At the same time, another those fermentation medium was incubated under static condition without shaking. These shaking culture and static culture were compared by using agar well diffusion assay method.

Results

Isolation of soil fungi

In this investigation, 29 fungi were isolated from six different soil samples between Yenanchaung and Magway Township, Magway Region. Three isolated fungi were collected from Gwaygone Village, four from Pawtaw, four from Nyaingbin Aing, thirteen from Kanthargyi, one from West Kanbyar and six fungi from Kanhla Village. These results were shown in Table 2 and Figure 2-3.

Table 2 Isolation of fungi from soil samples

No.	Samples Collected areas	BMEA Medium	MEA Medium	CZA Medium	Total
1	Gway gone	TT-3, TT-10, TT-22	TT-16	-	4
2	Pay taw	TT-1, TT-17, TT-27	TT-12	-	4
3	Nyaung bin aing	TT-8	TT-14, TT-15, TT-26	-	4
4	Kanthargyi	TT-4, TT-5, TT-6, TT-7, TT-9, TT-11, TT-18, TT-19, TT-23, TT-24, TT-28	TT-21, TT-29	-	13
5	West of Kanbya	TT-2,	-		1
6	Kanhla	TT-13, TT-20	-	TT-25	3
Total Isolated fungi		**21**	**7**	**1**	**29**

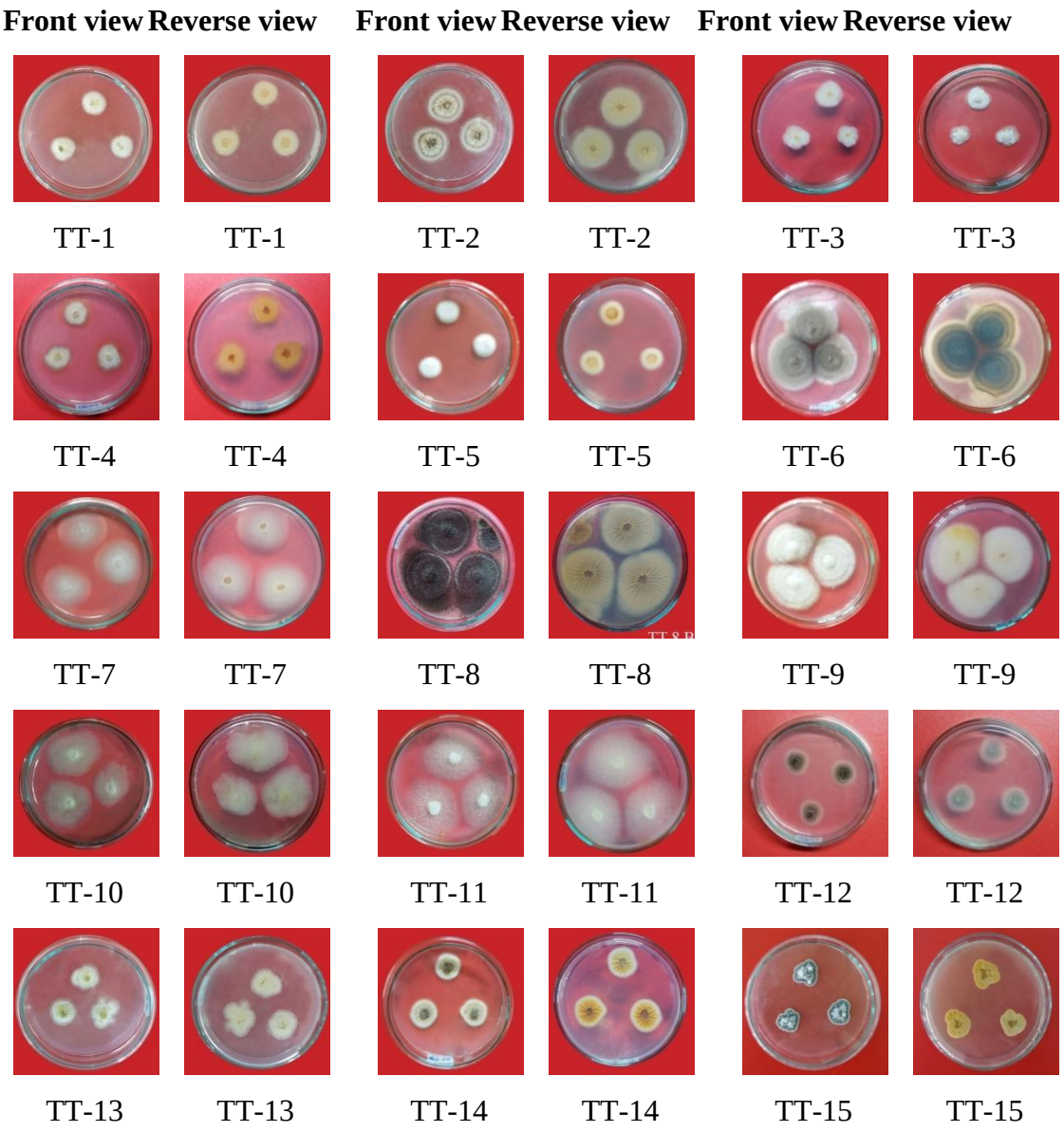
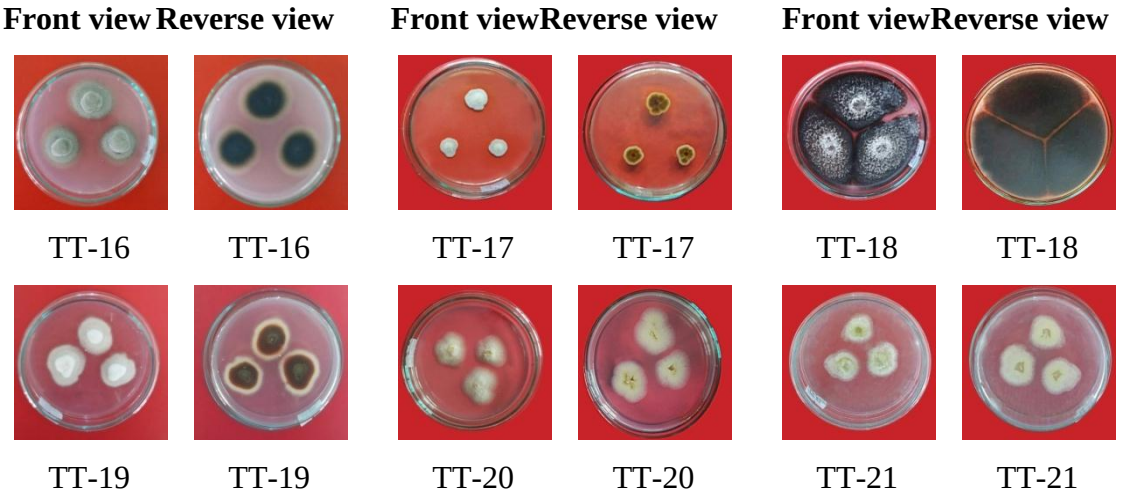


Figure 2 Mophology of isolated fungi on BMEA medium (TT-1 to TT-15)



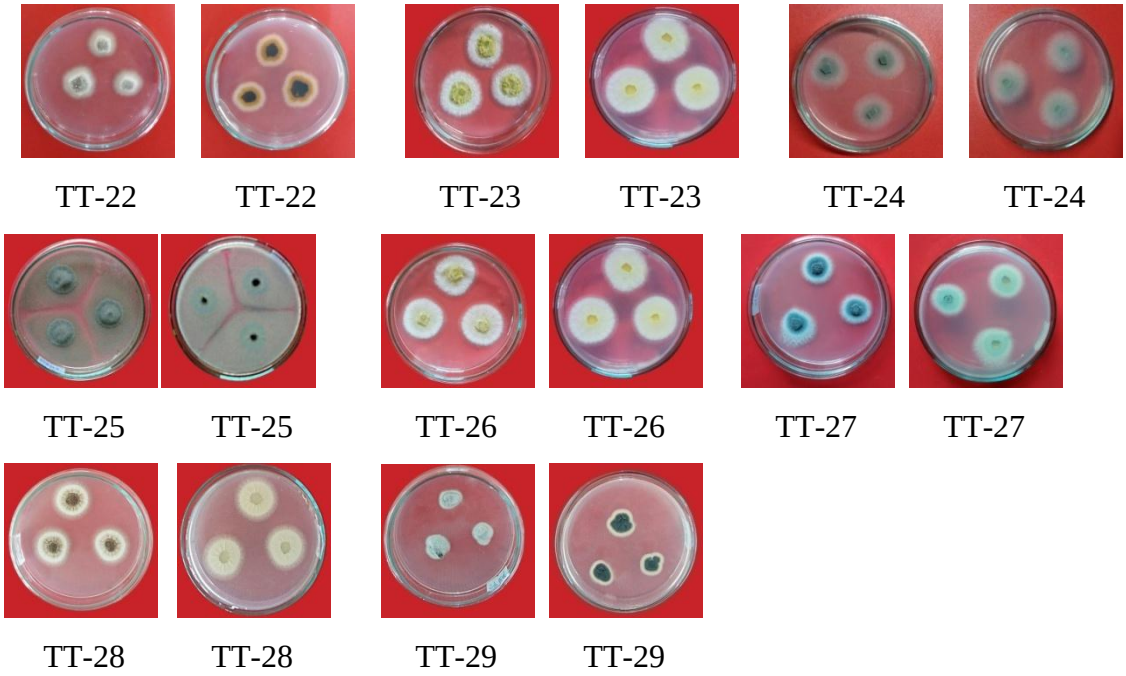


Figure 3 Mophology of isolated fungi on BMEA medium (TT-16 to TT-29)

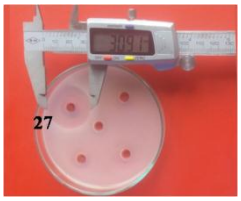
Antimicrobial activities of isolated fungal strains

Four isolated stains (TT-22, 23, 24 and TT-27) were tested for antimicrobial activity with seven test organisms. Agar well diffusion methods were employed for assay performance. Among them, TT-27 showed the maximum antimicrobial activities on all test organisms. TT-27 showed the highest antimicrobial activity (36.20 mm, 36.37 mm, 30.91 mm and 21.86 mm) at 5 days fermentation periods on *C. albicans*, *B. pumilus*, *B. subtilis* and *S. aureus* respectively. Moreover the highest antibacterial activities (39.10 mm, 34.92 mm and 30.95 mm) were found at 6 days fermentation periods on *E. coli*, *A. tumefaciens* and *P. fluorescens.* These results were shown in Table 3 and Figure 4.

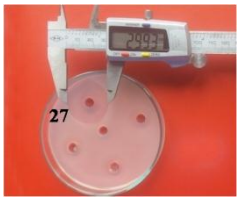
Table 3 Antimicrobial activity of isolated fungus TT-27 on the fermentation period

Fermentation period (days)	Test organisms and antimicrobial activity (mm)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
3	14.47	14.32	13.00	16.74	15.94	15.68	14.47
4	16.36	15.36	22.31	19.43	20.18	19.74	16.19
5	36.20	28.25	36.37	28.64	30.91	21.02	21.86
6	29.93	34.92	35.33	39.10	29.13	30.95	18.39
7	21.58	19.55	23.96	20.31	20.33	20.99	15.34
8	18.17	13.97	15.68	12.17	14.17	16.68	12.10

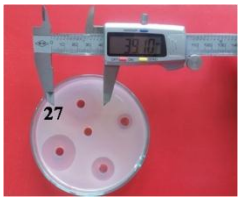
1-Candida albicans 4-*Escherichia coli* *7- Staphylococcus aureus*
2-*Agrobacterium tumefaciens* *5- Bacillus subtilis*
3-*Bacillus pumilus* 6-*Pseudomonas fluorescens*



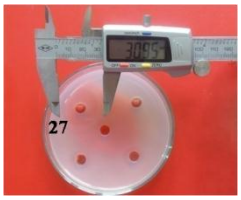
Bacillus subtilis



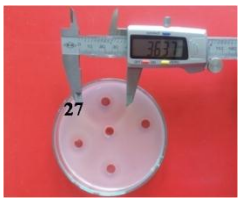
Candida alicans



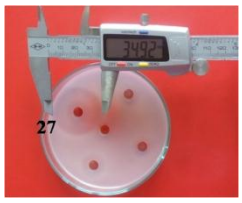
Escherichia coli



Pseudomonas fluorescens



Bacillus pumilus



Agrobacterium tumefaciens

Staphylococcus aureus

Figure 4 The Antimicrobial activity of isolated fungus TT-27 against seven test organisms

The effect of ages of inoculum of TT-27 on *Escherichia.coli*

In the effect of age of inoculum, TT-27 was investigated by using 48, 60, 72, 84, 96, 108, 120, 132 and 144 hrs old culture age of inoculums. The results showed that 108 hrs age of inoculum gave the highest activities (20.21 mm) followed by (19.81 mm) at 96 hrs and (19.53 mm) at 84 hrs age of inoculum.

Table 4 The effect of ages of inoculum

Sr. No	Age of Inoculum (hrs)	Antibacterial Activity (mm)
1	48	14.27 mm
2	60	16.68 mm
3	72	17.63 mm
4	84	19.53 mm
5	96	19.81 mm
6	108	20.21 mm
7	120	15.31 mm
8	132	14.90 mm
9	144	12.64 mm

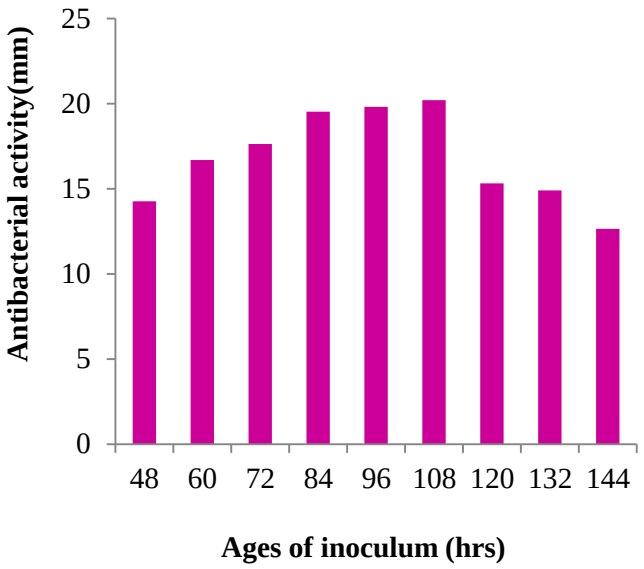


Figure 5 The effect of ages of inoculum

The effects of sizes of inoculums of TT-27 on *Escherichia. coli*

In this research work, the effect of size of inoculums was studied by using 5%, 10%, 15%, 20%, 25% and 30% inoculums (Table 5). Using 25% inoculums showed significantly higher (23.09 mm) than others, followed by 10% and 15% (20.05 mm and 21.09 mm) respectively in Figure 6.

Table 5 The effect of sizes of inoculum

Size of inoculums (%)	Antibacterial Activity (mm)
5	19.51mm
10	20.05mm
15	21.09mm
20	16.14mm
25	23.09mm
30	18.05mm

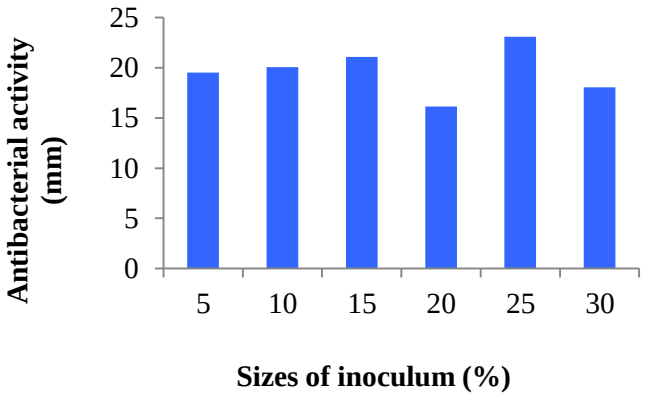


Figure 6 The effect of sizes of inoculum

Effects of Carbon and Nitrogen sources on TT-27

The different carbon sources such as potato, corn, carrot, oat, fructose, tapioca powder, lactose, soluble starch, mannitol, maltose, sucrose, glucose, glycerol, molasses and sweet potato were used for growth morphology of selected fungus TT-27. The selected fungus TT-27 showed that molasses, sweet potato and glucose were excellent growth and good growth on potato, carrot, fructose, mannitol, maltose, sucrose and glycerol but the other five carbon sources showed poor results. The nitrogen sources such as casein, $NaNO_3$, gelatin and asparagine showed the excellent growth and good growth on KNO_3 and urea but the remaining nine nitrogen sources were showed poor growth on TT-27.

Table 6 Growth of TT-27 on carbon and nitrogen sources

Carbon Sources	Growth Size (cm)	Nitrogen Sources	Growth Size (cm)
Potato	2.30 cm	NH_4Cl	1.40 cm
Corn	1.80 cm	NH_4SO_4	1.70 cm
Carrot	2.50 cm	NH_4NO_3	1.50 cm
Oat	1.90 cm	$NaNO_3$	3.70 cm
Fructose	2.70 cm	KNO_3	2.90 cm
Tapioca	2.10 cm	Soybean	2.20 cm
Lactose	1.90 cm	Peanut	2.20 cm
Soluble starch	2.00 cm	Meat	2.40 cm
Mannitol	2.60 cm	Peptone	2.40 cm
Maltose	2.90 cm	Gelatin	3.30 cm
Sucrose	2.80 cm	Urea	2.80 cm
Glucose	3.00 cm	Yeast Extract	2.40 cm

Carbon Sources	Growth Size (cm)	Nitrogen Sources	Growth Size (cm)
Glycerol	2.30 cm	Asparagine	3.10 cm
Molasses	3.40 cm	Casein	4.10 cm
Sweet Potato	3.00 cm	Fish cake	2.10 cm

3.00cm -3.40cm = Excellent
2.30cm -2.90 cm = Good
1.80cm -2.00 cm = poor

3.10cm -4.1cm = Excellent
2.60cm -3.00 cm = Good
1.40cm -2.50 cm = poor

Antibacterial activity of TT-27 on carbon and nitrogen utilization

There were variations in the level of antibacterial activity when the different carbon such as potato, corn, carrot, oat, fructose, tapioca powder, lactose, soluble starch, mannitol, maltose, sucrose, glucose, glycerol, molasses and sweet potato were used. The highest antibacterial activity was obtained by the addition of maltose and carrot (each 24.91mm), followed by sucrose (22.75mm), mannitol (22.75mm), corn (22.08mm), tapioca powder (21.68mm), glucose (21.36mm) and glycerol (20.79mm), oat (18.50mm), lactose (18.30mm), potato (18.27mm), molasses (17.87mm), sweet potato (17.40mm) and fructose (14.69mm) and soluble starch (14.24mm) respectively.

Similarly, when the various nitrogen sources were added, the significant inhibition zones (26.05 mm, 24.23 mm, 23.18 mm, 22.98 mm, 22.37 mm and 20.83 mm) were obtained in soybean, peptone, KNO_3, asparagine, meat and $NaNO_3$. TT-27 showed the moderate inhibition zones in peanut (19.95 mm), yeast (19.37 mm), NH_4NO_3 (19.31 mm), gelatin (19.04 mm), urea (18.81 mm), casein (17.80 mm), NH_4Cl (16.95 mm), fish cake (16.91 mm) and NH_4SO_4 (15.53mm) were regarded as poor inhibition zone. These results were shown in Figure (7) and (8).

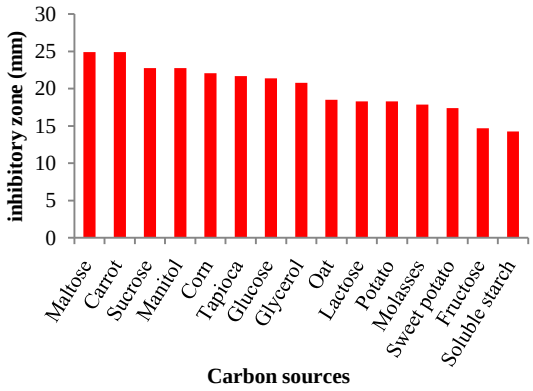


Figure 7 The effect of carbon utilization on fermentation

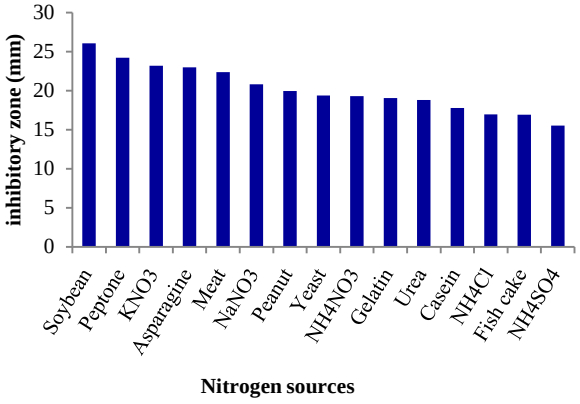


Figure 8 The effect of nitrogen utilization on fermentation

Antibacterial Activity medium of TT-27 on various fermentation media

In the fermentation medium (FM), the best antibacterial activity was obtained by using FM-1, (glucose and peanut, 29.17 mm) followed by FM-2, (maltose and meat, 26.79 mm) and FM-3, (corn and meat, 26.70 mm) respectively.

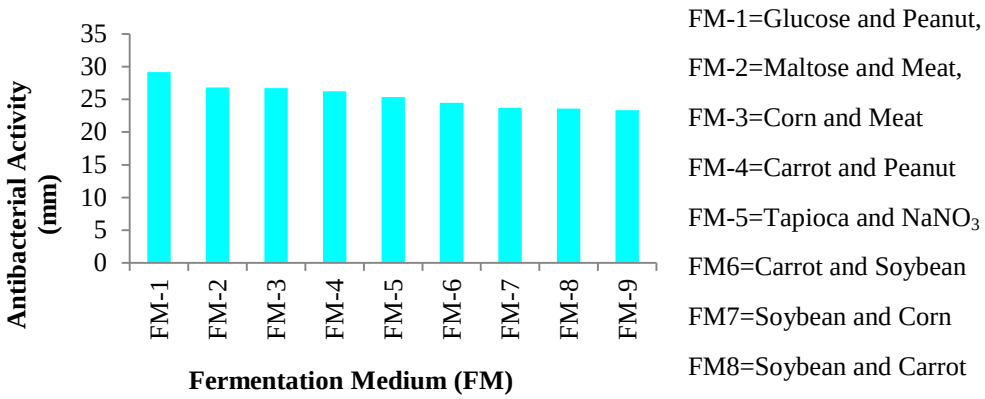


Figure 9 Antibacterial activity of TT-27 on various fermentation media

The effects of pH and temperature

Effect of pH was studied by varying pH 4, 5, 6, 7, 8, 9 and 10. The best antibacterial activity was found at pH-5 (22.10 mm).

In the temperature effect, TT-27 was incubated at changing temperature 20°C, 25°C, 30°C, 35°C and 40°C. The increased activity of antibacterial metabolite was recorded at temperature 25°C (18.44 mm).

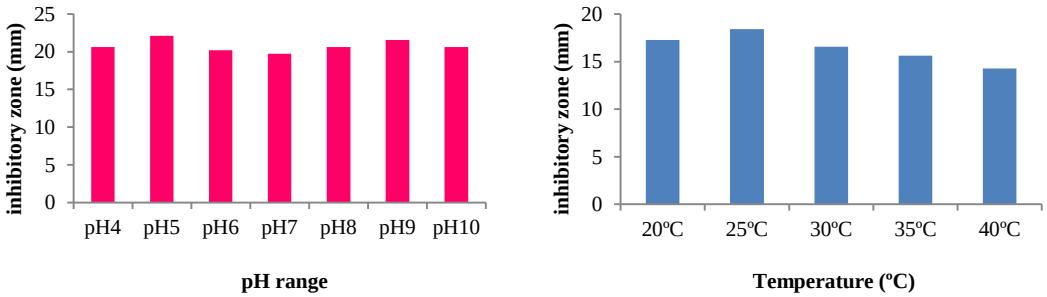


Figure 10 The effect of different pH and temperature

Comparison of static and shaking culture

In the comparison of shaking and static culture, the antibacterial activity of shaking culture (23.40 mm) was more than that of static culture (20.50 mm).

Static culture Shaking culture

Figure 11 Comparison of static and shaking culture

Discussion and Conclusion

The present research was focused by isolation of soil fungi between Yenanchaung and Magway township and preliminary antimicrobial activities with seven test organisms. In all

fungal strains, twenty strains showed different antimicrobial activities. Among them, fourteen selected fungi TT-1, 3, 4, 8, 15, 17, 18, 19, 22, 23, 24, 26, 27 and 29 exhibited moderate antimicrobial activities. TT-27 showed the best antibacterial activity (22.76mm) at five days on *E. coli.* Thus, TT-27 and *E. coli* was selected for the optimum fermentation conditions. TT-27 reached the highest activities (34.92mm) in 6 days fermentation period. To study the optimization of inoculums age, incubation time (48 ,60 ,72 ,84, 96, 108, 120, 132 and 144 hrs) were used and the highest antibacterial activity was found at 108 hrs. In the proper size of inoculums, 25% was the most suitable and the maximum activities (23.09mm) in TT-27 followed by 15% (21.09 mm) and 10% (20.05mm) respectively. In the carbon sources, the colony of TT-27 was the excellent growth on molasses, sweet potato and glucose.

The antibacterial substance production of TT-27 was influenced by addition of maltose and carrot reached the highest activity (24.91mm) followed by sucrose, mannitol and corn. Nitrogen is required by all organisms as an essential nutrient. The nature of the nitrogen source has notable effect on the production of antibacterial metabolites in TT-27. Especially, TT-27 showed the moderate growth on nearly all nitrogen sources. Colony morphology of TT-27 was excellent growth on casein, $NaNO_3$, gelatin and asparagine. Maximum antibacterial metabolite of TT-27 was found on soybean (26.05mm) followed by peptone, KNO_3, asparagines ad meat respectively as nitrogen sources. This result was in agreement with the description of EL-Gammal, 1986. He found that peptone has been reported by the suitability of nitrogen sources for the production of metabolites from microorganisms. The choice of a good fermentation medium is virtually as important to the success of an industrial fermentation as is the selection of an organism to carry out the fermentation (El-Tayeb *et al.,* 2004).

In the fermentation medium (FM), FM-1 gave the highest antibacterial activity (29.17 mm) by using glucose and peanut. Effects of pH was studied by varying pH-4, 5, 6, 7, 8, 9 andpH-10. The best antibacterial activity was found as pH- 5 (22.10mm). Effects of temperature was studied by varying from 20°C, 25°C, 30°C, 35°C and 40°C. The best antibacterial activity for temperature was found at 25°C (18.44mm), followed by 20°C(17.28mm), 30°C(16.59mm), 35°C(15.63mm) and 40°C(14.29mm) respectively. Thus the results of optimum fermentation conditions indicated that antibacterial metabolites of TT-27 were obtained by optimally 6 days fermentation period, in the presence of maltose and soybean, 108 hrs age of inoculums, 25% inoculums size, FM-1, pH-5, temperature 25°C and shaking culture. It was concluded that the present study revealed to observe the fermentation period of four isolated fungi and to investigate the optimization parameters of fermentation conditions on TT-27 against *E coli*.

Acknowledgements

I would like to record my deep thank to Professor Dr Kay Thi Mya, Head of Botany Department, University of Pathein and Professor Dr Wah Wah Lwin, Department of Botany, University of Pathein for their suggestion and kind understanding during this study. Many thanks are due to my supervisor Dr Zar Zar Yin, Associate Professor, Department of Botany, University of Pathein, for her valuable instructions, encouragement and overall supervision for the successful completion of this research paper.

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