ISOLATION AND FERMENTATION CONDITIONS OF SOIL FUNGUS PP-15 FROM GAWECHAUNG FORT

Phyu Phyu Aung[1], Zar Zar Yin[2]

Abstract

Soil samples were collected from six different places of Gawechaung fort, Magway Township, Magway Region. Thirty fungi were isolated from these six different soil samples. Isolations of fungi were undertaken by the serial dilution method and cultured by using Blakeslee's Malt Extract Agar (BMEA Medium), Czapek-Dox Agar (CZA Medium) and Malt Extract Agar (MEA Medium). Isolated fungi were given as PP-1 to PP-30. Antimicrobial activities of these fungi were evaluated by agar well diffusion assay with seven test organisms. Among them, ten fungal strains showed the antimicrobial activity.Especially, PP-15 gave the best antibacterial activity on *Agrobacterium tumefaciens* NTTE 09678.Therefore, different fermentation parameters of PP-15 were studied by the fermentation period, proper age and size of inoculums, effect of various carbon and nitrogen sources, pH, temperature, fermentation medium, shaker and static on *Agrobacterium tumefacines*.

Key words: Soil fungi, Antimicrobial activity, Fermentation

Introduction

Life on earth would have been impossible without microorganism in nature. There are numerous varieties which are living on earth and are deeply involved with human life. Microorganisms have significant function in ecosystems and are found in all kinds of habitats. Soil microbiology is the study of organisms in soil, their function and how they affect soil properties. Soil microorganisms can be classified as bacteria, actinomycetes, fungi, algae and protozoa. Soil sample is the most effective and popular materials for especially isolating a number of microorganisms such as fungi (Harayama, *et al.,* 2002).

Soil fungi play an important role as major decomposer in the soil ecosystem. There are about 75,000 species of soil fungi in the world (Finlay, 2007). Antibiotic is a drug used to treat infection caused by bacteria that can cause illness to humans and animals. Antibiotic functions to inhibit or destroy the bacterial cells that cause certain disease (Duerden,1993).The fermentation process is basically dependent on the transformation of carbohydrate, proteins and lipids to acidic, alcoholic and organic metabolites. Production of antibiotic metabolite has been known to be influenced by media components and cultural conditions, such as aeration, agitation, pH, temperature and glycerol concentration, which vary from organism to organism (Iwai *et al*.,1982).

This research paper aims to investigate the isolation of soil microorganisms and to study the different soil microorganisms from various soil samples and to investigate the effect of fermentation, pH, temperature, static and, shaker culture of selected fungus on *Agrobacteriumtunefaciens.*

Materials and Methods

Collection of Soil Samples

Soil samples were collected from Gawechaung Fort in Magway Township, Magway Region. Soil samples of six different places were collected during July 2017 to August 2017.

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Table 1 Six Different Soil Samples Collected from Gawechaung fort

Soil samples	Sample collected areas	pH	Soil Type	Location
S-I	South of Fort	10.15	Loamy Sand	N 19˚ 57.863' E 95˚ 3.93'
S-II	Front of fort landmark	8.66	Sandy Loam	N 19˚ 57.942' E 95˚ 3.951'
S-III	Entry Street of fort	7.59	Loamly Sand	N 19˚ 57.509' E 95˚ 4.76'
S-IV	West of fort	7.79	Loamly Sand	N 19˚ 57. 905' E 95˚ 3.906'
S-V	North East of fort	7.63	Sandy loam	N19˚ 57. 905' E 95˚ 3.906'
S-VI	Short landmark of Gawechaung	7.77	Sandy Loam	N19˚ 57.873 ' E 95˚ 4.519'

Isolation of Fungi From the Soil Samples

The soil fungi were enumerated by serial dilution method (Dubey, 2002) and media such as Blakeslee's Malt Extract Agar (BMEA Medium), Czapek-Dox Agar (CZA Medium) and Malt Extract Agar (MEA Medium).

Serial dilution Method

1g of soil sample was introduce into a conical flask containing 99 mL 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 petri dishes under aseptic condition. Chloramphenicol was added to the sterilized medium for preventing bacterial growth before pouring into petri plate. The sterilized medium in conical flask was cooled down to about 45ºC and separately poured into each of the petri dish containing the respective soil dilutions. The inoculated plates were shaken clock-wise and anticlock- wise 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 27ºC-30ºC for 3-6 days(Dubey , 2002).

Preliminary study for antimicrobial activity

The isolated fungi were grown on BMEA medium for 5 days. The isolated fungi were inoculated into 25 mL seed medium and incubated at room temperature for 3 days. After 3 days, 20 mL seed culture was transferred into the 80 mL of fermentation medium and incubated at room temperature. Fermentation was carried out for 3-10 days (Ando, 2004).

Screening of Antimicrobial Activity by Agar Well Diffusion Method

1 day old culture test broth (0.01mL) was added to 25mL of assay medium and thoroughly mixed and poured into plate.After solidification, cork borer was used to make the wells (wells - 8 mm). The fermented broth (20µL) was carefully added into the wells and incubated at room temperature for 24-48 hours. The diameter of the zones of inhibition around each well was measured and recorded after 24-48 hoursincubation (Collins, 1965).

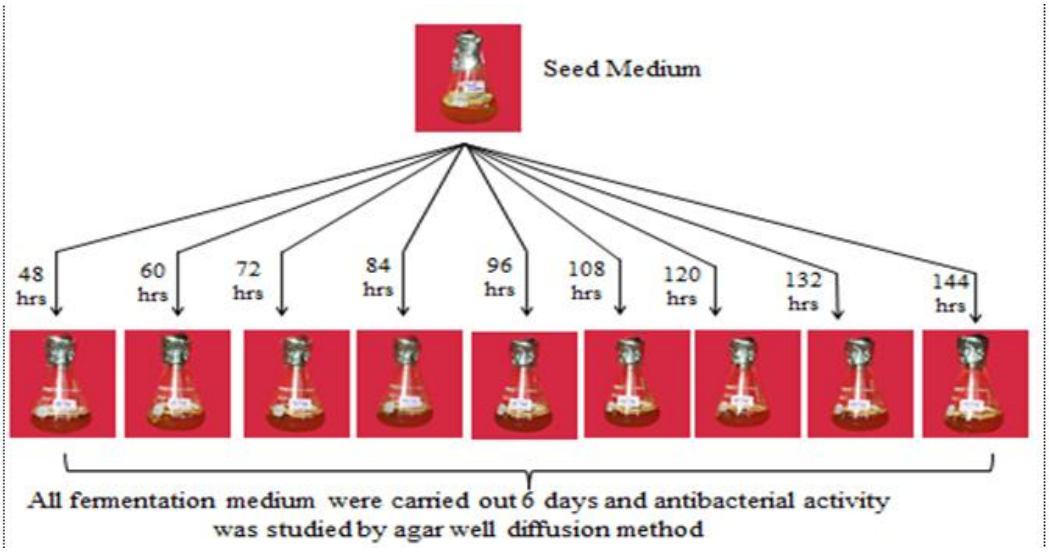


Figure 1 Study on the effects of ages of inoculums of PP-15

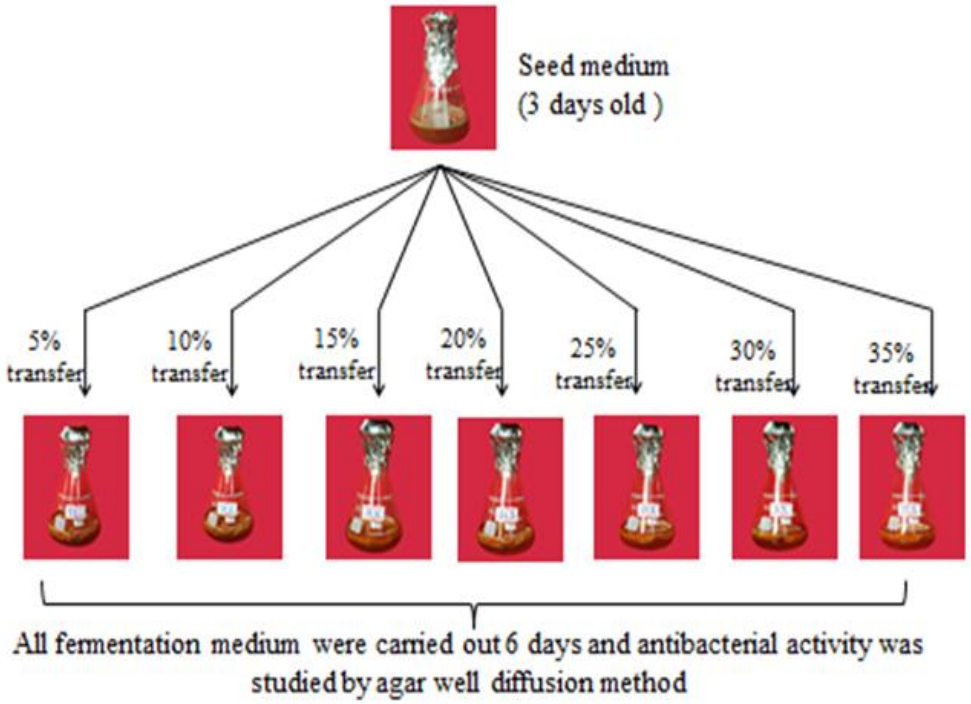


Figure 2 Study on the effects of sizes of inoculums of PP-15

Effect on the Carbon and Nitrogen Utilization of selected fungus(PP-15)

Optimal fermentations are very important for maximal productivity metabolites. In this study, carbon and nitrogen sources were employed in the fermentation for the production of antibacterial metabolites. Carbon sources such as carrot, corn powder, dextrose, fructose, glycerol, glucose, lactose, maltose, mannitol, molasses, oat, potato, rice powder, soluble starch, sucrose, xylose and tapioca powder were used. Nitrogen sources such as asparagine, casein, fish cake, gelatin, KNO_3 malt extract, meat extract, $NaNO_3$, NH_4N_3, $(NH_4)_2SO_4$, peanut cake, NH_4CL, peptone, polypeptone, rice bran, soybean, urea and yeast extract were also used.

The effect of pH on fermentation

Effects of different pH were used for antibacterial activity of pH 4, 5, 6, 7, 8 and 9.These different pH were adjusted by NaOH and HCL.

The effect of temperature on fermentation

The selected fungus PP-15 was inoculated and incubated at five different temperature by using 20ºC, 25ºC, 30ºC, 35ºC and 40ºC.

Study on the fermentation media of PP-15

Fermentation was undertaken with suitable conditions of 10% sizes and 72 hrs ages of inoculum with fourteen different media. Fermentation was carried out for 6 days and antibacterial activity test was carried out every 24 hrs.

Comparison of static culture and shaking culture

100 mL conical flask containing 50 mL of the best fermentation medium was incubated on the rotary shaker (100 rpm) for 6 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 fungi from soil samples

In this investigation, 30 fungi were collected from the six different soil samples of Gawechaung Fort, Magway Township, Magway Region. Isolated fungi PP-1 to PP-5 were collected from south of Fort , PP-6 to PP-10 from front of Fort landmark, PP-11 to PP-15 from entry street of Fort, PP-16 to PP-20 from west of Fort, PP-21 to PP-25 from north east of Fort and PP-26 to PP-30 from short landmark of Gawechaung Fort.

Table 2 Isolated Fungi from Soil Samples

Soil Samples	Isolated Fungi
S –I	PP- 1 to PP-5
S -II	PP- 6 to PP-10
S -III	PP- 11 to PP-15
S -IV	PP- 16 to PP-20
S –V	PP-21 to PP-25
S-VI	PP-26 to PP-30

Front view Reverse view Front view Reverse view Front view Reverse view

(PP-1) (PP-2) (PP-3)

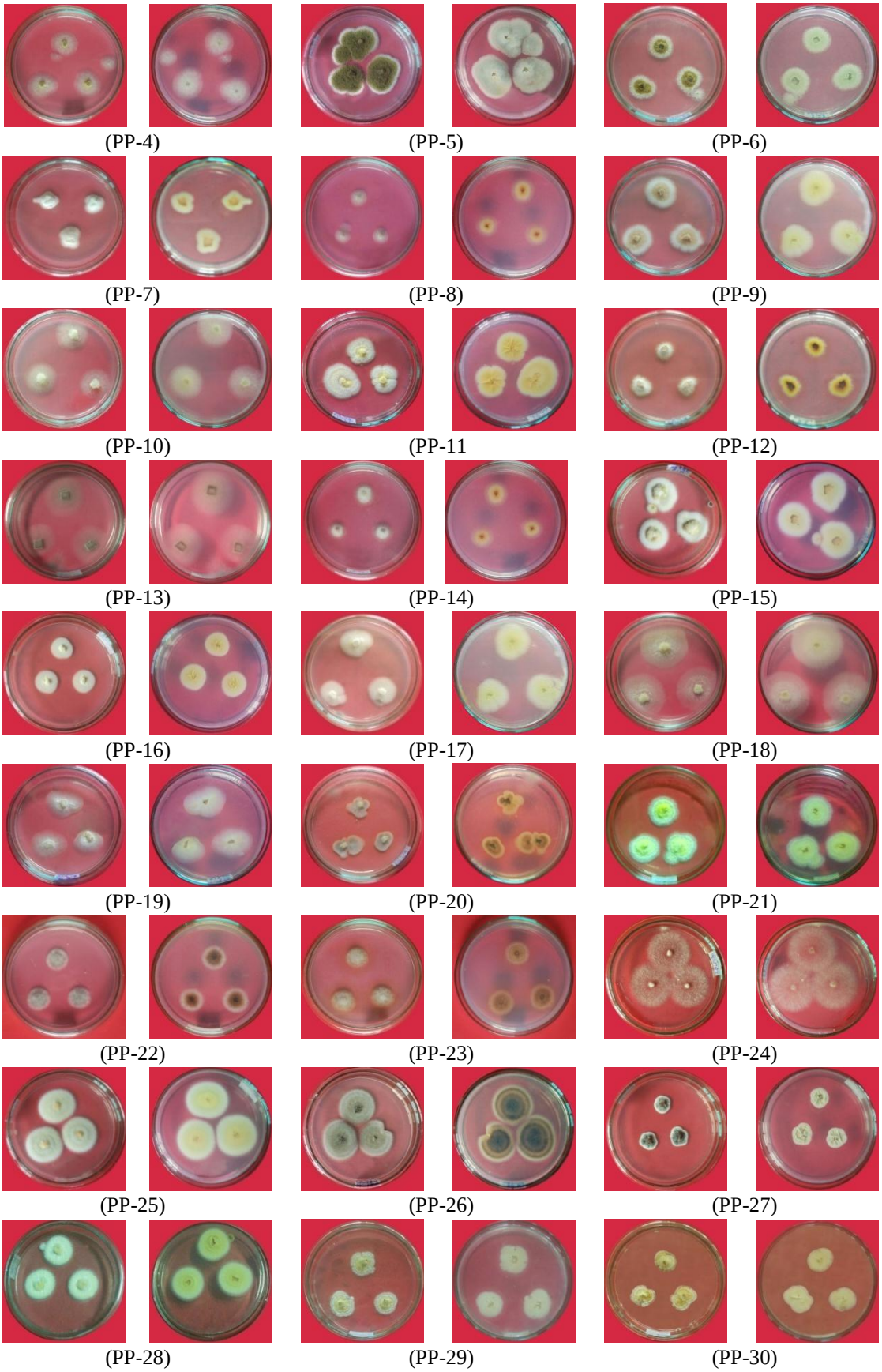


Figure 3 Morphological Character of Isolated Fungi PP-1 to PP-30

Isolated Fungi and their Antibacterial Activity

In this study, ten fungi strains were tested with *Agrobaterium tumefaciens* by agar well diffusion method. PP-15 gave the best activity on *Agrobaterium tumefaciens*.

Table 3 Isolated Fungi and their Antimicrobial Activity

No	Isolated fungi	Fermentation period of (days) and inhibition zone (mm)				
		3 days	4 days	5 days	6days	7days
1	PP-2	-	-	-	-	-
2	PP-3	-	-	-15.51	21.37	14.72
3	PP-8	17.34	12.79	16.68	20.02	-
4	PP-12	-	-	-	-	-
5	PP-14	15.22	17.97	19.09	15.32	-
6	PP-15	14.56	18.96	25.48	27.02	24.18
7	PP-21	14.29	12.34	16.16	-	-
8	PP-23	12.41	21.42	14.`11	24.50	17.25
9	PP-27	-	13.73	16.36	18.28	15.84
10	PP-28	14.30	15.13	21.97	23.30	23.29

Figure 4 Antibacterial Activity of Ten Selected Fungi Against *Agrobaterium tumefaciens*

The effects of ages of inoculum on the fermentation

In the effect of age of inoculum, PP-15 was investigated by using 48, 60, 72, 84, 96, 108, 120, 132 and 144 hrs old culture age of inoculums. The results showed that 72hrs age of inoculum gave the highest activity (25.35 mm) followed by (23.01 mm) at 96 hrs and (22.66 mm) at 84 hrs age of inoculum.

Table 4 The Effects of Ages of inoculums on the Fermentation for PP-15

Sr. No	Fermentation Period (hrs)	Antibacterial activity (mm)
1	48	20.21
2	60	21.78
3	72	25.53
4	84	22.66
5	96	23.01
6	108	22.35
7	120	17.46
8	132	16.32
9	144	16.04

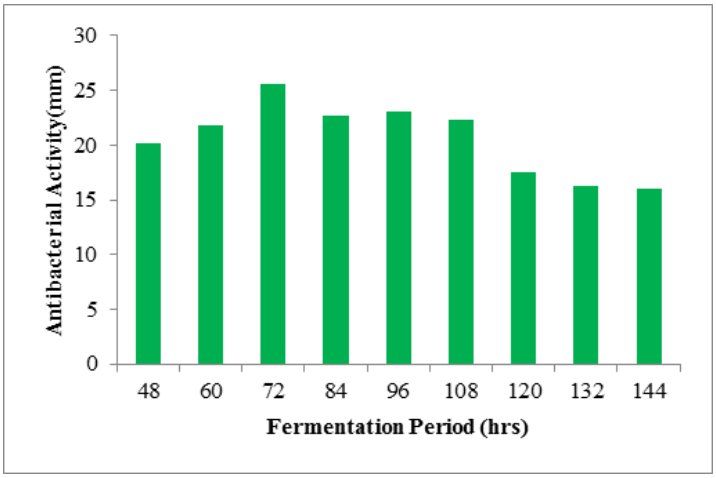


Figure 5 The Effects of Ages of inoculums on the Fermentation for PP-15

The effects of sizes of inoculums on the fermentation for PP-15

In this research work, the effect of size of inoculums was studied by using 5%, 10%, 15%, 20%, 25%, 30% and 35% inoculum. Using 10% inoculums showed significantly higher (27.95 mm) than others, followed by 30% and 20% (23.52 mm and 22.05 mm) respectively.

Table 5 The Effects of Size of inoculums on the Fermentation for PP-15

Sr. No	Size of inoculums (%)	Antibacterial activity (mm)
1	5	21.10
2	10	27.95
3	15	19.22
4	20	22.05
5	25	20.24
6	30	23.52
7	35	18.47

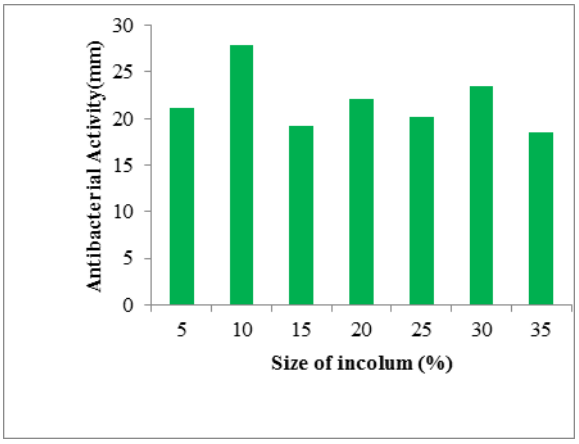


Figure 6 The Effects of Size of inoculums on the Ferment

The effects of carbon and nitrogen sources utilization for growth of PP-15

The selected fungus PP-15showed that the carbon sources such as glucose, and tapioca powder were excellent growth, good growth on starch, poor growth on corn powder, while other nine carbon sources showed moderate growth .The excellent growth were found on gelatin and yeast extract, good growth on Asparagine, moderate growth on malt extract, peptone, NH4CL, KNO_3, $(NH_4)_2SO_4$ and left four nitrogen sources were poor growth.

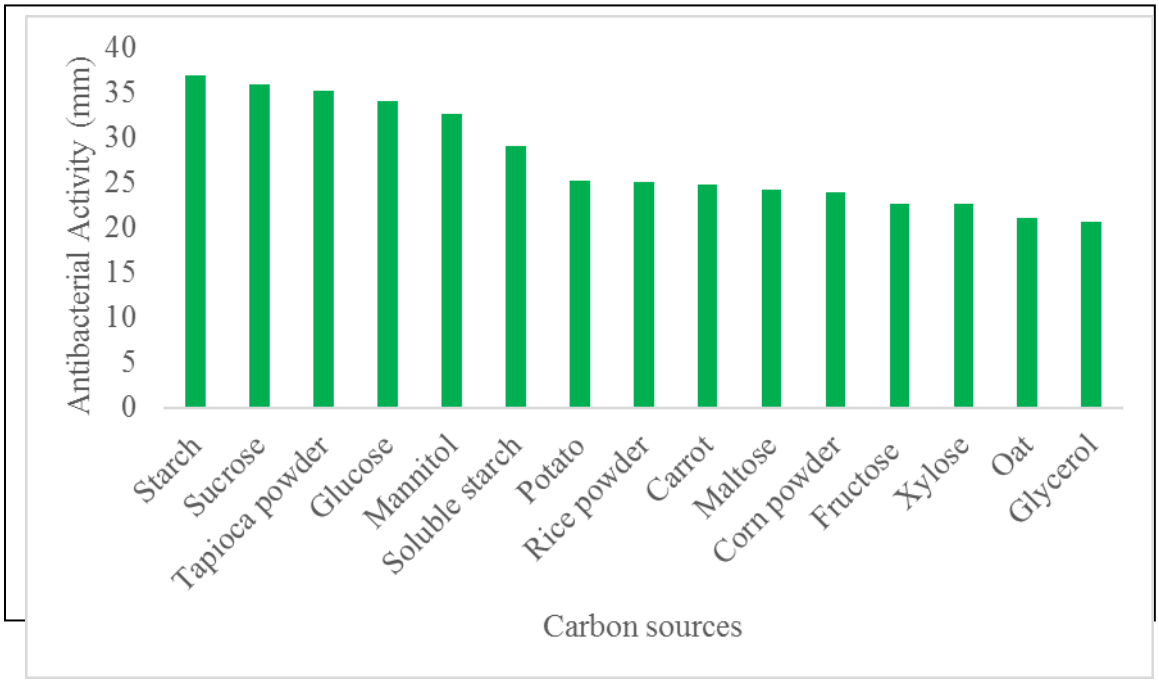
Table 6 Growth of PP-15 on carbon and nitrogen sources

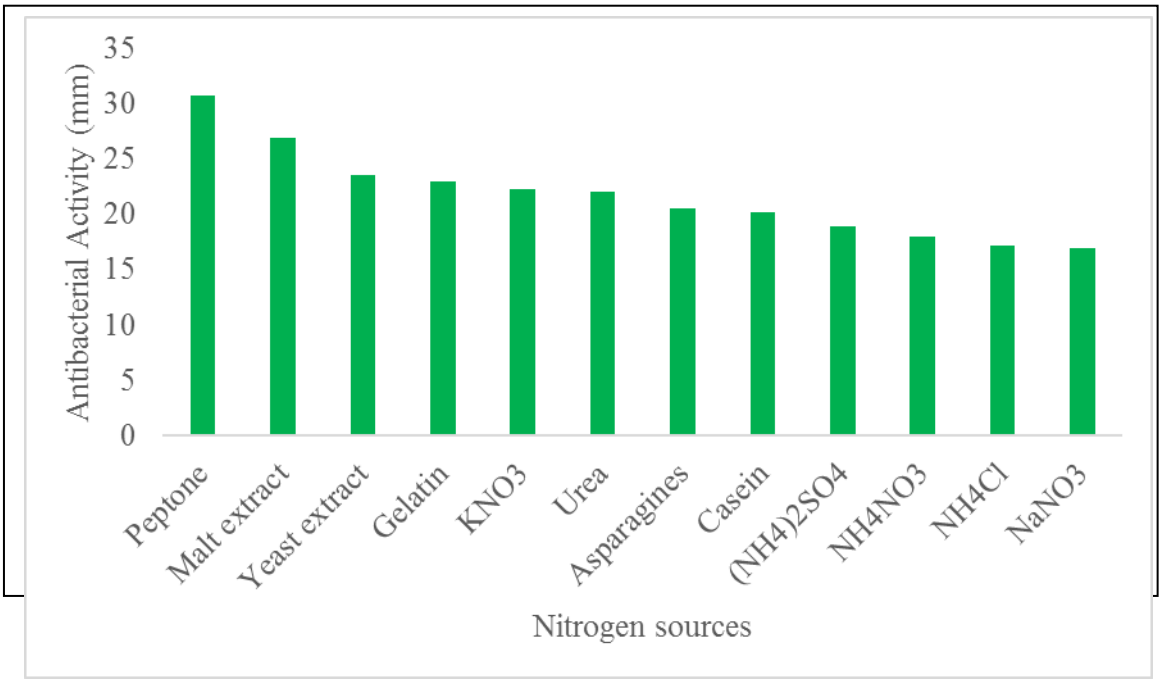
Sr. No	Carbon sources	Growth	Nitrogen Source	Growth
1	Glucose	5.5cm(Excellent)	Gelatin	4.5cm(Excellent)
2	Tapioca powder	4.5cm(Excellent)	Yeast Extract	4.5cm(Excellent)
3	Starch	4cm(Good)	Asparagine	3.9cm(Good)
4	Sucrose	3.5cm(Moderate)	Malt extract	3cm(Moderatet)
5	Soluble starch	3cm(Moderate)	Peptone	3cm(Moderate)
6	Potato	2.8cm(Moderate	NH_4Cl	2.7cm(Moderate)
7	Rice powder	3cm(Moderate)	KNO_3	3cm(Moderate)
8	Glycerol	2.5cm(Moderate)	$NH_4(SO_4)_2$	2.1cm(Moderate)
9	Carrot	2.7cm(Moderate)	Casein	1.7cm(Poor)
10	Fructose	3cm(Moderate)	NH_4NO_3	1.5cm(Poor)
11	Xylose	2.5cm(Moderate)	Rice bran	1.5cm(Poor)
12	Corn powder	2cm(Poor)	Urea	1cm(Poor)

1cm to 2cm = Poor, 2.1cm to 3cm = Moderate,
3.1cm to 4cm = Good 4.1cm to above = Excellent

Effect of carbon and nitrogen utilization on fermentation of PP-15

The significant inhibition zone (36.85mm, 35.84mm, 35.24mm, 34.03mm and 32.58mm) were obtained in starch, sucrose, tapioca powder, glucose and mannitol. Soluble starch (29.01mm), potato (25.21mm), rice powder (25.12mm), carrot (24.72mm), maltose (24.15mm), corn power (23.97mm), fructose (22.67mm), xylose (22.61mm), oat (21.06mm) and glycerol (20.66mm) showed moderate inhibition zone. Similarly, the addition of peptone exhibited the greatest activity (30.79mm) followed by malt extract (26.97mm), yeast extract (23.55mm), gelatin (22.95mm), KNO_3 (22.27mm), urea (22.05mm), asparagines (20.52mm), casein (20.21mm). The poor inhibition zone (18.97mm, 18.04mm, 17.23mm and 16.97mm) were obtained $(NH4)_2SO_4$, NH_4Cl, $NaNO_3$.





The effect of pH on the fermentation conditions of PP-15

In this study, the highest antibacterial activity was obtained at pH 6 (27.78 mm) against *Agrobacterium tumefaciens*.

Table7 The Effects of pH on the Fermentation Conditions of PP-15

Sr. No	pH	Antibacterial activity (clear zone , mm)
1	4	24.41
2	5	22.28
3	6	27.78
4	7	25.86
5	8	21.75
6	9	21.01

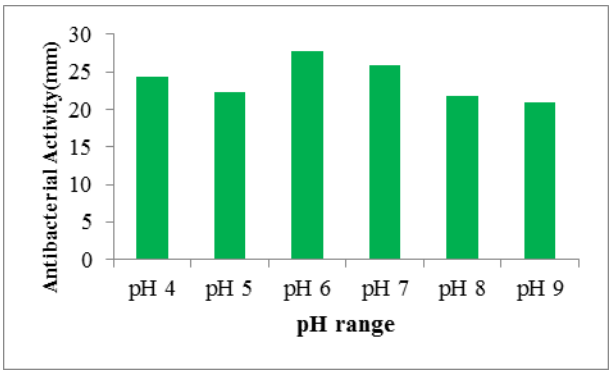


Figure 9 The Effects of pH on the Fermentation Conditions of PP-15

The effect of temperature on the fermentation condition

In this investigation, temperature 25˚C showed the highest antibacterial activity (28.83 mm) against on *Agrobacterium tumefaciens*.

Table 8 The Effects of Temperature on the Fermentation Conditions of PP-15

Sr. No	Temperature (˚C)	Antibacterial activity (clear zone, mm)
1	20	20.98
2	25	28.83
3	30	25.57
4	35	20.57
5	40	19.65

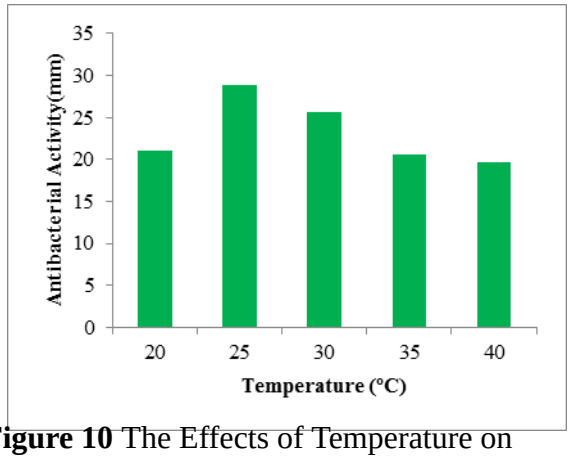


Figure 10 The Effects of Temperature on the Fermentation Conditions of PP-15

Antibacterial activity of PP-15 on Fermentation media

In the fermentation medium (FM), the best antifungal activity was obtained by using starch and peptone in FM-1 (30.89 mm) followed by 29.71 mm, FM-2 (starch and malt extract), 29.04 mm, FM-10 (glucose and peptone) and 28.14 mm, FM-3 (starch and gelatin) respectively.

Table 9 Antibacterial activity of PP-15 on various fermentation medium

Fermentation medium (FM)	Antibacterial activity (mm)
FM-1	30.89
FM-2	29.71
FM-3	28.14
FM-4	27.29
FM-5	28.10
FM-6	27.21
FM-7	25.82
FM-8	26.19
FM-9	24.29
FM-10	29.04
FM-11	27.72
FM-12	26.43
FM-13	25.76
FM-14	26.52

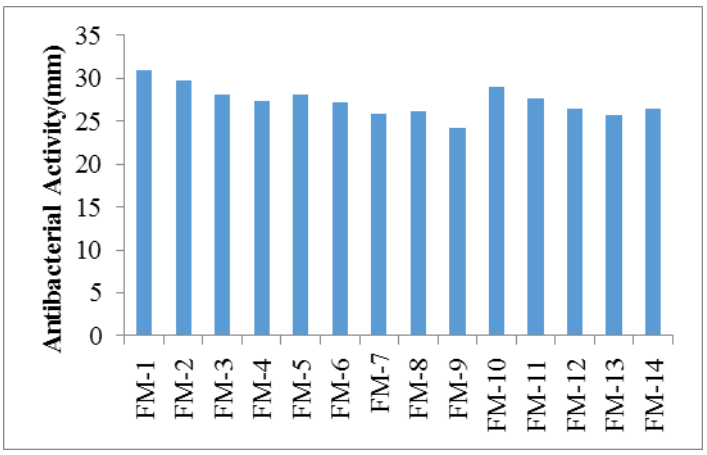


Figure 11 Antibacterial activity of PP-15 on various fermentation medium

Comparison of static culture and shaking culture

When comparing the static culture and shaking culture on fermentation medium of PP-15 antibacterial activity from shaking culture is better than (30.92 mm) than that of static culture (20.01 mm).

Table 12 TheAntibacterial Effects on Static and Shaking Culture of PP-15

Sr. No	Fermentation condition	Antibacterial activity (clear zone , mm)
1	Static	20.01
2	Shaker	30.92

Static Shaker

Figure 12 The Antibacterial Effects on Static and Shaking Culture of PP-15

Discussion and Conclusion

The soil serve as a reservoir for many microbial communities of plants and herbs which can be producing CO_2 and nitrogen cycle. The microorganisms plays major role in soil ecosystem. Microbial composition and functioning changes the soil quality through decomposition of organic matter, recycling of nutrients and biological control (Stefanis, *et al.*, 2013). Soil samples were collected from six inches depth after removing the surface soil for the isolation of fungi. The color of soil samples were red, brown and pale brown. In general the majority of microbial population is found in the upper six to twelve inches of soil and the number decreases with depth (Cattle, *et al*., 2002). Ten isolated fungi (PP-2, 3, 8, 12, 14, 15, 21, 23, 27

and 28) were tested with one test organism by agar well diffusion method. Among them, the selected fungus PP-15 showed potent antibacterial activity against *Agrobacterium tumefaciens*.

Therefore, PP-15 was selected for the study of the optimum fermentation condition. In the fermentation period, PP-15 reached the moderate activity (23.23mm) in 6 days fermentation period. To study the optimization of inoculum age, inoculation time (48, 60, 72, 84, 96, 108, 120, 132 and 144 hrs) were used and the highest antibacterial activity was found at 72 hrs (25.53 mm). In the proper size of inoculum, 10% was the most suitable and the maximum activities of PP-15 reached up (27.95 mm) followed by 30% and 20% respectively. According to Tomita (1988) in the fermentation studies, 72 hrs age and 10% size of inoculum were optimized for the production of antibacterial metabolite. The highest biomass and antibiotic activity was observed at an incubation time of 72 hrs by some other investigators (Srinivasulu *et al*., 2002).In addition, effects of variation of carbon and nitrogen sources were observed for the growth of colony morphology and maximum antimicrobial metabolite production. In the carbon source, the colony of PP-15 was the excellent growth on glucose, starch and tapioca powder. There was a high degree of variation in the level of antimicrobial activity in the present study when the different carbon sources were tested in the fermentation medium. Moderate growth and the antimicrobial substance production of PP-15 were influenced by addition of starch reaching the highest activity 36.85mm, followed by sucrose (35.84mm) and tapioca powder (35.84 mm).Katoke*et al* 1992 studied that different carbon sources like sucrose, glycerol, starch, dextrose, lactose and fructose have been reported to be suitable for production of secondary metabolite in different organisms. Fungi have 40-55% carbon use efficiency so they store and recycle more (C) compared to bacteria (James *et al*., 2011).

The nature of the nitrogen source has notable effect on the production of antibacterial metabolite in PP-15. Especially PP-15 showed the moderate growth on almost all nitrogen sources. Maximum production of antibacterial metabolite of PP-15 was observed on peptone (30.79mm) followed by malt extract, yeast extract and gelatin respectively as nitrogen sources. El-Gammal AA, 1986 described that peptone has been reported by the suitability of nitrogen sources for the production of metabolites from microorganisms. Effect of pH was studied by varying from pH 4, 5, 6, 7, 8 and 9. The best antibacterial activity was found at pH-6 (27.78 mm). The change of pH is also important for the enzyme activity of microorganisms, for the intermediate products, their dissociation and solubility (Rizk*et al*.,2007).

The maximum production of antibacterial metabolite (28.83 mm) was obtained at 25ºC. Physical factors incubation such as temperature, can exert different effect on the growth and production phases of secondary metabolism (Rizk*et al*.,2007).

Fermentation media (FM) were studied and FM-1 gave the highest activity (30.89mm). The choice of the 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 (E1-Tayeb*et al*., 2004).In the comparison between shaking and static culture, the antibacterial activity of shaking culture PP-15 more than the static culture. Stevens *et al*., 1975 indicated that adequate agitation was found to increase antibiotic metabolite production. Thus, the results of the optimum fermentation tests indicated that antimicrobial metabolites obtained from PP-15 may be produced optimally in the presence of 6^{th}days fermentation period,72 hrs age of inoculums and 10% inoculums size, glucose and starch in the carbon source, gelatin and peptone in the nitrogen source, pH-6, temperature 25ºC, FM-1 and shaking culture. It was concluded that the present

study revealed to observe the fermentation period of isolated fungi and to investigate the optimization parameters of fermentation condition on PP-15 against *Agrobacterium tumefaciens.*

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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