ISOLATION AND FERMENTATION CONDITIONS OF SELECTED SOIL BACTERIUM (YN-51) FROM LAPUTTA TOWNSHIP AND THEIR ANTIMICROBIAL ACTIVITIES

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Abstract

Soil samples from three different areas of Laputta Township, Ayeyawady Region were cultured on five kinds of media. A total of 79 bacterial colonies were isolated from these soil samples. The purpose of this study was to screen for antimicrobial activities and fermentation conditions for selected bacterium. In this study, antimicrobial activities of all isolated strains were examined by agar well diffusion assay with seven test organisms. Among them, ten strains namely YN-1, 6, 7, 35, 51, 55, 56, 57, 59, and 63 showed different levels of antimicrobial activities. Especially, YN-51 showed the highest activity on *Candida albicans.* The fermentation conditions of YN-51 were optimized by the studies of fermentation period, proper size, age, different carbon and nitrogen sources utilization, pH, temperature, static and shaking culture on antimicrobial metabolite production on *Candida albicans.* In the fermentation period, YN-51 showed the highest activity (22.31 mm) in 3 days followed by (21.82 mm) in 2 days against *Candida albicans.* In the investigation, YN-51 was found that 15% of size of inoculum and 72 h of age of old culture were suitable conditions. The addition of glucose as a carbon source resulted better growth of YN-51 and the inhibition zone reached 27.07 mm in sucrose. In the nitrogen source, the maximum growth of YN-51 was found in yeast extract and the highest antifungal activity (30.49 mm) was found in beef extract. And then, effects of pH range and temperature were also determined. According to the results, pH 7.5 and temperature 40 °C were found to be the best activities (29.64 mm and 27.29 mm) on *Candida albicans* respectively. In the comparison between shaking culture and static culture, the antifungal activity reached (25.61 mm) in shaking culture and (21.70 mm) in static culture respectively.

Keywords: antimicrobial activity, antifungal activity, soil bacterium, fermentation medium, *Candida albicans*

Introduction

Soil microorganism are the studies of organisms in soil, their functions and how they effect soil properties. It is believed that between two and four billion year ago, the first ancients bacteria and microorganisms came about in Earth's primitive seas. These bacteria could fix nitrogen, in time multiplied and as a result released oxygen into the atmosphere. This release of oxygen led to more advanced microorganisms. Microorganisms in soil are important because they affect the structure and fertility of different soils (Subba, 1999).

In their natural habitats, bacteria utilize the antibiotics they produce as protective substances by rendering the invasion of other bacterial species. Protection is not the only function of antibiotics. Hence, antibiotics also act as signaling molecules that bacteria use as a means of communication between cells (Linares *et al.,* 2006). The natural products that had been developed into drugs, many come from plant resources, but there had been a considerable number of important drugs harvested from microorganisms the production of antimicrobial substances depends upon the substrate medium for their optimal growth, temperature, pH and the concentration of nutrients in the medium (Leifert *et al*., 1995).

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In the presence of various carbon and nitrogen sources microorganisms have diverse levels of phosphate solubilization activity. Carbon source is an important parameter for active proliferation of organisms and production of organic acids and nitrogen source is important for the production of organic acids (Kucey, 1989). Carbon and nitrogen sources together with fermentation time have been reported to play significant roles in the determination of the final morphology of the culture. Carbon sources in the media play a very critical role in the production of antimicrobial substances by the bacteria (Papagianni, 2004). The aim of this research is to screen for antimicrobial activities and fermentation conditions for selected bacterium.

Materials and Methods

Collection of Soil Samples

Three different soil samples were collected from three different sites of Laputta Township, Ayeyawady Region in June, 2017. These samples were collected in depth 6 inches from soil surface. The samples were placed in plastic bag. The experiment was carried out at the laboratory of Biotechnology and Development Centre of Pathein University. Soil type, pH, moisture and location of these soil samples are shown in Table 1.

Table 1 Some Physiochemical Parameters of Three Different Soil Samples Collected from Laputta Township

Soil samples	Soil type*	Soil pH	Moisture %	Location
Aung-Taw-Mu (S-1)	Sandy Loam	6.83	1.77	N 16º 8.867" E 94º 45.658"
Tapin-Shwe-Tee (S-2)	Loam	6.06	3.02	N 16º 9.3" E 94º 48.359"
Laputalote (S-3)	Sandy Loam	6.83	0.93	N 16º 9.811" E 94º 42.604"

*Soil type was classified at Department of Agriculture, Insein Township, Yangon.

Isolation of Bacteria from Soil Samples

The soil bacteria were enumerated by serial dilution method on media such as CAS medium, Centenum medium, Nutrient Agar medium, Dextrose Casein Peptone Agar medium and Glucose Peptone Agar medium.

Serial dilutions method

Serial dilutions of fermented, plating and streaking techniques described by Salle (1948), Collins (1965) and Pelezar and Chan (1972) were used for the isolation of microorganisms from soil. An appropriate amount (1 g) of soil was introduced into a conical flask containing 99 mL of distilled water to make a soil-water dilution ratio of 1: 100. The flask was then shaken for about 30 min in order to make the soil particles free from each other. This solution was then serially diluted into 10^{-3} to 10^{-7} dilution in separate test tubes and 1 mL each of the above dilutions was separately transferred into sterile petridishes under aseptic condition. A sterile pipette was used for each transfer. The sterilized medium in conical flask was cooled down to about 45°C and

separately poured into each of the petridish containing the respective soil dilutions. The inoculated plates were shaken clock-wise and anti-clockwise direction for about 5 min so as to make uniform distribution of the bacterial inoculums. When the agar was solidified, the inoculated plates were inverted and incubated at 30°C for 24 h. Various types of colonies developed on the inoculated plates. They were separately streaked over another set of pertridishes containing the same sterile medium. Each of the discrete colonies visible in the second set of inoculated plates was separately transferred to sterile nutrient agar medium. The isolates were maintained in nutrient agar medium for further experiments.

Preliminary Study on Antimicrobial Activities of Isolated Bacteria

The isolated soil bacteria were inoculated into seed medium and incubated for 1 day at 27 ºC. After one day, the seed culture (1%) was transferred into the fermentation medium and carried out by static culture. Then, the fermented broth was used to check the antimicrobial activity by agar well method (Collins, 1965). Agar well having 8 mm in diameter were utilized for antimicrobial activity.

Screening of Antimicrobial Activity by Agar Well Diffusion Method

One day old culture test broth (0.2 mL) was added to 25 mL warm assay medium (glucose 1.0 g, yeast extract 0.3 g, peptone 0.2 g, agar 1.8 g, DW 100 mL) 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 h. The diameter of the zones of inhibition around each well measured and recorded after 24-48 h incubation.

Effect of Sizes and Ages of Inoculums for Fermentation

The cultivation times (24, 48, 72, 96, 120, 144 and 168 h) were employed for the production of antimicrobial metabolite. In the investigation of sizes of inoculums 5%, 10%, 15%, 20%, 25% and 30% were used for the antifungal activity of YN-51. Seed culture was inoculated in the 150 mL conical flask and incubated at room temperature. In the investigation of ages of inoculums, the incubation of seed culture times (24, 48, 72, 96, 120, 144 and 168 h) were used and transferred into the fermentation media. Fermentations were carried out for 7 days and antifungal activity was tested by agar well diffusion method.

Effects of Different Carbon Sources Utilization

Carbon sources (each 1.0 g) such as glucose, xylose, sucrose, mannitol, lactose, starch, fructose, maltose, glycerol, tapioca powder, molasses, soluble starch, potato, dextrose, corn, oat, carrot and rice were used. Fermentation media were incubated at 25 ºC for 2 days.

Effects of Different Nitrogen Sources Utilization

Nitrogen sources (each 1.0 g) such as ammonium chloride, ammonium nitrate, ammonium oxalate, asparagine, ammonium sulphate, beef extract, casein, fish cake, gelatin, meat extract, milk, malt extract, peanut, peptone, soy bean, sodium nitrate, potassium nitrate, urea and yeast extract were also used. Fermentation media were incubated at 25 ºC for 2 days.

Effect of Incubation pH and Temperature on the Selected Bacterium YN-51

Effects of different pH were used for antifungal activity of pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9. These different pH were adjusted by NaOH and HCl. The selected bacteria YN-51 was inoculated and incubated at six different temperatures 20 °C, 25 °C, 30 °C, 35 °C, 40 °C and 45 °C.

Antifungal Activity on Shaking and Static Culture of the Selected Bacterium YN-51

100 mL conical flask containing 50 mL of the best fermentation medium was incubated on the shaker (100 rpm) for 3 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 ager well diffusion assay method.

Results and Discussion

Isolation of Bacteria

In the course of the isolation of bacteria, three different samples were collected from Laputta Township, Ayeyawady Region. A total of 79 strains were isolated from these soil samples. 27 isolates were obtained from CAS medium, 9 isolates were formed from Centenum medium, 10 isolates from Nutrient Agar medium, 13 isolates from Dextrose Casein Peptone Agar medium and 20 isolates from Glucose Peptone Agar medium. These results are shown in Table 2 and Figure 1.

Table 2 Isolated Bacteria from Soil Samples

Soil Sample No.	CAS medium	Centenum medium	Nutrient Agar	Dextrose Casein Peptone Agar medium	Glucose Peptone Agar medium	Total strains (Soil Samples)
S-1	YN 1-24= 24	YN28-35= 8	YN37-39= 3	YN47-51= 5	YN60-76= 17	57
S-2	YN 25-26= 2	-	YN40-46= 7	YN52 = 1	-	10
S-3	YN 27 = 1	YN36 =1	-	YN53-59= 7	YN77-79= 3	12

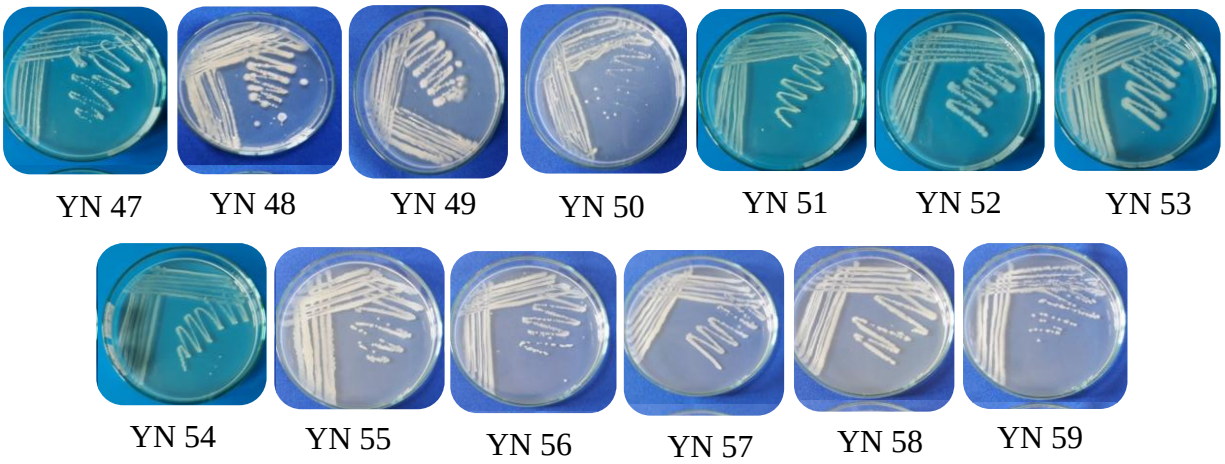


Figure 1 Morphological characters of isolated bacteria (YN 47-59) on dextrose casein peptone agar medium

Antimicrobial Activities of Isolated Bacterial Strains

Preliminary studies of antimicrobial activity of isolated bacteria (YN 1-79) were tested by seven test organisms with agar well diffusion assay method. 57 strains showed different level of antimicrobial activity. Among them, ten strains showed the moderate antimicrobial activity on almost seven test organisms. YN-7 showed the antibacterial activity (15.58 mm) at 1 day followed by YN-1 (15.56 mm) at 2 days and YN-6 (13.90 mm) at 3 days against *Agrobacterium tumefaciens*.

Especially, YN-56 gave the respective antibacterial activity (26.16 mm, 23.23 mm and 21.63 mm) at 1 day against *Bacillus pumilus*, *Bacillus subtilis* and *Escherichia coli,* respectively. Moreover, YN-56 exhibited the antifungal activity (23.02 mm) at 1 day on *Candida albicans* followed by YN-51 (21.47 mm) and YN-7 (16.81 mm). And then, YN-51 and 59 gave the antibacterial activity (17.28 mm and 16.80 mm) against *Staphylococcus aureus* and *Pseudomonas fluorescens.* Thus, YN-51 was selected for further studies (Table 3 and Figure 2-5).

Table 3 Antimicrobial Activity of Selected Bacteria against Seven Test Organisms

No.	Test Organisms	Strains and Inhibition zone (mm)					
		YN-1	**YN-6**	**YN-7**	**YN-51**	**YN-56**	**YN-59**
1.	*Agrobacterium tumefaciens*	15.56	13.90	15.58			
2.	*Bacillus pumilus*			-	-	26.16	-
3.	*Bacillus subtilis*			-	-	23.23	-
4.	*Candida albicans*			16.81	21.47	23.02	-
5.	*Escherichia coil*			-	-	21.63	-
6.	*Pseudomonas fluorescens*			-	-	-	16.80
7.	*Staphylococcus aureus*			-	17.28	-	-

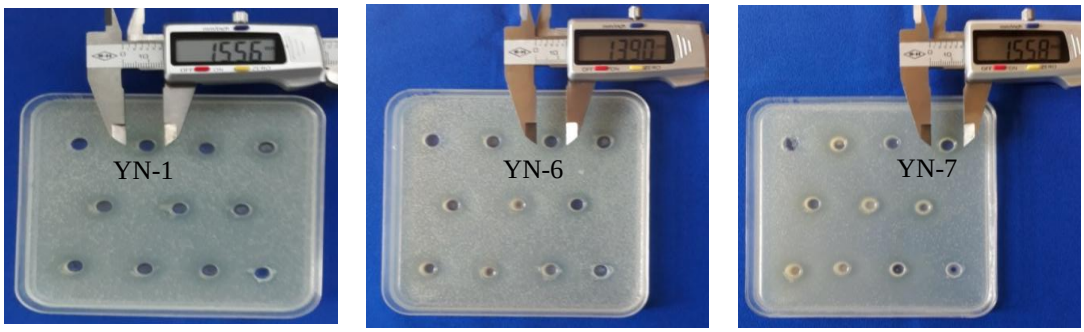


Figure 2 Antibacterial activity of selected bacteria against *Agrobacterium tumefaciens*

Figure 3 Antibacterial activity of selected bacteria against (a) *Bacillus pumilus* and (b) *Bacillus subtikis*

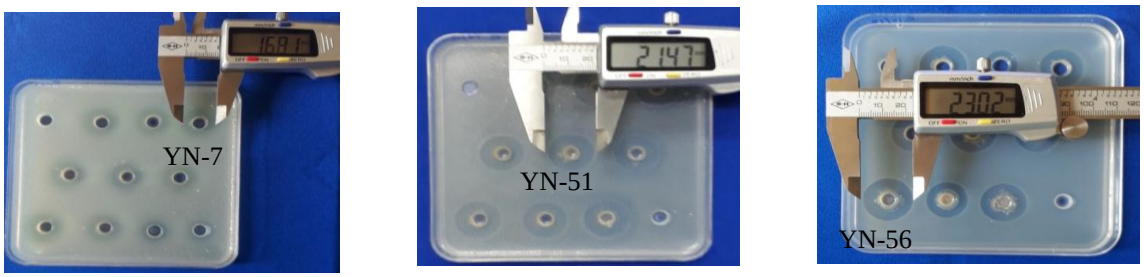


Figure 4 Antifungal activity of selected bacteria against *Candida albicans*

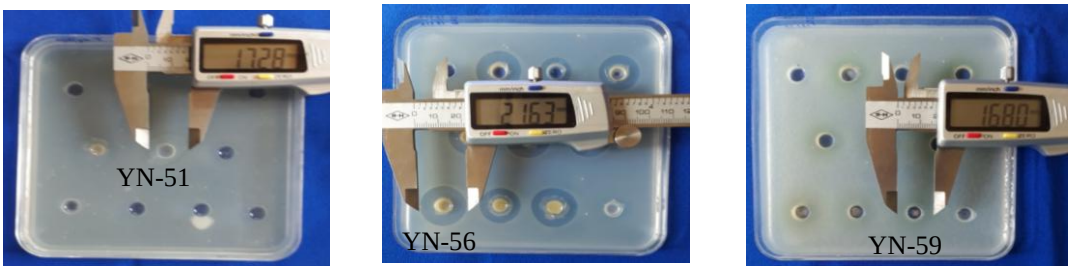


Figure 5 Antibacterial activity of selected bacteria against (a) *Staphylococcus aureus* (b) *Escherichia coil* and (c) *Pseudomonas fluorescens*

Effect of Fermentation Periods on Selected Strain YN-51

In the study of fermentation periods, the incubation zone indicated that if increased from 2 days to 3 days and the maximum highest activity showed in 3 days of fermentation period (Table 4 and Figure 6).

Table 4 Effect on Fermentation Periods of Selected Bacterium YN-51 Against *Candida albicans*

Fermentation period (Day)	Antifungal activity (mm)
1	20.42
2	21.82
3	**22.31**
4	19.58
5	18.79

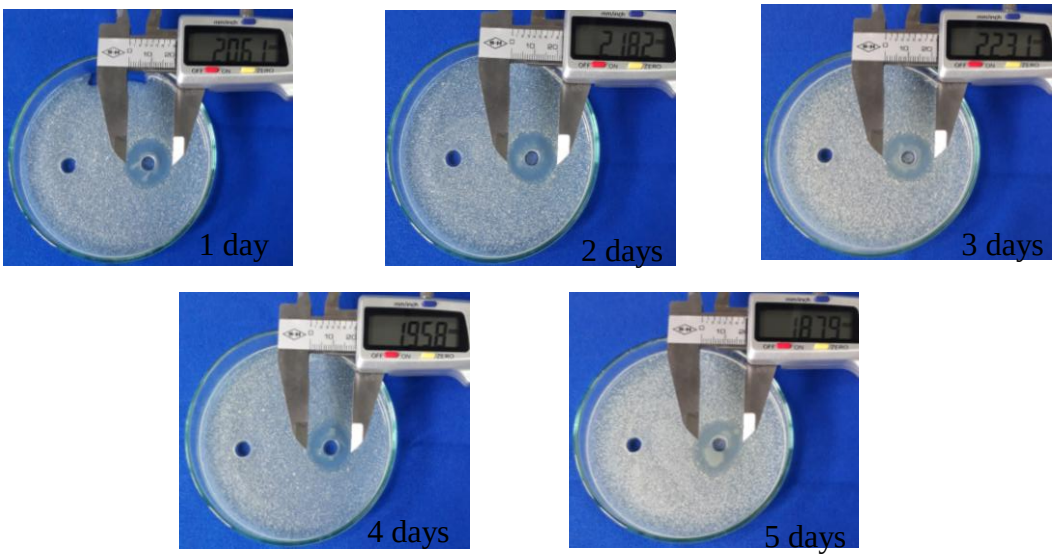


Figure 6 Effect on fermentation periods of selected bacterium YN-51 against *Candida albicans*

Effect of Sizes of Inoculums for YN-51

In this study, the effect of sizes of inoculums was studied by using 5%, 10%, 15%, 20%, 25% and 30% inoculums for the maximum production of antifungal activity. Using 15% inoculums exhibited significantly higher activity (23.17 mm), followed 25% (22,64 mm) and showed in 5%, !0%, 20%, 30% respectively. The result found that significant antifungal activity was increased at 15% for the optimal formation of antifungal activity (Table 5 and Figure 7).

Table 5 Effect of Sizes of Inoculums for YN-51

Sizes of Inoculums (%)	Antifungal activity (mm)
5	20.49
10	22.37
15	**23.17**
20	21.47
25	22.64
30	22.15

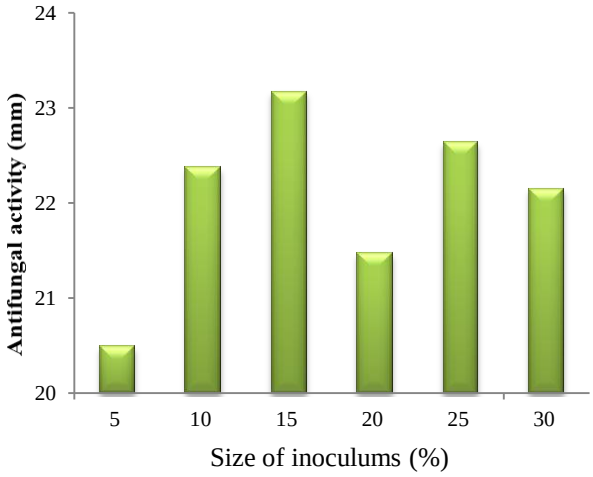


Figure 7 Effect of sizes of inoculums

Effect of Ages of Inoculums for YN-51

In the study of the effect of inoculums age, it was observed that from (27.20 mm) on 72 h, followed (26.76 mm) 96 h, (26.38 mm) 120 h, and showed in 144 h, 168 h, 48 h and 24 h respectively. The result indicate that antifungal activity of YN-51 reached the highest activities (27.20 mm) in 72 h age of inoculums on *Candida albicans* (Table 6 and Figure 8).

Table 6 Effect of Ages of Inoculums for YN-51

Ages of Inoculums (hrs)	Antifungal activity (mm)
24	20.92
48	26.24
72	**27.20**
96	26.76
120	26.38
144	22.29
168	20.37

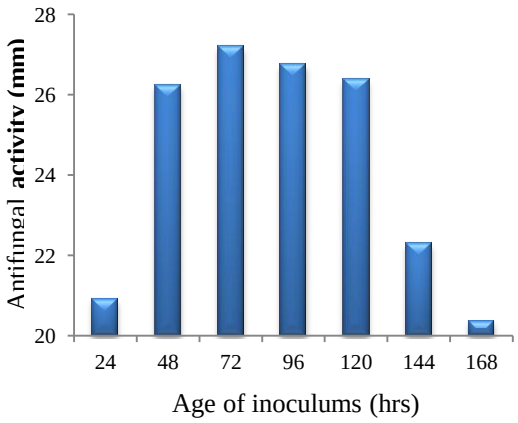


Figure 8 Effect of ages of inoculums

Investigation of Carbon and Nitrogen Sources Utilization

The effects of different carbon sources were observed for the growth rate. The addition of glucose, xylose, starch, corn and rice gave excellent growth, fructose, glycerol, tapioca powder, molasses and carrot were moderate growth and other sources showed good results. For the growth with nitrogen utilization, it was found that excellent growth of YN-51 on beef extract, milk, peanut and yeast, moderate growth on ammonium chloride, asparagine, casein, gelatin, meat extract, soy bean, sodium nitrate and urea and other nitrogen sources showed good results (Table 7).

Table 7 Growth of YN-51 on Carbon and Nitrogen Sources

Carbon sources	Growth (mm)	Nitrogen sources	Growth (mm)
Glucose	**excellent (10.43)**	Ammonium chloride	moderate (3.19)
Xylose	good (6.82)	Ammonium nitrate	good (6.81)
Sucrose	excellent (7.58)	Ammonium oxalate	good (5.38)
Mannitol	excellent (7.56)	Asparagine	moderate (4.12)
Lactose	good (6.66)	Ammonium sulphate	good (6.35)
Starch	excellent (7.25)	Beef extract	excellent (7.90)
Fructose	moderate (4.51)	Casein	moderate (4.47)
Maltose	good (5.60)	Fish cake	good (5.39)
Glycerol	moderate (4.51)	Gelatin	moderate (3.78)
Tapioca powder	moderate (3.78)	Meat extract	moderate (3.95)
Molasses	moderate (3.19)	Milk	excellent (7.94)
Soluble Starch	good (5.60)	Malt extract	good (5.60)
Potato	good (6.35)	Peanut	excellent (7.02)
Dextrose	good (6.79)	Peptone	good (6.49)
Corn	excellent (7.69)	Soy bean	moderate (4.64)
Oat	good (5.55)	Sodium nitrate	moderate (4.82)
Carrot	moderate (3.99)	Potassium nitrate	good (5.81)
Rice	excellent (8.26)	Urea	moderate (4.51)
-	-	**Yeast extract**	**excellent (9.83)**

1 – 2.9 mm = poor 3 – 4.9 mm = moderate
5 – 6.9 mm = good 7 & above = excellent

Effect of Different Carbon and Nitrogen Sources on the Antifungal Activity of YN-51 Against *Candida albicans*

The effects of different carbon sources were observed for the maximum antimicrobial metabolites production. The results indicated that sucrose showed the highest activity (27.07 mm), followed by molasses (27.07 mm) and potato (23.40 mm) in carbon sources (Table 8 and Figure 9)and Beef extract reaching the highest activity (30.49 mm), followed by malt extract (25.45 mm) and fish cake (24.34 mm) in nitrogen sources (Table 9 and Figure 10).

Table 8 Effect of Different Carbon Sources on the Antifungal Activity of YN-51 Against *Candida albicans*

No	Carbon sources	Inhibitory Zone (mm)	No	Carbon sources	Inhibitory Zone (mm)
1.	Glucose	17.16	10.	Tapioca powder	20.90
2.	Xylose	20.34	11.	Molasses	27.02
3.	**Sucrose**	**27.07**	12.	Soluble Starch	13.18
4.	Mannitol	23.39	13.	Potato	23.40
5.	Lactose	18.16	14.	Dextrose	19.44
6.	Starch	11.39	15.	Corn	16.57
7.	Fructose	19.22	16.	Oat	19.05
8.	Maltose	18.53	17.	Carrot	22.57
9.	Glycerol	24.84	18.	Rice	12.46

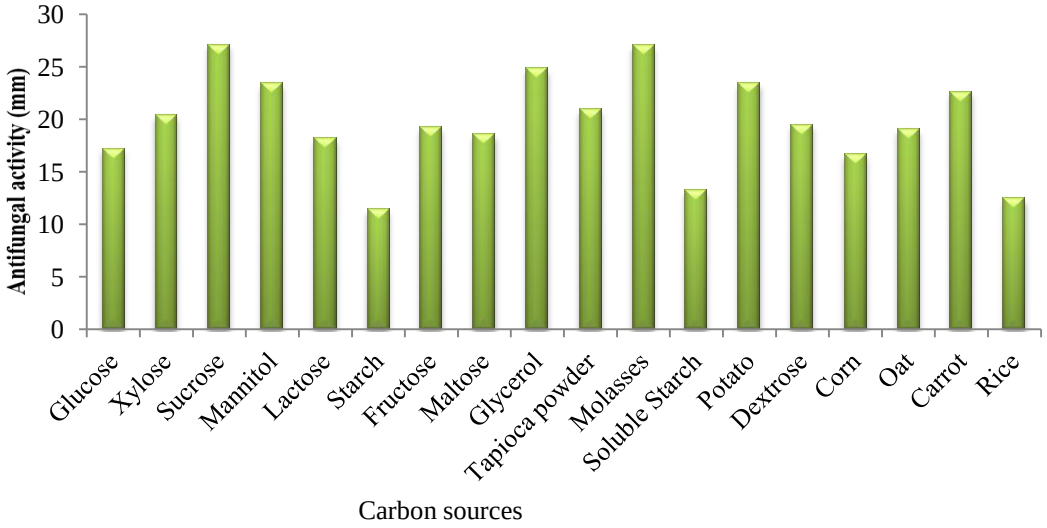


Figure 9 Effect of different carbon sources on antifungal activities of YN-51

Table 9 Effect of Different Nitrogen Sources on the Antifungal Activity of YN-51 Against *Candida albicans*

No	Nitrogen sources	Inhibitory Zone (mm)	No	Nitrogen sources	Inhibitory Zone (mm)
1.	Ammonium chloride	13.65	11.	Milk	19.53
2.	Ammonium nitrate	17.63	12.	Malt extract	25.45
3.	Ammonium oxalate	12.94	13.	Peanut	12.26
4.	Asparagine	23.51	14.	Peptone	17.46
5.	Ammonium sulphate	13.00	15.	Soy bean	18.45
6.	**Beef extract**	**30.49**	16.	Sodium nitrate	15.60
7.	Casein	-	17.	Potassium nitrate	23.86
8.	Fish cake	24.34	18.	Urea	18.86
9.	Gelatin	14.81	19.	Yeast extract	15.08

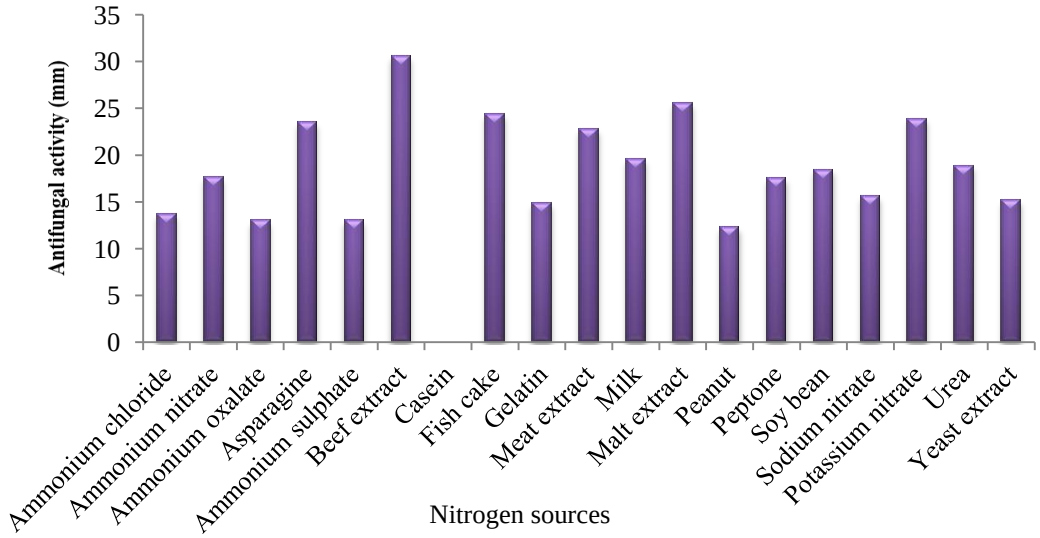


Figure 10 Effect of different nitrogen sources on antifungal activities of YN-51

Effects of pH and different temperature utilization of YN-51 against *Candida albicans*

The effect of pH and temperature were tested with pH levels (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) and different temperature ranges (20°C, 25°C, 30°C, 35°C, 40°C and 45°C). Maximum inhibitory zone was occurred in pH 7.5 (29.64 mm) and it was followed by pH 7 (29.06 mm) and pH 8 (28.52 mm) (Table 10 and Figure 11). Maximum antifungal activity was recorded at 40°C (27.29 mm), followed by 35°C (26.91 mm) (Table 11 and Figure 12).

Table 10 Effects of pH Utilization of YN-51 Against *Candida albicans*

pH range	Inhibitory zone (mm)
4.0	-
4.5	-
5.0	-
5.5	23.95
6.0	24.19
6.5	24.59
7.0	29.06
7.5	**29.64**
8.0	28.52
8.5	26.76
9.0	26.63

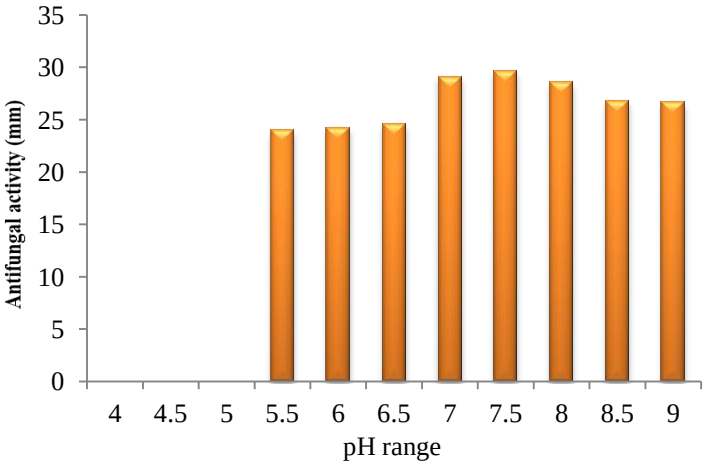


Figure 11 Effects of pH utilization of YN-51 against *Candida albicans*

Table 11 Effects of Different Temperature Utilization YN-51 Against *Candida albicans*

Temperature range (ºC)	Inhibitory zone (mm)
20	22.79
25	23.66
30	24.90
35	26.91
40	27.29
45	23.03

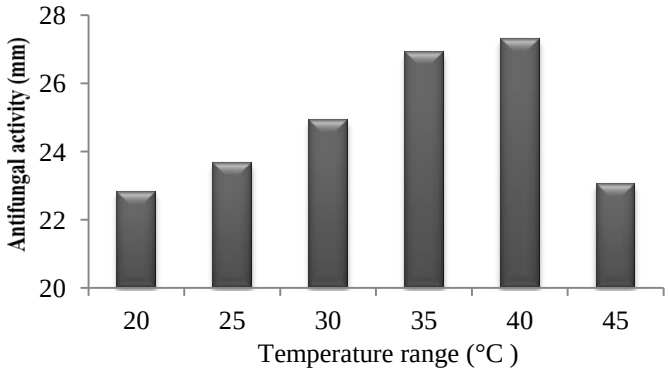
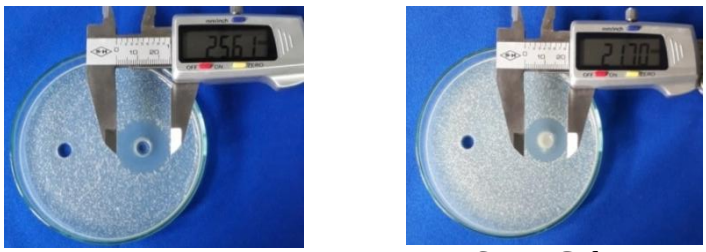


Figure 12 Effects of different temperature utilization YN-51 against *Candida albicans*

Comparison between Shaking Culture and Static Culture of YN-51

In this investigation, the comparison between shaking culture and static culture were observed. The shaking culture showed the inhibitory zone 25.61 mm and the static culture showed that 21.70 mm for 2 days fermentation (Figure 13).



Shaking Culture Static Culture

Figure 13 Comparison between shaking culture and static culture of YN-51

Conclusion

In the course of the isolation of bacteria, 79 strains were isolated from three different samples collected from Laputta Township, Ayeyawady Region. Five different media employed in the investigation of the isolation of bacteria, it was found that 27 isolates were obtained from CAS medium, 9 isolates were formed from Centenum medium, 10 isolates from Nutrient Agar medium, 13 isolates from Dextrose Casein Peptone Agar medium and 20 isolates from Glucose Peptone Agar medium. The isolated bacteria were designated as YN-1 to YN-79.

Some isolated bacterial strains were tested for antimicrobial activities by seven test organisms with agar well diffusion method and these strains showed different levels of antimicrobial activities. YN-7 showed the antibacterial activity (15.58 mm) at 1 day followed by YN-1 (15.56 mm) at 2 days and YN-6 (13.90 mm) at 3 days against *Agrobacterium tumefaciens*. Especially, YN-56 gave the respective antibacterial activity (26.16 mm, 23.23 mm and 21.63 mm) at 1 day against *Bacillus pumilus*, *Bacillus subtilis* and *Escherichia coli*, respectively. Moreover, YN-56 exhibited the antifungal activity (23.02 mm) at 1 day on *Candida albicans* followed by YN-51 (21.47 mm) and YN-7 (16.81 mm). And then, YN-51 and 59 gave the

antibacterial activity (17.28 mm and 16.80 mm) against *Pseudomonas fluorescens* and *Staphylococcus aureus*

In the investigation to optimize the fermentation, it was found that 72 h ages of inoculums and 15% of size of inoculum were suitable for fermentation. The effects of variation of carbon and nitrogen sources were observed for the growth and maximum metabolite production. The addition of glucose, xylose, starch, corn and rice as carbon sources were better growth and the maximum inhibition zone resulted in sucrose (27.07 mm) followed molasses (27.02 mm). Potato showed good growth but optimum inhibition zone was 23.40 mm.

In the utilization of nitrogen sources, the maximum production of antifungal metabolite of YN-51 was found in the present of beef extract (30.49 mm). The supplement of yeast extract and milk were excellent growth in the morphology but the optimum metabolite production were the former (15.08 mm) and the latter (19.53 mm) respectively. In the study of different pH and temperature utilization for the fermentation, the highest activity was found at pH 7.5 (29.64 mm) and the optimum temperature at 40°C (27.29 mm). Comparison between shaking culture and static culture showed shaking culture (25.61 mm) and static culture (21.70 mm). Hence, the antifungal activity of shaking culture was larger than the static culture in metabolite production.

The present study concluded that the optimal conditions required for the production of bioactive metabolites by selected bacterium YN-51 were determined and metabolites showed better antifungal activity against human pathogen, *Candida albicans*.

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