ISOLATION AND OPTIMIZATION PARAMETERS OF FERMENTATION CONDITIONS OF SELECTED SOIL BACTERIUM (SY-17) FROM MYINT THA TOWNSHIP, MANDALAY REGION

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Abstract

Soil samples were collected from six different areas of Yoar Thit village, Kin Sein Zay and Pauk Myang village of Myint Tha Township, Mandalay Region. These sample were cultured on Flo Medium, Nutrient. Agar medium, CAS medium, Glucose Peptone Agar medium and Centenum medium. The experiments were carried out at the Microbiology Laboratory of Botany Department in Mandalar Degree College. A total of 36 bacterial colonies were obtained from these soil samples. These isolated bacteria were designated as SY-1 to 36. Antimicrobial activity of all strains were carried out by agar well diffusion assay with five test organisms. Among them, SY -12, 13, 17, 22, 27, 29 and 32 strains showed different levels of antimicrobial activities. Especially, SY-17 showed the highest antifungal activity (39.06 mm) on *Candida albicans.* Therefore, SY-17 was selected and the fermentation condition of this bacterium was carried out by the study of fermentation period 3 days (27.77 mm), proper age 72 hrs (26.02 mm), size 20 % (16.86 mm), different carbon sources maltose (38.05 mm) and nitrogen sources sodium nitrate (48.19 mm), effect of fermentation medium FM 5 (29.98 mm), pH 7 (21.95 mm), temperature 25°C (39.65 mm), and shaking (34.01 mm) and static culture (26.02 mm) on antimicrobial activity against *Candida albicans*. It result of this work can provide the knowledge of nature of soil bacteria and how to select active strains exhibited against some test organisms.

Keywords: Soil bacteria, antimicrobial activity, fermentation

Introduction

Microorganisms are frequency present in soil, manure and decaying plant tissues which are able to degrade wastes that are correlated with the substrate organic matter (Alexander, 1977).

In general the majority of microbial population if found in the upper six to twelve inches of soil and the number decreases with depth.

Then number and kinds of organisms found in soil depend upon the nature of soil, depth, season of the year, state of the cultivation, reaction, organic matter, temperature moisture, aeration, etc. Soil is a primary source of microorganisms (Omalu *et al.,* 2011).

Microbes in the soil are the key to carbon and nitrogen recycling. A teaspoon of production soil generally contains between 100 million and 1 billion individual bacteria. That is as much as two cows per-acre. A ton of micrscopic bacteria may be action per core and there may be over one million species of bacteria present.

Natural products from microorganisms have been the most successful source that has found many application in the fields of medicines, pharmacy and Agriculture. Microorganisms were found to produces secondary metabolites with a diverse chemical structure and antimicrobial activities (Stachelhaus *et al.,* 1995).

Microorganisms play important roles on nutritional chains that are important for biological balance in the life on our planet being essential for the closing of nutrient and geochemical cycles such as the carbon, nitrogen, sulfer and phosphorus cycle. (Madsen, 2008).

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Materials and Methods

Collection of Soil Samples

Six different soil samples were collected from three different places of Yoar Thit village, Kin Sein Zay village and Pauk Myaung village in Myint Tha Township, Mandalay Region in the month of August 2017.

Isolation of Bacteria from the Soil Samples (Altas, 1993)

The soil Bacteria were enumerated by physical and chemical treatment dilution method or media such as FLO Medium, Nutrient Agar Medium, Chrome azural S **(**CAS) Medium, Glucose Peptone Agar Medium (GPA medium), Centenum Medium.

Media used for isolation of Bacteria

FLO Medium (Altas, 1993)

Casein	10.0 g
Peptone	10.0 g
K_2HPO_4	1.5 g
$MgSO_4.\ 7H_2O$	1.5 g
Agar	15.0 g
Distilled Water	1000 mL
pH	5.0

Nutrient Agar Medium (Altas, 1993)

Peptone	5.0 g
NaCl	5.0 g
Yeast extract	2.0 g
Agar	15.0 g
Beef extract	1.0 g
Distilled Water	1000 mL
pH	5.0

CAS Medium (Chrome azural S) (Altas, 1993)

Casein	10.0 g
$MgSO_4.\ 7H_2O$	1.0 g
K_2HPO_4	0.25 g
Agar	18.0 g
Distilled Water	1000 mL
pH	6.8

Glucose Peptone Agar Medium (GPA medium) (Altas, 1993)

Peptone	20.0 g
Glucose	10.0 g
NaCl	5.0 g
Agar	18.0 g
Distilled Water	1000 mL
pH	7.2

Centenum Medium (Altas, 1993)

Yeast extract	10.0 g
Sodium pyruvate	2.2 g
K_2HPO_4	1.0 g
$MgSO_4.\ 7H_2O$	0.5 g
Vitamin B12	0.02 g
Agar	20 g
Distilled Water	1000 mL
pH	7.0-7.2

Nutrient Broth Medium

(Dubey and Mahesh Wari 2007)

Peptone	5.0 g
Beef extract	3.0 g
Sodium chloride	5.0 g
Yeast extract	10.0 g
Distilled Water	1000 mL
pH	6.8-7.02

Medium used for Antimicrobial Activity

Seed and Fermentation Medium Glucose Yeast Peptone (GYP) Medium (Atlas, 1993)	
Glucose	10 g
Yeast extract	3 g
Peptone	2 g
Distilled water	1000 mL
pH	6.5

Assay Medium Glucose Yeast Peptone (GYP) Medium (Atlas, 1993)	
Glucose	10 g
Yeast extract	3 g
Peptone	2 g
Agar	16 g
Distilled water	1000 mL
pH	6.5

Serial Dilution Method of Soil Samples (Collins, 1964)

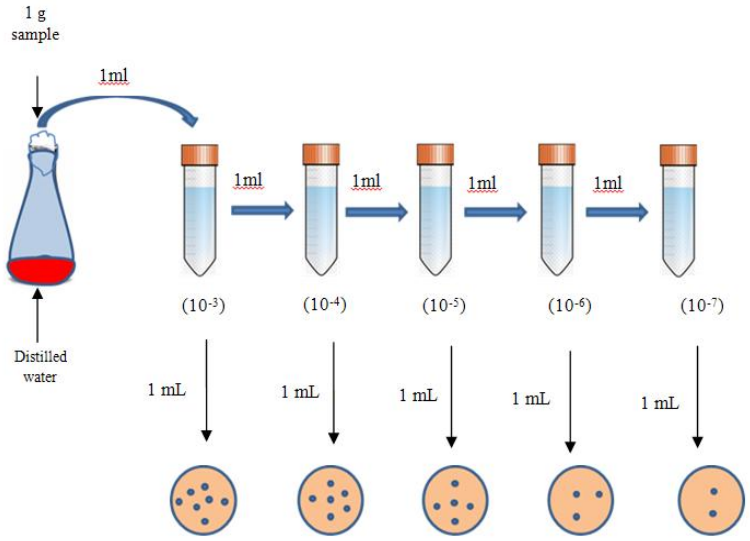

Figure 1 Serial dilution method for soil samples (Collin, 1964)

Scanning for Antimicrobial Activity of Isolated Bacteria

The isolated soil bacteria were inoculated into seed medium and incubated for 1 day at 27 ºC. Seed culture were transferred to the fermentation medium. 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, 1964). Agar well having (8 mm in diameter) were utilized for antimicrobial activity.

Scanning of Antimicrobial by Agar well method (Collins, 1965)

This method was used for the antimicrobial activity by five test organisms. The assay medium (glucose – 1.0 g, yeast extract – 0.3 g, peptone – 0.2 g, agar – 1.6 g) was utilized for these bacteria. Isolated strains were subjected with antimicrobial activity by agar well method. Cork borer was used to make the wells (8 mm in diameter) in the autoclave basal antimicrobial test medium.

Well impregnated with 1-5 days old culture fermented broth (0.1 mL) were incubated at room temperature for 24-48 hours. After 24-48 hours of incubation, the clear zones were

measured. Clear zone surrounding the test wells indicated the presence of antimicrobial activities which inhibit the growth of the test organisms selectively.

Fermentation medium with various carbon sources (NITE, 2005)

The initial basal medium contained yeast extract (0.5 g), K_2HPO_4 (0.001 g), $MgSO_4$ (0.001 g), $CaCO_3$ (0.1 g), DW (100 mL), pH 7. After that glucose (1 g, 1.5, 2, 2.5 and 3g) was added to the initial basal medium. The medium were sterilized by autoclaving at 121ºC for 30 min, and were cooled before inoculation. And then, SY-17 was inoculated. Fermentation was carried out for 5 days and antimicrobial activity was tested by agar well diffusion method.

Fermentation medium with various nitrogen sources (NITE, 2005)

The initial basal medium used contained glucose (1g), soluble starch (0.5g), K_2HPO_4 (0.001g), $MgSO_4$ (0.001g), $CaCO_3$ (0.1g), DW (100 mL), pH 7. Peptone (1, 1.5, 2, 2.5 and 3g) was added to the initial basal medium. The medium were sterilized by autoclaving at 121°C for 30 min, and were cooled before inoculation. And then, SY-17 was inoculated. Fermentation was carried out for 5 days and antimicrobial activity was tested by using agar well diffusion method.

Fermentation Medium (FM Medium)

Fermentation was undertaken with suitable conditions of 20 % size and 72 hrs ages of inoculum with different media. Fermentation was carried out for 7 days and antifungal activity test was carried out every 24 hrs. Composition of fermentation media use on the present study. All the ingredient well dissolved in 1 liter distilled water and adjusted to pH 7.0.

Fermentation Medium -1			**Fermentation Medium-2**	
Maltose	10 g		Maltose	10 g
Sodium nitrate	3 g		Ammonium phosphate	3 g
Agar	16 g		Agar	16 g
Distileld water	1000 mL		Distilled water	1000 mL
pH	7		pH	7

Fermentation Medium -3			**Fermentation Medium-4**	
Maltose	10 g		Maltose	10 g
Potassium nitrate	3 g		Urea	3 g
Agar	16 g		Agar	16 g
Distileld water	1000 mL		Distilled water	1000 mL
pH	7		pH	7

Fermentation Medium -5			**Fermentation Medium-6**	
Sucrose	10 g		Sucrose	10 g
Sodium nitrate	3 g		Ammonium phosphate	3 g
Agar	16 g		Agar	16 g
Distileld water	1000 mL		Distilled water	1000 mL
pH	7		pH	7

Fermentation Medium -7			**Fermentation Medium-8**	
Sucrose	10 g		Sucorse	10 g
Potassium nitrate	3 g		Urea	3 g
Agar	16 g		Agar	16 g
Distileld water	1000 mL		Distilled water	1000 mL
pH	7		pH	7

Fermentation Medium -9			**Fermentation Medium-10**	
Soluble starch	10 g		Soluble starch	10 g
Sodium nitrate	3 g		Ammonium phosphate	3 g
Agar	16 g		Agar	16 g
Distileld water	1000 mL		Distilled water	1000 mL
pH	7		pH	7

The effect of pH on fermentation conditions (Hernadez*, et al*., 2005)

The pH sensitivity of the culture supernatant recovered during stationary growth phase of the isolates, pH values were adjusted ranging from 4-10 by using 0.1 M NaOH or 0.1 M HCl. The medium constituents were sterilized by autoclaving at 121°C for 30 min, and were cooled before inoculation. And then, the strain was cultured. Fermentation was carried out for 5 days and antimicrobial activity was tested by agar well diffusion method.

The effect of temperature on fermentation (Hernadez*, et al*., 2005)

The medium constituents were sterilized by autoclaving at 121°C for 30 min, and were cooled before inoculation. And then, the strain was cultured. The selected bacteria were incubated at 20°C, 25°C, 30°C, 35°C , 40°C and respectively. Fermentation was carried out for 5 days and antimicrobial activity was tested by using agar well diffusion method.

The static and shaker of fermentation conditions

Using all optimized medium components, the shake-flasks was done using (1.5g) of sucrose as a carbon sources and (1 g) of sodium nitrate as nitrogen source. The flasks were

placed in (TS-2000 A VDRL) shaker. After 5 days, the fermented broth was tested on agar well diffusion method. Precisely, the static fermented broth was also tested on the above method.

Results

Isolation of bacteria from soil samples

In the investigation, 36 bacterial strains were isolated from the six different soil sample of Myint Tha Township. Isolated bacteria SY-1-27 were obtained from Yoar Thit village, SY 28-31 from Kin Sein Zay village and SY-32-36 from Pauk Myang village. The results were shown in Table 1. The isolated bacteria were designated as SY 1-36. (The colony morphology of soil bacteria were small, medium and large in size and color were white yellow and cream. The margin of colonies were entire, undulate, lobate, rhizoid, curled and the elevation were raised and flat.) The colony morphology of soil bacteria SY-17 was circular, large, entire, cream flat, cream colour. The strain were rod shape and gram positive. The results were show in Table (2).

Table 1 Chemical Analysis of Six Different Soil Samples Collected from Myint Tha Township

Soil sample	Collected place	location	Soil Type	pH	Moisture	Total N %	Available p (ppm)	Exchangeable k (me/100g)	Available K_2OMg/ 100
S I	Yoar Thit Village	N22º25'54" E 96º 19'54"	Silty loans	8.83	3.95	0.28	3.52	0.29	13.61
S II	Yoar Thit village	N22º25'54" E 96º 99'56"	Loans	8.62	4.4	0.22	3.41	0.15	7.04
S III	Kin Sein Zay Village	N22º25' 36" E 96º 07'03"	Silty clay	8.78	2.95	0.18	2.22	0.14	6.57
S IV	Kin Sein Zay Village	N22º25' 48" E 96º 06'48"	Silty clay loans	8.20	2.99	0.32	14.23	1.16	54.43
S V	Pauk Myang village	N22º26' 02" E 96º 06'00"	Silty clay	8.49	4.23	0.22	12.08	0.37	17.36
S VI	PaukMyaung village	N22º26'12" E96º05'40"	Silty clay	9.81	4.83	0.18	13.16	1.29	60.53

Table 2 Isolation of Soil Bacterium on five different media

Soil Sample	FLO Medium	Nutrient Agar medium	CAS medium	Glucose peptone Agar medium	Centenum medium
S -1	SY-1-9 = 9	SY-16-21 = 6			
S-2	SY-10-15= 6	SY-22-27 = 6			
S-3			SY -28 = 1		
S-4				29-31 = 3	
S-5				32-33 = 2	
S-6					34-36 = 3
Total	15	12	1	5	3

Figure. Chracteristic of Isolated Bacteria SY-17

Isolated bacteria and their Antimicrobial Activity

In this study, seven selected bacterial strains were tested with five test organisms by agar well diffusion method. There are *Agrobacterium tumefaciens, Bacillas Pumilus, Bacillus subtilis, Candida albicans, Staphylococcus aureus.* Among them, SY-17 showed the best activities on *Candida albicans* (Table 3)

Table 3 Antimicrobial activity of isolated bacteria SY-17 against five test organism

No.	Isolated bacteria	Test organism and Antimicrobial activity (mm)				
		Agrobaterium tumefaciens	***Bacillus pumilus***	***Bacillus subtilis***	***Candida albicans***	***Stuphylococcus aureus***
1	SY-12	28.24	22.24	26.01	+	23.09
2	SY-13	29.97	+	+	18.54	27.55
3	SY-17	+	30.90	31.51	**39.06**	25.81
4	SY-22	+	+	16.01	22.70	20.78
5	SY-27	+	+	25.45	+	+
6	SY-29	21.28	+	23.90	+	25.45
7	SY-32	+	17.48	+	-	23.37

Effect of fermentation period on the antifungal activity of SY-17 on *Candida albicans*

SY-17 reached the highest activities 27.77 mm in 3 day period of inoculum on *Candida albicans* (Table 4, Figure 3)

Table 4 Effect of fermentation period on the antifungal activity of SY-17 on *Candida albicans*

No	Fermentation period days	Activity (clear zone , mm)
1	1	14.72 mm
2	2	16.67 mm
3	3	**27.77 mm**
4	4	22.66 mm
5	5	16.71 mm

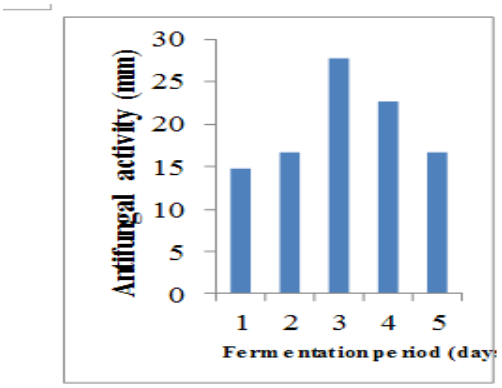

Figure 3 Effect of fermentation period on the antifungal activity of SY-17 on the *Candida albicans*

Effects of ages of inoculum on the antifungal activity of SY-17 on the *Candida albicans*

SY-17 showed the highest activities (26.02 mm) in 72 hrs age of inoculum on *Candida albicans* (Table 5 and Figure 4).

Table 5 Effects of ages of inoculum on the antifungal activity of SY-17 on the *Candida albicans*

No	Age of	Antifungal
1	24 hrs	14.99 mm
2	48 hrs	17.08 mm
3	72 hrs	**26.02 mm**
4	96 hrs	13.62 mm
5	120 hrs	13.23 mm
6	144 hrs	11.61 mm

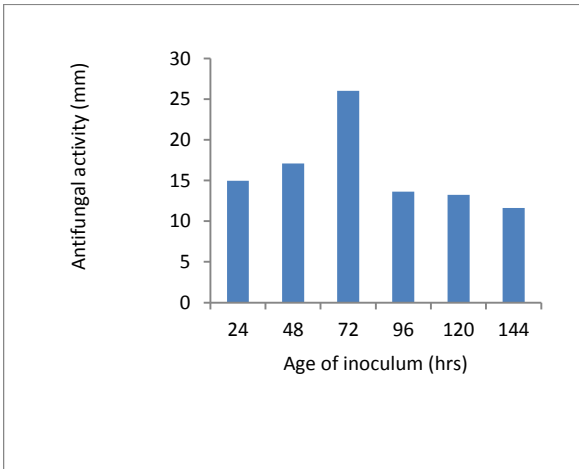

Figure 4 Effects of ages of inoculum on the antifungal activity of SY-17 on the *Candida albicans*

Effects of sizes of inoculums on the antifungal activity of SY-17 on the *Candida albicans*

In the proper size of inoculums, 20% was the most suitable activity 16.86 mm followed by 25% and 30% respectively (Table 6 and Figure 5).

Table 6 Effects of sizes of inoculums on the antifungal activity of SY-17 on the *Candida albicans*

No	Sizes of	Antifungal
1	5%	14.66
2	10%	14.70
3	15%	16.01
4	20%	**16.86**
5	25%	16.50
6	30%	16.31

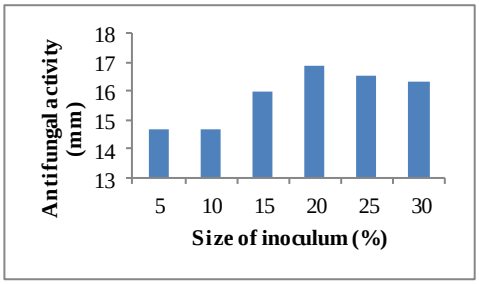

Figure 5 Effects of sizes of inoculums on the antifungal activity of SY-17 on the *Candida albicans*

Effects of carbon and nitrogen sources on the antifungal activity of SY-17 on *Candida albicans*

The maximum antifungal activity of SY-17 was influenced by addition of maltose and sodium nitrate reaching the moderate antifungal activity (38.05 mm and 48.19 mm). These results were shown in Table 7 and 8.

Table 7 Effects of carbon and nitrogen sources on the antifungal activity of SY-17 on *Candida albicans*

Sr. No	Carbon sources	Antifungal Activity(mm)	Sr. No	Carbon sources	Antifungal Activity (mm)
1	Carrot	13.14	8	Mannitol	15.79
2	Lactose	13.62	9	Xylose	15.70
3	Potato	14.77	10	Soluble starch	31.39
4	Oat	16.21	11	Rice	18.76
5	Molassess	15.93	12	Glycerol	16.41
6	Maltose	**38.05**	13	Glucose	17.11
7	Sucrose	35.18	14	Corn	16.35

Table 8 Effects of Nitrogen Sources on the antifungal activity of SY-17 on *Candida albicans*

Sr. No	Nitrogen sources	Antifungal Activity	Sr. No	Nitrogen sources	Antifungal Activity
1	Soybean	11.02	8	Peptone	15.26
2	Meat extract	14.30	9	Fish cake	14.66
3	Asparagine	14.03	10	Yeast	14.72
4	Urea	31.98	11	Ammonium sulphate	19.36
5	Ammonium phosphate	43.32	12	Gelatin	16.52
6	Sodium nitrate	**48.19**	13	Potassium nitrate	40.81
7	Casein	17.47	14	Ammonium chloride	24.16

Effects of various fermentation media on the antifungal activity of SY-17 on *Candida albicans*

In this study, FM 5 showed the maximum antifungal activity (29.98 mm) against *Candida albicans* (Tables 9).

Table 9 Effects of various fermentation media on the antifungal activity of SY-17 on *Candida albicans*

No	Fermentation media	Antifungal activity (mm)
1	FM-1	21.35
2	FM-2	15.80
3	FM-3	16.19
4	FM-4	15.18
5	FM-5	**29.98**
6	FM-6	15.58
7	FM-7	17.22
8	FM-8	15.02
9	FM-9	20.77
10	FM-10	14.82

Effects of different pH on the antifungal activity of SY-17 on *Candida albicans*

In this study, the highest antifungal activity was obtained at pH 7 (21.95 mm) against *Candida albicans* (Table 10 and Figure 6).

Table 10 Effects of different pH on the antifungal activity of SY-17 on *Candida albicans*

No	pH	Antifungal activity (mm)
1	4	-
2	5	14.13
3	6	17.31
4	7	**21.95**
5	8	21.07
6	9	18.21
7	10	14.82

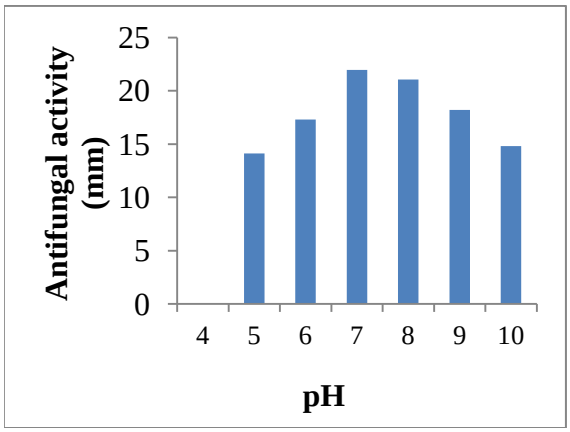

Figure 6 Effects of pH on the antifungal activity SY-17 on *Candida albicans*

Effects of different temperature on the antifungal activity of SY-17 on *Candida albicans*

In this investigation, temperature 25˚C showed the highest antifungal activity (39.69 mm) against *Candida albicans* (Table 11 and Figure 7).

Table 11 Effects of different temperature on the antifungal activity of SY-17 on *Candida albicans*

No	Temperature	Antifungal
1	20ºC	24.07
2	25ºC	**39.69**
3	30ºC	36.65
4	35ºC	35.65
5	40ºC	21.47

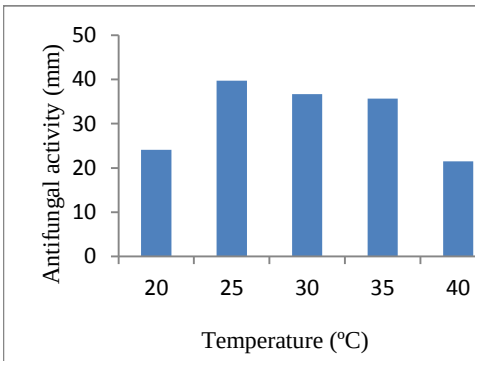

Figure 7 Effects of different temperature on the antifungal activity of SY-17 on *Candida albicans*

Effects of Static and shaking culture on the production of antifungal activity of SY-17 against *Candida albicans*

In this investigation, the antifungal activity between shaking culture and static culture were observed. The shaking culture showed the inhibitory zone 34.01 mm and the static culture showed 26.02 mm for 3 days fermentation.

Table 12 Effects of Static and shaking culture on the production of antifungal activity of SY-17 against *Candida albicans*

No	Fermentation	Antifungal activity
1	Static	**26.02**
2	Shaking	**34.01**

Static Shaking

Figure 8 Effects of Static and shaking culture on the production of antifungal activity of SY-17 against *Candida albicans*

Discussion and Conclusion

Soil contains varieties of microorganism including bacteria that can be established in any natural environment. Bacteria are the most important and abundant microorganism which is present in surrounding environment. These are very small, unicellular, primitive and non chlorophyll containing microorganisms. Dilution is one of the most important method to isolate the soil bacterium (Benson, 2001).

Soil samples were collected from six different places of Myint Tha Township, Mandalay Region. 36 bacteria were isolated from six different soil. The isolated bacteria were designated as SY-1 to SY-36.

In the present study, the antifungal activity of SY-17 was investigated by various fermentation conditions. In the fermentation period, SY-17 was found to be optimum fermented incubation period in 3 days.

Das 2006 stated that Antibacterial metabolites production by the strain was studied 1 to 4 days of fermentation. The highest amount was obtained on 3rd day of fermentation and then production was declined gradually. In the present work the isolated SY-17 was allowed to incubate for 72 hrs for the maximum production of antibacterial metabolites. And then, 20% inoculum size and 72 hrs age were the most suitable condition. The effect of various carbon and nitrogen sources were observed for the growth and maximum metabolite production. The addition of xylose, sucrose, molasses and glucose as carbon sources provided better growth and

the maximum inhibition zone resulted in maltose (38.05 mm) followed by sucrose (35.18 mm) and soluble starch (31.39 mm).

Among the nitrogen sources, the best growth of SY-17 was found on the ammonium sulphate and maximum antifungal activity was obtained in the sodium nitrate (48.19 mm) follow by ammonium phosphate (43.32 mm) and potassium nitrate (40.81 mm).

Yang *et al.,* 2006 reported that the carbon and nitrogen sources were the important constituents to be considered highly influenced on the antibiotic production by bacteria.

Ten kinds of fermentation media (FM) were utilized and the highest activity was found in FM-5 (29.98 mm). Microorganisms growing in the soil are influenced by factors such as moisture, temperature pH, carbon sources and nitrogen sources biotic factor and inhabiting factors (Davidson, *et al.,* 1998).

In the study of different temperature and pH utilization for the fermentation, the optimum temperature was found at 25°C (39.69 mm) and the highest activity was obtained at pH7.(21.95mm).

Lilly *et. al.,* 1951 reported that the maximum temperature of bacteria is 40°C and the optimum pH is 8. In the comparison between shaking culture and static culture, the antifungal activity of shaking culture (34.01 mm) was more than that of the static culture (26.02 mm). It was concluded that the present study was to observe the fermentation period of SY-17 against *Candida albicans* and to optimize the parameters of fermentation conditions of selected bacterium SY-17.

The present study concluded that the optimum conditions required for the production of bioactive metabolites by selected soil bacterium SY-17 were determined and metabolites showed better antifungal activity against human pathogen, *Candida albicans*.

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