FERMENTATION OPTIMIZATION AND SELECTION OF SOLVENT TO EXTRACT ANTIBACTERIAL COMPOUND FROM ISOLATED SOIL FUNGUS NLF-12

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Abstract

The present paper was studied by the fermentation optimization and selection of solvent to extract antibacterial compound from isolated soil fungus NLF-12. Twenty fungi were isolated from four different soil samples of Beikthano Ancient City, Taungdwingyi Township, Magway Region. Antimicrobial activity of all fungi was tested by agar well method on four test organisms and nine fungal strains showed the activity. Among them, soil fungus NLF-12 showed the highest antibacterial activity (30.54 mm) on *Micrococcus luteus*. Therefore, it was selected and the fermentation optimization of fungus NLF-12 were carried out by proper age, size, different carbon and nitrogen sources, fermentation medium (FM), pH, temperature, static and shaking culture. In the results of paper chromatography, ethyl acetate was the most suitable solvent for extraction of antibacterial secondary metabolites from fermented broth of fungus NLF-12 and R_f value had 0.92 on *Micrococcus luteus*. These results indicated that the selected soil fungus may be utilized by the optimal fermentation conditions for the screening of antibacterial activity.

Keywords: fermentation optimization, bioautography, metabolite

Introduction

Some microbe can perform an immense range of metabolic function. Fungi are considered as a good natural source for a production of biotic secondary metabolite that contains different bioactive agents including antibiotic, antitumor and antioxidant. Generally, the reason why they produce such metabolites is not known, but it is believed that many of these metabolites may act as chemical defense of microbes competing for substrates (Kumar *et al.*, 2012).

Paper chromatography separates dried liquid samples with a liquid solvent (mobile phase) and a paper strip (stationary phase). Separation of components depends on both their solubility in the mobile phase and their differential affinity to the mobile phase and stationary phase.

Solvent extraction is widely used during early purification of fermentation derived products and indeed, of all natural product matrices for initial and intermediate purification prior to final purification by chromatography, crystallization, or precipitation (Schugerl, 1999).

The aims and objectives of this research are to isolate the soil fungi from four different places, to observe the optimal fermentation conditions of soil fungus NLF-12 and to select the best solvent for extraction of antibacterial compound by paper chromatography.

Materials and Methods

Study Area and Collection of Soil Samples

Soil samples were collected from four different places of Beikthano Ancient City Taungdwingyi Township, Magway Region from July, 2016 to August, 2016. These samples were isolated by using two media (LCA and MEA).

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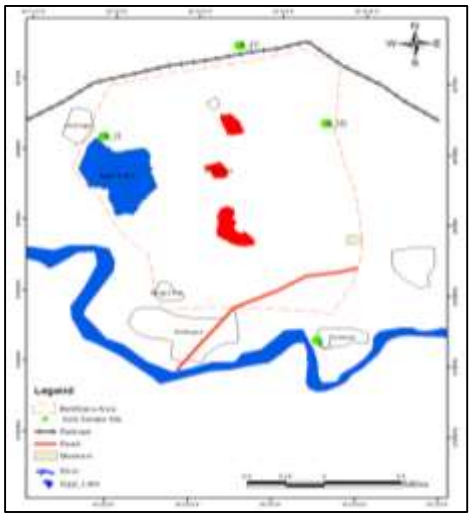

Map Sources: Beikthano Museum)

Table 1 Four different soil samples collected from Beikthano Ancient City

Samples Collected areas	pH	Soil type	Location
Gokkone village	9.54	Loam	N 19º 59.421' E 95º 24.432'
Shwe-yaung-taw compound	6.87	Sandy Loam	N 20º 0.874' E 95º 23.371'
Nan-twin-taw ya Monastery	9.04	Sandy Loam	N 20º 0.852' E 95º 23.277'
Beikthano railwaystation	6.80	Sandy Loam	N 19º 59.646' E 95º 23.353'

Figure 1 Four different sites of Beikthano Ancient City for soil sample collection

Chemical Treatment Dilution Method

The collected soil was air-dried at room temperature for 5 days. The soil sample was grounded and sieved in 2 mm screen. The sample was placed in the hot air oven at 120 ˚C for 1 hrs. The dried soil sample was suspended with 1.5 % phenol and diluted with sterile water. The dilution series were cultured on LCA medium. 30 µL of soil suspension was cultured on plates containing LCA medium and incubated for 5-10 days. Pure colonies were picked up to start containing in PGA medium (Phay and Yamamura, 2005).

Preliminary Study for Antimicrobial Activity

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

Screening of Antimicrobial Activity by Agar Well Diffusion Method

1 day old culture test broth (0.01 mL) was added to 25 mL 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 (20 µL) 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 hours incubation (Collins, 1965).

Test organisms

Agrobacterium tumefaciens NITE09678, *Aspergillus paraciticus* IFO5123, *Micrococcus luteus* NITE83297, *Pseudomonas fluorescens* IFO94307 were obtained from NITE (National Institute of Technology and Evaluation, Kisarazu, Japan)

Effects of ages of inoculums on the fermentation

The selected fungus NLF-12 was grown on PGA medium for 5 days at room temperature. After 5 days incubation period, seed cultures (48, 54, 60, 66, 72, 78, 84 and 90 hours) incubation was inoculated into the flasks containing fermentation medium. Fermentation medium was carried

out from 48 to 90 hours and antibacterial activity was tested by agar well diffusion method (Crueger, 1989).

Effects of sizes of inoculums on the fermentation

The selected fungus NLF-12 was grown on PGA medium for 5 days at room temperature. After 5 days incubation period, this fungus was inoculated into 150mL seed medium. Based on the result of the ages of inoculums of NLF-12, (5%, 10%, 15%, 20%, 25%, and 30%) of 72 hours seed cultures were utilized for the fermentation. All fermentation media were carried out 5 days and antibacterial activity was investigated by agar well diffusion method (Crueger, 1989).

Effect of different carbon and nitrogen sources on fermentation medium

Optimal fermentation is 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 dextrose, fructose, lactose, maltose, sucrose, manitol, molasses and glycerol and various nitrogen sources such as casein, gelatin, potassium nitrate, malt extract, sodium nitrate, ammonium chloride, ammonium sulphate and peptone were supplemented with the basal medium. The fermented broth containing these medium were assayed for antibacterial activity against *Micrococcus luteus* using agar well diffusion method.

Carbon sources in basal fermentation medium			**Nitrogen sources in basal fermentation medium**		
Peptone	-	0.5g	Glucose	-	1.0g
Yeast extract	-	0.5g	Sucrose	-	0.5g
K_2HPO_4	-	0.001g	K_2HPO_4	-	0.001g
$CaCO_3$	-	0.01g	$CaCO_3$	-	0.01g
$MgSO_47H_2O$	-	0.001g	$MgSO_47H_2O$	-	0.001g
DW	-	100mL	DW	-	100mL
pH	-	6.5	pH	-	6.5

Media selection in the fermentation study

Eight fermentation media (FM-1 to FM-8) were prepared for the fermentation study. In the preparation of eight fermentation media best carbon sources (molasses, glycerol and dextrose) and best nitrogen sources (peptone, NH_4Cl, and malt extract) were utilized as suitable ratios and compositions. The medium constituents were sterilized by autoclaving at 121ºC for 30 min, and were cooling thoroughly before inoculation. Fermentation was carried out 5 days and antibacterial activity was tested by agar well diffusion method.

Medium composition (gram per liter)

FM- 1		**FM- 2**		**FM- 3**		**FM- 4**	
Glycerol	10 g	Dextrose	10 g	Maltose	10 g	Molasses	0.5 g
NH_4Cl	10 g	Malt extract	10 g	$NaNO_3$	10 g	$NaNO_3$	10 g
K_2HPO_4	0.01 g	K_2HPO_4	0.01 g	K_2HPO_4	0.01 g	K_2HPO_4	0.01 g
$CaCO_3$	0.01 g	$CaCO_3$	0.01 g	$CaCO_3$	0.01g	$CaCO_3$	0.01 g
$MgSO_47H_2O$	0.01 g	$MgSO_47H_2O$	0.01 g	$MgSO_47H_2O$	0.01 g	$MgSO_47H_2O$	0.01 g
pH	6.5	pH	6.5	pH	6.5	pH	6.5

FM- 5		**FM- 6**		**FM- 7**		**FM- 8**	
Molasses	10 g	Molasses	15 g	Molasses	20 g	Molasses	25 g
Peptone	10 g	Peptone	10 g	Peptone	10 g	Peptone	10 g
K_2HPO_4	0.01 g	K_2HPO_4	0.01 g	K_2HPO_4	0.01 g	K_2HPO_4	0.01 g
$CaCO_3$	0.01 g	$CaCO_3$	0.01 g	$CaCO_3$	0.01 g	$CaCO_3$	0.01 g
$MgSO_47H_2O$	0.01 g	$MgSO_47H_2O$	0.01g	$MgSO_47H_2O$	0.01 g	$MgSO_47H_2O$	0.01 g
pH	6.5	pH	6.5	pH	6.5	pH	6.5

Effect of pH on fermentation conditions

The optimization of pH of the fermentation broth for antibacterial metabolite production was done by carrying out the fermentation at seven different pH values viz. 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0. For each pH value, desired pH by using either 0.1M NaOH or 0.1 M HCl was adjusted into fermentation medium (Furtado *et al*., 2005).

Effect of incubation temperature

The optimization temperature for antibacterial metabolite production was carried out at six different incubation temperatures viz. 20, 25, 30, 35, and 40 and 45°C. The fermentation medium was carried out 5 days and antibacterial activity was studied by agar well diffusion method (Cazar*et al*., 2004).

Comparison of static culture and shaking culture

250 mL conical flask containing 100 mL of the fermentation medium was incubated on the 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 method (Hassan *et al*., 2017).

Suitable synthetic fermentation medium

Based on the results of above fermentation parameters, composition of the suitable synthetic medium was prepared the following conditions. The effect of fermentation period for maximum production was observed up to 10 days by agar well diffusion method.

			Fermentation medium 7		
Ages of inoculum	-	72 hours seed culture	Molasses	-	20g
Size of inoculums	-	10%	Peptone	-	10g
Temperature	-	25ºC	K_2HPO_4	-	0.01g
pH	-	5	$MgSO_4$	-	0.01g
Fermentation period	-	5 days	pH	-	6.5
Agitation	-	100rpm	DW	-	1000ml

Paper chromatography (Tomita, 1998)

Paper chromatography of NLF-12 metabolite was carried out by the method of Tomita, 1998. The filter paper and four solvents; 20%NH_4CL, n-butanol saturated with water, n-butanol-acetic acid- water (3:1:1) and ethyl acetate saturated with water were used for preliminary characterization of antibiotics. The fermented broth samples were applied on the paper and allowed to dry. The papers were chromatographed in each solvent. Then, bioautography was done to check the antibacterial activity of each. Each paper was placed on assay agar plates. After one hour the

paper was taken out, and then the plates were incubated for 24-36 hours. The inhibitory zone was measured yielding R_f value for the corresponding bioactive compound. R_f value was calculated in the following equation.

$$R_f \text{ value} = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent}}$$

Results

Isolation of fungi from soil samples

In this study, 20 fungi were isolated from four different soil sample from Beikthano Ancient city, Taungdwingyi Township, Magway Region.

Table 2 Isolated fungi from soil samples

No.	Samples Collected areas	LCA Medium	MEA Medium	Total
1	Gokkone village	NLF-1, NLF-2	NLF-3,NLF-4, NLF-5	5
2	Shwe-yaung-taw compound	NLF-6	NLF-7, NLF-8, NLF 9,NLF-10	5
3	Nan-twin-taw ya Monastery	NLF-11, NLF-12	NLF-13, NLF-14, NLF-15	5
4	Beikthano railway station	NLF-16, NLF-17	NLF-18,NLF-19,NLF-20	5
	Total Isolated fungal strains	7	13	20

Screening on the antimicrobial activity of isolated fungi by agar well diffusion method

All isolated were tested for antimicrobial activity with four test organisms. Among them, NLF-12 showed the best activity against *Micrococcus luteus* NITE 83297 (30.54 mm) in 5 days fermentation period. According to these results, NLF-12 was selected further studies because it showed the highest antibacterial activity against *Micrococcus luteus* (30.54mm).

A B C

Figure 2 (A) Surface view of NLF-12, (B) Reverse view of NLF-12 and (C) The Antibacterial activity of selected fungus NLF-12 against *Micrococcus luteus*

Effects of ages of inoculums on the fermentation

In the effect of age of inoculums, NLF-12 was investigated by using 48, 54, 60, 66, 72, 78, 84 and 90 hours old culture age of inoculums. The results showed that 72 hours age of inoculums gave the highest activities (22.99mm) followed (22.95mm) at 66 hours and (22.92mm) at 60 hours age of inoculums.

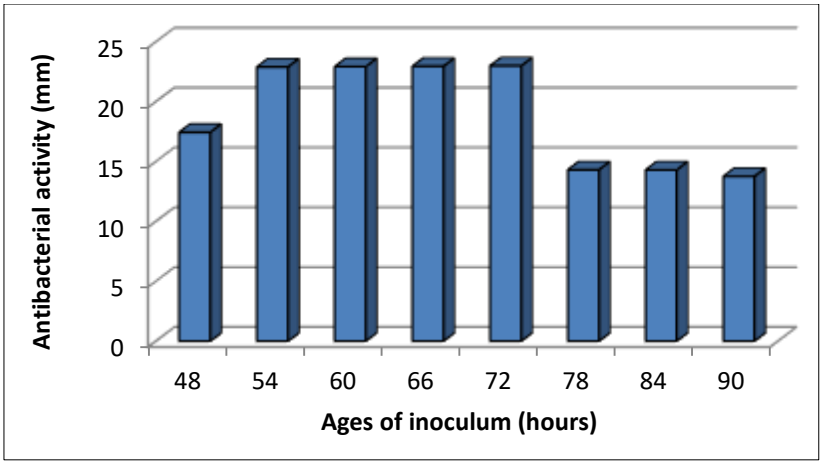

Figure 3 Effects of ages of inoculums of NLF-12 against *Micrococcus luteus* on the fermentation

Effects of sizes of inoculums on the fermentation

In this research work, the effect of size of inoculums were studied by using 5%, 10%, 15%, 20%, 25% and 30% inoculums. Using 10% inoculums showed the highest activities (26.53mm) than others, followed by 20% and 30% (25.06 mm and 25.10 mm).

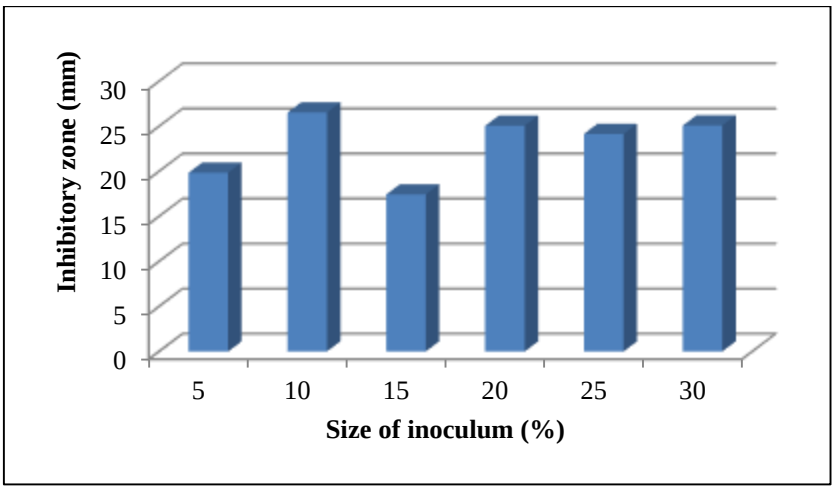

Figure 4 Effects of sizes of inoculums of NLF-12 against *Micrococcus luteus* on the fermentation

Effect of different carbon and nitrogen utilization on fermentation medium

There were variations in the level of antibacterial activity when the eight different carbon and nitrogen sources were used in the fermentation medium. The addition of the carbon sources showed the maximum antibacterial activities on molasses (31.36mm), followed by glycerol, dextrose and maltose and then fructose, lactose, sucrose, manitol were showed the minimum antibacterial activity. Similarly, when the addition of the various nitrogen sources, the maximum inhibition zone (30.94mm, 29.06mm, 28.71mm, 28.01mm) were obtained peptone, ammonium chloride, malt extract and sodium nitrate. Gelatin, potassium nitrate and ammonium sulphate showed moderate inhibition zone respectively. Casein as regarded as poor inhibition zone.

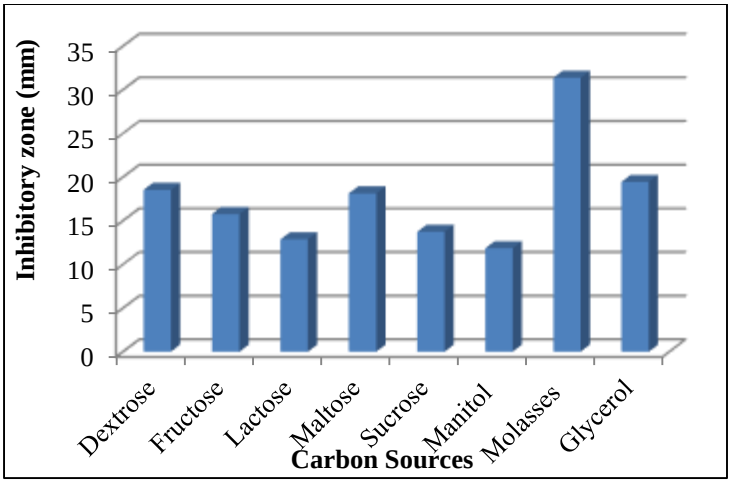

Figure 5 Effects of different carbon utilization on fermentation against *Micrococcus luteus*

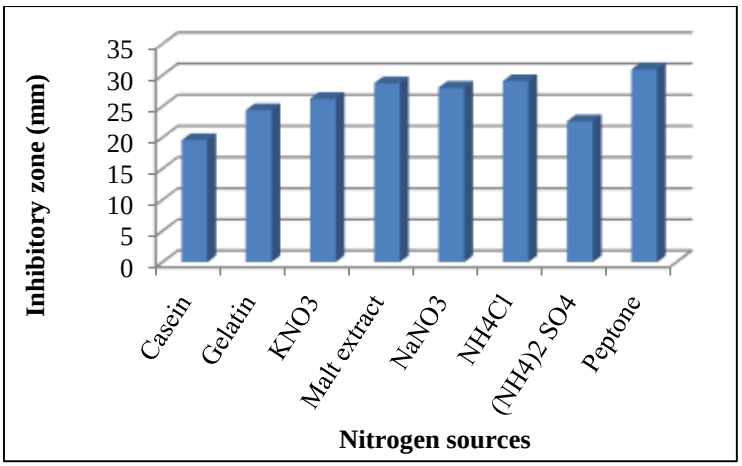

Figure 6 Effects of different nitrogen utilization on fermentation against *Micrococcus luteus*

Media optimization in the fermentation study

In the study of media optimization with eight fermentation media, FM-7 gave the highest antibacterial activity (29.33mm) followed by FM-8 (29.10mm), FM-6 (26.04mm) and FM-5 (24.35mm) respectively. It was determined that FM-7 showed the best activity on *Micrococcus luteus*, FM-7 was selected for the production of antibacterial metabolite.

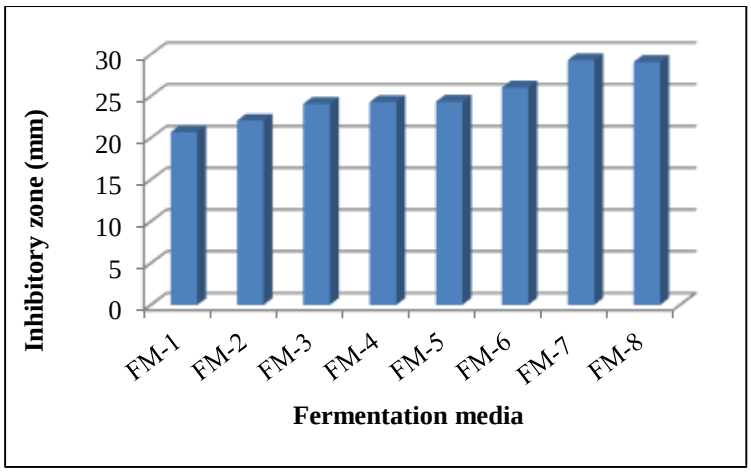

Figure 7 Antibacterial activity of selected fungus NLF-12 on the fermentation medium

Effects of different pH and temperature on bioactive metabolite production of selected fungus NLF-12 against *Micrococcus luteus*

The effect of pH and temperature were tested with different pH level (pH- 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) and different temperature ranges (20ºC, 25ºC, 30ºC, 35ºC, 40ºC and 45ºC). The best antibacterial activity was found at pH-5.0 (24.04mm) and temperature 25ºC showed the highest antibacterial activity (29.03mm) against *Micrococcus luteus*.

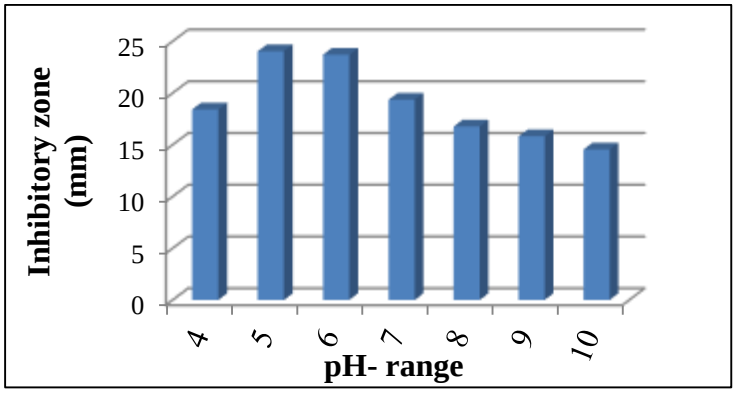

Figure 8 Effects of different pH on bioactive metabolite production of selected fungus NLF-12 against *Micrococcus luteus*

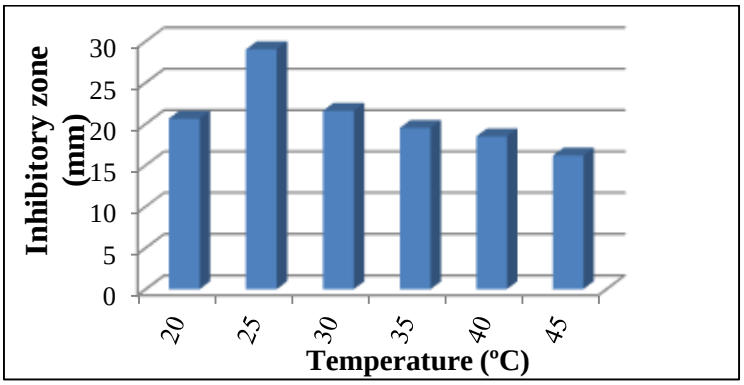

Figure 9 Effects of different temperature on bioactive metabolite production of selected fungus NLF-12 against *Micrococcus luteus*

Comparison of static culture and shaking culture

When comparing the static culture and shaking culture of fermentation medium, antibacterial activity from shaking is better than (28.19 mm) than that of static culture (26.76 mm).

Shaking culture

Static Culture

Figure 10 Comparison of static culture and shaking culture of of NLF-12 against *Micrococcus luteus*

The suitable synthetic fermentation medium

Based on the results of above fermentation parameters, such as 72 hours seed culture, 10% inoculums size, temperature 25ºC, pH 5.0 and shaking culture (100 rpm), FM-7, the maximum antibacterial activity of NLF-12 was observed by fermentation period 3 days to 10 days. In 5 days fermentation period, the NLF-12 showed the moderate antibacterial activity (29.32mm).

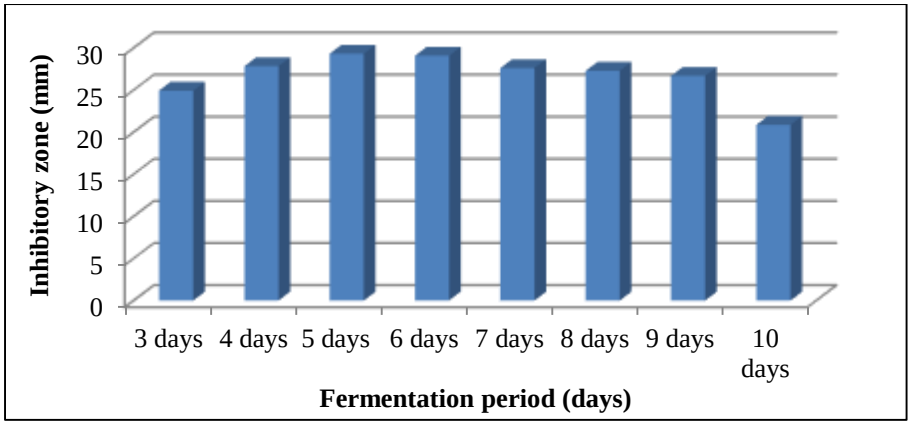

Figure 11 Antibacterial activity on suitable synthetic medium by selected fungus NLF-12 against *Micrococcus luteus*

Paper chromatography

In this study, four kinds of solvent 20%NH_4Cl, n-butanol saturated with water, n-butanol acetic acid with water (3:1:1) and ethyl acetate saturated with water were used. According to R_f value (0.92) was more extractable the antibacterial metabolites than other solvent, followed by n-butanol- acetic acid water (3;1:1)solvent (0.79), n-butanol (0.71) and the lowest R_f value at NH_4Cl (0.64).

1. 20%NH_4Cl
2. n-butanol saturated with water
3. n -butanol –acetic acid – water
4. Ethyl acetate saturated with water

Figure 12 Paper chromatography bioautographic assay

Discussion and Conclusion

Microorganism are able to synthesize secondary metabolites of various structure and hence bioactive (Demain, 1998). In the present research, fungus NLF-12 was focused by the fermentation optimization and selection of solvent systems. To study the optimization of inoculums age, incubation time (48, 54, 66, 72, 78, 84 and 90 hours) were used and the highest antibacterial activity was found at 72 hours (22.99 mm).In the inoculum size, 10% was the most suitable and gave the maximum activities (26.53 mm).

In the carbon sources, the maximum antibacterial metabolite of NLF-12 was found on molasses (31.36 mm). Kotake,(1992) described that the different carbon sources like sucrose, glycerol, starch, dextrose, lactose, and fructose to be suitable for production of secondary metabolite by different organisms. In the nitrogen sources, the maximum antibacterial metabolite

of NLF-12 was found on peptone (30.94 mm). El-Gammal, (1986) reported that peptone was the suitable nitrogen source for the production of metabolites from microorganism.

In the fermentation media (FM), eight kinds of fermentation media were used and FM-7 gave the highest antibacterial activity (29.33 mm). El-Tayeb *et al.*, (2004) described 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 of the fermentation.

In the effect of pH, the best antibacterial activity was found as pH-5.0 (24.04 mm). Jain and Pundir, (2011) reported that the pH level of the growth medium has a marked effect on secondary metabolite production with synthesis falling rapidly either side of an optimal level.

The various range of temperature were optimized by 20ºC, 25ºC, 30ºC, 40ºC and 45ºC and maximum inhibitory activity was recorded at the incubation temperature of 25ºC (29.03 mm). Temperature is one of important factors affecting microbial fermentation, due to it correlation with all biochemical enzyme catalysis processes (Zhou *et al.*, 2018). In the comparison of static culture and shaking culture, the antibacterial activity of static culture (26.76 mm) was less than that of shaking culture (28.19 mm). Mukhtar and Haq (2007) assumed that variation in the aeration level of the culture medium has specific effect on the growth of the microorganism and product formation during a submerged fermentation. The highest antibacterial activity was obtained by the suitable synthetic fermentation medium.

The rate of fermentation depends on the concentration of microorganisms, cells, cellular components as well as temperature and pH (Cruegar and Cruegar, 1989). According to the R_f value (0.92), ethyl acetate was the suitable solvent for NLF-12. Anuhya, (2017) described that the extraction of the secondary metabolite was effectively done with ethyl acetate.

Therefore in the next study, this solvent will use to extract bioactive compound and carry out purification of extracted bioactive compound from isolated soil fungus NLF-12.

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