ANTIBACTERIAL ACTIVITY OF ISOLATED ACTINOMYCETES FROM COLLECTED SOIL SAMPLES AT MAGWAY REGION

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Abstract

In this study, fifteen actinomycetes were isolated from the four soil samples collected at Pwint Phyu Township, Magway Region. Soil samples were carried out from June 2016 to July 2016. The isolation of actinomycetes was undertaken by the method of Chemical Treatment Dilution Method and Physical Treatment Serial Dilution Method. Soil actinomycetes were tested the antibacterial activities with five test organisms by using paper disc diffusion assay method. These isolated actinomycetes showed the antibacterial activities on test organisms. Among them, actinomycete A-01 showed the highest antibacterial activity on Bacillus subtilis (23.41 mm). Therefore, this actinomycete A-01 was selected for further investigation. In the fermentation studies of A-01, it was found that 46 hrs ages and 2% sizes of inoculum were optimized to produce the antibacterial metabolites.

Keywords: isolation, antimicrobial activity, ages and sizes of inoculum

Introduction

The Actinomycetes are aerobic, Gram-positive bacteria, which produce extensive branching vegetative (substrate) mycelium and aerial mycelium bearing chains of arthrospores. The substrate mycelium and spores can be pigmented, but also diffusible pigments are produced. On agar plates, they form lichenoid, leathery or burnous colonies. The GC-content of the DNA is 69-78 % (Williams *et al*., 1989).

Soil samples can be considered as a new source for isolation of microorganisms because there is much possibility of finding new microorganisms. Microorganisms have significant function in ecosystems and are found in all kinds of habitats. They produce numerous antimicrobial agents, including organic acids, enzymes and antibiotics. Microorganisms that live in the soil are essential to life on earth. The soil sample is the most effective and popular materials for the isolation of fungi and actinomycetes (Harayama & Isono, 2002).

Microbial secondary metabolites are important sources of natural compounds with potential therapeutic applications. As one of the versatile microorganisms, the streptomyces are the potent producers of secondary metabolites (Wux *et al*., 2007).

The percentage of actinomycetes and fungal strains which are showing antimicrobial activities. In standard agar diffusion assays ranges between 30- 80% depending on the ecological or taxonomic groups (Demain, 1999). Fermentation producers have to be developed for the cultivation of microorganisms under optimal condition and for the production of desired metabolites or enzyme by the microorganisms (Yamaie, 1984).

Antibiotic are the best known products of actinomycete. The present study in an attempt to produce antibiotics from actinomycetes, isolated from soil, by fermentation and the determination of their antimicrobial activity. *Bacillus subtilis* used as test organisms cause the food damage and fever.

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Materials and Methods

Collection of soil samples

Four different places of soil samples were collected at Pwint Phyu Township, Magway Region and were utilized for the isolation of soil actinomycetes. Soil samples were carried out from June 2016 to July 2016. In every collection site, soils were collected sample from different depths, between 1-6 inch. Four different places of soil samples were used for the isolation of actinomycetes and their location, soil pH and soil types are shown in Table 1.

Table 1 Soil samples collected at different places (Pwint Phyu Township)

Soil No.	Collected place		Texture	pH
S-1	Min Hla village	N 20º 21′ 45″ E 94º 40′ 08″	Silt Loam	7.2
S-2	Kwat Thit village	N 20º 21′ 43″ E 94º 40′ 08″	Silt Loam	7.4
S-3	Ywa Htaung village	N 20º 21′ 44″ E 94º 40′ 07″	Silt Clay Loam	7.8
S-4	Shwe Hlay village	N 20º 21′ 44″ E 94º 40′ 07″	Silt Clay Loam	7.9

Isolation of microorganisms from different soil samples

At the collecting different soil samples were dried at room temperature in the laboratory. Isolation of soil actinomycetes was undertaken by the methods of Chemical Treatment Dilution Method and Physical Treatment Serial Dilution Method (Phay and Yamamura, 2005).

Chemical treatment dilution method (Phay and Yamamura, 2005)

Soil sample 0.1 gram was added into the 9 mL of 1.5% phenol solution tube and shaking for 15 min. After shaking, 0.1 mL suspension was transferred to the 5 mL of sterile water tube. After that 0.5 mL of this suspension was transferred to the 4.5 mL sterile water tube and then 1 mL of this suspension was transferred to the 4 mL sterile water tube respectively. After taking 30 µL of the final soil suspension was placed on GSE medium and incubated at the temperature of 27°C. The inoculated plate was incubated for about 14 day.

Physical Treatment Serial Dilution Method

(Phay and Yamamura, 200 5)

One gram of soil was added into the 100 mL of water and heated at water batch (60°C) for 10mins. After that 0.1mL was transferred to 9.0 mL saline solution and then 1 mL was transferred to 9.0 mL of saline solution. In this way a series of 10 dilutions were prepared. After taking 0.1 mL of the final soil suspension tube was placed on GSE medium and incubated at the temperature of 27°C. The inoculated plate was incubated for about 14 day.

Medium Used for Screening of Actinomycetes

GSE Agar medium

Glucose	1.0g
Soy bean flour	0.5g
Nalidixic acid	0.01g
Humic acid	0.001g
$CaCO_3$	0.02g
NaH_2PO_4	0.5g
KCl	1.7g
$FeSO_47H_2O$	0.01g
Agar	1.8g
Soil extract + DW	50+50 mL
pH	7.2

After autoclaving, cycloheximide was added to this medium.

Preliminary study for antimicrobial activities by paper disc diffusion assay (NITE, 2005)

The isolated actinomycetes were grown at 27˚C for 14 days on ISP II medium. And then actinomycetes were inoculated on seed medium (glucose 2.0 g, Yeast extract 1.0 g, peptone 0.5 g, KNO_3 0.1g, K_2HPO_4 0.001 g, DW 100 mL at pH 7) and incubated at 27˚C for 3 days. Seed culture (4.0%) was transferred into the fermentation medium (glucose 2.0 g, glycerol 1.0 g, yeast extract 1.5 g, polypeptone 1.2 g, K_2HPO_4 0.001 g, $MgSO_4$ 0.001 g, $CaCO_3$ 0.1 g, DW 100 mL at pH 7.2) and incubated at 27˚C for 7 days. After seven days 30 µL sample was put on paper disc and dry. And then placed on assay agar plate containing test organism (Paper disc size = 8 mm). The test organisms used in paper disc diffusion assay were as followed.

Test organisms used in antibacterial activities

Five pathogenic bacteria, including two Gram positive bacteria (*Bacillus subtilis* IFO 90571, *Micrococcus luteus* NITE 83297) and three Gram negative bacteria (*Agrobacterium tumefaciens* NITE 09678, *Pseudomonas fluorescens* IFO 94307, *Escherichia coli* AHU 5436) were used to determined the antibacterial activity of the isolated actinomycetes strains.

Study on the Ages and Sizes of Inoculum for the Fermentation

A slant culture (8 days old) was transferred into seed medium. Seed cultures of 38 hrs, 42 hrs, 46 hrs, 50 hrs, 54 hrs and 58 hrs incubation was inoculated into the flasks containing fermentation medium. In the study of sizes of inoculum, 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% of seed culture at 46 hrs were transferred into the flasks containing the fermentation medium. Fermentation was carried out 7 days and antibacterial activity was tested by paper disc diffusion assay method (Omura, 1985).

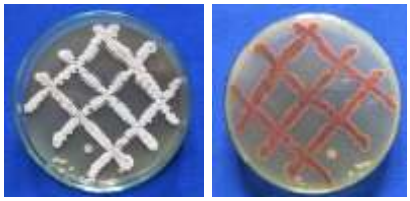
Results

Isolation of microorganisms from different soil samples

In this study, 15 kinds of soil actinomycetes were isolated from four different places of soil samples from Pwint Phyu Township, Magway Region, as shown in Table 2.

Table 2 Actinomycetes isolated from four different soil samples by Chemical and Physical Treatment Methods

Soil Sample No.	Collected Places	Chemical Treatment	Physical Treatment	No.
S-1	Min Hla village	2	2	A- 01,02,03,04
S-2	Kwat Thit village	2	1	A-05,06,07
S-3	Ywa Htaung village	3	1	A-08,09,10,11
S-4	Shwe Hlay village	3	1	A-12,13,14,15
Total Isolated Actinomycetes		10	5	15

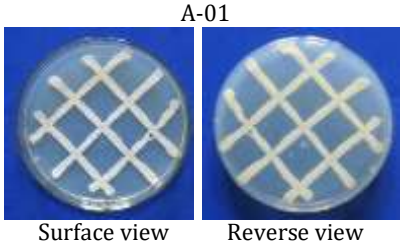


Surface view pigmentation Reverse view pigmentation

A-01

Surface view pigmentation Reverse view pigmentation

A-02



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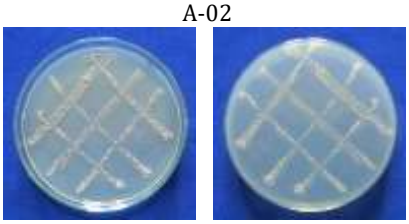

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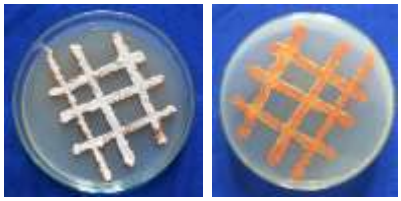
A-04



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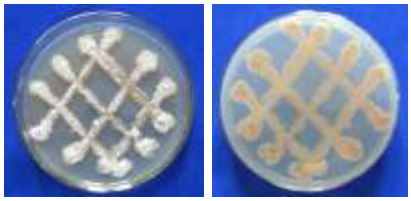
A-05



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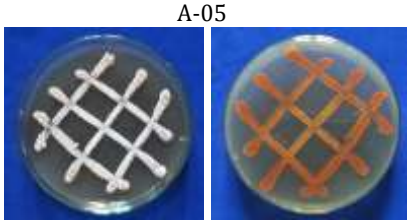
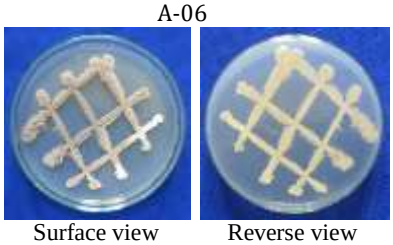
A-06



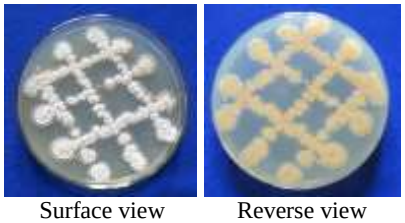
Surface view pigmentation Reverse view pigmentation

A-07



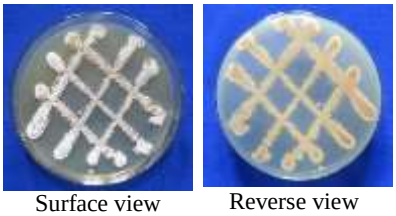
Surface view pigmentation Reverse view pigmentation

A-08



Surface view pigmentation Reverse view pigmentation

A-09



Surface view pigmentation Reverse view pigmentation

A-10

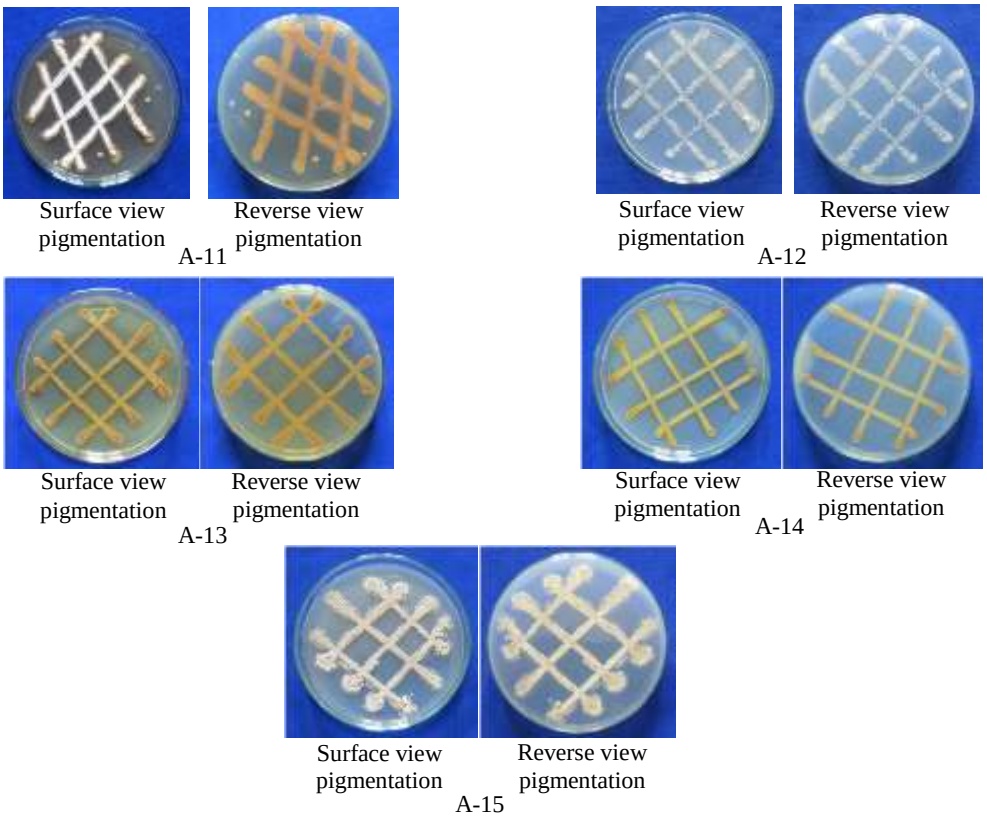


Figure 1 Aerial mass colour of actinomycetes strains on ISP II medium (7-14 days old culture)

Study on the antibacterial activities

In the study of antibacterial activitites of soil actinomycetes on five test organisms, A-01 and A-06 showed the activity against *Bacillus subtilis*. A-01 and A-08 showed the activity against *Agrobacterium tumefaciens,* A-04 and A-12 showed the activity against *Escherichia coli*. A-10 and A-15 showed the activity against *Pseudomonas fluorescens*. Among them, A-01 showed more highly antibacterial activity than others.

Table 3 Antimicrobial activities of isolated actinomycetes

Isolated Actinomycetes	*B. subtilis*	*M. luteus*	*A. tumefaciens*	*P. fluorescens*	*E. coli*
A-01	**23.41**	-	17.13	-	-
A-02	-	-	-	-	-
A-03	-	-	-	-	-
A-04	-	-	-	-	12.15
A-05	-	-	-	-	-
A-06	19.71	-	-	-	-
A-07	-	-	-	-	-
A-08	-	-	13.41	-	-
A-09	-	-	-	-	-
A-10	-	-	-	13.58	-
A-11	-	-	-	-	-
A-12	-	-	-	-	12.27
A-13	-	-	-	-	-
A-14	-	-	-	-	-
A-15	-	-	-	12.80	-

Figure 2 Antibacterial activity of isolated actinomycetes A-01 and A-06 against *Bacillus subtilis*

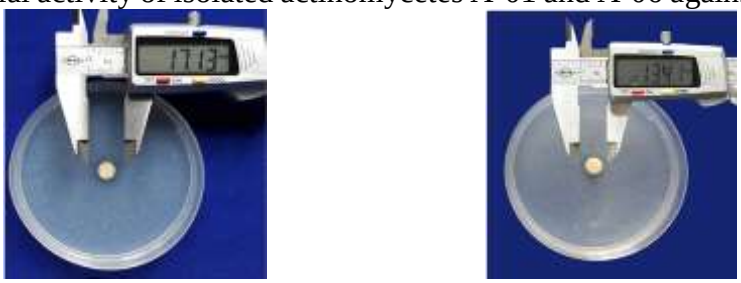


Figure 3 Antibacterial activity of isolated actinomycetes A-01 and A-08 against *Agrobacterium tumefaciens*

Figure 4 Antibacterial activity of isolated actinomycetes A-04 and A-12 against *Escherichia coli*

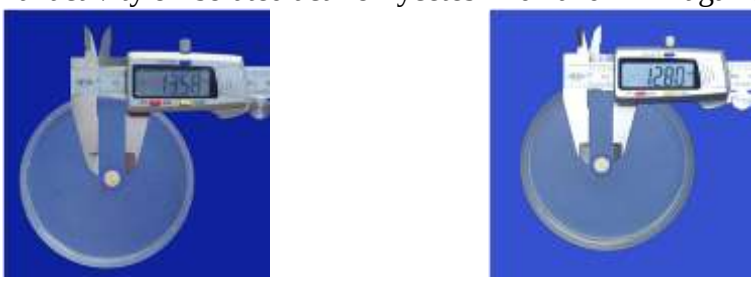


Figure 5 Antibacterial activity of isolated actinomycetes A-10 and A-15 against *Pseudomonas fluorescens*

Effect of ages of inoculums on the fermentation

In the investigation of the age of inoculums, six different hours of 38 hrs, 42 hrs, 46 hrs, 50 hrs, 54 hrs and 58 hrs were used and the results showed the inhibitory zone of 19.57 mm, 20.96 mm, 22.47 mm, 21.32 mm, 20.87 mm and 18.21 mm (Table 4 and Figure 6). According to these results, 46 hrs of culture was selected for the fermentation.

Table 4 Effect of Ages of Inoculums on Fermentation antibacterial activity against *Bacillus subtilis*

Culture Times (Ages of Culture, hrs)	Antibacterial activity (Clear zone, mm)
38	19.57
42	20.96
46	**22.47**
50	21.32
51	20.87
58	18.21

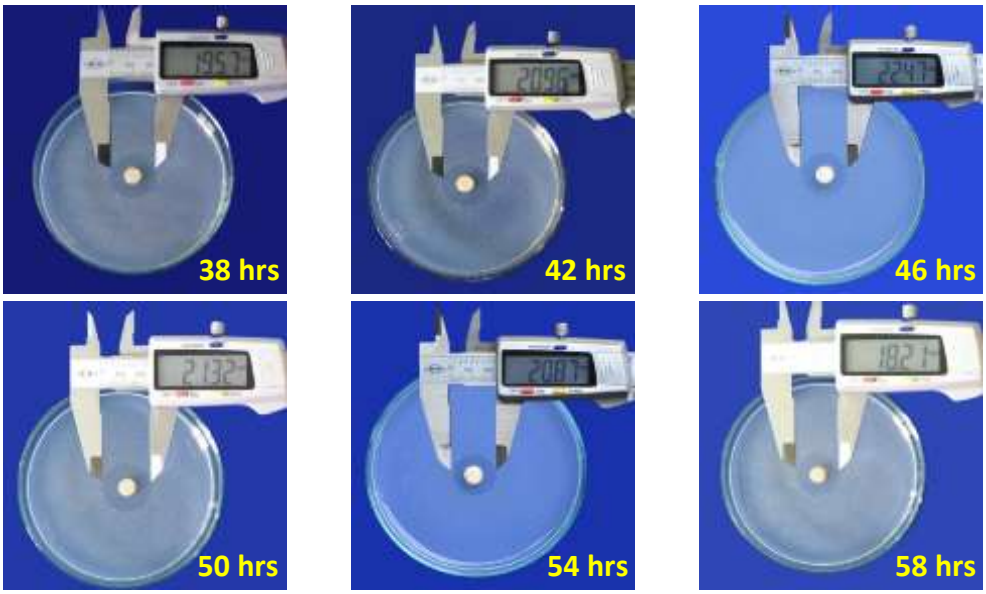


Figure 6 Effect of ages of inoculums on fermentation antibacterial activity against *Bacillus subtilis*

Effect of size of inoculums on the fermentation

The study of the size of inoculums 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% six different percentages were used and the results showed the inhibitory zone of 18.23 mm, 20.15 mm, 21.79 mm, 22.63 mm, 20.00 mm and 19.59 mm respectively (Table 5 and Figure 7). Depending on the results, it was determined that 2.0% inoculums was optimization for fermentation to produce the antibacterial metabolites.

Table 5 Effect of Sizes of Inoculums on Fermentation antibacterial activity against *Bacillus subtilis*

Culture Times (Sizes of Culture, %)	Antibacterial activity (Clear zone, mm)
0.5 %	18.23
1.0 %	20.15
1.5 %	21.79
2.0 %	**22.63**
2.5 %	20.00
3.0 %	19.59

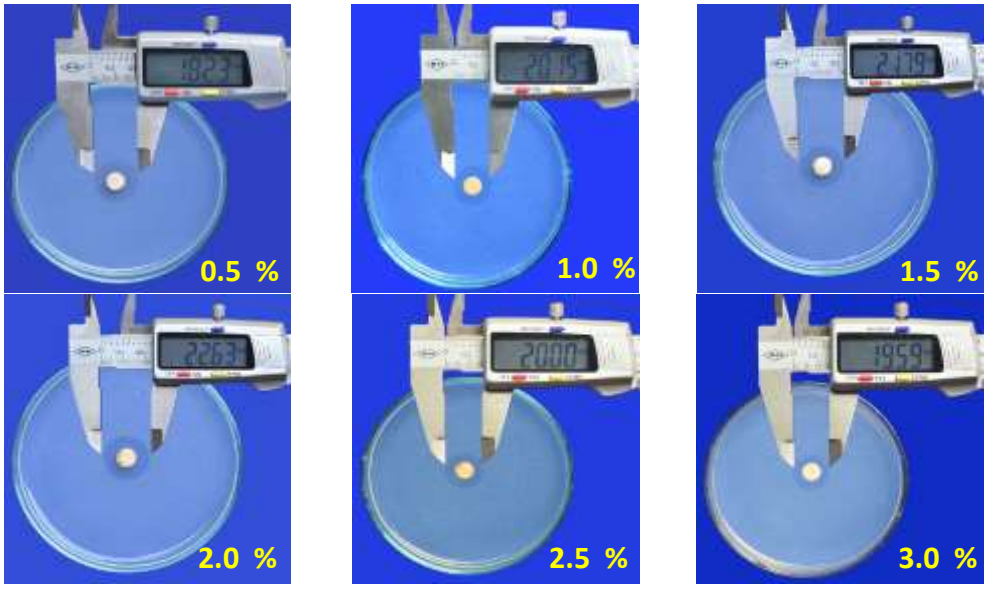


Figure 7 Effect of sizes of inoculums on fermentation antibacterial activity against *Bacillus subtilis*

Discussion and Conclusion

In the investigation for antibacterial metabolite producing actinomycetes, fifteen actinomycetes were isolated from the four soil samples collected at Pwint Phyu Township, Magway Region. Isolation of soil actinomycetes was undertaken by the methods of Chemical Treatment Dilution Method and Physical Treatment Serial Dilution Method (Phay and Yamamura , 2005). Actinomycets A-01, 02, 03 and 04 were isolated from Min Hla soil sample. A- 05, 06 and 07 were isolated from Kut Thit soil sample. A- 08, 09, 10 and 11 were isolated from Ywa Htaung soil sample. A- 12, 13, 14 and 15 were isolated from Shwe Hlay soil sample.

In the present work, 10 actinomycetes were isolated by Chemical Treatment Dilution Method and 5 actinomycetes were isolated by Physical Treatment Serial Dilution Method. By the result, the numbers of isolated actinomycete were greatly different between these two methods. Phay and Yamamura, 2005 reported that Chemical Treatment Dilution Method was more effective than Physical Treatment Serial Dilution Method for isolation of actinomycetes because the actinomycetes were released from the substrates depending on the isolation methods. So, similar result was obtained in this study. In the study of antibacterial activities, actinomycets A-01 and 06 showed antibacterial activities against *Bacillus subtilis.*

A-01 and A-08 showed antibacterial activities against Agrobacterium tumefaciens. A-04 and A-12 showed antibacterial activities against Escherichia coli. A-10 and A-15 showed antibacterial activities against Pseudomonas fluorescens. Among them, actinomycete A-01 exhibited the highest antibacterial activity against on Bacillus subtilis (23.41 mm). Therefore, this strain A-01 was selected for further studies such as ages of culture and sizes of inoculum. This actinomycete A-01 was isolated from the Min Hla village soil (Silt Loam, pH-7.2) of Pwint Phyu Township, Magway Region. In the study of the effects of ages and sizes of inoculum, it was observed that 46hrs seed culture and 2.0% sizes of inoculum were optimized for the fermentation. In conclusion, the isolated actinomycete A-01 will be intended to utilize in further processes like extraction, purification and identification of new bioactive metabolites.

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