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PURIFICATION OF ANTIMICROBIAL COMPOUND FROM *ACTINOMYCES* SP AGAINST *SALMONELLA TYPHIMURIUM*

Khin Swe Mya*[1], Thein Soe[1], Mu Mu Aung Soe[1], Thida Soe[1], Lei Lei Oo[1] and Myat Thandar Swe[2]

[1]Zoology, Department of Zoology, Pathein University.
[2]Zoology, Department of Zoology, Yangon University of Distance Education.

***Corresponding Author: Dr. Khin Swe Mya**
Zoology, Department of Zoology, Pathein University.

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ABSTRACT
In the study, isolation of microorganisms was done by dilution method. The strain was isolated from *Turritella carinifera*. The strains showed the antibacterial activity against *Salmonella typhimurium*. The results of the biochemical tests reveal that the strain was *Actinomyces* species. According to the results of paper chromatography (bioautography), it was considered that ethyl acetate was the best solvent to extract the bioactive compound from the fermented broth. Based on the results of thin layer chromatography (TLC), silica gel chromatography was undertaken. The fractions were collected and tested for the antibacterial activity against *Salmonella typhimurium*. The purified fractions of hexane from re-chromatography were collected and tested for the antibacterial activity. The purified and active fractions were collected and the biological activity was investigated. The results showed that 17.6 mg of purified bioactive compound could be isolated from 14 liters of fermented broth of *Actinomyces* species.

KEYWORDS: Isolation of bacteria, extraction and purification of antimicrobial compound.

1. INTRODUCTION

The need for new antibacterial was mainly caused by the emergence of antibiotic-resistant strains under selection pressure by antibiotics(De Lorenzo, 1985). The continuing success of a biotechnologist in the search for microbial metabolite for use as antimicrobial compounds (antibiotics) is critical to combating human, animal and plant disease. Microorganisms constitute an inexhaustible reservoir of compound with pharmacological, physiological, medical or agricultural applications (Smith, 1989, Bulock, 1987, Vandemme, 1988).

Many products can be purified by use of solvent extraction. A two- phase system is set up, using a solvent which is immiscible with the aqueous fermentation broth. After the desired product is concentrated in the solvent phase, it can be further purified. Solvent extraction has been widely used in the antibiotic industry using organic solvents such as butanol, ethyl acetate, butyl acetate (Schugerl, 1993). It is necessary to understand the character of metabolite such as hydrophobicity or hydrophilicity before the purification. A fermentation broth is a complex mixture of components that often contains only trace amounts of a desired product. Final purification that separates closely related impurities is typically achieved via preparative chromatography or crystallization (Patrick, 1998).

The antibacterial activity of a material depends on the destruction of the physical structure or the inhibition of reaction in a microorganism; it seems that the presence and the level of the antibacterial activity of the pigment varied significantly with the type of microorganism used (Martinko and Madigan 2006). Antibiotics have been extensively used in controlling bacterial infections in fish culture. Unfortunately, the abuse of antibiotics has been associated with diverse problems in the health of fish, terrestrial animals and human and for the environment (Alcaide *et al.*, 2005). Various bacteria have been confirmed to produce antimicrobial substances, with activities ranging from broad-spectrum to species-specific, including bacterial pathogens (Anand *et al.*, 2006).

2. MATERIALS AND METHODS

2.1. Isolation and Fermentation

The isolation of microorganisms was taken by dilution method (Crueger and Crueger, 1989). To prepare the Glucose Polypeptone Agar medium (GPA), glucose 1.0 g, polypeptone 0.5 g, yeast extract 0.5 g, agar 1.8 g were placed in a 250 mL conical flask. Then 100 mL of sterile distilled water was added and adjusted the pH 7.0.

Samples from the dilution series were inoculated on various culture media and then incubated at 27ºC for 3-7 days. Single colonies from the plates were picked up and purified by re-streaking. Pure strains were maintained as agar culture in test tubes.

The isolated microorganisms were incubated into conical flask containing the Soluble Starch Yeast Glucose seed medium at 27 ºC for 3 days. After incubation, the seed culture (1%) was transferred into the conical flask containing fermentation media. The fermentation period was 3 - 7 days. During the fermentation, the fermented broth was used for the paper disc diffusion assay.

2.2. Paper chromatography (Tomita, 1988)

The purpose of paper chromatography is to know how to extract the bioactive compound from fermentation broth by using which solvent system. 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, in the same method as paper disc assay, except that after one hour the paper was taken out, then the plates were incubated for 24-36 hours. The inhibitory zone was measured yielding a R_f value for the corresponding bioactive compound. R_f value was calculated in the following equation (Fig.1).

2.2. Preliminary extraction of the solvent with organic solvents

According to the result of paper chromatography, the fermented broth of *Actinomyces* species was adjusted to pH 6, 7, and 8. The metabolite was extracted with with ethyl acetate saturated in the same volume. The organic layers (ethyl acetate layer) and water layer of each pH-adjusted sample were tested the activity by paper disc diffusion assay using the test organism *Salmonella typhimurium* (Fig. 2).

2.4. Thin layer paper chromatography and bioautographic overlay assay

The extracted residue of the organic solvent is necessary to be separated and purified more. Thus, thin layer chromatography was developed. The solvent chloroform and chloroform-methanol mixture (9:1), (8:2) were used for the running of TLC. The inhibatory zone was measured yielding an R_f value for the corresponding antimicrobial bioactive compound. (Fig.3).

2.5. Silica gel column chromatography

Fourty liters of fermented broth was extracted with ethyl acetate in the same volume at pH 7.0. Water saturated ethyl acetate was concentrated *in vacuo*.

The silica gel was dissolved in chloroform and the silica gel column was packed. Water saturated ethyl acetate extract was then passed through silica gel column, and eluted with chloroform solvent and then tested the activity against *Salmonella typhimurium* (Fig.4).

The column volume and flow rate was the followings;

The eluting solvent	Chloroform / Chloroform-methanol (9:1) / Chloroform-methanol (8:2)
Column volume	1.9 cm x 49.5 cm
Flow rate	0.2 mL/ min

Each fraction 2 mL collected.

2.6. Silica gel Re-column chromatography

The active fractions of silica gel column chromatography with (chloroform-methanol, 9:1) were combined and concentrated in vacuo. The residue was re-chromatographed on silica gel with the eluting solvent hexane.

2.7. C_{18} Gel Column Chromatography

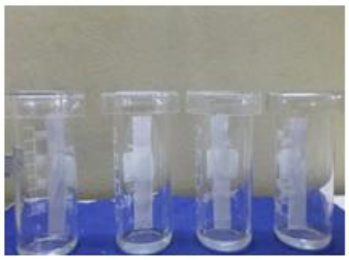
The C_{18} gel was dissolved in methanol, and then poured into the glass column. The C18 gel column chromatography was run with methanol-water mixture (8:2) based on the results of bioautography. The fractions were tested the antibacterial activity against *Salmonella typhimurium*. Each fraction 1.5 mLwas collected and tested. These active fractions were checked for purity on C18 gel TLC plate using the methanol-water (7:3) solvent and UV (254 nm).

2.8. Cerephrose gel column chromatography

The active fractions of C18 gel column chromatography were combined and concentrated in vacuo. 0.5 ml of the fractions were collected and tested the antibacterial activity against *Salmonella typhimurium*. These active fractions were checked for purity by using UV (254 nm).

1 2 3 4

A. Fermented broth samples applied on the paper



1 2 3 4

B. Running condition four solvents

1 2 3 4

C. Each paper on assay agar plates

1. 20% NH_4CL
2. Water saturated n-butanol
3. n-butanol acetic acid-water (3:1:1)
4. Water saturated ethyl acetate

Fig. 1: Procedure on paper chromatography.

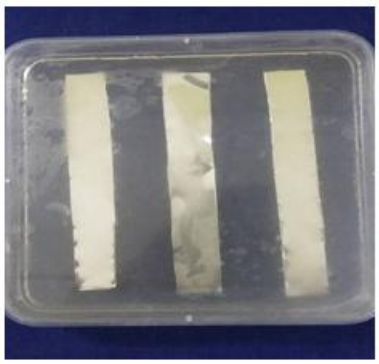
Fig. 2: Extraction of antimicrobial compound with ethyl acetate from the fermented broth.

1 2 3

A. Extracted applied on the paper

1 2 3

B. Running condition three solvents



C. Each paper on assay agar plates

Solvent 1= Chloroform

Solvent 2= Chloroform- methanol (9:1)

Solvent 3= Chloroform – methanol (8:2)

Fig. 3: Procedure for Thin Layer Paper Chromatography.

Fig. 4: Running condition in silica gel column chromatography.

3. RESULT

3.1. Isolation and Fermentation

The isolation for antimicrobial metabolite producing microorganisms, the strain was isolated from *Turritella carinifera*. In this study it was observed that ages of seed culture three days was the best for the fermentation on the basic of the antibacterial activity against *Salmonella typhimurium*. The effects of sizes of inoculums showed the activities, 1% sizes of inoculums exhibited higher antibacterial activity on *Salmonella typhimurium.*

3.2. Paper chromatography

According to the R_f value of the bioautography of paper chromatography, isolated *Actinomyces* sp. was suitable the solvent ethyl acetate. So, the solvent ethyl acetate was used for the extraction of antibacterial substance from the fermented broth (Fig.5)**.**

3.3. Thin layer chromatography and Bioautographi coverlay assay

In the study of thin layer chromatography, it was found that the R_f value in chloroform is, in $CHCL_3$, $CHCL_3$-MeOH (9:1) and $CHCL_3$-MeOH (8:2) (Fig. 6). It was selected for the development of silica gel column chromatography to separate the compound.

3.4. Silica gel column chromatography

Fractions (1-3) and (9-13) of eluting solvent chloroform did not show the activity. The active fractions (4-8) of eluting solvent chloroform exhibited the activity on *Salmonella typhimurium*. (Table 1, Fig. 7).

Fractions (14-16) and (37-41) of eluting solvent chloroform-metranol (9:1) did not show the activity. The active fractions (17-36) of eluting solvent chloroform-metranol (9:1) exhibited the activity on *Salmonella typhimurium*. (Table1, Fig. 8).

Fractions (42-45) and (50-55) of eluting solvent chloroform-metranol (8:2) did not show the activity. The active fractions (46-49) of eluting solvent chloroform-metranol (8:2) exhibited the activity on *Salmonella typhimurium*. (Table1, Fig. 9).

3.5. Silica gel Re-column chromatography

The active residues (fractions 17-36 of 9:1) were collected to make re-chromatography using silica gel column. It was observed that fractions (1-8) and (18-25) of eluting solvent hexane did not show the activity. However, fractions (9-17) exhibited the activity on *Salmonella typhimurium* and UV light. (Table 2, Fig. 10).

3.6. C_{18} Gel column chromatography

The active residues (fractions 9-17) of eluting solvent hexane were collected to make C_{18} Gel Column Chromatography. It was observed that fractions (1-6) and (14-25) of eluting solvent methanol water (8:2) did not show the activity. However, fractions (7-13) exhibited the activity on *Salmonella typhimurium* and UV light. (Table 3, Fig. 11).

3.7. Cerephrose gel column chromatography

The active residues (fractions 7-13) of eluting solvent methanol water (8:2) were collected to make Cerephrose gel column chromatography. It was observed that fractions (1-6) and (16-25) of eluting solvent methanol water (7:3) did not show the activity. However, fractions (7-15) exhibited the activity on *Salmonella typhimurium* and UV light (Table 4, Fig. 12).

The purified and active fractions were collected and the biological activity was investigated. Maximum 17.6 mg of compound could be isolated from 14 liters of fermented broth of *Actinomyces* sp.

Table 1: Antimicrobial activites of different fractions on *Salmonella typhimurium*.

Eluting Solvent	Fractions No.	Antagonistic Activity
Chloroform	1-3	No activity
	4-8	**Active**
	9-13	No activity
Chloroform-MeOH (9:1)	14-16	No activity
	17-36	**Active**
	37-41	No activity
Chloroform-MeOH(8:2)	42-45	No activity
	46-49	**Active**
	50-55	No activity

Table 2: Antimicrobial activites of hexane-fractions on *Salmonella typhimurium*.

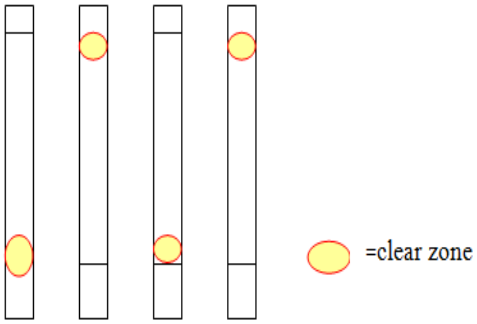
Eluting solvent	Fraction No.	Antagonistic activity
Hexane	1-8	No activity
	9-17	Active
	18-25	No activity

Table 3: Antimicrobial activites of methanolol-water (8:2) fractions on *Salmonella typhimurium*.

Eluting solvent	Fraction No.	Antagonistic activity
MeOH:Water (8:2)	1-6	No activity
	7-13	**Active**
	14-25	No activity

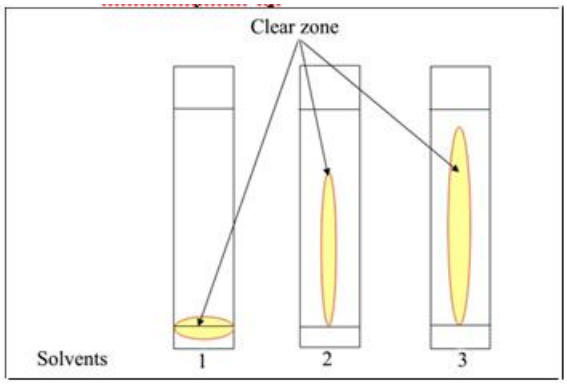
Table 4: Antimicrobial activites of methanol- water (7:3) fractions on *Salmonella typhimurium*.

Eluting solvent	Fraction No.	Antagonistic activity
MeOH: Water (7:3)	1-6	No activity
	7-15	**Active**
	16-25	No activity



Solvent 1 2 3 4
1. 20% NH_4CL
2. Water saturated n-butanol
3. n-butanol acetic acid-water (3:1:1)
4. Water saturated ethyl acetate

Fig 5: The bioautography of paper chromatography of bioactive compound produced by *Actinomyces* sp.



Solvents,
1 = chloroform;
2 = chloroform-methanol (9:1);
3 = chloroform-methanol (8:2)

Fig 6: The bioautography of *Actinomyces* sp bioactive compound in thin layer Chromatography.

Fig. 7: Active fractions (4 to 8) of silica gel column run with chloroform solvent.

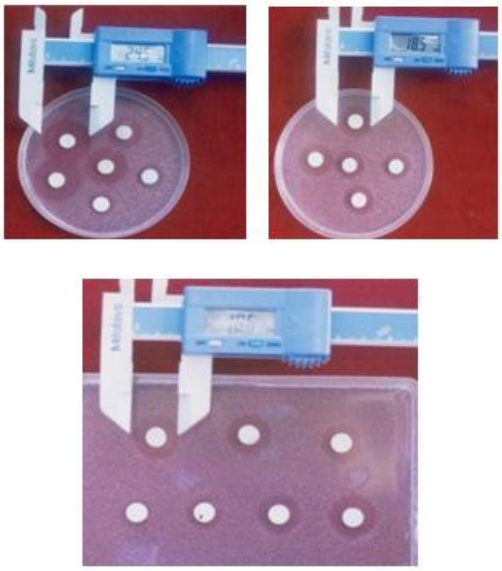


Fig. 8: Active fractions (17 to 36) of silica gel column run with chloroform methonal (9:1) solvent.

Fig. 9: Active fractions (46 to 49) of silica gel column run with chloroform-methonal(8:2).

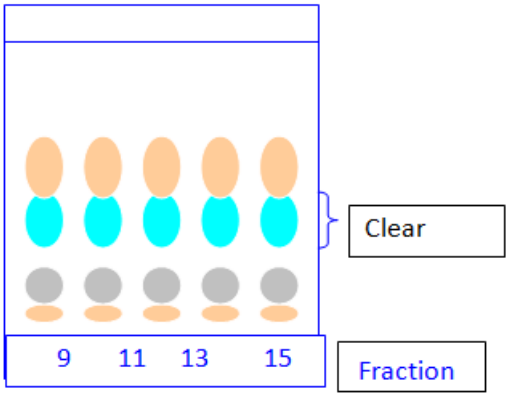


Fig. 10: Bioautography under UV (254 nm) after purification with silica gel using solvent hexane.

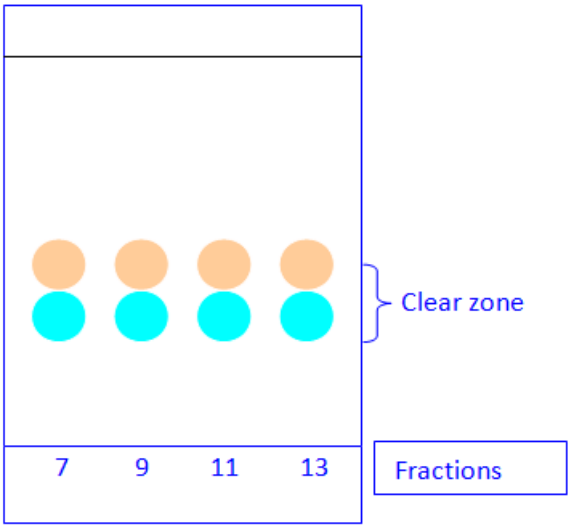


Fig. 11: Bioautography under UV (254 nm) after purification with C18 gel TLC using Solvent MeOH-water (8:2).

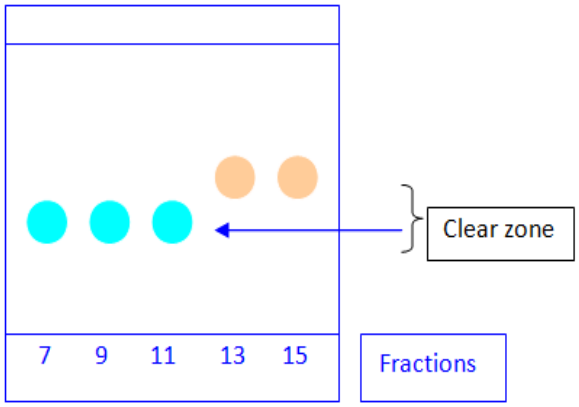


Fig. 12: Bioautography under UV (254 nm) after purification with cerephrose gel TLC using Solvent MeOH-water (7:3).

4. DISCUSSION AND CONCLUSION

In the present study, according to the results of the bioautography of paper chromatography, the fermented broth of isolated *Actinomyces* sp. was extracted with ethyl acetate with the same volume. Based on this result the extraction of antibacterial substances was undertaken with ethyl acetate from fourty liters of fermentated broth. According to the result of thin layer paper chromatography, it may be considered that isolated *Actinomyces* sp. product was isolated to purify by silica gel column chromatography with chloroform, chloroform-methanol (9:1) and chloroform-methanol (8:2). The active residues (fractions17-36 of 9:1) were collected to make re-chromatography using silica gel column. Fractions (9-17) of eluting solvent hexane exhibited the activity on *Salmonella typhimurium*. The active residues (fractions 9-17) of eluting solvent hexane were collected to make C_{18} Gel Column Chromatography. Fractions (7-13) of eluting solvent methanol water (8:2) exhibited the activity on *Salmonella typhimurium*. The active residues (fractions 7-13) of eluting solvent methanol water(8:2) were collected to make Cerephrose gel column chromatography. Fractions (7-15) eluting solvent methanol water (7:3) exhibited the activity on *Salmonella typhimurium*.

In the development of silica gel column chromatography with methanol eluting solvent, fractions (85-100) showed activity against *Micrococcus luteus*. In the development of silica gel column chromatography with chloroform-methanol (9:1) eluting solvent, fractions (36-54) showed activity against *Micrococcus luteus.* In the development of silica gel column chromatography with Toluene-methanol (8:2) eluting solvent, fractions (22-31) showed activity against *Micrococcus luteus*. Again, in the development of silica gel column chromatography with Toluene-methanol (8:2) eluting solvent, fractions (52-61) showed activity against *Micrococcus luteus.* Finally silica gel column chromatography was carried using Benzene-acetone (5:5). 20 mL pure compound was purified from 20 liters of fermented broth. The purification of bioactive metabolites from fermented broth of microorganisms largely depends upon the physiochemical properties of metabolite (Saxena *et al*., 2007).

The isolated substance from *Actinomyces* sp was examined by UV light. The functional groups of these active fraction compounds were checked. These results showed the posses the functional group of antibiotic compound.

5. ACKNOWLEDGEMENTS

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