Extraction and Purification of Antibacterial Metabolite Producing from *Trichoderma reeseii* against *Staphylococcus aureus*

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Abstract

In the course of my research work, 18 fungi were isolated from 6 different places of Swedaw Pagoda, Amarapura Township, Mandalay Region. Eighteen different fungi were isolated by using chemical treatment dilution methods and soil dilution methods. Eleven kinds of test organisms were used in paper disc diffusion assay method. In the screening program, fungus *Trichoderma* was more highly exhibited antibacterial activity against *Staphylococcus aureus* than others. The study of carbon and nitrogen utilization were investigated; soluble starch, molasses and potato powder were excellent for carbon sources and NZ amine type A, meat extract, KNO_3, peanut and fish cake were excellent for nitrogen sources. In the fermentation studies, the highest activity reached at 84 hrs ages of culture and 20% sizes of inoculum for the fermentation. In this study, FM-2 was excellent for the production of antibacterial metabolite. Based on the result of paper chromatography assay, ethyl acetate is suitable for the extraction of antibacterial metabolite at pH 5. In this research work, purification of secondary metabolites was carried out by various chromatography to get pure compound from fermented broth of *Trichoderma* strain.

Keywords : Extraction and Purification of Antibacterial Metabolite

Introduction

Nature is an attractive source of new therapeutic candidate compounds since a tremendous chemical diversity is found in millions of species of plants, animals, marine organisms and microorganisms. Microorganisms such as bacteria and fungi have been invaluable to discover drugs and lead compounds. These microorganisms produce a large variety of antimicrobial agents. Microorganisms have been used as a source for the production of variety of bioactive metabolites. Many of natural synthesized antibacterial and antifungal metabolite have been reported in clinical and agricultural uses (Phay, 1996).

In large scale of production, pH plays an important role in expresion activity of any biological system, it has great influence on radial growth of *Trichoderma*. Extraction is very common separation process, used to purifying product. One of the widely applied methods in the extraction and purification of desired product is chromatography. It can be used in a search for the optimum extraction solvents, for identification of known and unknown compounds.

Paper chromatography is one of the useful method for separation and identifying compounds in a mixture. Column Chromatography is a preparative technique used to purify compounds depending on their polarity or hydrophobicity.

TLC also plays the key role in preparative isolation of compounds, in purification of the crude extracts, and in control of the separation efficiency of the different chromatographic techniques and systems. Thin-layer chromatography or TLC, is a solid-liquid form of chromatography where the stationary phase is normally

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a polar absorbent and the mobile phase can be a single solvent or combination of solvents.

In column chromatography, a mixture of molecules is separated based on their differentials partitioning between a mobile phase and a stationary phase.

Antibiotic production by *Trichoderma* spp. was first described by Weindling (1934). *Trichoderma* have been known since at least the1920s for their ability to act as biocontrol agents against phytopathogenic fungi and some strains are able to produce metabolites (Howell, 1998; Harman, 2006; 2009; Nallathambi *et al*., 2009).

Materials and Methods

Extraction of Antibacterial Metabolite from the Fermented Broth

Based on the result of paper chromatography assay, ethyl acetate is suitable for the extraction of antibacterial metabolite at pH 5 from fermented broth 20 Liter.

Thin Layer Chromatography and Bioautographic Overlay Assay (Simon and Gray, 1998; Cannel, 1998)

The extract residue of the organic solvent is necessary to be separated and purified more. The obtained EtOAc extracted samples (20 µL) were applied on the TLC plate and allowed to dry. The TLC plates were developed in the solvent of Toluene, Toluene - MeOH mixture (9:1) and chloroform, chloroform - methanol mixture (9:1). Each TLC plate was placed on assay agar plates. After one hour, they were peeled off and the plates were incubated for 24 hrs. Then bioautography was done to check the antibacterial activity. In this case, the inhibitory zone was measured yielding an R_f value for the corresponding antibacterial metabolites. R_f value was calculated in the following equation.

$$R_f \text{ value} = \frac{\text{Distance of compound from origin}}{\text{Distance of Solvent front from origin}}$$

Silica Gel Column Chromatography by Using Solvent Chloroform-Methanol (Simon and Gray, 1998; Fair, *et al.*, 2008)

According to the results of TLC, it may be considered that *Trichoderma* extract can be isolated to purify by silica gel column chromatography with chloroform-methnol mixture as eluting solvent. Fermented broth was extracted with ethyl acetate in (1:1) volume at pH 5.0. Ethyl acetate extract was concentrated in *vacuo.* The silica gel (Wako gel 60 mersh) was dissolved in chloroform and silica gel column was packed. EtOAc extract was then passed through silica gel column, and eluted with chloroform and chloroform-methanol solvent (9:1, 8:2, 7:3); then tested the activity against *Staphgylococcus aureus*. Two mL of each fraction was collected and examined the activity against *S. aureus.*

Thin Layer Rechromatography by Using Solvent Hexane-Ethylacetate (Joseph, 1991).

Thin Layer Chromatography is to know for the separation of compound by column chromatography using the eluting system (Joseph, 1991). TLC plate (Wako, 10 mm) was utilized for this study. Active fractionated sample was performed TLC with the solvent of hexane-ethylacetate mixture (9:1). And then TLC plate was placed on assay agar plates. After one hour, the plate was taken out and the plate was incubated for 24 hours. Then bioautography was done to check the antibacterial

activity. In this case, the inhibitory zone was measured yielding an R_f value for the corresponding antibacterial metabolites.

Silica Gel Column Rechromatography by Using Solvent Hexane-Ethylacetate (Joseph, 1991 and Touchstone, 1992)

According to TLC result, Silica gel column chromatography was undertaken with the solvent of hexane-ethylacetate (9:1, 8:2, 7:3). Two mL of each fraction was collected and examined the activity. Silica gel (Wako, 60 mm) was utilized for this investigation.

Preparative Thin Layer Chromatography (Geiss, 1987; Jork *et al.*, 1994)

The active fractrions of silica gel rechromatography with hexane-ethylacetate (42-46) were combined and 20 uL were applied on TLC plate and allowed to dry. The TLC plate were developed in solvent of Benzene-Acetone mixture (8:2).

Then, TLC plate was seen under UV (324 nm). Silica gel from active spots were scrapped and collected. Silica gel with active compound were filtered with acetone and examined the antibacterial activity.

Results

Extraction of Antibacterial Metabolite from the Fermented Broth

Based on the results of bioautography (PPC), the extraction of antibacterial metatabolite was undertaken by adjusted pH. pH 5 was suitable for the extraction of antibacterial metabolite with ethyl acetate from the fermented broth (Figure-1).

Thin Layer Chromatography and Bioautographic Overlay Assay

According to the results of TLC, it may be considered that *Trichoderma* product was isolated to purify silica gel column chromatography with chloroform - methanol mixture as eluting solvents.

Silica Gel Column Chromatography by Using Solvent Chloroform Methonal (9:1) (Simon and Gray, 1998; Fair, *et al.*, 2008)

After column chromatography, the fractions were checked for the antibacterial activity using the test organism *Staphylococcus aureus* (Table -1, Figure 1). The activity was found at fraction No. 30-35 in the slovent system chloroform-methanol (9:1).

Table 1. Antibacterial activity shown by fractions on *S. aureus* by silica gel column chromatography with chloroform-methanol

Fraction No.	Activity	Eluting solvent
1 -24	No activity	Chloroform
25 -29	No activity	Chloroform-methanol (9:1)
30 -35	**Activity**	**Chloroform-methanol (9:1)**
36 -43	No activity	Chloroform-methanol (9:1)
44 -45	No activity	Chloroform-methanol (9:1)
46 -69	No activity	Chloroform-methanol (8;:2)
70 -93	No activity	Chloroform-methanol (7;3)

Figure 1. Antibacterial activity shown by fractions on *S. aureus* by silica gel column chromatography with chloroform-methanol (9:1)

Thin Layer Rechromatography by Using Solvent Hexane-Ethylacetate (Joseph, 1991)

After developing TLC with hexane-ethylacetate mixture (9:1), the antibacterial activity was examined.

Silica Gel Column Rechromatography by Using Solvent Hexane-Ethylacetate (9:1) (Joseph, 1991, Touchstone, 1992)

After developing silica gel column rechromatography by using solvent hexane-ethylacetate (9:1, 8:2, 7:3), the fractions were checked the antibacterial activity using the test organism *Staphylococcus aureus* (Table -2, Figure 2). The activity was found at fraction No: 42-46 in the slovent system hexane-ethylacetate (8:2).

Table 2. Antibacterial activity shown by fractions on *Staphylococcus aureus* by Silica Gel Column Re-chromatography with Hexane- Ethylacetate

Fraction No.	Activity	Eluting solvent
1- 30	No activity	Hexane- Ethylacetate (9:1)
31-40	No activity	Hexane- Ethylacetate (8:2)
42-46	**Activity**	**Hexane- Ethylacetate (8:2)**
47-60	No activity	Hexane- Ethylacetate (8:2)
61-80	No activity	Hexane- Ethylacetate (7:3)

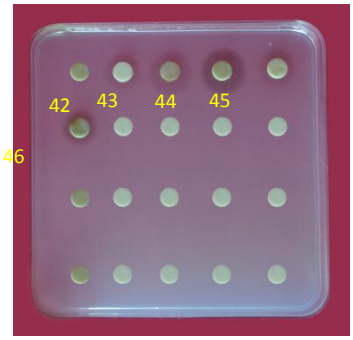

Figure 2. Antibacterial activity of isolated compound against *Staphylococcus aureus* with Hexane- Ethylacetate (8:2)

Preparative Thin Layer Chromatography (Geiss, 1987; Jork *et al.*, 1994)

After developing the preparative thin layer chromatography with Benzene : Acetone (7:3), the antibacterial activity was examined under UV 324 nm and active sports were marked and scratched.

Figure 3. Antibacterial activity of isolated antibacterial metabolite against *Staphylococcus aureus*

Discussion and Conclusion

Fungi are rich source of bioactive compounds that have drawn the attention of natural product chemists for combating the current bacterial resistance (Luzhetskyy *et al*, 2007). In the extraction of antibacterial metabolite, active metabolite was extracted with ethylacetate in the same volume at pH 5.0. Similar results are also described by Nishihara *et al*.2001. In the investigation of thin layer chromatography, four kinds of different eluting solvents were used. According to R_f value, Chloroform: Methanol showed the excellent activity than other. Therefore, there was selected for further investigation.

In this research work, the extracted residue was purified by silica gel column chromatography with Chloroform : Methnol (9:1, 8:2, 7:3); fraction No. 30-35 were shown antibacterial activity on *S. aureus* (Table 1, Figure 1). The active fractions (30-35) were combined for further rechromatography. According to the R_f value by TLC bioautography, silica gel column rechromatography was undertaken by hexane-ethylacetate l (9:1, 8:2, 7:3), fraction No. 42-46 (8:2) were shown the antibacterial activity on *S. aureus* (Table 2, Figure 2). After that, the active fractions (42-46) were combined and undertaken preparative thin layer chromatography (PTLC) with Benzene : Acetone (7:3). According to TLC bioautography, the active sports were marked and scratched. Finally, the collected scraps were combined and examined antibacterial activity on *S. aureus* (Figure 3).

Watt *et al* (1988) reported a new antifungal metabolite, richodermin of *Trichoderma reesei* and its activity against *Aspergillus flavus, A. niger, Cladosporium cucumerium, Rhizotonia solani, and Rhizopus stolonifer*. In 1998, Sunita and Nandi also

found that the cellulase production by *T. reeseii. Trichoderma reeseii* was utilized in the bio-ethanol production from rice straw residues (Belal, (2013).

According to literature reviews, cellulase, hemicellulase and beta-glucosidase were mostly produced by *Trichoderma reeseii*. It was only found that antifungal metabolite, Trichodermin, from the *Trichoderma reeseii*. However, it was not observed antibacterial metabolite against *Staphylococcus aureus*. Therefore, further research will be required for the determining the chemical structure of metabolite and to determine the cytotoxicity.

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