Universities Research Journal 2008
Vol. 1, No. 1

Editorial Board

Prof. Daw Thai Thai Aye, Head of Department of Botany, University of Yadanabon
Prof. Daw Tin Ye, Head of Department of Botany, University of Meiktila
Prof. Nay Win, Head of Department of Botany, University of Kyaukse
Prof. U Aung Myint Thein, Head of Department of Botany, University of Kalay
Prof. Swe Mar Tin, Head of Department of Botany, University of Lashio
Asso. Prof. Dr. San San Aye, Head of Department of Botany, University of Kyainge Tong

Contents

Page

Production of Antibacterial Metabolite by *Lecanicillium waksmanii* MKN-09

Moe Moe Aye[1], Khine Swe Nyunt[2], Nyunt Phay[3]

Abstract

In the screening program of antimicrobial metabolite, 54 fungi isolated from the soil collected at Chin State and Mandalay Division. In this study, 24 test organisms were utilized for antimicrobial activities. Among these fungi, one fungus showed selective highly antibacterial activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA). Therefore, this fungus was selected for further investigations such as fermentation, purification and biological properties. Fungus MKN-09 was isolated from the soil collected at Hpa-Lam area, Chin State. MKN-09 was identified as *Lecanicillium waksmanii*. In fermentation studies, metabolite production reached at 6 days fermentation with 20% sizes and 66 hrs ages of cultures. Antibacterial metabolite was purified by various chromatographies. It was observed that MIC of this metabolite is 0.3125 μg/ml on MRSA. This metabolite (MKN-09) affects on the growth of *S. aureus* at a concentration of 0.3125 μg/ml; any further increase in its concentration above 0.3125 μg/ml resulted in a more suppressive effect on growth. At a concentration of 1.25 μg/ml, cell lysis was observed that indicating that MKN-09 metabolite acts as a bacteriocidal compound.

Introduction

The typical materials for microbial sources are soil, living and fallen leaves, leaf litters, dung, insect, fresh water, marine water, and etc. The soil sample is the most effective and popular materials for especially isolating a number of microorganisms such as fungi and actinomycetes (Harayama, 2002).

Some pathogenic microbes are resistant to antibiotics. The worldwide occurrence of methicillin resistant *Staphylococcus aureus* (MRSA) is one of the most common causes of fatal infections and diseases in hospitals. Recently, the emergence of Methicillin resistant *S. aureus* is raising serious public health concerns. With the emergence of resistant organisms it is high time to continue search for novel antibiotics that inhibit pathogens resistant to other antibiotics (Zulaybae, 2005). During the past decade much effort

1. Assistant Lecturer, Botany Department, Pathein University
2. Biological Resources and Biotechnology Development Centre, Pathein University
3. Pro-Rector, University of Pathein and Head of BR-BDC

has been devoted worldwide to limiting the spread of methicillin-resistent *Staphylococcus aureus* (MRSA). In addition to MRSA, emergence of almost untreatable vancomycin-resistant enterococci and the threat of transfer of glycopeptide resistant to *Staphylococcus aureus* (Hiramatsu, 1997 & Hiramatsu, 1997) have led to a new and unexpected public health problem in hospitals and the community. Accordingly, the discovery and development of new anti-multi-drug resistant (MDR) bacterial agents have become urgent. (Sugie, 2001). Microbial metabolites are biodegradable in nature, giving less stress to the ecosystems than synthetic ones. Therefore, antimicrobial metabolites today are required to have potent activity and be safe to animals, human and ecosystems.

In the course of the screening for discovery of new antibiotics, a fungus was found to produce a novel antibiotic, which shows antibacterial activity against Gram-positive MDR bacteria. In this paper, it was reported the taxonomy of producing organism, fermentation, purification and biological properties.

Materials and methods

Isolation of fungi from soil samples and screening of selective highly active fungus

In the screening program of antimicrobial metabolite, 27 soil samples were collected at Chin State and 18 soil samples at Mandalay Division. Isolation of fungi was undertaken by physical and chemical treatment dilution method. In the investigation of antimicrobial activities, isolated soil fungi were tested using 24 test organisms.

Identification of the antibacterial metabolite producing fungus MKN-09

Morphological characters of fungus MKN-09 were studied culturing on potato dextrose agar, corn meal agar, malt extract agar, Oatmeal agar, and Czapek-yeast extract agar were used. Microscopical observation was also done.

Fermentation

The producing fungus MKN-09 was inoculated into a 300-ml Erlenmeyer flask containing 50 ml of a seed medium (glycerol 2.0%, Glucose 0.2%, Soybean flour 2.0%, Yeast extract 0.2%, NaCl 0.25%, $CaCO_3$ 0.4%, pH 6.5) and incubated for 96 hrs at 27°C on a rotary shaker

(200 rpm) to investigate the microbial growth kinetics measuring packed cell volume percent. Transfer of culture (sizes of inoculum) was also studies. After determination of ages and sizes of inoculum, suitable amount of seed culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of the identical medium. Fermentation was carried out for 8 days at 27°C on a rotary shaker (200 rpm).

Isolation and purification of antibacterial metabolite

Antibacterial metabolite was isolated and purified by various chromatographies from fermented broth.

Determination of the minimum inhibitory concentration and Bacteriocidal effect on *Staphylococcus aureus* (Phay, 1999)

The minimum inhibitory concentrations (MICs) of MKN-09 were determined in GYA liquid medium (10 ml) inoculated with approximately 1.6×10^7 CFU of test organism *S. aureus* and were introduced with the treatment of MKN-09 metabolite (0.1562 µg/ml, 0.3125 µg/ml, 0.625 µg/ml, 1.25 µg/ml and 2.5 µg/ml) into 5 tubes. The control was also done. After incubation at 27°C for 24 hr, the MICs were determined by selecting the lowest concentration of metabolite which caused complete inhibition of bacteriocidal growth. Experiments were done in triplicate.

Test organism *S. aureus* (0.1 ml) was separately inoculated into 5 Conical flasks containing medium (100 ml, 1.0% glucose, 0.3% Yeast extract, 0.3% NZ amine type, pH 7.0) and incubated for 60 hours. At 24 hr incubation, it was introduced with the treatment of MKN-09 metabolite (0.3125 µg/ml 0.625 µg/ml 1.25 µg/ml and 2.5 µg/ml) into 4 flasks. PCV % was measured in 12 hr intervals. One flask is for contained.

Results

Isolation of fungi from soil samples

In the screening program of antimicrobial metabolite, 54 fungi isolated from 45 soil collected at Chin State and Mandalay Division. In the study of antimicrobial activities, one fungus MKN-09 showed selective highly antibacterial activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA). Therefore, this fungus was selected for further

investigations such as fermentation, purification and biological properties. Fungus MKN-09 was isolated from the soil collected at Hpa-Lam area, Chin State.

Identification of the producing fungus MKN-09

Morphological characters of fungus MKN-09 are shown in Table 1. On potato dextrose agar, the colonies grew rapidly with white mycelia and attained a diameter of 5.2-6.3 cm after 10 days at 25°C. When sporulated, the surface of the colonies showed pale blue green, reverse pale pinkish and were slightly powdery. No diffusible pigments were produced. On cornmeal agar, the colonies were thin and pale green, attaining a diameter of 2.4 cm after 10 days at 25°C. On oatmeal agar, the colonies were pale blue green and powdery and attained 2.8-3.6 cm in diameter after 10 days at 25°C. On Czapek-yeast extract agar, the colonies were blue green and powdery, 3.7-4.5 cm diameter after 10 days at 25°C. The optimal temperature for growth was between 25 and 28°C. The maximum temperature for growth on these media was 32°C and no growth occurs at 37°C. The pH for growth was from 5.0 to 8.5, with an optimal between 5.5 and 6.5.

Aerial hyphae were smooth-walled, branches arising low on the conidiophores, Conidia long chain, hyaline, smooth-walled, globose to subglobose, 2.0-2.5 μm diameter, Based on these distinct characteristics, it was placed in the genus *Lecanocillium*. According to Domsch (1993), there are eight species of *Lecanocillium*,. Among them, fungus MKN-09 was consistent with the assignment to *Lecanocillium waksmanii* Zaleski 1927. Therefore this fungus MKN-09 was identified as *Lecanocillium waksmanii* (Figure 1).

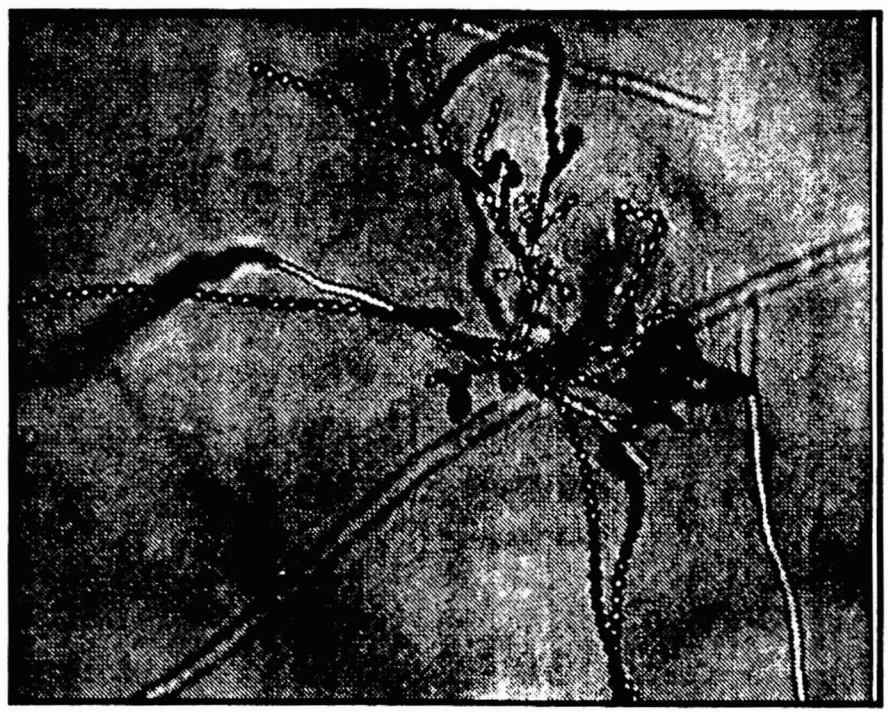

Figure 1. Photomicrograph of fungus MKN-09 (x 500)

Fermentation

On the basis of the results of microbial growth kinetics and transfer of inoculum, fermentation was carried out. In this study, it was observed that growth phase is between 48 hrs and 84 hrs and the optimal ages is 72 hrs to produce the metabolite (Table 1). It was also found that among 5%, 10%, 15%, 20%, 25% and 30%, 20% sizes of inoculum was the best for fermentation (Table 2). The producing fungus MKN-09 was inoculated into a 300-ml Erlenmeyer flask containing 50 ml of a seed medium (2.0% glycerol, 0.3% Glucose, 2.0% Soybean flour, 0.3% Yeast extract, 0.25% NaCl, 0.3% $CaCO_3$, pH 6.5) and incubated for 72 hrs at 27°C on a rotary shaker (200 rpm). The seed culture (40ml, 20.0%) was transferred to a 500-ml Erlenmeyer flask containing 160 ml of the identical medium. Maximum antibacterial activity reached at 6 days fermentation. Time courses of fermentation are shown in Table 3. Fermentation broth (7 liter) was collected for the purification of antibacterial metabolite.

Table 1. Effects of ages of inoculum for fermentation

Ages of inoculum (hrs)	Inhibitory zone (mm)
48	16.5
60	19.7
72	24.3
84	22.6
96	20.8

Table 2. Effects of sizes of inoculum for fermentation

Sizes of inoculum (%)	Inhibitory zone (mm)
5	15.6
10	18.8
15	22.1
20	24.3
25	21.7
30	20.4

Table 3. Time course of fermentation for the production of metabolite

Fermentation periods (days)	Inhibitory zone (mm)
1	-
2	14.8
3	16.5
4	19.4
5	21.7
6	24.4
7	22.6
8	21.4

Isolation and purification of antibacterial metabolite

The culture broth (7 liters) was centrifuged to give supernatant and mycelial cake. The supernatant was adjusted to pH 4.0 with 1N HCl and partitioned between EtOAC and water. The EtOAc layer was concentrated *in vacuo* and the dried residue was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH (10:1). The active fractions were concentrated and purified by C18 gel column chromatography with acetonitrile-water (8:2). The active fractions were further purified by Sephadex LH-20 column chromatography with $CHCl_3$-MeOH (7:3) to afford pure metabolite (24.5 mg).

Determination of the minimun inhibitory concentration and Bacteriocidal effect on *Staphylococcus aureus* (Phay, 1999)

It was observed that MIC of this metabolite is 0.3125 µg/ml on MRSA. This metabolite (MKN-09) affects on the growth of *S. aureus* at a concentration of 0.3125 µg/ml; any further increase in its concentration above 0.3125 µg/ml resulted in a more suppressive effect on growth. At a concentration of 1.25 µg/ml, cell lysis was observed that indicating that MKN-09 metabolite acts as a bacteriocidal compound.

Conclusion

In the screening program of antimicrobial metabolite, 54 fungi isolated from 45 soil collected at Chin State and Mandalay Division using physical chemical treatment dilution method.. In the study of antimicrobial activities with 24 test organisms, one fungus MKN-09 showed selective highly antibacterial activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA). Therefore, this fungus was selected for further investigations such as fermentation, purification and biological properties. Fungus MKN-09 was isolated from the soil collected at Hpa-Lam area, Chin State. The fungus MKN-09 was identified as based on the morphological and microscopical characters. In the microbial growth kinetics, it was observed that growth phase is between 48 hrs and 84 hrs. The optimal cultivation for fermentation was 72 hrs. It was also found that 20% sizes of inoculum was the best for fermentation. In fermentation studies, metabolite production reached at 6 days fermentation with 20% sizes and 66 hrs ages of cultures. Seven liters of fermented broth was collected for the isolation and purification. Antibacterial metabolite was purified by various chromatographies to afford pure metabolite (24.5 mg/ 7 liters). It was observed that MIC of this metabolite is 0.3125 μg/ml on MRSA. This metabolite (MKN-09) affects on the growth of *S. aureus* at a concentration of 0.3125 μg/ml; any further increase in its concentration above 0.3125 μg/ml resulted in a more suppressive effect on growth. At a concentration of 1.25 μg/ml, cell lysis was observed that indicating that MKN-09 metabolite acts as a bacteriocidal metabolite. The present study on antibacterial metabolite with expectations which have the potential to provide a therapeutic agent to control methicillin-resistant *Staphylococcus aureus* in clinical fields resulted in interesting compound from the soil fungus *Lecanicillium waksmanii* MKN-09.

Acknowledgement

We would like to express our gratitude to Pro-Rector U Hla Tint,, University of Pathein for his allowing us to do this research. We are also grateful to Minster and Deputy Minister of Ministry of Education and Director General, Department of Higher Education (Lower Myanmar) for their support to work the research. We thanks Ma Nant Khin Cho Aye and Nant San Gi Bwar, Biological Resources and Biotechnology Development Center for their kind help in this research.

References

Domsch, K. H., W. Gams., and T. H. Adserson. (1993): Compendium of soil fungi, vol 1

Harayama, T. and K. Isono. (2002): Sources of Microorganisms, J. Microbiology, 48, 46-50

Hiramatsu, K.; H. Hanaki & T. Ino. (1997): Methicillin resistant *Staphylococcus aureus* clinical strains with reduced vancomycin susceptibility. J. Antimicrobiol. Chemother. 40, 135-136

Hiramatsu, K.; N. Aritaka, H. Hanaki, S. Kawasaki, Y. Hosoda, S. Hori, Y. Fukuchi & I. Kobayashi. (1997): Dissermination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet 350, 1670-1673

Phay, N. (1999): Fistulosin, antifungal compound from Welsh onion, J. Phytochemistry, 52, 271-274

Sugie, Y.; H. Hirai, M. Ishiguro, Y. Shiomi, & Y. Kojima. (2001): CJ-15,801, a novel antibiotic from a fungus *Seimatosporium* sp., 1060-1065

Zulaybae, T. O.: (2005): Screening of anti-MRSA metabolites, Book of Abstracts, Bio-Thailand, 221,