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Research Article

STUDY ON ANTIMICROBIAL ACTIVITY OF *NOCARDIA* sp. STRAIN TP1 ISOLATED FROM TANGKUBAN PERAHU SOIL, WEST JAVA, INDONESIA

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ABSTRACT

Objective: The aim of this research was to investigate the growth profile of *Nocardia* sp. strain TP1, as well as antimicrobial acitvity of *Nocardia* sp. strain TP1 fermentation broth and extract.

Methods: Antimicrobial compound was produced by liquid fermentation method. Microbe used in this study was identified as *Nocardia* sp. strain TP1. *Nocardia* sp. strain TP1 was isolated from soil of Tangkuban Perahu volcano region, West Java Indonesia. This isolate was grown in several media composition composed of glucose, starch, tryptone, yeast extract, and calcium carbonate using 7 L fermentor. The fermentation broth was extracted by liquid-liquid extraction using n-butanol, ethyl acetate and chloroform solvents at pH 4.5, 7 and 9. Then, the active substance was detected by bioautography using ethyl acetate-toluene (8:2) as eluent.

Results: The highest activity of the culture against *methicillin sensitive Staphylococcus aureus* (MSSA) was shown after 36 and 38 hours fermentation and 36 hours after fermentation against *M. gypseum*. Antimicrobial activity of n-butanol extract at pH 7 was shown by MIC value of 128 ppm and 64 ppm against MSSA and *M. gypseum*, respectively. Bioautography examination on MSSA and *M. gypseum* showed antimicrobial activity was produced by active spot which gave Rf value of 0.85.

Conclusion: Secondary metabolite was produced by *Nocardia* sp. strain TP1 in fermentation broth active against MSSA and *M. gypseum*.

Keywords: *Nocardia* sp. strain TP1, Fermentation, Antimicrobial, MSSA, *Microsporum gypseum*.

INTRODUCTION

Antibiotics were mostly produced by *Actinomycetes* (70%) followed by fungi (10%) and bacteria(10%). Antibiotics produced by *Actinomycetes* are chloramphenicol (*Streptomyces venezuelae*), cephamycin (*Streptomyces clavuligerus*), kanamycin (*Streptomyces kanamyceticus*), nystatin (*Streptomyces nourse*), streptomycin (*Streptomyces griseus*), tetracycline (*Streptomyces aureofaciens*). Antibiotics derived from fungi are griseofulvin (*Penicillium griseofulvum), penicillin* (*Penicillium chrysogenum*), bacteria produce bacitracin (*Bacillus licheniformis*) and polymyxin B (*Bacillus polymyxa*)[1,2,3,4]. Nowdays in developing countries, main diseases are still dominated by infectious diseases, therefore antibiotic therapy is highly required[5].

However, the use of antibiotics is recently debated because many of diseases caused organisms getting more resistant. According to WHO, in 2009 Indonesia is ranked at 8th position out of 27 countries with high Multidrug Resistant (MDR) value. Therefore, it is important to explore brand new antibiotics.

Indonesia is a mega biodiversity country and the world second biggest biodiversity after Brazil. These biodiversities include microbial diversity in soil and water. The soil biodiversity of microbes have been explored recently to search antibiotic produced by microorganisms. Soil microorganisms were selected since soil is the best medium for *Actinomycetes.* Most of *Nocardia* usually grow on laterit soil and black chernozem soil contain organic compound[6].

Nocardia sp. strain TP1 was one of the four isolates of microbes isolated from soil around Tangkuban Perahu in West Java, Indonesia. The *Nocardia* sp. strain TP1 showed the highest activity against MSSA and *M. gypseum* compared to the other 3 microbes, therefore this isolate was further studied. The aim of this research was to investigate the growth profile of the *Nocardia* sp. strain TP1, to determine the fermentation process and examined the antimicrobial activity of the fermentation product and its extract.

MATERIAL AND METHODS

Microorganisms

Nocardia sp. strain TP1 isolated from soil around Tangkuban Perahu in West Java Indonesia was grown on Potato Dextrose Agar (PDA) for 3-5 days.

Bacteria used in this study were gram positive bacteria: *methicillin resistant Staphylococcus aureus* (MRSA), *methicillin sensitive Staphylococcus aureus* (MSSA), *methicillin resistant coagulase negative Staphylococcus* (MRCNS), v*ancomycin resistant Enterococcus* (VRE), and gram negative bacteria: *Escherichia coli* and *Pseudomonas* sp. While *Aspergillus niger, Candida albicans, Microsporum gypseum* were fungi used in this study. All microbes were obtained from culture collection of School of Pharmacy, Bandung Institute of Technology, Indonesia.

Modification of medium

Medium for fermentation was selected from 6 composition (medium I-VI).

Table 1: Composition of fermentation media

Medium I	Medium II	Medium III
▪ PDB 10 g/L ▪ Tryptone 5 g/L ▪ Yeast extract 2.5 g/L ▪ Na Thiosulfat 1 g/L	▪ PDB 10 g/L ▪ Tryptone 5 g/L ▪ Yeast extract 2.5 g/L ▪ $CaCO_3$ 1 g/L	▪ PDB 10 g/L ▪ Tryptone 5 g/L ▪ Yeast extract 2.5 g/L
Medium IV	**Medium V**	**Medium VI**
▪ Starch 10 g/L ▪ Glucose 10 g/L ▪ Tryptone 5 g/L ▪ Yeast extract 2.5 g/L ▪ Na thiosulfat 1 g/L	▪ Starch 10 g/L ▪ Glucose 10 g/L ▪ Tryptone 5 g/L ▪ Yeast extract 2.5 g/L ▪ $CaCO_{3\ 1}$ g/L	▪ Starch 10 g/L ▪ Glucose 10 g/L ▪ Tryptone 5 g/L ▪ Yeast extract 2.5 g/L

Fermentation

Isolate of microbe TPA was inoculated to sterile preculture medium in 250 mL erlenmeyer flask, then incubated in orbital shaker at room temperature for 12 hours. Then, the inoculum was inoculated to a sterile preculture medium in 1L erlenmeyer flask, and once more incubated in orbital shaker for 16 hours. After that, similar treatment was performed to 7L fermentor for 48 hours. The fermentation condition in fermentor was arranged at pH 7 with agitation at 150 rpm. Sampling was done every one hour to observe the growth profile of the microbe, antimicrobial activity of fermentation broth and biomass, starch and glucose consumption pattern.

Growth rate of microbes

Growth measurement of microbes produced antibiotics was done by nett dry cells weight method.

Study of fermentation broth activities

Sampling was done every hour, then centrifugated at 6000 rpm for 15 minutes. Antimicrobial activities of fermentation broth was studied using perforator method.

Study of fermentation products biomass activities

Fermentation biomass products was washed twice by sterile aquadest, then dissolved in solvent (acetone:methanol) for 24 hours, sonicated and centrifugated at 6000 rpm for 15 minute. Study of acitivities was carried out by Kirby Bauer method.

Determination of glucose and starch concentration in medium during fermentation process

Total glucose concentration was analyzed by hydrolizing fermentation broth, using 3% HCl, then boiled under cooler for 3 hours. After cooled down, it was neutralized by 30% NaOH solution using phenolphtalein as indicator. Then glucose solution was put into 50 mL volumetric flask. Nelson reagent was added to 1 mL of glucose solution (with various concentration), then heated in water bath. After cooled down, arsenomolybdate reagent was added to the solution. Then it was shaked an diluted with aquadest until final volume of 10 mL. Absorbance was read at wavelength of 520 nm.

The measurement of reduction glucose concentration was done by varying concentration of fermentation broth and addition of nelson reagent, then heated in water bath. After cooled down, arsenomolybdate reagent was added. Then it was shaked an diluted with aquadest until final volume 10 mL. Absorbance was read at wavelength of 520 nm[7].

Determination of starch concentration

Starch weight was obtained by multiplying the difference between total glucose concentration and reduction glucose concentration with 0.9 (obtained from comparison of glucose and starch MW).

Extraction

Fermentation products were centrifugated at 6000 rpm for 12 minutes. The filtrates were extracted by liquid-liquid extraction method with various solvents: n-butanol, ethyl acetate, and chloroform, at various pH (4.5, 7, 9). Then extracts were evaporated at 40^0C.

Minimum inhibitory concentration (MIC)

MIC measurement was done by microdillution method using ciprofloxacin and ampicillin (for bacteria) and ketoconazole and nystatin (for fungi) as comparison.

Bioautography

Bioautography was done by thin layer chromatography. Chromatogram plate that already showed separation of the spot, was adhered for 30 minutes on medium which contain pathogen microbe. Then it was incubated at 22-25^0C for 72 hours (for fungi) and at 37^0C for 24 hours (for bacteria).

RESULTS AND DISCUSSIONS

Fermentation medium modification

Fermented *Nocardia* sp. strain TP1 in medium VI showed optimum antimicrobial acitvities. It can be shown by the large and clear inhibitory diameter resulted from fermented microbe in medium VI. Based on this result, further fermentation was done using medium VI (table 2). *Nocardia* sp. strain TP1 possessed activities against MSSA and *M. gypseum*, while no activities shown on MRSA, MRCNS, *Pseudomonas*, *Escherichia coli*, VRE, *Candida albicans*, dan *Aspergillus niger*. Mineral content such as calcium in this medium was obtained from calcium carbonate. Calcium is not essential growth nutrient for most microorganisms, but it can help in stabilizing bacteria cell wall and important in heat stabilization for endospore[5]. In media I and IV, the mineral content was obtained from Na-Thiosulfat, but antibiotic was not produced. It was likely that the mineral used in these medium was not suitable for growth and production of tested microbes. The microbial and fermented products are largely composed of nitrogen and carbon source, therefore the optimization of fermentation medium is more important in selecting an organism to obtain antibiotic production[8,9].

Table 2: Fermentation medium modification

Microbe	Modification of medium	MSSA		*M. gypseum*	
***Nocardia* sp. strain TP1**		**24 hour**	**48 hour**	**24 hour**	**48 hour**
	M I	14	13	14	13
	MII	17	16	16	15
	M III	12	10	14	12
	M IV	15	14	14	13
	M V	12	10	14	12
	M VI	**19**	**18**	**18**	**17**

Growth profile of *Nocardia* sp. strain TP1

Fermentation was done in 7L fermentor. Optimum growth peak of *Nocardia* sp. strain TP1 in 7 L fermentor was achieved after 13 hour fermentation with the dry nett of the cells was 38.8 mg (figure 1).

Fermentation condition that was done at pH 7, agiation at 150 rpm, 1% v/v aeration and temperature 30^0C were suitable to support the growth of *Nocardia* sp. strain TP1. Generally *Actinomycetes* is incubated at temperature 25-30^0C because most of *Actinomycetes* group is mesophil which has optimum growth temperature ranged on that number[10]. Fermentation above 30^0C resulted the decline of bacterial growth and antiobiotic production. Antibiotic was not produced in 40^0C incubation[11,12].

Antimicrobial Activites of Fermentation Products

Based on the antimicrobial activities study of biomass intracellular liquid, no inhibitory diameters were observed on pathogen bacteria and fungi.

Optimum activites of fermentation broth of *Nocardia* sp. strain TP1 was found on 36 and 38 hours after fermentation for MSSA and 36 hours after fermentation for *M. gypseum*, where inhibitory diameter on MSSA and *M. gypseum* was 20 mm (figure 2 and 3). Secondary metabolite characteristic is not generally produced during growth phase (trophophase), but synthesized during idiophase. A study on liquid culture showed that secondary metabolite production started when the growth of microbe was restricted due to the lack of

medium nutrient such as carbon and nitrogen sources[13]. Changing pH influenced antibiotic production time of *Actinomycetes* spp.[14]. Filamentous *Actinomycetes* are known to be capable of producing various secondary metabolites[15,16].

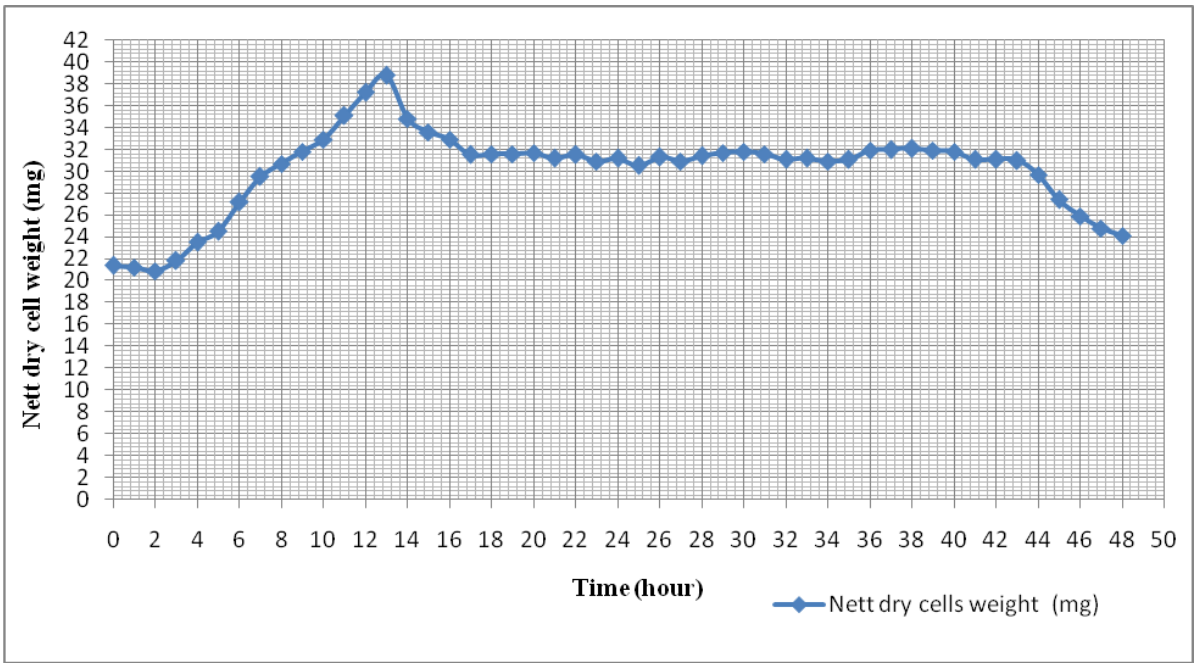

Fig. 1: Growth curve of *Nocardia* sp. strain TP1 (nett dry cells weight method) in 7L fermentor

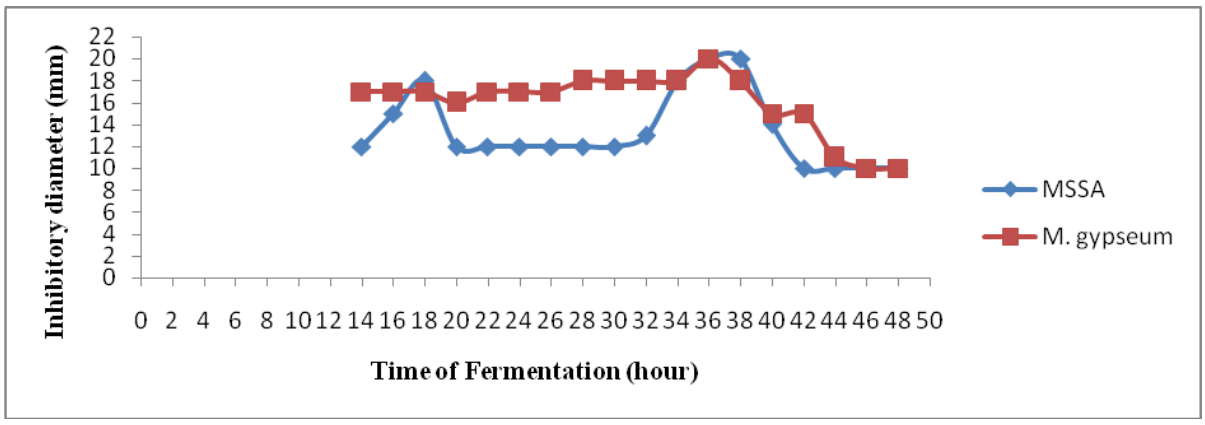

Fig. 2: Antimicrobial activities of *Nocardia* sp. strain TP1 of fermentation broth

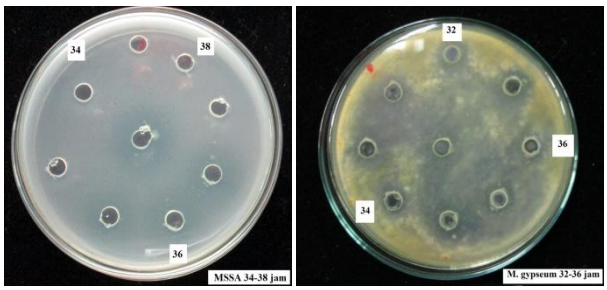

Fig. 3: Antimicrobial activities of fermentation broth of *Nocardia* sp. strain TP1 (Each sample were analyzed in triplo)

Glucose and Starch Consumption Pattern during Fermentation

Glucose consumption by *Nocardia* sp. strain TP1 was observed in early stage of fermentation process. It was differ compared to starch consumption pattern. The founding was shown after 2 hours of fermentation process, which measured glucose concentration was 9.74 mg/mL, whereas starch concentration was still about 9.98 mg/mL (figure 4).

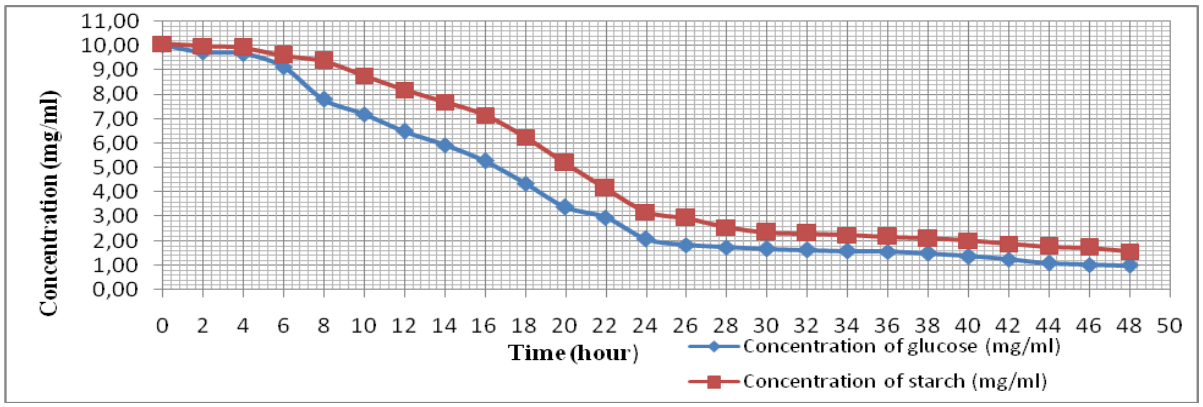

Fig. 4: Time course of *Nocardia* sp. strain TP1 fermentation

Glucose consumption during fermentation process by *Nocardia* sp. strain TP1 occured earlier than starch consumption. Starch is a macromolecule that consist of several glucose. Therefore, prior to microbe consumption, starch has to be broken down to a simple form such as glucose[17]. Decleva (1985) describe that carbon and other energy sources are entirely used during growth and not used to produce antibiotic. Component that is not entirely used during cells growth is more suitable for antibiotic production[18].

Extraction and bioautography

Liquid-liquid extracion results showed that n-butanol extract has antimicrobial activity. N-butanol extract at pH 7 showed optimum antimicrobial activites with MIC 128 ppm on MSSA and 64 ppm on *M. gypseum*. Bioautography study in n-butanol extract using ethyl acetate:toluene (8:2) as eluent, resulted separate spot with 0.85 Rf value. This spot showed antimicrobial activity against MSSA and *M. gypseum* (figure 5). Clear area around the spot indicated inhibition of microbe growth by extract component[19]. Based on the property of the used eluent, it can be predicted that the substance was semipolar.

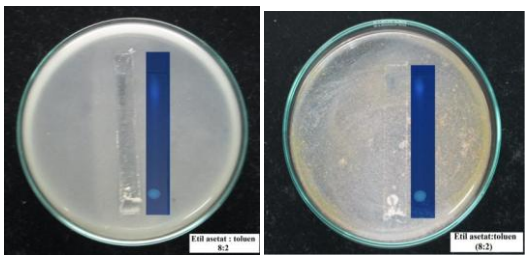

Fig. 5: Bioautography test results of n-butanol extract on uv light 366nm (a) in MSSA; (b) in *M. gypseum*

CONCLUSION

Secondary metabolite of *Nocardia* sp. strain TP1 was produced in fermentation broth and showed activities against MSSA and *M. gypseum*, whereas intracellular liquid from cell biomass did not show anitmicrobial activities. Growth peak occured after 13 hours fermentation. N-butanol extract at pH 7 actively against MSSA and *M. gypseum* with MIC about 128 ppm and 64 ppm, respectively. TLC study of this extract produced in one spot Rf value of 0.85.

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