

Screening of Antibacterial Activities of Actinomycetes Isolates from Indonesia

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Abstract: New outbreaks caused by bacteria or rickettsia occur almost every year with serious impacts for human health and global economy. Countermeasures against these problems can be solved by looking for new antibacterial alternatives which are relatively easy to be found in nature, efficacy and safety for patient. Actinomycetes are known as a source of new antibacterials. The aim of this study was to obtain actinomycetes isolated from Indonesian soils which producing antibacterial compound that inhibit 4 pathogens tested, *Salmonella* Typhimurium InaCC B.283, *Escherichia coli* InaCC B.285, *Staphylococcus aureus* InaCC B.286 and *Bacillus subtilis* InaCC B.289. A total of 299 actinomycetes was used during this research. As result of the screening of 299 isolates, 48 isolates were reproducible to inhibit pathogens tested. Actinomycetes InaCC A.413 that inhibit 4 pathogens tested with the largest inhibition zones were incubated for 2, 3, 4, 5, 6 and 7 days to find out the optimal timing to produce antibacterial. The optimum percentage of inhibition index of actinomycetes InaCC A.413 was obtained on the fifth day with percentage rate that was more than 80% in doses 30 µL on 37°C temperature. Morphological characteristics showed that the strain has long hyphae, white substrate mycelium, smooth and white colony surface. Actinomycetes InaCC A. 413 were identified using 16S rRNA gene analysis as *Streptomyces ginsengisoli*. Actinomycetes InaCC A. 413 has inhibitory effects against four pathogens Gram-positive and Gram-negative bacteria that allegedly have a broad spectrum.

Key word: Actinomycetes • Screening • Antibacterial

INTRODUCTION

Recently, emerging and re-emerging disease an alarming number of new viruses and other pathogens have been recorded. Many of them are the emerging disease comes from wildlife. New outbreaks caused by zoonotic pathogens (transmitted from animals to humans) occur almost every year with serious impacts for human health and global economy [1].

Emerging infectious diseases cases are dominated by zoonoses (60.3% of emerging infectious), the majority of them (71.8%) originate from wildlife and are increasing significantly over time. Approximately 54.3% of emerging infectious diseases are caused by bacteria or rickettsia, reflecting a large number of drug-resistant microbes [2].

Antibiotics are used in animals as in humans for therapy and control of bacterial infections [3]. Some of these pathogenic bacteria cause infectious disease are resistant to antibiotic. Nowadays, the up growing resistance of pathogenic bacteria to the conventional antimicrobial agents is a great concern to clinical microbiologists [4-5]. Meanwhile, the use of synthetic drugs has severe side effects especially with those tested for the infectious disease [6]. This became a problem that should be addressed immediately. Countermeasures to solve these problem can be done by looking for new antibacterial alternatives that are found in nature, efficacy and safety for patient. The current control measures are concentrated mostly on treatment of infectious diseases that uninfected to animals and humans by using the natural products [7].

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Actinomycetes are known as a source of antibiotics, herbicides, pesticides and anti-parasitic [8]. Nearly 8,000 actinomycete-derived antibiotics had been described by 1994, of which 80% were from *Streptomyces* species and 20% from other actinomycetes genera [9]. Actinomycetes are soil microorganisms that commonly found in various types of soil in the nature [10].

Research of Indonesia's actinomycetes local isolates has been conducted since 2003 in the Research Center for Biotechnology, Indonesian Institute of Sciences. The research was conducted by isolating actinomycetes from soils and leaf-litters at botanical gardens and other areas in Indonesia. In order to utilize actinomycetes collection, it need to conduct characterization test of bioactive compounds product, especially those with antibacterial characteristics from Indonesia's actinomycetes local isolates.

The aim of this study was to obtain actinomycetes local isolates from Indonesian soils which produce antibacterial bioactive compounds inhibiting *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*.

MATERIALS AND METHODS

Materials

Isolates: Actinomycetes collection and pathogens tested used in this research are the property of Applied Microbiology Laboratory, the Research Center for Biotechnology, Indonesian Institute of Sciences; which were 299 actinomycetes local isolates from Indonesian soil, *S. Typhimurium* InaCC B.283, *E. coli* InaCC B.285, *S. aureus* InaCC B.286 and *B. subtilis* InaCC B.289.

The Culture Media and Chemicals: The culture media and chemicals used in this research are humic acid-vitamin agar (HVA) agar medium and International Streptomyces Project-2 (ISP-2) agar medium to cultivate actinomycetes isolates; nutrient agar, nutrient broth, liquid production medium (soluble starch 20 g/L, CaCO₃ 30 g/L, soy bean 20 g/L, glucose 10 g/L, cooking oil 10 mL/L), Mueller Hinton agar medium to bioassay test; ethyl acetate to extract the bioactive compounds; PCR mixture, primer 9F and 1541R to identify actinomycetes and pathogens tested.

Methods

Validation of Pathogens Tested (*S. Typhimurium*, *E. Coli*, *S. Aureus* and *B. Subtilis*): *S. Typhimurium* InaCC B.283, *E. coli* InaCC B.285, *S. aureus* InaCC B.286

and *B. subtilis* InaCC B.289 were re-cultured on nutrient agar medium and incubated for 24 hours at a temperature of 35-37°C. Genus and species of bacteria were validated based on 16S rRNA gene analysis [11].

Actinomycetes Cultivation: A total of 299 isolates of actinomycetes was re-cultured in HVA media and incubated at 30°C [12]. After that, actinomycetes colony was purified on ISP-2 media and incubated at 30°C. Actinomycetes at the age of 4 days were used for screening of antibacterial producing actinomycetes against 4 pathogens tested.

First Phase Screening for Antibacterial Activity:

The principle bioassay method of agar diffusion is using two layers of agar medium (the lower layer and top layer). The lower layer was filled with 10-15 mL of 1 portion of Mueller Hinton agar medium and the top layer was filled with 5 mL of ½ portion of Mueller Hinton agar medium. Pathogens tested with transmittance value of 25% at a wavelength of 580 nm [13] were added to the top layer and poured in petri dishes which contain lower layer that has been harden. Each of four suspension of pathogens tested was inoculated in top layer as follows 0.1% of *S. Typhimurium* InaCC B.283 culture, 0.2% of *E. coli* InaCC B.285 culture, 0.1% of *S. aureus* InaCC B.286 culture and 0.1% of *B. subtilis* InaCC B.289 culture [14].

Actinomycetes isolates on ISP-2 agar medium, at the age of 4 days, were cut using sterile straw (diameter 6 mm), piece of this agar medium with actinomycetes isolates was aseptically placed on the top layer of Mueller Hinton agar media and were incubated at 30°C for 24 hours on all pathogens tested [15]. Each of the isolates was observed and measured for their ability to inhibit the four pathogens tested by the formation of a clear zone around the piece of agar. The actinomycetes that has higher inhibitory effects against all of the four pathogens tested (broad spectrum) in three repetitions were selected for further testing.

Extraction of Active Compounds from Actinomycetes:

Actinomycetes with the largest inhibition zone that inhibited the four pathogens tested (broad spectrum) were selected, re-cultured on ISP-2 agar medium and incubated at 30°C for 4-5 days. After that, actinomycetes colony was inoculated in 50 mL liquid production medium and incubated on a shaker in 200-220 rpm at room temperature for 2, 3, 4, 5, 6 and 7 days. Every 2, 3, 4, 5, 6 and 7 days actinomycetes in liquid culture medium were centrifuged in 4,000 rpm at 4°C for 15 minutes to get the supernatant.

The supernatant was added by ethyl acetate with the same volume ratio, shaken and then wait for 30 minutes to get a liquid fraction and ethyl acetate fraction. The ethyl acetate fraction was added by ethyl acetate with same volume and extracted again for three times. Then, the ethyl acetate fraction was fully evaporated up to 1 mL (filtrate). The filtrate was used for the second phase of the screening test. The observation was conducted to measure the inhibition zone (in mm) and inhibition index that was calculated by Valestine formula [16]:

$$\text{Inhibitory Index} = \frac{a}{b} \times \frac{b}{a}$$

a = Diameter of the clear zone

b = Diameter of colonies zone/discs

Second Phase Screening for Antibacterial Activity:

Bioassay test was conducted by using agar diffusion methods with disc paper in diameter 6 mm. Amount of 30 µL of filtrate was poured dropwise to paper disc and which allowed to dry. After that, paper disc was placed on the Mueller Hinton agar media surfaces inoculated with the pathogens tested. Ethyl acetate paper disk was used as a control negative. After that, the plate was incubated at 37°C for 24 hours and the diameter of inhibition zones were measured [19]. Actinomycetes candidate was tested for the optimum time to form inhibition zone on day 2, 3, 4, 5, 6 and 7 in two repetitions.

Identification of Actinomycetes Based on 16S rRNA Gene Analysis: Selected actinomycetes isolates were identified based on 16S rRNA gene analysis into the genus or species level [11].

RESULTS

First Phase Screening for Antibacterial Activity:

Screening results showed that 48 isolates from 299 isolates of actinomycetes formed reproducible inhibition zone on three repetitions. Eleven isolates inhibited only the growth of *S. Typhimurium* InaCC B.283, 5 isolates only *E. coli* InaCC B.285, 12 isolates only *S. aureus* InaCC B.286, 11 isolates only *B. subtilis* InaCC B.289, 3 isolates inhibited the growth of 2 pathogens tested (*S. aureus* and *B. subtilis*), 3 isolates inhibited the growth of 3 pathogens tested and 3 isolates inhibited 4 pathogens tested. Diameter of inhibition zone varied from 6.1 mm to 30.45 mm. Data of inhibition zone of 3 isolates (InaCC A.234, InaCC A.358 and InaCC A.413) that could inhibit 4 pathogens tested were shown in Table 1. Isolate of actinomycetes InaCC A.413 with the relatively larger inhibition zone were selected as candidate for second phase of antibacterial assay.

Second Phase Screening for Antibacterial Activity:

Figure 1 showed the screening of selected actinomycetes InaCC A.413 that was grown on liquid production medium. The result showed that all ethyl acetate paper disk as control negative did not showed any inhibition zone. Supernatant paper disk were showed inhibition zone with diameter from 6.00 mm to 9.50 mm. The disk that contained culture extracted actinomycetes before concentrated were showed inhibition zone with diameter from 6.00 mm to 25.00 mm. Meanwhile, the disk that contained filtrate of actinomycetes InaCC A.413 showed inhibition zone with diameter from 8.15 mm to 48.00 mm.

Table 1: Bioassay of actinomycetes against four pathogens tested.

Actinomycetes	Mean of Inhibition Zone (mm) (Inhibition Index %) Against			
	<i>S. Typhimurium</i> InaCC B.283	<i>E. coli</i> InaCC B.285	<i>S. aureus</i> InaCC B.286	<i>B. subtilis</i> InaCC B.289
InaCC A. 234	19.2 (69)	10.7 (44)	16.6 (64)	15.9 (62)
InaCC A. 358	8.4 (29)	7.8 (23)	12.7 (53)	16.6 (64)
InaCC A. 413	21.3 (72)	7.2 (17)	20.9 (71)	17.0 (65)

Table 2: Mean of inhibition index (%) antibacterial activity filtrate of actinomycetes InaCC A.413 with different incubation time against four pathogens tested with two repetitions.

Pathogens Tested	Inhibition Index (%)					
	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
<i>S. Typhimurium</i>	59.95	80.17	82.09	81.01	54.13	61.61
<i>E. coli</i>	37.69	83.89	84.40	81.47	74.58	73.33
<i>S. aureus</i>	59.27	72.20	76.83	83.61	34.64	28.83
<i>B. subtilis</i>	56.99	87.30	84.35	84.42	69.07	72.67

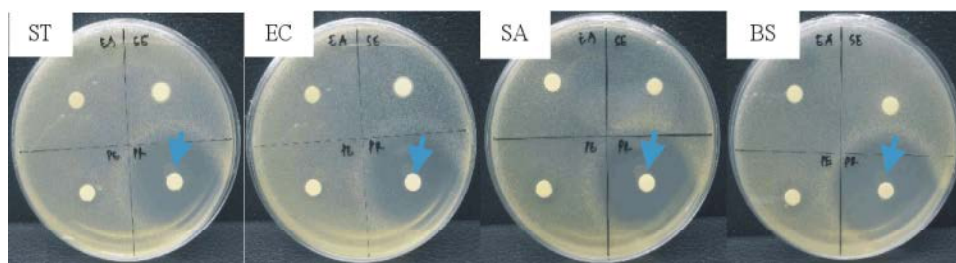


Fig 1: Screening of actinomycetes InaCC A.413 after incubation on third days against pathogens tested *S. Typhimurium* InaCC B.283 (ST), *E. coli* InaCC B.285 (EC), *S. aureus* InaCC B.286 (SA) and *B. subtilis* InaCC B.289 (BS). Left up: ethyl acetate as control negative (EA); right up: supernatant of actinomycetes cultures InaCC A.413 (SE); left down: culture extracted actinomycetes before concentrated (PE) and right down: filtrate of actinomycetes (PR). Petri dish diameter: 90 mm.

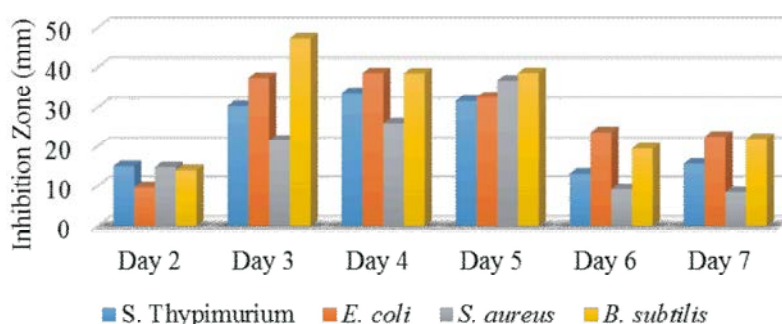


Fig 2: Antibacterial activity of filtrate actinomycetes isolate InaCC A.413 with different incubation times against four pathogens tested.

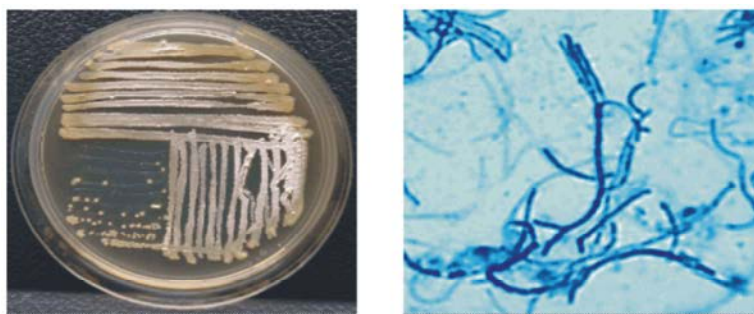


Fig 3: Isolate of actinomycetes InaCC A.413 on the ISP-2 medium (left) and its microscopic morphology on lactophenol cotton blue staining (right) (100x magnifications)

Optimum Time to Produce Antibacterial Compound:

The largest size of the inhibition zone was determined as the optimum time. Figure 2 showed the optimum inhibition zone of the actinomycetes InaCC A.413 filtrate against four of the pathogens tested. The result showed that the optimum time for the actinomycetes InaCC A.413 filtrate against *S. Typhimurium* and *E. coli* were obtained on day 4, meanwhile the optimum time for the actinomycetes InaCC A.413 filtrate against *S. aureus* was obtained on day 5 and the optimum time for the actinomycetes InaCC A.413 filtrate against *B. subtilis* was obtained on day 3.

The inhibition index (%) showed the ability of filtrate to inhibit the growth of pathogens tested. Table 2 showed the inhibition index filtrate of actinomycetes InaCC A.413, during 7 days of incubation times with doses 30 μ L. The highest inhibitions index of filtrate of actinomycetes InaCC A.413 was 87.30%, against *B. subtilis* obtained on day 3, following by the filtrate against *E. coli* (84.40%) on day 4, the filtrate against *S. aureus* (83.61%) on day 5 and lastly was the filtrate against *S. Typhimurium* (82.09%) on day 4.

Sequence Gene Analysis of Actinomycetes Based on 16S rRNA: Identification of actinomycetes isolate was performed using 16S rRNA gene analysis methods. Sequence partial of 16S rRNA gene from isolates actinomycetes isolate InaCC A.413 was compared to all bacterial sequence which registered in database of gene-bank using BLAST programme that can be accessed in website <http://www.ncbi.nlm.nih.gov/BLAST>. The result of sequence analysis showed that the actinomycetes InaCC A.413 was 99% homolog with *Streptomyces ginsengisoli* strain HBUM1174543.

DISCUSSION

The ability of actinomycetes to produce antimicrobial metabolite compounds made actinomycetes have an important role in pharmaceuticals industries. Based on this characteristic, many actinomycetes were developed and used as a therapeutics agent to cure any human or animal diseases [17]. Research to find new drugs based on actinomycetes metabolites do with many different approaches and methods, such as exploration for unique and specific area, development a new method for isolation and development genetically modified technics.

This research showed that 48 of 299 actinomycetes isolates indicates a reproducible inhibitory action against four pathogens tested. The absence of pathogens growth around isolates was started after 24 hours of incubation with specific diameter with specific diameter showed antibacterial activity [18]. The characteristic of bacterial inhibition varied for 48 isolates. A number of 39 isolates were able to inhibit only one pathogens tested (limited spectrum), 3 isolates inhibited 2 Gram-positive pathogens tested (narrow spectrum), 3 isolates inhibited 3 varied pathogens tested (narrow spectrum) and 3 isolates inhibited 4 pathogens tested (broad spectrum). Further, from 48 isolates, 32 isolates inhibited Gram-positive bacteria (*S. aureus* and *B. subtilis*), 22 isolates inhibited Gram-negative bacteria (*S. Typhimurium* and *E. coli*) and 6 isolates inhibited varied Gram-negative and Gram-positive bacteria. This result indicated that Gram-positive bacteria were more sensitive than Gram-negative bacteria as mentioned by Kariminik *et al.*, Pandey *et al.* and Thakur *et al.* [19-21]. In this study, the actinomycetes candidate with the largest inhibition zone and was able to inhibit the growth of four pathogens tested was actinomycetes InaCC A.413.

The filtrate of actinomycetes InaCC A.413 against four pathogens tested showed inhibitory action on bacterial growth. The inhibition assumed actinomycetes

secretion metabolite. During the screening of secondary metabolite, actinomycetes isolates was often encountered shown by antibiotic activity on medium agar [21]. More antibacteria were secreted, a larger inhibition zone were formed [22].

An active antibacterial compound was extracted in the supernatant of actinomycetes InaCC A.413. This result indicated that the active compound of actinomycetes InaCC A.413 was produced as an extracellular [23]. The filtrate has ability to inhibit the Gram-negative (*S. Typhimurium* and *E. coli*) and Gram-positive (*S. aureus* and *B. subtilis*) bacteria therefore bacteria compounds predicted as broad spectrum antibacteria.

The filtrate of actinomycetes InaCC A.413 that could inhibit 4 pathogens tested was extracted using ethyl acetate. Antibacterial active compound was non polar, it was shown by its ability to bind with ethyl acetate. To obtain extract containing non polar compound can be used non polar solvent such as ethyl acetate. The advantage of ethyl acetate is specific for non polar compound [24]. Supernatant paper disk were small inhibition zone. The *Streptomyces* spp. can be a poor secondary metabolite if the solvent used for extraction may not be suitable for it or the compound may not be properly extracted by the solvent [21].

Filtrate of actinomycetes InaCC A.413 showed optimum inhibition zone on third, fourth and fifth day of incubation. The optimum percentage of inhibition index of actinomycetes InaCC A.413 was obtained on the fifth day with percentage rate that was more than 80% in doses 30 μ L on 37°C temperature. The result of actinomycetes InaCC A.413 are consistent with Susilowati *et al.* [22] that reported the optimum inhibition zone occurs at 72 and 96 hours incubation and decline after 120 hours incubation. The maximal inhibition zone of *Streptomyces*, which produce antibiotics in synthetic medium performed it maximal inhibition zone on fifth day of incubation at 30°C [25-27].

During their late stationary phase, microorganism produced secondary metabolites such as antibiotics, vitamins and hormone [28]. The duration of production phase of each microorganism was varied, depend on their genetic factors and environment conditions. Actinomycetes and fungi have production phase longer than bacteria. When there were not any inhibition or other repression factors, actinomycetes and fungi were able to produce antibiotic for several days [29].

The colony growth of actinomycetes InaCC A.413 was relatively slow. The isolate produced spores with white colour and changed to grey as the incubation period increased. The morphology of actinomycetes

InaCC A.413 had long hyphae, formed mycelium, formed an aerial hyphae and substrate mycelium with white colour which penetrate agar medium (Figure 5). The presence of aerial hyphae and substrate mycelium are specific character of *Streptomyces* [30].

The identification using 16S rRNA gene analysis methods showed that actinomycetes InaCC A.413 had closest taxonomy classification with *Streptomyces* sp. The taxonomic position of *Streptomyces* is in the Class of Actinobacteria, Ordo Actinomycetales, Family Streptomycetaceae and Genus *Streptomyces*. The sequencing result using the 16S rRNA gene showed that the actinomycetes InaCC A.413 was 99% homolog with *Streptomyces ginsengisoli* strain HBUMI174543. Up to recently, study of *Streptomyces ginsengisoli* is only on their ability to produce L-asparaginase enzyme, with it several activity especially against cancer [31]. Therefore, the results of this research presented more information about *Streptomyces ginsengisoli*, especially its antibacterial activity against the four pathogens tested (*S. Typhimurium*, *E. coli*, *S. aureus*, and *B. subtilis*).

CONCLUSION

The screening for antibacterial activity shown 48 of 299 actinomycetes isolates showed inhibition zone. From 48 isolates, the largest diameter of inhibition zone was actinomycetes InaCC A.413. The isolate that was the highest inhibition index was 87.30%, where the filtrate of actinomycetes InaCC A.413 was against *B. subtilis* and obtained on day 3. The optimum percentage of inhibition index of actinomycetes InaCC A.413 was obtained on the fifth day with percentage rate that was more than 80% in doses 30 µL on 37°C temperature. Actinomycetes InaCC A.413 analysis using 16S rRNA gene were identified as *Streptomyces ginsengisoli*. Result of this reseach also shown that *streptomyces* predicted to have a broad spectrum action against Gram negative and Gram positive bacteria.

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