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RESEARCH ARTICLE

Antimicrobial Activity of Streptomyces sp. Isolated from Acidic Peatlands against Extended Spectrum Beta Lactamase (ESBL) producing *Escherichia coli*

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ABSTRACT:

The incidence of infection by extended spectrum of ß lactamase (ESBL)-producing bacteria is increasing throughout the world. Surveillance results in Indonesia (2012) showed that extended spectrum of ß lactamase (ESBL) producing *Escherichia coli* was increased (52%). Streptomyces has become an important source of important bioactive compounds with high commercial value and continues to be routinely screened to look for new bioactive substances. The aims of this research is to screen in vitro antibacterial activity of the free cell fermentation *Streptomyces sp.* of Kalimantan acidic peatland (isolate 2.1). *Streptomyces sp.* have been isolated from Kalimantan Tengah peat soil (Palangka Raya). The isolatation and fermentation process to obtain antibaterial substances were performed in the International Streptomyces Projects (ISP)-4 media on rotary shaker at 150 rpm, 28°C for 5 days. In vitro antibacterial testing of one to 5 days free cell fermentation broth (FCFB) of the *Strepromyces sp.* isolate 2.1 have been carried out by diffusion agar method. Antibacterial testing using the extended spectrum of ß lactamase (ESBL) *Escherichia coli* isolated from Dr. Soetomo Hospital patients in Surabaya as test microorganisms. The results of this research is the FCFB of *Streptomyces sp.* isolate 2.1 showed its activities againts ESBL producing *Escherichia coli* 6024 and 6110 with inhibition zone average 12,42 mm and 13,17 mm. Conclusion of this research is *Streptomyces sp.* isolated Kalimantan acidic peatland (isolate 2.1) potent to againts ESBL producing *Escherichia coli*.

KEYWORDS: *Streptomyces sp.*, free cell fermentation broth, ESBL producing *Escherichia coli*, diffusion agar method, inhibition zone.

INTRODUCTION:

Discovery and production antibiotics are one of the greatest successes of mankind (1). But now antibiotic resistance and side effects of antibiotics are the main problems in the field of medicine (2) (3) (4), which is quite worrying is the increasing incidence of infections caused by the appearance of ESBL-producing bacteria (5) (6).

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The prevalence of ESBL-producing bacteria varies in various countries and hospital cares (3). The problem of bacterial resistance caused by β lactamase enzyme activity can be reduced by looking for antibiotic compounds that are resistant to the enzyme activity of β lactamase, as well as looking for new β lactam molecules. Chemical modification of β lactam antibiotics is also needed, to improve the therapeutic effect against pathogenic bacteria producing β lactamase, and need to be considered to find compounds that can inhibit β lactamase enzyme activity.

Streptomyces has become an important source of bioactive compounds with high commercial value and

continues to be routinely screened for new bioactive substances (7). Recent research has found a secondary metabolite compound of 1,2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester (DMEHE) from marine *Streptomyces sp.* VITSJK8, which has the potential as an anti-ESBL (8). Antibiotics produced by *Streptomyces sp.* including Streptomycin.

Streptomyces are considered neutrophilic. They prefer a neutral pH to alkaline environment (9)(10). Streptomyces acidophilic and acidotolerant to date, based on biological properties, ecological functions and the role of these organisms in the community of soil microorganisms are still being explored (11). Acidophilic Streptomyces are aerobic and chemoorganotrophic organisms that grow in acidic environments and are potential sources of antimicrobial compounds that have acid-resistant enzymes. Optimal growth occurs at a pH of around 4.5-5.0 in the mesophilic temperature range (12).

The soil harbors diverse microorganisms which are more beneficial and promising habitat to find and isolate new natural products (13)(14). The microorganisms produce many secondary metabolites which are commercially exploited in many applications . One type of soil that has a unique character is peat soil with a high level of acidity. Peat is formed from the remains of plants that have died and decomposed into organic deposits with the help of aerobic and anaerobic bacteria (15). High accumulation of organic matter in peat soils provides various factors suitable for microbial growth and development (16). Several factors that influence microbial activity include soil moisture, temperature (17), nutrients and substrates availability (18), in combination with peat properties such as pH, cation, cation exchange capacity and soil dryness (19)(20).

Indonesia is the fourth country with the largest peat swamp area in the world which is around 20 million ha after Canada (170 million ha), the Soviet Union (150 million ha), and the United States (40 million ha). Peatlands in Indonesia are generally found in Sumatra, Kalimantan and Papua or around 10.8 percent of Indonesia's land area (21) (22). The spread of the area is around 5.7 million ha or 27.8% is found on Kalimantan island. One of the provinces on the island of Kalimantan is the province of Kalimantan Tengah, which has a peatland area of 3.01 million ha (21).

The extent of the peatlands in Kalimantan Tengah can be a great source for finding antibacterials that are useful for overcoming antibiotics that have been resistant so far. This research was carried out on unexplored peatland habitats to find new strains of Streptomyces that produce antibiotics.

MATERIAL AND METHODS:

Collection of samples and test bacteria:

Peat soil sample was taken from peat land in Palangka Raya, Kalimantan Tengah with a pH of <6 within the Alexander and Strete (23), respectively. The following clinical pathogenic bacteria strains (extended spectrum of ß lactamase (ESBL) producing *Escherichia coli*) were also collected from the dr. Soetomo Hospital, Surabaya: *Escherichia coli* 6024 and *Escherichia coli* 6110. Their identities were confirmed and maintenanced at 4°C till further analysis.

Isolation and identification of Streptomyces:

Isolation of *Streptomyces sp.* from peat soil composites carried out according to Alexander and Strete (23). 10 grams of peat soil put in 90mL phosphate buffer pH 7 and homogenize to obtain suspension. With a sterile 1-mL pippete, 1mL of the suspension dilute by adding 9 mL of phosphate buffer solution pH 7 (10^{-1}) to the test tube and diluting to 10^{-10}. Then, 1 mL of each dilution put into sterile petri dishes using a micropipette and then added 10mL of ISP-4 agar medium which has been thawed at 45°C. 1 ose of the colony that grows in isolation media which has the character *Streptomyces sp.* (macroscopic identification) then planted on solid ISP-4 media on petri dishes and incubated at 28°C for 4 days. *Streptomyces sp.* was transferred to the ISP-4 slant agar in order to ensure proper growth. *Streptomyces sp.* isolate stored at ISP-4 liquid containing 20% glycerol at -80°C (to maintenance bacterial morpholagic and physiology. Bacteria were grown in ISP4 medium and physiologically identified referring to Bergeys Manual Identification (24).

ESBL culture stock:

Culture of ESBL-producing *Escherichia coli* bacteria was obtained from dr. Soetomo hospital Surabaya, East Java Province. The isolate used is *Eschericia coli* 6024 and 6110 taken from the patient's urine sample. 1 ose of colony was cultured in MacConkey Agar media and incubated on 37°C for 24 hours. Furthermore, the preparation of the bacterial inoculum was tested by adding 2mL of sterile solution of phosphate buffer pH 7 to the test bacteria which had been incubated at 37°C for 24 hours, it go then shaken until the entire colonies on the surface were released and suspended in a pH phosphate buffer solution 7. Bacteria the dissolved test in pH 7 phosphate buffer was taken 2 mL and put into a sterile tube. The turbidity of the solution containing the test bacteria in the tube was adjusted to the turbidity of the standard Mc. Farland 0.5 (1.5x108 CFU / mL). After suitable turbidity, 1 mL of test bacteria in the liquid medium was put into Mueller Hinton Agar media as much as 20mL. The preliminary identification of *E. coli* isolates was done using EMB agar. Further the *E. coli isolates* were identified by microscopic, morphological

and biochemical characters referring to Bergeys Manual Identification (24).

***Streptomyces sp.* free cell fermentation broth:**

First step was made a starter by taken one ose needle of *Streptomyces sp.* colony and was planted in 8mL of ISP-4 slant medium and then incubated at 28°C for 2 days. After growth occurs, a suspension was made by taking 1 ose needle of *Streptomyces sp.* colony planted in a liquid ISP-4 medium and incubated in rotary shaker 150rpm with 28°C temperatur for 48 hours. Fermentation of *Streptomyces sp.* isolate done by removing as much as 10% of the *Streptomyces sp.* suspension. from the starter into 250mL ISP-4 liquid media then shaken using thermoshake at 150rpm at 28°C for 5 days. During fermentation, the 1st day to 5th day were taken 10mL of fermented broth and transferred to the tube and centrifuged at a speed of 5000rpm for 10 minutes. The supernatant was taken and tested for antibacterial activity compare to commercial antibiotics, Kanamycin (®Meiji).

Antibacterial test:

The selected bacteria in the sensitivity test observed antimicrobial activity using agar diffusion method. As much as 10% of the starter bacterial suspension is added to 250ml of liquid ISP-4 media. Medium is shaken using thermoshake with a speed of 150rpm at 28°C for 12 days. The supernatant from fermentation was taken as much as 10µL and put on disks that had been made in the test medium (NA 15mL medium, 1 inoculum ESBL-producing *Escherichia coli* by pour method on petri dishes, incubated on 28°C for 24 hours). Inhibition zones formed around culture colonies with calipers. As a positive control for the test bacteria, 250ppm Kanamycin was also added to the disk on NA media containing ESBL-producing *Escherichia coli*.

RESULTS:

The isolate (isolat 2.1) obtained from peat soil samples was composted to grow on ISP4 medium. The isolate was able to grow on medium with acidic pH. This acid resistance capability is in accordance with the peat environment conditions that tend to be acidic [18]. Bacteria that are able to grow in acidic environmental conditions in addition to their physiological properties are also due to their thicker wall structure than bacteria that grow at normal pH. Further the *Streptomyces sp.* isolate was identified by microscopic, morphological and biochemical characters.

This species found in Kalimantan Tengah peat soil grows well on ISP 4 medium with pH 5-6. This species has white chalk colonies, white round chain colonies cluster and turn black as time goes on and smell like typical soil, microscopically chain rod cells, Gram positive, and biochemically show oxidase (-), not motile, nitrate (+), fermentation test (-), and have tryptophan deaminase activity.

As despicted in Figure 1, free cell fermentation broth of *Streptomyces sp.* isolate showed antibacterial activities againts (ESBL) producing *Escherichia coli* 6024 and (ESBL) producing *Escherichia coli* 6110 with the highest inhibition zone diameter mean 12,50 (±0,52) mm and 13,17 (±1,19) mm. The commercial antibiotics Kanamycin (®Meiji) showed inhibitory action againts (ESBL) producing *Escherichia coli* 6024 and (ESBL) producing *Escherichia coli* 6110 with inhibition zone 10,00 (±0,60) mm and 9,97 (±0,45) mm.

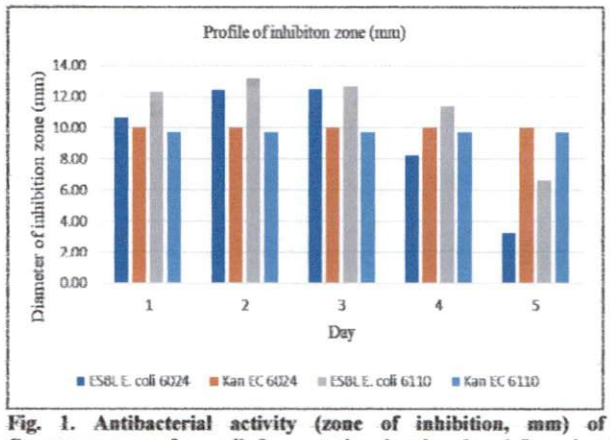

Fig. 1. Antibacterial activity (zone of inhibition, mm) of *Streptomyces sp.* free cell fermentation broth day 1-5 against (ESBL) producing *Escherichia coli* (ESBL *E. coli* 6024 and *E. coli* 6110) compared to Kanamycin

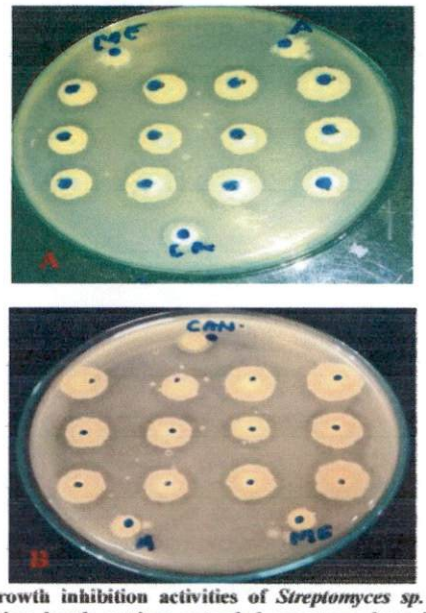

Fig 2. Growth inhibition activities of *Streptomyces sp.* free cell fermentation broth against extended spectrum beta lactamase (ESBL) producing *Escherichia coli* 6024 (A) and (ESBL) producing *Escherichia coli* 6110 (B) A: Aquadest, ME: Methanol, CAN: Kanamycin

The two types of extended spectrum beta lactamase (ESBL) producing *E. coli* strains are able to be inhibited by *Streptomyces sp.* (isolation bacteria on peatlands) free cell fermentation broth. Antibacterial activity of *Streptomyces sp. products* against ESBL producing *Escherichia coli* 6024 and 6110 starting from the first day, and increasing on day 2 and showing the peak activity on day 3 (againts against ESBL producing *Escherichia coli* 6024) and day 2 (againts against ESBL producing *Escherichia coli* 6110) with the diameter of the largest inhibition zone. Antibacterial activity began to decline on the 4th day and decreased on the 5th day (the diameter of the inhibition zone was getting smaller). Antibacterial activity of *Streptomyces sp.* products. against ESBL producing *Escherichia coli* 6024 on day 1 to day 3 higher than Kanamycin. Antibacterial activity of *Streptomyces sp.* Products. against ESBL producing *Escherichia coli* 6110 on day 1 to day 4 higher than Kanamycin.

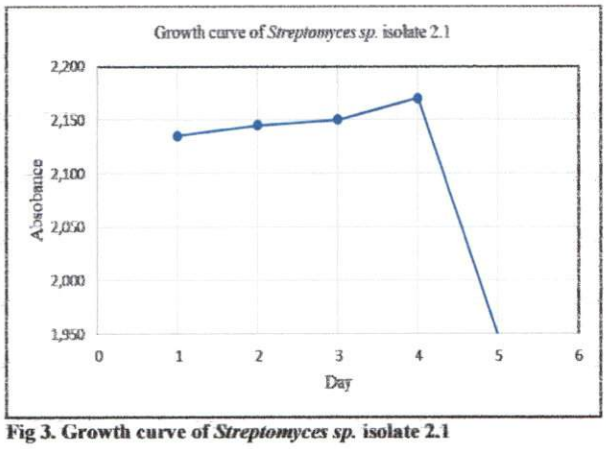

Fig 3. Growth curve of *Streptomyces sp.* isolate 2.1

Growth of bacteria began to decline the 5th day. (the diameter of the inhibition zone was getting smaller). Antibacterial activity of *Streptomyces sp.* products. against ESBL producing *Escherichia coli* 6024 and 6110 began to decline on the 4th day and decreased on the 5th day (the diameter of the inhibition zone was getting smaller). It was shown that *Streptomyces sp.* products was starting decline on the 5th day. In conclusion, Growth of bacteria assosiated with antibacterial activity, if growth bacteria was starting decline, so antibactial activity will be decrease (25).

DISSCUSION:

Peat soil generally has a relatively high level of acidity with a pH range of 3-4. Oligotropic peat, like many found in Kalimantan, has very low base cations such as Ca, Mg, K and Na, especially in thick peat. The thicker the peat, the lower bases it contains and the soil reaction becomes more acidic. Oligotropic peat which has quartz sand substratum in Palangka Raya, Kalimantan Tengah has a range of pH 3.25 - 3.75. The acidity level of peat soil is closely related to the content of organic acids (humic acid and fulvic acid). Decomposed organic matter has carboxyl and phenol reactive groups which are as weak acids. It is estimated that 85-95% of the acidity of peat soil is caused by both carboxyl and phenol groups. The acidity of peat soils tends to decrease along with the depth of the peat. The upper layers of shallow peat tend to have a higher pH than thick peat.

The isolate (isolat 2.1) obtained from peat soil samples was composted to grow on ISP4 medium. The isolate was able to grow on medium with acidic pH. This acid resistance capability is in accordance with the peat environment conditions that tend to be acidic (26). Bacteria that are able to grow in acidic environmental conditions in addition to their physiological properties are also due to their thicker wall structure than bacteria that grow at normal pH.

Extended spectrum beta-lactamases (ESBLs) are defined as certain bacteria that produce enzymes that are able to hydrolyze extended spectrum cephalosporins. Bacteria that produce this enzyme are very effective in fighting the work of β-lactam antibiotics such as ceftazidime, ceftriaxone, cefotaxime and oxyimino monobactam (27).

Streptomyces is a decomposer group bacteria. These bacteria are able to remodel litter, leaves or pieces of wood on the forest floor and turn it into humus. Therefore several methods of growing the group of actinobacter use a lot of humic vitamin medium. Streptomycetes have the capacity primarily to produce various kinds of different bioactive compounds that have broad spectrum activity (28). It was reported that 45% of known bioactive microbial metabolites are still isolated from various species of Actinomycetes and Streptomyces. A total of 7600 bioactive compounds are produced from Streptomyces (29).

Species are found from Kalimantan Tengah 's peat soil to grow well on the medium ISP 4 with pH 5-6. This species has white chalk colonies, white round chain colonies cluster and turn black as time goes on and smell like typical soil, microscopically chain stem cells, Gram positive, and biochemically show oxidase (-), not motile, nitrate (+), fermentation test (-), and have tryptophan deaminase activity.

Antibacterial activity test was carried out with isolates 2.1. Fermentation aims to produce microbial cells in large quantities so that the resulting secondary metabolites become more optimal. The fermentation process uses liquid media because fermentation with a liquid medium is more effective for producing biomass and bioactive compounds than fermentation in solid media (30).

Antibacterial activity test showed that *Streptomyces sp.* originating from the peat soil of Palangka Raya, Kalimantan Tengah is a potential species. The results of the test showed inhibition of two ESBL producing *Escherichia coli* species. Selection of ESBL producing *Escherichia coli* as a test bacterium based on the number of bacterial findings in nosocomial infections in the world (31), including in Indonesia. ESBL producing *Escherichia coli* 6024 and 6110 used in this study is a collection of the Department of Microbiology, Dr. Soetomo Surabaya. ESBL producing *Escherichia coli* 6024 was isolated from urine specimens (middle stream), female patients (52 years) and ESBL producing *Escherichia coli* 6110 were isolated from urine specimens (catheters), female patients (53 years) were proven to be resistant to beta-lactam penicillin (astreonam, amoxicillin -clavulanic acid, ampicillin, and ampicillin-sulbactam) and beta-lactam cephalosporin generation-1 (cephazolin) and generation-3 (ceftazidime, cefotaxime, and ceftriaxone). 16s rRNA examination showed that the *Escherichia coli* sample above was included in the ESBL group and one of them directed to TEM-1.

It is estimated that more than 90% of ampicillin resistance to E. coli is related to the presence of TEM-1. Majority of ESBLs are derived from the amino acid substitutions of their parent enzymes TEM and SHV ß-lactamases, but there are several other types of acquired ESBLs found in Enterobacteriaceae. Among these ESBLs, TEM, SHV and CTX-M types of are of being major clinical concern and predominated in Gram-negative bacteria (32). Resistance also occurs in mutant TEM enzymes called TEM-resistant inhibitors (IRTs), resulting in resistance to inhibitors of TEM ß-lactamase such as clavulanic acid and sulbactam (33).

The first β-lactamase enzyme was found in gram-negative bacteria, mediated by plasmids in Greece in the 1960s. This enzyme is named TEM, according to the patient's name from this enzyme-producing bacterial isolate, Temoniera (34). Then TEM-2 was found and very identical in its biochemical structure to TEM-1, differing only from one amino acid which caused a difference in the isoelectric point of these two enzymes. TEM-1 enzymes are spread throughout the world and are now found in many different species of Enterobacteriaceae, *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Neisseria gonorrhoeae* (34)(35). Amino acid substitutions responsible for the ESBL phenotype sequence around the enzyme's active location and changing configuration, allow access to the oxyimino-β-Lactum substrate. Opening the active site for the b-lactam substrate also usually increases the susceptibility of the enzyme to β-lactamase inhibitors, such as clavulanic acid. The substitution of single amino acids at positions 104, 164, 238, and 240 produces ESBL, but broad spectrum ESBLs usually have more than one amino acid substitution. Based on different combinations, there are currently around 140 types of TEM enzymes known. TEM-10, TEM-12, and TEM-26 are the most common in the United States (35).

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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