Exploration of Potential Actinomycetes from CIFOR Forest Origin as Antimicrobial, Antifungus, and Producing Extracellular Xylanase

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Abstract
This study aimed to isolate and explore the actinomycetes of CIFOR forest origin as an antimicrobial and antifungal agent, to produce an extracellular xylanase, and to identify isolates based on 16S rRNA gene sequences. Actinomycetes were isolated using Humic-acid Vitamin-B agar (HV) media. Actinomycetes colonies that grow on the medium HV was subsequently purified by growing them on yeast malt agar (YMA) media, then an antagonistic test of selected bacteria against Bacillus sp., Escherichia coli, Fusarium oxysporum, and Sclerotium sp was performed. Xylanase activity test was detected by observing a clear zone, followed by identification. Total of 35 isolates of actinomycetes isolated based on their colony morphology characteristics and diverse types of spore chains showed Streptomyces spp. of isolates CFR-06, CFR-15, CFR-17, CFR-18, and CFR-19 were able to inhibit the growth of Bacillus sp.. The highest inhibition zone has a diameter of 10.1 mm (isolate CFR-17). Isolates CFR-01 and CFR-15 were able to inhibit the growth of E. coli with the highest inhibition zone diameter of 5.1 mm (isolate CFR-15). Isolates CFR-29 and CFR-12 were able to inhibit the growth of F. oxysporum while isolate CFR-35 were able to inhibit the growth of Sclerotium sp.. Xylanase activity test showed that isolates CFR-12, CFR-20, CFR-22, CFR-24, CFR-25, CFR-30, CFR-33, CFR-34 have an ability to produce extracellular xylanase enzyme. Actinomycetes isolate (Xyl_22) as a potential xylanase enzyme producer was closely related with Streptomyces drozdowici by the maximum similarity of 99%.

Keywords:
actinomycetes; antimicrobial, antifungus; xylanase extracellular

How to Cite
INTRODUCTION

Indonesia is a country with abundant natural resources. Forest Center for International Forestry Research (CIFOR), which is located in Situ Gede, Sindang Barang, West Bogor, is one of the forests with the high richness of flora, fauna, and microbial germplasm. This forest is still well-maintained because it is protected by the firm laws and regulations. Nutrient availability found in the soil of forest area is a factor that determines the diversity of microbes in this habitat. One of the microbes which are dominantly found in nature is actinomycetes. The existence of actinomycetes in the environment is very abundant, especially in the rhizosphere because this layer provides many nutrients needed by these microbes.

Hemicellulose is a plant cell wall component apart cellulose and lignin in with its composition reaches 25-30% of the total dry weight of the wood (Perez et al., 2002). The largest component of hemicellulose in the plant cell is xylan. Xylan is the second most abundant polysaccharide in nature after cellulose (Saha, 2003; Subramaniyan and Prema, 2002). Xylan is the complex heteropolysaccharide with a backbone or basic chain composed of homopolymer units D-xylopyranose that linked by β-1,4-D-xylene (Saha, 2003; Tseng, et al., 2002). Its backbone or basic chain may also contain some substituents which later became the side chains such as O-acetyl, α-L-arabinofuranosyl, D-glucuronyl, and the residue Omethyl-D-glucuronyl (Silveira et al., 1999). Thus to effectively hydrolyze xylan into its constituent monomers, a system of enzymes that can work as synergy and have a special function was needed (Ryan et al., 2003).

Xylan hydrolysis involves two main types of enzymes i.e. endo-1,4-β-xylanase (1,4-β-xylan xylanohydrolase) and β-xylisidase (β-D-xylisida xylohydrolase) (Sunna and Antranikian, 1997; Ali et al., 2004). Endo-1,4-β-xylanase polymerizes xylan through the hydrolysis of β-1,4 to xylooligosaccharide and xylose randomly, and β-xylisidase hydrolyzes xylooligosaccharide from the non-reducing end into free xylose (Gilbert and Hazlewood, 1993). Meanwhile, the side groups that exist on the xylan are released by α-L-arabinofuranosidase, α-D-glucuronidase, acetyl xylan esterase and galactosidase (Subramaniyan and Prema, 2002).

Some groups of microbes have been found to produce xylanase enzyme, including fungi (Lin et al., 1999; Saha, 2003), bacteria (Sunna et al., 1997; Beg et al., 2001), and protozoa (Devilllard et al., 2003). One group of potential xylanase-producing bacteria is the actinomycetes, especially *Streptomyces* group (Ruiz-Arribas et al., 1995; Georis et al., 2000; Kaneko et al., 2000; Wang et al., 2007; Kansoh and Nagieb, 2004).

Actinomycetes is a Gram-positive of filamentous bacteria that can form mycelia and spores (Madigan et al, 2000). Actinomycetes can live in the soil aerobic zone, as saprophyte with a wide range of organic substrates, in freshwater and marine sediments. Some species of actinomycetes groups have an important role in decomposition of some types of polymers. The ability to degrade macromolecules is supported by the production of various extracellular enzymes. Some types of *Streptomyces* are known to produce xylanase enzyme that degrades complex lignocellulose.

Instead of producing extracellular xylanase enzyme, actinomycetes group especially *Streptomyces* was reported to have antimicrobial activity. Research conducted by Oskay et al., (2004) has been succeeded to isolate 50 isolates of actinomycetes from agricultural soil. Seventeen of the 50 isolates were able to inhibit the growth of bacteria *Erwinia amylovora*, *Pseudomonas viridiflava*, *Agrobacterium tumefaciens*, *Clavibacter michiganensis* subsp. *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC10031, *Enterococcus fecalis* ATCC 10541, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 29 998, *Sarcina lutea* ATCC 9341. Ambarwati & Trisnawati (2009) reported actinomycetes from rice field soil in Klaten that has antibacterial activity against *Staphylococcus aureus* ATCC 25923. The other potency of actinomycetes is as antifungal. Many of actinomycetes bacteria have the ability to produce antifungal. *Streptomyces*, for example, can produce the antifungal amphotericin B isolated from *Streptomyces nodosus*, *Kandidisin* from *Streptomyces griseus*, and nystatin from *Streptomyces noursei* (Aghighi et al., 2004).

Based on the background above, it is necessary to explore actinomycetes origin from the soil of Center for International Forestry Research (CIFOR) Situ Gede, Sindang Barang, West Bogor, which has potential roles as antimicrobial, antifungal, and producer of extracellular xylanase. It also needs to perform identification of selected isolates based on 16S rRNA gene sequences.

METHODS

Isolation and cultivation of soil microbes

Soil samples were used for actinomycetes resources taken from Center for International Forestry Research (CIFOR) Situ Gede, Sindang
Barang, West Bogor, Indonesia. Soil taken from the field was dried for one night at room temperature and incubated at 60 °C for 2 hours. Furthermore, 1 g of a soil sample was dissolved in 9 mL of saline (0.85% NaCl), and serial dilution up to 10⁻⁴ was performed. A total of 100 mL solutions at 10⁻⁴ and 10⁻⁵ dilution was spread in a petri dish containing agar medium that made by humic acid vitamin B (media HV) with cycloheximide (50 mg / L media), and nalidixic acid (20 mg / L media). Furthermore, it was incubated at room temperature (28 °C) for 1-2 weeks until actinomycetes colony growth can be observed well.

Purification and microscopic observation of actinomycetes

Isolated soil microbes that have grown well on media HV were purified on agar Yeast Malt Extract (YMA). Purification of soil actinomycetes was done by two repetitions. The next step was the incubation of isolates for seven days at a room temperature. After that, observation of morphological of spore chain diversity was performed. Actinomycetes isolates were placed on the objective glass with a drop of water on it, then observed at 100x and 400x magnification.

Antagonistic test of actinomycetes against Bacillus sp., E. coli, Sclerotium sp. and F. oxysporum

The direct antagonistic test was performed using the dual culture method. The first step was pouring the semisolid media with four mL E. coli and Bacillus sp. by the optimum concentration of 10⁸ cells / mL on the top of nutrient media that has been solidified. Actinomycetes colonies which have grown on YMA medium for seven days were taken by the sterile suction with a diameter of 8 mm and placed on the semisolid NA medium that has been solidified, the colony was in the upside down position. Furthermore, cultures were incubated for 24 hours at a temperature of 37 °C. A clear zone formed was observed and the inhibition value against the target bacteria was measured. The same method was carried out to test the antagonistic ability against Bacillus sp. The level of inhibition was determined from the difference between the clear zone (') and the diameter of the tested isolates ('') by the equation Δ = ') - ('', where +++ if Δ ≥ 20 mm, ++ if Δ = 10-19 mm, + if Δ = 5-9, and no inhibitory activity (-) if Δ = 5 mm (El-Tarabily et al., 2000). The streaked of actinomycetes did antibiotic test against Sclerotium sp. and F. oxysporum isolates onto four separate areas on the YMA media which has been added to nalidixic acid (1 mg / l) and incubated for four days at room temperature. Sclerotium sp. and F. oxysporum colonies were taken by sterile suction with a diameter of 5 mm, placed in the middle of the petri dish, and incubated at room temperature four days (Sclerotium sp.) and 5-7 days (F. oxysporum).

Screening and identification of actinomycetes producing extracellular xylanase enzyme

Screening of isolates producing extracellular xylanase enzyme was done by growing the actinomycetes isolates on oat spelled xylan media and incubated for four days at room temperature. After three days, colonies of bacteria that grow were recultured on plates containing xylan medium and 0.1% congo red for observing the clear zone easier. Furthermore it rinsed with NaCl 5 M. The determination of the potential xylanase-producing isolates was done by selecting colonies that formed the clear zone with the widest diameter. Colonies that have a clear zone were purified by the quadrant method and rejuvenated by streaking it on YMA agar. Selected actinomycetes isolates were further identified using 16S rRNA gene. Isolates were grown on yeast malt broth media for 48 hours and then total genomic of bacteria were isolated by special bacterial isolation kit (Genaid) following manufacturer’s instruction. Furthermore, The Genomic DNA was amplified by using specific prokaryotic primers (Marchesi et al., 1998) i.e. forward primer 63f (5’-CAG GCCCACTAAGT CATGCAAA-3’) and reverse primer 1387r (5’-GGGGGGGTAWGTCAAGGC-3’). The PCR reaction containing 0.5 mL of DNA polymerase enzyme LaTaq (long amplification Taq polymerase), 25 mL of 2x GC buffer, 8 mL of dNTP mixture, 1.5 mL of each primer (10 pmol), 9.5 mL of ddH₂O, and 4 mL of DNA template. PCR condition used was pre-denaturation (94 °C, 4 minutes), denaturation (94 °C, 45 seconds), annealing (55 °C, 1 min), elongation (72 °C, 1 min 10 sec), and post-PCR (72 °C, 7 minutes) for 30 cycles. The raw sequencing data was trimmed and assembled using ChromasPro program version 1.5. Assembling data was further analyzed using Blast from NCBI/ national center for biotechnology information. Furthermore, data was aligned using MEGA 5.0 (Tamura et al., 2011). Construction of phylogenetic tree was performed to show the affiliation of actinomycetes isolate Xyl_22 and other non-actinomycetes using Neighbor Joining method with bootstrap 1000x replications (Felsenstein, 1985).
RESULTS AND DISCUSSION

Total of 35 actinomycetes isolates origin from the soil can grow well on YMA media with the variation of colony morphology presented in Figure 1. Actinomycetes colonies mostly have a rough morphology which grown on the agar, in contrast to other microbial colonies that appear in the soft morphology on the agar media.

The diversity of 15 colonies of actinomycetes showed in Figure 1 were able to form aerial and substrate mycelia varied from white, beige, brown, gray. All of these isolates belonged to genus *Streptomyces* sp. Overall, those isolates have a widely fungi-like branching mycelia, and the spores have variation including curly, hook, and spiral (Figure 1). According to Ghadin et al., (2008), the growth of *Streptomyces* sp. on the solid media showed aerial mycelia with white, brown, and gray spores. The spore of *Streptomyces* sp. has spirales (S), recti flexibles (RF), and retinaculaperti (RA) types of chain arrangement (Shirling and Gottlieb, 1996). Based on the type of spore chain arrangement, all isolates showed *Streptomyces* spp. characteristics of RF and RA spore types.

The result of the direct antagonistic test on actinomycetes isolates to the growth of *Bacillus* sp. showed the formation of a clear zone indicating the inhibitory activity of actinomycetes isolates in the age of 7 days against *Bacillus* sp. (Figure 2). Total of 35 isolates tested against *Bacillus* sp., only five isolates showed significant inhibition based on criteria proposed by (El-Tarabily et al., 2000). From the five isolates, inhibitory activity with the largest clear zone was produced by isolates 17 with the clear zone of 10.1 mm (++) (Table 1).

The results were almost similar to direct antagonistic test of actinomycetes isolates on the growth of *E. coli*. Figure 2 showed the formation of a clear zone indicating the inhibitory activity of actinomycetes isolates with age of 7 days against *E. coli*. Total of 35 isolates were tested against *E. coli*. However, only two of them showed a significant inhibitory activity based on the criteria stated by El-Tarabily et al., (2000). Those isolates produced inhibition zone of 5.0 mm (+) to isolate CFR-01 and 5.1 mm (+) to isolate CFR-15 (Table 1).

From 35 isolates tested against *F. oxysporum* and *Sclerotium* sp., three isolates have the ability to inhibit those fungi. Both isolates CFR-29, and 12 CFR have an inhibitory activity on the growth of *F. oxysporum* while CFR-35 specifically inhibited *Sclerotium* sp. The highest inhibition zone with a diameter greater than or equal to 5 mm (+) was showed by isolate CFR-29 (Figure 2). This was proved by the ability of that isolate to inhibit the growth of *F. oxysporum* up today 21 (3 weeks) after the antagonistic test. Cao et al., (2005) reported *Streptomyces* was able to inhibit the endophytic *F. oxysporum*. Taechowisan et al., (2005) was also reported that *Streptomyces aureofaciens* CMUAc130 was able to inhibit *F. oxysporum*. Research conducted by Rochmawati et al., (2015) reported that the growth of the *Escherichia coli* was able to be inhibited by the active com-

![Figure 1](image-url). Variation of colony morphology of *Streptomyces* sp. from CIFOR forest origin after seven days of incubation at YMA media (upper figure). The type of spore chains *Streptomyces* sp. was observed by a light microscope using 400x magnification (lower figure).
pounds extracted from the scallop shells. Growth inhibition of the bacteria tested was predicted as the activity of bioactive compounds produced by actinomycetes isolates which will enter into bacteria through the cell wall, and then inhibit the process of protein, purines and nucleic acids synthesis that cause the damage to the proteins structure, denaturation of the cell wall, and eventually leading to the death of bacteria.

Besides having antibacterial and antifungal activity, previous studies showed that *Streptomyces* spp. has an ability to produce extracellular xylanase enzyme. *Streptomyces olivaceoviridis* A1 (Wang et al., 2007), *Streptomyces thermonitrificans* NTU-88 (Cheng et al., 2008), *Streptomyces* sp. S9 isolated from soil (Li et al., 2008), and *Streptomyces* sp. SWU10 isolated from rice straw (Deesukon et al., 2011), have been reported to produce xylanase enzymes that can be applied in various fields such as food, textiles, and waste handling.

The test results of extracellular xylanase activity on xylan media suggested the formation of the widely clear zone; it indicated the activity on producing extracellular xylanase enzyme by actin-
omycetes (Figure 3). From 34 isolates, six isolates have the ability to produce extracellular xylanase enzyme, they were isolated CFR-12, CFR-20, CFR-22, CFR-24, CFR-25, CFR-30, CFR-33 and CFR-34 (Table 2).

![Figure 3. Clear zone produced by CFR-22 isolates after stained by congo-red.](image)

### Table 2. The diameter of clear zone produced by some actinomycetes isolates on xylan media before stained by congo-red.

<table>
<thead>
<tr>
<th>Number of isolates</th>
<th>Diameter of isolate (r) mm</th>
<th>Diameter of clear zone (r₀) mm</th>
<th>Δr = r₀-r mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFR-12</td>
<td>10.5</td>
<td>26.0</td>
<td>15.5</td>
</tr>
<tr>
<td>CFR-20</td>
<td>11.5</td>
<td>20.75</td>
<td>9.25</td>
</tr>
<tr>
<td>CFR-22</td>
<td>14.0</td>
<td>45.5</td>
<td>31.5</td>
</tr>
<tr>
<td>CFR-24</td>
<td>9.75</td>
<td>24.75</td>
<td>15</td>
</tr>
<tr>
<td>CFR-25</td>
<td>11.5</td>
<td>18.0</td>
<td>7.5</td>
</tr>
<tr>
<td>CFR-30</td>
<td>12.75</td>
<td>41.5</td>
<td>29.25</td>
</tr>
<tr>
<td>CFR-33</td>
<td>10.75</td>
<td>28.0</td>
<td>17.25</td>
</tr>
<tr>
<td>CFR-34</td>
<td>10.25</td>
<td>28.5</td>
<td>18.25</td>
</tr>
</tbody>
</table>

CFR-22 (Xyl_22) was the isolate that has an ability to produce extracellular xylanase enzyme with the widest clear zone (clear zone). The amplification of 16S rRNA gene of isolate CFR-22 (Xyl_22) using primers 63F and 1387R with a size of ~1300 bp DNA fragment was performed. The result of the sequence alignment isolate Xyl_22 using Blast.N program showed that this isolate has similar sequences with *Streptomyces* sp, *Streptomyces drozdowiczii*, *Streptomyces setonensis*, *Streptomyces sangleiri*, *Streptomyces atratus*, and *Streptomyces sporovirgulis* with identity 99% (Table 3) and E. value 0.0 within 2289 nucleotides.

Several studies showed that *S. drozdowiczii* isolated from the soil has an important role in producing cellulose enzymes (Semedo et al., 2004). *S. drozdowiczii* showed gray aerial mycelium with a smooth surface and colorless substrate mycelia. Type of spore chains are rectiflexible (RF). This statement supported the data from this study which is showed that isolate Xyl_22 has gray aerial mycelium, colorless substrate mycelia, and reflexible (RF) type of spore chain. But further research of that species as the xylanase enzyme producer has not been widely reported, so that the data in this study are believed to be the initial data that reported *S. drozdowiczii* extracellular xylanase producer.

Analysis of phylogenetic tree with bootstrap 1000x showed that isolate Xyl_22 was closely related to *S. drozdowiczii* HBUM 175 063, with a value of 98% bootstrap. Based on phylogenetic tree analysis (Figure 4), it can be seen that isolate Xyl_22 belonged to the cluster of *Streptomyces*, and separated from *Kocuria sediminis* and *Arthrobacter* that belonged to actinomycetes group. In addition, isolate Xyl_22 was also separated from the out group species i.e. Gram-positive (*Bacillus*) and Gram-negative (*Pseudomonas* and *Agrobacterium*). It indicated that the isolate CFR 22 (Xyl_22) can be expressed as *Streptomyces*.

This research is the preliminary study to get information about potential actinomycetes isolated from protected forests CIFOR, Bogor. From this preliminary data, a clear picture about the actinomycetes isolates with their capabilities

### Table 3. Percentage of 16S rRNA gene sequence similarity of isolate Xyl_22

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species Affiliation (GenBank)</th>
<th>Accession number</th>
<th>E-value</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aktinomiset CFR-22</td>
<td><em>Streptomyces drozdowiczii</em> HBUM175063</td>
<td>FJ486467.1</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td>Xyl_22 (Xyl_22)</td>
<td><em>Streptomyces</em> sp. FZ12</td>
<td>KF803308.1</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td><em>Streptomyces setonensis</em> strain 17-1</td>
<td>EU367980.1</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td><em>Streptomyces sangleiri</em> strain NRBC 100784</td>
<td>NR041417.1</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td><em>Streptomyces</em> sp. 80134</td>
<td>AY9996829.1</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td><em>Streptomyces atratus</em> strain IHB B 8031</td>
<td>KF475815.1</td>
<td>0.0</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td><em>Streptomyces sporovirgulis</em> strain UrC05</td>
<td>KF218589.1</td>
<td>0.0</td>
<td>99</td>
</tr>
</tbody>
</table>
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Figure 4. Phylogenetic tree of isolate CFR-22 (Xyl_22) based on 16S rRNA gene. The figure showed the 16S rRNA sequence of isolate Xyl_22 closely related to the bacteria inside or outside the group. This phylogenetic tree was constructed using Neighbor Joining method with bootstrap 1000x replications.

CONCLUSION

Based on the result, it can be concluded that 35 actinomycetes isolates have been successfully isolated from CIFOR forest soil which can be antibacterial, anti-fungal, and extracellular xylanase produce. The highest inhibitory activity against bacteria *Bacillus* spp. was showed by isolate CFR-07, while isolate CFR-15 showed the highest inhibitory activity against *E. coli*. The antagonistic test showed that isolate CFR-29 has potential ability to inhibit the growth of *F. oxysporum* while isolate CFR-35 showed inhibitory activity against the growth of *Sclerotium* sp. Enzyme activity test of extracellular xylanase showed that isolate CFR-22 identified as *S. drozdowicii* has a potential role in producing extracellular xylanase enzyme that can be used in industrial applications.

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