Isolation of Lignocellulolytic Bacteria From Ereki-erek Geoforest Ijen Geopark, Indonesia

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Abstract. Ijen Geopark is one of the UNESCO Global Geoparks in Indonesia. The Ereki-erek Geofoorest (EEG) is one of the biosites in the Ijen Geopark. The EEG has an abundant biomass which is composed of lignocellulosic complex. The abundance of biomass has the potential as a source of lignocellulolytic microorganisms that can degrade lignocellulose material. The purpose of this research was to isolate lignocellulolytic bacteria from the Ereki-erek Geofoorest. This research method was soil sampling, physicochemical analysis of soil, isolation, and purification of bacteria, growth capability in lignocellulose media, and pathogenicity-test. The results showed that a total of 180 were isolated from EEG. There are 93 bacteria growth in lignin media and 142 bacteria growth in cellulolytic with the isolated code EIB (erek-erek Ijen Banyuwangi) under aerobic conditions which had various bacterial morphologies. To ensure the safety of using these isolates, it is necessary to do a pathogenicity test of these bacteria using a blood agar plate. Indicated pathogenic bacteria will form a clear zone around the bacterial colony on the blood media. The pathogenicity test of the highest lignocellulolytic bacteria showed that the 3 ligninolytic and 3 cellulolytic EIB bacteria isolates were non-pathogenic. Further, non-pathogenic bacteria from the soil litter of ereki-erek forests can be tested for their potential as biological agents for the industry.

1 Introduction

Use Ereki-erek Ijen geoforest is a primary tropical rainforest located in Licin, Banyuwangi, East Java, Indonesia. Ereki-erek geoforest is located on the eastern slope of Mount Ijen and is part of the Ijen Geoforest. This forest is classified as a primary forest because it has complete stratification with a forest floor rich in biodiversity Marchantiophyta, Bryophyta, Pteridophyta, and several grass species such as reeds [1]. The diversity of these plants shows a high level of soil fertility which indicates that the decomposition process in the soil is going well, especially lignocellulose decomposition as part of the carbon cycle in nature. Lignocellulose is a constituent component of plant cell walls, so the abundance of biomass available in Ereki-erek is positively correlated with its lignocellulose abundance. Litter decomposition in forests occurs as a form of degradation of lignocellulose components.

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Lignocellulose is composed of three main polymers, namely cellulose (35-50%), hemicellulose (20-35%) and lignin (10-25%) [2]. The structure of lignocellulose is very compact where the bond between lignin and cellulose and hemicellulose is inserted in the cellulose, making lignocellulose a very complex substrate for several enzymes. The bond between lignin with hemicellulose and cellulose plays a major role in composing plant cell walls, impermeability, and maintaining plant resistance to microbial attack and oxidative stress. Some factors that cause lignocellulose to be classified as a recalcitrant material are lignin content that protects cellulose, cellulose-hemicellulose complex, crystallinity, and high degree of polymerization of cellulose and low accessibility of cellulose due to fiber strength [3]. The fibril cellulose matrix complex is embedded in hemicellulose and lignin, which reduces the accessibility of cellulase and hemicellulase to bind to their substrate thereby reducing the degradation activity of plant biomass [4]. Therefore, it is necessary to research microbes that can degrade lignin and cellulose, both crystalline and amorphous, so that the utilization of lignocellulose waste as a carbon source can be optimal.

Bacteria are one of the microbes that play a role in lignocellulose biodegradation in forest floor litter. In the decomposition process, the advantage of bacteria compared to other microbes is a rapid growth rate and a relatively small enzyme molecule size compared to fungi so it is easier to diffuse into plant tissues [5]. Some bacteria have been reported to have lignocellulose degrading abilities including *Pseudomonas poae*, *Streptomyces thermoviolaceus*, *Klebsiella oxytoca*, *Paenibacillus polymyxa* ND25, *Pantoea ananatis* Sd-1, and *Sphingobacterium* sp. KSN-11. There are also several genera of *Bacillus*, *Paenibacillus*, *Actinobacteria*, *Pseudomonas*, *Arthrobacter*, *Pseudoxanthomonas* [6]–[11]. The research aims to characterize lignocellulolytic bacteria, especially lignin degradation or ligninolytic bacteria, and cellulose-degrading or cellulolytic bacteria from Erek-erek Ijen Geoforest, Banyuwangi, Indonesia.

## 2 Materials and Methods

### 2.1 Sampling

Soil samples (± 500 grams) were collected from Erek-erek Geoforest Banyuwangi using a soil drill. The samples underwent physicochemical analysis, including pH and temperature measurements.

### 2.2 Isolation and Screening of Lignocellulolytic Bacteria

#### 2.2.1 Isolation of Bacteria

Bacterial isolation was carried out using Nutrient Agar (NA) media at dilution results stratified to 10⁻⁷ and incubated at 30 °C for 24 hours. Bacterial colonies that have different morphologies were purified using the NA media quadrant method.

#### 2.2.2 Screening of ligninolytic bacteria

Screening of ligninolytic bacteria is carried out by growing isolates on a minimum of M9 + 0.25% alkaline lignin media. The ligninolytic activity of bacterial isolates was shown by the ability of these bacterial isolates to grow on alkaline lignin media.
2.3 Pathogenicity test

Pathogenicity tests were carried out on 5 isolates of ligninolytic bacteria that can degrade dyes and 10 isolates of cellulolytic bacteria that had the best growing ability on seulose media. The pathogenicity test was performed by inoculating each isolate on blood agar media and incubating it at room temperature for 24 hours. Isolates that can hydrolyze blood agar indicated pathogenic.

2.4 Data analysis

This study used descriptive statistical analysis. Data was displayed in the form of tables and histograms. The best results were determined by ranking values from highest to lowest.

3 Results and Discussion

The isolation of lignocellulolytic bacteria was conducted by collecting litter samples from six different locations in Erek-erek Ijen Geopark Banyuwangi. Additionally, abiotic conditions of the sample location i.e. humidity, temperature, pH, and vegetation analysis were measured (Table 1). The environmental data was evaluated for optimisation on the isolation and screening process, while the type of vegetation was evaluated for the analysis of lignocellulolytic bacterial potential. The composition of biomass, which serves as a substrate for the activity of decomposer bacteria on the forest floor litter, is affected by the type of vegetation.

### Table 1. Environmental conditions at the sampling location

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<th>Geographical Coordinates</th>
<th>Humidity (%RH)</th>
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### 3.1 Isolation and screening of ligninolytic and cellulolytic bacteria

Plant diversity shows the level of soil fertility which indicates the decomposition process in the soil is going well. One of the microorganisms that play a role in the decomposition process of plant biomass on the forest floor of Erek-erek Ijen Geopark Banyuwangi is bacteria. The results showed that a total of 180 bacteria had been successfully isolated from six sampling locations. Bacteria isolation is performed using nutrient agar media, an enrichment medium. This method allows for obtaining a higher number of culturable bacteria.

These bacterial isolates become a source of bacterial isolate to obtain potentially lignocellulose-degrading bacteria. The results of lignocellulolytic activity tests are expected...
to be additional material for culture collection of biodiversity of microorganisms. The diversity of bacteria from the Erek-erek forest floor plays an important role, especially in the process of lignocellulose decomposition as part of the carbon cycle in nature. The abundance of biomass available in Erek-erek is positively correlated with its lignocellulose content. Lignocellulose is a constituent component of plant cell walls.

Forest litter decomposition involves the degradation of lignocellulose components. To analyze lignocellulolytic degradation activity, bacteria were inoculated on alkaline lignin media for lignin degradation and on carboxymethyl cellulose (CMC) media for cellulose degradation. The initial screening involved testing the ability to grow bacteria that can degrade lignin and utilize cellulose as the sole source of carbon. In this study, a total of ninety-three bacterial isolates were positively degrading lignin based on the results of the ability-to-grow test on selective media. Additionally, a total of 142 isolates were able to utilize cellulose as their sole source of carbon. (Table 2, Table 3, and Figure 1).

**Table 2.** Screening of ligninolytic bacteria based on ability to grow on alkaline lignin media

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as their sole source of carbon. (Table 2, Table 3, and Figure 1).

Three bacterial isolates were positively degrading lignin to degrade lignin and utilize cellulose as the sole source of carbon. In this study, a total of ninety isolates were screened for lignin degradation and on carboxymethyl cellulose (CMC) media for cellulose degradation. The initial screening involved testing the ability to grow bacteria that are lignocellulolytic degradation activity, bacteria were inoculated on alkaline lignin media for lignin degradation and on carboxymethyl cellulose (CMC) media for cellulose degradation. The initial screening involved testing the ability to grow bacteria that are lignocellulolytic degradation activity, bacteria were inoculated on alkaline lignin media for lignin degradation and on carboxymethyl cellulose (CMC) media for cellulose degradation.

Lignocellulose is a constituent component of plant cell walls. Forest litter decomposition involves the degradation of lignocellulose components. To understand the diversity of bacteria from the Erek forest floor plays an important role, especially in the process of lignocellulose decomposition as part of the carbon cycle in nature. The abundance of biomass available in Erek forest floor is positively correlated with its lignocellulose content.

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Description: (-) no growth, (+) grows rarely, (++) grows well, (+++) grows very well

Table 3. Screening of cellulolytic bacteria based on ability to grow on CMC media
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Description: (-) no growth, (+) grows rarely, (++) grows well, (+++) grows very well
Bacteria degrade lignocellulose to obtain organic carbon both as an energy source and as a constituent of cellular components. Lignocellulose consists of 3 main components, namely lignin, cellulose, and hemicellulose. The three main polymers are bound in hetero matrix bonds with abundant presence varying depending on the type of biomass. Lignin, hemicellulose, and cellulose are bound to form a matrix that blocks hemicellulose and cellulose activity, where lignin is called glue by filling the gap between cellulose and hemicellulose. Microorganisms can degrade lignin to utilize lignin as a carbon source. However, some microorganisms carry out lignin degradation activity as a co-metabolism. The microorganism initially breaks down lignin to enhance the accessibility of its hydrolytic enzymes to cellulose substrates, making them both ligninolytic and cellulolytic. The study results showed that 85 bacteria had both ligninolytic and cellulolytic activity (Table 4).

**Table 4.** Bacterial growing ability of lignin and cellulose

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Table 4: Bacterial isolates and their activities.

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Description: (-) no growth, (+) grows rarely, (+++) grows well, (+++) grows very well

Pathogenicity tests were conducted on 63 isolates of ligninolytic and cellulolytic bacteria based on their hemolytic characteristics on blood agar media. This was indicated by the presence of a clear zone produced around the bacterial colony. Beta-hemolysis and alpha-hemolysis bacteria are bacteria that can lyse red blood cells on blood agar media and classified as pathogenic bacteria. Based on pathogenic tests on 63 isolates of lignocellulose-
degrading bacteria, 39 were found to be pathogenic due to the formation of a clear zone, while the remaining 24 isolates were non-pathogenic lignocellulose-degrading bacteria, as indicated by the absence of a clear zone (Table 4). These 24 bacterial isolates were classified into gamma-hemolysis types or nonpathogenic bacteria. Therefore, these 24 bacterial isolates can be developed for use as organic material decomposer agents [13], plant biocontrol agents [14], and probiotics [15]. The isolate number EIB 180 presented alpha hemolysis type i.e. isolates were not able to form clear zones, but there was a change in the blood agar medium around the colony (Table 4). This bacterial isolate was indicated as a pathogenic bacteria because it can lyse red blood cells in blood media even though it undergoes incomplete lysis [16]. The proportion of non-pathogenic lignocellulolytic bacteria was about 38.1 % or 24 (Figure 2).

**Table 5.** Analysis of hemolysis of bacterial isolates of Erek-erek Forest Ijen Geopark

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**Fig. 2.** Percentage of hemolysis type of bacterial isolates from Erek-erek Ijen Geopark
4 Conclusion

A total of 180 bacterial isolates had been isolated from the forest floor of Erek-erek Ijen Geopark Banyuwangi whereas a total of 93 bacterial isolates were ligninolytic, 142 bacterial isolates were cellulolytic and a total of 85 bacteria had both ligninolytic and cellulolytic activities or lignocellulolytic. Based on the results of the hemolysis test, a total of 3.68% of lignocellulolytic bacteria from Erek-erek forest soil litter were gamma-hemolysis which indicated the nature of non-pathogenic-bacteria.

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