Characterization of xylanolytic bacteria *Bacillus* sp. EM24 from mangrove forest sediment, Kahyapu, Enggano Island

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Abstract. Xylanases are extracellular enzyme that hydrolyze xylan into xylooligosaccharide and xylose. Group bacteria that have ability to hydrolyze xylan are called xylanolytic bacteria. This study aimed to characterize xylanase produced by *Bacillus* sp EM24 isolated from mangrove forest sediment in Kahyapu, Enggano island. The mangrove forest sediment samples were collected from Kahyapu village, Enggano island. Isolation of bacteria was done by using 0.5% xylan beechwood agar medium. The potential xylanase producing bacteria were qualitatively investigated by using 0.5% congo red solution. Colonies which produced clear zone were presumed as xylanolytic bacteria furthermore they were selected for determination of xylanase enzyme activity. The potential xylanolytic isolate was identified by Gram staining and biochemical tests. The result of isolation showed that a total number of 30 isolates were grew well on medium. Based on clear zone screening, EM24 isolate had widest clear zone. Xylanase produced by EM24 isolate had highest activity at 40th hours after incubation with the enzyme activity of 0.484 U/mL. Characterization of xylanase measurement showed that xylanase produced by EM26 isolate can optimally work at pH 6 and temperature 35 °C. Based on identification result, EM24 isolate had close relationship with *Bacillus* sp.

1. Introduction

Enggano island is one of the outer islands that located in North Bengkulu Regency, Bengkulu Province. Administratively, Enggano island has six villages, namely Kahyapu, Kaana, Malakoni, Apoho, Meok, and Banjar Sari [1]. Potentially, this island has high biodiversity of flora, fauna, germ plasma, and natural resource, one of the them is mangrove forest.

Mangrove forest in Enggano island had been conserved since a long time ago, and some of them had been produced lots of litters and sediments that consisted of polysaccharide including xylan. Xylan is the second most abundant polysaccharide in nature. It is found in larger quantities in hardwoods from angiosperms (15-30% of cell wall content) and lower abundance in softwoods from gymnosperms (7-12%). Xylans occur as a complex polysaccharide comprising a homopolymeric backbone chain of β-1,4 linked D-xylanopyranosyl units substituted based on varied degrees and become the side chains, such as O-acetyl, α-L-arabinofuranosyl, D-glucuronylpyranosyl, feruloyl and/or p-coumaroyl group. Complete hydrolysis of xylan requires a large variety of cooperative enzyme actions, such as endo-1,4-β-D-xylanases, β-D-xylosidases, α-L-arabinofuranosidases, α-D-glucuronidases, ferulic acid esterases, and p-coumaric acid esterases [2]. In addition, according to [3] xylanases show good potential applications in food, beverage, pharmaceutical, feed, and paper industries.
Xylanases can be produced by many microorganisms, including bacteria, fungi and protozoa. Bacteria is the most widely used as a source of enzymes production due to their rapidly growth, easy to grow and regulate the production, and easy to be genetically engineered [4]. The study of xylanase producing bacteria have been reported by several researchers, such as *Bacillus safensis* LBF P20 [5], *Bacillus subtilis* LBF M8 [6], and *Streptomyces drozdowici* [7]. However, the study of xylanase producing bacteria isolated from mangrove forest sediment in Bengkulu province has never been reported, thus this study aims to obtain and characterize the xylanase produced by *Bacillus* sp. isolated from mangrove forest sediment in Enggano island.

2. Methods

2.1. Sampling of mangrove forest sediment
Mangrove forest sediment collection was done by using tube (0.5 inch and 10 cm long) from Kahyapu, Enggano island. Enggano island is about 35 km long from east to west and south 26 km wide from north to south. Administratively, Enggano island is located on North Bengkulu Regency. Sampling was conducted at three different areas with 10 m distance. Samples were taken three time as replication at each area with 1 m distance.

2.2. Isolation and screening of xylanolytic bacteria
The isolation was done by making serial dilution of 1 g of sample. Then, 0.1 mL of diluted sample was inoculated to 0.5% beechwood xylan agar medium by pour plate technique and incubated at 27 oC for 48 hours. Isolates which grew on medium were identified based morphological characters and purified for further assay. Purified isolates were continued for enzymatic activity assay qualitatively by investigating clear zone formation after adding 0.5% congo red solution in the medium, followed by measurement of xylanolytic index.

2.3. Xylanase activity assay
Isolate with the highest xylanolytic index was selected for xylanolytic activity assay quantitatively. 2.5 mL of pre-grown culture was inoculated into 250 mL of 0.5% xylan broth medium containing 0.5% xylan, 0.5% peptone, 0.1% K2HPO4, 0.5% yeast extract, and 0.02% MgSO4.7H2O and incubated in shaker at 125 rpm for 48 hours at 27 oC. The culture was centrifuged at 10000 rpm for 10 minutes and supernatants were used as crude enzyme. To this solution, 0.5 mL of crude enzyme and 0.5 mL of xylan beechwood as substrate was added. Then, the mixture was incubated at 27 oC for 30 minutes. The xylanase activity was assayed by determining the concentration of reducing sugars liberated by the enzyme activity on the xylan substrate using 3,5-dinitrosalicylic acid (DNS) [8] and xylose as standard. Colour was developed by boiling at 100 oC fro 15 minutes and read by using spectrophotometer at 540 nm. One unit of enzyme activity was defined as 1 mM equivalent xylose produced per minute under given condition. Protein concentration was measured by Bradford method [9] and Bovine serum albumin (BSA) was used as the standard solution.

2.4. Characterization of pH and temperature
The optimum pH was obtained by assaying xylanase activity at different pH from 4 to 9. Optimum temperature for xylanase activity was determined by putting crude enzyme substrate mixture at the selected temperatures from 25-75 oC for 30 minutes incubation.

2.5. Identification of potential xylanolytic bacteria
Potential xylanolytic bacteria were identified by using Gram staining and biochemical test, including citrate, urease, catalase, motility, sucrose, lactose, glucose, and maltose test.
3. Result and discussion

3.1. Isolation of bacteria from mangrove forest sediment
Total of 30 bacterial isolates from mangrove forest sediment can grow well on 0.5% beechwood xylan agar medium with the variation of colony morphology presented in Figure 1.

Figure 1 shows that different morphological characters including colony surfaces, shapes, elevations, edges, and colours. Each of the isolates were named with EM 1-30, EM meant sampling location and sample name (Enggano, Mangrove), while 1-30 meant number of isolates.

3.2. Screening of xylanolytic bacteria
Screening of xylanolytic bacteria was carried out with the aim to obtain isolates that have ability to degrade xylan containing medium. The isolates that formed clear zone were presumed as xylanolytic
bacteria (Figure 2). Based on clear zones screening, there are five isolates had xylanase activities on 0.5% beechwood xylan agar with various xylanolytic index as presented in Table 1.

Table 1. The measurement of xylanolytic index from eight isolates based on ratio of clear zone diameter to colony diameter.

| Isolate code | Xylanolytic index |
|--------------|-------------------|
| EM7          | 0.76              |
| EM24*        | 1.53              |
| EM26         | 0.016             |
| EM29         | 0.9               |
| EM30         | 0.021             |

* = selected isolate

![Figure 2. Clear zone formed by eight isolates after adding 0.5% congo red.](image)

Tabel 1 and Figure 2 show EM24 isolate has highest xylanolytic index compared to the other isolates, furthermore this isolate is selected for further xylanase activity assay quantitively. Formation of clear zone around a bacterial colony indicated the presence of xylanolytic activity. According to Teather [10], congo red strongly attached to regions of polysaccharide (such as xylan) where there is β-1,4-D-glycosidic linkage, thus the hydrolysis of xylan by working of xylanase produced clear zone.

3.3. Xylanase activity assay

Xylanase activity was assayed by using DNS according to Miller [8] for 48 hours. In this work, EM24 isolate was cultured in 0.5% beechwood xylan broth medium, checking the xylanase activity in the culture for each 2 hours intervals. In the time interval of 0 until 20 hours, EM24 isolate produced a low xylanase activity as shown in Figure 3.

Based on the curve of xylanase activity, there are three peaks of xylanase activity, they are 24th, 30th, and 40th hour. The highest activity occurred at 40th hour of the incubation time. Three peaks are indicated the stationary phase of EM24 isolate growth. In that phase, growth and death rate were equal due to the lack of nutrients, furthermore induced EM24 isolate produced xylanase, then hydrolysed xylan containing medium into xylose and xylooligosaccharide [11]. On the other hand, according to Wilkinson [12], presence of more than one peak was caused by the presence of isoenzymes and several different enzymes in hydrolysing the substrate.
3.4. Characterization of pH and temperature

The effect of pH on xylanase activity was examined at various pH level ranging from 4 until 9. The result showed that optimum pH for xylanase activity was 6 (neutral condition) with the unit 0.289 U/mL as shown in Figure 4. The optimum pH level for xylanase activity in this study similar with optimum pH of xylanase from *Bacillus* sp. reported by Sunna and Antranikian [13] that maximum xylanase activity occurred at pH 6 until 10.

![Figure 4](image-url)  
*Figure 4. Effect of pH on xylanase activity produced by EM24 isolate. Measurement was done at temperature 27 °C using various pH level ranging 4 until 9.*
The effect of temperature on xylanase activity was examined at the temperature range from 25-75 °C. The result showed there are two optimum temperatures on xylanase activity produced by EM24 isolate as presented in Figure 5. These temperatures were 35 °C and 55 °C with the activity equal to 0.127 U/mL and 0.125 U/ml, respectively.

Based on the characterization result, xylanase produced by EM24 isolate work stably at temperature 35 °C and 55 °C with neutral condition. This result had similarity with study reported by Subramaniyan and Prema [14] that Bacillus SSP-34 produced xylanase with optimum pH and temperature at 6 until 8 and 50 °C, respectively.

3.5. Identification of potential xylanolytic bacteria

Potential xylanolytic isolates were continued with identification using Gram staining and biochemical tests. The result of these identification approach were presented in Figure 6 and Table 2. Based on identification by using Gram staining method, five potential xylanolytic bacteria were Gram positive shaped bacil as presented in Figure 6.

![Figure 5. Effect of temperature on xylanase activity produced by EM24 isolate at pH 5 using 0.5% beechwood xylan. Measurement was done at temperature range among 25 °C until 75 °C.](image_url)

**Figure 5.** Effect of temperature on xylanase activity produced by EM24 isolate at pH 5 using 0.5% beechwood xylan. Measurement was done at temperature range among 25 °C until 75 °C.

![Figure 6. Gram staining of five potential xylanolytic bacterial isolates.](image_url)

**Figure 6.** Gram staining of five potential xylanolytic bacterial isolates.
Table 2. Result of biochemical tests from five potential xylanolytic bacterial isolates.

| Isolate code | Biochemical tests | Genus       |
|--------------|-------------------|-------------|
|              | G    | L    | S    | M    | C    | Mot | Cit | Ur  |       |
| EM7          | +    | -    | +    | +    | +    | -   | -   | -   | Listeria |
| EM24         | +    | -    | +    | +    | +    | -   | +   | -   | Bacillus |
| EM26         | +    | -    | +    | -    | +    | +   | -   | -   | Listeria |
| EM29         | +    | -    | +    | +    | +    | +   | +   | -   | Corynebacterium |
| EM30         | +    | -    | +    | +    | +    | +   | +   | -   | Bacillus |

Note: G = Glucose, L = Lactose, S = Sucrose, M = Maltose, C = Catalase, Mot = Motility, Cit = Citrate, Ur = Urease

The results of biochemical tests showed that five potential xylanolytic bacteria had different physiological characters as shown on Table 2. EM7 and EM26 had close relationship with genus *Listeria*, EM24 and EM30 had close relationship with genus *Bacillus*, and EM29 has close relationship with genus *Corynebacterium*.

According to Barrow and Feltham [15], *Bacillus* is Gram positive bacteria and abundantly distributed on the ground. Morphological and biochemical tests revealed that *Bacillus* has rod-shaped body, anaerobic and aerobic, thermophilic, has ability to degrade organic compounds and produce multiple enzymes, such as cellulase, amylase, protease, and xylanase. The other *Bacillus* group that produce xylanase are *Bacillus circulans* and *Bacillus pumilus* PU4-2 that successfully isolated by Septiningrum K and Chandra [16] and Haryati [17], respectively.

4. Conclusion

There are 30 isolates were successfully isolated from mangrove forest sediment. Five isolates have ability to produce xylanase showed by clear zone formation on xylan agar medium which EM24 isolate had widest clear zone. EM24 isolate had close relationship with *Bacillus* sp. based on Gram staining and biochemical tests identification, which produced highest xylanase activity at pH 6 and temperature 35 °C.

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