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Isolation and Molecular Characterization of Gelatinase-Producing Bacteria from Mangrove Sediment

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ABSTRACT

Protease is an important enzyme widely produced by microorganisms applied in food, health, and industry. Mangrove ecosystem, a rich microorganism habitat, accounted as a new resource for isolating the proteolytic bacteria. The purpose of this study was to identify protease-producing bacteria from mangrove ecosystems in the Tuban area, Indonesia. Three isolates that produced the gelatinase was successfully isolated from mangrove sediments. Bacterial isolates were then tested for extracellular gelatinase. The results showed that isolate T1 had high gelatinase activity. Two isolates (isolates T2 and T3) produced moderately gelatinase enzymes. Molecular identification revealed that isolate T1 is *Enterobacter hormaechei*.

Keywords: 16S rRNA; *Enterobacter hormaechei*; molecular identification; protease; Tuban-Inland

INTRODUCTION

Enzymes are biocatalysts produced by living cells to produce specific biochemical reactions that occur in the metabolic processes of living things. Enzymes are widely used by the modern biotechnology industry to produce environmentally friendly products (Rupali, 2015; Singh *et al*., 2016). The proteolytic enzyme can be found in all living organisms, hence vital for cell growth (Clausen *et al*., 2002; Souza *et al*., 2015), division (Langer, 2000; Adan *et al*., 2016), transcription (Matsushima *et al*., 2010; Gibbs *et al*., 2014), differentiation (Lamkanfi *et al*., 2007; Guenther *et al*., 2019), synthesis, protein homeostasis (Alexopoulos *et al*., 2012), and trigger-specific signaling pathways (Turk *et al*., 2012; Penna *et al*., 2015; Salvesen *et al*., 2016).

Microbial protease is one of the three largest industrial enzyme groups and traditionally holds the dominant part of the industrial enzyme market account for around 20% of total enzyme sales worldwide and valued at $2.767 million on the pharmaceuticals industry by 2019 (Rao *et al*., 2009; Singhal *et al*., 2012; Jabalia *et al*., 2014; Razzaq *et al*., 2019). These bacteria found in mangrove sediment has an inherent capability to produce the gelatinase enzyme (Gupta *et al*., 2017; Haldar & Nazareth, 2018; Balakrishnan *et al*., 2019). They utilize organic material and total nitrogen in mangrove sediment as a metabolism energy source and enrich benefits by mangrove succession seeing as microbial-nutrient relationships (Kumar *et al*., 2007; Mendes & Tsai, 2014; Chen *et al*., 2016; Luo *et al*., 2017).

Mangrove ecosystems provide a diversity of microorganisms, as well as the ability to produce extracellular enzymes, including gelatinase as the proteolytic enzyme (Dias *et al*., 2009; Kathiresan *et al*., 2011; Thatoi *et al*., 2013), likewise in Tuban-Inland, Indonesia. Therefore, the bacteria isolated in this study were bacteria capable of producing gelatinase enzymes.

Research on the gelatinase-producing bacteria was carried out by isolating potential bacteria as a producer of proteases and analyzing their identities. Phenotype identification of bacteria has frequent errors as a significant weakness in the differentiation of species and strains (Ochman, 2005). In contrast, the molecular identification method by amplifying the bacterial 16S rRNA region can

answer the weaknesses of the phenotype identification process (Poretsky *et al*., 2014). The purpose of this study was to obtain and identify gelatinase-producing bacteria from mangrove areas in Tuban-Inland.

MATERIALS AND METHODS

Sampling in Field. Sediment samples were taken from mangrove located in Jenu Tuban Beach, Tuban-Inland using a spoon, put in plastic, and placed in a cool box, maintained at 4°C.

Screening of bacteria. One gram of sample was dissolved in 9 mL Na-Fis then mixed using a vortex. Samples were diluted at 10-3-10-5 dilutions. A total of 100 µL sample was planted in LB agar medium, then cultured for 24 hours at 35°C. Bacteria that grew on the media were purified by using the three quadrants streak method. The pure isolate was then stored temporarily at 4°C until further testing.

Proteolytic Assay. The ability of proteolytic testing is carried out using the liquefaction gelatin method (Prihanto & Nursyam, 2018).

16s rRNA Molecular Analysis. Using a method based on Prihanto *et al*. (2018), molecular identification was provided by sequencing 16s rRNA genes. DNA extraction was carried out with DNA Purification Kit Wizard following the company's standard protocol. Forward 533F primers (5' GTGCCAGCAGCCGCGGTAA-3´) and reverse primers 1492R (5'-GGTTACCTTGTTACGACTT-3´) were used for sequencing. A comparison of sequences with gene sequence databases was carried out using the BLAST program via the NCBI. Phylogeny tree analysis was describe following the method of Dereeper *et al*. (2008).

RESULT AND DISCUSSION

Isolation and Screening of gelatinase-producing bacteria. Isolation of mangrove sediment bacteria showed three isolates of the gelatinase enzyme bacteria. Each isolate was coded to be able to distinguish. The morphological characteristics of isolates can be seen in Table 1.

Table 1. The morphological characteristics of bacterial colonies from mangrove sediments

Code	Colony Shape	Elevation	Colony Colour
T1	Rounded	Flat	White
T2	Irregular	Flat	Brownish white
T3	Irregular	Flat	White

The three isolates were subsequently tested for extracellular gelatinase using the liquefaction gelatin method. This test is based on the gelatinase enzyme's ability in melting gelatin media that have been incubated at cold temperatures. The results of gelatinase activity are expressed in terms of strong, moderate, and weak.

Mangrove ecosystem is a reservoir of organic matters which lead microorganism to pool. Based on metagenomic analysis also revealed that the mangrove ecosystem is a rich source for microorganisms (Gomes *et al*., 2011; Pessoa *et al.*, 2017).

The analysis showed that isolate with T1 code had high gelatinase activity (Table 2). Whereas the other two isolates (T2 and T3 isolates) only produced a moderate amount of the gelatinase enzyme. Considering the high ability to isolate T1, only T1 was followed by analyzing the species using molecular methods.

Table 2. Gelatin liquefaction test results

Code	Enzyme Activity	Activity Result
T1	++	strong
T2	+	moderate
T3	+	moderate

Molecular Identification. The results of DNA extraction of T1 bacteria produce genomic DNA weighing of 1200 bp (Figure 2). In line with Shahimi *et al*. (2019), who successfully amplified DNA bands gelatinase candidate above 1000 bp. Furthermore, this genomic DNA was amplified using universal primers to amplify 16s rRNA. The electrophoresis results shown in the figure show that DNA bands are formed but are very thin, due to the lack of extraction of DNA concentrations. Smeared band intensity ratio and ominously low purity DNA was indicative of poor quality DNA (Aranda *et a*l., 2012; Dilhari *et al*., 2017).

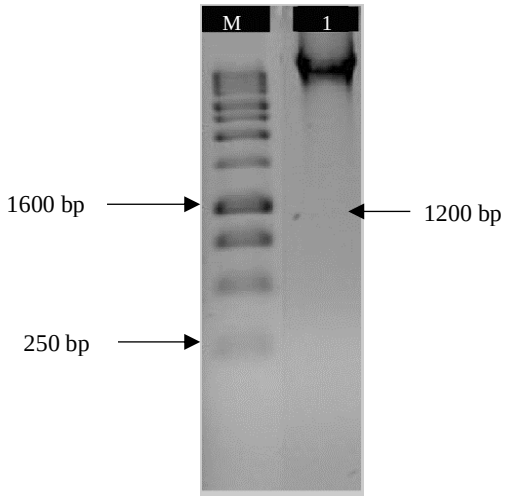

Figure 2. DNA extraction results of T1 bacterial genome:
M= molecular marker 1 kb DNA ladder; Lane 1= T1 sample

The phylogenic tree shows two main groups (Figure 3). The first group is divided into three sub-branches. The first sub-division is divided into two sub-branches filled by *Enterobacter hormaechei* strain E890 and *E. hormaechei* strain RPK2. In comparison, the second sub-branch is *E. mori* strain R3_3 to *E. hormaechei* strain 0992_77. *E. hormaechei* strain UB4 has a kinship that is not too close to other types of *Enterobacter*. The Bootstrap test is carried out by a repetition of 500-1000 times to get a high level of confidence. The bootstrap test is stated to be stable if it has a value higher than 70% (Baldauf, 2003). The meaning of the bootstrap value of 70% is *E. hormaechei* strain UB4 with *E. hormaechei* different strains that have kinship are not to close but still in kinship with *Enterobacter* sp. Therefore, isolate T1 can be identified as *Enterobacter hormaechei*.

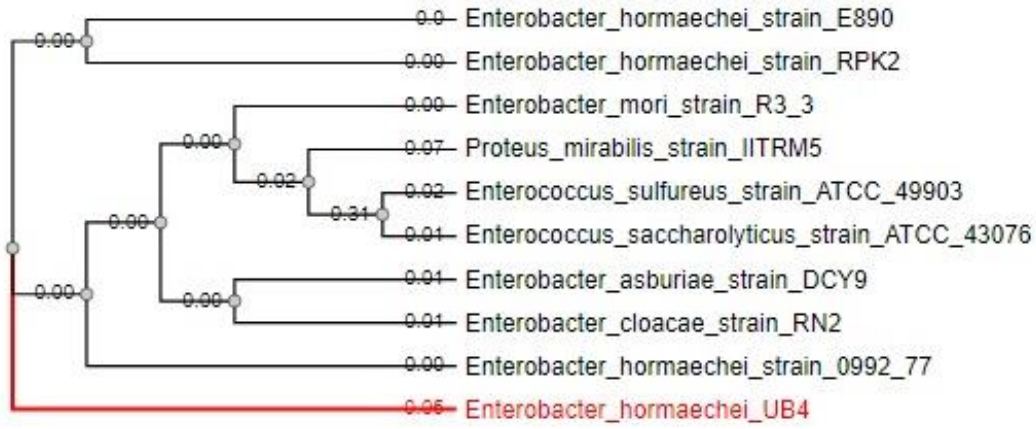

Figure 3. The phylogenetic tree represented kinship of *Enterobacter hormaechei*_UB4

CONCLUSION

Sediment mangrove from Jenu, Tuban Regency, is a source for three proteolytic isolates capable of hydrolyzing gelatin substrate. Isolate T1 produced the highest gelatinase enzyme. Based on molecular analysis, it is *Enterobacter hormaechei*.

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