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Abstract. Xylanases are hydrolytic enzymes that degraded xylan into xylooligosaccharide and xylose. Nowadays, the xylanases play a major important role in the industrial products, in the field of paper, pulp, food, beverage, pharmaceutical and animal feed. This study was to characterize and optimize xylanase produced by *Stenotrophomonas* sp. EL-8 isolated from the seagrass substrates in Enggano Island. The seagrass substrates were collected from Banjar Sari Beach, Enggano Island. Isolation of bacteria from seagrass substrates using 0.5% beechwood xylan agar medium. The isolates were screened by morphological characters. Colonies which produced clear zone were presumed as xylanolytic bacteria furthermore they were selected for determination of xylanase enzyme activity. The selected potential xylanolytic isolate was identify based on 16S rRNA. The results of this study showed that a total of 22 bacteria were isolated. Based on clear zone screening, EL-8 isolate indicated more potentially than the other 21 isolates. The crude enzyme production of EL-8 showed the highest activity at 18 hours incubation with the enzyme activity of 0.831 U/mL. This crude enzyme optimally worked at pH 5, temperature 45 ^{o}C with the enzyme activity 2.836 U/mL. EL-8 isolate has a close relationship with *Stenotrophomonas* sp. EL-8 based on 16S rRNA gene identification.
Keywords: *Enggano island, Seagrass substrates, Stenotrophomonas* sp. *EL-8, Xylanase*

1. Introduction

Seagrass beds are one of the most globally productive and dynamic ecosystems. It provides habitat and nursery grounds for many marine animals and acts as substrate stabilizer. Seagrass beds are influenced by substrate production and all biological activities of marine biota. Seagrass substrates comprise of broken leaves, root, rhizome, flowers, sand, and mud, that are composed of polysaccharides, such as cellulose, lignin, and hemicellulose, including xylan [1]. Xylan is the major structure of polysaccharides in plant cells and the second most abundant polysaccharides in nature. Xylan is in angiosperms (15-30% of cell wall content), gymnosperms (7-10%), and annual plants (<30%). Xylan is the complex heteropolysaccharides with homopolymeric backbone chain of 1,4- β-D-xylanopiranosil units, that can be substituted based on varying degrees and become the side chains,

such as O-acetyl, α-L-arabinofuranosil, D-glucuropyranosil, feruloyl and/or *p*-coumaroyl group. Complete hydrolysis of xylan requires a large variety of cooperative enzyme action, i.e. endo-1,4-β-D-xylanases, β-D-xylosidases, α-L-arabinofuranosidases, α-D-glucuronidases, ferulic acid esterases, and *p*-coumaric acid esterases due to the heterogeneity and complexity characteristic [2].

In addition, many microorganisms can produce multiple xylanases [2]. Some groups of microbes have been found to produce xylanases, including fungi, bacteria [3], and protozoa [4]. Xylanases show a good potential application in food, feed, pharmaceutical and paper industries [5].

Enggano island is one of the regions in Indonesia that has a high diversity of flora, fauna, and germ plasma. The island is still well-maintained because it has not been exposed to human exploitation. One of the biodiversity objects in Enggano island is preserved seagrass ecosystem. This condition makes the seagrass ecosystem has the potential to provide habitat for xylanase-producing microorganism. In addition, a study of potential xylanolytic bacteria in Enggano island has never been done and published. Thus, this study aimed to isolate and characterize xylanase produced by *Stenotrophomonas* sp. EL-8 isolated from seagrass substrates in Enggano Island.

2. Methods

2.1. Seagrass substrate collection

Seagrass substrates were collected about 100 g from Banjar Sari Beach, Enggano Island. Enggano is about 35 km (22 miles) long from east to west and about 16 km (9.9 miles) wide from north to south. Potentially, it is subdistrict of North Bengkulu Regency of Bengkulu province. Collection of samples was done at three different areas with 100 m distance. Substrates were taken three times as replications in each area with 10 m distance. Then, the collection was brought to the laboratory.

2.2. Isolation, purification, and screening of xylanase producing bacteria

The isolation of bacteria from seagrass substrates was done by making serial dilution until 10^{-3}. The diluted substrate was taken 0.1 ml and inoculated to 0.5% beechwood xylan agar medium by spread plate technique, and then incubated at 27 ^{o}C for 48 hours. Isolates grew well on xylan media were screened based on different morphological characters and purified to be prepared for the next stage.

Screening of xylanase producing isolates was done by using 0.5% congo red staining for 15 minutes. Isolates were then rinsed with NaCl 1 M. Potential xylanase producing isolates were determined by selecting colonies that formed the widest diameter of the clear zone.

2.3. Enzymatic activity assay

The isolate, which had the widest clear zone was selected for further assay. The isolate was inoculated as much as 1% inoculum into the fermentation medium (0.5% of xylan, 0.5% of peptone, 0.1% of K_2HPO_4, 0.5% of yeast extract, and 0.02% of $MgSO_4.7H_2O$) and incubated in a shaker at 160 rpm for 48 hours at 27 ^{o}C. Culture medium was centrifuged at 10000 rpm for 10 minutes, then supernatants were used as a crude enzyme. The xylanase activity was measured by determining the concentration of reducing sugars liberated by the enzyme activity on the xylan substrate using dinitrosalicylic acid (DNS) [6].

Specific xylanase activity was obtained by measuring protein concentration using Bradford method [7]. Bovine serum albumin (BSA) was used as the standard solution stock.

2.4. Characterization of pH and temperature parameters for xylanase activity

The optimum pH was found by monitoring xylanase activity at pH values between 4-9 using citrate buffer (0.05 M, pH 4), phosphate buffer (0.05 M, pH 7), and citrate-phosphate buffer (0.05 M, pH 5, 6, 8 and 9). The optimum temperature for xylanase activity was examined by putting crude enzyme-substrate mixture at the selected temperatures from 25-75 ^{o}C for 30 minutes incubation.

2.5. 16S rRNA gene identification

The selected isolate was further identified using 16S rRNA gene. Total genomic isolation of selected isolate was done using a special bacterial isolation kit (*Genaid*) based on the manufacture instruction. The 16S rRNA gene of genomic DNA was amplified by using specific prokaryotic primers [8], i.e. forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC-3'). The PCR composition of the PCR consisted of 25 µL 2x GotaqGreen Buffer, 2 µL forward primer (10 pmol), 2 µL revere primer (10 pmol), 4 µl DNA template, and 17 µL Nuclease free water. PCR process was done by pre-denaturation (94ºC, 4 minutes), denaturation (94 ºC, 45 seconds), annealing (55 ºC, 1 minute), elongation (72ºC, 1 minute 10 seconds), and post PCR (72 ºC, 7 minutes) with 30 cycles. Sequencing analysis result was determined using *ChromasPro ver. 1.5* program and compared with genomic data that has been registered to NCBI using BLASTn (http://www.ncbi.nlm. nih.gov/BLASTn). Some sequential data from BLASTn results which has the closest species and the strain type species was inserted in Gene Bank data at NCBI. Data were re-analyzed by aligning the sequence using MEGA 6.1 program [9]. The phylogenetic tree of selected isolate was constructed to show the relationship of isolates with other species using *Neighbor-Joining Tree* method with 1000x bootstrap replication.

3. Result and discussion

3.1. Isolation and purification of bacteria from seagrass substrates

Collected seagrass substrates were used in the dilution method. Bacterial isolates grew on the media were identified based on morphological characters. Based on the identification of morphological characters, twenty-two bacterial isolates grew well on 0.5% beechwood xylan agar after 48 hours incubation with varied colony morphology (figure 1). Each isolate named EL, which meant as sampling location (Enggano) and sample name in Bahasa (Lamun). Furthermore, twenty-two isolates were purified for the next stage.

3.2. Screening of bacteria producing xylanase

The test results of xylanase activity on xylan media suggests the formation of wide clear zone indicating the activity of xylanase production by bacterial isolates (figure 2). Ten isolates from twenty-two isolates could produce xylanase, i.e. EL-1, EL-3, EL-4, EL-7, EL-8, EL-10, EL-12, EL-14, EL-15 and EL-19 (table 1). Based on clear zones screening, EL-8 isolate form the widest clear zone. Therefore, EL-8 isolate was selected for further assay by dinitrosalicylic acid (DNS) and identified based on the 16S rRNA gene.

3.3. Enzymatic activity assay

Xylanase from EL-8 showed a maximum peak of xylanase activity at 18 hours of incubation time that indicated the early stationary phase with 0.831 U/mL xylanase activity (figure 3). The stationary phase began at 16 hours and ended at 48 hours of incubation time, which growth and death rate were equal. This could be caused by the lack of nutrients. This condition induced EL-8 isolate to synthesize xylanase, thus capable of degrading xylan into xylose and xylooligosaccharide [10].

3.4. Characterization of pH and temperature for xylanase activity

The characterization of pH on xylanase activity produced by EL-8 was investigated at various pH level ranging from 4 to 9 as shown in figure 4. The measurement results showed that optimum pH for xylanase activity was 5 with the unit was 2.621 U/mL.

The other peak of xylanase activities were found at pH 6 and 7 with the units were 2.583 U/mL and 2.589 U/mL, respectively. Characterization results of pH on xylanase activity showed that xylanase produced by EL-8 was active at a wide range of pH from 5-7 (acidic to neutral condition). Different pH observation on xylanase production may indicate that the organism can induce multiple xylanases at different pH [11]. The optimum pH level for xylanase activity in this study was similar to the

optimum pH of xylanase from other marine bacteria, such as *Halomonas meridian* NBRC 15608 [12], *Bacillus safensis* P20 [13], and *Acinetobacter baumannii* [14], with optimum pH at 6, 7 and 8, respectively.

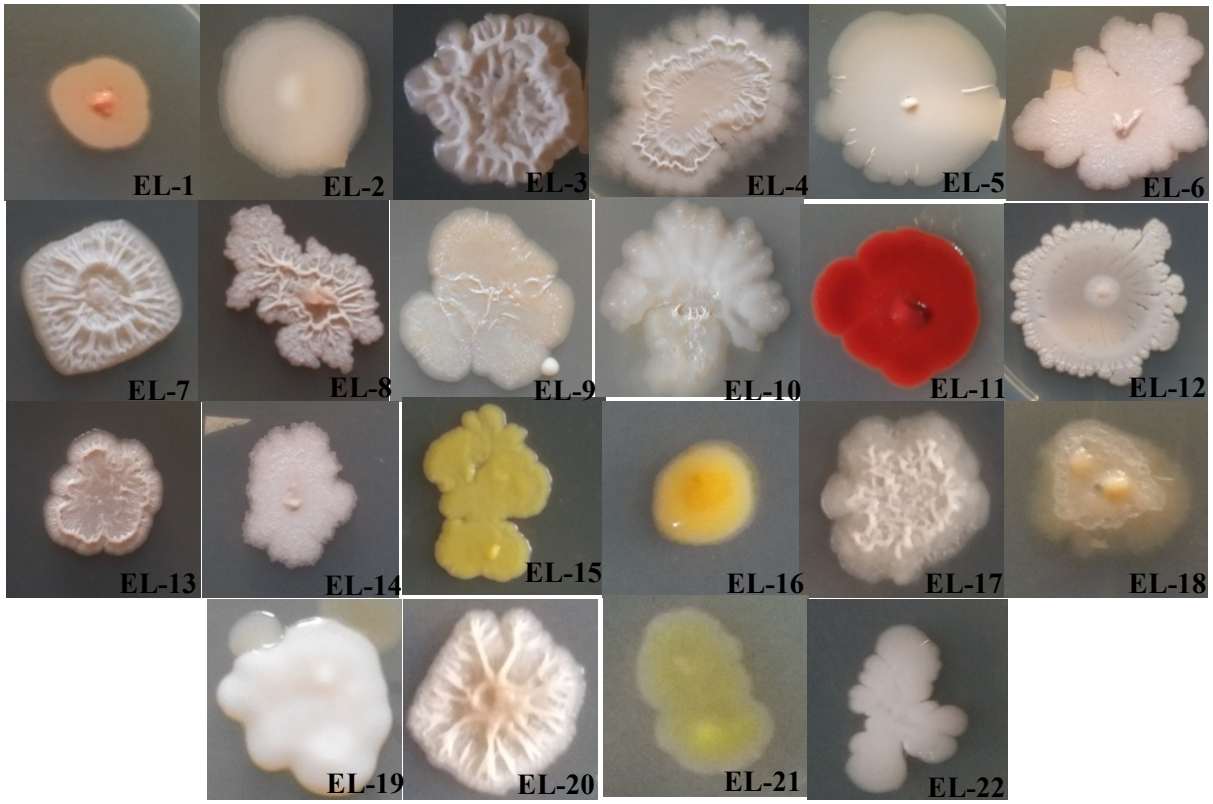

Figure 1. Morphology of bacterial colonies isolated from seagrass substrates after incubation for 48 hours at 27 ^{o}C.

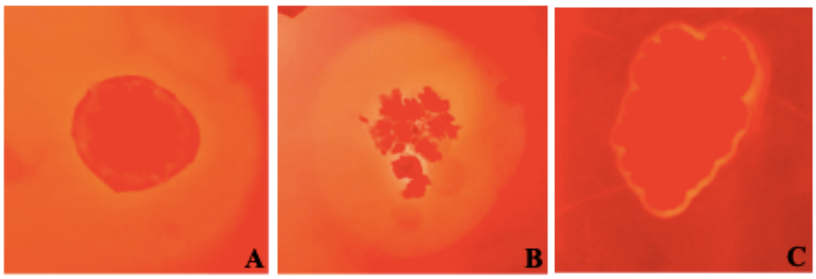

Figure 2. Clear zone produced by EL- 4 (A), EL-8 (B) and EL-12 (C) after staining with congo-red 0.5% solution on 0.5% beechwood xylan agar after incubation for 48 hours at 37 ^{o}C.

Temperature effect on xylanase activity was examined at the temperature range among 25-75 ^{o}C (figure 5). Temperature can influence rate of enzyme reaction. The higher temperature will lead to higher enzyme activity as shown when enzyme activity increased at 35 ^{o}C compared to 25 ^{o}C. However, this activity will be limited by the thermal denaturation as the enzyme is composed of protein. Xylanase produced by EL-8 showed maximum activity at 45 ^{o}C with the acidic condition. Xylanase from EL-8 is classified as a thermoenzymes or thermophilic enzyme because it can produce optimum xylanase at the temperature range of 45-85 ^{o}C [15].

Table 1. The diameter of the clear zone produced by ten isolates on 0.5% beechwood xylan media congo-red staining.

Isolates	The diameter of the clear zone with isolate (a) mm	The diameter of isolate (b) mm	Diameter of clear zone (c)= a-b mm	Xylanolitic index (IX)= c/b
EL-1	10	8	2	0.25
EL-3	23	15	8	0.53
EL-4	20	10	10	1.00
EL-7	15	7	8	1.14
EL-8*	20	7	13	1.86
EL-10	10	7	3	0.42
EL-12	13	11	2	0.18
EL-14	18	10	8	0.8
EL-15	14	10	4	0.4
EL-19	19	15	4	0.27

*: Selected isolate

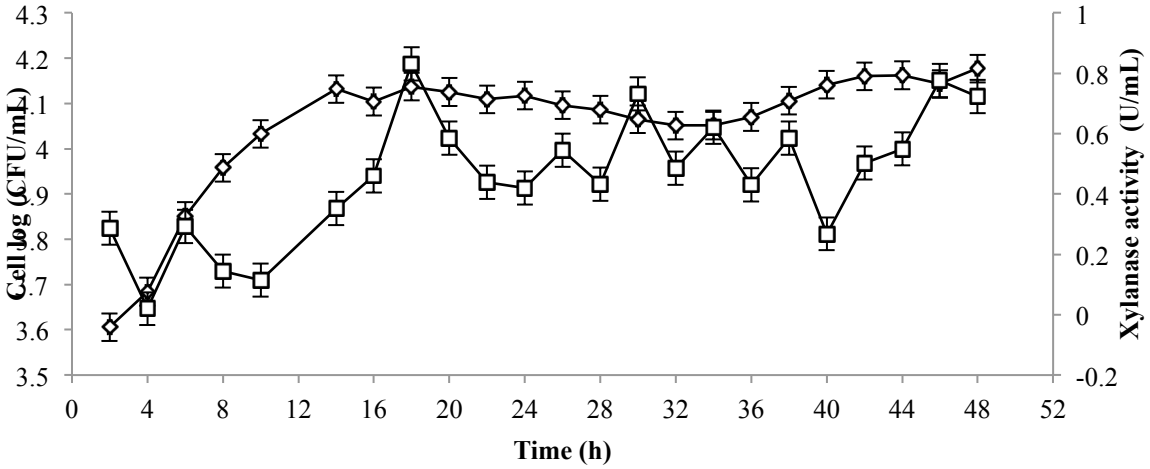

Figure 3. Growth phase and xylanase activity wereere produced by EL-8 isolate using 0.5% xylan broth-medium for 48 hours. —◇— Cell log (CFU/mL), —□— Xylanase activity (U/mL).

3.5. Identification of 16S rRNA gene

The amplification of 16S rRNA gene from EL-8 was done using 63f and 1387r primer. The amplification result indicated ~1300 bp DNA fragment size was performed (figure 6). The results of sequence alignment using BLASTn program showed that EL-8 isolate has similar sequences with *Stenotrophomonas* sp. HPC1294, *Stenotrophomonas rhizophila* strain USBA 843H, *Microvirga* sp. NCCP-1162, *Bradyrhizobium* sp. TUTMa33 and *Methylobacterium suomiense* TNAU4 as shown in Table 2. BLASTn analysis resulted EL-8 isolate had 16S rRNA gene sequence which was homologous with *Stenotrophomonas* sp. HPC1294 within 80.91% similarity. EL-8 isolate was closest to *Stenotrophomonas* sp. HPC1294, as *Stenotrophomonas* sp. HPC1294 was located at the earliest position when analyzed. Furthermore, the construction of phylogenetic tree that shows the kinship level of isolates with other bacterial species using *Neighborhood Joining Tree* method with 1000x bootstrap replication value is presented in figure 7, where EL-8 isolate has the closest relationship with *Stenotrophomonas* sp. HPC1294.

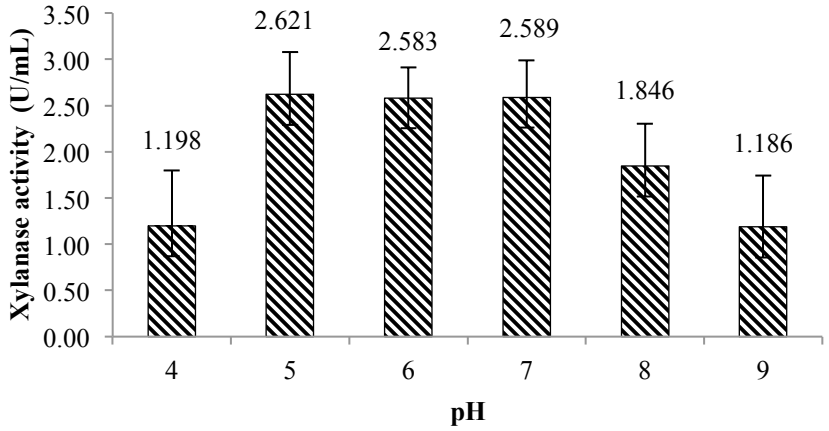

Figure 4. pH effect on xylanase activity produced by EL-8 isolate. Measurement was done at temperature 27 ^{o}C using various buffer solution at pH 4 until 9 using 0.5% beechwood xylan.

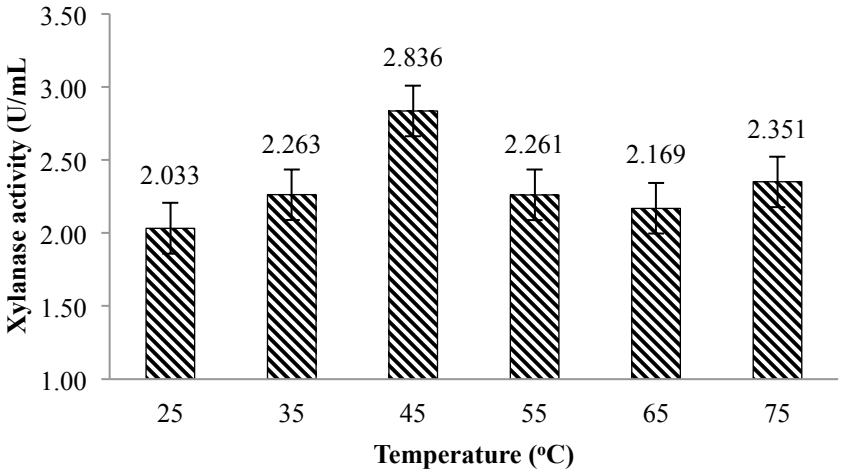

Figure 5. Temperature effect on xylanase activity produced by EL-8 isolate at pH 5 using 0.5% beechwood xylan. Measurement was done at temperature range among 25 ^{o}C until 75 ^{o}C.

Studies of xylanase produced by *Stenotrophomonas* sp. are still limited to explore. Xylanase produced by *Stenotrophomonas* sp. has been published by [16], isolated from a saw-dust dump, which produced crude xylanase enzyme in the range of 10.4-23.21 IU/mL. Morphological and biochemical assays showed that *Stenotrophomonas* sp. has a rod-shaped body, Gram-negative or Gram variable [17], motile, catalase-positive and oxidase-positive with optimum temperature and pH are 35 ^{o}C and 8, respectively. The similar study of xylanase producing microorganism has been published by Sipriyadi *et al*. [18], it showed that actinomycetes (CFR-22) isolate had the ability to produce xylanase. Based on identification 16S rRNA gene, CFR-22 isolate had similar sequences with *Streptomyces drozdowiczii*.

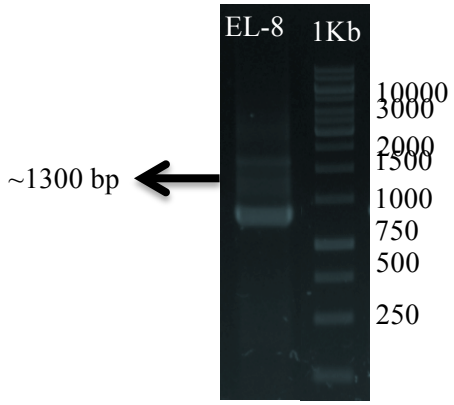

Figure 6. Result of 1% agarose gel electrophoresis visualization of EL-8 isolate with 1 kb ladder marker.

Table 2. BLASTn result sequences of 16S rRNA gene EL-8 isolate compared to other bacterial species at Gene Bank

Isolate	Species Affiliation (Gene Bank)	Similarity (%)	Accession Number
EL-8 isolate	*Stenotrophomonas* sp. HPC1294	80.91%	DQ299189.1
	Stenotrophomonas rhizophila strain USBA 843H	80.84%	LT627107.1
	Microvirga sp. NCCP-1162	78.47%	LC065189.1
	Bradyrhizobium sp. strain TUTMa33	75.15%	MK611719.1
	Methylobacterium suomiense strain TNAU4	74.63%	EF116584.1

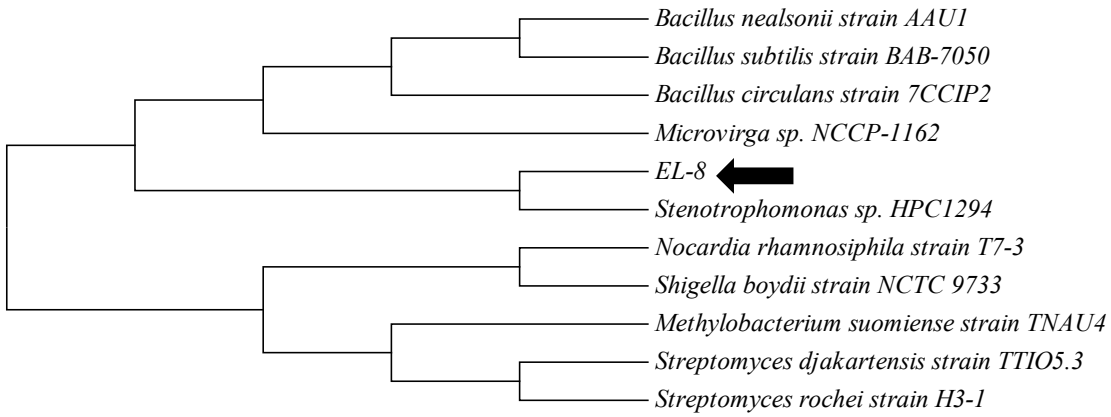

Figure 7. The phylogenetic tree that describes the closeness of EL-8 isolate to other bacteria in one clade and other clades (outer group). Construction is based on the *Neighbor-Joining Tree* method with 1000x bootstrap replication value

4. Conclusion

Twenty-two bacterial isolates were successfully isolated from seagrass substrates with ten isolates of them potentially produced xylanase. EL-8 isolate was the most potential isolate on producing xylanase based on clear zone screening. The identification results of 16S rRNA gene from EL-8 isolate showed that EL-8 isolate had close relationship with *Stenotrophomonas* sp., which produced maximum xylanase activity at pH 5 and 45^{o}C with the unit 2.836 U/mL.

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