Isolation of Cellulolytic Bacteria from Mangrove Sediment in Dumai Marine Station Riau and the Antibacterial Activity against Pathogens

N Nursyirwani1*, F Feliatra1, A Tanjung1 and F Harjun2
1 Lecturer at the Marine Science Department, Faculty of Fisheries and Marine Science, Universitas Riau
2 Postgraduate Student at the Marine Science Study Program, Faculty of Fishery and Marine Science, Universitas Riau
*Corresponding Author: nursyirwani_adnan@yahoo.com

Abstract. Cellulolytic bacteria are a group of bacteria that have an ability to degrade material containing cellulose and have potency to inhibit the growth of pathogenic bacteria. The bacteria can be found in soil or sediment in the mangrove ecosystem. This research aimed to: 1) isolate cellulolytic bacteria from mangrove sediments in Dumai Marine Station, of Riau, Indonesia, 2) to examine the antagonism against pathogenic bacteria (Escherichia coli, Pseudomonas aeruginosa, Vibrio alginolyticus), and 3) to identify and the phenotype and genetic characters of the potential isolates. The bacteria were isolated on Zobell Marine Agar 2216 added with carboxymethyl cellulose (CMC). Cellulolytic index value was obtained by reducing clear zone diameter with the bacterial colony diameter. Disc diffusion agar method was used to examine antagonism of selected isolates against pathogens. The isolates were observed for the colony and cell morphology, biochemical and genetically characters. Twenty four isolates showed cellulolytic activity and index values ranged from 1.00 to 2.86 and 0.01 to 2.12, respectively. Nine of selected isolates performed ranges of zone inhibition against E. coli, P. aeruginosa and V. alginolyticus from 2.38 ± 0.21 mm to 3.58 ± 0.83 mm, 2.75 ± 0.59 mm - 4.81 ± 0.57 mm and 2.28 ± 0.45 mm - 4.68 ± 1.40 mm, respectively. Based on the 16S rDNA sequence analysis, three cellulolytic isolates indicated similarity to Bacillus toyonensis (99.53 %).

Keywords: Cellulolytic bacteria, mangrove, sediment, antagonism, pathogen, 16S rDNA

1. Introduction
Mangrove plant, grows in coastal region, contains complete organic compounds such as starch and cellulose which are composed mainly in the leaves and stems. Cellulose is the major component of plant biomass [1]. Mangrove leaf litter in addition to metabolic waste of organism and input from inland will be accumulated di the sediment of mangrove areas. These sources of organic matter would be degraded by microorganism such as of fungi and bacteria to produce detritus. Detritus is rich in enzymes, protein and contains large microbial population [2], and detritus from mangrove constitutes a large reservoir of carbon and energy potentially available to the estuarine food web. Bacteria in mangrove ecosystem actively take part in bio-mineralization and biotransformation of minerals [3]
Among the bacterial types, cellulolytic bacteria, a group of cellulose-degrading bacteria is part of bacteria participate in the carbon, sulphur, nitrogen and phosphorous cycles in mangrove forest [4].

Cellulose degrading bacteria have been isolated and characterized for obtaining more effective cellulases from variety sources such as soil [5], kitchen waste [6], municipal waste [7], marine environment [8] and gastrointestinal tract of marine fishes [9]. Cellulase enzyme produced by these microorganism has diversity application in industry. The major industrial application of cellulose are bio-polishing, bio-stoning, bio-finishing, etc. in textile industry, starch processing, grain alcohol fermentation, malting and brewing in beer and wine industry, extraction and processing of fruit and vegetable juices, etc. in food industry, in pulp and paper industry, house hold laundry detergents for improving fabrics softness and brightness [10, 11], controlling plant pathogen and disease in agriculture as well as bacterial and fungal pathogens [6, 7].

Some previous researches had found cellulolytic bacteria and their activities against pathogens. In mangrove sediment of Ilha do Cardos Brazil cellulolytic bacteria had been identified and grouped into classes of Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, Firmicutes or Bacteroidetes [12]. From mangrove soil of Mahanadi River Delta in Odhisa, India, cellulolytic bacteria were identified as Micrococcus spp., Bacillus spp., Pseudomonas spp., Xanthomonas spp. and Brucella spp. [13]. Meanwhile, Bacillus cereus JD0404 was found in mud of mangrove forest in Rayong River, Thailand [14]. Four species of cellulolytic bacteria were isolated from the decayed mangrove stems in the Waai Seashore of Ambon Island, Indonesia, namely Micrococcus luteus, Bacillus pumilus, Planococcus mycoides and B. cereus [15].

Many research on cellulolytic bacteria from mangrove ecosystem in Indonesia and the activity had been done. From mangrove area in Mangrove area of Peniti West Kalimantan, cellulolytic bacteria had been identified as Pseudomonas, Plesiomonas, Pasturella, Neisseria, Actinobacillus, Corynebacterium, Aeromonas, Vibrio, Bacillus, Alcaligenes, Actinobacillus, Listeria and Chromobacterium [16, 17]. Four cellulolytic bacterium from the mangrove soil of Gunung Anyar River Estuary in Surabaya were identified as Bacillus subtilis, Pseudomonas diminuta, Micrococcus luteus and Pseudomonas shigelloides, however, only B. subtilis and P. diminuta were able to inhibit the growth of Edwardsiella tarda [18]. Four and nine isolates each from Sungai Liat and Tukak Sadai, Bangka Belitung had been isolated and identified as Bacillus pumilus, Pseudomonas sp., B. amyloliquefacien, B. alvei dan B. coagulant were identified, and the most active produced cellulase indicated by Pseudomonas aeruginosa [19]. However, there was not data on the present of cellulolytic bacteria from mangrove area in Dumai Marine Station of Riau Province. Therefore, this research aimed to isolate, identify and evaluate the antagonism of selected isolates against bacterial pathogens (Escherichia coli, Pseudomonas aeruginosa and Vibrio alginolyticus).

2. Materials and Methods

This survey research was conducted from March until July 2019. Isolation of cellulolytic bacteria and antagonism test against pathogen were performed in the Marine Microbiology Laboratory of the Faculty of Fishery and Marine, and isolation of the bacterial isolates was conducted in the Genetic Laboratory of Biology Department at the Faculty of Math and Natural Science in Universitas Riau. Sequent of 16S rRNA of the bacterial isolates was analyzed by PT. Genetika Science Indonesia in conjuction with the First Base Malaysia.

2.1. Sampling location

Surface sediment samples were collected from three sampling points around the mangrove area of Dumai Marine Station. Sampling point 1, 2 and 3 and the geographical positions of each were in the mangrove area (N : 01°42’ 50.57”; E : 101°23’ 19.17”), at the ship house within the mangrove area (N : 01°42’ 54.39”; E : 101°23’ 15.31”) and mangrove area in the estuary of Masjid River (N : 01°43’ 11.03”; E : 101°23’ 14.05”). The sediment samples were collected using stirile scoop, and ±500 gram of the sediment was put in sterile plastic bags of 1 kg capacity. All of the sample bags were kept in an ice box during transportation to the laboratory.
2.2. **Bacterial isolation and identification**

Ten grams of each sediment samples was suspended with sterile seawater of 20 ppt in an erlenmeyer flask up to a volume of 100 mL. The suspension was prepared for a dilution series from $10^{-1}$ until $10^{-3}$ in test tube containing 9 mL of seawater. One mL of each dilution was inoculated on Zobell Agar medium added with 1% of carboxymethyl cellulose (CMC). All inoculated dishes were incubated at 30°C for 48 hours. Grown colonies were then identified based on morphological characters, these were the colour, shape and size. Different colonies were reinoculated and purified on the same fresh medium to get single-cell colonies.

2.3. **Activity of enzyme cellulase**

Selected isolates of cellulolytic bacteria were tested for the activity of enzyme produced. The isolates were grown on fresh Zobell agar + CMC, then flooded with congo red of 0.1%. Clear zone (a) observed around the grown colonies (b) was measured (Figure 1). Enzymatic activity was measured from the ratio of clear zone diameter over the colony zone diameter. While, the cellulolytic activity index = (diameter of clear zone – diameter of colony) / diameter of clear zone.

![Figure 1. Colony of cellulolytic bacteria indicating clear zone surrounding](image)

2.4. **Antagonism test**

Activity of cellulolytic bacterial isolates against pathogenic bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Vibrio alginolyticus*) was examined by the diffusion agar method following the procedures of Wolf and Gibbons [20]. A volume of 25 µL of each of the pathogen suspension in nutrient broth (NB) was inoculated and spread onto Mueller Hinton Agar (MHA, Difco) medium. Meanwhile, 50 µL of suspension of each cellulolytic bacterial isolate was dropped on a sterile paper disc. Chloramphenicol of 250 mg solution and NB were used as positive and negative controls, respectively. All triplicate treatments were incubated at 30°C for 24 hours. Antagonism activity of the cellulolytic bacteria was indicated by inhibition zones around the paper disc. Diameter of the inhibition zones were measured by using a caliper.

2.5. **Bacterial identification based on 16S rDNA**

Genotype characters of three cellulolytic bacterial isolates were analyzed based on molecular characterization of 16S rDNA gene sequence. Bacterial DNA was extracted following the procedures
of Geneaid® Gel/PCR DNA Fragment Extraction Kit. Genome of bacterial 16S rDNA was amplified with polymerase chain reaction (PCR) using thermal cycler (Eppendorf, Master cycler Personal). DNA of the isolates was extracted followed by amplification using polymerase chain reaction (PCR). Amplification was run by using two universal primers: 24F (5'-AGAGTTTGATCCTGGCT-3') and 1541R (5'-AAG GAGGTGATCCTGGCT-3') at the following condition: pre-denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds; annealing at 50°C for 45 seconds; extension at 72°C for 90 seconds; final extension at 72°C for 10 minutes. The PCR products were detected from electrophoresis in 0.5 % agarose agar. Purified PCR products were sequenced in First Base Malaysia and then were sequenced. The sequenced products were analyzed by using the BLAST (Basic Local Alignment Search Tool) at the NCBI GenBank. Phylogenetic tree was constructed by using Mega 06 and Bio-edit program.

2.6. Data analysis
Data of cellulolytic bacterial isolates, cellulose activity, antagonism against pathogens and genetic analysis of selected isolates were presented in tables and figures. The data were then analyzed descriptively and compared to previous related and similar researches.

3. Results and Discussion

3.1. Cellulolytic activity of bacteria
Twenty four bacterial isolates had the ability to grow on medium containing carboxymethyl cellulose (CMC), a polysaccharide functioning as cellulose indicator. Morphological characters of the isolates showed that all isolates have round with smooth and elevated colonies. Some of the colonies were white, white to reddish and white yellowish in colour. Bacteria producing extracellular enzyme cellulase was indicated by the formation of clearing zone around the colonies flooded with Congo red on the agar plates. The average of the bacterial cellulolytic indexes was presented in Table 1.

Data in Table 2 shows that three isolates, each of three sampling points indicating high cellulolytic activity and index. The highest cellulolytic activity and index was performed by isolate S.St2.8 (2.12 ± 1.04). The ratio values indicated the ability of enzyme cellulose produced by the isolates to use and degrade cellulose-containing CMC. Cellulose consists mainly of long polymers of β-1-4, linked glucose units and forms a crystalline structure [21]. Cellulase enzyme, which can hydrolyze cellulose forming glucose and other commodity chemicals, can be divided into three types: endoglucanase (endo-1, 4-β-D-glucanase); cellobiohydrolase or exoglucanase (exo-1, 4-β-D-glucanase) and β-glucosidase (1,4-β-D-glucosidase) [22, 23]. However, cellulolytic activity obtained in current research was lower than that found formerly [7].

Nine cellulolytic isolates indicating high cellulolytic activity and index were selected for further antagonism test against pathogenic bacteria. Cell morphology and biochemical characters of the isolates were as presented in Table 2. All isolates were Gram positive and bacil bacteria, only one isolate was in-motile. All isolates did not produced indole but catalase was produced. Only one isolate used citrate as carbon source for nutrition, and two isolates produced H2S from amino acid in the growth medium. All isolates were able to produced acid on the use of triple sugar iron agar (TSA).

3.2. Antagonism to pathogens
The ability of nine cellulolytic bacteria to inhibit the growth of pathogenic bacteria (E. coli, P. aeruginosa and V. alginolyticus) was different among the bacteria (Table 3). The highest inhibition against E. coli, P. aeruginosa and V. alginolyticus was indicated by isolates S.St2.3 (3.58 ± 0.83 mm), S.St3.1 (4.81 ± 0.57 mm) and S.St2.8 (4.68 ± 1.40 mm), respectively. From the highest inhibition zone value and as seen in Table 3, it is shown that isolate S.St3.1 was the most active in inhibiting the growth of pathogens, particularly against P. aeruginosa. This finding was different from previous study which reported that cellulose degrading bacteria were not able to inhibit the growth of P.
and *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Enterococcus faecalis* [7].

3.3. Genetic character of cellulolytic bacteria

Three isolates exhibiting high cellulolytic activity (S.St1.7, S.St2.8 and S.St3.4) were selected to be identified for the DNA sequence analysis. All isolates indicated similarity (99.53%) relationship to *Bacillus toyonensis* BCT-7112 (Figure 2). Strain BCT-7112 was one of soilborne organisms [24] and known as Toyocerin® which had been used as probiotic for animal feed [25].

4. Conclusion

Cellulolytic bacteria had been isolated from sediment of mangrove ecosystem in Dumai Marine Station. Isolate BS.St2.8 performed the highest cellulolytic activity and antagonism against bacterial pathogens, mainly *Pseudomonas aeruginosa*. The isolate was categorized into *Bacillus toyonensis*.

| Table 1. Cellulolytic activity and index of bacterial isolates on CMC 1% containing medium |
|---------------------------------------------|---------------------------------------------|
| No. | Isolate code | Diameter of Inhibition Zone (mm) | Diameter of Colony Zone (mm) | Cellulolytic activity (Ratio of IZ/CZ) | Cellulolytic index (IZ - CZ)/IZ |
|-----|--------------|---------------------------------|-----------------------------|--------------------------------------|-------------------------------|
| 1   | S-ST1.1      | 32.07                           | 31.68                       | 1.01                                 | 0.01                          |
| 2   | S-ST1.2      | 20.42                           | 8.68                        | 2.35                                 | 1.36                          |
| 3   | S-ST1.3      | 40.45                           | 29.18                       | 1.39                                 | 0.67                          |
| 4   | S-ST1.4      | 36.28                           | 26.82                       | 1.35                                 | 0.58                          |
| 5   | S-ST1.5      | 12.10                           | 12.05                       | 1.00                                 | 0.00                          |
| 6   | S-ST1.6      | 19.05                           | 11.42                       | 1.69                                 | 0.67                          |
| 7   | S-ST1.7      | 22.58                           | 9.28                        | 2.43                                 | 1.46                          |
| 8   | S-ST1.8      | 11.07                           | 10.38                       | 1.08                                 | 0.06                          |
| 9   | S-ST2.1      | 15.72                           | 10.02                       | 1.57                                 | 0.59                          |
| 10  | S-ST2.2      | 14.32                           | 9.65                        | 1.48                                 | 0.53                          |
| 11  | S-ST2.3      | 15.98                           | 9.38                        | 1.70                                 | 0.79                          |
| 12  | S-ST2.4      | 16.25                           | 15.02                       | 1.08                                 | 0.10                          |
| 13  | S-ST2.5      | 27.50                           | 17.60                       | 1.56                                 | 0.64                          |
| 14  | S-ST2.6      | 20.98                           | 12.85                       | 1.63                                 | 0.69                          |
| 15  | S-ST2.7      | 4.42                            | 3.68                        | 1.20                                 | 0.19                          |
| 16  | S-ST2.8      | 21.15                           | 2.86                        | 2.86                                 | 2.12                          |
| 17  | S-ST3.1      | 19.15                           | 8.05                        | 2.38                                 | 1.38                          |
| 18  | S-ST3.2      | 26.15                           | 14.78                       | 1.77                                 | 0.95                          |
| 19  | S-ST3.3      | 18.05                           | 16.25                       | 1.11                                 | 0.15                          |
| 20  | S-ST3.4      | 19.32                           | 7.22                        | 2.68                                 | 1.76                          |
| 21  | S-ST3.5      | 9.68                            | 8.65                        | 1.12                                 | 0.12                          |
| 22  | S-ST3.6      | 18.12                           | 17.05                       | 1.06                                 | 0.10                          |
| 23  | S-ST3.7      | 11.52                           | 8.68                        | 1.33                                 | 0.34                          |
| 24  | S-ST3.8      | 19.25                           | 9.28                        | 2.07                                 | 1.10                          |

Note: highlight values indicates high cellulolytic activity

| Table 2. Cell morphology and biochemical characters of cellulolytic bacterial isolates |
|---------------------------------------------|---------------------------------------------|
| Character                          | Strain code | Bacil/short motile | Bacil/short motile | Bacil/short motile | Bacil/short motile | Bacil/pendek motile | Bacil/short motile | Bacil/short motile |
| Shape of cell | Bacil/short motile | Bacil/short motile | Bacil/short motile | Bacil/short motile | Bacil/short motile | Bacil/short motile | Bacil/short motile | Bacil/short motile |
| Motility                          | +           | +                   | +                   | +                   | +                   | +                   | +                   | +                   |
| Gram staining                     | -           | +                   | -                   | -                   | -                   | -                   | -                   | -                   |
| Use of                           | -           | +                   | -                   | -                   | -                   | -                   | -                   | -                   |
citrate Prod. of Indole Prod. of H₂S gas Prod. of catalase Test on TSIA  
- - - - - - - -  
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Table 3. Average of inhibition zone of cellulolytic bacterial isolates against pathogens  

| Isolate code | Diameter of inhibition zone (mm) | Escherichia coli | P. auroginosa | V. alginolyticus |
|--------------|---------------------------------|-----------------|--------------|-----------------|
| S.St1.2      | 2.38 ± 0.25                     | 4.61 ± 1.52     | 2.75 ± 0.56 |
| S.St1.6      | 2.38 ± 0.21                     | 4.18 ± 0.85     | 2.48 ± 0.75 |
| S.St1.7      | 2.45 ± 1.00                     | 3.51 ± 0.12     | 2.85 ± 1.48 |
| S.St2.3      | **3.58 ± 0.83**                 | 3.35 ± 0.51     | 3.45 ± 0.46 |
| S.St2.6      | 1.92 ± 0.72                     | 2.75 ± 0.59     | 2.28 ± 0.45 |
| S.St2.8      | 2.78 ± 0.35                     | 4.11 ± 0.49     | **4.68 ± 1.40** |
| S.St3.1      | 3.02 ± 0.99                     | **4.81 ± 0.57** | 4.32 ± 1.50 |
| S.St3.4      | 2.91 ± 1.94                     | 3.41 ± 0.81     | 3.58 ± 0.65 |
| S.St3.8      | 2.48 ± 0.58                     | 3.11 ± 0.35     | 4.15 ± 0.46 |

Figure 2. Phylogenetic tree of cellulolytic bacterial isolates (BS.St2.8, BS.St3.4, BS.St1.7) constructed by the UPGMA program  

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