Preliminary Screening of Carbohydrase-Producing Bacteria from Chaetomorpha sp. in Sepanjang Beach, Yogyakarta, Indonesia

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Abstract. Enzyme is a biocatalyst that has been known for its function in various industrial applications. One of the potential natural producers of enzymes is seaweed associated bacteria. Seaweed associated bacteria has been studied as a natural source of carbohydrase such as carrageenase, alginate lyase, and agarase. The purpose of this study was to determine the potential of seaweed associated bacteria from Gunung Kidul, Yogyakarta, Indonesia as a source of carbohydrase enzymes. A total of 13 bacterial isolates were successfully isolated from Chaetomorpha sp. in Sepanjang Beach. Enzymatic activity was measured through cultivation of each bacterium on semi-solid media with addition of substrate of each enzyme. The results showed that 3 isolates (GK.6.10; GK.6.11; GK.6.12) had clear zones around the growing colonies in medium containing 0.2% starch and 2% κ-carrageenan. Meanwhile, 4 isolates (GK.6.3; GK.6.10; GK.6.11; and GK.6.12) showed clear zones in medium containing 0.5% alginate and 2% agar indicating the production of alginate lyase and agarase enzyme. Bacteria GK.6.10; GK.6.11; and GK.6.12 were identified as \textit{Salinicola zeshunii}, \textit{Bacillus piscis}, and \textit{Bacillus licheniformis} with BLAST homology 95.23%, 99.46%, and 99.26%.

1. Introduction
Research on enzymes isolated from marine organisms was a topic that has been widely studied in recent decades. Marine enzymes are known for their high stability and great potential in the industrial sector. Marine enzymes can be isolated from various marine organisms such as animals, plants, and microorganisms [1-14]. Previous studies have reported that several types of enzymes (mostly carbohydrase) such as cellulase, alginate lyase, carrageenase, and agarase were isolated from the marine environment [1,3,5,10-13]. Marine enzymes are also known to have many advantages over enzymes from terrestrial areas such as pH tolerance, thermostability, barophilicity, and tolerance to...
other extreme conditions [2]. These unique enzyme properties are caused by extreme environmental conditions in marine ecosystems such as high salinity, tides, low temperatures, and high pressure which can affect the variation of enzymes produced by marine organisms [2]. These advantages lead to the use of marine enzymes which are widely used in various fields such as the textile industry, food industry, detergent formulations, pharmaceuticals, and other fields [1,2,4,13]. Previous research showed that enzymes isolated from microorganisms had better stability than enzymes from plants and animals [1-3]. One group of microorganisms that showed potential as a natural producer of enzymes is the seaweed association bacteria. Research by Zilda et al. [5] showed that the alginate lyase isolated from seaweed associated Bacillus sp. has thermostability up to 50°C and pH tolerance up to pH of 9. Unfortunately, the study on marine enzymes from Indonesia marine ecosystems is still rarely reported. For this reason, this study aims to isolate seaweed-associated bacteria in Sepanjang Beach, Yogyakarta, Indonesia and to screen the bacteria that produce extracellular carbohydrate (amylase, agarase, alginate lyase, and κ-carrageenase) enzymes.

2. Materials and Methods

2.1. Seaweed Sampling
Seaweed sample was collected from Sepanjang Beach, Yogyakarta, Indonesia (N 8° 08' 12.9", E 110° 34' 01.6") in June 2020. After collection, seaweed species were identified by matching their morphology with the seaweed identification book by Coppejans et al. [21].

2.2. Isolation and Purification
Isolation was carried out using serial dilution method according to Sibero et al. with several modifications [15]. Identified seaweed sample was ground and diluted to a concentration of $10^{-3}$, $10^{-4}$, and $10^{-5}$. The 100 μl of sample from each concentration was then inoculated and spread into ISP 4 medium and incubated at room temperature (27°C). The single colonies with different morphology then purified using streak method on ISP 2 medium.

2.3. Gram Staining
A thin smear of purified bacteria prepared on a dry glass slide and fixed using gentle heat. Gram staining method was carried out according to the Gram staining protocol using the gram staining kit Himedia K001. The slides were then air-dried and examined under a microscope using a 400× magnification [16].

2.4. Screening of Enzymatic Activity
Screening of enzymatic activity was carried out in 4 media containing different substrates of each enzyme. Each isolates dotted in 4 solid media namely starch medium (2% agar, 0.1% yeast extract, 0.5% peptone, and 0.2% starch), agar medium (2% agar, 0.1% yeast extract, and 0.5% peptone), alginate medium (2% agar, 0.1% yeast extract, 0.5% peptone, and 0.5% alginate), and κ-carrageenan medium (2% κ-carrageenan, 0.1% yeast extract, and 0.5% peptone). The isolates were incubated for 4 days at room temperature (27°C) then poured with 10% iodine solution. The clear zone around the colonies indicated the production of enzyme from the isolates [3-6].

2.5. Molecular Identification
Molecular identification was carried out according to the DNA extraction protocol from Zymo Quick-DNA Miniprep Kit. Bacterial identification was carried out through DNA barcoding in 16S ribosomal RNA region. Amplification was performed using 27F and 1492R primers. Bacterial species were identified based on NCBI BLAST homology [22].

3. Results and Discussion
Seaweed GK.6.22 (Fig 1.b) has a green filament-like body structure without any branching from each filament. Based on the results of morphological matching, the seaweed sample was identified as green
seaweed from the genus Chaetomorpha. Although several researchers had studied the properties of the enzyme from seaweed, nevertheless only a few studies reported the enzymes from Chaetomorpha sp. and their associated bacteria [17]. In this study, the incubation time in isolation step was applied in a longer period with the expectation of obtaining slow-growing bacteria which had not been widely reported.

Figure 1. Sampling site (A) and Chaetomorpha sp. (GK.6.22) from Sepanjang Beach (B)

The isolation step was carried out using various concentrations of dilution to obtain a more diverse species of bacteria. The purification results showed a total of 13 isolates were successfully isolated from various dilution concentrations. The morphology of each isolate can be seen in Figure 2. Each of the pure bacterial isolates then went through the gram staining step.

Figure 2. Bacteria isolated from Chaetomorpha sp. cultured on ISP 2 agar medium

The results of gram staining were obtained from observations of bacteria after going through the gram staining process. The results of the slide observation showed that 4 isolates, namely GK.6.3;
GK.6.7; GK.6.11; and GK.6.12 (Fig 3), had a purple colour after going through the gram staining step which showed that the four isolates were gram-positive bacteria. Meanwhile, 9 other isolates showed a pink colour, indicated that these isolates were classified as gram-negative bacteria.

![Figure 3. Result of gram staining observed using a light microscope (400×)](image)

Enzymatic activity is measured based on the presence of a clear zone around the bacterial colony. The clear zone can be observed after adding the 10% iodine solution to the medium. The results showed that 3 isolates (GK.6.10; GK.6.11; GK.6.12) had clear zones around the growing colonies in medium containing 0.2% starch and 2% κ-carrageenan (Fig 4 and Fig 5). The results above indicated that these three isolates produced amylase and κ-carrageenase enzymes. On the other hand, 4 isolates (GK.6.3; GK.6.10; GK.6.11; and GK.6.12) showed clear zones in medium containing 0.5% alginate and 2% agar (Fig 6 and Fig 7). The presence of clear zones indicated the production of alginate lyase and agarase enzyme. This result shows that GK.6.10; GK.6.11; and GK.6.12 were more potential as carbohydrase producing bacteria. Previous research showed that bacteria isolated from seaweed *Chaetomorpha linum* has a potential as a chitinase-producing bacteria. These bacteria come from the genus *Enterococcus* [17]. Unfortunately, no research has studied the carbohydrase enzyme produced by *Chaetomorpha* sp. associated bacteria.
Figure 4. Clear zone around isolates grown in κ-carrageenan medium

Figure 5. Clear zone around isolates grown in starch medium

Figure 6. Clear zone around isolates grown in alginate medium

Figure 7. Clear zone around isolates grown in agar medium
Figure 8. The phylogenetic tree of GK.6.10; GK.6.11; and GK.6.12 that exhibited extracellular enzymes according to 16S rRNA analysis

Molecular identification was carried out via DNA barcode on the 16S ribosomal RNA region. The phylogenetic tree of the most active isolates (GK.6.10; GK.6.11; and GK.6.12) is shown in Figure 8. According to NCBI BLAST homology, GK.6.10 was identified as *Salinicola zeshunii* with 95.23% similarity to *Salinicola zeshunii* 16S ribosomal RNA gene partial sequence (NR 132717.1:652-1466). Interestingly, previous study has never reported the enzymes produced by *Salinicola zeshunii*. On the other hand, GK.6.11 and 12 are known to belong to the genus *Bacillus*. GK.6.11 was identified as *Bacillus piscis* with 99.46% similarity to *Bacillus piscis* 16S ribosomal RNA gene partial sequence (NR 165685.1:351-1459). Meanwhile, GK.6.12 was identified as *Bacillus licheniformis* with 99.26% similarity with *Bacillus licheniformis* 16S ribosomal RNA gene partial sequence (MT242553.1). Based on previous research, bacteria from the genus *Bacillus* known as various types of enzymes producer such as amylase, lipase, alginate lyase, and protease [5, 18-20].

4. Conclusion
A total of 13 bacteria were successfully isolated from *Chaetomorpha* sp. in Sepanjang Beach, Yogyakarta, Indonesia. The results of gram staining showed that GK.6.3; GK.6.7; GK.6.11; and GK.6.12 identified as gram-positive bacteria. Meanwhile, 9 other isolates were classified as gram-negative bacteria. 4 isolates (GK.6.3; GK.6.10; GK.6.11; and GK.6.12) showed a clear zone in solid medium indicating the presence of enzymatic activity. According to NCBI BLAST homology, GK.6.10; GK.6.11; and GK.6.12 were identified as *Salinicola zeshunii*, *Bacillus piscis*, and *Bacillus licheniformis* with 95.23%, 99.46%, and 99.26% similarity.
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