Characterization of the gene encoding chitinase enzyme from bacillus isolates insulated from some locations in Southeast Sulawesi

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Abstract. This study aimed to determine the isolates of local Bacillus that have potential chitinolytic activity and to know the characteristic of the gene encoding chitinase enzyme from local Bacillus isolates from Southeast Sulawesi that were selected to have chitinolytic activity. Selection of chitinolytic bacteria based on bacterial ability to form clear zone on chitin agar medium which was grew by spread method and incubated for 4 days. From 5 test isolates used, one isolate which had chitinolytic activity was isolate Bacillus sp. Rh 3.8. The amplification of the gene encoding chitinase enzyme selected bacterial isolates was done by PCR (Polymerase Chain Reaction) technique using Chitbac F and Chitbac R primers. Sequence analysis was conducted by BLASTn, mapping of restriction enzyme using Bioedit software, analysis of amino acid using expasy software, analysis of hydrophobicity using Bioedit software, phylogenetic tree construction using MEGA software. The results showed that the characters of the gene encoding chitinase enzyme was a gene measuring 804 bp. Based on BLASTn analysis, the gene has 100% similarity with Bacillus thuringiensis SCG0402 (CP017577). The gene has 9 restriction enzyme cutting sites. Based on hydrophobicity analysis shows that the amino acid sequence of chitinase enzyme is dominant exist on hydrophilic region. The results of phylogenetic tree construction show isolates of the Bacillus sp. Rh 3.8 is a group with Bacillus thuringiensis so this strain is a species of Bacillus thuringiensis.

1. Background

Chitin is the second largest biopolymer after cellulose, which is composed of N-acetylglucosamine residues through β (1-4) bonds with a very abundant supply in nature [1]. Chitin is a component of the body of shrimp, crabs, insects, shellfish, squid and other arthropod animals. Chitin is also a component of cell walls of many fungi and algae. The size of chitin is relatively large and the solubility of chitin is low and difficult for the human body to absorb, so that the application of chitin is limited and causes chitin to become the main source of pollution of organic compounds. The pollution can be overcome by managing chitin into a product that has economic value [2]. Chitin processing efforts can be carried out enzymatically or chemically. Enzymatic chitin processing can be done using chitinolytic microorganisms.

Chitinolytic microorganisms are microorganisms that are able to produce chitinase enzymes that can degrade chitin. These microorganisms can be obtained from various sources such as rizosphere,
soil phyllosphere or from aquatic environments such as sea, lake, and pond. Currently there are many studies that discuss the isolation, screening, and characterization of the chitinase enzyme from various sources. Chitin degrading microorganisms are generally derived from a group of bacteria [3]. Some bacteria that have been reported to have chitinolytic activity are *Aeromonas* sp., *Pseudomonas* sp., *Bacillus* sp., *Serratia* sp., and *Vibrio* sp.

Based on several research results, one of the most potential bacterial groups to produce the enzyme chitinase is from the genus *Bacillus*. It has been reported that chitinolytic bacteria isolated from Pancuran Tujuh, Baturaden, Central Java are *Bacillus* sp. PT23 [1]. In another study, *Bacillus licheniformis* MB-2 was obtained from Tompaso Manado hot springs and *Bacillus coagulans* LH 28-38 isolated from Lahendong, North Sulawesi, was also able to produce chitinase enzymes [4, 5].

Bacteria from the genus *Bacillus* have also been widely isolated from the Southeast Sulawesi region. Some local *Bacillus* species from Southeast Sulawesi that have been successfully isolated include *Bacillus* sp. Rh 3.6, *Bacillus* sp. Rh 3.8 and *Bacillus* sp. Sn 1.1 which was isolated from Rhizosper mangrove Kendari Bay of Southeast Sulawesi [6], *Bacillus* sp. LIII C isolated from the gold mining area of PT. SUN in Wumbu Bangka Village, North Karowatu District Bombana Regency [7] and *Bacillus megaterium* PSA 10 isolated from sago flour obtained from sago processing locations in Sampara District, Konawe Regency, Southeast Sulawesi [8].

Some local *Bacillus* isolates from Southeast Sulawesi had previously been known to be a probiotic bacterium, bioplastic producer and as a mercury degrading agent. Utilization of isolates from the local *Bacillus* genus from Southeast Sulawesi can still be expanded its development potential, one of which is by knowing its ability to produce chitinase enzymes. Chitinase enzymes are encoded by specific genes that distinguish them from other bacteria that do not have chitinolytic abilities. The chitinase enzyme coding gene is a gene that codes for the formation of chitinase so that bacteria can express the chitinase enzyme in their cells.

The chitinase enzyme coding gene has been successfully isolated from several species of *Bacillus*, the chitinase enzyme coding gene of *Bacillus thuringiensis* strain YPOO3665928 measuring 1200 bp, *Bacillus cereus* measuring 766 bp and *Bacillus thuringiensis* UG measuring 1129 bp [9, 10]. These results indicate that the chitinase enzyme coding gene has a variety of sizes.

Exploration of local *Bacillus* can be done through qualitative tests based on its ability to express the chitinase enzyme on chitin agar media and through isolation of the chitinase enzyme coding gene using the PCR (Polymerase Chain Reaction) technique using specific primers of the chitinase gene. In addition, through the PCR process it can also be seen the differences in the character of the gene encoding chitinase enzyme each of the local *Bacillus* isolates. The chitinase enzyme coding gene in the future can be used in industrial, pharmaceutical, molecular and biotechnology fields such as genetic engineering [11].

In this research, we can find local *Bacillus* isolates which have chitinolytic activity and can know the characteristics of the chitinase enzyme coding gene in *Bacillus* isolates.

2. Materials and Methods

2.1. Media Making

2.1.1. Chitin Agar Media

The media used for chitinolytic testing is chitin agar media. Making chitin agar media begins with the preparation of the source of chitin from shrimp shells. Furthermore, the shrimp shell is dried then mashed using mortar and pestle. Shrimp shells that have been mashed weighed as much as 12 g and put into an erlenmeyer with the addition of 60 mL NaOH, then heated on a hot plate for 2 hours at 60°C. The precipitate formed is then filtered using filter paper and washed using distilled water to a neutral pH. The precipitate is dried in the oven for 4 hours at 60°C. The dried precipitate is then weighed as much as 9 g, then added to 90 mL concentrated HCl, and allowed to stand for 2 days to
form a precipitate. The precipitate formed is washed with distilled water to a neutral pH. The precipitate is then dried in an oven for 4 hours at 60°C and pure chitin will be obtained [12].

2.1.2. **NA (Nutrient Agar) Media**
The media used for the rejuvenation of local Bacillus isolates was NA (Nutrient Agar) media. The process of making NA media is as much as 1.6 g NB and 4 g agar added with 200 mL of distilled water, then heated on a hot plate and homogenized using a magnetic stirrer and then sterilized by autoclaving pressurized 1 atm 121°C.

2.1.3. **NB (Nutrient Broth) Media**
The composition of NB media is 8 g NB dissolved in 1000 mL of distilled water. The process of making media that is as much as 0.8 g NB is dissolved in 100 ml of distilled water. The media is heated on a hot plate and homogenized using a magnetic stirrer and then sterilized with a 1 atm 121°C pressurized autoclave.

2.2. **Equipment and Media Sterilization**
The equipment and materials to be used in the study are sterilized by the wet heating method. The equipment was sterilized using a 1 atm 121°C pressurized autoclave, for 30 minutes. Sterilized tools are generally made of glass. Media were sterilized using pressurized autoclaves of 1 atm 121°C, for ± 15-20 minutes.

2.3. **Chitinolytic Bacteria Selection**

2.3.1. **Source of Test Bacteria Isolates**
The local Bacillus isolates used in this study are presented in Table 1.

| No. | Isolate Code | Information                      | Reference |
|-----|--------------|----------------------------------|-----------|
| 1   | *Bacillus* sp. Rh 3.8 | Isolate 8 *Rhizophora apiculata* III | [6]       |
| 2   | *Bacillus* sp. Rh 3.6 | Isolate 6 *Rhizophora apiculata* III | [6]       |
| 3   | *Bacillus* sp. Sn 1.1 | Isolate 1 *Sonneratia alba* I | [6]       |
| 4   | *Bacillus* sp. L III C | Isolate C Location 3 | [7]       |
| 5   | *Bacillus megaterium* PSA 10 | Isolate 10 sago starch flour | [8]       |

2.3.2. **Rejuvenation of Local Bacillus Bacteria Isolates**
The five local Bacillus isolates were inoculated on oblique NA media using a round ose needle. The isolate was inoculated using the scratch method, then incubated at room temperature for 24 hours.

2.3.3. **Chitinolytic Activity Test**
Chitinolytic activity tests were carried out using the spread method. Local Bacillus strain bacteria were grown using specific media, namely chitin agar media and incubated for 4-5 days.
2.4. Molecular Characterization of Chitinase Gene

2.4.1. Isolation of Genomic DNA from Local Bacillus Strains

DNA isolation of the bacterial genome of the local Bacillus strain was carried out using the modified DNA isolation method of Doyle (1998) [13]. The steps in isolation of the local Bacillus genome DNA are bacteria were cultured in NB media for 24 hours at room temperature. As much as 1.5 mL of bacterial culture was put into eppendorf, centrifuged at 5000 rpm for 2 minutes. Pellet is taken and added with solution I (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA) as much as 100 µL and incubated in ice for 5 minutes. Then the sample was added to solution II (0.2 N NaOH, 1% SDS) as much as 200 µL and turned back 5 times and then in vortex for 2 minutes. Subsequently the sample was added with solution III (5 mM Sodium acetate, 11.5 mL glacial acetic acid, 28.5 H2O) as much as 150 µL and centrifugation at a speed of 10000 rpm for 10 minutes at 8°C. Supernatant is taken and added 1x Volume Phenol Chloroform. Subsequently the sample was centrifuged at 10,000 rpm, temperature 4°C, for 10 minutes. The supernatant was taken and added with 0.1 volume of sodium acetate 3 M pH 5.2 then added with 1 x volume of 100% ethanol then incubated at -20°C for 30 minutes and centrifuged 10,000 rpm, temperature 4°C for 20 minutes. Furthermore, DNA pellets are washed with 70% alcohol as much as 500 µL, then dried and dissolved in 20-50 µL H2O.

2.4.2. Primer Design

The primer design is carried out by aligning 10 chitinase gene sequences of Bacillus genus bacterial (Table 2) and then determining the sequences that are conserved around the 5' and 3' ends as forward and reverse primers, respectively. The recommended primer sequences are 5’-CTCTCGTACTCGTGAACCAT-3’ (Chitbac F) and 5’-ACACACCTGTRTCACCGGTAG-3’ (Chitbac R).

Table 2. Bacteria reference strains used for specific primer designs

| No | Accession number | Species Name       | Strain Code |
|----|------------------|--------------------|-------------|
| 1  | CM000759         | B. thuringiensis   | IBL 4222    |
| 2  | NZ_CP017577      | B. thuringiensis   | SCG04-02    |
| 3  | NC_012472        | B. cereus         | 03BB102     |
| 4  | AP007209         | B. cereus         | NC7401      |
| 5  | CP009331         | B. anthracis      | K3          |
| 6  | CM002402         | B. anthracis      | 8903-G      |
| 7  | CP001598         | B. anthracis      | A0248       |
| 8  | CP014282         | B. thuringiensis  | Bt185       |
| 9  | CP006863         | B. toyonensis     | BCT-7112    |
| 10 | CM000724         | B. cereus         | BDRD-ST26   |

2.4.3. Amplification of Chitinase Gene Fragments by PCR Technique

PCR (Polymerase Chain Reaction) is a technique for multiplying DNA sequences. Local Bacillus chitinase gene fragments were amplified by PCR technique. The total PCR volume is 10 µL consisting of 100 ng genomic DNA, each 0.5 µM forward primer and reverse primer, 1x master mix, and dH2O up to a volume of 10 µL. The PCR program consisted of initial denaturation at 94°C for 5 minutes; 35 cycles of PCR process which include denaturation of 94°C for 1 minute, annealing 59°C for 30 seconds, elongation of 72°C for 90 seconds; and final extension at 72°C for 5 minutes.

2.4.4. DNA sequencing

Sequencing is done by using a DNA sequencer tool. The DNA sequencing process follows the Sanger method.
2.4.5. Sequence Analysis
Data obtained from the results of isolation and characterization of chitinase-coding genes were analyzed using 3 types of programs namely BLAST (Basic Local Alignment Search Tools), MEGA (Molecular Evolutionary Genetics Analysis), and BioEdit. The BLAST program is used to analyze local nucleotide alignments, the MEGA program is used for phylogenetic tree analysis based on nucleotide sequences and the BioEdit program is used to analyze restriction enzymes.

3. Results and Discussion

3.1. Chitinolytic Activity in Local Bacillus Isolates
Determination of local Bacillus isolates which have chitinolytic activity is done in vitro based on their ability to grow on chitin agar media and the formation of clearer zones around the scratching of bacterial cells. Based on the test results of 5 isolates used showed that 1 isolate which has the ability of chitinolytic activity, namely isolate Bacillus sp. Rh 3.8. The results of the testing of chitinolytic activity of local Bacillus isolates are presented in Figure 1.

Based on the results in Figure 1 shows that the isolate Bacillus sp. Rh 3.8 can grow on chitin agar media while the four other isolates cannot grow. The ability of isolates Bacillus sp. Rh 3.8 grows on chitin agar media indicating that the isolate can use chitin as a source of nutrition for its growth while the other four isolates cannot use chitin. Chitinolytic bacteria during their growth will utilize chitin contained in the media where it grows [14]. The presence of chitin in the media will stimulate bacterial isolates to produce the enzyme chitinase. The resulting chitinase enzyme will hydrolyze chitin around the colony which is then used by bacteria as a source of carbon and energy for its growth.

The second indicator besides its ability to grow on chitin media, isolate Bacillus sp. Rh 3.8 can also form clearer zones around its growth. A clearer zone is formed because chitin has been hydrolyzed by the enzyme chitinase. The clear zone around the colonies in chitin agar media is formed due to the presence of the chitinase enzyme that is secreted by bacterial cells to break down chitin macromolecules (chitin polymers) into smaller chitin molecules (oligomers and N-acetyl glucosamine) that can be absorbed by microbes as a carbon source for chitin metabolic processes and growth [15]. Chitin degradation and use of the result of degradation by bacteria caused the composition of chitin in the medium to decrease and cause the medium to appear clear especially around the growing colonies.

![Figure 1. Qualitative Chitinolytic Activity Test Results (clearer zone on chitin agar media): isolates Rh 3.6, SN 1.1 and Rh 3.8 (Bacillus sp. from mangrove rhizosphere), PSA 10 (Bacillus megaterium from sago pulp), L III C (Bacillus sp. from post-mining land).](image-url)
Clear zone formed indicates chitin degraded to produce N-acetyl-D-glucosamine. The clear zone is formed due to the breaking of $\beta$-1,4 bonds from the N-acetylglucosamine homopolymer in chitin by chitinase to the N-acetylglucosamine monomer. The size of the clear zone produced depends on the amount of N-acetylglucosamine monomer produced from the chitin hydrolysis process. The greater the amount of N-acetylglucosamine monomer produced, the greater the clear zone formed around the colony [16].

Based on the results of chitinolytic activity tests showed that only isolates of Bacillus sp. Rh 3.8 which has the ability of chitinolytic activity. This is likely influenced by the environment of the local Bacillus isolate source. To find bacteria that have chitinolytic activity must be isolated from the environment containing chitin substrate [17]. Rhizosfer Mangrove is one of the places where marine sediments are accumulated such as shellfish and a breeding ground for various marine biota including fish, shrimp, crabs and other marine biota [18].

Chitin is the main component of the body of shrimp, shellfish and crab so that Rhizosfer mangrove has great potential as a source of chitinolytic bacteria. This is evidenced by the presence of chitinolytic activity in isolates of Bacillus Rh 3.8 isolated from Rhizosfer mangrove. Unlike the case with isolates Rh 3.6 and SN 1.1 there is no chitinolytic activity even though it also comes from Rhizosfer mangrove. In addition to environmental factors, chitinolytic activity is also influenced by its ability to produce chitinase enzymes. The ability of microorganisms to produce enzymes to break down chitin differs from species to species [3]. Each microorganism produces different enzymes so that the activity produced is also different. Bacillus sp. Rh 3.8 which has been known to have chitinolytic activity is then carried out DNA isolation to obtain the chitinase enzyme coding gene.

3.2. DNA Isolation of Isolate Bacillus sp. Rh 3.8
Isolation of genomic DNA isolates of Bacillus sp. Rh 3.8 is carried out through 3 stages: bacterial lysis, DNA extraction and DNA purification. Bacterial lysis is done by adding solutions I, II and III. The three solutions can work optimally in damaging cells when in hot conditions. Warming can cause the cell pores to stretch so that the solution can enter the cell. The solution that enters the cell will cause the bacterial cell membrane to degrade, so that all cell contents can come out including DNA. The second stage is DNA extraction or DNA cleaning that aims to separate DNA mixed with polysaccharides, proteins and other impurities. Furthermore, the centrifugation process aims to separate molecules based on molecular size and weight. The centrifugation process will cause larger and heavier components to settle to the bottom of the tube called the pellet, while molecules which are smaller in size and weight will be in the upper layer called the supernatant. The final stage is DNA purification aimed at removing chloroform residues from the extraction stage. DNA purification is also carried out to separate compounds that are still attached to DNA and RNA contaminants so that it can produce pure DNA that is free of RNA and is ready to be used as a DNA template during PCR.

3.3. DNA Quality Test of Bacillus sp. Rh 3.8
Quality of DNA isolated from Bacillus sp. Rh 3.8 can be determined by testing using the electrophoresis method. Electrophoresis is the process of migrating DNA in an electric field. DNA will migrate from negative charges to positive charges [19]. The results of DNA quality testing of Bacillus sp. Rh 3.8 is presented in Figure 2. The electrophoresis results in Figure 2 show the DNA genome of the isolate Bacillus sp. Rh 3.8 has been successfully isolated. DNA bands formed on the results of electrophoresis show that DNA has good quality because there is no visible pattern of degraded DNA bands. This is indicated by the presence of a firm band concentrated at the bottom of the well. DNA degrades when the DNA band is not concentrated but spreads along the well [20]. The results in Figure 2 also show that the DNA bands of the genome formed are quite thick. The presence of thick DNA bands indicates that DNA has a high quantity value. The thickness of the DNA band can indicate genomic DNA concentration [21].
Figure 2. Hasil elektroforesis DNA genom isolat Bacillus sp. Rh 3.8 pada gel agarose 1%.

The value of genomic DNA concentration of Bacillus sp. Rh 3.8 is 1,460 µg/µl (data not shown). DNA concentration is categorized as high concentration if it has a concentration value of ≥ 1000 µg/µl [22]. This shows that the DNA concentration of the genome of the isolate Bacillus sp. Rh 3.8 is high because it has a concentration value of 1,460 (µg/µl). The DNA will then be used as a template to amplify the target DNA in the PCR process. DNA purity determines the success of the PCR process. Low purity, for example due to the presence of contaminants in the form of proteins and other organic compounds, can inhibit primer attachment to the DNA template [21]. In addition, a low level of DNA template purity can affect the amplification reaction and can inhibit the work of the DNA polymerase enzyme [23].

3.4. Amplification of Chitinase Gene Fragment of Isolate Bacillus sp. Rh 3.8

Based on the visualization of the electrophoresis results showed that the chitinase gene fragment of Bacillus sp. Rh 3.8 has been successfully amplified by PCR technique using Chitbac F and Chitbac R primers. This is marked by the formation of clear bands on the electrophoretic gel. Electrophoresis results of chitinase gene fragment amplification of Bacillus sp. Rh 3.8 are presented in Figure 3.

Figure 3. Electrophoresis results of chitinase gene fragment amplification of Bacillus sp. Rh 3.8 uses the Chitbac F primer and Chitbac R on 1% agarose gel. M: Marker 1 kb ladder, 1: Isolate Bacillus sp. Rh 3.8.
Figure 3 shows that the chitinase enzyme coding gene fragment is well amplified which is characterized by the formation of a single band. The results of chitinase gene fragment amplification by PCR technique showed that the isolate Bacillus sp. Rh 3.8 has a nucleotide sequence of around 800 base pairs. This is because the Chitbach F and Chitbach R primers used in the PCR technique are designed with a size of about 800 base pairs.

A DNA sample is said to be specific and successfully amplified if the results of the electrophoresis analysis show the presence of a single band of DNA of an appropriate size based on previously known markers [24]. The success of the chitinase gene fragment amplification process is determined by optimal PCR conditions. The optimal PCR condition is related to the composition of the PCR reaction components (DNA concentration, primers, dNTP and Mg\(^{2+}\)), primer specificity and number of PCR cycles.

### 3.5. DNA Sequencing and Sequence Analysis of Chitinase Enzyme Encoding Genes

The chitinase enzyme coding gene obtained from this study was 804 bp in size (Figure 4). Other researchers have succeeded in isolating chitinase coding genes in molds of around 766 bp [25]. The sequence of chitinase enzyme coding gene fragments is presented in Figure 4.

![Figure 4](image-url)

**Figure 4.** Sequence of chitinase enzyme encoding gene fragments *Bacillus* sp. Rh 3.8

#### 3.6. BLAST analysis

BLAST (Basic Local Alignment Sequence Tools) analysis is done by comparing the data of the nucleotide sequence of samples with the nucleotide sequences contained in GeneBank. BLAST analysis aims to determine the similarity of *Bacillus* sp. Rh 3.8 with other species of the genus Bacillus. The results of the sequence alignment analysis are presented in Figure 5.

The results of the BLASTn analysis at the NCBI website showed that gene sequences isolated from Bacillus sp. Rh 3.8 has a high level of similarity with Bacillus thuringiensis SCG0402 in GeneBank. The similarity can be seen from the magnitude of the similarity value of the nucleotide base sequence of the chitinase enzyme coding gene. The analysis shows a maximum score of 1451, 100% Query coverage value and 100% identity value. Two species are said to be the same if the nucleotide
sequence has a Query coverage value and Max identity is close to 100% with an error value close to 0 [26]. Maximum score values above 100 indicate the validity of the same nucleotide sequence. Theoretically if the range of scores > 50 bits with E-value > e-04 shows a high degree of similarity [27]. Based on these data, it can be ascertained that the gene sequence coding for the chitinase enzyme of isolate Bacillus sp. Rh 3.8 has high similarity with Bacillus thuringiensis SCG0402 species.

Figure 5. The result of sequence alignment of chitinase enzyme coding gene of isolate Bacillus sp. Rh 3.8 with nucleotide sequences on Genebank using the BLASTn program

3.7. Phylogenetic Tree Analysis Using the MEGA Program

Phylogenetic tree analysis aims to determine the relationship between the organisms density with each other based on the similarity of DNA, genes, amino acids, proteins and other similarities. Phylogenetic analysis in this study was carried out based on the nucleotide sequence of the chitinase gene isolate Bacillus sp. Rh 3.8 with strains of Bacillus from GeneBank. The phylogenetic tree construction in this study uses the Neighbor-joining 1000x replication algorithm method. The value shown in the phylogenetic tree branching is a bootstrap value that shows the level of branching tree accuracy in the phylogenetic tree [13].
Figure 6. Phylogenetic trees constructed based on the Neighbor-joining algorithm which shows the kinship between isolates of Bacillus sp. Rh 3.8 with strains of members of the genus Bacillus on the basis of chitinase gene fragment sequences. The number at the branch indicates the bootstrap value based on a Neighbor-joining analysis with 1000 replications.

Sequences used in the analysis of phylogenetic trees were 11 sequences. The sequence consisted of 1 sample sequence of isolate Bacillus sp. Rh 3.8 and 10 chitinase gene sequences of the genus Bacillus obtained from GeneBank (Table 2). The ten species of the Bacillus strain consist of Bacillus thuringiensis strain SCG0402, Bacillus thuringiensis strain IDL4222, Bacillus cereus strain BDRD-ST26, Bacillus anthracis strain K3, Bacillus cereus strain 03BB102, Bacillus thuringiensis strain Bt185, Bacillus anthracis strain 8903, Bacillus cereus strain NC7401, Bacillus anthracis strain A0248 and Bacillus toyonensis strain BCT-7112 (Figure 6).

Figure 6 shows that the results of the phylogenetic tree construction of Bacillus sp. Rh 3.8 with 10 comparative isolates based on sequence sequence formed 3 clades. The first clade consisted of isolates Bacillus sp. Rh 3.8, Bacillus thuringiensis strain SCG0402, Bacillus thuringiensis strain IDL4222, Bacillus anthracis strain K3 and Bacillus cereus strain BDRD-ST26. The second clade consists of Bacillus cereus strain NC7401, Bacillus anthracis strain 8903 Bacillus cereus strain 03BB102 and Bacillus thuringiensis strain Bt185. The third clade consists of Bacillus anthracis strain A0248 and Bacillus toyonensis strain BCT-7112.

Bacillus sp. Rh 3.8 has the closest kinship to the Bacillus thuringiensis species compared to other comparative isolates (Figure 6). Bacillus sp. Rh 3.8 formed a group with the isolate Bacillus thuringiensis strain SCG0402. This shows that the gene sequence coding for the enzyme chitinase isolate Bacillus sp. Rh 3.8 has almost the same character as chitinase gene sequences owned by isolate Bacillus thuringiensis strain SCG0402.

The first clade in the phylogenetic tree formed three subclades, namely the first subclade consisted of isolates Bacillus sp. Rh 3.8 and Bacillus thuringiensis strain SCG0402 with a bootstrap value of 96%, the second subclade consisted of members of the first subclade with Bacillus thuringiensis strain IDL4222 bootstrap value of 94%, the third subclade consisted of isolates Bacillus cereus strain BDRD-ST26 and Bacillus anthracis strain K3 with a bootstrap value of 94%, the third subclade consisted of isolates of Bacillus cereus strain BDRD-ST26 and Bacillus anthracis strain K3 with a bootstrap value of 95%. Members of the third subclade join the second subclade with a bootstrap value of 100%. This shows that the isolate Bacillus sp. Rh 3.8 has a higher level of similarity with Bacillus thuringiensis (SCG0402), compared to other Bacillus isolates.
The second clade consists of three subclades, namely the first subclade consists of *Bacillus anthracis* strain 8903 and *Bacillus cereus* strain NC7401 with a bootstrap value of 94%, the second subclade consists of members of the first subclade with *Bacillus thuringiensis* strain Bt185 with a bootstrap value of 100% and the third subclade consisting of *Bacillus cereus* strain 03BB102 with members of the second subclade bootstrap value of 55%. The third clade consisted of *Bacillus anthracis* strain A0248 and *Bacillus toyonensis* strain BCT-7112 with a bootstrap value of 93%.

Based on the analysis of phylogenetic tree construction, it can be concluded that the gene sequence encoding the chitinase enzyme from the *Bacillus* sp. Rh 3.8 has a high similarity with *Bacillus thuringiensis* SCG0402. This is indicated by the formation of the same node with a bootstrap value of 96% at the branching. The knot symbolizes a taxonomic unit that can be a species, population, individual, gene, or sequence (nucleic acid or protein). The bootstrap value ≥95% in a branch can indicate the level of trust of a branch [28].

The phylogenetic tree analysis also shows that strains of bacteria from the same species are not in one clade, but are in different subclade (Figure 10). The difference in the position of the *Bacillus* strain in the phylogenetic tree proves that the chitinase coding gene sequence in each species is different. This shows that chitinase enzyme coding gene sequences vary in each bacterial species.

### 3.8. Analysis of the Restriction Enzyme Cutting Site for Chitinase Enzyme Encoding Genes

Restriction enzymes are enzymes that have the ability to cut DNA fragments specifically in certain nucleotide base sequences. The main characteristic of restriction enzymes is that each enzyme has a specific nucleotide sequence and cuts DNA in that specific sequence, but does not cut in other sequences [23]. The restriction enzyme cutting site analysis aims to determine the characteristics of the chitinase enzyme coding gene from the isolate *Bacillus* sp. Rh 3.8.

The results of the analysis (Table 3) show nine types of restriction enzymes found in the chitinase enzyme coding gene fragment of the isolate *Bacillus* sp. Rh 3.8. The site of restriction enzyme cutting of some members of the genus *Bacillus* is different, both in the same species and in different species [23]. The difference in cutting patterns is due to differences in the nucleotide order of each species. The cutting position that can be recognized by each restriction enzyme is unique. Therefore, restriction enzyme cutting site can be used to identify certain organisms.

Based on restriction enzyme analysis shows that the *Bcl*I and *Age*I enzymes can be used as an enzyme that distinguishes between *Bacillus thuringiensis* and other species. If the PCR product added to the *Bcl*I and *Age*I enzymes causes the PCR product to not be cut off, then the sample is similar to *Bacillus thuringiensis*. The results of analysis of the restriction enzyme the gene encoding chitinase enzyme of *Bacillus* sp. Rh 3.8 and several other isolates in GeneBank are presented in Table 3.
Table 3. Results of analysis of restriction enzyme cutting sites in chitinase gene fragments of several species of the genus Bacillus using the BioEdit

| No | Restriction Enzyme | Recognition Sites | Nucleotide Position |
|----|-------------------|-------------------|--------------------|
|    |                   |                   | I     | II    | III   | IV    | V     |
| 1  | AcI               | AACG\_TT          | -     | -     | -     | 444   | 360   |
| 2  | AfeI              | AGCGCT            | -     | -     | -     | -     | 64    |
| 3  | AgeI              | A\_CCCG\_T        | -     | -     | 15    | 787   | 15    |
| 4  | AseI              | ATTA\_AT          | 497   | 497   | 307   | 375   | 307   |
| 5  | BclI              | T\_GATC\_A        | -     | -     | 349   | 453   | 349   |
| 6  | BfrBI             | ATG\_CAT          | -     | -     | -     | 376   | 430   |
| 7  | BseVI             | C\_CCAG\_C        | 579   | 573   | 223   | 579   | 223   |
| 8  | NsiI              | A\_TGCA\_T        | -     | -     | -     | 378   | 432   |
| 9  | PvuII             | CAG\_CTG          | -     | -     | -     | -     | 92    |

Note: Restriction enzymes for special markers of Bacillus sp. Rh 3.8. (I) Bacillus sp. Rh 3.8; (II) B. Thuringiensis SCG04-02; (III) B. thuringiensis Bt185; (IV) B. cereus BDRD-ST26; (V) B. cereus NC7401.

The data in Table 3 shows that the AseI endonuclease restriction enzyme can be used as a molecular marker in species identification of the genus Bacillus because the AseI restriction enzyme has a similar cutting site with other Bacillus species members (Table 3). Bacterial isolate Bacillus sp. Rh 3.8 has a special endonuclease restriction enzyme, BclI and AgeI.

4. Conclusions
One local Bacillus isolate which has the capability of chitinolytic activity is Bacillus sp. Rh 3.8. Chitinase enzyme coding fragments from isolates Bacillus sp. Rh 3.8 has been successfully amplified by PCR technique using Chitbach F and Chitbach R primers. Characteristics of the chitinase enzyme coding gene are 804 bp in size with 267 amino acids, similar to Bacillus thuringiensis chitinase gene (SCG0402) with 100% identity value, has 9 sites Retriction enzyme cutting with BclI and AgeI enzymes specifically cuts the chitinase gene from Bacillus thuringiensis.

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