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Characterization of chitinolytic bacteria isolated from shrimp and crab waste producing chitinase and anti- fungal enzymes to overcome saprolegniasis in aquaculture

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Abstract

Chitinolytic bacteria was proposed in this study as an effective solution to overcome the growth of *Saprolegnia* sp in aquaculture practices. *Saprolegnia* sp. causes Saprolegniasis, a disease that affects aquaculture species characterised by white filamentous mycelium on the body or fins of freshwater fish. The use of probiotic bacteria as an alternative strategy to traditional chemical remedies may be applied. Potential bacteria may be isolated from shrimp and crab waste since these organic materials were known to harbor these organisms.

The search for the specific bacteria was conducted through the research activity namely isolation and cultivation of bacteria, chitinolytic activity test, anti-fungal activity test using *Saprolegnia* sp and finally characterization through molecular analysis of 16S rRNA gene and chitinase encoding fragments. Cultivation of isolated bacteria and general characterization used Gram staining while the results of the chitinolytic activity test showed that the selected candidates (species 1 dan 2) obtained the highest chitinolytic indexes of 2.12 mm and 1.99 mm, respectively. Results of the *invitro* anti-fungal activity test using *Saprolegnia* sp using species 1 and 2 obtained the zone of inhibition respectively 6.00 mm and 11.59 mm. Species 1 and 2 were identified with a similarity of 96% to *Bacillus thuringiensis* (no accession YP 003665928.1) and 99% identical to *Bacillus cereus* (without accession WP 000932552.1).

Keywords: anti *Saprolegnia* sp., chitinolytic bacteria, shrimp and crab waste, 16S rRNA

1. Introduction

Chitin is a natural biopolymer that is commonly found as the building structure of arthropod exoskeleton, molluscan shells, and also cell walls of fungal organisms [1, 2]. The total yield of this specific polysaccharide structure in nature may reach 1 billion tonnes per year [3]. Large amounts are commonly found in the waste of aquatic animals such as shrimp, crabs, and molluscan shells. Chitin found in many organisms has similar structure except the bond between protein and calcium carbonates which are mainly found in shrimp shells [4]. Many microorganisms that exist in nature produce hydrolytic enzymes such as chitinases. Hence, chitinolytic bacteria may be found in shrimp shell waste.

The research topic regarding these organisms focuses on the advantage of the microorganisms since its enzyme produced could potentially have an important role as a biocontrol agent in aquaculture to control *Saprolegnia* sp infection. These microorganisms are known as chitinolytic agents that are able to degrade fungal cells [5]. Chitin may be degraded through chitinolytic mechanisms which hydrolyze β -1,4-glycoside bonds thus or polymers go through deacetylation which the first step is hydrolyzation by the chitosanase enzyme.

Saprolegnia itself is one of the main pathogens in aquaculture that may affect fertile eggs, larvae, juvenile or larger fish. The infections in fish commonly start with an indication of fungi attaching the dorsal region. If not treated, these fungi will spread in the culture water while fish will have irritation symptoms such as flashing. The region affected with *Saprolegnia* will further develop lesions that destroy mucosa cells. Fish may further show cotton-like parasites and pimple-like structures throughout the body mainly affecting fins and also likely upon their body. Cotton-like colonies when observed through microscopic analysis

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of *Saprolegnia* will feature mycelium with a brownish, red, or greenish color. The treatment of *Saprolegnia* sp. should be conducted as soon as possible since its infection rate is quite rapid. When fish are not treated properly, the fish's health rapidly deteriorates and may cause fish to lose their appetite for food. Extreme infections may inhibit recovery although medical treatments are given.

Well known effective methods of *Saprolegnia* sp. treatment used chemical agents such as *Malachite Green* formalin and hydrogen peroxide [6]. Other remedies such as herbal treatments may improve survival although their effectivity may not be equal to chemical remedies. Throughout the development of fungal infection treatment in aquaculture, concerns in using chemical remedies remain high since these agents are known to affect human health and also chemical residual may develop in the environment. Thus, alternatives such as probiotics may become a potential remedy since these organisms possess chitinase enzymes that are able to degrade the chitin walls of *Saprolegnia*. Application in aquaculture has been conducted where chytinolytic microorganisms were able to reduce fungal *Aspergillus* in *Clarias batrachus* and *Oreochromis niloticus* [7].

This study will focus on the identification of potential microorganism using recent 16S rRNA gene characterization since the method to observe is common to amplify bacterial DNA [8]. Further, results of amplification were then sequenced and identified using bioinformatics protocols. The microorganisms isolated from shrimp waste are then discussed on how it could be optimized as a natural remedy to overcome *Saprolegnia* infections in fish. Previous studies have shown that not all isolated bacteria could produce a positive effect against *Saprolegnia*. Therefore it is important to screen the bacterial candidates appropriately. According to past research, a standardized test was conducted by assessing the chitinolytic activity of microorganisms in an in-vitro environment using agar media added with 0.5 gr colloidal chitin. Using these methods, a clear zone could be observed and measured. Thus, selected bacterial strains found in this research may potentially have the advantage of possessing *chitinase* enzymes. Antagonism test upon 24 bacterial candidates upon *Saprolegnia* sp in previous studies, showed that only 10 species had the ability to resist *Saprolegnia* sp. in a 7 day incubation period [9]. This may inform that not all chitinolytic bacteria could overcome *Saprolegnia*.

2. Material and Methods

2.1 Preparation of bacterial isolates

Bacterial candidates were sampled from location nearby the Nusantara Fishery Fishing Ports at Cirebon Indonesia, while further laboratory activity was conducted at the Biotechnology Laboratory at the Fisheries and Marine Science, Universitas Padjadjaran. Samples of shrimp and crab waste were cold preserved in 0.9% NaCl solution to reduce bacterial activity.

2.2 Media preparation and bacterial culture

Colloidal chitin was made during research following the method of Nasran [10]. As much as 10 gram of shrimp shells were diluted in 200 ml HCl and incubated overnight at 4°C. This solution was then filtered using glass wool and afterwards added with 100 ml of cool aquadest. The solution was neutralized from acidic state by adding 10 N NaOH then centrifugated at 8000 rpm for 20 minutes at 4 °C. The

precipitate was collected and washed using cool aquadest dan recentrifugated. This washing step is repeated until a brownish colored precipitate was obtained. Bacterial candidate strains were isolated and purified using agar medium enhanced with colloidal chitin. The composition of the media were 2 gram of colloidal chitin, 0.05 gram of yeast extract, 3 gram NaCl, 0.7 gram of (NH₄)₂SO₄, 0.01 gram of MgSO₄.7H₂O, 0.1 gram of K₂HPO₄ and 2 gram of agar. These ingredients were diluted in 100 ml aquadest and boiled until made into a sterile solution by autoclaving procedures. Bacterial samples were cultivated for 48 hours in this media. Bacteria that were growing were selected and harvested and recultured based on difference of morphologic characters. This process was conducted until pure strains were obtained. Examination of bacteria was conducted under microscope using standardized bacterial staining protocols.

2.3 Chitinolytic activity test

Chitinolytic activity tests were conducted in agar media containing 0.5 gram colloidal chitin. The steps of this test were preparing a solid chitin agar media previously described. One ose of bacteria from the selected pure bacterial cultures were inoculated in chitin agar using the spot method. Bacteria were incubated for 24 hours. Clear zones developed were observed and measured since chitinolytic bacteria are known to react upon colloidal kitin. Among all the pure bacterial strains, only two were selected with the highest chitinolytic activity index. The index was measured the formula proposed by Gohel [11] which was

$$\text{Chitinolytic index} = \frac{\text{average diameter of clear zone}}{\text{average diamter of bacterial colony}}$$

2.4 Anti fungal activity upon *Saprolegnia* sp.

Saprolegnia sp. was cultured by preparing PDA agar media (3.9 gram of PDA media added with 100 ml aquadest). The media was prepared in hot plate magnetic stirrer and added with chloramfenicol before solid (at around 45°C) and afterwards was cooled down in room temperature. *Saprolegnia* sp. was obtained from infected fish and cultured in the middle of the previously prepared plate and incubated for 48 hours. The antifungal activity of selected bacterial strain upon *Saprolegnia* sp. was conducted based on the method proposed by Elkahoui *et al.* [12]. The following steps were selected chitinolytic bacteria were streaked on PDA medium near fungal organism with a distance of 2 cm forming a rectangle surrounding the fungi. The bacteria inoculated plate was incubated at 30°C for 4 days. The resistance/clear zone was measured using a digital caliper.

2.5 Molecular Analysis of 16S rRNA

This step was conducted to identify bacterial isolates that possess the highest chitinolytic activity and able to prevent growth of *Saprolegnia* sp. The steps were culturing selected bacterial strains in liquid broth enhanced with colloidal chitin as much 2 gram in 1.3 gram of nutrient broth. These ingredients were added with 100 ml aquadest and mixed in a hot plate magnetic stirrer until homogenized. Liquid medium was autoclaved for 15 minutes in 121 °C. Two selected bacterial strains which had the best performance were cultured in this media and incubated for 48 hours at 37°C. This liquid culture was placed on an incubator shaker at 270 rpm. Bacterial DNA genome from the harvested bacteria was

isolated using the Protocol Wizard Genomic DNA Purification Kit from Promega with the following procedure. As much as 1 ml of the liquid cultured bacteria was placed in 1.5 ml microtube. The tube was centrifuged at 13.000 rpm for 2 minutes. Afterwards, the supernatant was removed and the pelleted result was processed. As much as 480 μ l 50Mm EDTA and 120 μ l lysozyme was added to this pellet. The mixture was incubated for 30-60 minutes at 37 °C. This mixture was then centrifuged for 2 minutes at 13.000 rpm. The supernatant was then removed and afterwards added with 600 μ l nuclei lysis solution. The next step was this mixture was incubated for 5 minutes at 80 °C and afterwards cooled down until room temperature. 3 μ l of RNase solution was then added and reincubated at 37 °C for 15-60 minutes. As much as 200 μ l protein precipitation solution was added afterwards the mixture was incubated on ice for 5 minutes. This mixture was then centrifuged at 13.000 rpm for 3 minutes. The supernatant was moved into a new sterile tube then added 600 μ l of isopropanol. The DNA pellet was then centrifuged for 2 minutes at 13.000 rpm. Afterwards 600 μ l of 70% ethanol was added and further centrifugation was conducted for 2 minutes at 13.000 rpm. The microtube was air dried in sterile room condition for 10-15 minutes. As much as 100 μ l rehydration solution was added then the mixture was incubated in a water bath at the temperature of 65°C for 40 minutes. Afterwards the sample was processed through an electrophoresis stage to identify the bacterial DNA genome from the selected isolates.

2.6 Amplification of DNA using PCR

Amplification of 16S rRNA encoding gene was conducted using the Polymerase Chain Reaction (PCR) to obtain material for further sequencing for bacterial identification purposes. The primer used was the universal 16S rRNA primer [13] with the nucleotide sequence as follows;

Primer Nucleotide Sequence (5'-3')

Forward primer 27 F:AGAGTTTGATCCTGGCTCAG

Reverse primer 1492 R: GGTTACCTTGTTACGACTT

The amplification of the 16S rRNA gene sequence from the DNA genom of the selected bacteria strains used the reaction formula of Nuclease Free Water 8 μ l, 2x KAPA2G Fast Ready Mix 12,5 μ l, Primer forward 27 F 0,5 μ M 1,25 μ l; Primer reverse 1492 R 0,5 μ M 1,25 μ l, DNA template 2 μ l. Total volume of the mixture was 25 μ l. Fragment amplification was conducted on the PCR with following program; Initialization 95 °C for 2 minutes (1 cycle), Denaturation 95 °C for 1 minute, Annealing 55 °C for 1 minutes, Extension 72 °C for 1 minute. Denaturation, annealing and extension was conducted for 30 cycles. Final hold was at 4°C.

2.7 Characterization of the Encoding Fragments of Chitinase from Bacterial DNA

In order to copy the encoding gene sequence of the chitinase enzyme from the DNA bacterial genom, the CHiE-F primer forward and CHiE-R primer reverse were used [14]. The sequence for these primers (5'-3') were

ChiE-F CTAGACAACCTTTTGTATAGGAGTGTGATATG

ChiE-R CGATTGATGAGGGCTAATTATAGTTTTACTTTG

The formula for reaction components of Chitinase encoding genes were Nuclease Free Water as much as 8 μ l, Go Taq

Green Master Mix (Promega) 12,5 μ l, Primer forward ChiEF (0,5 μ M) as much as 1,25 μ l, Primer reverse ChiER (0,5 μ M) as much as 1,25 μ l and DNA template as much as 2 μ l. Total volume obtained was 25 μ l. The steps to amplify encoding genes of chitinase enzymes was based on the following PCR program Initialization 94 °C for 3 minutes (1 cycle), Denaturation 95 °C for 1 minute, Annealing 55 °C for 40 seconds, Extension 72 °C for 1 minute. Denaturation, annealing and extension was conducted for 32 cycles. Final extension was at 72 °C for 5 minutes (1 cycle) while the final hold was at 4 °C (1 cycle).

2.8 Electrophoresis

Electrophoresis is a molecular separation technique which measure the size of DNA fragments. The stages for conducting electrophoresis were preparation of electrophoresis included a preparation of a total of 0.4 grams of agarose gel which is made at a concentration of 1% then put into a Schott bottle. A total of 40 ml TBE was added to the Schott bottle. Agarose gel was prepared using a hot plate magnetic stirrer until dissolved. Liquid agarose gel was poured into the existing trays and afterwards gel was conditioned with a perspex (comb). Comb-shaped perspex was removed when the agarose gel solidify. Solid agarose gel into the electrophoresis tank which contains TBE. Electrophoresis work was conducted by inserting markers and DNA amplification results in each agar wells. Tanks were closed and the electricity was activated by plugging wires into the to the socket. The side containing the amplification was given a negative current. The amount of current electrophoresis should not exceed 75 volts for 70 minutes to obtain the best electrophoresis result of genomic DNA isolates. When finished, the agarose gel is soaked in EtBr for 10-15 minutes. Agarose gel was then soaked in sterile distilled water for 15 minutes. After finishing the process, the agarose gel is removed using a spatula and drained. Agarose gel was observed using UV transilluminator. The amplification product electrophoresis process used DNA Ladder 1 kb (Geneaid) which could identify fragments ranging from 500 to 10,000 bp. PCR amplification using a 16S rRNA primer has an amplification target area of 1,500 bp followed by amplification of targeted chitinase encoding fragments at around 1120 bp amplification.

2.9 Data Analysis

After obtaining the data from sequencing process, the analysis was carried out using bioinformatic devices (Bioedit). Sequences were aligned with the sequence data at www.ncbi.nlm.nih.gov through the BLAST (Basic Local Alignment Search Tool). BLAST is a free web based program for searching sequence similarity and is a tool in gene identification and genetic character. BLAST can perform sequence searches via comparison with the DNA database in record time (<http://www.ncbi.nlm.nih.gov>). The data that has been obtained from the research results are then analyzed in a descriptive manner. BLASTN program was used to determine the type of the 2 best isolates which has a clear zone and can inhibit fungal growth *Saprolegnia* sp. and BLASTX to find out which bacteria have genes chitinase enzyme coding.

3. Results

3.1 Bacterial isolation

The result of the isolation of chitinolytic bacteria of waste shrimp and crab was successfully conducted with the 10⁻¹

dilution method. Bacterial strains observed showed several different colors of bacterial colonies which were colonies with different colors such as white, cream, milky white, yellow in one petri dish.

Based on the isolation process and bacterial purification, we obtained eight chitinolytic bacterial isolates, four isolates chitinolytic bacteria from shrimp waste and four isolates from chitinolytic bacteria from origin crab waste. To make it easier to distinguish, the chitinolytic bacteria from shrimp waste were coded as isolates A1, A2, B1, B2. Meanwhile, chitinolytic bacteria from crab waste were coded C1, C2, D1, D2.

The purification of chitinolytic bacteria was carried out. From this process, the selected pure strains were then collected and analyzed. Colony obtained in showed different colors of bacterial colonies. One bacterial colony were outgrowing the streak pattern (B2) while some bacteria colonies were similar although isolated from different source. Although possessing the same colors, some colonies had different intensity in white colors for example the color of B1 was more distinct compared to D2. In order to characterize further, Gram staining was conducted. Gram staining of bacterial candidates were a standard protocol in microbiology to detect and identify bacteria. Results of staining showed different characteristics of obtained pure strains of bacteria where some was showing Gram negative and also Gram positive. While shapes varied between coccus and rod shaped. Hence, bacteria showed potential differentiation of species and was possible to take further steps in bacterial identification.

3.2 Chitinolytic Activity Test

Chitinolytic activity test was conducted to observe bacterial isolates which possess chitinase enzymes by measuring clear zones produced in each sample. Measurements showed that from eight isolates of chitinolytic bacteria, there were three isolates possessing chitinolytic activity. Isolate B1 reached the highest value of 2.12 mm while D1 isolate was at 1.99 mm (Figure 1).

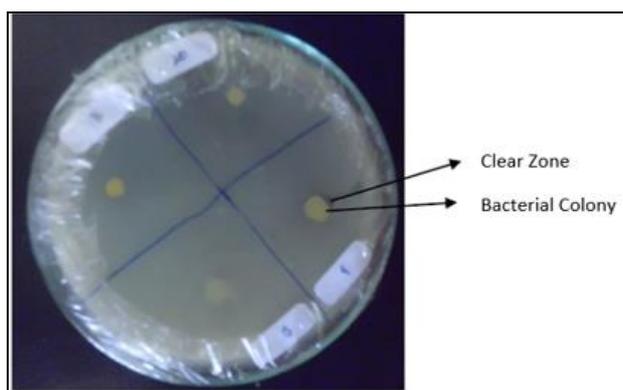


Fig 1: Test Result of Chitinolytic Activity

3.3 Anti *Saprolegnia* Activity Test

The anti *Saprolegnia* sp. activity test was conducted with the dual culture method. Results of clearance zone measurements upon *Saprolegnia* sp. Chitinolytic bacteria test upon *Saprolegnia* sp. showed that only 2 bacterial isolate namely strain B1 and D1 produced significant clear zones with values of 6.005 mm and 11.59 mm respectively. These chitinolytic bacteria was able to inhibit growth as shown in Figure 2. Other isolates namely A1, A2, B2, C1, C2 dan D2 was unable to inhibit growth, possibly caused by the difference of chitinase enzymes produced by each isolates. Isolates that

unable to inhibit growth showed that bacterial streaks were overgrown by *Saprolegnia* sp.

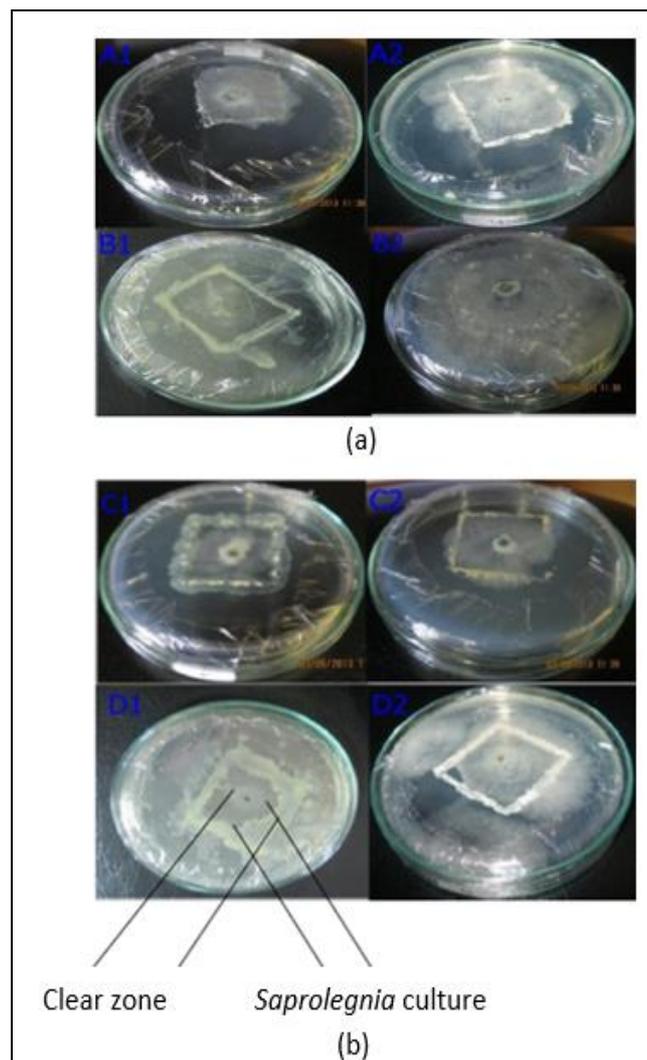


Fig 2: (a) *Saprolegnia* Inhibition Activity of Chitinolytic Bacteria Isolated from Shrimp Waste; (b) *Saprolegnia* Inhibition Activity of Chitinolytic Bacteria Isolated from Crab Waste

3.4 DNA Genome Isolation from Chitinolytic Bacteria Candidates

DNA genome isolation of bacterial candidates was conducted using Promega genome kit with the method of *Quick Protocol Wizard Genomic DNA Purification*. We have identified DNA bands with the potential candidates of B1 and D1 showed bands identified were relatively long fragments (above 10.000 bp compared to the 1 kb marker) containing exon and introns. The bacterial genome (DNA) isolates of B1 and D1 was then used for further identification which was the amplification of 16S rRNA genes.

3.5 16S rRNA Gene Amplification and Sequencing

The amplification of DNA was conducted with the *in vitro* PCR method using the 16S rRNA gene utilizing the designated primer. The primer for the 16S rRNA genes were universal primers with amplicon target of 1500 bp and 32 cycles during the PCR process (Figure 3). The final product of the 16S rRNA PCR process was then visualised using the electrophoregram. Based on the positive result in this stage, the further step of sequencing could be conducted.

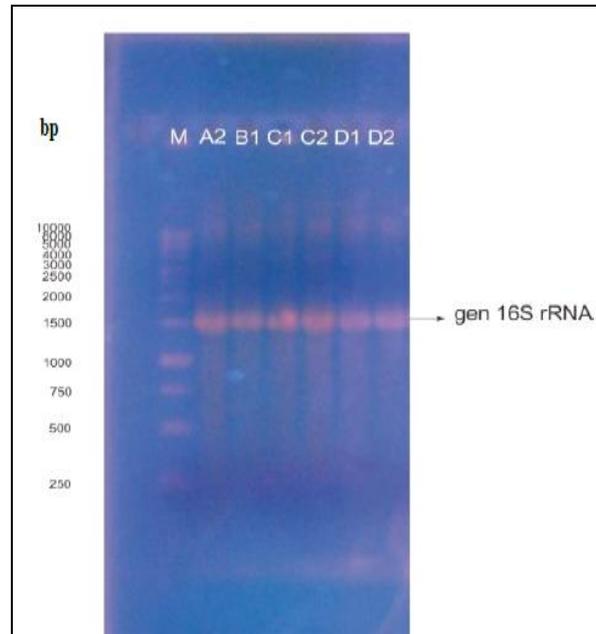


Fig 3: Electrophoregram from the PCR 16S rRNA Gene, with M as Marker, A2, B1, C1, C2, D1 and D2 Bacterial Isolate.

The further stage of this research was DNA sequencing to identify the nucleotides from the amplification process. Sequencing is important to decide the similarity of chitinolytic bacterial isolates based on 16S rRNA genes thus an identity of a certain gene with a gene sequence that has been thoroughly identified with library based data (gene bank). Two of the potential chitinolytic bacteria (sample B1 and D1) was isolated from crab waste. This sample was chosen since it possess a high anti *Saprolegnia* sp activity. The sequencing process was conducted at 1st BASE

Singapore with the standard operating procedure of product purification to eliminate contaminants. Purification of sample B1 and D1 conducted at 1st BASE was clarified and successfully showed clear bands. The purified PCR product (processed at 1st BASE) of the B1 and D1 showed that the DNA band was exactly at 1500 bp according to the target of the amplified 16S rRNA gene primer. This purified sample was then used for the sequencing processing. The 1500 bp nucleotide sequence of this product was able to identified since it was found registered in the existing gene bank.

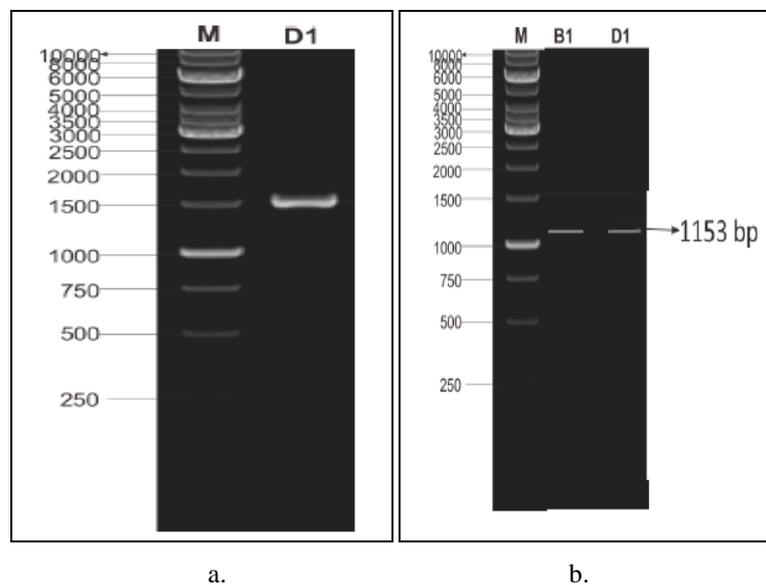


Fig 4: Electrophoregram of a the purified PCR sample of (a) B1 and (b) D1.

3.6 Bioinformatic Analysis of the 16S rRNA Gene for the Bacterial Candidates

The sequencing result obtained from sample B1 originating from shrimp waste and D1 originating from crab waste was further processed using the Bioedit software. After processing using the Bioedit software, we then read the results using the sequence scanner software version 1.0 (*Applied Biosystem, USA*). The nucleotide sequence obtained from the DNA fragment sequencing process was presented based on the

consensus shown in the *bioedit* program.

Result of alignment with the BLASTN program to verify identities with the chitinase gene from various bacteria that have been registered in the gene bank showed sample B1 (from shrimp waste) had 96% homology to *Bacillus thuringiensis* 960 bp (NR 043403.1) while sample D1 (crab waste) had 98% homology to *Bacillus cereus* (accession number NR 074540.1.).

3.7 Amplification and Sequencing of Chitinase Encoding Fragments

The functional domain of the chitinase coding gene in the original chitinolytic bacteria shrimp waste was at the position of 90-1050 bp, where this domain is located in the amplified sequence using the ChiE-F and ChiE-R primers. Through BLASTX program we respectively identified sample B1 dan D1 to *Bacillus thuringiensis* and *Bacillus cereus*. The PCR electrophoregram process of the encoding fragments of chitinase enzyme indicates that chitinolytic bacteria candidates of isolate B1 and D1 could correctly be amplified at a fragment size of 1153 bp. The fragment size of the chitinase coding gene is appropriate with the results of research conducted by Usharani [14] which was 1153 bp. Thus the genes successfully isolated from the bacteria from the shrimp and crab waste were referred to the chitinase coding gene.

4. Discussion

4.1. Anti *Saprolegnia* sp activity test.

Bacterial candidates which was identified as *Bacillus cereus* and *Bacillus thuringiensis* in this research showed high activity for anti *Saprolegnia*. The clearance zone between bacterial colonies and *Saprolegnia* was developed due to the chitinase enzymes which were secreted by bacteria to breakdown chitin macromolecules into smaller chitin molecules [15]. This is necessary for bacteria to obtain nutrition from smaller molecules. The chitinase enzymes are bounded to chitin particles hence chitin were degraded. However, difference in chitinolytic index among bacterial isolates was thought to be caused by the different rates of chitinase enzymes activity.

4.2. DNA Characterization

During the PCR process, correct primers should function in obtaining the target DNA intended to be amplified. Other than the primers, the temperature for the PCR process must be exact since temperature may affect process such as the annealing of primers. Inappropriate temperature may inhibit the primer to bond with the DNA or may cause primers to non specific location. The PCR Electrophoregram of 16S rRNA gene in showed that all bacterial isolated from shrimp and crab waste showing chitinolytic potential had positive results with genes amplified clearly at the fragment size of 1500 bp. The size of the gene was in accordance to the amplification target of the 16S rRNA primer [16]. However only the successful candidates were processed further. The 16S rRNA gene is often used to study taxonomy and phylogenetic since this gene is found in almost all bacterial species. The function of this gene does not change throughout history, thus it is categorized as a conserved sequence. Isolates that had a similarity more than 97% of the 16S rRNA sequence may be associated to the species in the Genbank [17]. However if the similarity was between 93% to 97%, the identification should only be limited to the genus level since it is possible that species may differ.

4.3. Chitinolytic Potential of *Bacillus thuringiensis* and *Bacillus cereus* Bacteria

The genus *Bacillus* is a bacterium that has a variety kinds of capabilities that can be developed on an industrial scale. *Bacillus* has great potential to be developed in the biotechnology industry because it has properties such as a wide temperature range for growth, capable for spore-

forming, cosmopolitan, resistant to antiseptic compounds, aerobic or facultative anaerobes, has various enzymatic abilities and some species are capable of biodegradation of many recalcitrant and xenobiotic compounds [18]. The main thing is that the *Bacillus* genus does not need relatively expensive growth medium. The enzymes produced by *Bacillus* including the enzyme alanine and formates, α -amylase, isoamylase, β -amylase, glucoamylase, chitinase, and cholesterol oxidase [19]. The types of *Bacillus* that are capable of producing the chitinase enzyme are *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus licheniformis* and *Bacillus subtilis* [20]. *Bacillus* is classified into the heterotrophic class of bacteria, namely protists which are unicellular, included in the group of microorganisms redusen or commonly referred to as decomposers [21]. *Bacillus thuringiensis* found in this study is a rod-shaped vegetative cell gram positive, are aerobic but generally facultative anaerobes, have flagella and forming spores [22]. Production of chitinase from *Bacillus thuringiensis* was possible due to shrimp waste as a carbon source [23]. The chitinolytic enzymes produced by *Bacillus thuringiensis* was also known to inhibit the fungus *Sclerotium rolfsii*. The chitinase enzyme is part of that important in fungal biological control because of the ability of chitinolytic bacteria that can degrade the fungal cell wall which has the main component in the form chitin [24]. *Bacillus cereus* identified in this study is a gram-positive rod-shaped bacteria with large cells and the length of 3-5 microns and width of 1 micron. These bacteria produce spores that are elliptical in shape and are located in the center of the cell. Spores only formed when there is oxygen in the environment (facultative aerobes). *Bacillus cereus* is a mesophilic organism that can grow on optimal temperature 30-35°C. *Bacillus cereus* has a locomotion in the form of two or more flagella which may surrounds the entire surface of the bacterial cell [25]. The chitinolytic potential of *Bacillus cereus* has been widely reported, of which can inhibit the growth of the fungus *Rhizoctonia solani* on plants [12]. Both bacteria were capable to inhibit *Saprolegnia* sp since it is a fungus with hyphae made of chitin. Chitinolytic enzymes in fungi play a role in physiological regulation at the time of cell division, differentiation, and microparasitic activity. Both bacterial candidates should play an important role to medicate or inhibit *Saprolegnia* sp in aquaculture.

5. Conclusions

Bacterial candidates obtained from crab and shrimp waste were successfully characterized in this study. *Bacillus cereus* and the *Bacillus thuringiensis* both possess the capability as chitinolytic bacteria and acts as a potential biological agent to overcome *Saprolegnia* through in vitro studies. Further steps for this study should observe the effects of these bacteria in *in vivo* experimental conditions upon fungal diseased fish. Hence, increasing the potential of these bacteria in aquaculture practices.

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