Identification of chitinolytic bacteria isolated from shrimp pond sediment and characterization of their chitinase encoding gene

A U Triwijayani¹, I D Puspita¹, Murwantoko¹ and Ustadi¹

¹Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora Gd. A4, Bulaksumur, Yogyakarta, Indonesia

Abstract. Chitinolytic bacteria are a group of bacteria owning enzymes that able to hydrolyze chitin. Previously, we isolated chitinolytic bacteria from shrimp pond sediment in Bantul, Yogyakarta, and obtained five isolates showing high chitinolytic index named as isolate PT1, PT2, PT5, PT6 and PB2. The aims of this study were to identify chitinolytic bacteria isolated from shrimp pond sediment and to characterize the chitinase encoding gene from each isolate. The molecular technique was performed by amplification of 16S rDNA, amplification of chitinase encoding gene and sequence analysis. Two chitinolytic bacteria of PT1 and PT2 were similar to Aeromonas bivalvium strain D15, PT5 to Pseudomonas stutzeri strain BD-2.2.1, PT6 to Serratia marcescens strain FZSF02 and PB2 to Streptomyces misionensis strain OsiRt-1. The comparison of chitinase encoding gene between three isolates with those in Gen Bank shows that PT1 had similar sequences with the chi1 gene in Aeromonas sp. 17m, PT2 with chi1 gene in A. caviae (CB101) and PT6 with chiB gene in S. Marcescens (BJL200).

1. Introduction

Chitinolytic bacteria are microorganisms enable to degrade chitin using chitinase enzymes. Chitinolytic bacteria can be found in the various type of environment, such as rhizosphere, phyllosphere, soil and water environment includes oceans, lakes and shrimp ponds [1]. Shrimp pond sediment is a distinct environment for chitinolytic bacteria bioprospecting, as it contains high chitinous substrate, as well as other organic residues, resulted from the degradation of crustacean shells during an intensive culture. Chitinolytic bacteria isolated from shrimp pond sediment had been reported, such as Vibrio alginolyticus [2], Bacillus licheniformis [3] and Chitinimonas taiwanensis [4]. Previously we isolated 46 chitinolytic bacteria from the pond sediment of local shrimp aquaculture plant of PT. Indokor Bangun Desa located in Bantul, Yogyakarta, in which five of them showed high chitinolytic index [5].

Chitinase, an enzyme that catalyzes the breakdown of β-1,4-glycosidic bond in chitin polymer, is produced by bacteria to hydrolyze chitin into its monomer or dimer that will be further used as a nutrient for growth [6]. Chitinase is also used to produce chitin hydrolysate products that have been widely applied in the fields of health, food and agriculture [1]. Therefore, the productions of bacterial chitinase gain much interest and have been explored by many researchers. Chitinase encoding genes, called the chitinase gene (chi gene), from various bacteria, have been studied and used to construct recombinant enzyme to understand the characteristics of the enzyme. Three chitinase encoding gene chiA, chiB and chiC from Serratia marcescens was reported previously [7]. The chitinase encoding gene chiA in Aeromonas caviae was also reported [8]. This study aimed to identify chitinolytic bacteria from shrimp pond sediments and characterize the chitinase encoding genes of each isolate.
2. Materials and Methods

2.1. Bacterial cell preparation
Five isolates of chitinolytic bacteria were obtained from the collection of Laboratory of Quality and Safety of Fisheries Product, Universitas Gadjah Mada. The isolates were previously isolated from shrimp pond sediment in PT. Indokor Bangun Desa located in Bantul, Yogyakarta [5]. One loop full of bacteria suspension in glycerol stock was transferred into 7 mL of chitin medium and incubated at 37 °C for 24 h.

2.2. DNA isolation
As much as 1 mL of cell culture from the previous step was inserted into a 1.5 mL microtube and centrifuged at 14,000 × g for 1 min. The supernatant was removed and cell pellet was used for genomic DNA isolation using Presto TM Mini gDNA Bacteria Kit (Geneaid) following manufacturer’s protocol. The cell pellet was added with 200 μL of Gram + buffer solution that was previously mixed with lysozyme 4 mg mL⁻¹. The mixture was homogenized and incubated for 30 min at 37 °C (inverted every 10 min). Then, 20 μL Proteinase K solution was added to the mixture, homogenate and incubated for 10 min at 60 °C (inverted every 3 min). A 200 mL GB Buffer solution was added to the sample solution and homogenate for 10 sec. The sample solution was incubated at 70 °C for 10 min (inverted every 3 min). Then, 200 μL of ethanol absolute was added to the sample solution and mixed gently. The sample was moved into the GD column then centrifuged at 14,000 × g for 2 min. Collection tubes containing liquids were discarded. The GD column was assembled into a new 2 mL collection tube. A 400 μL W1 buffer solution was added to the GD column, centrifuged at 14,000 × g for 30 sec, discarded the liquid in the collection tube and placed back the GD column into the collection tube. Then, wash buffer (600 μL) solution was added to the GD column, centrifuged at 14,000 × g for 30 sec, discarded the liquid and continued to centrifuge the column for 3 min at a rate of 14,000 × g. The GD column was moved into a clean 1.5 mL microtube. The pre-heated Elution Buffer solution of 100 μL was added to the GD column and allowed to stand for 3 min until the solution was absorbed. The sample was centrifuged at 14,000 × g for 30 sec to obtain the purified DNA.

2.3. Amplification of 16S rDNA fragment
The purified DNA from the previous step was amplified by means of PCR using the Kapa Biosystem® PCR kit. The PCR mixture consisted of 6 μL ready PCR mix (consisted of Taq polymerase, dNTP, Mg²⁺ and loading dye), 5 μL ddH₂O, 0.5 μL forward primer, 0.5 μL reverse primer and 0.5 μL DNA template. The universal primer of 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') was used in this step. The mixture was inserted into the thermocycler (T100 TM Biorad) following the process of pre-denaturation (95 °C for 3 min), denaturation (95 °C for 30 sec), annealing (55 °C for 30 sec), elongation (72 °C for 1.5 min), final extension (72 °C for 5 min) and infinite hold (until 12 °C). The process of denaturation, annealing and elongation were repeated for 30 cycles.

2.4. Electrophoresis
The size of resulting amplicon was confirmed by electrophoresis using 1 % agarose gel in Tris Boric EDTA (TBE) solution added with 0.75 μL fluorosafe. The formed agarose gel is transferred to mini-gel electrophoresis. A 3 μL of the marker and 5 μL of amplicon were incorporated into the well. The process was held for 15 min at 100 Volts. The band formed on the gel was visualized using UV Transilluminator. The size of the band produced from the sample was obtained by comparing with the size of the marker.
2.5. Molecular identification of the 16S rDNA sequence
The 16S rDNA fragment was sent to First Base Laboratory Singapore for sequence analysis and matched with the nucleotide database available at Gen Bank using BLAST tool in NCBI and further processed using MEGA version 5. The identified bacterial species provided basic information for the design process of primers.

2.6. Molecular identification of the putative chitinase encoding gene
The specific primers were designed for amplification of the putative chitinase encoding gene of each isolate. The nucleotide sequences of a chitinase gene from five reference bacteria of the same genus were obtained from Gen Bank. The sequence was analyzed using BLASTX (NCBI) to identify the active site of each reference gene to be included for the consideration for primer design. Then the chitinase genes of references were aligned using BioEdit software and the primers were designed based on the conserved domain. Primers characteristic was analyzed by Oligocalculator software to identify the GC content and melting temperature (T_m). All designed primers were confirmed not to have self-complementary and self-annealing sequence that potential to form a hairpin loop. The design of primers was sent to First Base Laboratory Singapore to be assembled.

Table 1. The primers used for amplification of the putative chitinase encoding gene.

| Genus    | Primer                                           | T_m (°C) | % GC |
|----------|--------------------------------------------------|----------|------|
| Aeromonas| 1218F (5'-GAGAAACAACAGCCTACGC-3')               | 53.8     | 55   |
|          | 2450R (5'-CCTCGTCATGCCGGTTG-3')                 | 51.9     | 65   |
| Serrata  | 4F (5'-TCCRMACGYAAACGTTATGG-3')                 | 53.5–58.8| 43–57|
|          | 1475R (5'-TCATTAYGCGYASRCGGCCACYTTCAGCC-3')     | 62.9–68.8| 52–66|

The amplification of the putative chitinase encoding gene was performed by means of PCR using the Kapa Biosystem® PCR kit following the manufacturer’s protocol as mentioned previously. Genomic DNA was used as the template and specific primers (table 1) was used for each isolate. The thermal cycle used for amplification was as follow: pre-denaturation (95 °C, 3 min), denaturation (95 °C, 30 sec), annealing (56–57 °C for Aeromonas and 58 °C for Serratia, 30 sec), elongation (72 °C, 1.5 min), final extension (72 °C, 5 min) and infinite hold (12 °C). The process of denaturation, annealing and elongation were repeated for 30 cycles. The size of the amplicon was confirmed by electrophoresis on 1 % agarose gel and sent to First Base Laboratory Singapore for sequence analysis.

2.7. Characterization of the putative chitinase encoding gene
The sequence of putative chitinase encoding gene from each isolate was matched with the reference gene database in BLAST (NCBI) for identification. Further, the characteristic of putative chitinase encoding gene was also determined using BLATSX.

3. Results and Discussion

3.1. Identification of chitinolytic bacteria with 16S rDNA
Five chitinolytic bacteria showing high chitinolytic index value from the previous experiment was identified [5]. The visualization of PCR product from 16S rDNA fragment of all isolates on agarose showed a single band of 1,500 bp. Molecular identification based on BLAST results on 16S rDNA sequences in Gen Bank showed that PT1 and PT2 were similar to Aeromonas bivalvium strain D15, PT5 to Pseudomonas stutzeri strain BD-2.2.1, PT6 to Serratia marcescens strain FZSF02 and PB2 to Streptomyces misionensis strain OsiRt-1 (table 2).

An isolate having a sequence similarity of 16S rDNA greater than 97 % with the reference sequence represent the same species, meanwhile a sequence similarity between 93–97 % may represent the similar identity at the genus level but differ at the species level [9]. All five bacteria isolates identified in this research showed a similarity of 99 % or more with the reference, however
further morphological and biochemical identification is necessary to support the molecular identification results.

**Table 2.** Molecular identification of five chitinolytic bacteria isolated from pond sediment.

| Isolates | Species reference | Identity | Accession number |
|----------|-------------------|----------|-----------------|
| PT1      | *Aeromonas bivalvium* strain D15 | 99 %     | KF500920.1      |
| PT2      | A. *bivalvium* strain D15        | 99 %     | KF500920.1      |
| PT5      | *Pseudomonas stutzeri* strain BD-2.2.1 | 100 %   | LC125170.1      |
| PT6      | *Serratia marcescens* strain FZSF02 | 99 %     | KU145144.1      |
| PB2      | *Streptomyces misionensis* strain OsiRt-1 | 99 %   | KU321340.1      |

3.2. Identification of the putative chitinase encoding gene

The primers for the amplification of putative chitinase encoding gene were designed based on the conserved domain in chitinase genes from five reference bacteria of the same genus. In this research, we only observed the putative chitinase encoding gene of three isolates, namely PT1, PT2 and PT6, because the sequence of a chitinase gene from reference bacteria of other two isolates was not available by the time when the research was conducted. Figure 1 shows that the target gene was amplified. The length of the targeted putative chitinase encoding gene from PT1 and PT2 was 1,157 bp, and PT6 was 1,500 bp (figure 1).

![Agarose gel electrophoresis of the chitinase encoding gene](image)

**Figure 1.** Agarose gel electrophoresis of the chitinase encoding gene. Lane M: DNA Marker, lane 1: PT1 strain, lane 2: PT2 and lane 3: PT6.

Bioinformatics analysis of chitinase encoding genes of PT1, PT2 and PT6 is shown in table 3. The chitinase encoding gene of isolate PT1 had a similarity of 85 % to *chil* gene of *Aeromonas* sp. 17m and PT2 showed 84 % similarity to *chil* gene of *A. caviae* CB101. The low percentage of similarity indicates that chitinase encoding gene possessed by PT1 and PT2 was not similar to chitinase gene of the reference bacteria. Previously, PT1 and PT2 were identified as *A. bivalvium*. This result indicates that the variation of chitinase encoding gene might be presented at the species level. By the time of this research was conducted, the reference of chitinase encoding gene from *A. bivalvium* was not available in Gen Bank.
The chitinase encoding gene of isolate PT6 showed the similarity of 99% to chiB gene of Serratia marcescens BJL200. This result was in accordance with the result of molecular identification in which PT6 was identified as Serratia marcescens. The ChiB of S. marcescens is an exochitinase catalyzing the hydrolysis of chitin into GlcNAc and (GlcNAc)2 [7]. S. marcescens possesses three types of chitinase, namely ChiA (exochitinase), ChiB (exochitinase) and ChiC (endochitinase), in which each of them has a specific characteristic and exhibits different mechanisms to hydrolyze chitin [7]. In the chitinolytic system of S. marcescens, these three types of chitinase work synergistically to produce the final product of GlcNAc from the hydrolysis of chitin.

**Table 3.** Identification the putative chitinase encoding gene of PT1, PT2 and PT6.

| Isolates | Chitinase Gene | Identity | Accession Number |
|----------|----------------|----------|-----------------|
| PT1 | Aeromonas sp. 17m chi1 gene | 85 % | JN987186.1 |
| PT2 | A. caviae CB101 chi1 gene | 84 % | AJ534329.1 |
| PT6 | S. marcescens BJL200 chiB gene | 99 % | Z36295.1 |

Characteristics of each putative chitinase encoding gene were further analyzed by comparing the deduced amino acid with the amino acid sequence of the reference chitinase protein provided from BLASTX (table 4). The deduced amino acid from PT1 and PT2 putative chitinase encoding gene were similar to GH18 chitinase from Aeromonas molluscorum, however, the similarity of the deduced amino acid sequence from PT1 was higher (93 %) than PT2 (86 %). Meanwhile, the deduced amino acid from PT6 putative chitinase encoding gene had a 93 % similarity to Glyco18 of chitinase B from S. marcescens. The deduced amino acid sequence from all isolates showed 10 active sites with a different position between PT6 and other two isolates, however, they had the identical amino acid residues in the active sites. The amino acid residues located at the active sites were tyrosine, phenylalanine, aspartic acid, glutamic acid, methionine and tryptophan. The identical amino acid residues at the active sites indicate that they may have a similar mechanism of hydrolyzing chitin polymer.

**Table 4.** Characteristics of the putative chitinase encoding gene from three chitinolytic bacteria isolated from pond sediment.

| Isolates | Region name chitinase | Identity | Active site | Amino acid residues | Accession number |
|----------|-----------------------|----------|-------------|---------------------|-----------------|
| PT1 | GH18 chitinase (Aeromonas molluscorum) | 93 % | 163, 191, 311, 313, 315, 388, 390, 391, 444, 539 | Y, F, D, D, E, M, Y, D, Y, W | WP_005896739.1 |
| PT2 | GH18 chitinase (A. molluscorum) | 86 % | 163, 191, 311, 313, 315, 388, 390, 391, 444, 539 | Y, F, D, D, E, M, Y, D, Y, W | WP_005896739.1 |
| PT6 | Glyco 18 (Chitinase B Serratia marcescens) | 93 % | 10, 51, 140, 142, 144, 212, 214, 215, 292, 403 | Y, F, D, D, E, M, Y, D, Y, W | AIP98318.1 |

**4. Conclusion**
Five chitinolytic bacteria obtained from shrimp sediments were identified as Aeromonas bivalvium (PT1 and PT2), Pseudomonas stutzeri (PT5), Serratia marcescens (PT6) and Streptomyces misionensis (PB2). The comparison of the putative chitinase encoding gene between three isolates with chitinase gene in Gen Bank shows that PT1 had similar sequences with the chi1 gene in Aeromonas sp. 17m, PT2 with chi1 gene in A. caviae (CB101) and PT6 with chiB gene in S.
marcescens (BJL200). The deduced amino acid from PT1 and PT2 putative chitinase encoding gene was similar to GH18 chitinase from Aeromonas molluscorum and PT6 was similar to Glyco18 of chitinase B from S. marcescens. The deduced amino acid sequence showed that they had the identical amino acid residues in the active sites.

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