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Research Article

Screening and Identification of Sponge-Associated Chitinolytic Bacteria by Forming Chitosan from Manado Bay, Indonesia

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Abstract

Background and Objective: Chitosan can be produced through the enzymatic process catalyzed by chitin deacetylase which can be produced by bacteria. The biotransformation of chitin to chitosan by bacteria is interesting because the process is economical and environmentally friendly. This study described the potential of sponge-associated bacterium capability in degrading chitin and forming chitosan. **Materials and Methods:** The bacteria were isolated from sponge *Cribrochalina* sp. at Manado Bay, Indonesia. In the screening of the chitinase activity of bacteria, chitin media was used. Meanwhile, the transformation of chitin to chitosan was tested by using Chitinase Degrading Activity media. Molecular identification of bacteria was based on 16S rRNA gene sequences. **Results:** The results showed that the SS1, SS2, SS3, SS4 and SS5 bacterial isolates could degrade chitin based on chitinolytic indexes. These five bacteria could also form chitosan exhibited through the presence of chitosan in the form of precipitation in the fermented broth of bacteria. SS1 had the highest chitinase activity based on the chitinolytic index identified as *Bacillus subtilis* (100% identity), hence it is called *B. subtilis* strain SS1. The partial rRNA gene sequences data were deposited at GenBank under accession number MN999892. **Conclusion:** The bacteria strain isolated from *Cribrochalina* sp. can be utilized in degrading chitin and form chitosan which could be a promising candidate for an economical and eco-friendly process of chitosan.

Key words: *Bacillus*, chitin deacetylase, chitinolytic bacteria, chitosan, identification, screening, sponge

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Chitin is a linear chain of long polysaccharides which comprises of β-(1-4)-linked D-glucosamine, while chitosan is N-acetyl-D-glucosamine residues obtained by deacetylation of chitin[1-3]. Chitosan can interact with other molecules in solution and have great economic value because of their biological activities and biomedical applications such as antifungal, antibacterial, anti-inflammatory and anti-cancer activities, antioxidant and chelating abilities[4,5] as well as fat-binding and film-forming capacities[6]. Chitosan is widely used as a preservative, a packaged addictive, supplement for diets and encapsulation for nutrients[7]. Chitosan is also used for protecting plants from bacteria, fungi and viruses and can further be used as a regulator for plant growth and additive on fertilizer[8-10]. In treating the wastewater, the fat-binding and chelating potential of chitosan are used to remove fat dyes and heavy metals in water[11]. Moreover, the medical application uses it due to its biocompatibility, biodegradability and non-toxic properties[12,13].

Currently, the chemical NaOH pyrolysis method is used to produce chitosan from chitin. There are notable problems for this method, such as high energy consumption, environmental pollution and the poor quality of the resulting chitosan. Thus, an economical and environmentally friendly method for producing chitosan with good quality is the best alternative. The economical and environmentally friendly method can utilize Chitin Deacetylase (CDA). Chitin deacetylase is the enzyme that catalyzes the conversion of chitin to chitosan using the deacetylation of N-acetyl-D-glucosamine residues. The acetyl groups ($COCH_3$) in chitin are removed through a process called deacetylation to yield chitosan[3]. The chitin degradation technique by chitin deacetylase offer the possibility of being controlled, well-defined chitosan oligomers and polymers and produce high yields with minimal environmental impacts[14-19].

The existence of chitin deacetylase activity had been reported in several fungi[20-22], some insects[23-25] and bacteria[14,26-28]. Bacteria are often found living in symbiosis with various marine organisms, such as marine sponges[29-34]. Bacterial biomass can comprise up to 40% of the sponge tissue. They fulfill diverse functions influencing the sponge's physiology and ecology and greatly contributed to the evolutionary success of the sponges[35]. They play key roles in nutrient cycling[28,36] and could produce bioactive secondary metabolites[37-41] which can in turn be used by the sponge as chemical defences[42] and to adapt in the environmental stresses[43,29-31].

Sponge-associated bacteria also have the potential to produce chitinase and forming chitosan by their chitin deacetylase's function. Sulawesi Sea in Indonesia, specifically Manado Bay, is home to a variety of sponges, one of them is *Cribrochalina* sp. However, so far bacteria associated with *Cribrochalina* sp. are still poorly investigated; especially their capabilities to produce chitinase and forming chitosan have not yet been reported. Whereas, the new sources of chitin deacetylase bacteria is necessary to be found and applied on economical and eco-friendly processes to produce chitosan. In this present study, we reported our attempts to screen and identify sponge-associated chitinolytic bacteria from Manado Bay and its capability of forming chitosan.

MATERIALS AND METHODS

This study was conducted from May, 2019-February, 2020 at the Laboratory of Marine Molecular Biology and Pharmacology, Faculty of Fisheries and Marine Sciences, Sam Ratulangi University Manado, North Sulawesi, Indonesia.

Bacterial isolation: The research used *Cribrochalina* sp. sponge-associated bacteria that were isolated previously in our laboratory. The sponge was taken from Manado Bay, Indonesia. There were 5 bacterial isolates, associated with sponge *Cribrochalina* sp. denoted as SS1, SS2, SS3, SS4 and SS5. Before the analysis, the bacteria were re-cultured on Nutrient Agar (NA-Merck) and the morphological characteristics of the colony, such as color, shape, edge and elevation were observed to confirm the individual colony of bacteria growing on agar medium.

Screening the chitinase-producing ability: The ability of the bacteria to produce chitinase was observed by the capability of the bacteria to degrade chitin on chitin medium (NA contains 2% colloidal chitin). Each of the bacteria was inoculated into a chitin medium followed by incubation at 37°C for 24 hrs. The clear zone formed around the colony after incubation indicated the capability of the bacteria to produce chitinase and be avowed as a chitinolytic index. The chitinolytic index was determined by dividing the diameter of the clear zone with the diameter of bacterial colony[44].

Transformation of chitin to chitosan by bacteria: The ability of bacteria to degrade chitin into chitosan was tested by using Chitinase Degrading Activity (CDA) media (1 g of yeast extract, 0.4 ammonium sulphate and 0.15 g of potassium dihydrogen phosphate, 50 mg of colloidal chitin). Fifty milliliter of CDA

media was filled in 250 mL capacity flasks, then sterilized. One milliliter of 0.1 O.D.600 suspensions of each bacterial isolate was inoculated on the CDA medium. As a control one of the CDA media was not inoculated with bacteria. All the flasks were incubated at the rotary shaker for 48 hrs at 25°C. After that, all the flasks were taken for further analysis[14].

Chitosan recovery from cultured media of bacteria: The cultured media broth (CDA medium) was centrifuged at 12000 rpm for 15 min. Obtained pellets comprised of a mixture of bacteria, chitin and chitosan. The pellet is then transferred to the tube. A total of 10 mL of NaOH 0.1 N was added, mixed thoroughly and autoclaved at 121°C for 60 min. The tubes were centrifuged again, at 12000 rpm for 15 min, the pellet was taken afterward. The pellet was then mixed with 10 mL of 2% acetic acid in the clean tube and incubated on a rotary shaker for 12 hrs at 25°C. The tubes were centrifuged at 12000 rpm for 15 min. Then, the pellet was removed and 10 mL of the supernatant was neutralized using 1 N NaOH. Upon adding 1 N NaOH, white precipitate appeared indicating the formation of chitosan[14].

Chitosan estimation qualitatively: The tubes contained the white precipitate were centrifuged at 5000 rpm for 15 min. After white precipitates were obtained, distilled water was used for neutralization to pH 7. The precipitates in 0.5 mL of distilled water at pH 7 were then re-suspended and dried at 55°C for 2-4 hrs. Twenty-three drops of potassium iodide solution were added to dry precipitate and then mixed. Two-three drops of 1% H_2SO_4 solution were then added. After the addition of iodine/potassium iodide, the precipitate color changed to dark brown and when 1% of H_2SO_4 was added, the precipitate color changed to dark purple. This showed the presence of chitosan[14,44,45].

Identification of bacteria: Molecular identification was carried out to identify the bacteria. The isolation of the genomic DNA bacterium used QIAprep Miniprep kit (Qiagen, Germany, Hilden). The genomic DNA was used as the template for the PCR-amplification of 16S rRNA gene region with the universal primer pair (Integrated DNA Technologies-IDT, Singapore) 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACT-3')[3,46-47]. The PCR condition consisted of initial denaturing at 95°C for 6 min followed by 35 cycles of 95°C for the 30 sec, 52°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 10 min. Electrophoresis on 1% agarose gel was used to observe the size-corrected PCR products with a 1 kB DNA ladder as a marker (Solis Bio Dyne, Tartu, Estonia). The PCR product was then sent to the First-Base Co., Selangor, Malaysia for sequencing. DNA sequences of the 16S rRNA gene quality were assessed using Sequence Scanner version 2.0 Software (Applied Biosystem) and the sequence traces were trimmed, assembled and edited using MEGA 7. The consensus DNA was compared with the sequence databases using the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), aligned with similar sequences and then constructed the phylogenetic tree by neighbor-joining using MEGA 7 program[48].

RESULTS AND DISCUSSION

All of the SS1, SS2, SS3, SS4 and SS5 bacterial isolates have chitinase activity which is characterized by the formation of clear zones around bacterial colonies when the bacteria are grown on media containing chitin (Fig. 1). The chitinolytic indexes of the SS1, SS2, SS3, SS4, SS5 were 2.40, 2.00, 1.75, 2.00 and 1.60, respectively. Chitinolytic index showed the ability of the bacteria to degrade chitin or indicated chitinase activity that is produced from the bacteria to break-down chitin compound in medium[49]. Each bacterium showed different chitinolytic activity, therefore the chitinolytic index are also varied, from weak to strong[49-51]. The chitinolytic index of SS1 was above 2 which is considered a strong activity[52].

All of the bacteria also could degrade chitin and form chitosan. This is shown through the presence of chitosan in the form of precipitation in the fermented broth after specified incubation. The precipitation is chitosan which is

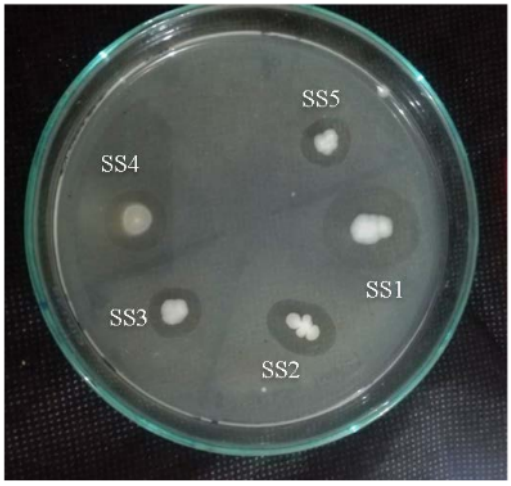

Fig. 1: Spot test of bacteria to produce chitinase on chitin medium

characterized by a reaction that gives rise to a dark purple colorization when 1% H_2SO_4 was added[14,52]. The mechanism of color-binding of chitin is through the absorption and it depends on the pH. In normal conditions, the amine group of chitins has both neutral charge (-NH_2) and cationic (-NH_3^+). In acidic conditions, chitin polymer has a positive charge and the amine group (NH_2) is protonated. To keep a neutral condition, the polymer of chitin will have ion exchange with negative ions from anionic colorant to form electrostatic interaction[52]. The smallest unit of chitin, i.e., chitobiose, which consists of two molecules of N-acetyl-D-glucosamine has two hydroxyl groups, one carbonyl group and one amine group, which is the group that will form a reactive linkage with iodide to form color complex[53].

The ability of the bacteria to degrade chitin and form chitosan indicated that the ability of the bacteria to produce chitin deacetylase (CDA)[14] that catalyzed the conversion of chitin to chitosan by the deacetylation of N-acetyl-D-glucosamine residues. Hence, the bacterial isolates from this research could be classified as bacteria strain of CDA-producing abilities. Deacetylation is the most important step in chitosan synthesis[3]. Therefore, the key to chitosan quality is the deacetylation process of chitin. The deacetylation process of chitin by bacteria could replace the degradable chitin to chitosan by chemicals process which used NaOH for deacetylation.

Several fungi[20-22] and insects[23-25] have reported producing chitin deacetylase. Using CDA-producing fungi for chitin N-deacetylation theoretically could also avoid the problem of chitin degradation to chitosan by chemicals process. However, the capabilities of most fungal strains to produce CDA were weak and their fermentation requirements were tricky[14]. Therefore, the bacteria strain function of CDA-producing is better than the fungal strains' function. Bacteria are easy and quick to grow in a large-scale fermentation system and cost-effective as well. Process modification and optimization can be done very easily and for application, it is not necessary to purify the enzyme[54,55]. Therefore, the bacterial isolates from this research could be an effective technique for degrading chitin and form chitosan.

In this present study, SS1 was chosen to be identified, in which the bacterium had the biggest chitinolytic index. The colony of SS1 on the NA has a filamentous shape, white with a fusiform edge and flat elevation. The DNA gene of the SS1 was successfully carried out and the 16S-rRNA gene was successfully amplified. Amplicon 16S rRNA sequences of SS1 on 1% agarose gel was shown in 1500 bp (Fig. 2). Its length was enough for informatics purpose[46,47]. Based on the 16S rRNA gene sequences that was compared with the

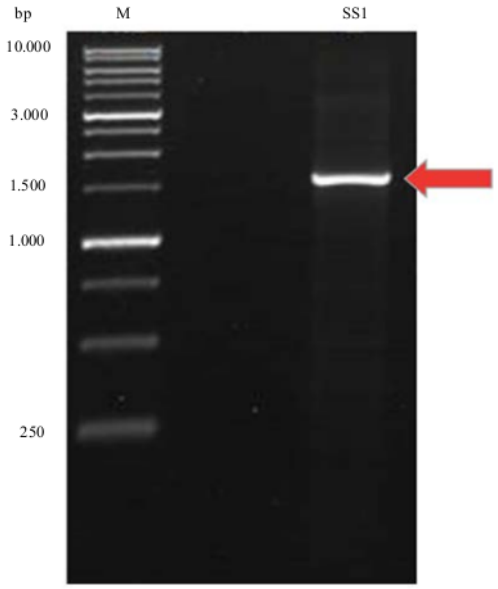

Fig. 2: Agarose gel electrophoresis (1%) of the 16S-rRNA gene PCR product of the SS1 bacterial isolate

sequence databases using the BLASTN program, SS1 indicated similarity (100% identity) to *Bacillus subtilis*. A similar result was seen on the phylogenetic analysis as shown in phylogenetic tree (Fig. 3). It showed that the SS1 and *B. subtilis* were in the same group. Therefore, SS1 is called *B. subtilis* strain SS1. The 16S rRNA gene sequence data of the *B. subtilis* strain SS1 have been deposited at NCBI/GenBank under accession number MN999892.

The genus *Bacillus* is a rod shape, phenotypically large, heterogeneous collection of Gram-positive or Gram-variable spore-forming, aerobic or facultative anaerobic bacteria[56]. *Bacillus* spp. maybe divided into nine groups (groups I-IX), based on phenotypic features linked to 16S rRNA gene sequence similarity or their high-level phylogeny. *Bacillus subtilis*, *B. licheniformis*, *B. pumilus*, *B. altitudinis*, *B. amyloliquefaciens*, *B. mojavensis*, *B. safensis*, *B. atrophaeus* and *B. circulans* belongs to group I[57-59].

Various species of *Bacillus* populate on marine and coastal environments[60-62]. *Bacillus* sp. have been successfully isolated from various habitats such as shrimp waste[49] and marine[63] and are known to have the highest chitinolytic activity[64,49,63]. Furthermore, *B. subtillis* was associated with the sponge and could be a good source to produce cytotoxic metabolites[65], antibacterial[66] and anticholinesterase compounds[67]. Additionally, *B. subtilis* has potent chitinase to bio-convert of chitin-containing wastes efficiently.

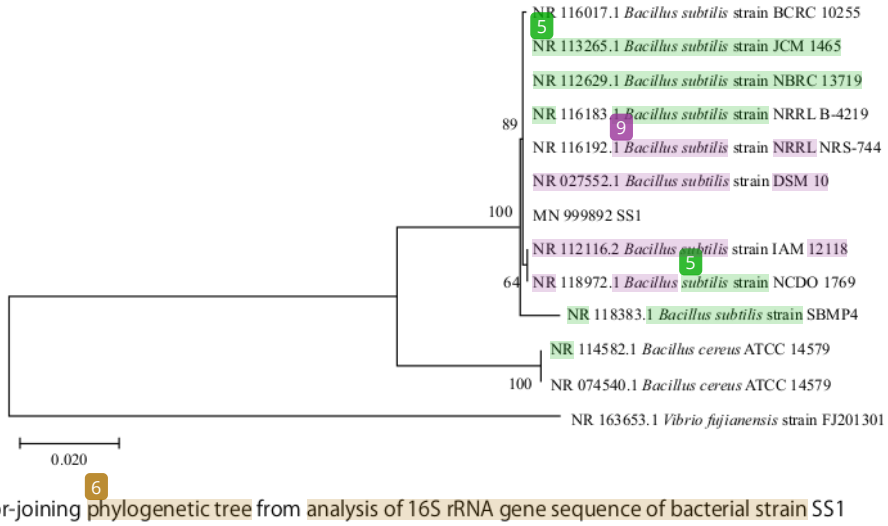

Fig. 3: Neighbor-joining phylogenetic tree from analysis of 16S rRNA gene sequence of bacterial strain SS1

Chitinase that was produced from *B. subtilis* was found to be more excellent than the well-known chitinase A from *Serratia marcescens* and the commercial chitinase that was prepared from *Streptomyces griseus* by degrading crude crab shell using grinding techniques and protease pretreatment[68].

The finding of this study presented the ability of the bacteria strain isolated from *Cribrochalina* sp. which includes *B. subtilis* strain SS1 to produce chitinase and form chitosan. It will be useful in many industrial applications such as pharmacy and medicine, agriculture, water treatment, food sciences, etc where it will be able to be applied for chitosan production by economical and eco-friendly process. Furthermore, the characterization of chitosan which is formed by these bacteria will be conducted at our laboratory.

CONCLUSION

The results obtained from the present study confirmed that SS1, SS2, SS3, SS4, SS5 bacterial isolates that were associated with sponge *Cribrochalina* sp. had the capability of degrading chitin and form chitosan. The SS1 was indicated as *B. subtilis* strain SS1. All the bacteria could act as CDA-producing in forming chitosan and be a promising candidate for an economical and eco-friendly process of chitosan.

SIGNIFICANCE STATEMENT

This study discovered that bacterial isolates that were associated with the sponge *Cribrochalina* sp. from Manado Bay, North Sulawesi showed chitinase activity and were capable of degrading chitin to form chitosan. This study will assist researchers in developing a method that utilizes bacteria biologically within the production of chitosan. Thus, the production of chitosan in an economical and eco-friendly processes may be arrived at.

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