Isolation and Molecular Identification of Chitinolytic Bacteria from Ronto

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Abstract. Ronto is a traditional food from South Kalimantan made of processed *rebon* shrimp. The production of Ronto relies on the fermentation process done by various types of microorganisms, especially bacteria. With shrimp as the main ingredient, Ronto has a high salt concentration and low pH level. Therefore, there is a potential in the exploration of chitinolytic bacteria with specific characteristics in Ronto. This study aimed to isolate and identify chitinolytic bacteria in Ronto. Ronto that was used in this experiment had a pH of 5. The isolation of chitinolytic bacteria using chitin agar obtained 10 isolates, namely RNT1, RNT2, RNT4, RNT5, RNT6, RNT7, RNT8, RNT9, RNT10, and RNT11. The chitinolytic index of these isolates ranged from 1.1 to 2.8. The highest chitinolytic index was shown by isolate RNT8. Molecular identification using a partial 16s rRNA gene shows that all isolates were similar to *Bacillus* sp.

1 Introduction

Shrimp is the main commodity of fisheries in Indonesia. However, the high demand for processed shrimp products, in turn, results in the increased amount of shrimp waste in the form of shrimp shells. Shrimp shells contain chitin as much as 15-20%. Chitin is a polysaccharide, a linear polymer composed of its monomers, namely β-1,4-N-acetylglucosamine [1]. Chitin can be used in various fields of pharmacy and biotechnology by simplifying its compounds using the enzyme chitinase produced by chitinolytic bacteria. Various explorations of chitinolytic bacteria from numerous sources have been carried out, but the majority of isolation of chitinolytic bacteria comes from nature.

Few research is done on the isolation of chitinolytic bacteria from processed fishery products. One processed fishery product that has the potential as a source of chitinolytic bacteria is Ronto. Ronto is a fermented product made from *rebon* shrimp, salt and rice. This product has the form of a paste, pink to brownish-purple in color, has a shrimp paste-like aroma, but tends to be acidic and has a sour and salty mixture [2]. Ronto has the same characteristics as *rusip*. Research on the identification of chitinolytic bacteria from *rusip* has been conducted by [3]. In the study, molecular identification was used and the results were suspected to be isolates in *rusip* which were chitinolytic bacteria in the form of *Bacillus*

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cereus, *Bacillus thuringiensis*, *Stenotrophomonas maltophilia*, *Enterobacter cloacae*, and *Pseudomonas stutzeri*.

Figure 1 shows what Ronto looks like. This study aimed to isolate and identify chitinolytic bacteria from Ronto. The best bacteria generated from this study can be used to degrade chitin into simpler compounds, and may even have further and wider implications in the fields of biotechnology and pharmacy.

Fig.1. Ronto

2 Materials and methods

2.1 The preparation of chitin

The shrimp shell waste that has been obtained must be washed thoroughly and then dried under the hot sun until it is completely dry before it can be used for deproteination. The deproteination stage is done by soaking the shrimp shells into the NaOH solution while immediately stirring them. What follows next is the immersion process, in which the shells are processed using a hot plate stirrer at a temperature of 60-70ºC for 60 minutes. After 60 minutes, the shrimp shells are washed again to neutral pH, weighed, and dried in the oven for 24 hours at 60ºC. The next stage is the demineralization stage. This stage is done by immersing the shells into a concentrated HCl solution for 120 minutes at a temperature of 25-30ºC. Stirring is done every 10 minutes for 1 minute. The results are filtered for re-demineralization. The shells that have been demineralized are used in the depigmentation stage, which is carried out by placing the demineralized shrimp in the Erlenmeyer containing NaOCl, soaking it for 5 minutes at room temperature, and washing it with water to neutralize the pH. Then the shell was weighed and dried in the oven for 24 hours at 60ºC. The results obtained were in the form of chitin powder, and the chitin made was examined by the FTIR test.

2.2 Preparation of chitin colloidal

Shells that had passed the deproteination, demineralization, and depigmentation stages were used in the manufacture of colloidal chitin. Colloidal chitin was made by mixing 20 grams of chitin into 37% concentrated HCl, and then homogenized at room temperature for 60 minutes. Once homogeneous, it was filtered using glass wool, and then the supernatant was poured into 800 ml of distilled water at 4ºC. Next, the chiffon process was done every day until the pH was neutral. After the neutral pH was obtained, a centrifuge was set at a speed of 6000 rpm for 20 minutes at 4ºC. Furthermore, the colloidal chitin which had been centrifuged could be stored in the refrigerator and ready to use as an ingredient for making the chitin agar medium.

2.3 Isolation of chitinolytic bacteria from Ronto

Chitinolytic bacterial isolation was carried out using a direct method proposed by [4]. A total of 10 grams of sample Ronto were mixed in 90 ml of 0.9% NaCl. Then, the mixture was diluted three times (10-1, 10-2, and 10-3), and as much as 0.1 ml of suspension was taken from each dilution on the microtube by using a spuite and it is spread on the chitin agar medium. The chitin making was based on the research conducted by [5] in which the material for making chitin consisted of 0.1% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.1% NaCl, 0.7% $(NH_4)_2SO_4$, 0.05% yeast extract, 2% colloidal chitin, and 1% agar. Furthermore, the suspended medium was incubated for 48 hours at 37ºC and every single colony formed was purified by being transferred to the chitin medium, so that it was subsequently cultured on the Tryptone Soya Broth (TSB) medium. The purified isolates were stored in glycerol as a stock culture and in TSB as a working culture.

2.4 Screening of chitinolytic bacteria

The chitinolytic bacterial screening was carried out to determine the chitinolytic index produced from each isolate obtained. The first step was to measure Optical Density (OD) on each isolate that had been cultured for 24 hours in the TSB medium. The OD measurements were carried out using a wavelength of 600 nm. Previously, the chitin medium was prepared, so that the paper dish was placed on the top of four points in one plate. Then the culture in each isolate was taken using a white tip and then placed on the paper dish. Observations were carried out every 24 hours for three days by measuring the colony diameter (DK) with the diameter of the clear zone (DZ). The chitinolytic induction calculation formula used was IK = DZ/DK. Then, the results obtained were recorded.

2.5 Molecular identification

The DNA extraction was carried out using the PrestoTM Mini gDNA Bacteria Kit Protocol. The isolation began with the sample preparation. The sample in the form of bacterial culture from each isolate was put into 1.5 ml of the microtube, and then centrifuged for 1 minute at a speed of 14,000-16,000 x g. Furthermore, the supernatant was removed, and a Gram l Buffer solution of 200 µl/sample was added and mixed with 4 mg/ml lysozyme into the microtube. Afterward, the solution isortexed until mixed, and then incubated at 37ºC for 30 minutes (inverted every 10 minutes). Next, 20 µl of Proteinase K solution was added to the microtube containing the sample solution, and it was vacuumed until dissolved. Then the solution was incubated for 15 minutes at 60ºC and inverted every 3 minutes. As much as 200 µl of GB Buffer solution was added to the sample solution and vortexed for 10 seconds. The homogeneous solution is then incubated for 10 minutes at 70ºC (inverted every 3 minutes). The solution produced from the lysis stage was added to 200 µl of absolute ethanol, and it was mixed slowly. If sediment appeared, the precipitate was dissolved through pipetting. The GD column was attached to the collection tube. The sample was moved into the GD column. Moreover, the sample solution was centrifuged for 2 minutes at a speed of 14,000-16,000 x g. Then, the collection tube was removed and replaced with a new collection tube. Then, a total of 400 l of W1 buffer solution was added to the sample solution and centrifuged for 30 seconds at a speed of 14,000-16,000 x g.

Then the solution at the bottom was removed before a 600 µl wash buffer solution was added to the sample solution in the GD column. The sample solution was again centrifuged at a speed of 14,000-16,000 x g for 30 seconds. Then the bottom solution was again discarded and centrifuged for 3 minutes at a speed of 14,000-16,000 x g so that the sample in the GD column was dried. The dried sample on the GD column was then transferred to a 1.5 ml

sterile microtube. As many as 100 µl of the pre-heated Elution Buffer solution was added to the sample. The sample was then allowed to stand for 3 minutes so that it permeated. The sample was then centrifuged at a speed of 14,000-16,000 x g for 30 seconds to obtain pure DNA. The sample that had gone through the DNA extraction stage was used for 16S rDNA gene amplification by employing the PCR technique by using a thermal cycler machine. The solutions included in the PCR tube are as follows:

PCR Mix	: 6 µl
Dye loading	: 5 µl
Primary 27F	: 0.5 µl
Primary 1492R	: 0.5 µl
Samples	: 0.5 µl
Total volume	: 12.5 µl

The primer use is a pair of universal primers consisting of 27 F (5 '- GAGTTTGATCMTGGCTCAG-3') and 1492R (5 '- CGGTTACCTTGTTACGACTT-3') with a target of 1500 bp amplicon. Furthermore, the solution in the sample must be homogenized by spinning it until there was no solution attached to the tube wall. After that, the PCR tube was inserted in the thermal cycler. Temperature and time settings for each PCR process and the number of cycles carried out during the experiment are presented below.

Predenaturation	: 3 minutes at 95^{o}C
Denaturation	: 30 seconds at 95^{o}C
Annealing	: 30 seconds at 55^{o}C
Elongation	: 1.5 minutes at 72^{o}C
Final extension	: 5 minutes at 72^{o}C
Infinite hold	: 12^{o}C until the results were obtained

The pre-denaturation, denaturation, annealing, and elongation stages were repeated 30 times, and then the obtained PCR products were confirmed by using the electrophoresis stage by visualizing under UV light. The electrophoresis stage used agarose gel made by mixing 0.15 grams of 1% agarose gel into 15 ml of TBE solution. Then, the solution was homogenized and heated to boil. The boiling solution was cooled, but before it firmed, 0.75 µl fluorosafe was added. Then, the solution was slowly mixed again and then poured into an agar mold with masking tape attached on both sides. A comb was put in the solution to make eight holes. Then, the solution was let set to form a gel and to make sure there were no bubbles and dirt in the solution.

Furthermore, the formed agarose gel was transferred into the electrophoresis mini-gel. Then, as many as 3µl markers consisting of 2µl markers and 1µl loading dye were mixed by pipetting and put into a well. The process was carried out for 15 minutes at 100 Volt. Then the gel was visualized with UV light using a UV transilluminator. Visualization produced a single band which indicated that a positive result with the target length matched the size of the specified DNA fragment. After the electrophoresis process was completed, the PCR product was ready for the sequencing stage. Nitrogen base sequence was sent to the 1st BASE Singapore. The sent samples contained 30 µl of PCR DNA in each sample. Sequenced data, in the form of soft-files, were sent through an email. The data obtained from sequencing results were processed using the Bio-edit application. Then, the sequencing results were confirmed using BLAST-N on the Gene Bank. The results showed similar sequences of chitinolytic bacterial isolates with the results of the reference bacterial species sequences found in the database.

3 Results and discussion

Before the chitinolytic bacterial isolation, the pH of the Ronto is measured using the pH paper, and the result of pH Ronto is 5. As many as 10 pure isolates of chitinolytic bacteria

were resulted from the isolation of chitinolytic bacteria. Purification is done by transferring a single colony formed into chitin medium to be sterile using an ose. Then Ronto was incubated at room temperature for 3 days, and 10 isolates were identified, namely RNT1, RNT2, RNT4, RNT5, RNT6, RNT7, RNT8, RNT9, RNT10, and RNT11. These 10 pure isolates were later screened to find out their highest chitinolytic index. The results of screening show that isolate RNT8 has the highest chitinolytic index of 2.8, while RNT9 has the lowest chitinolytic index of 1.1 mm.

Fig. 2. Pure Isolate of RNT8

Fig. 3. Pure Isolate of RNT5

After that, all isolates were identified molecularly. The results of chitinolytic bacterial isolates which have been extracted from DNA were used as a template at the 16S rDNA fragment amplification. The results of the amplification are in the form of PCR products which have been electrophoresed and then detected by ultraviolet (UV) light. The results of visualization can be said positive if a single band that matches the size of the target DNA fragment is formed. The target amplicon of the 16S rRNA gene utilized is 1500 bp. The following is a picture of the amplification of the 16S rDNA fragment as seen in Figure 4.

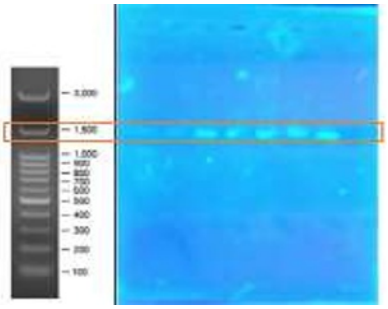

Fig. 4. The results of electrophoresis on RNT1, RNT2, RNT4

Figure 4 shows that the left part is the marker acting as a control, then followed by replicated 1 of RNT1, replicated 2 of RNT1, replicated 1 of RNT2, replicated 2 of RNT2, replicated 1 of RNT4 and replicated 2 of RNT4. From the results, it is apparent that a single band is formed on RNT1, RNT2, and RNT4 isolates. In addition to that, Figure 5 shows the results of the electrophoresis on RNT5, RNT6, RNT7, RNT8, RNT9, RNT10, and RNT11.

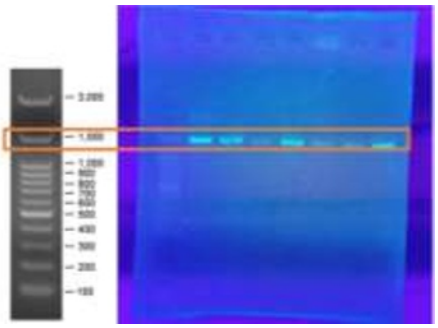

Fig. 5. The Results of the Electrophoresis on RNT5, RNT6, RNT7, RNT8, RNT9, RNT10, and RNT11, RNT2, RNT4

Figure 5 shows the marker as a control in its left part, and RNT5, RNT6, RNT7, RNT8, RNT9, RNT10 and RNT11 in the right part. The visualization results display that all isolates produced a single band, and they appear according to the target used, namely at the length of 1500 bp. The result of the electrophoresis 1500 bp has been in accordance with previous research, in which [6] state the size of the 16s rRNA gene amplicon in bacteria is between 1500-1600 bp. Isolates that form a single band were used at the sequencing stage. The results of the 16S rRNA gene sequencing are in the form of a chromatogram, and therefore to find out the results, the data were analyzed by using the BioEdit application and BLAST analysis. Table 1 shows the BLAST analysis results.

Table 1. The results of the BLAST analysis

Isolate	Species	% Identity	Accession Number
RNT1	*Bacillus cereus* strain ZCGT07	99.93	MK267335.1
RNT2	*Bacillus mycoides* strain A18	99.93	MK267335.1
RNT4	*Bacillus anthracis* strain SAK4	99.82	MG706137.1
RNT5	*Bacillus mycoides* strain A18	100	MG598443.1
RNT6	*Bacillus cereus* strain EC3	99.93	MK894129.1
RNT7	*Bacillus cereus* strain SSW1	100	MK533796.1
RNT8	*Bacillus thuringiensis* strain QZL38	100	CP032608.1
RNT9	*Bacillus cereus* strain SSW1	100	MK533796.1
RNT10	*Bacillus cereus* strain ZCGT07	100	MK267335.1
RNT11	*Bacillus mycoides* strain A18	100	MG598443.1

The results of the identification showed that all isolates were bacteria with the genus *Bacillus spp.* which shows different forms of the colony on agar medium. Colony colors are generally white to yellowish or gloomy white, and the edges of colonies vary, but generally are uneven. Besides, the surface is rough and not slimy, some even tend to be dry, wide, and not shiny. The shape of the colonies and their size varies greatly depending on the type. In addition, each type also shows different abilities and resilience in dealing with environmental conditions, such as resistance to heat, acid, salt, and so on. *Bacillus spp.* is very potential to be developed in the biotechnology industry because it has properties such as a wide temperature range of growth, spore-forming, cosmopolitans, resistance to antiseptic compounds, aerobic or facultative anaerobes, diverse enzymatic abilities, and capability of biodegradation against many compounds in some species.

In addition, the main feature of *Bacillus spp.* is that it does not need a relatively expensive growth factor [7]. *Bacillus sp.* is a bacterium that often contaminates food, causing food poisoning. Research conducted by [8] reveals that the type of bacteria from the genus *Bacillus* includes *B. Cereus* and *B. Huringiensis* found in rice-based products. The chance of *B. Cereus* and *B. Thuringiensis* in Ronto can be very large because Ronto is also a rice-based product with shrimp and salt in. Besides, the results of the molecular identification indicate the possibility of the chitinolytic bacteria in Ronto, namely *B.cereus*, *B.mycoides*, *B.thuringiensis*, and *B.anthracis*. According to [9]), the four types of these bacteria have a close kinship and to distinguish them biochemical tests can be carried out. Therefore, the results of this molecular identification seem to be certainly unconfident because it needs further research.

4 Conclusion

The results of isolations in Ronto, a food product based on shrimp, rice and salt, obtain 10 isolates, namely RNT1, RNT2, RNT4, RNT5, RNT6, RNT7, RNT8, RNT9, RT10, and RNT11 all of which are bacteria in genus *Bacillus spp.*

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