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Isolation and production of proteases from bacteria isolate from Bekasam and Rusip (Traditional Food from South Sumatera, Indonesia)

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Abstract

The research aims to assay enzyme activity of bacteria from traditional fermentation product (bekasam and rusip). The bacteria were inoculated in skim milk agar (SMA), and will produce clear zone. The best isolates from 9 isolates of bekasam and 9 isolates from rusip will be choosen, and the best result from bekasam and rusip were A3K2 and B3K2, respectively. Index proteolitic of A3K2 and B3K2 isolates were 10.0 and 7.0, respectively. Optimum time production of protease from A3K2 and B3K2 were 16 h and 32 h, respectively.

Keywords: Bekasam; Isolation; Production; Protease; Rusip

1. Introduction

Indonesia has many regional fisheries products that are very rich in benefits, in addition to increasing the selling value and maintaining storability, also contain many nutritional values that are good for our body. One of the provinces of South Sumatra was famous for its fishery products which use fermentation methods, such as bekasam and rusip

Bekasam was a fermented fishery product that utilizes lactic acid bacteria and salt as a selection of microorganisms. In general, used products were made by mixing fish, the main ingredients were added rice and salt, which were then placed in tightly closed containers at room temperature and then stored for five to seven days. Lactic acid bacteria contained in used products were very important in the fermentation process because lactic acid bacteria are antimicrobial which can inhibit pathogenic bacteria and decomposing bacteria[1].

Rusip was a fermented fish product, using anchovy raw material. In general rusip was made on a household scale, namely during the fish season. Besides salt, another ingredient added was palm sugar which can function as a source of energy and nutrients needed by bacteria that play a role in the fermentation process. Rusip was usually consumed as a mixture for chili sauce, either by being cooked first or directly consumed as a side dish without cooking (raw). Rusip was ready to be consumed after being stored for at least 1 week[2]. During the bacterial fermentation process that grows a lot in rusip products is the group of lactic acid bacteria and the rest of the other halophilic bacteria and sulfur bacteria such as *Sulfulobus* sp and *Bagiotoa* sp which are resistant to high salt levels[3].

Some microorganisms that have been known to produce proteases for commercial applications were *Bacillus, Lactobacillus, Pyrococcus, Termonospores Rhizopus, Mucor, Endothia* and *Aspergillus*[4]. So important was this enzyme that it is necessary to look for enzymes from microbes with different habitats so that the resulting enzyme is expected to have a unique character to meet the industrial needs of agricultural, chemical and medical products. Protease studies of microbes have been carried out such as proteases from swamp water bacteria[5], bacteria from swamp soil[6], *Bacillus*

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liceniformis F11 bacteria from shrimp waste[7], bacteria from the soil[8]. One source of this enzyme is the microbes from fermantation of rusip and bekasam which allow to produce enzymes. Research on proteases of bacteria from traditional food fermetation products from South Sumatera (bekasam and rusip) has not been done, so this research is important to do

2. Materials and methods

2.1. Isolation of Bacteria from Bekasam and Rusip

Isolation bacteria from bekasam and rusip was carried out according to the method of Baehaki et al.[9], samples from bekasam and rusip prepared and diluted from 10-1 to 10-4. For the first dilution, the used sample and rusip were weighed as much as 1 gr each, then put in a test tube, add 9 ml aquadest and homogenize. Then 1 ml was taken to put in the second test tube and 9 ml of aquadest was added, and so on until the 4th tube (dilution 10-4). From the 4th tube, 1 ml was taken to spread on LB media, then incubated at an incubator at 37°C, then tested the ability of the protease activity.

2.2. Proteolytic Index

Protease index was measured by method of Setiawan[10]. The bacterial isolate was then taken one ose using a toothpick and then inserted into the Lauria Bertani (LB) + skim milk (SMA), bacteria were grown and incubated at an incubator at 37 °C for 24 h. Bacteria that produce extracellular prosthetic enzymes are bacteria that have clear zones around bacterial colonies. The diameter of the clear zone and colony was measured using a ruler in units of millimeters (mm). The measurement of the diameter of the clear zone and colony was carried out 3 times the measurement of different diameters and the results of the measurements were summed and averaged. Proteolytic activity of bacterial isolates was determined by calculating the Enzyme Activity Index (IAE) by measuring the diameter of the clear zone and diameter of the colony.

2.3. Protease Assay

Protease activity was measured by method of Bergmeyer et al.[11] using the Hammerstein casein substrate 2% (b/v). The procedure for testing protease activity is: reacting 0.2 ml of the enzyme with 1 ml of casein Hammerstein substrate and 1 ml of buffer. The reaction mixture was incubated at 37 °C for 10 min, then 0.2 M TCA was added. Then the solution was re-incubated at 37 °C for 10 min, followed by centrifugation at a speed of 5.000 rpm 10 min. The supernatant was taken from the centrifugation mixture and added to the test tube containing 0.4 M Na_2CO_3 then added folin ciocalteu reagent (1: 2) and incubated at 37 °C for 20 min. The incubation results were measured by a spectrophotometer at 578 nm. One unit of protease activity is defined as the amount of enzyme that can produce one µmol of tyrosine products per min at the optimum measurement conditions.

3. Results and discussion

3.1. Microbial Isolation from Bekasam and Rusip

Microbial isolation was carried out on bacteria found in used and used fishery products. Samples were obtained from three different places with sample codes A1, A2, A3, B1, B2, B3. Samples were taken to the laboratory for dilutions from 10-1 to 10^{-4}, samples were spread in petri dishes using NA media to determine the presence of bacteria in the sample, incubation for 24 h on the sample. Bacterial isolates from bekasam and rusip can be seen in Table 1.

Table 1 Bacterial colonies from Bekasam and Rusip

Sample	Bacteria Isolate
Bekasam	A1K1, A1K2, A1K3, A2K1, A2K2, A2K3, A3K1, A3K2, A3K3
Rusip	B1K1, B1K2, B1K3, B2K1, B2K2, B2K3, B3K1, B3K2, B3K3

Based on Table 1, the bacteria that grow in each colony have a different shape and size of growth, while the results of the measurements will be taken one of the best treatments for each sample.

3.2. Proteolytic Index

Proteolytic index were carried out to determine whether or not a bacterium produced proteases which were characterized by the presence of clear zones around the colony using skim milk agar, which then measured proteolytic indices by measuring the diameter of the clear zone and diameter of the colony[12]. Protein hydrolysis is indicated by the presence of a clear zone around bacterial growth. Measurement of proteolytic index by compare the diameter of the clear area divided by the diameter of the colony. Based on the results of the study of 9 bacterial isolates, 7 bacterial isolates were obtained which had a proteolytic enzyme activity index on while 2 other isolates did not show proteolytic activity. The proteolytic index of bacteria isolate from bekasam can be seen in Table 1, which has an index of proteolytic enzyme activity namely A1K1, A1K2, A2K1, A2K2, A3K1, A3K2 and A3K3.

The highest index of proteolytic enzyme activity was shown in A3K3 isolates. The activity of proteolytic enzymes characterized by the presence of clear zones around the colonies can be seen in Figure 2. Baehaki et al.[6], that the proteolytic activity of swamp bacteria grown on skim milk agar was seen from the presence of clear areas around the colonies formed. Proteolytic index of bacterial isolates from bekasam can be seen in Table 2.

Table 2 Proteolytic Index of Bacteria Isolate from Bekasam

Bacteria Isolate	Proteolitic Index
A1K1	2.3
A1 K2	5.4
A1 K3	-
A2 K1	2.3
A2 K2	3.6
A2 K3	-
A3 K1	6.9
A3 K2	10.0
A3 K3	9.6

Whereas in rusip of 9 bacterial isolates, 3 isolates had proteolytic activity, namely B1K1, B2K2 and B3K2, which had the highest proteolytic enzyme activity index in B3K2 bacterial isolates, the results can be seen in Table 3.

Table 3 Proteolytic Index from Bacteria Isolate from Rusip

Bacteria Isolate	Proteolitic Index
B1 K1	3.0
B1 K2	-
B1 K3	-
B2 K1	-
B2 K2	4.7
B2 K3	-
B3 K1	-
B3 K2	7.0
B3 K3	-

Table 3 shows that not all isolates of used have protease activity, 18 isolates from bekasam and rusip taken from each selected isolate based on the size of the clear zone produced from each of these isolates. Selected isolates were A3K2 from bekasam with proteolytic index was 10.0 and B3K2 from rusip with proteolytic index was 7.0, differences between bekasam and rusip, from the results of the study it could be seen that the protease activity in bekasam was higher than rusip, this was due to differences in materials, manufacturing methods and product locations. even though the results of the fishery products are fermented with the same harvest time range.

The proteolytic test results showed that samples from the three different sites that had the largest proteolytic index existed in the A3K2 and B3K2 samples. This was due to differences in place with temperature, time and storage in different bekasam and rusip products so that it could affect the level of proteolytic growth on the product.

3.3. Protease Production from A3K2 and B3K2 Isolates

Production of protease was carried out on Lauria Bertani (LB) media after being cultured in the same medium until the absorbance value reached 0.8. Observations were carried out every 8 h for 56 h at 37°C at a speed of 120 rpm. The enzyme that has been produced by bacteria was separated from bacterial cells by centrifugation using a cold centrifuge. Through this technique, deposits will be seen due to the presence of gravity, while the enzyme will remain in the supernatant. Further testing of protease activity, while for bacterial growth was observed in absorbance values with λ = 578 nm. The time for protease production from A3K2 isolate was shown in Figure 1.

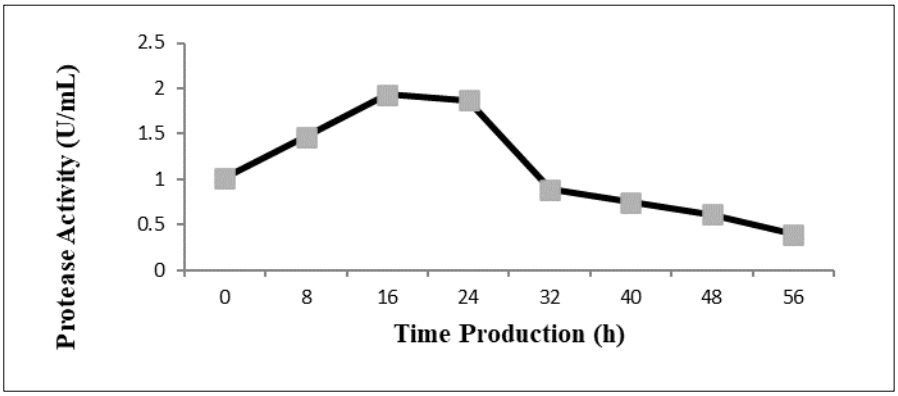

Figure 1 Optimum Production Time of Protease from A3K2 Isolate from Bekasam

Determination of optimum production time was carried out at the production time of 0, 8, 16, 24, 32, 40, 48, and 56 h by isolates used in A3K2. Based on the measurement results can be seen in Figure 1, the optimum production time of the protease enzyme used in each selected isolate, the A3K2 isolate is known to have the highest optimum activity occurring at 16 h at 1.933 IU/ml when the bacteria reach the stationary phase. so that from this result it can be determined when the next enzyme harvest was at the 16 h. While the optimum activity of B3K2 rusip isolates occurred at 32 h at 3.040 IU/ml can be seen in Figure 2. When the next enzyme harvest at the 32 h of B3K2 activities from rusip. The time for production from B3K2 isolate can be seen in Figure 2.

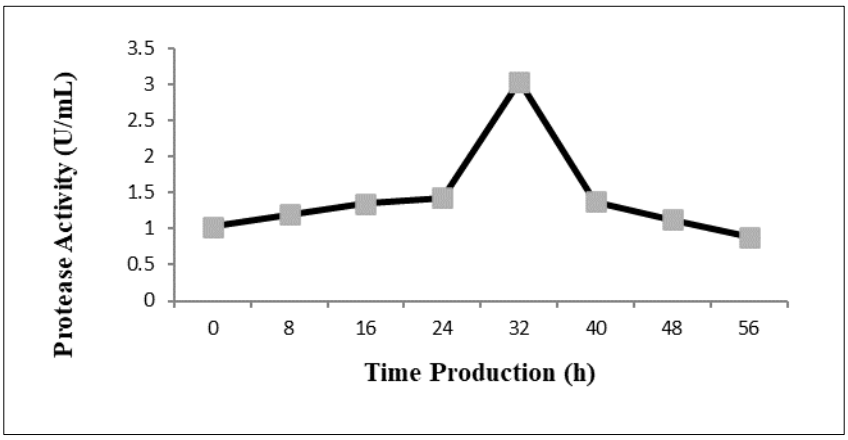

Figure 2 Optimum Production Time of Protease from B3K2 Isolate from Rusip

Protease was produced by cell growth, the more cells grow, the higher the protease. Figure 1 and 2 shows the relationship between incubation time the bacteria grows to the activity of the protease enzyme produced by the formation of the log phase where in the log phase all cells undergo division. Every generation passed, the number of cells doubled to the peak at the 16th hour for A3K2 isolate and 32nd hours for B3K2 isolate so that the stationary phase was formed.

The growth and production of proteases is observed by observing the data on cell tubules at a wavelength of 600 nm which was measured every time interval. The growth of *L. plantarum* SK beginning with the log phase was the phase of the synthesis of enzymes by cells used for metabolizing metabolites[13]. Pommerville[14] states the log phase occurs when all cells in a culture experience binary fission. Every generation passed, the number of cells increases twice and the graph increases in the form of a straight line or logarithmic graph. The activity of the protease enzyme increases until it reaches its maximum point at the 12th hour which represents the stationary phase of 3.18 U/mg protein. Proteolytic activity in lactic acid bacteria was carried out at 37 °C for 12 h[15].

4. Conclusion

The bacterial isolates from bekasam and rusip produced the best isolates of each bacterial isolate containing proteases namely A3K2 and B3K2. The optimum production of A3K2 isolates was at 16 h, and protease activity was 1.933 IU/ml. The optimum production of B3K2 isolates was at 32 h, and protease activity was 3.040 IU/ml.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Data Availability

All datasets generated or analyzed during this study are included in the manuscript and/or the Supplementary Files.

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