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RESEARCH ARTICLE

ISOLATION AND MOLECULAR IDENTIFICATION OF PROTEOLYTIC BACTERIA IN WADI FERMENTATION PRODUCTS OF DIGESTIVE ORGANS OF EEL (*Anguilla* sp.) BASED ON 16SrRNA GENE

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Abstract

In the field of food and health, protease enzymes have an important function in the process of tenderizing meat, reducing glut and treating type II diabetes. Utilization of biological resources such as plants, animals and microorganisms such as bacteria is carried out to produce new sources of protease enzymes. This study aims to isolate the protease enzyme-producing bacteria contained in the wadi fermented product of the digestive organs of eel after 7 days of fermentation. Bacterial isolation was carried out by culture method using Nutrient Agar (NA) media and obtained 5 bacterial colonies with unique morphology. The selection test for protease-producing bacteria was carried out using Skim Milk Agar (SMA) media and 3 isolates of proteolytic bacteria were obtained, with the highest protein hydrolysis index of 1.85 owned by isolate WFA51 (Wadi-fermented Anguilla 51). ntification of WFA51 isolates was carried out molecularly using olymerase Chain Reaction (PCR) with the target of amplification of the 16S rRNA gene using the Polymerase Chain Reaction (PCR) method with the result of 1 band measuring ~1500. The results of the 16S rRNA gene sequencing of WFA51 bacterial isolates using the Sanger method showed 1206 bp DNA sequences with 83% similarity and 5 query cover with Acidovoraxlacteus strain M36 bacteria. From the results of this study, it can be concluded that the wadi fermented product of the digestive organs of eel (Anguilla sp.) is a source of proteolytic bacteria, one of which is Acidovorax sp. WFA51.

Introduction:-

Technological advances in the field of genetic engineering, enzyme applications, biotechnology and fermentation technology have led to an increase in the use of enzymes in the food and health industries.(1)Proteases are one of three groups of commercial enzymes whose total sales are up to 60% as catalytic agents and others.(2)Consumption

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of enzymes in Indonesia in 2017 reached 2,500 tons with import costs of 200 billion, plus an increase of up to 7% per year.[3]

The increasing need for enzymes continues to occur, but Indonesia has not been able to produce protease enzymes on a large scale for these needs.[2] The wide market and biodiversity in Indonesia are opportunities for the development of protease enzymes.[4]Therefore, in an effort to overcome dependence on imports of protease enzymes, the use of biological resources in Indonesia.[5]

New protease enzymes can be produced by plants, animals and microorganisms such as bacteria and fungi. Plants are the largest source of protease enzymes (43.85%), then bacteria (18.09%), then fungi (15.08%), animals (11.15%) and viruses (4.41%).[6] One of the processes in obtaining protease enzymes in microorganisms is to isolate proteolytic bacteria.[7] Utilization of wadi fermentation in the digestive organs of eel is carried out with high concentrations of salt without the addition of microorganisms.[4]So that enzymes will be produced in the digestive organs of eels in controlled microorganisms.

Bacteria can produce protease enzymes through bacterial isolation using Skim Milk Agar (SMA) media.[7] One of the most frequently used methods for DNA-based molecular detection is Polymerase Chain Reaction (PCR). PCR is an enzymatic method for amplifying DNA in vitro.[1,7–9]

Research on the activity of protease enzymes in the digestive organs of eel has been published by (10). However, the isolation of bacteria from the wadi fermentation products of the digestive organs of eel and the identification of bacteria obtained molecularly have not been reported. Based on this reason, it is necessary to isolate and identify molecular with 16S rRNA gene against proteolytic bacteria in wadi eel fermentation. It is hoped that in this study new proteolytic bacterial strains as protease enzyme producers can be obtained.

Material and Methods:-

The type of research conducted is descriptive exploratory research. The research sample used was bacterial isolates in the wadi fermented products of the digestive organs of eel.

Sample Preparation Samples

The samples of wadi fermented products from the digestive organs of eel (Anguilla sp.) were made directly from fresh fish. A total of 500 g of the digestive organs were fermented by wadi according to the basic procedure for making wadi.[11]

Isolation of Proteolytic Bacteria

The samples of the wadi fermentation of the digestive organs of eel were diluted in stages with a dilution of 10^{-1} to 10^{-5} using physiological NaCl solution. The results of dilution with well-separated colonies were selected to be followed by observations of colony morphology and colony purificat on Nutrient Agar (NA) media according to the previous method.[8] After purification of each culturable colony, Gram staining was performed on the bacterial cells. Furthermore, on the purified colonies, proteolytic bacteria were selected using media containing protein substrates, namely Skim Milk Agar (SMA) and observations were carried out following the procedure of [12].

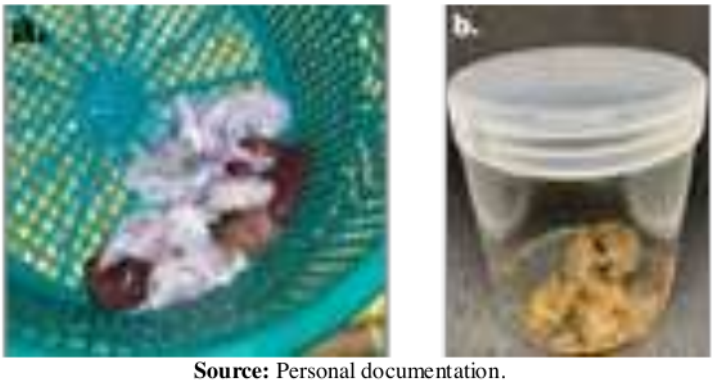
Identification of Proteolytic Bacteria with PCR (Polymerase Chain Reaction)

Isolates with the highest proteolytic index were identified using the PCR method with the target gene of 16S rRNA using specific primers 47F and 1492R. The DNA isolation procedure, the purity test of the extracted DNA and the determination of its concentration followed the previous research protocol.[8] Bioinformatics analysis of 16S rRNA DNA sequences of proteolytic bacterial isolates was carried out with BLASTn from NCBI according to the previous procedure[1,9].

Results and Discussion:-

In this study, bacterial samples were obtained from wadi fermented products made from the digestive organs of the eel Anguilla sp. The fermentation process is carried out to stimulate the degradation of fish substrates in general, including protein degradation involving proteolytic bacteria. The wadi sample products made in this study are shown in Figure 1.

Figure 1:- Fermented products of eel digestive organs (a) before fermentation and (b) after becoming a product of wadi fermentation.



Source: Personal documentation.

The wadi product obtained was then diluted by graded dilution using physiological NaCl solvent. From this process can be obtained a total of 5 bacterial colonies that can be cultured with a unique morphology or different from each other. These five different colony forms are an early indication of the presence of different bacterial species in the sample. The appearance of each colony purified on NA media can be seen in Figure 2 and Table 1. The five isolates were given the prefix WFA strain which stands for Wadi-Fermented Anguilla.

Figure 2:- Five bacterial colonies with different colony morphology that can be cultured from the wadi fermentation products of the digestive organs of eel, namely isolates A. WFA41, B. WFA42, C. WFA51, D. WFA52, and E. WFA53 (WFA = Wadi-Fermented Anguilla) Source: Personal documentation.

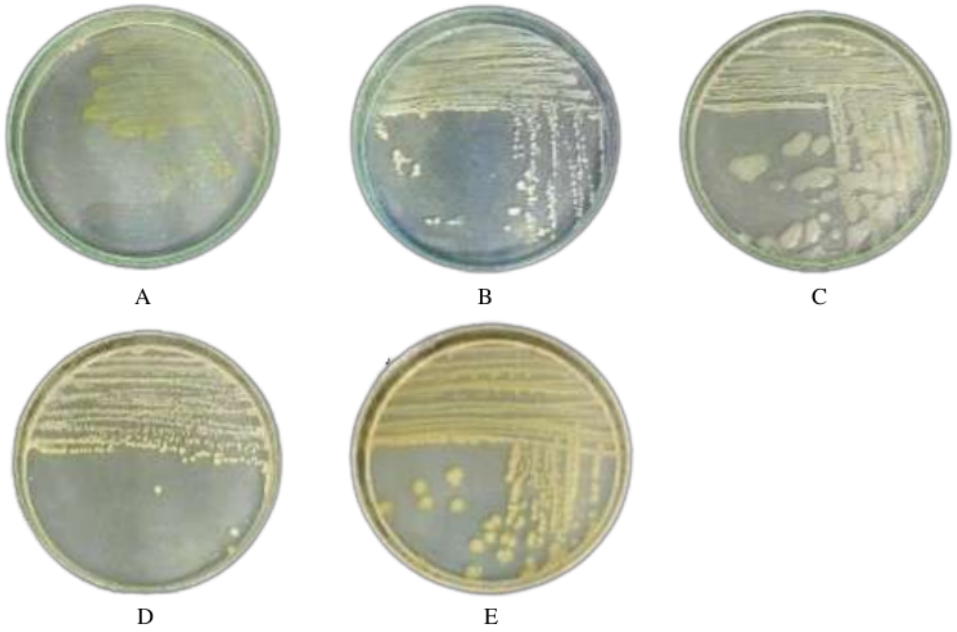
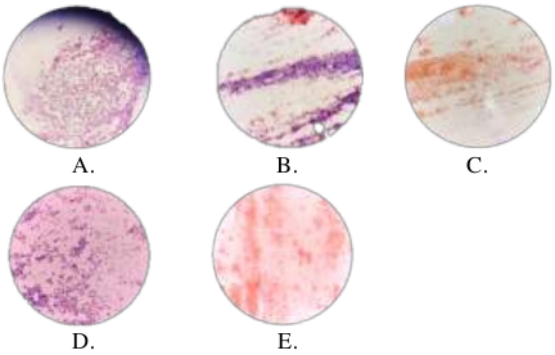


Table 1:- The purified colonies were on Nutrient Agar (NA) media.

Name Isolate	Shape	Color	Size	Edge	Elevation
WFA41	Circular	Yellow	0.5 mm	Entire	Convex
WFA42	Irregular	White	2 mm	Entire	Flat
WFA51	Irregular	White	3 mm	Entire	Raised
WFA52	Circular	Yellow	1 mm	Entire	Convex
WFA53	Circular	Yellow	2 mm	Entire	Flat

Figure 3:- The results of the gram staining of 5 bacterial colonies colony purification results, isolated from the wadi fermented products of the digestive organs of eels Source: Personal documentation.



Each successful unique bacterial colony found and then given Gram staining to obtain data on Gram properties and bacterial cell characteristics. The results of the Gram staining of the 5 bacterial colonies in question are shown in Figure 3, while the results of the observations on the characteristics of the bacteria observed by Gram staining are shown in Table 2.

Table 2:- Gram characteristics of bacterial colonies isolated from fermented eel wadi products.

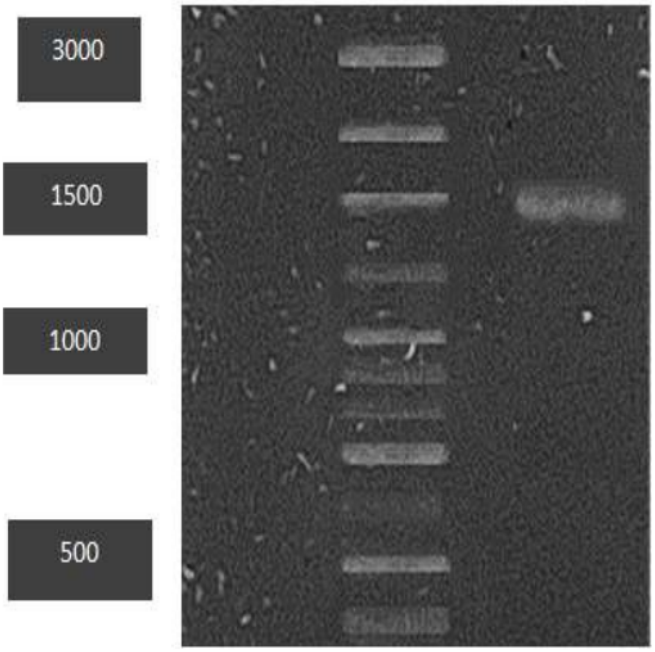
Name of Isolate	Form	Gram stain
WFA41	Gram	Gram-positive
bacilli WFA42	bacilli	-positive
WFA51	bacilli	Gram-negative
WFA52	Gram	Gram-positive
cocci WFA53	bacilli	-negative

Figure 4:- Three bacterial isolates were selected on the selective medium of Skim Milk Agar (SMA) and produced a proteolytic clear zone around each colony. Note A. WFA41 isolate, B. WFA51, C. WFA53. Source: Personal documentation.

Bacterial colonies that have been isolated and identified characteristics of bacteria. Furthermore, enzymatic tests were carried out to determine the ability of bacteria to break down proteins. The results can be seen in Figure 4.

Size (bp) of I. WFA51

Figure 5:- Results of 16S rRNA gene amplification of bacterial isolate WFA51 with primer 27F-1492R visualized on agarose gel electrophoresis and showed a single band measuring ~1500 bp based on Marka (M).



Based on the calculation of the protein hydrolysis index on SMA media, the colony used for the DNA isolation stage was WFA51 with a size of 1.85 cm. The WFA51 colony with the highest protein hydrolysis index was then identified molecularly, starting with the DNA isolation step using the Spin Column method with PrestoTM Mini G-DNA Bacteria Kit (Geneaid).

The purity of the product from DNA isolation was determined using absorption at 260 nm and 280 nm with a Nanodrop 2000 Spectrophotometer. The purity of the extracted WFA51 genomic DNA obtained was an A26/280 ratio of 1.9 with a concentration of 7.87 g/mL. DNA is said to be pure if the absorbance ratio of purity 260/280 ranges from 1.8 to 2.0. Thus the bacterial DNA isolates obtained in this study are in the pure category so that they can be used as DNA templates in the PCR process for the 16S rRNA gene.[13]

The next step of bacterial molecular identification is PCR amplification which targets the bacterial 16S rRNA gene using isolated bacterial genomic DNA as a template whose purity is known. The amplification results were tested for size by agarose gel electrophoresis method. The visualization is shown in Figure 5.

The results of the sequencing process carried out by a third party, namely PT. Indolab Utama is the nucleotide form of the 16S rRNA gene. Sequence analysis the nucleotide form of the 16S rRNA gene was performed on Geneious and the analysis was carried out online at http://blast.ncbi.nlm.gov/Blast.cgi. BLAST results will show the level of similarity in the nucleotide sequence of the 16S rRNA gene obtained with the nucleotide sequence of the 16S rRNA gene that has been registered in GenBank.[14]

From the alignment results of the 1206 bp sequencing results that have been carried out, it can be seen that the16S rRNA gene fragment in the WFA51 isolate obtained has a Query Cover of 57% and the highest percentage of similarity is 83% with the Acidovoraxlacteus strain M36 species.

This study used wadi fermented products of the digestive organs of eel (Anguilla sp.) following the procedures reported.[11] Samples were taken 0.5 g and then diluted with 4.5 mL physiological NaCl in the mother tube, then the dilution was 10-1 and then diluted up to 10-5. The results of the dilution were then planted on NA media. The previous dilution was carried out so that it was hoped that there would be no accumulation of bacterial colonies and their morphology could be clearly observed.[13] The 10-4 and 10-5 dilutions were grown on NA media, respectively. Five colonies that grew on NA media with 2 colonies each in 10-4 dilution and three colonies on NA medium 10-5 dilution, colony morphology with different bacteria were then taken for purification, the bacterial colonies were coded WFA41, WFA42, WFA51, WFA52 and WFA53. Purification aims to obtain pure isolates from bacteria that have been isolated and besides that it can also be used as stock.[15]

The purified bacterial colonies were then tested by Gram staining to determine the Gram characteristics and cell uniformity. Furthermore, the bacterial cells were observed with an optical microscope with a magnification of 100x [8,12]. This stage was followed by testing the ability to produce protease enzymes on SMA media on 5 isolates that had been obtained.

Based on the test results, it is known that the isolates WFA41, WFA51 and WFA53 are bacteria that have the ability to produce protease enzymes. The diameters of the three bacterial isolate colonies were measured, then the value of the protein hydrolysis index was calculated. Based on the results of the WFA41 protein hydrolysis index value, the hydrolysis index value was 1.08. While isolates WFA51 and WFA53 each produced a hydrolysis index value of 1.85 and 1.83. Based on the results of the protein index of the three colonies, WFA51 isolate had the highest value, this indicates that the isolate was able to produce better protein than other isolates.

Furthermore, the genomic DNA of the selected bacterial isolates was extracted. The obtained extracts were measured for purity and concentration based on the principle of DNA absorbance using the NanoDrop 2000 tool.[12] After obtaining a DNA extraction product that is pure DNA and its concentration is known, the DNA isolate can be used as template and then visualized on 2% agarose electrophoresis to display thick genomic DNA bands.[16]

In this study, the results of the identification of bacteria based on the 16S rRNA gene in the form of determining the species name were declared acceptable if the size of the isolated 16S rRNA gene sequence was at least 1250 bp, the BLASTn query was between 95-100% with anidentity97-100%. The results of the sequencing that have been carried out only obtained 1206 bp with a query cover of 57% with a similarity level of 83% with Acidovoraxlacteus strain M36. The results of the microscopic test showed similarities with Acidovorax sp.

Acidovorax sp. is a Gram-Negative bacterium with a rod shape and grows at a temperature of 15-42°C. In the aquatic environment, members of this genus have temperature-resistant proteins that allow aquatic animals to grow under thermophilic conditions. Another previously reported use, that of the bacterium Acidovorax sp. is a saprophytic bacterium that can stimulate plant growth.[17] The results of this study indicate that in the wadi fermented product of the digestive organs of eel, isolates of the proteolytic bacteria Acidovorax sp. WFA51. To find out bacterial species molecularly more accurately, bacterial genome DNA sequencing can be performed using the NGS (Next Generation Sequencing) method.[18]

Conclusion:-

Based on the results of this study, in the wadi fermentation product, the digestive organs of eel produced 5 unique colonies of bacteria that could be cultured on NA media with 3 isolates of which were protease producers. The isolate with the highest protein hydrolysis index was WFA51 (Wadi-Fermented Anguilla 51) of 1.85. The results of the molecular identification process for WFA51 isolates showed that this proteolytic isolate was Acidovorax sp. WFA51.

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