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Isolation and Molecular Identification of Proteolytic Bacteria from Rusip an Indonesian Fermented Food

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Abstract. Processing fish with the fermentation method can increase the nutritional content, bring out a distinctive taste, and make the fish last longer. Rusip is a type of complementary food from Bangka Belitung in the form of fish sauce that has been rotten or fermented. Rusip is made from fermented anchovy (*Clupeoides borneensis*). The concept of fermentation in Rusip is the breakdown of complex protein molecules by proteolytic bacteria into simple amino acid components. The purpose of this study was to determine the presence of proteolytic bacteria that play a role in rusip fermentation. The media used in screening proteolytic bacteria is skim milk agar (SMA) media. Then it was incubated at 37ºCfor 24 hours. Bacterial colonies that form a clear zone on the media indicate that these bacteria can degrade protein. After the isolation process and isolates were obtained, the bacteria were characterized by Gram staining method and molecular identification. The results showed that there were proteolytic bacteria in Rusip food, and the 3 best isolates that produced protease enzymes were identified as *Bacillus cereus*.

Keywords: Rusip; Proteolytic Bacteria; Protease Enzymes; Fermentation; Marine Fish

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INTRODUCTION

Based foods contain high protein, essential amino acids, and other nutrients that play an important role in human health. Fish is a source of essential amino acids and polyunsaturated fatty acids that play an important role in physiological functions for the maintenance and development of the fetus, neonate, and baby's brain. The nutritional content of fish can be used to prevent and overcome food insecurity and malnutrition (Malulu et al, 2021; Ağagündüz et al, 2022; Dahiya and Nigam, 2022; Sun et al, 2022). Fish contains 18-20% protein and contains eight essential amino acids including sulfur containing lysine, methionine, and cysteine (Khalil et al, 2019).

Processing fish by fermentation allows food to be stored longer, changes the texture and flavour of food, and increases the nutritional value and functional properties of the food (Xu et al, 2020; Hadjimbei et al, 2022). One example of fish food that is processed by a fermentation process is Rusip. Rusip is a type of complementary food from Bangka Belitung in the form of fish sauce that has been rotten or fermented. Rusip is made from fermented various types of small fish such as anchovies (*Clupeoides borneensis*), anchovies (*Stolephorus commersonnii*), and rebon shrimp (*Acetes indicus*). This fish is fermented and after emitting a sour smell, the next processing is carried out with the addition of onions, chilies, brown sugar, rice crust, salt, and key oranges (Palupi et al., 2018; Sanjaya, 2023).

Fermented foods are safe for consumption and nutritious for health. This is because, during the fermentation process, some anti-nutritional and toxic compounds will be reduced or converted into useful and non-toxic compounds. This makes fermented food safe for consumption and has specific compounds that function as functional compounds that can regulate the metabolism of other compounds so as to maximize the digestive process (Ma et al., 2022). In rusip fermentation, proteolytic bacteria that play a role in the fermentation process degrade proteins into amino

acids and peptides and then convert them into other simpler components. During the fermentation process, complex biochemical reactions occur and cause significant changes in the characteristics of fish tissues. Proteolysis causes the formation of new peptides and amino acids. The occurrence of this proteolysis is due to the presence of protease enzymes produced by proteolytic bacteria during the fermentation process (Xu et al, 2020).

Seeing the important role of proteolytic bacteria in fish fermentation, it is necessary to conduct research on the isolation and identification of proteolytic bacteria in rusip fermentation. This research is an early stage to determine the presence of proteolytic bacteria in fermented fish food. Starting from the isolation process to characterization to determine the presence of proteolytic bacteria in fish fermentation. For the continuation of this research, a fibrinolytic activity test on bacterial isolates will be carried out. These fibrinolytic bacteria will later be used as an agent for the prevention of cardiovascular disease.

METHODS

Isolation

Bacteria were isolated from rusip which was sold commercially under the brand name Cap Tiga Bintang. Bacteria were isolated using SMA (*skim milk agar*) media. SMA media (*skim milk agar*) was made by mixing 52.5 grams of instant powdered SMA medium with 1L of distilled water. Then sterilized by autoclaving for 15 minutes, pressure of 1 atm, and temperature of 121°C. Bacterial isolation started with the spreadplate method on SMA media. After that, it was purified again using the streak plate method on SMA media. Colony purification was continued until a single bacterial colony was obtained (Pandey et al., 2022).

Cell Morphological Staining with Gram Stain

The gram staining method used was in accordance with Harun et al. (2023). Before use, the slides were sterilized with 70% alcohol. Bacterial isolates were placed on a glass slide along with a few drops of distilled water and then passed over the fire several times to fixate. Then the isolate was dripped with Crystal violet and allowed to stand for one minute, rinsed with distilled water. Next, the isolate was dripped with iodine, left for one minute, then rinsed with distilled water. Dropped with 96% alcohol, left for 30 seconds, rinsed with distilled water. Dropped with safranin, left for 30 seconds then rinsed with distilled water. The isolates were observed with a microscope with a magnification of 100x. Observations were made on the shape and color of the cells in the isolates. Gram positive bacteria are marked with a purple color because they are able to bind crystal violet. Gram-negative bacteria are characterized by the formation of a pink color because they are unable to bind to crystal violet and are only stained by safranin Harun et al. (2023).

DNA Isolation

Isolation DNA isolation using the Quick-DNA Fungal/Bacterial Miniprep Kit protocol (Zymo Research, D6005).

Measurement of DNA purity with nanodrops

Measurement of DNA purity with nanodrops aims to determine the level of DNA purity quantitatively. Before carrying out the nanodrop process, DNA isolation was carried out first. Open NanoDrop 2000 application and select nucleic acid. The pedestal on the nanodrop is cleaned with a tissue. Then the nanodrop calibration was carried out with the solvent used to dissolve the DNA. The solvent used is ddH_2O. 0.1 L ddH_2O was added to the nanodrop pedestal for calibration. After calibration, the DNA purity test was started by dripping 0.1 L of DNA sample on the pedestal. The results will appear on the computer screen.

According to Begum et al. (2022), a quantitative test of DNA samples was carried out to determine the concentration and purity of DNA. The absorbance value of DNA concentration can be measured by light at a wavelength (λ) of 260 nm. To determine the purity of DNA, a comparison of the absorbance values was measured at 260/280 and 260/230. Based on what was written by Hindash and Hindash (2022), DNA was declared pure if it had a value ranging from 1.8 to 2.0 when the absorbance value was detected at a wavelength of 260/280 nm. If the DNA purity shows a value of less than 1.8, it means that the DNA sample is declared contaminated with protein, while the DNA purity shows a value of more than 2.0, which means that the DNA sample is declared contaminated with RNA. At a wavelength of 260/230 nm, DNA is said to be pure if it has a value of 2-2.2. If the absorbance value shows a number less than 2, it means that the DNA is contaminated with organic matter, carbohydrates, or other chemicals.

PCR Amplification of 16S rDNA gene

Polymerase chain reaction amplification using 2X MyTaq HS Red Mix (BIO-25048). PCR Master Mix and PCR conditions can be seen in table 1 and table 2.

Table 1. PCR Master Mix

Component	1 × 25 L
Dd H_2O	9.5
MyTaq Red Mix, 2x	12.5
10 L 27F Primer*	1
10 M 149R Primer**	1
DNA Template	1

*Sequence 27F Primer: 5' –AGAGTTTGATCMTGGCTCAG– 3'
**Sequence 1492R Primer: 5' – GGTTACCTTGTTACGACTT– 3'

Table 2. PCR Condition

Step	Temperature (ºC)	Duration	Cycles
Initial Denaturation	95	3 min	1
Denaturation	95	15 sec	
Annealing	52	30 sec	35
Extension	72	45 sec	
Final Extension	72	3 min	1
Hold	4	Electrophoresis	1

Electrophoresis

Electrophoresis is carried out to determine the desired target DNA band length. 1 L PCR product was assessed by electrophoresis with 0.8% TBE agarose (Pahriyani and Wardani, 2020).

Sequencing

The DNA sample that has been PCR is sent to PT Genetika Science for sequencing. Sequencing using the Sanger sequencing methode.

Raw Sequence Data Processing

Homology analysis and phylogenetic analysis were carried out using BioEdit, Mega6, Clustal X, Miceosoft Word and Notepad applications. The device specifications used for this analysis are ASUS UM462.

RESULTS AND DISCUSSION

Bacterial Isolation Results

Isolation of bacteria using Skim Milk Agar media with the spreadplate method. The dilution used is 10^{-1} to 10^{-6}. The results showed that bacteria with dilutions of 10^{-1}, 10^{-2}, and 10^{-3} could grow well on SMA media and were detected to form a clear zone. Colonies with the best growth were at a dilution of 10^{-3}. Then the bacteria purification process was carried out to form a single colony using the Streakplate method. The results obtained were 22 bacterial dishes with pure colonies and forming a clear zone. Isolates were coded R1 to R22. However, the bacteria that formed the clear zone well and were not contaminated were found in isolates R7, R10, and R20. The formation of a clear zone on SMA media indicates that the isolated bacteria produce extracellular protease enzymes. So it can be concluded that the bacterial isolates with codes R7, R10, and R20 isolated from rusip were proteolytic bacteria.

This is in accordance with Saikh *et al.* (2023) that to select proteolytic bacteria it is necessary to test to determine the production of protease enzymes from these bacteria. To determine the enzymatic activity of proteolytic bacteria, it can be done by growing bacteria on differential *skim milk agar* (SMA) media. SMA media contains nutrients in the form of a carbon source from lactose and a nitrogen source from casein. Casein is a milk protein composed of phosphoproteins and binds to calcium to form casein micelles or white colloids. Rasheed (2022) and Zeyneb et al (2023) explained that the casein content in SMA media was high enough to cause this medium to be yellowish white or bone white. Proteolytic bacteria grown in SMA media will form a clear zone around the colony due to casein hydrolysis by extracellular proteases produced by these bacteria. The results of bacterial isolation can be seen in Figure 1.

Figure 1. Bacterial Isolation Results from Rusip samples in SMA media (*skim milk agar*) with a dilution of 10^{-3}

Three of this bacterial isolates, namely R7, R10, and R20 had a yellowish white colony morphology, with a smooth and flat surface, and the edges of the colony are round and uneven. Based on gram staining, it was found that the bacterial isolates R7, R10, and R20 had a rod shape. The cell color of the three bacterial isolates was purple which indicated that the isolate was a gram-positive bacterium. Tripathi and Sapra (2020) stated that the reaction in Gram staining is based on the composition of the bacterial cell wall. Gram-positive bacteria have cell walls composed of 90% peptidoglycan and the remainder composed of tekoic acid. Gram-positive bacteria have purple cells when Gram staining is performed because the cells can form complex bonds with crystal violet which has a purple color. Gram-negative bacteria have cell walls composed of 5-20% peptidoglycan and the rest are polysaccharides. Giving 95% alcohol solution at the time of gram staining aims to increase the porosity of the cell wall. Alcohol can dissolve lipids in the outer membrane of the cell, so that in Gram-negative bacteria, the purple complex will fall off and the cell will be colorless. Cells in Gram-negative bacteria are red in color as a result of the comparison dye safranin.

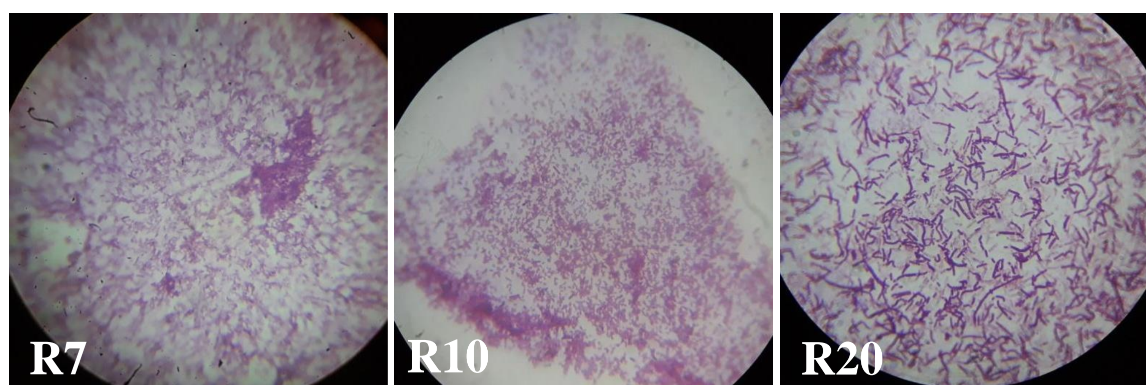

Figure 2. Results of Gram Staining on Isolate R7, R10, R20

DNA Isolation, Quantitative Test, and DNA Qualitative Test

Extraction of genomic DNA using Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). Quantitative tests were carried out to see the purity of DNA. The tool used is nanodrop. The nanodrop results can be seen in table 3.

Table 3. Nanodrop Results

No.	Sample Name	Conc. (ng/µL)	A260/280	A260/230	Volume (µL)
1	R7	30.4	1.95	0.26	30
2	R10	27.7	1.98	1.26	30
3	R20	29.3	1.94	0.67	30

These results indicate that the DNA in samples R7, R10, and R20 has a concentration of 30.4 ng/µL; 27.7 ng/µL; and 29.3 ng/µL. The results of DNA purity are seen at the A260/280 wavelength. DNA R7, R10, and R20 had a purity of 1.95; 1.98; and 1.94, which indicated that the DNA R7, R10, and R20 had good purity. Hindash and Hindash (2022) explained that the DNA quantitative test with nanodrops can be used to see the purity and concentration of DNA. DNA purity was seen by the absorbance value at a wavelength with a ratio of 260/280. DNA is known to be of good quality if it has an absorbance value of 1.8-2.0 when measured at a wavelength of 260/280 and has a concentration above 100 ng/µL.

Qualitative test is done by electrophoresis process. Electrophoresis results from DNA samples of each isolate can be seen in the image below.

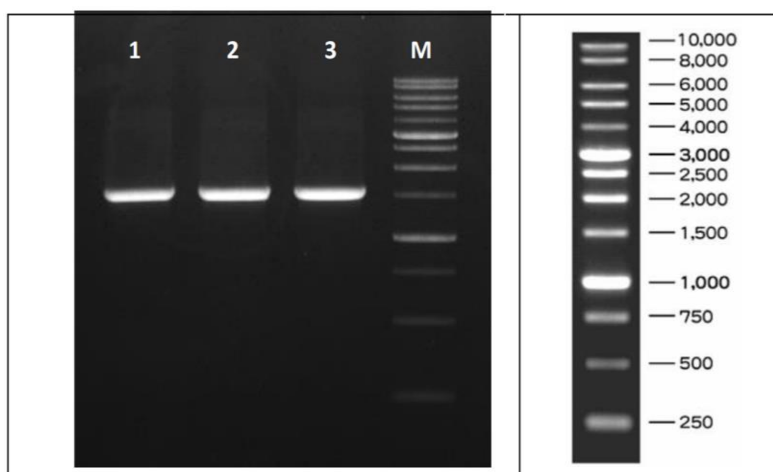

Figure 3. DNA electrophoresis results R7, R10, and R20

Explanation:
1 : DNA band from electrophoresis isolate R7
2 : DNA band from electrophoresis isolate R10
3 : DNA band from isolate R20
M : DNA marker

Electrophoresis results show that DNA bands are clearly visible and can be read. The marker used is 1 Kb bp DNA ladder. After being compared with markers, it was found that the DNA R7, R10, and R20 had a size of 2,000 Kb.

Sequence DNA Processing

Sequencing results from isolate R7, R10, R20 can be seen in Figure 5, Figure 6, and Figure 7.

```
CAAGTCGAGCGAATGGATTAGGAGCTTGCTCTGATGAAGTTAGCGGCGGACGGGTGAGTAAC
ACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAA
CATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCC
GCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTG
AGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTT
CGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGA
CGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTG
```

Figure 4. The nucleotide sequence of the forward 16S rRNA and reverse 16S rRNA contigs on DNA R7

```
TGCTAATACTGCAAGTCGAGCGAATGGATTAGGAGCTTGCTCTTATGAAGTTAGCGGCGGAC
GGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTA
TGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCG
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
ATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAA
```

Figure 5. The nucleotide sequence of the forward 16S rRNA and reverse 16S rRNA contigs on DNA R10

```
CCTTAGGCGGCTGGCTCCAAAAGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCGTGGT
GTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTA
CTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTA
TGAGATTAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTACCGTCCATTGTAGCACGTGTGTA
GCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCG
GCAGTCACCTTAGAGTGCCCAACTTAATGATGGCAACTAAGATCAAGGGTTGCGCTCGTTGC
GGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTG
CTCCCGAAGGAGAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCT
TCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTT
GAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTA
AAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCT
AATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTT
CGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGAATTCCACTTTCC
TCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCAC
ATCAGACTTAAGAGACCACCTGCGCGCGCTTTACGCCCAATAATTCCGGATAACGCTTGCCA
CCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCTGTGGCTTTCTGGTTAGTACCGTCAA
GGTGCCAGCTTATTCAACTAGCACTTGTTCTTCCCTAAC
```

Figure 6. The nucleotide sequence of the forward 16S rRNA and reverse 16S rRNA contigs on DNA R20

The nucleotide sequence of the sequencing results that have been processed with the *contig was* analyzed by BLAST (*Basic Local Alignment Search Tool*). BLAST (*Basic Local Alignment Search Tool*) is a software that contains a database of biological sequences from around the world. BLAST is used to search for DNA or protein sequence data that is similar to the sequence being analyzed. BLAST is useful for finding information from the sequence in question. This is in accordance with Moritania et al. (2019) statement in his module which states that BLAST is one of the standard DNA, RNA, and protein database software used worldwide. The BLAST database was used to obtain information and identify nucleotide sequence homology. The accession number of the consensus sequences obtained can be seen in table 4.

Table 4. Accession number of isolates R7, R10, and R20

No.	Sample Name	Accession Number
1	R7	OP946515
2	R10	OR342125
3	R20	OQ299556

Table 5. BLAST Result

Isolate Code	Bacteria Name	*Max score*	*Total Score*	*Query Cover(%)*	E-Value	*Percent Identity*
R7	*Bacillus cereus*	985	985	100	0.0	99.63
R10	*Bacillus cereus*	784	784	99	0.0	99.77
R20	*Bacillus cereus*	1890	24443	100	0.0	99.71

Based on the BLAST results (Table 5), it can be seen that isolates R7, R10, and R20 were declared as Bacillus cereus because they had a high percent identity value. According to Moritania et al. (2019), BLAST results with a homology value (percent identity) of more than

97% can represent similarities at the species level. The homology (percent identity) value of 93% - 97% can represent identity at the genus level but differs at the species level. Percent identity with a value below 93% means the possibility of a new species whose nitrogen base sequence has not been included in the Genbank database. According to Lamdo et al. (2023), the maximum score and total score are the number of alignment segments from the database sequence that match the nucleotide sequences. The score value indicates the accuracy of the alignment of the unknown nucleotide sequence with the data in GenBank, the higher the value the higher the homology level of the two sequences. The E value is an estimated value that provides a statistically significant measure of both sequences. A higher E-value indicates a lower level of homology between sequences, while a low E-value indicates a higher level of homology between sequences. The E-value is 0 (zero) indicating that the query sequence is similar to the sequence in GenBank. Query coverage shows the percentage of database covered by queries.

Next step is to reconstruct the phylogenetic tree using the *neighbor-joining* using the MEGAX application. *Neighbor-joining* is a *Neighbor Joining tree* reconstructed using *Test of Phylogenybootstrap* 1000 replication (Asih and Kartika, 2021). The results of the phylogenetic tree reconstruction can be seen in the image below.

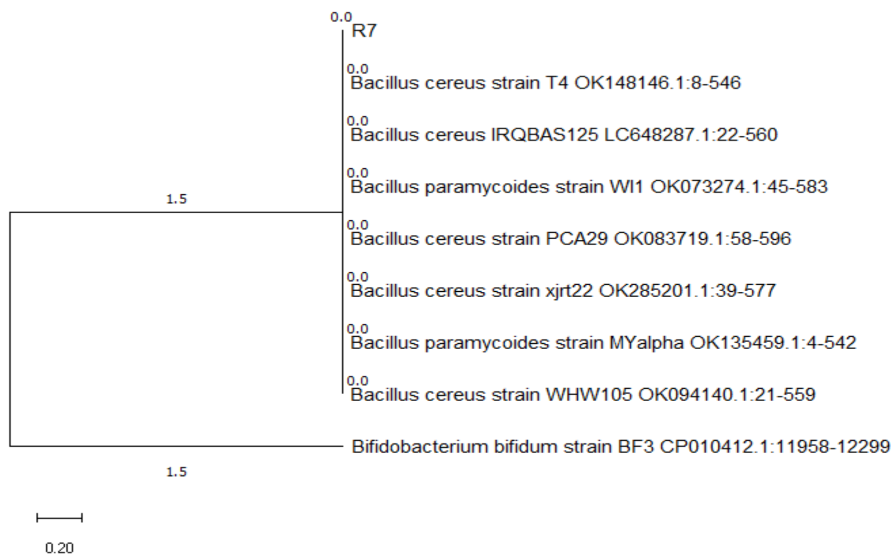

Figure 7. Phylogenetic tree of R7 isolate using *Neighbor-joining* test *bootstrap* 1000 with *outgroup Bifidobacterium bifidum*

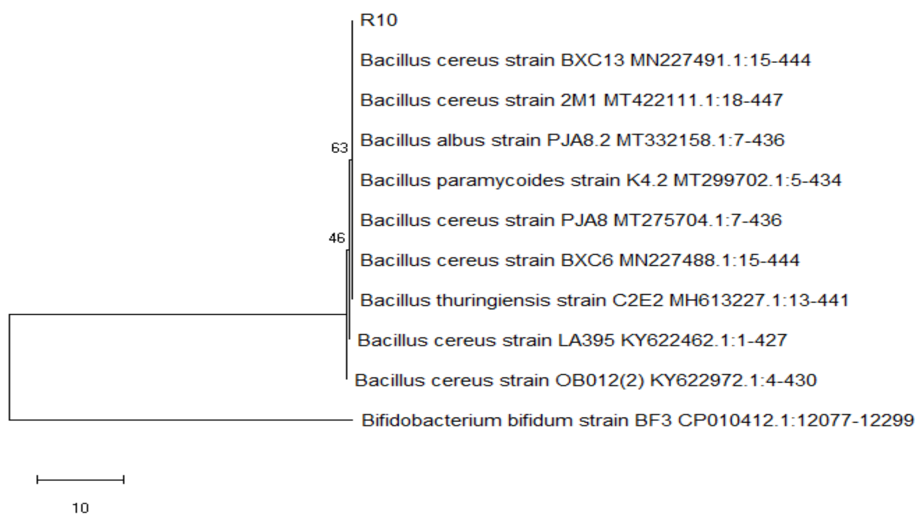

Figure 8. Phylogenetic tree of isolate R10 using *Neighbor-joining* test *bootstrap* 1000 with *outgroup Bifidobacterium bifidum*

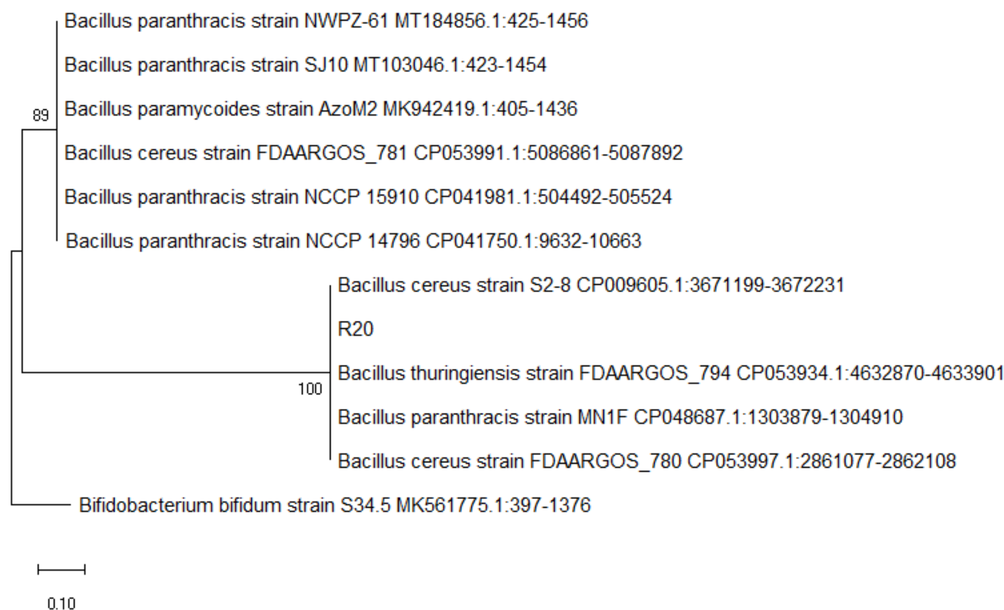

Figure 9. Phylogenetic tree of isolate R20 using *Neighbor-joining* test *bootstrap* 1000 with *outgroup Bifidobacterium bifidum*

Based on the results of processing the DNA sequence data obtained, the results show that isolates R7, R10, and R20 have the closest relatives with *Bacillus cereus*. The results of this study can be adapted to the research of Purwadi et al. (2019) who isolated protease bacteria from tiger prawn rusip. After morphological observations and molecular identification were carried out in this study, the results showed that in the tiger shrimp rusip there was the bacterium *Bacillus cereus* which had protease activity.

Bacillus cereus is known for its pathogenic potential. Even though *Bacillus cereus* is a food borne pathogen that causes diarrhea. In this Rusip there is *B. cereus* even with three different strains. According to Ehling-Schulz et. Al. (2019), generally *B. cereus* is known as a toxic agent in food, a cause of local wounds, eye infections, and systemic diseases. *B. cereus* is a ubiquitous and highly resistant food poisoning bacterium which is an important food safety problem (Rahnama et al, 2023; Tirloni et al, 2022). Even after the processing of fish by means of fermentation such as rusip cannot kill *B. cereus*. For this reason, the process of processing rusip needs to be evaluated again.

Rusip processing by fermenting raw fish and without prior sterilization such as heating. Processing fish without heating can leave pathogenic bacteria in fish. The fermentation process that occurs in rusip may not be able to kill existing pathogenic bacteria such as *B. cereus*. *B. cereus* has a variety of stain types with different levels of pathogenesis. There are things that can still be tolerated by the human body and not. The content of *B. cereus* in rusip will also affect the eligibility of rusip as food that can be consumed as well.

CONCLUSION

There are proteolytic bacteria that play a role in the rusip fermentation process. This is evidenced by the appearance of a clear zone on bacteria from rusip isolated using SMA (skim milk agar) media. After isolation, species identification was carried out by morphological to molecular observations on isolates R7, R10, and R20. The results showed that isolates R7, R10, and R20 were species of *Bacillus cereus*.

For further research, it is expected to examine the pathogenicity and benefits of *Bacillus cereus* in food products. especially in fermented foods such as rusip. It is also necessary to know the bioactive compounds produced by Bacillus and their role in the process of fermented food processing.

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