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Characterization and molecular identification of bacteria from mackerel *bekasam* in Sorong City, Southwest Papua Province, Indonesia

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Abstract. *Sukmawati S, Ratna R, Sipriyadi, Yunita M. 2023. Characterization and molecular identification of bacteria from mackerel bekasam in Sorong City, Southwest Papua Province, Indonesia. Biodiversitas 24: 4967-4977. Bekasam* is traditional food type produced by traditional fermented fish. Microbes that grow through fermentation play an important role in forming the product's aroma, texture, and overall quality. The study aimed to determine the biochemical characteristic of bacteria from mackerel (*Scomberomorus* sp.) *bekasam* in Sorong City and identify bacteria at the molecular level. This research was a descriptive study, which described the results of the characterization of bacteria from fermented mackerel fish and the results of molecular identification to the species level through the PCR (Polymerase Chain Reaction) technique. Then, the DNA sequences were further analyzed using the agarose gel electrophoretic separation method to visualize the bacterial DNA profile. The biochemical characterization of bacterial isolates from mackerel showed that all isolates were negative indole, and eight isolates were positive in reducing nitrate. In comparison, four isolates were negative in reducing nitrate, then all isolates had proteolytic activity except the FST 3.1 and FST 3.2 isolates. Eleven isolates were positive in hydrolyzing fat, and one isolate could not hydrolyze fat. According to the DNA patterns seen in electrophoresis and alignment of the 16 sRNA gene sequences, several types of bacteria had been identified as *Bacillus paramycoides* strain 2883 FST 1.1, *Bacillus paramycoides* strain 3665 FST 2.1, *Bacillus mobilis* strain ICA-144 FST 3.1, *Bacillus cereus* strain ATCC 14579 FNT 1.1, *Bacillus mobilis* strain ICA-144 FNT 2.1, and *Bacillus cereus* strain ATCC 14579 FNT 3.1.

Keywords: *Bekasam,* identification, lactic acid bacteria

INTRODUCTION

Indonesia is a country that has a lot of diversity of traditional food, one of which is the *bekasam* product. *Bekasam* is a processed fish product made through a fermentation process. *Bekasam* has a sour and salty taste, so this product has a distinctive taste. *Bekasam* is widely known in South Sumatra and Kalimantan and is commonly made from freshwater fish (Adawyah 2016). *Bekasam* is a fermented fisheries product that utilizes the activity of lactic acid bacteria. It is typically created through spontaneous fermentation, the amount and type of bacteria found in it vary (Purwati et al. 2019).

The *bekasam* is made through several stages, fish cutting, cleaning, mixing with rice and salt, and fermentation for seven days in a closed container. During fermentation, environmental conditions should remain controlled and airtight (Murtini 2017). The *bekasam* fermentation occurs spontaneously and only relies on salt as a selector for microorganisms (Lestari et al. 2018). Microorganisms that grow on the *bekasam* are lactic acid bacteria that belong to the amylotic group. According to Ardilla et al. (2022), starch, as the main carbohydrate, becomes the initial substrate for lactic acid bacteria, which is then hydrolyzed into simple carbohydrates (Arfianty et al. 2017; Zang et al. 2020). Therefore, in this study, we used the combination of sago with the sample code FST & FNT and rice as carbohydrate sources which also became this study's novelty. In addition, the is study's fish fermented into *bekasam* differed from common *bekasam*; seawater fish, namely mackerel, were used in this study. The addition of sago as a source of carbohydrates is something new and unusual in the *bekasam* processing, where only rice is generally used as a source of carbohydrates for the fermentation process.

Fermentation is a biochemical process that involves microorganisms involving microorganisms such as bacteria and yeast to process raw materials into more stable products and produce a distinctive taste (Luo et al. 2020). In the mackerel *bekasam*, microorganisms play an important role in fermentation, giving the final product a distinctive taste and aroma (Luo et al. 2020).

Lactic acid bacteria (LAB) are commonly the most prominent bacteria in fermented products. When fermented foods are made, LAB is a crucial fermentation agent. LAB converts lactose into lactic acid, which gives the product its sour taste and unique texture. The human digestive tract, especially the intestines, also contains lactic acid bacteria.

They support intestinal health and the maintain the microbial balance in the intestine, which boosts the immune system and helps recover from harmful bacterial infections (Mikelsaar et al. 2016).

Lactic acid bacteria (LAB) have antimicrobial properties that can be used as natural preservatives in food. During fermentation, they produce organic acids and other antibacterial substances that prevent the growth of dangerous bacteria and increase the shelf life of food products. LAB also plays a role in silage processing, a preserved animal feed component. The fermentation process by LAB helps maintain the nutritional quality of forage during storage and makes it a better feed for livestock. LAB also produces polylactic acid (PLA), a biodegradable plastic material. It can be used as an environmentally friendly alternative to conventional plastics. LAB is also often used in probiotic supplements to improve gut health and the immune system. They can help with digestive problems such as diarrhea, constipation, or lactose intolerance (Taib et al. 2023).

Several previous studies have identified the bacteria in the basic ingredients of tilapia fish, milkfish, and tuna. Bacterial morphological and biochemical characterization of those three *bekasam* products resulted in the genera of *Leuconostoc*, *Lactobacillus*, and *Pediococcu*s, and after being identified using the API CH50 Kit, they were identified as *Lactobacillus pentosus*, *Lactobacillus plantarum*, and *Pediococcus pentosaceus* (Wikandari et al. 2012), *Staphylococcus* sp., *Lactobacillus* sp. (Melizah et al. 2018). In comparison, the identification of bacteria in mackerel fish shells has never been carried out, referring to the results of related reference searches.

Characterization and molecular identification of bacteria in mackerel *bekasam* in Sorong City was aimed to determine the biochemical characteristics of bacteria from mackerel *bekasam* in Sorong City and identify bacteria at the molecular level. The characterization and molecular identification of bacteria in mackerel *bekasam* have important implications for developing better fermentation production processes for fish products, with a deeper understanding of the patterns of the microbes involved. Therefore, producers may also optimize the fermentation conditions to improve the quality and safety of the *bekasam* products. In addition, this research would also provide insight into the local microbial diversity associated with certain traditional products, which has the potential to be used in other food product innovations.

MATERIALS AND METHODS

Research design

This study was a descriptive study, which described the results of the characterization of bacteria and revealed the results of molecular identification at the species level of bacterial species from mackerel (*Scomberomorus* sp.) fish *bekasam* in Sorong City. This research was conducted from April to July 2023 at the Laboratory of the Faculty of Fisheries, Universitas Muhammadiyah Sorong and the Biology Laboratory, Bengkulu University, and the sequencing was carried out at PT. First Base in Malaysia via PT. Genetics Science Indonesia, Jakarta.

Characterization of Lactic Acid Bacteria (BAL)

Indole test

Bacterial isolates were inoculated into test tubes containing tryptone broth and incubated at 37ºC for 24 hours. After incubation, 0.5 mL of kovacs reagent was added. If a red color appears, it indicates a positive indole test (Procop et al. 2020).

Nitrate reduction test

Bacterial isolates were inoculated into nitrate broth. After incubation at 37ºC for 48 hours, three drops of sulfanilic acid and three drops of dimethyl alpha-naphthylamine were added. Nitrate reduction occurs if a red color is formed (Buxton 2011).

Proteolytic activity test

Bacterial isolates were streaked on skim milk agar (SMA) medium and then incubated at 30ºC for 48 hours. The bacteria are proteolytic if a clear zone surrounds the colony (Freundt 2012).

Fat hydrolysis test

Bacterial isolates were streaked on nutrient agar (NA) medium + 1% fat (butter) and neutral red indicator as a substrate. The isolates were then incubated at 30ºC for 48 hours. If a red color is formed at the bottom of the colony, then it is positive for fat hydrolysis (Yanuar et al. 2017).

Molecular identification of bacteria from mackerel fish *bekasam*

Bacterial genomic DNA isolation (modified from Sambrook and Russell 2001)

A total of 1.5 mL of bacterial culture was centrifuged at 8,000 rpm for 10 minutes, then the pellet was washed with STE buffer and then centrifuged at 8,000 rpm for 10 minutes. The pellets obtained were added with STE buffer and lysozyme and then incubated at 55ºC for 1 hour to form protoplasts. Furthermore, proteinase-K was added and incubated at 55ºC for 60 minutes. Subsequently, 10% CTAB was added to 0.7 M NaCl solution and incubated at 65ºC for 30 minutes. The solution was then added with 1 volume of phenol: chloroform (25:24) and centrifuged at 12,000 rpm for 10 minutes. The clear phase was added 0.6 times the volume of isopropanol and sodium acetate, then incubated at -20ºC overnight and centrifuged at 12,000 rpm for 10 minutes. The resulting pellets are washed with 70% alcohol. DNA was dried for 1 hour to remove alcohol and then dissolved in sterile ddH2O, then the results of isolated DNA were stored at 4ºC.

Bacterial 16s rRNA gene amplification

The 16S rRNA gene from genomic DNA was amplified using the Polymerase Chain Reaction (PCR) machine and prokaryotes-specific primers (Marchesi et al. 1998), namely forward primer 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and reverse primer 1387r (5′-GGG CGG WGT GTA CAA GGC-3′). The PCR reaction composition

consisted of Gotaq Green Master mix (2X) µL, each forward and reverse primer (10 pmol) 1.5 µL, Nuclease free water (NFW) 18.0 µL, and DNA template 4.0 µL. The PCR conditions used were pre-denaturation, denaturation, annealing, elongation, and post PCR, with 30 cycles. PCR product DNA was separated in a mini-gel electrophoresis machine using 1% agarose at 75 Volts for 45 minutes. DNA was visualized using Ethidium Bromide (EtBr) dye on a UV transilluminator.

The sequencing results were trimmed and assembled using the ChromasPro version 1.5 program, the data was BLAST with genomic data registered with NCBI/National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The data were further analyzed by aligning the sequences using the MEGA 5.0 program (Tamura et al. 2011). Phylogenetic trees were then constructed to show the degree of kinship of isolate Xyl_22 with actinomycetes and other non-actinomycetes using the Neighbor-Joining Tree method with bootstrap values using 1000 replications (Felsenstein 1985).

RESULTS AND DISCUSSION

The characterization of all bacterial isolates from samples of mackerel *bekasam* in Sorong City showed all negative results for the indole test, while the results of the nitrate reduction test showed that 8 isolates were positive in reducing nitrate, while 4 isolates were unable to reduce nitrate (Table 1; Figures 1 and 2). Proteolytic test results of bacterial isolates from mackerel *bekasam*, 10 isolates showed proteolytic activity and 2 isolates showed negative proteolytic activity (Table 1; Figure 3). The results of the fat hydrolysis test on bacterial isolates from the mackerel *bekasam* showed that eleven isolates could hydrolyze fat, and only one isolate could not hydrolyze fat (Table 1; Figure 4). Bacterial genome DNA amplification from mackerel *bekasam* using the PCR method with primers of 63f and 1387r produced DNA fragments of approximately 1300 bp in size (Figure 5).

Table 1. Biochemical characterization of bacterial isolates from mackerel *bekasam*

Sample	Indole test	Nitrate reduction test	Proteolytic activity test	Fat hydrolysis test
FST 1.1	Negative	Positive	Positive	Positive
FST 1.2	Negative	Positive	Positive	Negative
FST 2.1	Negative	Positive	Positive	Positive
FST 2.2	Negative	Positive	Positive	Positive
FST 3.1	Negative	Negative	Negative	Positive
FST 3.2	Negative	Negative	Negative	Positive
FNT 1.1	Negative	Positive	Positive	Positive
FNT 1.2	Negative	Positive	Positive	Positive
FNT 2.1	Negative	Negative	Positive	Positive
FNT 2.2	Negative	Negative	Positive	Positive
FNT 3.1	Negative	Positive	Positive	Positive
FNT 3.2	Negative	Positive	Positive	Positive

Table 2. The results of 16S rRNA gene sequences of bacterial isolates from mackerel *bekasam* according to the data available at NCBI (BLASTX)

Isolate name	Description	Max score	Total score	Quary cover	E value	Identity	Access number
FST 1.1	*Bacillus paramycoides* strain 2883	2183	2871	100 %	0.00	99.92%	MT611845.1
	Bacillus nitratireducens strain AB304	2183	2871	100 %	0.00	99.92%	MT436100.1
	Bacillus cereus strain 65 gite	2183	2871	100 %	0.00	99.92%	MT378165.1
FST 2.1	*Bacillus paramycoides* strain 3665	2206	2206	100 %	0.00	99.59%	MT538529.1
	Bacillus cereus strain NRC215	2206	2206	100 %	0.00	99.59%	MT229271.1
	Bacillus albus strain NA-18	2206	2206	100 %	0.00	99.59%	MN882644.1
FST 3.1	*Bacillus mobilis* strain ICA-144	2294	2871	100 %	0.00	99.92%	OQ062548.1
	Bacillus cereus strain ATCC 14579	2294	2871	100 %	0.00	99.92%	OQ028258.1
	Bacillus thuringiensis strain AFS064504	2294	2871	100 %	0.00	99.92%	OP986979.1
FNT 1.1	*Bacillus cereus* strain ATCC 14579	2283	2283	100 %	0.00	99.76%	OQ028258.1
	Bacillus mobilis strain ICA-144	2283	2283	100 %	0.00	99.76%	OQ062548.1
	Bacillus thuringiensis strain AFS064504	2283	2283	100 %	0.00	99.76%	OP986979.1
FNT 2.1	*Bacillus mobilis* strain ICA-144	2266	2283	100 %	0.00	99.92%	MT538528.1
	Bacillus cereus strain ATCC 14579	2266	2283	100 %	0.00	99.92%	MT229271.1
	Bacillus thuringiensis strain AFS064504	2266	2283	100 %	0.00	99.92%	MN882644.1
FNT 3.1	*Bacillus cereus* strain ATCC 14579	2266	2266	100 %	0.00	99.92%	OQ028258.1
	Bacillus mobilis strain ICA-144	2266	2266	100 %	0.00	99.92%	OQ062548.1
	Bacillus thuringiensis strain AFS064504	2266	2266	100 %	0.00	99.92%	OP986979.1

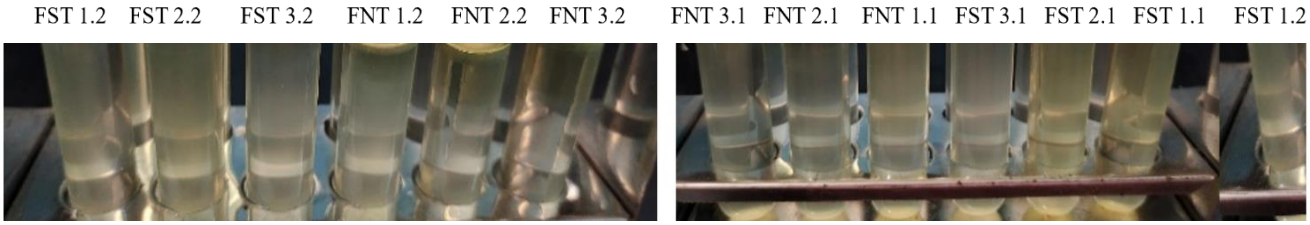

Figure 1. The results of the indole test of bacterial isolates from the mackerel *bekasam*

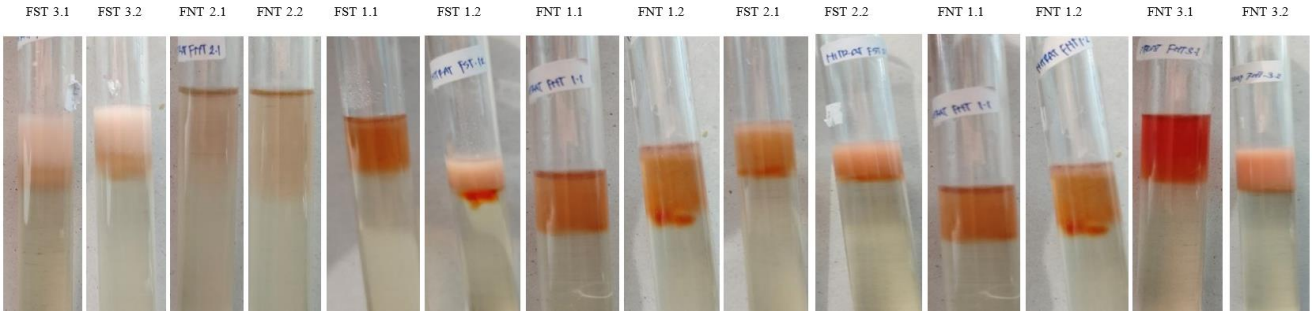

Figure 2. Results of the nitrate reduction test of bacterial isolates from mackerel *bekasam*

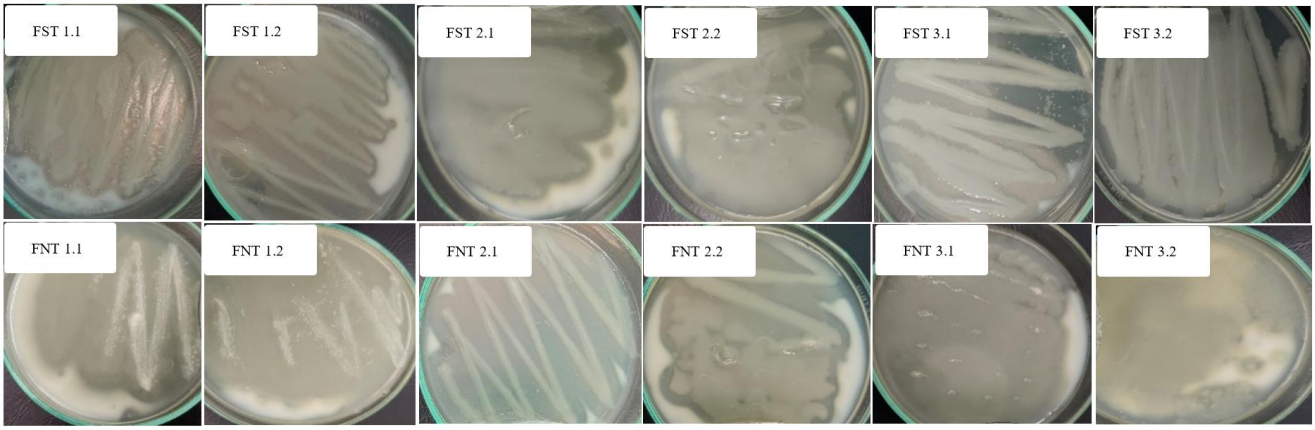

Figure 3. Proteolytic test results of bacterial isolates from mackerel *bekasam*

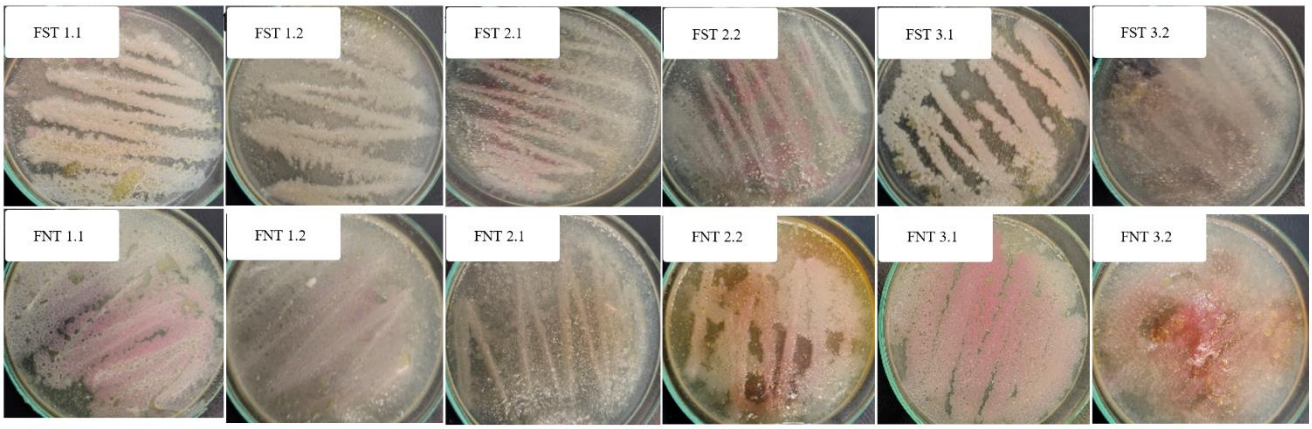

Figure 4. Fat hydrolysis test of bacterial isolates from mackerel *bekasam*

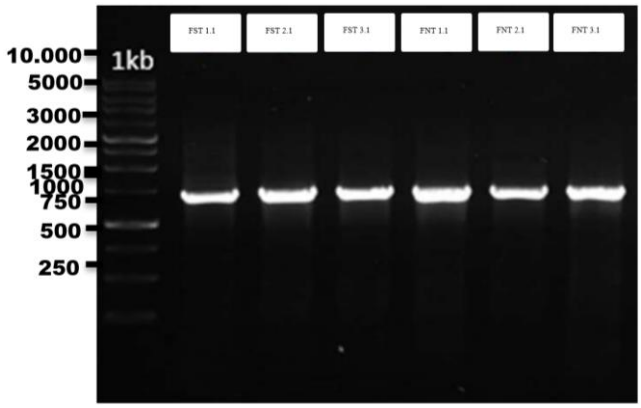

Figure 5. PCR amplification of 16 sRNA genes with primer 63f and 387r; M = marker 1 Kb ladder

Of the 12 isolates that have been characterized, 6 were selected to be identified molecularly, including isolates FST 1.1, FST 2.1, FST 3.1, FNT 1.1, FNT 2.1, and FNT 3.1. Only 6 bacterial isolates were identified at the molecular level, and these isolates were representatives of each isolate code because all isolates had similar biochemical characteristics. The results of bacterial 16S rRNA gene sequence alignments are presented in Table 2, and the construction results of the phylogenetic trees according to the Neighbor-Joining Tree of isolates FST 1.1, FST 2.1, and FST 3.1 are presented in Figure 6, while isolates FNT 1.1, FNT 2.1, and isolate FNT 3.1 in Figure 7.

Gene sequence order 16 sRNA isolate FST 1.1

TGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGA
TAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTT
GAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGT
CACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATG
AAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAA
CAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGA
AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG
GAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTG
TAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTG
GCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGT
TTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTC
CGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA
TCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGA
GCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAG
GTGGAGCTAATCTCATATTCGAAGCAACGCGAAGAACCT
TACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGG
GCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGT
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTTA
GTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA
CCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAG
CTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGT
TCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATG

Gene sequence order 16 sRNA isolate FST 2.1

TGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGA
TAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTT
GAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGT
CACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATG
AAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAA
CAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGA
AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG
GAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTG
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GCGAAGGCGACTTTCTGGTCTGTGACTGACACTGAGGCG
CGAAAGCGTGGGGAGCACACACGAGAAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGT
TTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTC
CGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA
TCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGA
GCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTGATCTTAGTTGCCATCATTCAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAG
GTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTG
TA

Gene sequence order 16 sRNA isolate FST 3.1

TGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGA
TAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTT
GAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGT
CACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATG
AAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAA
CAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGA
AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG

GAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTG
TAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTG
GCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGT
TTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTC
CGCCTGGGGAGTACGGCCGCCAGGCTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA
TCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGA
GCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAG
GTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTG
TAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAG

Gene sequence order 16 sRNA isolate FNT 1.1

TGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGA
TAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTT
GAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGT
CACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATG
AAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAA
CAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
ATTTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGA
AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG
GAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTG
TAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTG
GCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGT
TTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTC
CGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA
TCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGA
GCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAG
GTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTG
TAGGCTGCAACTCGCCTACATGA

Gene sequence order 16 sRNA isolate FNT 2.1

TGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGA
TAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTT
GAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGT
CACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATG
AAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAA
CAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGA
AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG
GAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTG
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GCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGT
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CGCCTGGGGAGTACGGCCGCCAGGCTGAAACTCAAAGGA
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GCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAG
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TAGGCTGCAACTCGCCTACATGA

Gene sequence order 16 sRNA isolate FNT 3.1

TGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGA
TAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTT
GAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGT
CACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
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CAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
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AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG
GAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTG
TAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTG
GCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCG
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GTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGT
TTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTC
CGCCTGGGGAGTACGGCCGCCAGGCTGAAACTCAAAGGA
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GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
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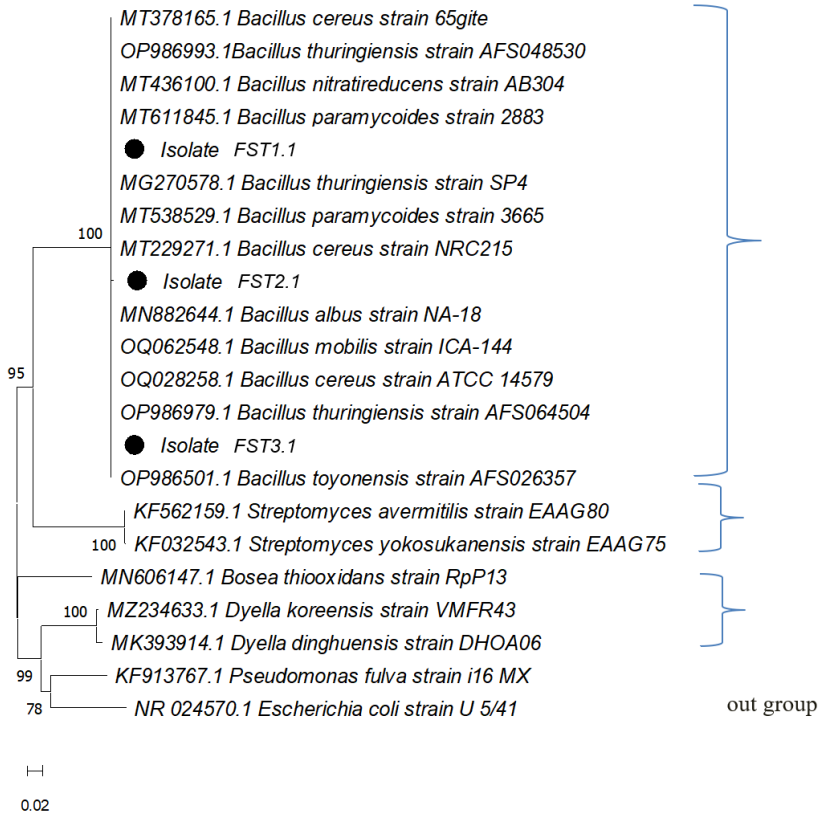

Figure 6. Phylogenetic tree construction of isolates FST 1.1, FST 2,1, and FST 3.1 according to Neighbor-Joining Tree using the Tamura 2-parameter method with 1000 bootstrap replications

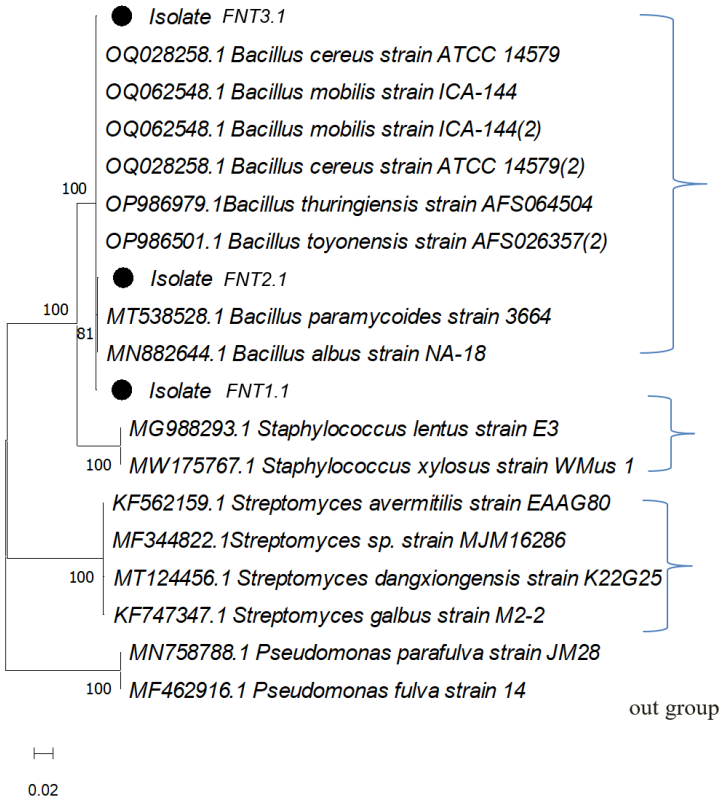

Figure 7. Phylogenetic tree construction of isolates FNT 1.1, FNT 2.1, and FNT 3.1 according to the Neighbor-Joining Tree by the Tamura 2-parameter method with 1000 bootstrap replications

Discussion

The results of the biochemical characterization showed that all bacterial isolates showed negative results (Table 1; Figure 1). This was indicated by the absence of a color change after administration of kovacs reagent to bacterial isolates grown on tryptone broth media, so it can be said that these bacterial isolates did not produce tryptophanase enzymes. The usefulness of the tryptophanase enzyme is that it can hydrolyze tryptophan, which has an indole side group; if kovacs reagent is given, the indole will react with the reagent and form a red indole ring (Shabeer et al. 2016).

The characterization also showed that 8 isolates were able to reduce nitrate, namely isolate FST 1.1, isolate FST 1.2, isolate FST 2.1, isolate FST 2.2, isolate FNT 1.1, isolate FNT 1.2, isolate FNT 3.1, isolate FNT 3.2, while isolate FST 3.1, isolate FST 3.2, FNT 2.1 isolate, and FNT 2.2 isolate were unable to reduce nitrate (Table 1; Figure 2). Bacteria capable of reducing nitrate can produce the enzyme nitrate reductase (Kraft et al. 2011; Tourova et al. 2022). Bacteria with an anaerobic metabolic mechanism require an electron acceptor other than oxygen (O_2). Some anaerobic bacteria use nitrate as an electron acceptor in their metabolic processes (Müller 2001; Shan et al. 2012; Zhang et al. 2019; Zhang et al. 2020; Zhang et al. 2022). Bacterial isolates capable of reducing nitrate in this study a lign with the origin of these bacterial isolates, namely from mackerel *bekasam* a fermented product.

The results of the proteolytic activity test showed that of the 12 isolates, only 2 had no proteolytic activity, namely isolate FST 3.1 and isolate FST 3.2 (Table 1; Figure 3). Bacterial isolates that have proteolytic activity are bacteria that are capable of producing protease enzymes, which indicates the breakdown of peptide bonds into simpler molecules (Donkor et al. 2007; Moslehishad et al. 2013; Zhong et al. 2021; Zhu et al. 2021). It is relevant to the origin of the bacterial isolate, *bekasam*, which is the fermentation product. It is known that the bacteria crucial in the fermentation process are group of bacteria with proteolytic properties. Proteolytic bacteria generally come from the genus *Bacillus*, *Pseudomonas*, *Proteus*, *Staphylococcus*, and *Steptobacillus* (Todorov and Dicks 2009; Yani et al. 2022).

The fat hydrolysis test revealed that 11 bacterial isolates isolated from mackerel fish shells showed a positive reaction in hydrolyzing fat except for FST 1.2 isolate. Eleven bacterial isolates (Table 1; Figure 4) were categorized as capable of producing lipase enzymes. Lipase enzyme is an enzyme that can break down fats into fatty acids and glycerol. This is in line with the origin of the bacterial isolate, a product of the mackerel's former sour fermentation.

Bacteria that are crucial in the fermentation process can hydrolyze complex compounds into simpler compounds, including lipolytic bacteria that can hydrolyze fat (Dueramae et al. 2017; Daroonpunt et al. 2018; Tatiyaborworntham et al. 2022). Fats are broken down into fatty acids, and glycerol makes it easier for the digestive system to absorb nutrients (Wang et al. 2013; Saha et al. 2021). In addition, the breakdown of fat into simpler compounds contributes to the formation of aroma and taste (Ding et al. 2020; Liu et al. 2021). This was confirmed by the origin of the bacterial isolate, namely mackerel *bekasam*. The ability of bacteria to secrete extracellular enzymes is an adaptation mechanism to the environment in which bacteria grow. Furthermore, extracellular lipase secretion for the breakdown of fat into fatty acids and glycerol allows bacteria to use it as a carbon source (Patnala et al. 2016; Adetunji et al. 2021).

Moreover, degraded fatty fish may form unsaturated fatty acids Eicosapentaenoic Acid (EPA) and Docosaexanoic Acid (DHA), which can reduce the amount of very low-density lipoproteins (VLDLs) in the blood. The process of fat degradation into fatty acids and glycerol can potentially prevent hypertension in the human body (Susanto and Fahmi 2012; Oliphant and Allen 2019). Lipolytic bacteria, members of the lactic acid group, are also present in food fermentation's by-food fermentation's by-products and are important for giving unique texture, flavor, and nutritional value (Hu et al. 2022).

Amplification of the genomic DNA of bacterial isolates from mackerel ex-acid products resulted in DNA fragments that were about 1300 bp in size (Figure 5). The 6 selected isolates were identified, only 6 bacterial isolates were identified at the molecular level, and these isolates were representatives of each isolate code because all isolates had similar biochemical characteristics. The results of the 16S rRNA gene sequence alignment of the bacterial isolates (Table 2) and the results of the phylogenetic tree construction of the bacterial samples were isolates FST 1.1, isolate FST 2.1, and isolate FST 3.1 (Figure 6), and isolates FNT 1.1, FNT 2.1, and isolate FNT 3.1 (Figure 7), according to Neighbor-Joining Tree in addition, isolate FST 1.1 identified as *Bacillus paramycoides* strain 2883, isolate FST 2.1 identified *Bacillus paramycoides* 3665, isolate FST 3.1 identified as *Bacillus mobilis* strain ICA-144, isolate FNT 1.1 identified as *Bacillus cereus* strain ATCC 14579, isolate FNT 2.1 identified as *Bacillus mobilis* strain ICA-144, and FNT 3.1 isolate identified as *Bacillus cereus* strain ATCC 14579.

Isolate FST 1.1 was identified as *Bacillus paramycoides* strain 2883, and isolate FST 2.1 as *Bacillus paramycoides* strain 3665. According to the research results, *Bacillus paramycoides* could be used as a bioremediation agent. *Bacillus paramycoides* can degrade the heavy chromium VI contaminating water (Gu et al. 2023). Chromium is a toxic and carcinogenic industrial waste; Chromium that is often found in nature is Cr^{6+} and Cr^{3+}. Chromium VI is more dissolved in water, while chromium III is more abundant in soil. Chromium III is more stable than chromium VI. However, chromium VI is more carcinogenic and toxic than other forms of heavy metal chromium (Shekhawat et al. 2015; Hossini et al. 2022). The chromium metal industry includes, petroleum refining, electroplating, inorganic chemical production, leather tanning, and textile processing (Srinath et al. 2002; Kerur et al. 2021). It was also reported that *Bacillus paramycoides* MW876249 was able to degrade insoluble chicken feather keratin (CFK) into amino acids (AA) through the keratinase system (Moussa et al. 2021).

Bacillus paramycoides MZ676667 BL2 is reported to be able to produce ligininolytic enzymes and can degrade complex pollutants originating from paper pulp industry waste (PPIE), where PPIE toxicity is reduced by up to 75% (Verma et al. 2022).

Bacillus paramycoides PBG9D and *Bacillus paramycoides* BCS10 isolated from *Channa punctatus* and *Channa striatus* have potential as probiotics. These bacterial strains showed antimicrobial activity against the fish pathogen, including *Aeromonas hydrophila*. These bacteria also show tolerance to acidic and basic pH (2, 3, 4, 7, and 9) and bile salts (0.3%). In addition, food supplements by administering *Bacillus paramycoides* strains were reported to increase the specific growth rate and disease resistance to *Aeromonas hydrophila* in *Labeo rohita* (Yousuf et al. 2023). *Bacillus paramycoides* spp. has also been reported to be effective in treating hospital wastewater (Rashid et al. 2021; Khan et al. 2021).

Isolate FST 3.1 was identified as *Bacillus mobilis* strain ICA-144, and isolate FNT 2.1 was included in the same strain as *Bacillus mobilis* strain ICA-144. *Bacillus mobilis* 34T was reported to be able to degrade 2,4,5-Trichlorophenoxyacetic acid, which contaminates the soil (Korobov et al. 2019).

Isolate FNT 1.1 was identified as *Bacillus cereus* strain ATCC 14579, and isolate FNT 3.1 as *Bacillus cereus* strain ATCC 14579. The genus *Bacillus* is a multifunctional microorganism in its application, in traditional fermentation and modern biotechnology. *Bacillus* spp. functions as a starter culture in various traditional fermented foods and biotechnology as a producer of enzymes, bioactive peptides, and antibiotics. *Bacillus* is also used as a probiotic (Todorov et al. 2022).

The *Bacillus* strain tolerated pH 2.5 for 2.5 hours, resistant to 0.3% bile salts and 0.1% to pancreatin for 5 hours. *Bacillus* can attach to intestinal epithelial cells and shows good proteoliytic activity against food proteins such as gelatin and milk potential probiotic proteolytic strains for food proteins (Wang et al. 2021).

Bacillus species can also be used as a bacterial concentrate to reduce odors and kill pathogenic bacteria, as well as potential as probiotics (Łubkowska et al. 2023). However, *Bacillus cereus* is also considered a foodborne pathogen that commonly causes gastrointestinal diarrhea and emetics (Sornchuer et al. 2022). *Bacillus cereus* originating from soil can cause food poisoning because this bacterium is an enteropathogenic strain that produces enterotoxins in the intestine (Jessberger et al. 2020). The implications of this research include providing information about the diversity of bacteria involved in the mackerel fermentation process and assisting in food safety. They can be used to develop new or existing food products with unique properties produced by certain bacteria during fermentation. They provide scientific support for developing a local food industry based on mackerel fish paste and serve as a basis for more extensive research.

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