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Diversity and protease production potential of haloalkaliphilic bacteria isolated from the Kesongo Mud Volcano of Indonesia

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ABSTRACT

Aims: Extremophiles are microorganisms capable of surviving in extreme environments and producing stable enzymes called extremozymes, such as proteases. One example of extremophilic bacteria is haloalkaliphilic bacteria, which can thrive under high salinity and alkaline pH conditions. The Kesongo Mud Volcano in Blora, Central Java, Indonesia, offers potential as a source of haloalkaliphilic bacteria with protease-producing capability. The aim of this study was to isolate, characterize morphologically, screen proteolytic haloalkaliphilic bacteria, determine the optimal temperature and pH for protease activity of the best isolate, and identify the best isolate using molecular methods.
Methodology and results: Samples were collected from two points in the Kesongo Mud Volcano, and bacterial isolation was performed using modified Luria Bertani media. Proteolytic activity was screened using Skim Milk Agar media, and the best isolate was selected based on its proteolytic index. Further tests were conducted to optimize protease production in terms of temperature and pH. Molecular identification involved 16S rRNA gene amplification and phylogenetic analysis. Out of 35 isolates, 17 showed proteolytic activity, with BK4 exhibiting the highest activity (proteolytic index 1.66). Optimization showed BK4 produced maximal protease at pH 11 and 37 °C. Molecular analysis revealed 99.07% similarity of BK4 with *Halomonas* sp. SSL14.
Conclusion, significance and impact of study: These findings highlight the potential of haloalkaliphilic bacteria from extreme environments as a source of industrially relevant enzymes, emphasizing the value of microbial bioprospecting in geothermal ecosystems.

Keywords: haloalkaliphilic bacteria, *Halomonas* sp., Kesongo Mud Volcano, protease enzyme

INTRODUCTION

Extreme environments are habitats characterized by physical and geochemical extremes. Physical extremes include temperature, radiation, and pressure, while geochemical extremes involve dryness, salinity, pH, reactive oxygen species, or redox potential (Rothschild and Mancinelli, 2001). Microorganisms that are able to thrive in extreme environments are known as extremophiles (Zilda, 2008). Among these extremophiles, haloalkaliphiles represent a unique group capable of surviving under conditions of high salinity and extreme alkalinity. Due to their remarkable adaptations, haloalkaliphiles have gained increasing attention as promising sources of biomolecules with potential applications in biotechnology and industry (Zhao *et al*., 2014).

Extremophilic microorganisms possess specialized proteins and enzyme systems that remain functional under harsh conditions without denaturation (Kaushik *et al*., 2021). These highly stable enzymes, known as extremozymes, exhibit unique industrially relevant properties (Zilda, 2008). Proteases are among the important extremozymes with significant potential for various biotechnological and industrial applications (Gomes and Steiner, 2004).

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Proteases are enzymes that break down proteins into simpler compounds such as amino acids or peptides (Rao *et al*., 1998). Proteases have broad industrial applications, and their production continues to expand with advances in biotechnology (Mótyán *et al*., 2013). Protease enzymes are widely applied in detergents, leather tanning, textiles, food processing, pharmaceuticals, biotechnology, and waste treatment (Song *et al*., 2023). Microorganisms are widely used as protease sources because they can be cultured rapidly in large quantities, are cost-effective, and can be maintained as isolate stocks (Sharma *et al*., 2019).

Research on bacteria that produce protease enzymes continues to expand. One potential geothermal environment for isolating such bacteria is the mud volcano. Mud volcanoes are surface expressions of mud that originates from beneath the ground. The topography of mud volcanoes can take the form of cone-shaped domes or low mud pies (Kopf, 2002). Mud volcano eruptions are driven by pressurized methane-rich sediments beneath the Earth's surface and typically release clay-rich material, water, and methane gas (CH_4) through tectonic fractures (Jusfarida and Abdilbar, 2020).

Mud volcanoes are commonly found in the region stretching from Purwodadi Regency to Blora Regency. One of these is the Kesongo Mud Volcano, located in Gabusan Village, Jati District, Blora Regency, Central Java Province, Indonesia. Mud volcanoes have existed for a long time, and local communities have become accustomed to their activity, even utilizing it for various purposes such as oil mining, salt production, and tourism. Several traditional hydrocarbon mining sites operated by local residents are also found on the western side of the Kesongo Mud Volcano. Although these mud volcanoes have been established for a long time, research on mud volcanoes in this area remains very limited (Novian *et al*., 2013).

Reports on halo-alkaliphilic protease-producing bacteria from the Kesongo Mud Volcano are still very limited, and this study provides new data on the isolation and protease-producing potential of bacteria from this extreme environment. Therefore, the aim of this study was to isolate and screen haloalkaliphilic proteolytic bacteria from the Kesongo Mud Volcano, evaluate the effects of pH and temperature on protease activity, and identify selected isolates using 16S rRNA gene analysis.

MATERIALS AND METHODS

Mud sampling

Mud samples were collected from the Kesongo Mud Volcano, located in Gabusan Village, Jati District, Blora Regency, Central Java, Indonesia (Figure 1). Sampling points were selected from active areas, as indicated by mud bubbling, and were free from visible waste to minimize the risk of contamination. Samples were obtained from two different locations at a depth of approximately 10-20 cm below the mud surface. Sampling was performed aseptically using sterile gloves and disinfected equipment. Mud samples were transferred into sterile bottles and transported under cooled conditions to prevent contamination. Environmental parameters were measured *in situ*, including temperature (°C) using a thermometer, pH using pH paper and a portable pH meter, and salinity (ppt) using a refractometer.

Figure 1: Kesongo Mud Volcano, Blora Regency, Central Java Province, Indonesia.

Isolation of haloalkaliphilic bacteria

The isolation of bacteria from the Kesongo Mud Volcano was carried out following the procedures of Asy'ari *et al*. (2014, 2015). The isolation medium used was modified Luria Bertani (LB), prepared at concentrations of 0.1× and 0.01×, adjusted to pH 9 and supplemented with 4% NaCl to mimic the natural alkaline and saline conditions of the Kesongo Mud Volcano. The use of modified LB medium at pH 9 and 4% NaCl not only supported bacterial growth but also served as an initial selective step for haloalkaliphilic isolates, ensuring that only bacteria tolerant to high salinity and alkaline conditions were obtained for further studies. Modified LB 0.1× consisted of 1 g/L tryptone (0.1%), 0.5 g/L yeast extract (0.05%), and 40 g/L NaCl (4%). Modified LB 0.01× contained 0.1 g/L tryptone (0.01%), 0.05 g/L yeast extract (0.005%), and 40 g/L NaCl (4%). Both media were adjusted to pH 9 using 1 M NaOH. For agar preparation, 15 g of agar was added per litre of solution.

The bacterial isolation process commenced with the measurement of 2 g of mud samples (comprising 1 g from each of the two sampling sites) using an analytical balance. The sample was added to two Erlenmeyer flasks containing 200 mL of modified LB broth at 0.1× and 0.01× concentrations and then incubated in a shaker incubator at 37 °C for 72 h. The next step involved serial dilution from 10^{-1} to 10^{-7}. Briefly, 1 mL of the incubated mud sample in 0.1× modified LB broth was added to 9 mL of the same medium for dilution. The same procedure was applied to the sample in 0.01× modified LB broth. From each dilution, 1 mL was spread on the surface of the corresponding modified LB agar-0.1× samples on 0.1× LB agar and 0.01×

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samples on 0.01× LB agar, using a sterile spreader. Each dilution was plated in duplicate. Plates were allowed to dry on the surface before incubation at 37 °C for 120 h.

The incubation resulted in mixed colonies, each exhibiting distinct morphological characteristics. Colonies with different morphologies on each plate were marked for identification. Purification was performed by picking each distinct colony with a sterile toothpick and spotting it three times onto modified LB agar at 0.1× and 0.01×, followed by incubation at 37 °C for 120 h. Subculturing of the purified isolates was carried out on LB agar with the standard (1×) composition. Each pure isolate was picked using a sterile inoculating loop, streaked onto the surface of a Petri dish, and incubated at 37 °C for 48 h.

Morphological identification of haloalkaliphilic bacterial isolates

The bacterial isolates were characterized using both macroscopic and microscopic observations to complement molecular identification. Macroscopic characterization included assessment of colony shape, texture, margin, color, and elevation (Dwi *et al*., 2023). Microscopic characterization was performed on Gram-stained preparations using standard procedures. A small amount of bacterial suspension was spread on a sterile glass slide, heat-fixed, and sequentially stained with Crystal Violet (1 min), iodine (1 min), alcohol (10 sec), and Safranin (1 min). Gram-positive bacteria appeared purple, whereas Gram-negative bacteria appeared red (Dewi, 2013). These conventional macroscopic and microscopic methods were performed alongside molecular analysis of the 16S rRNA gene to ensure comprehensive bacterial identification. Further biochemical and metabolic analyses could also be performed to strengthen the identification in future studies.

Screening of proteolytic Bacteria

Proteolytic bacteria were screened using selective Skim Milk Agar (SMA) medium. SMA was prepared in two steps. First, 4.8 g of agar was dissolved in 125 mL of distilled water and sterilized by autoclaving. Second, 80 mL of sterile distilled water was combined with 4 g of skim milk powder and pasteurized at 70 °C for 30 min for three consecutive days. After pasteurization, the skim milk solution was mixed with the sterilized agar to produce the final SMA medium (Cahyaningrum *et al*., 2021). Initial screening was performed in duplicate using the spot method, where all isolates were dotted onto the SMA surface with sterile toothpicks. Isolates that tested positive were further screened using the disc method, in which 10 µL of bacterial suspension was placed onto paper discs on the SMA, and the procedure was repeated in duplicate. Plates were incubated at 37 °C for 48 h. Proteolytic activity was indicated by clear zones around colonies, measured three times per isolate (horizontally, vertically, and diagonally) before calculating the average. The proteolytic index was calculated by comparing the diameter of the clear zone to the diameter of the colony (Suhartono and Artika, 2017).

Temperature and pH optimization test

The temperature and pH optimization tests were conducted using the disc method only on the bacterial isolate that exhibited the highest proteolytic index. Temperature variations were 30 °C, 37 °C, and 40 °C, while pH variations were 5, 7, 9, and 11. The isolate was inoculated into 1 mL of LB broth medium in a microtube and incubated at 37 °C for 48 h in a shaker incubator. After incubation, 10 µL of the grown bacterial suspension was applied onto the center of SMA plates adjusted to the respective pH values, with a paper disc placed in the center. Plates were incubated at the three temperatures for 48 h. Each treatment was performed in triplicate. Data on the interaction between temperature and pH were analyzed using SPSS software, after testing for normality and homogeneity. A two-way ANOVA was conducted at a 95% confidence level (α = 0.05), followed by Duncan's multiple range test if a significant effect was observed.

Molecular identification of the best proteolytic bacterial isolate

The bacterial isolate with the highest potential for protease enzyme production was selected for genomic DNA extraction. DNA was extracted using the InstaGene Matrix Kit following the procedure recommended by Bio-Rad Laboratories. DNA concentration and purity were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) at 260/280 nm. The 16S rRNA gene was amplified using universal primers 27F and 1492R (Raji *et al*., 2008). PCR was performed in a 50 µL reaction mixture containing 25 µL MyTaq polymerase, 2 µL of each primer, 2 µL DNA template, and 19 µL ddH_2O. Amplification was carried out for 35 cycles with initial denaturation at 95 °C for 3 min, followed by denaturation at 95 °C for 45 sec, annealing at 54 °C for 1 min, extension at 72 °C for 90 sec, and a final extension at 72 °C for 10 min. PCR products (3 µL) were confirmed by 1% agarose gel electrophoresis in 1× TAE buffer at 100 V for 25 min. The PCR products were sequenced, and the resulting sequences were analyzed using BLAST against the NCBI GenBank database to determine species identity.

Phylogenetic analysis

Phylogenetic analysis was performed using MEGA XII with ClustalW alignment and a Neighbor-Joining tree based on 1000 bootstrap replications (Pangestika *et al*., 2015).

RESULTS AND DISCUSSION

Isolation of haloalkaliphilic bacteria from the Kesongo Mud Volcano

The environmental parameters at the sampling site showed a temperature of 34 °C, pH 9, and salinity of 40 ppt (4%), indicating alkaline and hypersaline conditions that support the presence of haloalkaliphilic microorganisms. Similar alkaline and saline conditions have also been

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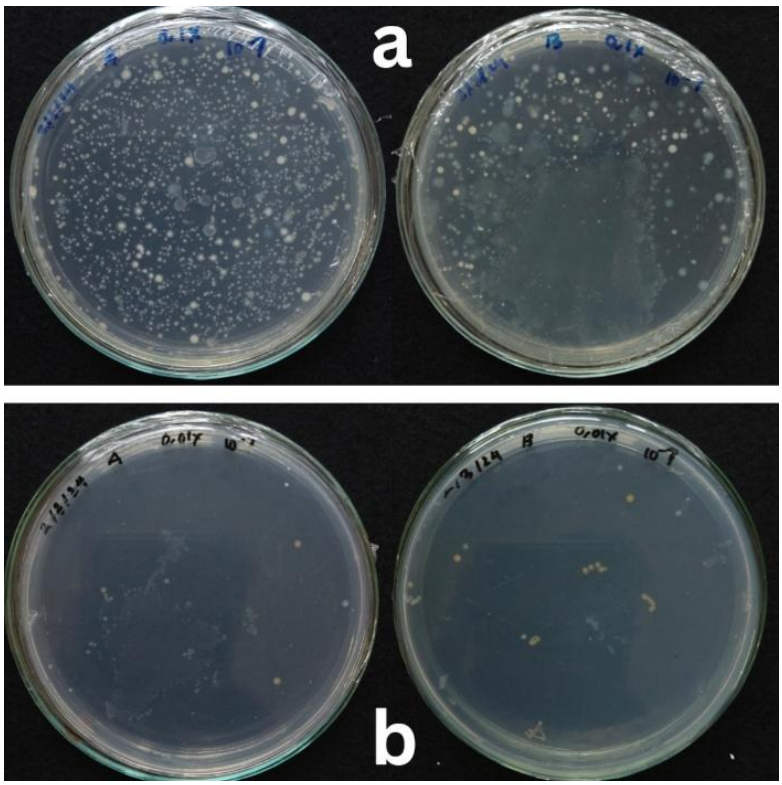

Figure 2: Bacterial isolation results at 10^{-7} dilution on (a) 0.1× modified LBA medium; (b) 0.01× modified LBA medium.

reported in other Indonesian mud volcanoes, such as Bledug Kuwu (Siregar and Siregar, 2016; Sabdaningsih and Lunggani, 2018) and the Sidoarjo Mud Volcano (Lapindo) (Puspitasari, 2014). Isolation was performed using serial dilution on modified 0.1× and 0.01× LB media (Figure 2), following approaches commonly applied for cultivating halophilic bacteria from Indonesian mud volcano environments (Asy'ari et al., 2015). Morphologically distinct colonies were purified, resulting in a total of 35 pure isolates, labeled BK1-BK35.

Morphological identification of haloalkaliphilic bacteria from the Kesongo Mud Volcano

The haloalkaliphilic bacterial isolates were characterized morphologically, and the results are presented in Table 1 and Figure 3. Macroscopic observations showed that most isolates formed circular colonies with smooth margins and raised elevations. Moist colony texture was predominant, and cream-colored colonies were the most frequently observed among the 35 isolates. Microscopic examination revealed that the isolates consisted of both Gram-positive and Gram-negative bacteria, with cocci as the dominant cell shape. Overall, Gram-positive isolates were more frequently observed than Gram-negative ones. This morphological diversity indicates that the Kesongo Mud Volcano environment supports a varied community of haloalkaliphilic bacteria.

Similar findings have also been reported from Bledug Kuwu, where bacterial isolates showed diverse colony characteristics and were dominated by coccoid forms with both Gram-positive and Gram-negative properties (Sabdaningsih and Lunggani, 2018). The presence of diverse morphological types in Kesongo Mud Volcano suggests that this hypersaline and alkaline habitat may represent a potential source of bacteria with biotechnological relevance, including protease-producing strains.

Protease screening of haloalkaliphilic bacterial isolates

Proteolytic activity of the isolates was screened using Skim Milk Agar (SMA), where casein hydrolysis is indicated by the formation of clear zones around bacterial growth (Soeka and Sulistiani, 2014). Screening was conducted in two stages. Initial screening using the pick colony method showed that 17 out of 35 isolates were able to produce detectable proteolytic activity (Table 2). Isolates that tested positive were further evaluated using the disc method to identify the most active protease producer (Figure 4). Among the tested isolates, BK4 exhibited the highest proteolytic index (1.66), calculated as the ratio between the

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Table 1: Results of macroscopic and microscopic characterization.

Isolate code	Macroscopic characteristics					Microscopic characteristics	
	Colony colors	Colony shape	Colony margin	Colony elevation	Colony texture	Cell shape	Gram
BK1	Brown with transparent edges	Round	Smooth	Raised	Moist	Rod	+
BK2	Light brown cream	Rhizoid	Irregular	Raised spreading edge	Moist	Coccus	+
BK3	Brown with white edges	Round	Smooth	Raised	Moist	Coccus	+
BK4	Orange	Round	Smooth	Convex	Moist	Rod	-
BK5	Dark orange	Round	Smooth	Convex	Moist	Coccus	-
BK6	Creamy white	Round	Smooth	Raised	Moist	Coccus	+
BK7	Dark orange	Round	Smooth	Convex	Moist	Rod	-
BK8	Brown with transparent edges	Irregular	Lobate	Raised spreading edge	Moist	Coccus	+
BK9	Cream	Irregular	Irregular	Raised spreading edge	Mucoid	Rod	+
BK10	Brown white	Round	Smooth	Convex	Mucoid	Coccus	+
BK11	Cream with transparent edges	Filamentous	Irregular	Raised	Moist	Coccus	+
BK12	Transparent cream	Filamentous	Filamentous	Raised	Moist	Coccus	-
BK13	Bright yellow	Round	Smooth	Raised	Moist	Rod	+
BK14	Creamy white	Filamentous	Lobate	Raised	Moist	Coccus	+
BK15	Cream with transparent edges	Round	Smooth	Raised Spreading Edge	Dry	Coccus	-
BK16	Cream	Filamentous	Filamentous	Raised	Dry	Coccus	+
BK17	Light brown with cream edges	Round	Smooth	Umbonate	Moist	Coccus	+
BK18	Cream with white edges	Round	Smooth	Raised	Mucoid	Rod	-
BK19	Yellow	Round	Lobate	Raised	Moist	Rod	+
BK20	Cream	Rhizoid	Irregular	Raised	Dry	Coccus	+
BK21	Orange	Irregular	Lobate	Umbonate	Moist	Coccus	-
BK22	Creamy white	Irregular	Irregular	Umbonate	Dry	Coccus	+
BK23	Brown with white edges	Filamentous	Lobate	Raised spreading edge	Moist	Rod	+
BK24	Cream	Filamentous	Filamentous	Raised	Dry	Coccus	+
BK25	Creamy white	Round	Irregular	Raised	Moist	Rod	+
BK26	Cream	Filamentous	Filamentous	Raised	Dry	Coccus	+
BK27	Cream	Round	Smooth	Raised	Moist	Coccus	+
BK28	Transparent cream	Irregular	Irregular	Flat	Dry	Coccus	+
BK29	Brown with cream edges	Irregular	Lobate	Umbonate	Moist	Rod	+
BK30	Yellowish cream	Round	Irregular	Raised	Moist	Rod	+
BK31	White	Rhizoid	Rhizoid	Flat raised margin	Dry	Coccus	+
BK32	Pink with cream edges	Round	Irregular	Convex	Moist	Coccus	+
BK33	White with transparent edges	Round	Smooth	Flat	Dry	Rod	+
BK34	Transparent brown	Round	Irregular	Umbonate	Moist	Coccus	+
BK35	Light brown	Round	Irregular	Raised	Moist	Coccus	+

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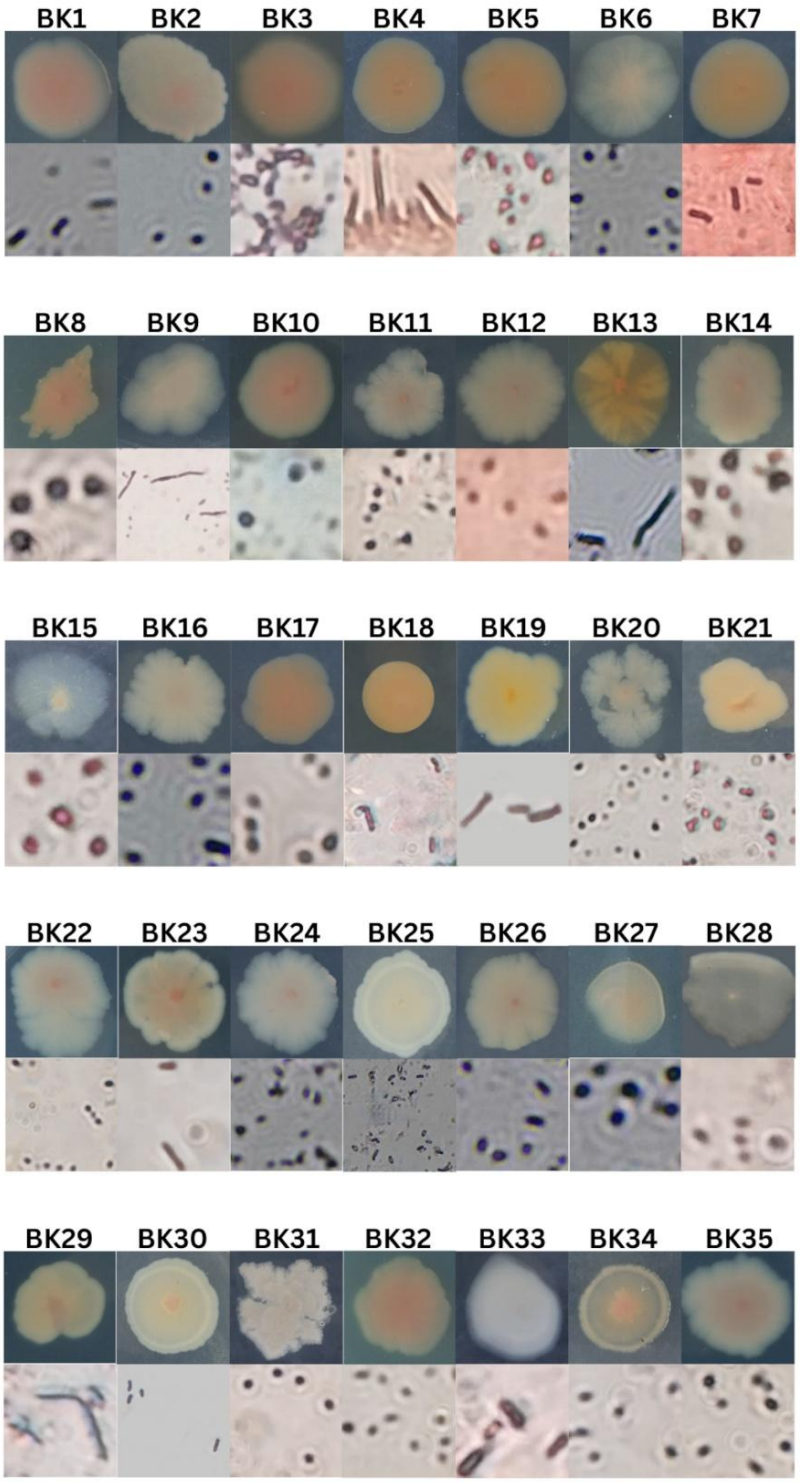

Figure 3: Macroscopic and microscopic characterization of haloalkaliphilic bacterial isolates BK1-BK35.

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Table 2: Screening of proteolytic activity of 35 isolates using the pick colony method.

Isolate code	Clear zone	Proteolytic index	Isolate code	Clear zone	Proteolytic index
BK1	+	1.06	BK19	-	-
BK2	-	-	BK20	-	-
BK3	-	-	BK21	+	2.54
BK4	+	2.84	BK22	-	-
BK5	+	1.68	BK23	-	-
BK6	-	-	BK24	-	-
BK7	+	1.96	BK25	+	0.67
BK8	+	2.84	BK26	+	0.84
BK9	-	-	BK27	-	-
BK10	-	-	BK28	+	0.69
BK11	-	-	BK29	+	1.34
BK12	+	1.78	BK30	+	0.66
BK13	-	-	BK31	+	1.04
BK14	+	2.53	BK32	-	-
BK15	-	-	BK33	+	0.64
BK16	-	-	BK34	-	-
BK17	+	2.5	BK35	+	1.09
BK18	-	-			

Note: (+) isolates capable of producing protease, (-) isolates not capable of producing protease

Table 3: Screening of proteolytic activity of 35 isolates using the paper disc method.

Isolate code	Clear zone	Proteolytic index
BK1	+	0.58
BK4	+	1.66
BK5	+	0.96
BK7	+	0.74
BK8	+	1.58
BK12	+	0.65
BK14	+	0.79
BK17	+	1.58
BK21	+	1.32
BK25	+	0.83
BK26	+	0.84
BK28	+	0.49
BK29	+	1.04
BK30	+	0.76
BK31	+	0.56
BK33	+	0.77
BK35	+	0.3

Note: (+) isolates capable of producing protease

clear zone diameter and colony diameter (Table 3). Similar casein-based screening approaches have been widely applied as a semi-quantitative method to assess extracellular protease production on solid media (Anagnostakis and Hankin, 1975; Lopes *et al.,* 2011). Based on these results, BK4 was selected for subsequent optimization of temperature and pH conditions, as well as molecular identification using the 16S rRNA gene.

Although protease screening (both spot and disc assays) was performed on SMA under standard conditions, all isolates had been previously enriched and purified under alkaline (pH 9) and saline (4% NaCl) conditions during the isolation stage. Therefore, the screening assay was intended to evaluate the proteolytic potential of isolates previously selected from haloalkaline conditions. The effects of pH and temperature on protease activity, including highly alkaline conditions (up to pH 11), were subsequently examined during the optimization stage.

Optimization test of temperature and pH

Temperature and pH are key factors affecting extracellular protease production (Baharuddin *et al*., 2014; Herlet *et al*., 2017). The results of temperature and pH optimization for the best proteolytic isolate, BK4, are shown in Table 4. Two-way ANOVA indicated that the interaction between temperature and pH significantly influenced protease activity ($p < 0.05$).

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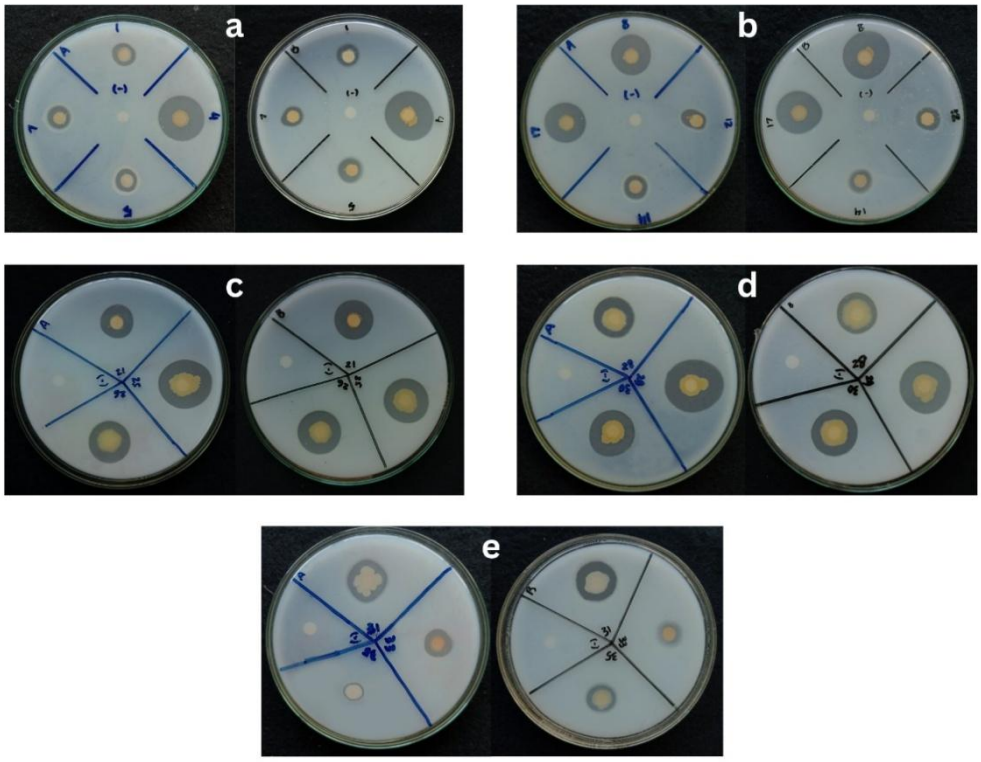

Figure 4: Protease screening results using the paper disc method in duplicate for (a) BK1, BK4, BK5, BK7; (b) BK8, BK12, BK14, BK17; (c) BK21, BK25, BK26; (d) BK28, BK29, BK30; (e) BK31, BK33, BK35.

Table 4: Proteolytic index for temperature and pH variation treatments.

	Replication	pH 5	pH 7	pH 9	pH 11
Temperature 30°C	A	1.66	1.60	1.19	1.87
	B	1.25	1.58	1.26	1.90
	C	1.39	1.50	1.63	1.90
Temperature 37°C	A	1.43	1.80	1.59	2.06
	B	1.37	1.81	1.68	1.67
	C	1.31	1.70	1.72	1.96
Temperature 40°C	A	1.22	1.18	1.26	1.51
	B	1.57	1.19	1.36	1.68
	C	1.34	1.26	1.30	1.61

Note: (A) first replication, (B) second replication, (C) third replication

Based on Duncan's multiple range test, the highest proteolytic index was obtained at 37 °C and pH 11, with a value of 1.897 (Table 5; Figure 5). These conditions agree with previous studies. Behera *et al*. (2024) reported that *Streptomyces barkulensis* RC1831 exhibited maximal protease production at 37 °C and pH 11. Similarly, Dodia *et al*. (2008) found that purified protease from the Haloalkaliphilic bacterium AH-6 showed catalytic activity and stability across pH 8-13, with peak activity at pH 9-11 and 37 °C. Protease enzymes active at 37 °C and pH 11 have potential applications in industries such as detergents (Sayaniya and Patel, 2021) and the production of concentrated latex (DPNR) for sphygmomanometers (Siswanto *et al*., 2009). Notably, the skim milk agar at pH 11 remained solid throughout the experiment, enabling reliable evaluation of protease activity under strongly alkaline conditions, consistent with previous studies that have employed skim milk-based agar media for screening alkaline protease-producing bacteria at high pH (Mahakhan *et al.,* 2023).

Molecular identification of the best proteolytic bacterial isolate

Molecular identification of the best proteolytic isolate, BK4, was carried out by amplifying the 16S rRNA gene DNA.

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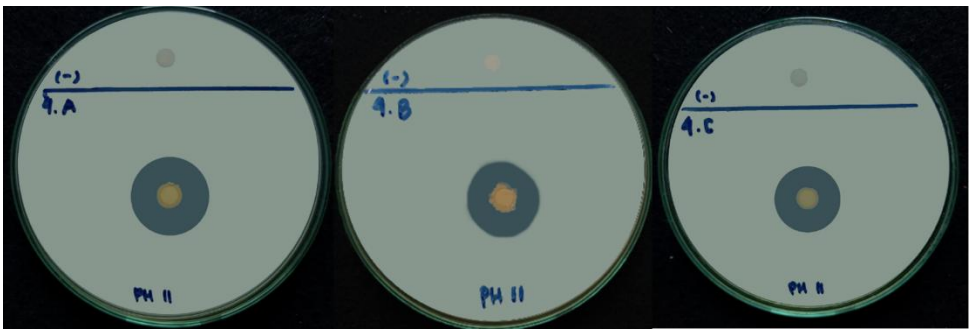

Figure 5: Results of optimal condition test at the interaction of 37 °C temperature and pH 11.

Table 5: Duncan test for isolate BK4.

pH	Temperature		
	30 °C	37 °C	40 °C
5	1.433 ± 0.208^{abc}	1.370 ± 0.060^{ab}	1.377 ± 0.178^{ab}
7	1.560 ± 0.053^{bcd}	1.770 ± 0.061^{de}	1.210 ± 0.044^{a}
9	1.360 ± 0.236^{ab}	1.663 ± 0.066^{cde}	1.307 ± 0.050^{a}
11	1.890 ± 0.017^{e}	1.897 ± 0.202^{e}	1.600 ± 0.085^{bcd}

Note: Numbers followed by different letters indicate significant differences between temperature and pH treatments. Bold text: the best temperature and pH interaction for BK4 in producing protease enzyme.

was extracted following standard protocols and showed high purity with an A260/A280 ratio of 1.98 (Table 6). PCR amplification of the 16S rRNA gene produced a clear band of approximately 1500 bp, indicating successful amplification (Figure 6). The band was bright and clearly visible (Hidayati *et al*., 2016), intact, thick, and non-smearing (Nugraha *et al*., 2014), indicating successful DNA isolation.

The PCR amplification product was sent to PT. Saraswanti Indo Genetech, Bogor, Indonesia, for sequencing. The obtained sequence was then analyzed using BioEdit software. It was compared to the GenBank database using BLAST via NCBI, revealing 99.07% similarity to *Halomonas* sp. SSL14. The BK4 sequence has been deposited in GenBank under accession number PV076946 as *Halomonas* sp. strain BK4 16S ribosomal RNA gene, partial sequence.

Table 6: Results of DNA purity and concentration measurement.

DNA sample	Concentration (ng/ µL)	A260	A280	A260/A280
BK4	315.1	6.301	3.175	1.98

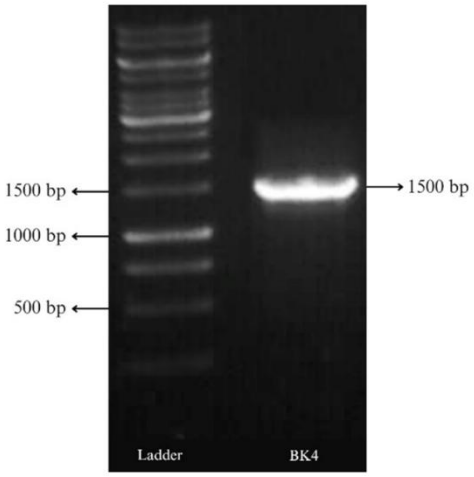

Figure 6: Electrophoresis results of 16S rRNA gene amplification of isolate BK4 showing a band length of 1500 bp.

Phylogenetic tree analysis

The phylogenetic tree (Figure 7) shows that BK4 clustered with *Halomonas* sp. SSL14 on the same branch, supported by a bootstrap value of 100%, indicating strong confidence in the relationship. According to Simpson (2010), bootstrap values of 70% or higher generally indicate that a node is strong and reliable. The ingroup included BK4, *Halomonas* sp. SSL14 (the closest relative based on BLAST), four additional protease-producing species from mud volcanoes (Venkadesaperumal *et al*., 2014; Sepanian *et al*., 2018), and three species from deep-sea environments (Xiong *et al*., 2007; Zhou *et al*., 2009). The outgroup used in this phylogenetic analysis was *Gemmata obscuriglobus* strain UQM 2246.

The BLAST results and phylogenetic tree showed that isolate BK4 is similar to *Halomonas* sp. SSL14. BK4 colonies are yellow to orange, round, smooth-edged, and moist, with bacillus-shaped, Gram-negative cells. This is in accordance with Perumal *et al*. (2024), who stated that *Halomonas* sp. colonies are yellow, round-shaped, translucent, and Gram-negative. Mnif *et al*. (2009) further reported that *Halomonas* sp. colonies can range in colour

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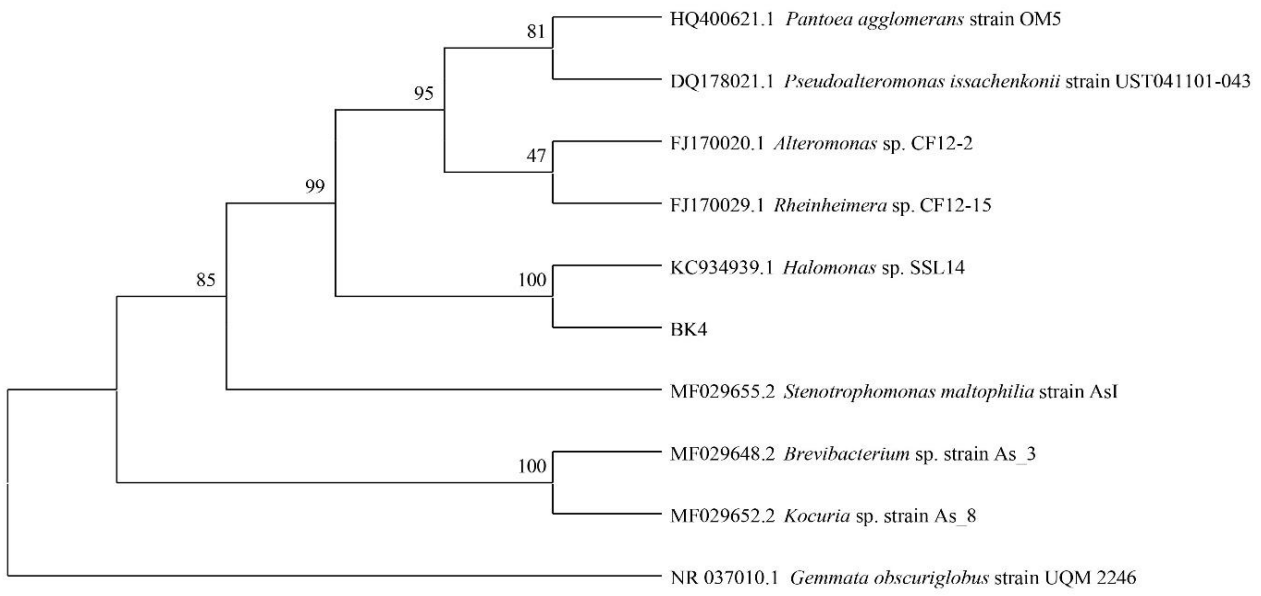

Figure 7: Phylogenetic tree analysis result of isolate BK4.

from cream to golden yellow, are small, smooth, aerobic, highly halotolerant, bacillus-shaped, and Gram-negative.

Potential applications of *Halomonas* sp.

Since BK4 is closely related to *Halomonas* sp., it may have similar biotechnological potential. *Halomonas salifodinae* IM328, for instance, has a high capability for producing compatible solutes (Wang *et al*., 2024), such as hydroxyectoine (Yang *et al*., 2024), a valuable raw material for pharmaceutical and cosmetic industries (Parwata *et al*., 2022). *Halomonas meridiana* BK-AB4, isolated from Bledug Kuwu, shows great potential in biotechnology, particularly as a biosurfactant producer (Sari *et al*., 2020). *Halomonas* TD01 can produce polyhydroxyalkanoates (PHA) with titers and yields comparable to sterile systems using *Escherichia coli* or *Ralstonia eutropha* (Fu *et al*., 2014).

CONCLUSION

A total of 35 haloalkaliphilic bacterial isolates were recovered from the Kesongo Mud Volcano, of which 17 exhibited protease-producing potential based on clear zone formation on skim milk agar. Among these, BK4 emerged as the most proteolytic isolate, with optimal enzyme production observed at 37 °C and pH 11. Molecular analysis revealed that BK4 is closely related to *Halomonas* sp. SSL14, indicating its potential relevance for enzyme-based applications in alkaline systems. However, further quantitative evaluation and optimization in liquid and industrially relevant environments are required before practical implementation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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