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by Elvy Like Ginting 13

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Identification of Proteolytic Thermophiles from Moinit Coastal Hot-Spring, North Sulawesi, Indonesia

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ABSTRACT

We have isolated thermophilic bacteria from Moinit coastal hot-spring, North Sulawesi, Indonesia. Of 12 seawater samples collected, we obtained 20 thermophilic bacterial isolates. Sixteen of the isolated thermophiles were found to exhibit protein-degrading ability as indicated by the presence of clearing zones surrounding their colonies on skim milk-containing plates at 65 °C. Five of them with larger proteolytic indexes, designated as 2b, 4b, 9a, 9b, and 12L, were selected for further studies. The ability of these five strains to hydrolyze casein at high temperatures was indicated by relatively high activity of their extracellular proteases at 60 °C. Partial protease-encoding genes were amplified from each isolated strain and sequenced. Further bioinformatics analysis suggests that the extracellular proteases that they produced belong to serine proteases. The integrated morphological, biochemical, and genetic analyses showed the identity of the strains 2b, 4b, and 9a as members of *Bacillus*, 9b as *Pseudomonas*, and 12L as *Aneurinibacillus*. To the best of our knowledge, so far thermophilic *Pseudomonas* and *Aneurinibacillus* remain poorly investigated in term of extracellular protease production, suggesting the biotechnological potential of the strains 9b and 12L as the producer of a unique extracellular thermostable protease.

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KEYWORDS
Coastal hot-spring; extracellular proteases; proteolytic thermophiles

Introduction

Proteases are one of the most important classes of industrial enzymes, which account for 60% of the total world-wide enzyme sales (Rao et al. 1998). They have found a wide range of applications, including food, pharmaceutical, and detergent industries (Namjoshi et al. 2008; Rao et al. 1998; Toplak et al. 2013). Proteases are produced by a wide range of microbial groups, including bacteria, molds, and yeasts. Among microorganisms, bacteria especially have attracted more attention as the source for protease production, because they tend to grow more rapidly and easier to maintain and access for genetic modifications (Singh et al. 2016). In the marine ecosystems, highly diverse heterotrophic bacteria produce proteases extracellularly to decompose larger macromolecules or high molecular weight dissolved organic matter (DOM) into smaller molecular sizes (Arnosti 2013; Arnosti et al. 2014), making organic nutrients available for themselves and other marine organisms (Cunha et al. 2010). Extracellular proteases produced by bacteria within complex microbial communities can inhibit biofilm formation by competitive microorganisms. Since extracellular proteins can represent a major component of the biofilm's dry mass (Jiao et al. 2010; Lasa and Penadés 2006), bacterial proteases hold promising applications as anti-biofilm agents for the treatment and prevention of biofilm-associated infections (Blackledge et al. 2013; Boels 2011; Fleming and Rumbaugh 2017; Nahar et al. 2018).

Various types of unique marine habitats can be considered as the reservoirs of bacterial proteases with unusual properties (e.g., relatively high stability and activity) which could be useful for industrial processes (Fulzele et al. 2011). Bacteria living in marine hot-springs or hydrothermal vents, called thermophiles, have especially been recognized as the ideal producers of thermostable enzymes (Dalmaso et al. 2015). The thermostability of their cell components at highly elevated temperatures has led to their extensive use in various biotechnological and industrial applications (Dumorné et al. 2017; Raddadi et al. 2015). Several advantages of thermophilic bacteria or thermostable enzymes for biotechnological applications include (1) lower fermentation/feedstock cost, (2) decreased viscosity of culture broth, (3) sterility requirements not as stringent as for mesophiles, (4) possibility that low biomass leads to high product, (5) secreting many enzymes, (6) simplified recovery of volatile products, (7) stability of enzymes, (8) more resistant than mesophilic to detergent and denaturants, and (9) having higher specific activities than mesophilic enzymes (Berquist et al. 1987).

Thermophilic bacteria have been isolated from a wide range of environmental habitats, including marine hydrothermal vents (Gugliandolo et al. 2012; Harmensen et al.

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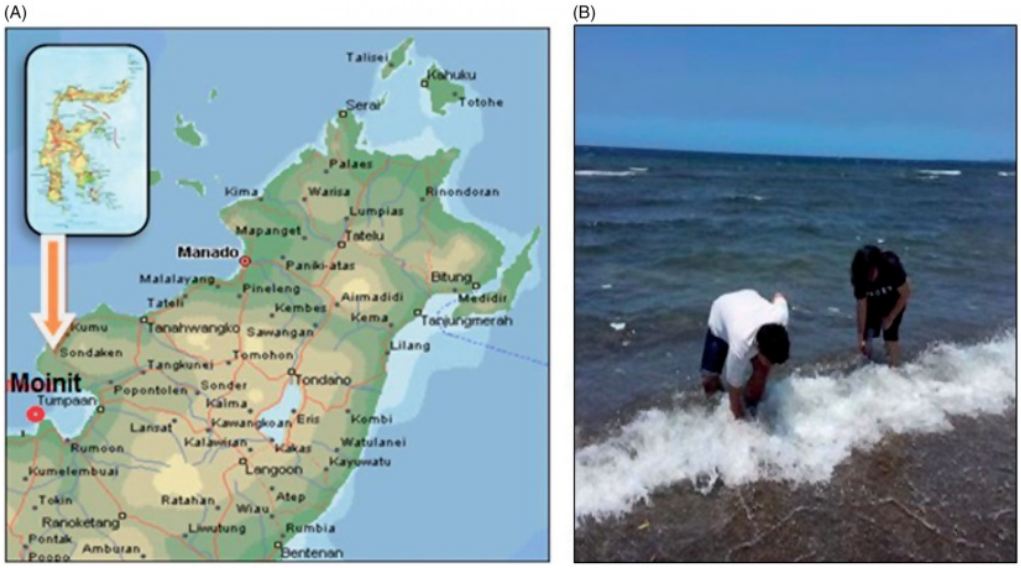


Figure 1. Sample collection at Moinit coastal hot-spring in North Sulawesi, Indonesia. (A) Google map of North Sulawesi showing Moinit, Amurang district as indicated by an arrow. (B) Sampling activities at several sites along Moinit hot-spring coast.

1997; Nicolaus et al. 2002). Although marine hydrothermal spots can be found in many places throughout Indonesia archipelago, so far only a few studies were reported about thermostable protease-producing bacteria originated from Indonesian hot-springs. Notable examples are protease-producing thermophiles isolated from hot-springs in Padang Cermin and Banyu Wedang Bali (Wang et al. 2012; Zilda et al. 2013, 2014, 2018). So far proteolytic thermophiles originated from hot-springs in North Sulawesi remain poorly investigated. In this study, we reported our attempts to isolate and identify thermophilic bacteria with the ability to produce proteases extracellularly from a coastal hot-spring in North Sulawesi. The coastal hot-spring chosen in our study is located at Moinit, Amurang district, North Sulawesi. We discuss briefly the importance of proteolytic strains isolated in this work from ecological and biotechnological perspectives.

Materials and methods

Materials

Polymerase chain reaction (PCR) primers were purchased from Integrated DNA Technologies (IDT), Singapore. QIAprep Miniprep kit was from Qiagen (Hilden, Germany), and the DNA ladder marker was from Solis BioDyne (Tartu, Estonia). All other reagents, including agarose LE and chemicals for making medium, were the highest quality obtained from Sigma Chemicals Co. (St. Louis, MO).

Sampling and screening of protease-producing thermophilic bacteria

Samples of seawaters were obtained from Moinit coastal hydrothermal region located in Amurang District, South Minahasa Regency, North Sulawesi, Indonesia (Figure 1) using sterile glass tubes. Environmental parameters (e.g., temperatures, pH, and salinity) of the sampling sites were measured. The temperature of the sampling location was about 60 °C. The pH recorded was in the range of 7–8. For bacterial isolation, each seawater sample was serially diluted and plated on Thermus Medium Modified Agar (TMMA) consisting of 0.01% $MgSO_4.7H_2O$, 0.1% K_2HPO_4, 0.35% $(NH_4)_2SO_4$, 0.1% NaCl, 0.5% Yeast extract, 0.05% peptone, and 2% agar. TMMA plates were incubated at 55 °C for 24–48 h. Single growing colonies exhibiting diverse morphological characteristics (e.g., colony, color, edges, and growth shape) were individually isolated, grown on new TMMA agar plates, and subsequently observed more carefully in term of their morphology. To screen for their ability to produce protease extracellularly, each isolate was transferred onto the middle of a TMM agar plate containing 2% skim milk, followed by incubation at 45–65 °C for 24–48 h. A clear zone formed around the colony indicated the ability of the isolate to produce protease extracellularly. The proteolytic index was calculated by comparing the diameter of the clear area (zone around colony + colony diameter) with that of the corresponding bacterial colony (Cheeseman 1963; Montville 1983). The isolates exhibiting a high proteolytic index with the ability to grow at the high temperature were selected for further studies.

Protease activity assay

Bacterial strains were individually cultivated in liquid TMMB containing 2% casein at 65 °C for 18 h in an orbital shaking water of 200 rpm. Free-cell supernatant was obtained by centrifugation at 5000 rpm for 15 min, and subsequently used for measuring protease activity. Protease

activity was measured according to the method originally described by Anson (Anson 1938) with some modifications (Lanoë and Dunnigan 1978; Shimogaki et al. 1991; Takami et al. 1989). The following components were mixed in a glass tube: 1 ml of 1% (w/v) casein in 0.02 M NaOH, 2 ml of 0.4 M phosphate buffer pH 6, and 1 ml of cell-free medium (suitably diluted). This reaction mixture was incubated at 65 °C for 10 min. The reaction was subsequently stopped by adding 3 ml of 10% trichloroacetic acid. After 5 min of the reaction termination, it was centrifuged at 12,000 ×*g* for 5 min. The supernatant (0.5 ml) from each sample was incubated with 2.5 ml of 0.5 M NaOH in 2% (w/v) Na_2CO_3 for 10 min. Thereafter, 0.25 ml of Folin phenolic reagent (commercial solution diluted 1:1 in distilled water) was added, mixed and kept for 30 min at room temperature. The absorbance value was measured at 750 nm in an 1-cm quartz cuvette, which was subsequently converted to mg of tyrosine/l using a tyrosine calibration curve (mg tyrosine/l *vs.* absorbance). One unit of protease (EU/ml) was defined as the amount of enzyme that produced an absorbance at 750 nm equivalent to 1 µmol of tyrosine/min under the assay conditions.

Isolation and bioinformatic analysis of partial protease-encoding genes

Genomic DNA from each strain was prepared using QIAprep Miniprep kit (Qiagen, Hilden, Germany). The partial genes coding for proteases were PCR-amplified from the genome DNA of individual proteolytic isolates using the primer pair KerF (5′-TAYAYHGTNGGNTTYAAR-3′) and KerR (5′-NARNARYTTNACNCCRTA-3′) (Zilda et al. 2018). The target PCR product of ~331 bp was run on 1% agarose, extracted from the gel, and sequenced at the First Base Asia (Malaysia). All of the protease-encoding DNA sequences isolated in this work were subjected to BLASTx to predict their function (Altschul et al. 1990; Altschul et al. 1997). These gene sequences were translated into amino acid sequences using the Web-based translation tool ExPASy (http://www.expasy.org/tools/dna.html), and subsequently aligned with representatives of protease sequences retrieved in GenBank using ClustalW or Clustal Omega (Sievers and Higgins 2018; Thompson et al. 1994). Based on the multiple alignments, the conserved catalytic triads for protease activities were identified.

Identification of proteolytic thermophiles and phylogenetic analysis

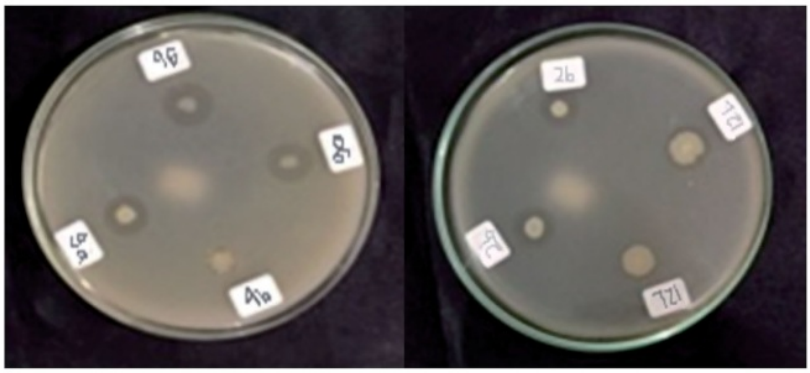
Bacterial isolates with high proteolytic indexes were morphologically and biochemically characterized according to the Bergey's manual of Determinative Bacteriology (Bergey et al. 1994). Morphological examination was carried out on TMMA media, followed by Gram staining and microscopic observation (Coico 2006). The combined cellular, biochemical, and molecular analyses were taken into consideration in determining the taxonomic status of each isolated strain. Molecular analysis of almost complete *16S rRNA* gene (~1400 bp) was conducted (Weisburg et al. 1991), which included the preparation of genomic DNA from each sample using QIAprep Miniprep kit (Qiagen, Hilden, Germany) and subsequent PCR amplification of 16S rRNA gene region using the universal primer pair 8 F (5′-AGAGTTTG ATCCTGGCTCAG-3′) (Turner et al. 1999) and 1492 R (5′-ACCTTGTTACGACTT-3′) (Weisburg et al. 1991; Wilson et al. 1990). The PCR condition consisted of initial denaturing at 95 °C for 6 min followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. The size-corrected PCR products were observed by electrophoresis on 1% agarose gel, and subsequently sent to the First Base Asia (Malaysia) for DNA sequencing.

Partial 16S rRNA gene sequences obtained in this work were subjected to BLAST analysis to predict its taxonomic affiliation (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990; Altschul et al. 1997). These sequences along with representative 16S rRNA gene sequences retrieved from the GenBank (http://www.ncbi.nlm.nih.gov/Entrez/) were aligned using MUSCLE Software (Edgar 2004) and imported to Geneious, a bioinformatics desktop software package produced by Biomatters Ltd. (Auckland, New Zealand) (http://www.biomatters.com). A phylogenetic tree was constructed in Geneious using the neighbor-joining method (Saitou and Nei 1987) and Tamura–Nei genetic distance model (Tamura and Nei 1993). Resampling method with boot-strapping values inferred from 1000 replicates was set up on each tree branch to estimate the reliability of phylogenetic tree reconstruction. Based on this phylogenetic tree, we determined the taxonomic affiliation of proteolytic bacterial strains isolated in this work.

Results and discussion

Extracellular enzymes produced by heterotrophic microbial communities play key ecological roles in carbon and nutrient cycling in marine ecosystems (Arnosti et al. 2014). One of the most important classes of extracellular enzymes is proteases secreted by proteolytic bacteria to hydrolyze proteinous compounds into smaller molecular sizes, which subsequently enter the cells, thereby making dissolved organic nutrients available for themselves and other marine organisms (Hoffman and Decho 2000; Tamaki et al. 2004). From the biotechnological point of view, proteolytic bacteria living in unique marine habitats have attracted much attention as the potential sources of industrially relevant proteases. So far ecologically important bacteria inhabiting marine hot-springs in Indonesia remain poorly investigated. We therefore aimed at investigating protease-producing bacteria specifically isolated from a coastal hot-spring in North Sulawesi. The sampling location chosen in this work was Moinit hot-spring, a marine tourism destination located in Amurang District, South Minahasa Regency, North Sulawesi (Figure 1(A)). Sample collection was conducted at several sites along the coast with the water temperature of approximately 60 °C (Figure 1(B)). We describe below our results in detail.

(A)



(B)

Strain		Proteolytic index				Protease activity 60 °C, 10 min (U/mL)
		45 °C, 24h	55 °C, 24h	55 °C, 48h	65 °C, 48h	
2b	A	2.81	3.86	3.97	1.80	65.60
	SD	0.13	0.06	0.08	0.12	
4b	A	1.67	ND	3.00	2.00	57.65
	SD	0.25	ND	0.62	0.30	
9a	A	1.17	1.57	1.44	4.00	54.60
	SD	0.20	0.19	0.,21	0.46	
9b	A	1.17	1.33	1.22	6.00	60.49
	SD	0.25	0.20	0.10	0.49	
12L	A	1.92	ND	2.98	1.32	63.30
	SD	0.06	ND	0.11	0.06	

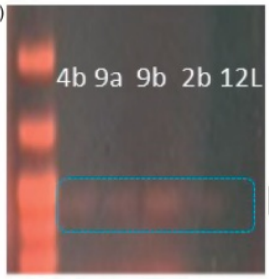
Figure 2. Screening of several thermophilic bacterial strains capable of producing extracellular proteases. (A) Bacterial isolates were placed on TMMA containing 2% skim milk. Positive colonies hydrolyzing protein were indicated by the presence of clearing zones around bacterial growth. (B) The ability to degrade protein at the temperature range of 45–65 °C for up to 48 h was measured as proteolytic index. In addition, the protease activity of the cell-free supernatant was measured as units/ml. A: average; SD: standard deviation.

Twelve seawater samples were individually plated in serial dilution on TMMA. After 1–2 d of incubation at 55 °C, phenotypically different colonies appeared on the agar plate surfaces. Twenty bacterial isolates were further screened based on the ability to produce protease extracellularly on skim milk-containing plates at 65 °C. It was found that there were 16 thermophilic bacterial strains exhibiting protein-degrading ability as indicated by clearing zones surrounding their colonies. Five of them with relatively larger proteolytic indexes, designated as 2b, 4b, 9a, 9b, and 12L (Figure 2(A)) were subsequently subjected to protease activity assay (Figure 2(B)). Among the five selected strains, 2b exhibited the highest PI at 45–55 °C with the extracellular protease activity of 65.60 U/ml. However, the PI of 2b decreased dramatically at 65 °C for 48-h incubation. In contrast, the PI of 9b was relatively low at 45–55 °C, but it was significantly high at higher temperature (65 °C for 48 h), suggesting the higher thermostability of the protease extracellularly produced by this strain compared with those from other strains investigated in this study. The ability of bacterial isolates to degrade protein at 65 °C suggests that thermostable proteases were secreted outside their cells. The presence of protease activity observed in the cell-free supernatants prepared from these five strains (see the section Materials and methods) suggests that the casein hydrolysis was catalyzed by the proteases extracellularly produced by such isolated bacteria.

To verify that the secreted proteases are genetically encoded in their genomes, we employed PCR-amplification using degenerate primers targeting protease genes (Figure 3(A)). Sequencing and BLASTx analysis showed the target PCR products were parts of genes coding for proteases. The amino acid sequences deduced from the isolated partial genes showed high similarity with many serine proteases, either serine alkaline proteases or subtilisin-like proteases (Figure 3(B)), which are typically characterized by the presence of a serine residue in their catalytic site (Rao et al. 1998). Multiple alignments of them with three representatives of characterized serine proteases retrieved from GenBank showed homology indicated with many conserved motifs (Figure 3(C)). Based on the alignment, we particularly identified the presence of one of the five catalytic triads of serine protease activities as previously reported (Geng et al. 2016).

The taxonomic status of five protease-producing isolates was determined based on the integrated data of cell morphology, biochemical properties, and 16S rRNA gene analysis (Figure 4). Gram staining and microscopic observation

(A)



(B)

Str.	Protein homology (BLASTx)				Putative Function
	Homolog	Organism	Acc. nu.	I/S (%)	
2b	Subtilisin /alkaline protease	*B. subtilis*	APB87957	96/98	Ser protease
4b	Subtilisn E	*B. subtilis*	KIO56488	98/99	Subtilisin
9a	Ser alkaline protease	*B. subtilis*	AGL34969	97/98	Ser protease
9b	Subtilisin-like protease	*Bacillus* sp. CN	ABU93240	99/100	Subtilisin
12L	Ser alkaline protease	*B. subtilis*	BBG71956	93/96	Ser protease

(C)

Figure 3. Bioinformatic analysis of partial protease genes isolated in this work. (A) The amplified partial protease genes of ~331 bp were individually purified from the agarose gel and subsequently sequenced. (B) The sequence data were subjected to BLASTx to predict the function. (C) The amino acid sequences deduced from partial protease gene sequences (designated as Pro2b, Pro4b, Pro9a, Pro9b, and Pro12L) were aligned with three representatives of serine alkaline protease or subtilisin obtained from GenBank (Sep1, Bace16, and Suba). The conserved motifs in the multiple alignments were marked by asterisks (*). Three of the five conserved catalytic triads for serine protease activities (Geng et al. 2016) are indicated by open red boxes. Abbreviations and description: Sep1, Serine protease from *B. firmus* (EWG10090.1); Bace16, alkaline serine protease from *B. subtilis* B16 (AAV30845.1); Suba, subtilisin from *B. amyloliquefaciens* (AEK88155.1); Str: strain; Ser: serine. Serine proteases can be classified as serine alkaline proteases and subtilisins (Rao et al. 1998).

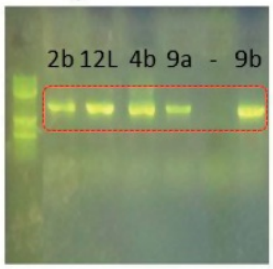
(Figure 4(A)) showed that the cells of all five strains were rod in shape. Four of them (except 9b) exhibited Gram-positive. The physiological properties of the three isolated strains (2b, 4b, and 9a) as listed in Figure 4(A) suggest their identity as *Bacillus*. The partial rRNA genes of the three isolated strains were amplified (Figure 2(C)), and subsequently sequenced. The partial 16S rRNA gene sequence data were deposited with Accession Numbers of KY038329 (strain 2b), KY966258 (strain 4b), and KY992592 (strain 9a). BLASTn search and further phylogenetic analyses, as shown in Figure 5, indicated that strain 2b was very closely related to *Bacillus cereus* (99% identity). Other two *Bacillus* strains 4b and 9a showed the highest level of sequence homology with *Bacillus halotolerans* (100% identity) and *Bacillus megaterium* (100% identity), respectively. Members within *Bacillus* have been used for the production of alkaline proteases, mainly due to their chemoorganotrophic characteristics and ability to secrete various types of alkaline proteases (Rasmussen and Morrissey 2007).

Most of thermostable proteases reported so far were produced by members of the genus *Bacillus* (Contesini et al. 2018), exemplified by *Bacillus licheniformis* (Yildiz et al. 2016), *Bacillus stearothermophilus* (Abdel-Naby et al. 2017; Kim et al. 2002; Latiffi et al. 2013), *Bacillus subtilis* (Marathe et al. 2018), *B. cereus* (Ghorbel et al. 2003; Kotlar et al. 2015; Prakash et al. 2005), and *Bacillus mojavensis* (Haddar et al. 2009). *Bacilli* belong to the most interesting producers of commercial extracellular proteases because of their stability at elevated temperatures (Kumar and Takagi 1999; Nigam 2013). This suggests the potential application of bacterial isolates obtained in this work from a bioindustrial viewpoint. One of the *Bacillus* strains (strain 4a) obtained in this study seemed to be very similar to those isolated from hot-springs at Padang Cermin (Lampung) and Banyu Wedang (Bali), in which *B. subtilis* was found to produce thermostable proteases (Choi et al. 1999; Zilda et al. 2013, 2014, 2018).

(A)

Str.	Morphology		Biochemical tests												
	Shape	Gram	Mot	Ind	Cat	Gas	Carbohydrates fermentation				Lys	MR	VP	H_2S	Cit
							Glu	Suc	Lac	Fruc					
2b	Rod	+	+	-	+	-	+	-	-	-	+	+	+	-	+
4b	Rod	+	+	-	+	-	+	-	-	+	+	+	+	-	-
9a	Rod	+	+	-	+	-	+	-	-	-	-	+	+	-	+
9b	Rod	-	+	-	+	-	+	-	-	-	+	+	+	-	-
12L	Rod	+	-	-	+	-	+	-	-	+	+	+	+	-	-

(B)



(C)

Str	L (bp)	16S rRNA gene Sequence homology (BLASTn)			Predicted affiliation
		Organism	Acc. nu.	I (%)	
2b	1430	*Bacillus cereus* BC-2	JQ799048	99	*B. cereus*
4b	1450	*B. halotolerans* AH38	MH040966	100	*B. halotolerans*
9a	1398	*B. megaterium* SICAU-PV1	MG515173	100	*B. megaterium*
9b	1456	*Pseudomonas balearica* Z8	KX495190	98	*P. balearica*
12L	1464	*Aneurinibacillus migulanus NBRC 15520*	NR_113764	99	*A. migulanus*

Figure 4. Cellular, biochemical, and molecular analyses of five proteolytic bacterial thermophiles isolated in this work. (A) Cell morphology and biochemical characteristics of individual isolates. (B) The amplified partial *16S rRNA* genes (~1400 bp) were extracted from the gel and subjected to DNA sequencing. (C) DNA sequence analysis by BLASTn predicted the taxonomic affiliation of each strain based on the best search hits. Mot: motility; Ind: indole; Cat: catalase; Glu: glucose; Suc: sucrose; Lac: lactose; Fruc: fructose; Lys: lysine; MR: methyl red; VP: Voges–Proskauer reaction; Cit: citrate; Acc. Nu.: accession number.

The strain 9b was rod-shaped, Gram-negative, aerobic, and motile. It was able to ferment glucose, but not sucrose, lactose, and fructose. The partial 16S rRNA gene of 9b was amplified, sequenced, and deposited in GenBank with the accession number of MF062680. The combined cell morphological, biochemical properties and 16S rRNA gene analysis suggest the identity of 9b as *Pseudomonas*, a member of ω-proteobacteria (Figure 4). Phylogenetic analysis (Figure 5) showed that strain 9b shared the highest homology with *Pseudomonas balearica* (98% identity) (Figure 4(C)). To the best of our knowledge, thermophilic *Pseudomonas* remains poorly investigated for extracellular enzyme production. Only a few reports have been published so far about *Pseudomonas* capable of producing thermostable proteases, such as *Pseudomonas fluorescens* (Allison et al. 1984; Kohlmann et al. 1991), *Pseudomonas aeruginosa* (Geok et al. 2003; Tang et al. 2010), and *Pseudomonas putida* (Singh et al. 2011). Other members of ω-proteobacteria known as predominant producers of proteases include *Alteromonas, Halomonas, Marinobacter, Pseudoalteromonas,* and *Shewanella* (Zhang et al. 2015).

The isolated strain 12L was Gram-stain-positive, motile and rods. It exhibited positive reactions for glucose assimilation, lysine decarboxylase, and citrate utilization, as shown in Figure 4(A). The partial ~1400-bp 16S rRNA gene of strain 12L was PCR-amplified, sequenced, and deposited in GenBank with the accession number of MK828190. BLASTn search showed that it shared the high level of sequence homology with members of the genus *Aneurinibacillus*. In particular, it showed the highest *16S rRNA* gene sequence identity (99%) with that of *Aneurinibacillus migulanus* NBRC 15520, as shown in the phylogenetic tree (Figure 5). The biochemical characteristics of 12L mentioned above, except catalase test, were similar with those of *Aneurinibacillus thermoaerophilus* reported by Lee et al. (2014).

In summary, we reported the isolation of proteolytic bacterial thermophiles from a coastal hot-spring in North Sulawesi. Three of them belong to *Bacillus*, and other two strains are members of *Pseudomonas* and *Aneurinibacillus*, respectively. Their ability to produce thermostable protease extracellularly suggests their critical ecological roles in decomposition and mineralization of nutrients. From ecological perspective, thermostability of the proteases secreted by these bacteria represents a form of their adaptive strategies to survive in elevated temperatures, thereby contributing to the ecosystem functioning, especially nitrogen cycling in Moinit coastal hot-spring. In the nitrogen cycling, protease-producing bacteria decompose particulate organic nitrogen into dissolved organic nitrogen (peptides and amino acids), which subsequently taken up by other bacteria for subsequent metabolic processes, such as ammonification, nitrification, and denitrification (Hunter et al. 2006; Li et al. 2017). From biotechnological point of view, high activities of thermostable proteases at high temperatures can decrease the risk of contamination by common mesophiles in fermentation, suggesting the potential development of serine proteases from these strains toward applications in biotechnological and industrial processes. In pharmaceutical industry, serine proteases in combination with antimicrobial

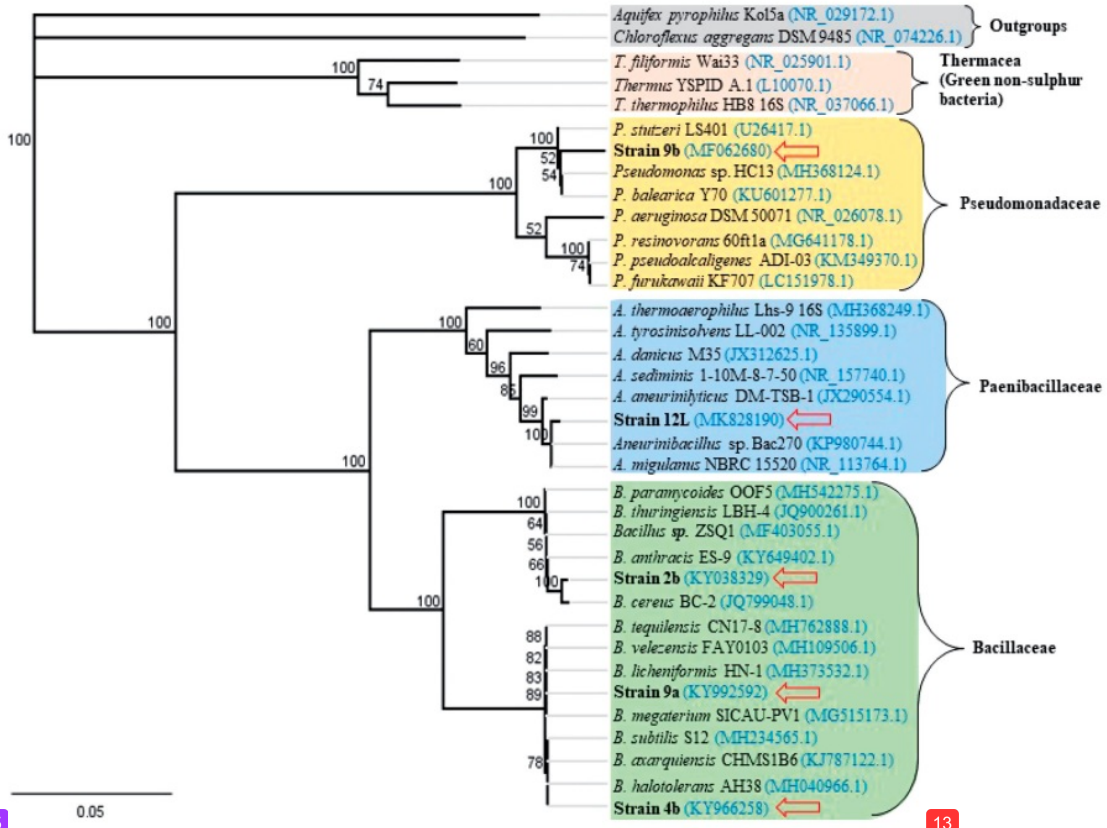


Figure 5. A phylogenetic tree showing the taxonomic affiliation of five proteolytic bacterial thermophiles isolated in this work. It was constructed using the neighbor-joining method (Saitou and Nei 1987) and Tamura–Nei genetic distance model (Tamura and Nei 1993). Resampling method with boot-strapping values inferred from 1000 replicates was set up on each tree branch to estimate the reliability of phylogenetic tree reconstruction. *Aquifex pyrophilus* and *Chloroflexus aggregans* used as the 'outgroups.' *A. pyrophilus* is an unusual group of thermophilic bacteria growing well at 95 °C, which is able to produce thermostable proteases (Choi et al. 1999; Deckert et al. 1998). GenBank accession numbers of 16S rDNA sequences are shown in each species.

agents promise potential applications in the total removal of pathogenic biofilms (Mitrofanova et al. 2017).

Conclusion

We have isolated a number of proteolytic bacterial strains from Moinit coastal hot-spring, North Sulawesi, Indonesia. Morphological, biochemical, and 16S rRNA gene analyses indicated their identities within the genera *Bacillus*, *Pseudomonas,* and *Aneurinibacillus*. Their ability to produce extracellular proteases, as indicated by having high protease activity and protease-encoding genes, suggest that they are a part of microbial communities that play important ecological roles in hydrolyzing proteinous compounds in the hot-spring environment, thereby making dissolved organic nutrients available for themselves and other marine organisms. Furthermore, the thermostability of the proteases secreted by these isolated bacteria suggests their biotechnological potential, especially in the food and pharmaceutical industries. Further research about the optimal production condition and characterization of thermostable proteases secreted by these thermophiles are necessary to be pursued.

Disclosure statement

We declare that there is no conflict of interest with any financial organization regarding the materials discussed in the manuscript.

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