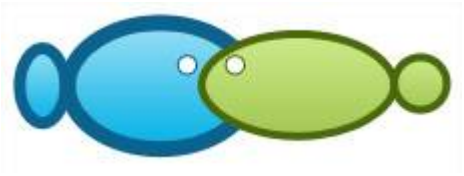

Endophytic bacteria isolated from brown seaweed *Chnoospora* sp. as potential producer of therapeutic protease

[1]Nurhilaliyah Nurhilaliyah*, [2]Dewi S. Zilda*, [3]Wijanarka Wijanarka, [2]Ayu R. Sulistyaningtyas, [1]Stalis N. Ethica

[1] Clinical Laboratory Science, Postgraduate Program, Universitas Muhammadiyah Semarang, 50273 Semarang, Central Java, Indonesia; [2] Research Center for Deep Sea, Earth Sciences and Maritime Research Organization, National Research and Innovation Agency (Badan Riset dan Inovasi Nasional, BRIN), Pademangan, North Jakarta City, Jakarta 14430, Indonesia; [3] Faculty of Biology, Universitas Diponegoro, 50275 Semarang, Indonesia. Corresponding author: S. N. Ethica, norma@unimus.ac.id
*Equally contributed as first author.

Abstract. Marine bacterial proteases, both as epibiont or symbiont, have gained attention for their stability in harsh condition as well as their potential as therapeutic (thrombolytic and fibrinolytic) agents. Indonesia with high algae biodiversity has a great opportunity to provide such therapeutic protease-producing symbiont bacteria. Brown algae in particular contained higher protein levels compared to red and green algae. Brown algae are widely found in Panjang Island, Karimunjawa, Central Java, Indonesia. Yet information about the potential of such seaweed as rich source of endophytic bacteria producing thrombolytic protease is scarce. This study aimed to obtain selected endophytic bacterial isolates of the brown alga symbiont *Chnoospora* sp. capable of thrombolysis, followed by molecular bacterial identification based on the 16S. As results, three endophytic bacterial strains CS-1, CS-2 and CS-3 (CS refers to "*Chnoospora* sp. symbiont") showing ability to form proteolytic zones on skim milk agar (SMA) medium and thrombolytic activity were successfully isolated. Isolate CS-2 showed the best thrombolytic activity and its molecular identification results based on BLAST (Basic Local Alignment Search Tool) analysis confirmed that the bacterium shared 99.46% homology level with the species *Metabacillus indicus* strain Sd/3 (Accession code: NR_029022.1.). The constructed phylogenetic tree based on 16S rRNA gene supported the closest relationship of strain CS-2 with *Metabacillus* group.
Key Words: *Chnoospora* sp., macroalgae symbiont, marine bacterium, *Metabacillus indicus*, therapeutic protease.

Introduction. Indonesia is a country with exceptionally high biodiversity. Such diversity mainly comes from marine biological resources, including groups of algae. In almost all coastal waters in Indonesia, macroalgae can be found (Sahri et al 2020; Farobie et al 2022). Macroalgae or seaweeds contain bioactive compounds including polysaccharides, unsaturated fatty acids, polyphenolic compounds, peptides, vitamins, essential minerals and enzymes, where these macromolecules are said to have activity as anticoagulants, antivirals, anti-cancers, antioxidants and immunomodulators (Ganesan et al 2019).

There are 3 main groups of algae namely brown algae (Phaeophyceae), red algae (Rhodophyceae) and green algae (Chlorophyceae). It was reported that among those groups of seaweeds, the highest protein levels were recorded in brown algae and lowest in green algae (Parveen & Nadumane 2020; Fauziee et al 2021). In the world several types of brown algae have been reported to have a high protein content such as *Ericaria amentacea* from the Middle East with a protein content of 141.4 g kg^{-1}, nevertheless this algae species has not been found in Indonesia. The type of brown algae which has a high protein content is *Sargassum* sp. with 16 g kg^{-1} which is largely obtainable in Indonesia, yet the bacteria associated with *Sargassum* sp. have been intensively studied (Wulansari et al 2019; Fauziee et al 2021; Nurhilaliyah et al 2022). Meanwhile, the brown algae

Chnoospora sp., when compared to the average protein content of other brown algae found in Indonesia, has a relatively high protein content of 11.3-12.3 mg g^{-1} (Parveen & Nadumane 2020).

Protease is a large group of enzymes of which utilization is urgently needed in the health industry as fibrinolytic and thrombolytic agents (Fuad et al 2021; Hidayati et al 2021; Ayanti et al 2022). Such therapeutic proteases can be sourced from animals, plants, fungi and microorganisms. Various studies on endophytic bacteria producing therapeutic proteases have been developed (Mamangkey et al 2022). Therapeutic proteases from bacteria are more profitable in industry due to easy-to-control production, faster cell growth (which is not affected by the season), and the easy cell genetic manipulation. In particular, marine bacterial proteases are favorable for their stability in harsh condition and for their potential as therapeutic (thrombolytic and fibrinolytic) agents (Barzkar 2020; Gemechu et al 2020; Nurhilaliyah et al 2022).

Isolation of thrombolytic protease from endophytic bacteria with fibrinolytic abilities from *Chnoospora* sp. inhabiting Karimunjawa seawater of Indonesia is yet to be reported. Isolation of thrombolytic protease from the algal symbiont *Chnoospora* sp. usually is started by screening protease-producing bacteria using skim milk agar (SMA) medium based on the presence of a proteolytic clear zone formed. Next, the thrombolytic activities of bacterial proteases can be tested using the gravimetric method. To study the biodiversity of protease producing bacteria isolated from *Chnoospora* sp., it is necessary to carry out 16S rRNA gene-based identification (Hidayati et al 2021; Zafrida et al 2022).

Molecular analysis of the bacterial 16S rRNA gene aiming to identify bacteria and study the phylogenetic relationship of bacteria is generally carried out by the polymerase chain reaction (PCR) (Japri et al 2019; Iskandar et al 2021). PCR is a molecular identification method for bacteria with a high level of sensitivity and specificity (Yang et al 2020). The 16S rRNA gene is a functional marker gene commonly analyzed to determine the taxonomy of bacteria and to support the establishment of a novel bacterial species (Jones et al 2019). The 16S rRNA gene sequence was then used as the basis of phylogenetic analysis (Ethica & Sabdono 2021).

Phylogenetic analysis or so-called evolutionary tree is a series of analyzes to determine how nucleotide or amino acid sequence families are derived during the evolutionary process. Relationships are described by grouping certain bacterial DNA sequences based on the level of homology in a phylogenetic tree (Coleman et al 2021). The purpose of phylogenetic analysis is to construct relationships between organisms and estimate differences from an ancestor to its offspring.

Finding new sources of therapeutic proteases with thrombolytic activity from endophytic bacteria of brown seaweeds is interesting yet important to do to support health sector. The higher level of proteins in such macroalgae can increase the possibility to obtain proteolytic bacteria, since protein in general is a specific substrate of protease (Nurhilaliyah et al 2022). Next, phylogenetic analysis of endophytic bacteria in *Chnoospora* sp. producing proteases thrombolytic activity needs to be carried out to determine the kinship relationship with other reported bacteria. The aim of this study was to identify selected endophytic bacterial isolates of the brown algal symbiont *Chnoospora* sp. Thrombolysis is possible, followed by 16S-based molecular bacterial identification.

Material and Method. The sample used in this study was brown seaweed *Chnoospora* sp. collected from seawaters surrounding Panjang Island of Karimunjawa Islands, Central Java Indonesia, at a depth of 3-7 m. The sampling has been conducted during 10-12th of January, 2022.

Algal sampling. The macroalgae sample was photographed under water (Figure 1), and then taken and put into a zipper bag. The sample was then brought to the surface and put into a cool box and then taken to the Microbiology Laboratory, Universitas Muhamamdiyah, Semarang, at the same day.

Figure 1. Underwater photograph and of *Chnoospora* sp. inhabiting seawater of Panjang Island, Karimunjawa, Central Java, Indonesia and its aliquoted sample in laboratory.

Endophytic bacterial isolation. Endophytic bacterial isolation was conducted following steps reported from previous study (Wulansari et al 2019). As much as 1 g of algal sample was weighed which surface was first sterilized using 70% alcohol for 1 min, and then soaked in 5.25% sodium hypochlorite for 3 min before rinsing it with running sterile water. Samples were air-dried, and then crushed. After that, bacterial isolation was carried out by serial dilution with physiological NaCl. The diluted samples were inoculated into nutrient agar (NA) medium enriched with 0.01% Nystatin by spreading technique, and then incubated for 24 h at 37°C. Each distinct colony was individually purified in the same medium in triplicates (Japri et al 2019).

Bacterial protease production test. The obtained endophytic bacterial isolates from previous step were dotted on SMA medium before incubation for 24 h at 37°C. The proteolytic clear zones formed were observed until the 7^{th} day of incubation. The formation of such clear zones was observed as indication of protease production by bacteria (Harun et al 2018). To visually clarify the formed proteolytic clear zone on solid medium, 5% Lugol was pured on the surface of each plate of solid medium (Hidayati et al 2021).

Isolation of bacterial crude protease. Enzyme production was carried out using minimal synthetic medium (MSM) starter medium with the addition of 1% skim milk powder. A loopful of bacterial single colony was put into 300 mL of starter medium and placed in an incubator shaker at 37°C for 24 h. As much as 10% of the starter was inoculated into 300 mL of liquid production medium and incubated for 48 h under similar conditions as the starter. The obtained sample was then centrifuged after 48 h at 6000 rpm for 30 mins at 4°C to separate the bacterial cells. Supernatant was regarded as bacterial crude protease, and then subjected to thrombolytic activity test (Harun et al 2018; Fuad et al 2021).

Thrombolytic activity test of bacterial crude protease. *In vitro* thrombolytic activity test was conducted using fresh blood from blood bank (without anticoagulant addition) as reported from previous study (Hidayati et al 2021). Each of five 1.5-mL empty microtubes was weighed and coded as negative control, positive control and samples. A total of 600 µL of blood was added to each tube, then allowed to stand for 30 mins until blood clots occurred. The serum was removed by centrifugation. Each microtube containing the blood clot was then weighed. The weight of the clot could be calculated by subtracting the weight of the tube containing the clot from the empty tube (clot weight = weight of the clot in the tube - weight of the empty tube) (Fuad et al 2021). As much as 100 µL of water was added to the negative control tube, while 100 µL of Natokinase was put into the positive control tube. Into each of sample tubes, 100 µL of bacterial crude protease was added. All of the tubes were then incubated at 37°C for 30 mins.

Molecular identification of bacteria

Bacterial genomic DNA extraction. Cells of proteolytic bacteria were diluted and cultivated in brain heart infusion broth (BHIB) agar. After 24-h incubation, the total DNA of the bacteria was extracted from the cells using Presto™ Mini gDNA Bacterial extraction Kit (Geneaid) (Zhang et al 2020).

Cloning and amplification of bacterial 16S rDNA. Cloning was performed using the Qiagen PCR Cloning Kit (UA-cloning, Qiagen) and the purified amplicons were ligated into the *p*Drive cloning vector and transformed into Qiagen EZ-competent *Escherichia coli* cells according to the manufacturer's instructions (Zhang et al 2020). Amplification of the cloned 16S rRNA gene fragment using the extracted DNA as template was done following previously reported procedure using Go Tag Green Master Mix (Promega) (Hidayati et al 2021). The primers used in amplification process were universal pair for 16S rDNA: 27-F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492-R (5'-GGT TAC CTT GTT ACG ACT T-3') (dos Santos et al 2019). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen).

Cloned 16S rRNA sequencing. PCR amplification product of 16S rRNA gene fragment from bacterial isolates and clones was sequenced at PT. Genetika Science Indonesia by Sanger method. The obtained DNA sequences in Fasta format were then analyzed using bioinformatics tools (Ethica & Sabdono 2021).

Results and Discussion

Isolation and characterization of endophytic bacteria. Of the total endophytic bacteria found in the *Chnoospora* sp. samples, 8 purified bacterial isolates with distinct colony characteristics could be obtained (Table 1). These isolates were coded CS-1 to -8 referring to "*Chnoosopora* sp. Symbiont". Based on Table 1, 5 out of 8 obtained endophytic bacterial isolates were Gram-positive and the rest were Gram-negative. Cell shapes of most isolates were bacilli. Colonies of all of these endophytic bacterial isolates were then subjected to proteolytic assay on SMA (Fuad et al 2021). Gram staining results of the 8 isolates are displayed in Figure 2.

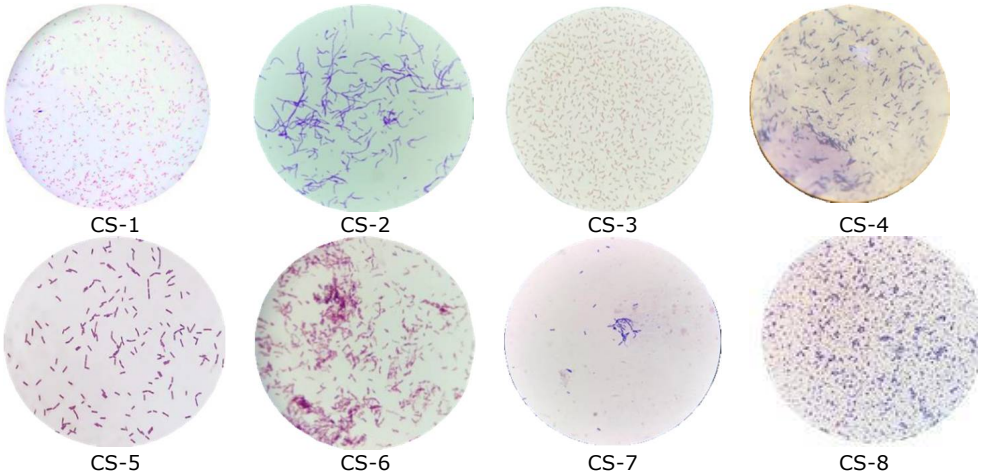

Figure 2. Eight different types of cells of endophytic bacterial isolates obtained from brown seaweed *Chnoospora* sp. in Karimunjawa after Gram-staining.

Table 1

Morphology of endophytic bacterial colonies that are symbiont with brown seaweed *Chnoospora* sp. purified on nutrient agar (NA) medium

No.	*Isolate code (strain)*	*Colony morphology*				
		Arrangement	*Cell shapes*	*Gram +/-*	*Color*	*Edge*
1	CS-1	Solitary	Bacilli	-	White	Entire
2	CS-2	Chain	Bacilli	+	Yellow	Entire
3	CS-3	Solitary	Bacilli	-	Cream	Lobate
4	CS-4	Chain	Bacilli	+	Creamy white	Undulate
5	CS-5	Chain	Bacilli	+	White	Entire
6	CS-6	Chain	Bacilli	-	Cream	Entire
7	CS-7	Chain	Bacilli	+	White	Entire
8	CS-8	Cluster	Cocci	+	White	Undulate

Note: CS = C*hnoospora* sp. symbiont; (+) = Gram-positive; (-) = Gram-negative.

Proteolytic assay of endophytic bacteria. Results of proteolytic assay on 8 endophytic bacterial isolates showed that only 3 isolates can produce protease. These 3 proteolytic isolates along with the clear zones they produced on SMA medium after 1st and 7th day of observation are displayed in Table 2. The presence of a clear zone around the colony in the SMA medium indicated that the bacteria utilized casein protein contained in the medium. The diameter size of the proteolytic hydrolysis zones qualitatively represented the protease production capacity of the bacteria.

Table 2

Morphology of endophytic bacterial colonies that are symbiont with brown seaweed *Chnoospora* sp. purified on nutrient agar (NA) medium

Incubation period on skim milk agar medium	*Bacterial code*		
	CS-2	*CS-4*	*CS-5*
1 day			
7 days			

As seen on Table 2, the size of proteolytic clear zone of CS-2, CS-4 and CS-5 isolates on SMA medium with 0.5% lugol staining after 7 days of incubation revealed that isolate CS-2 increased. It indicates that by time more casein contained in the medium has been successfully converted by bacteria into short chain peptide compounds and amino acids.

Thrombolytic assay of endophytic bacterial crude protease. Selected endophytic bacteria were grown in SMB medium to obtain crude enzyme. After centrifugation, the resulted crude enzyme was tested for its thrombolytic acidity. Three of 8 obtained endophytic bacterial isolates producing protease (CS-2, CS-4 and CS-5 isolates) in this study showed thrombolytic activity. A commercial supplement Nattokinase (Natto-10) was used as a positive control in blood clot lysis test. Nattokinase is protease produced by *Bacillus natto*, which has been widely used as antithrombosis agent with a prominent

thrombolytic activity. A thrombolytic agent has a function to increase blood flow by either inhibiting platelet aggregation or preventing thrombus formation. The use of such positive control is also useful to determine if thrombolytic activity of protease studied can compete with that of commercial protease.

Based on Table 3 and Figure 3, the results of the thrombolytic activity test show that crude proteases from three bacterial isolates (CS-2, CS-4 and CS-5) positively demonstrated ability to lyse blood clot. The highest percentage of clot lysis (%) of all belongs to CS-2 (sample code C) by 53.47±0.10, which value was higher than that of positive control by 49.82±0.08. This shows that protease of CS-2 isolate has the most competitive value in terms of *in vitro* thrombolytic activity and therefore more characterization should be done on the particular isolate. Based on gravimetry results, molecular identification of CS-2 isolate was then done to support the scale up of crude protease production of the isolate. This step was also necessary to molecularly characterize protease of CS-2 bacterium before it can be developed as commercial antithrombosis agent in the future.

Table 3

Gravimetri assay results representing *in vitro* thrombolytic activity potential of crude protease of endophytic bacteria isolated from brown seaweeds *Chnoospora* sp.

Sample code	*Percentage of clot lysis (%)*
Negative control (untreated blood)	4.12±0.12
Positive control (Natto-10)	49.82±0.08
CS-2 crude protease	53.47±0.10
CS-4 crude protease	47.22±0.06
CS-5 crude protease	49.30±0.09

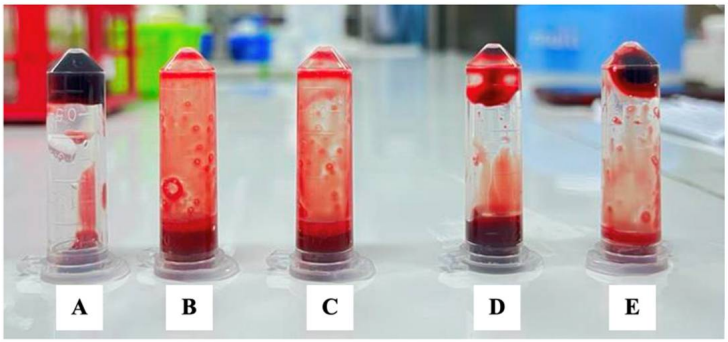

Figure 3. Blood clot-lysis (gravimetry) test results of crude enzyme of 3 endophytic proteolytic bacteria isolated from *Chnoospora* sp. Tubes were filled with blood clot with: A. No addition of enzyme (negative control), B. 100 μL commercial Nattokinase (Natto-10) (positive control), C. 100 μL crude enzyme 100% from CS-2 isolate, D. 100 μL crude protease 100% from CS-4 isolate, E. 100 μL crude protease 100% from CS-5 isolate.

Molecular identification of CS-2 bacterium. Bacterial DNA cloning and amplification. In this study, genomic DNA of CS-2 bacterium with the best and competitive *in vitro* thrombolytic activity of all endophytic bacteria isolated from *Chnoospora* sp. was successfully extracted. The extracted genomic DNA was then purified before subjected to gene cloning and PCR amplification.

Using 27-F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492-R (5'-GGT TAC CTT GTT ACG ACT T-3') primers, PCR products were confirmed as a single DNA band 2% agarose electrophoresis gel sized ~1500 bp as indicated by the DNA marker (Figure 4). This size matched the expected size of the bacterial 16S rRNA genes, which size is around 1500 bp (Ethica & Sabdono 2021).

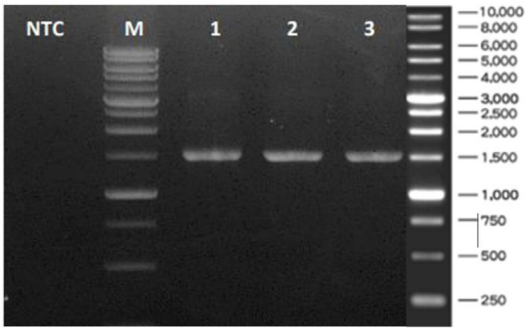

Figure 4. Electrophoresis visualization result of 16S rRNA PCR gene products of endophytic bacterial isolate CS-2 with UV Transluminator. M = Marker, 1 = CS-2, 2 = CS-4, 3 = CS-5 bacterial isolates. Each lane of samples on gel showed a single band sized ~1500 bp.

In this study, the obtained clones harboring 16S rRNA gene were sent to PT. Genetika Science Indonesia for sequencing using the Sanger method. The overall sequence of bases from the results of the 16S rRNA CS-2 gene cloning products can be seen in Figure 5. Sequenced DNA was then further analyzed using bioinformatics tool to determine taxonomy of CS-2 bacterium.

CTGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCC
CTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGG
GCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTG
GGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACT
GGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAA
TGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTCCATTCGCCATTCAGGCTGCG
CAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGAT
TAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTA
TAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCCAATACTTGGTTA
CCTTGTTACGACTTCACCCCAATCATCTACCCCACCTTAGGCGGCTGGCTCCTTGCGGTTACCCCACCGACTTCGGGTG
TTGCAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTAC
TAGCGATTCCAGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAATGGTTTTATGGGATTGGCTAAACCTCG
CGGTCTCGCAGCCCTTTGTACCATCCATTGCAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATC
CCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGC
TCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTTTGTCCCCCGAAGG
GGAACCTTCTATCTCTAGAAGTGGCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATG
CTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATG
CGTTAGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTA
ATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCA
CATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTTCCCAGTTTCCAATGACCCT
CCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGCGCGCTTTACGCCCAATAATTCCGGACAACG
CTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTCAGGTACCGTCAAGGTGCCGGCA
GTTACTTCGGCACTTGTTCTTCCCTGACAACAGAGTTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGT
CAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCC
GATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATGCGCCGCGGGCCCA
TCTGTAAGTGACAGCCGAAACCGTCTTTGAATGTCAAACCATGCGGTTTGACATAGTATCCGGTATTAGCTCCGGCTTCC
CGGAGTTATCCCAGTCTTACAGGCAGGTTGCCCACGTGTTACTCACCCGTCCGCCGCTGATCTCCGAAGAGATCCGCTCG
ACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAACTCTAGTATTGGGAATTCCTGCAGCCCG
GGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCG
CTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAA
GCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAG
TCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTC
CGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATAC
GGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAG
GCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCG
AAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGC
TTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCG
GTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTA
TCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT
ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCT
CTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTT
TTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACG
CTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAAT
TAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGC
ACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGG
GCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAG
CCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGC
TAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGT
TTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTT
AGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAA
TTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTA
TGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATC

Figure 5. The overall sequence the cloned products of 16S rRNA gene of endophytic bacterial isolate CS-2 gene: Note: Yellow highlighted = Primer Forward T7 (TAATACGACTCACTATAGGG); Green highlighted = Primer Reverse T3 (CCCTTTAGTGAGGGTTAATT).

The 16S rRNA gene cloning step aimed to obtain the full-length sequence of the marker gene. The cloning process basically consisted in a series of processes consisting of ligation reactions, transformation and isolation of recombinant DNA from transformants (Glick & Patten 2022). Ligation reaction is a combination of a target DNA molecule with a vector to produce recombinant DNA. The 16S rRNA gene is the target DNA which was

previously obtained by PCR method using pTA2 vector (Zafrida et al 2022). Successful cloning was indicated by the presence of white colonies as signed that recombinant DNA is already transformed in *E. coli* Zymo-5α bacteria as clones carrying recombinant DNA (data not shown).

Bioinformatics analysis. Bioinformatics analysis was carried out to obtain consensus of forward and reverse sequenced 16S rRNA gene with the DNA Baser Sequence Assembler Program (Heracle BioSoft SRL, Mioveni, Romania). The consensus sequence was then further analyzed to match with analog DNA sequences retrieved in the GenBank using Basic Local Alignment Search Tool (BLAST) of NCBI (National Center for Biotechnology Information). BLAST was accessed from https://blast.ncbi.nlm.nih.gov/Blast.cgi (Fuad et al 2021).

The results of BLAST analysis of endophytic bacterium producing thrombolytic protease strain CS-2 were obtained. The obtained consensus sequence in fasta type of file along with its BLAST results are displayed in Figure 6.

>*Metabacillus indicus* CS-2 16S ribosomal RNA (rRNA), cloned
GTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACG
TGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGT
TGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACC
CGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGAC
CTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGA
AGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCG
GTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCT
TAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTT
GAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAG
GAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTG
GGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGT
TAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTA
CGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTA
GAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAACGCAACCCTTGATCTTAGTTGCCAGC
ATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG
TCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAG
GGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGT
CTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTG
AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGA
AGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAA

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Metabacillus indicus strain Sd/3 16S ribosomal RNA, partial sequence	Metabacillus indicus	2712	2712	99%	0.0	99.46%	1505	NR_029022.1
Metabacillus dongyingensis strain BY2G20 chromosome, complete genome	Metabacillus dongyingensis	2647	29093	99%	0.0	98.60%	5053952	CP082944.1
Metabacillus sp. strain KIGAM252 16S ribosomal RNA gene, partial sequence	Metabacillus flavus	2536	2536	99%	0.0	97.26%	1539	ON394536.1
Metabacillus sp. strain KIGAM252 16S ribosomal RNA gene, partial sequence	Metabacillus flavus	2531	2531	99%	0.0	97.20%	1539	ON394542.1
Metabacillus sp. strain KIGAM252 16S ribosomal RNA gene, partial sequence	Metabacillus flavus	2531	2531	99%	0.0	97.20%	1539	ON394541.1
Metabacillus sp. strain KIGAM252 16S ribosomal RNA gene, partial sequence	Metabacillus flavus	2531	2531	99%	0.0	97.20%	1539	ON394540.1
Metabacillus sp. strain KIGAM252 16S ribosomal RNA gene, partial sequence	Metabacillus flavus	2525	2525	99%	0.0	97.13%	1539	ON394539.1
Metabacillus mangrovi strain AK61 16S ribosomal RNA, partial sequence	Metabacillus mangrovi	2497	2497	97%	0.0	97.48%	1464	NR_157636.1
Metabacillus indicus strain LMG 22858 16S ribosomal RNA gene, partial sequence	Metabacillus indicus	2495	2495	92%	0.0	99.28%	1379	MN543833.1
Bacillus sp. Marseille-P9898 partial 16S rRNA gene, strain Marseille-P9898	Metabacillus schmidteae	2442	2442	99%	0.0	96.02%	1548	LR797940.1

Figure 6. Consensus of sequenced 16S rRNA gene of endophytic bacterial isolate CS-2 sized 1417 bp was obtained by Sanger Method and assembled by DNA Baser Sequence Assembler Program (Heracle BioSoft SRL, Mioveni, Romania) along with its BLAST (Basic Local Alignment Search Tool) result.

The data in Figure 6 showed that 16S rRNA gene sequence of strain CS-2 shared 99.93% similarity level with that of *Metabacillus indicus* strain Sd/3 (NCBI Accession code: NR_029022.1.). After taxonomy determination of CS-2 strain by BLAST was complete,

the bacterial 16S rRNA gene sequence was then used as base to construct a phylogenetic tree. Phylogenic tree of CS-2 isolate designed using the MEGA X program (Kumar et al 2018; Stecher et al 2020) is displayed in Figure 7. The Neighbor-Joining Tree algorithm was used to design the phylogenic tree (Tamura et al 2004; Iskandar et al 2021).

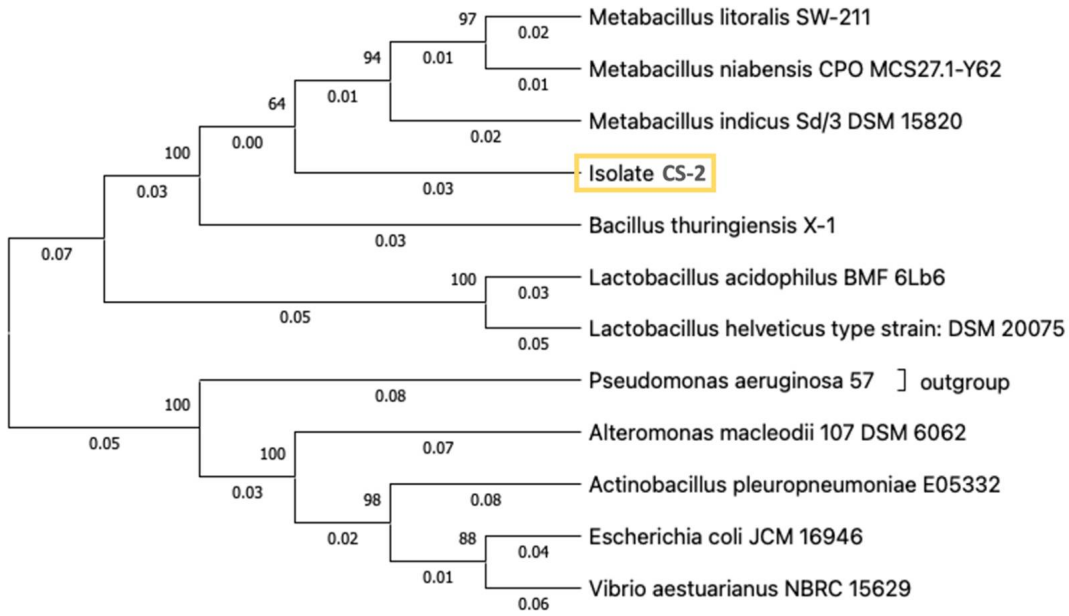

Figure 7. Phylogenetic tree of endophytic bacterium CS-2 isolated from brown macroalgae *Chnoospora* sp. with a competitive *in vitro* thrombolytic activity. The tree was constructed using Neighbour-Joining algorithm (Kumar et al 2018; Stecher et al 2020).

From the phylogenic tree (Figure 7), it can be seen that the CS-2 strain has the closest relationship with *Metabacillus indicus* Sd/3. In more detail, strain CS-2 appeared in the same phylogenic branch with *Metabacillus* cluster group although with only a bootstrap value of 64. The bootstrap value is less than 70% meaning that the relationship is actually weakly supported. However, in terms of sequence divergence, there are only 3 bases differences in every 100 bases (0.003 scale bar) between isolate CS-2 and *M. indicus* Sd/3 meaning the number of mutation/evolutionary events between compared species is relatively low.

BLAST and phylogeny analysis results obtained basically can support that isolate CS-2 has DNA similarity and the closest relationship with *Metabacillus indicus* (formerly *Bacillus indicus*). *M. indicus* was first reported from West Bengal, India in 2004 as an arsenic-resistant with strain name Sd/3T (=MTCC 4374T=DSM 15820T). The bacterium was rod-shaped, Gram-positive, immobile, endospore-forming, with yellowish-orange color. It had all the characteristics corresponding to the genus *Bacillus*. Based on its chemotaxonomic and phylogenetic characteristics, the Sd/3T strain was identified as a species of the genus *Bacillus* (Suresh et al 2004).

In this work, observation results on colony morphology of isolate CS-2 are in line with the molecular analysis results, and both matched to the reported description of *M. indicus* bacterial species. Isolate CS-2 has yellow color (see Figure 8) similar to that of *M. indicus*. Steiger et al (2015) reported that the yellow color of *M. indicus* cells was due to their ability to produce carotenoid, which contains yellow pigment. Genotypically, the genome of *M. indicus* contains the genes coding for C30 carotenoid pathways. Carotenoids are natural dyes that can be used as textile dyes that are non-toxic and have anti-bacterial properties (Brunt & Burges 2018).

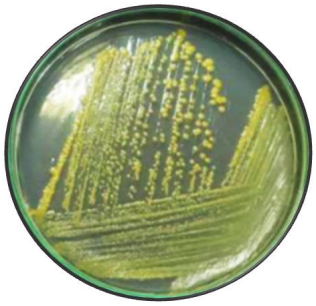

Figure 8. Yellow colonies of *Metabacillus indicus* strain CS-2 bacterium on nutrient agar medium isolated from brown macroalgae *Chnoospora* sp. inhabiting seawater around Panjang Island, Karimunjawa, Indonesia.

Conclusions. Three of 8 endophytic symbiont bacteria with proteolytic activities can be isolated from protein-rich brown macro algae *Chnoospora* sp. Among 3 endophytic bacteria producing protease coded CS-2, CS-4 and CS-5, only the CS-2 isolate could show a competitive *in vitro* thrombolytic protease when compared to commercial supplement Nattokinase as standard. Isolate CS-2 was morphologically and molecularly identified as *Metabacillus indicus* CS-2. Overall, efforts to obtain a new source of therapeutic protease from symbiont (endophytic) bacteria living in *Chnoospora* sp. tissue inhabiting Karimunjawa seawater in this study showed positive outcomes. Three endophytic bacterial isolates namely CS-2, CS-4 and CS-5 producing protease with *in vitro* thrombolytic activity were obtained. Among the 3, an isolate with the best and most competitive thrombolytic activity was morphologically and molecularly identified as *Metabacillus indicus* CS-2 (CS is referring to "Chnoospora sp. Symbiont"). In the next future, further research needs to be done to test whether protease produced by strain CS-2 could be confirmed by in vivo and clinical assays. In addition, it is also important to characterize protease from the bacterium in terms of substrate specificity, pH and temperature stabilities.

Acknowledgements. This work was conducted in cooperation between Universitas Muhammadiyah Semarang with Badan Riset Inovasi Nasional (BRIN), Indonesia.

Conflict of interest. The authors declare that there is no conflict of interest.

References

Ayanti B. P., Ethica S. N., Sulisytaningtyas A. R., Dewi S. S., Zilda D. S., 2022 Prospective purification and assay of thrombolytic protease from *Bacillus* sp. HSFI-10 isolated from sand sea cucumber for antithrombotic agent development. Proceedings of the 7th International Conference on Biological Science, pp. 404-415.

Barzkar N., 2020 Marine microbial alkaline protease: an efficient and essential tool for various industrial applications. International Journal of Biological Macromolecules 161:1216-1229.

Brunt E. G., Burgess J. G., 2018 The promise of marine molecules as cosmetic active ingredients. International Journal of Cosmetic Science 40(1):1-15.

Coleman G. A., Davín A. A., Mahendrarajah T. A., Szánthó L. L., Spang A., Hugenholtz P., Szöllősi G. J., Williams T. A., 2021 A rooted phylogeny resolves early bacterial evolution. Science 372(6542):eabe0511.

dos Santos H. R. M., Argolo C. S., Argôlo-Filho R. C., Loguercio L. L., 2019 A 16S rDNA PCR-based theoretical to actual delta approach on culturable mock communities revealed severe losses of diversity information. BMC Microbiology 19(1):74.

Ethica S. N., Sabdono A., 2021 Phylogenetic analysis and screening of hydrolytic bacteria with hydrolase enzyme activity from hospital wastewater of Semarang, Central Java, Indonesia. Ecological Engineering & Environmental Technology 22(2):81-86.

Farobie O., Syaftika N., Hartulistiyoso E., Amrullah A., Bayu A., Moheimani N. R., Matsumura Y., Karnjanakom S., 2022 The potential of sustainable biogas production from macroalgae in Indonesia. IOP Conference Series: Earth and Environmental Science 1038:012020.
Fauziee N. A. M., Chang L. S., Mustapha W. A. W., Nor A. R. M., Lim S. J., 2021 Functional polysaccharides of fucoidan, laminaran and alginate from Malaysian brown seaweeds (*Sargassum polycystum*, *Turbinaria ornata* and *Padina boryana*). International Journal of Biological Macromolecules 167:1135-1145.
Fuad H., Hidayati N., Darmawati S., Munandar H., Sulistyaningtyas A. R., Ernanto A. R., Muchlissin S. I., Zilda D. S., Nurrahman N., Ethica S. N., 2021 Exploration of bacteria isolated from" rusip" fermented tissue of sand sea cucumber *Holothuria scabra* with fibrinolytic, anticoagulant and antiplatelet activities. AACL Bioflux 13(3):1242-1258.
Ganesan A. R., Tiwari U., Rajauria G., 2019 Seaweed nutraceuticals and their therapeutic role in disease prevention. Food Science and Human Wellness 8(3):252-263.
Gemechu G., Masi C., Tafesse M., Kebede G., 2020 A review on the bacterial alkaline proteases. Journal of Xidian University 14(11):264-274.
Glick B. R., Patten C. L., 2022 Molecular biotechnology: principles and applications of recombinant DNA. 6th edition. John Wiley & Sons, 896 pp.
Harun A., Muchlissin S. I., Mukaromah A. H., Darmawati S., Ethica S. N., 2018 [Isolation of *Staphylococcus hominis* protease enzyme producing bacteria on post-fermentation red oncom for 120 hours]. Prosiding Seminar Nasional Edusainstek FMIPA UNIMUS 1(1):23-30. [in Indonesian]
Hidayati N., Nurrahman N., Fuad H., Munandar H., Zilda D. S., Ernanto A. R., Samiasih A., Oedjijono O., Ethica S. N., 2021 *Bacillus tequilensis* isolated from fermented intestine of *Holothuria scabra* produces fibrinolytic protease with thrombolysis activity. IOP Conference Series: Earth and Environmental Science 707(1):012008.
Iskandar A. U., Ethica S. N., Sukeksi A., Mukaromah A. H., Sulistyaningtyas A. R., Darmawati S., 2021 Molecular systematic and phylogenetic analysis of indigenous bacterial isolates with potential as bioremediation agent based on 16S rRNA gene analysis. IOP Conference Series: Earth and Environmental Science 743(1):012010.
Japri N. A., Sulistyaningtyas A. R., Darmawati S., Ethica S. N., 2019 Isolasi dan identifikasi molekuler bakteri proteolitik *Staphylococcus cohnii* strain IRLV5 pada rusip udang putih *(Litopeaneus vannamei*) pasca fermentasi 120 jam berdasarkan analisis gen 16S rRNA. Prosiding Mahasiswa Seminar Nasional Unimus 2:325-333. [in Indonesian]
Jones H. J., Kröber E., Stephenson J., Mausz M. A., Jameson E., Millard A., Purdy K. J., Chen Y., 2019 A new family of uncultivated bacteria involved in methanogenesis from the ubiquitous osmolyte glycine betaine in coastal saltmarsh sediments. Microbiome 7(1):120.
Kumar S., Stecher G., Li M., Knyaz C., Tamura K., 2018 MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35(6):1547-1549.
Mamangkey J., Mendes L. W., Harahap A., Briggs D., Kayacilar C., 2022 Endophytic bacteria and fungi from Indonesian medicinal plants with antibacterial, pathogenic antifungal and extracellular enzymes activities: a review. International Journal of Science, Technology and Management 3(1):245-255.
Nurhilaliyah N., Ethica S. N., Hersoelistyorini W., Ernanto A. R., Wijanarka W., 2022 A literature review on the potential of the biodiversity of thrombolytic protease-producing bacteria isolated from brown seaweeds *Chnoospora* sp. Proceedings of the 7th International Conference on Biological Science, pp. 416-423.
Parveen S., Nadumane V. K., 2020 Anti-angiogenesis and apoptogenic potential of the brown marine alga, *Chnoospora minima*. Future Journal of Pharmaceutical Sciences 6:19.
Sahri A., Mustika P. L. K., Dewanto H. Y., Murk A. J., 2020 A critical review of marine mammal governance and protection in Indonesia. Marine Policy 117:103893.

Stecher G., Tamura K., Kumar S., 2020 Molecular evolutionary genetics analysis (MEGA) for macOS. Molecular Biology and Evolution 37(4):1237-1239.
Steiger S., Perez-Fons L., Cutting S. M., Fraser P. D., Sandmann G., 2015 Annotation and functional assignment of the genes for the C30 carotenoid pathways from the genomes of two bacteria: *Bacillus indicus* and *Bacillus firmus*. Microbiology 161(1): 194-202.
Suresh K., Prabagaran S. R., Sengupta S., Shivaji S., 2004 *Bacillus indicus* sp. nov., an arsenic-resistant bacterium isolated from an aquifer in West Bengal, India. International Journal of Systematic and Evolutionary Microbiology 54(4):1369-1375.
Tamura K., Nei M., Kumar S., 2004 Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences of the USA 101(30):11030-11035.
Wulansari A., Aqlinia M., Wijanarka W., Raharja B., 2019 Isolasi bakteri endofit dari tanaman bangle (*Zingiber cassumunar* Roxb.) dan uji aktivitas antibakterinya terhadap bakteri penyebab penyakit kulit *Staphylococcus epidermidis* dan *Pseudomonas aeruginosa*. Berkala Bioteknologi 2(2):25-36. [in Indonesian]
Yang Y., Li M., Li H., Li X. Y., Lin J. G., Denecke M., Gu J. D., 2020 Specific and effective detection of anammox bacteria using PCR primers targeting the 16S rRNA gene and functional genes. Science of The Total Environment 734:139387.
Zafrida S., Ethica S. N., Ernanto A. R., Wijanarka W., 2022 Optimization of crude protease production from *Bacillus thuringiensis* HSFI-12 and thrombolytic activity its enzyme dialysate. Trends in Sciences 19(23):1952.
Zhang W., Chen Y., Shi Q., Hou B., Yang Q., 2020 Identification of bacteria associated with periapical abscesses of primary teeth by sequence analysis of 16S rDNA clone libraries. Microbial Pathogenesis 141:103954.

Received: 30 January 2023. Accepted: 07 April 2023. Published online: 20 May 2023.
Authors:
Nurhilaliyah Nurhilaliyah, Clinical Laboratory Science, Postgraduate Program, Universitas Muhammadiyah Semarang, 50273 Semarang, Central Java, Indonesia, e-mail: nurhilaliyah025@gmail.com
Dewi Seswita Zilda, Research Center for Deep Sea, Earth Sciences and Maritime Research Organization, National Research and Innovation Agency (Badan Riset dan Inovasi Nasional, BRIN), Pasir Putih Raya, street, Pademangan, North Jakarta City, Jakarta 14430, Indonesia, e-mail: dewi052@brin.go.id
Wijanarka Wijanarka, Faculty of Biology, Universitas Diponegoro, Semarang, Central Java 50272, Indonesia, e-mail: wijanarka1810@gmail.com
Ayu Rahmawati Sulistyaningtyas, Research Center for Deep Sea, Earth Sciences and Maritime Research Organization, National Research and Innovation Agency (Badan Riset dan Inovasi Nasional, BRIN), Pasir Putih Raya, street, Pademangan, North Jakarta City, Jakarta 14430, Indonesia, e-mail: norma@unimus.ac.id
Stalis Norma Ethica, Clinical Laboratory Science, Postgraduate Program, Universitas Muhammadiyah Semarang, 50273 Semarang, Central Java, Indonesia, e-mail: norma@unimus.ac.id

How to cite this article:
Nurhilaliyah N., Zilda D. S., Wijanarka W., Sulistyaningtyas A. R., Ethica S. N., 2023 Endophytic bacteria isolated from brown seaweed *Chnoospora* sp. as potential producer of therapeutic protease. AACL Bioflux 16(3):1372-1383.