ORIGINAL ARTICLE

Spongejaspis sp-Associated Bacteria Producing Protease Inhibitor

DEDE MAHDIYAH[1,2], ARIS TRI WAHYUDI[3], WIDANARNI[3], HELMIA FARIDA[4]
[1] *Sari Mulia University, Banjarmasin, Indonesia*
[2]*Doctoral Programm, Faculty of Medicine, Diponegoro University, Semarang, Indonesia*
[3]*Bogor Agricultural University, Bogor, Indonesia*
[4]*Faculty of Medicine, Diponegoro University, Semarang, Indonesia*
Correspondence to Dr. Dede Mahdiyah, Email :mahdiyahdede@yahoo.co.id, Telp: +62-82250812565

ABSTRACT

Background: Proteaseinhibitors are important in medicine, particularly in disabling proteases in the pathogenic processes of human diseases such as arthritis, cancer,HIV/AIDS and other infections. Sponges are excellent sources forbioactive compounds such as enzyme inhibitors, antiviral, antimicrobial.
Aim: To explore sponge-associated bacteria as sources of protease inhibitors.
Methods: This was an explorative descriptive study. Bacteria isolated from sponge *Jaspis sp* were screened using sea water complete and skim-milk double-layer plate. When the screening test positive, the activity of protease inhibitors was assessed using three substrates i.e proteinase-K, crude extract of protease (CEP) from *Staphylococcus aureus, Pseudomonasaeruginosa, Enteropathogenic Escherichiacoli*(EPEC) K11, and subtilisin. Optimum incubation time, temperature, and pH were determined to measure the activity ofthe protease inhibitors. Phenotypical characterization was performed using Gram and Microbact-kit. 16S-rRNA sequencing was done for identification.
Results: Out of 136 isolates screened, three were positive for their potency as protease inhibitor producers, including oneGram-negative coccus and two Gram-positive cocci. The isolates showed protease inhibitor activity up to 90% toward the three substrates. The optimum time incubation toward three substratesranged 12-24 hours. The optimum temperatures for the three isolates were 30C^{o}-60°C, 20°-30°C, and 30°C respectively. The optimum pH ranged widely from pH 4 to 7. 16S-rRNA analysis identified the isolates-producing proteinase inhibitors, as *Bacillus pumilus* strain 210_50, *Bacillussubtilis* strain DURCK11, and *Paracoccus sp*.Jx9.
Conclusion:*B.pumilus* strain 210_50, *B.subtilis* strain DURCK11, *Paracoccus sp*.Jx9 were potential sources of protease inhibitors.
Keywords:protease inhibitor,sponge-associated bacteria,*Jaspis*sp, *Bacillus pumilus* strain 210_50, *Bacillussubtilis* strain DURCK11, *Paracoccus sp*.Jx9

INTRODUCTION

Protease inhibitors are non-protein compounds that inhibit enzyme activities or may negatively affect enzyme activities by inhibiting catalytic and regulators.[1] Recently protease inhibitors have received much attention not only as a tool for studying enzyme structures and mechanisms of actions but also as potential compounds in pharmacology. For example, protease inhibitors are useful in the diagnosis and treatment of inflammation, pancreatitis, and other diseases caused by protease-producing bacteria.[2] Protease inhibitors are also usefulto prevent excessive destruction of the tissue target of proteases in the pathogenic process of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular dystrophy, cancer. They also a potentialantiviruses such as HIV.[3]

Protease inhibitors have also been found from marine microbes.[2,4,5]Sponges are a great source of natural products ofbioactive compounds, such asenzyme inhibitors, cell division inhibitors, antimicrobes, antiinflammatory, antitumor, or cytotoxic.[6]An example of a sponge that produces a bioactive compound is *Achantellasp* the producer of the calibinol-a compound which functions as an antibiotic. Other sponge, *Jaspis jhonstoni* is known to be able to produce jasplakinolide compounds which have cytotoxic property.[7]

The metabolites produced by sponges were the results of their symbiotic-bacterial biosynthesis.[7] Thus, the sponges contain the same bioactive components as the symbionts. *Micrococcus sp*. produces a diketopiperazine component, which has previously been reported to be generated by its host, *Tedania ignis*.[8] This information shows that many sponge symbionts have been identified as to produce the same bioactive components as the host. However, a specific bioactive component, namely the protease inhibitor has never been reported to be produced by sponge symbion bacteria.This study aimed to explore bacterial isolates associated with *Jaspis sp* sponge from Raja Ampat island, Indonesia, which havethe ability to produce protease inhibitor compounds, as well as to learn the activity of protease inhibitor compounds on various substrates, temperature, and pH.

METHODS

Bacterial Isolation from Sponge Samples: Superficial sponge bacterial isolation was done by swabbing the surface of the sponge on a 1cm^{2}witharea at three different parts of the sponge using a sterile swab. The swabs were then dipped into three tubes containing sterile PBS (Phosphate Buffer Saline). Bacterial suspension in eachtubewas serially diluted from 10^{-1} to 10^{-5}. The last three dilutions stage were spread on the SWC (*Sea Water Complete*) agar medium and incubated at room temperature for 24 hours.

Screening Bacteria Associated with Sponges Jaspis which Produce Protease Inhibitors: Screening was carried out using a two-layer skim milk plate agar (modified by Imada 1985) of which the lower layer consisted of the

medium for the SWC, and the upper layer consisted of 1.5% skim milk nutrient agar (NA). The sponge associated bacterial isolates were stabbed into the lower layer (SWC) using a sterile loop, then incubated 24 and 48 hours at 30⁰C to let them produce biochemical compounds including protease inhibitors. The skim milk-NA then poured on the SWC as thick as 2 mm. Protease-producing pathogenic bacteria, i.e..*Staphylococcus aureus, Pseudomonas aeruginosa* and Enteropathogenic *Escherichiacoli* (EPEC) K11 each was planted on the skim milk-NA layer,and incubated for 24 hours at 37⁰C. The presence of proteaseinhibitors was indicated by the absence or reduction of the diameter of clear zones around the colonies of the protease-producing bacteria.

Determination of Production Time of Protease Inhibitors: Isolates that give positive results in the screening test were then tested for their ability in producing protease inhibitors. The media used was marine broth. The propagation stage is carried out at 30⁰C, by shaking at 150 rpm in shaker water-bath to reach the logarithmic phase followed by cultivation for 32 hours under the same conditions. Observations for the determination of optimum production time of protease inhibitors were done every four hours.

Activity of Protease Inhibitors: The activity of protease inhibitors were tested against three different proteases as the substrates, i.e., proteinase-K, crude extract of protease (CEP) from three pathogenic bacteria (*S. aureus*, *P. aeruginosa*, *EPEC K11*), and subtilisin. A mixture of 0.5 ml CEP and 0.5 ml suspension of the alleged isolate-producing protease inhibitors was preincubated at 30ºC for 12 min. Then, 1 ml of casein Hammerstein 2% (w /v) in Tris-HCl 50mM buffer solution with pH 8 was added, followed by incubation for 12 minutes at 30ºC. Afterward, 2ml oftrichloroacetic acid (TCA) 5% (w/v) was added to stop the enzymatic reaction. This mixture was kept for 20 minutes at 30ºC to precipitate undigested casein, followed by centrifugation at 3000g for 10 minutes. The supernatant was measured with 280nm absorbance.One unit of protease inhibitor activity is defined as the number of inhibitors that can inhibit protease activity by 50%.

Optimum Temperature test.: Suspension of isolates-containing protease inhibitors was tested at various temperatures from 30ºC to 70ºC with a 10ºC intervals and the protease inhibitor activity was measured in each temperature interval.[2]

Optimum pH test.: Suspension of isolates-containing protease inhibitors were measured at different pHs ranging from pH 3-12 with an interval of 1 and incubated for 10 min at 30ºC. After incubation, the activity of the protease inhibitor was measured.[2]

Identification of Bacteria (Microbact, 16SrRNA): Theisolates capable of producing protease inhibitor were identified based on phenotypicalcharacteristics, including Gram staining, spore staining, and biochemical tests with MicrobactTM kit GN A ID and GN B ID.An *16S rRNA*followed by sequence analysis with BLAST program was conducted as previously described.[9]The phylogenetic tree was constructed using TreeCon program.[10]

RESULTS

Isolation of Bacteria Associated with Sponges: The isolation yielded 66 isolates surface bacteria. The isolates were encoded SAB S (Sponge-Associated Bacteria Surface).

Screening Bacteria Producing Protease Inhibitors: Out of the 136 isolates, three isolates gave positive results in the screening test using*S.aureus, P. aeruginosa,* and *EPEC K11*. These pathogenic bacteria produce proteases which are able to degrade or lyse protein in skim milk-NA media, resulting in the presence of a clear zone around the colonies. When the tested isolates produced protease inhibitors, the proteolytic process was inhibited, resulting in the absence or reduced the clear zone diameters compared to the control as shown in Figure 1. The three isolates were SAB S-17 (strongest effect on the protease of *S. aureus*), SAB S-21 (strongest effect on the protease of *EPEC K11*), and SAB S-43 (strongest effect on the protease of *P. aeruginosa*) (Table 1).

Determination of Production Time of Protease Inhibitors: From Figure 2, it was known that SAB S-21 isolates had very large protease inhibitor activity against the three types of substrates used. Protease inhibitor activity against subtilisin substrate was very low for all the three isolates compared to proteinase-K substrate and CEP. In the protease inhibitor activity tests using proteinase-K substrate, it wasfound that SAB S-17 isolates and SAB S-21 had the largest protease inhibitor activity after an incubation time of 20 hours. Meanwhile, SAB S-43 isolates had the largest protease inhibitor activity after incubation for 12 hours.

Characterization of Protease Inhibitors:

Optimum Temperatures: The protease inhibitors had maximum activity at a certain temperature; its activity increased along with the increase in temperature until it reached the optimum temperature. Afterward, a further temperature rise caused its activity to decrease (Figure 3).SAB S-21 isolate had the greatest protease inhibitor activity with an optimum temperature of 20-30⁰C against all three substrates.There was an exception for SAB S-17 isolates, for which the activity of the inhibitor was achieved at high temperature (60⁰C). This was an interesting finding to study further.

Optimum pH: pH may affect enzyme activities. The enzyme catalysis power becomes low at low or high pH, due to the denaturation of enzyme proteins. This was consistent with the results obtained from the three isolates that tested for the activity of their protease inhibitors on various pH with the three different substrates (Figure 4).The SAB S-17 isolate showed greatest inhibitory activity against the three substrates in acidic environments; even for subtilisin substrate.The largest inhibitor activity occurredat pH 4. SAB S-21 isolate showed greatest inhibitor activity against CEP at alkaline pH (pH 8), whereas for proteinase K, the highest protease inhibitor activity was achieved at acidic pH (pH 5).

Identification of Protease Inhibitor-producing Bacteria: Gram and spore staining revealed that SAB S-17 was Gram-negative coccus;whileSAB S-21 and SAB S-43 were spore-formingGram-positiverod.Microbact-kit test found that SAB S-17 was *Paracoccussp*, SAB S-21 was *Bacillus pumilus*, and SAB-43 was *Bacillus subtilis*. Molecular identification with 16S-rRNA gene amplification of the three

isolates tested resulted in a specific band fragment measuring 1300 bp (Figure 5).The DNA sequence analysis of 16S rRNAs of the three positive protease inhibitor isolates showed kinship with other bacteria with different percentages of similarity (Table 2). Phylogenetic tree of the three isolates is depicted in Figure 6.

Table 1.Isolates Capable of Producing Protease Inhibitors (on Screening Test)

Isolates	Source of CEP	% Reduction in the diameter of clear zone
SAB S-17	*S. aureus**	55.5%
SAB S-21	EPEC K11*	100.0%
SAB S-43	*P. aeruginosa**	62,5%

*Only test bacteria with the greatest reduction is shown in this table

Table 2 Results of 16S rRNAs on the Three Isolates Capable of Producing Protease Inhibitors

Isolate	Homology with	Identity	Access number
SAB S-17	*Paracoccus* sp. Jx9	86%	FJ539115
SAB S-21	*Bacillus pumilus*strain 210_50	100%	GQ199752
SAB S-43	*Bacillussubtilis* strain DURCK11	98%	FJ430065

Figure 1 (A, B, C) *S.aureus, P.aeruginosa,*and EPEC K11 showed proteolytic activities on the 1.5% skimmed milk-NA resulting clear zone around the colonies with diameters of proteolysis of 3.5, 4.0, and 4.5 mm respectively. (A', B', C') Results of positive screening tests, in which proteolysis was inhibited, resulting in the absence or diameter reduction of clear zone, suggesting that the isolates produced substance capable of inhibiting proteolysis by a protease of the test bacteria.
(A) Percentage of protease inhibition reached 100% (SAB S 21), (B) percentage of protease inhibition reached 62.5% (SAB S 43), (C) percentage of protease inhibition was 55 55% (SAB S 17).

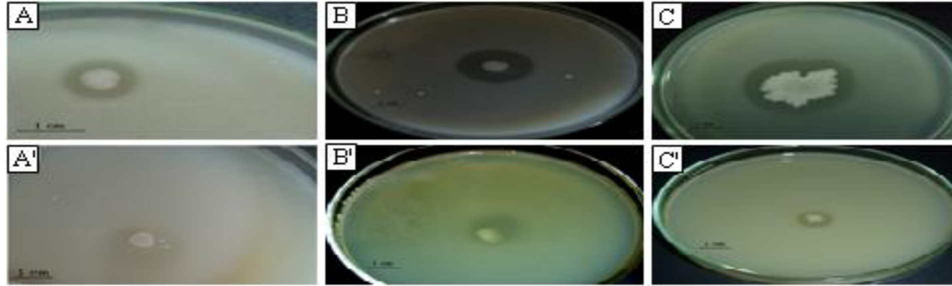
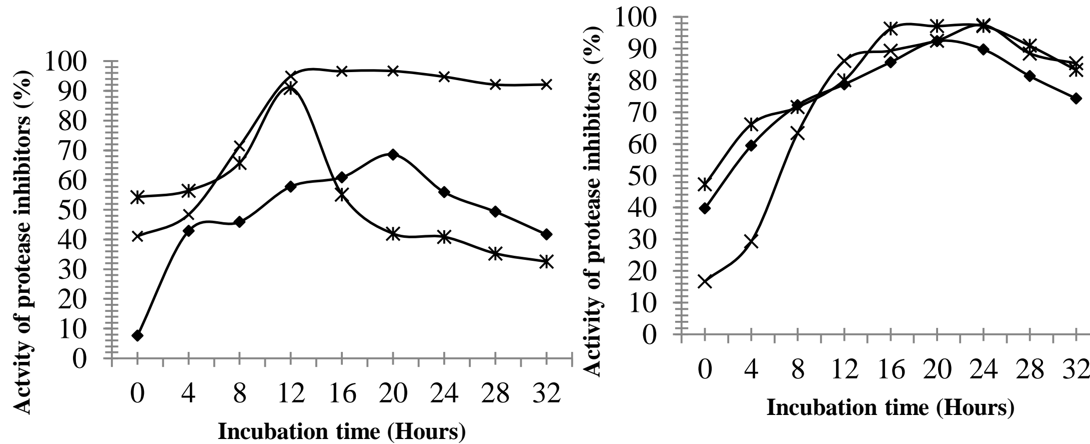


Figure 2. The activities of protease inhibitors during incubation time for 32 hours with substrates (A) proteinase K, (B) Crude extract Protease (CEP) from pathogenic bacteria, and (C) subtilisin.



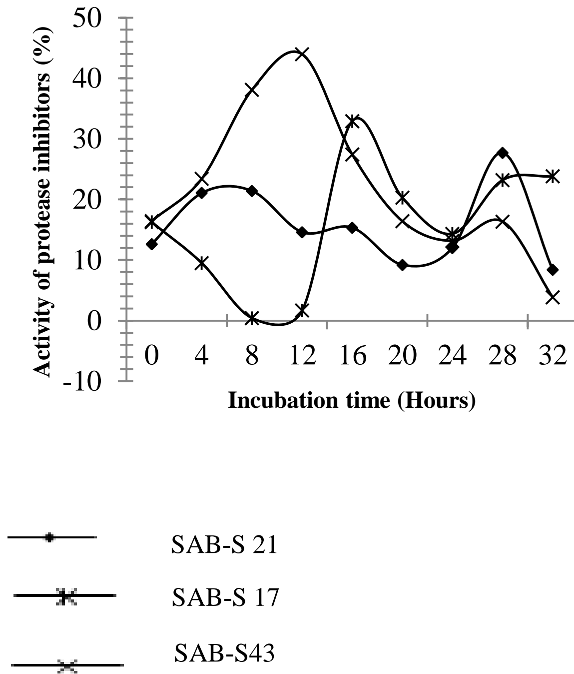
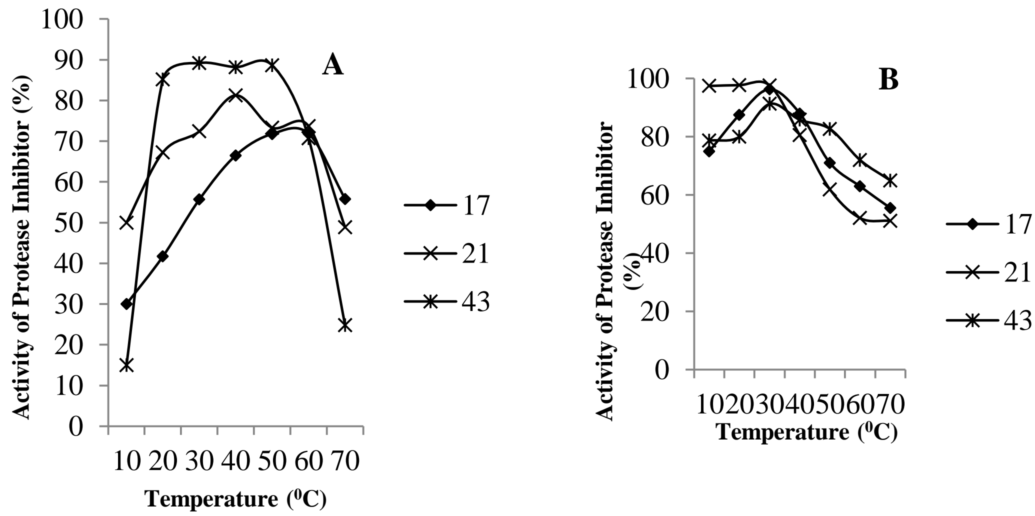


Figure 3. Protease inhibitor activities at various temperatures using substrate (A) proteinase K, (B) Crude Extract of Protease (CEP) from pathogenic bacteria and (C) subtilisin.



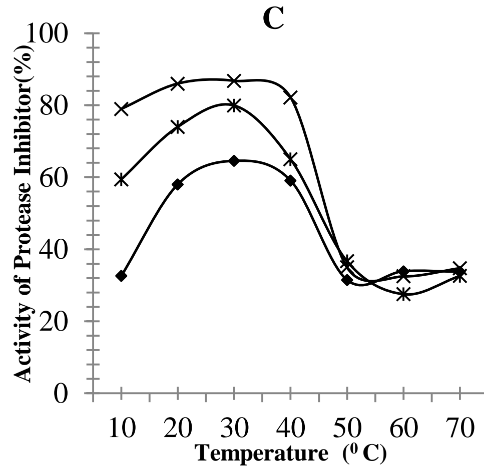


Figure 4. Activities of protease inhibitor at various pH with substrate (A) proteinase K, (B) crude extract of protease (CEP) and (C) subtilisin.

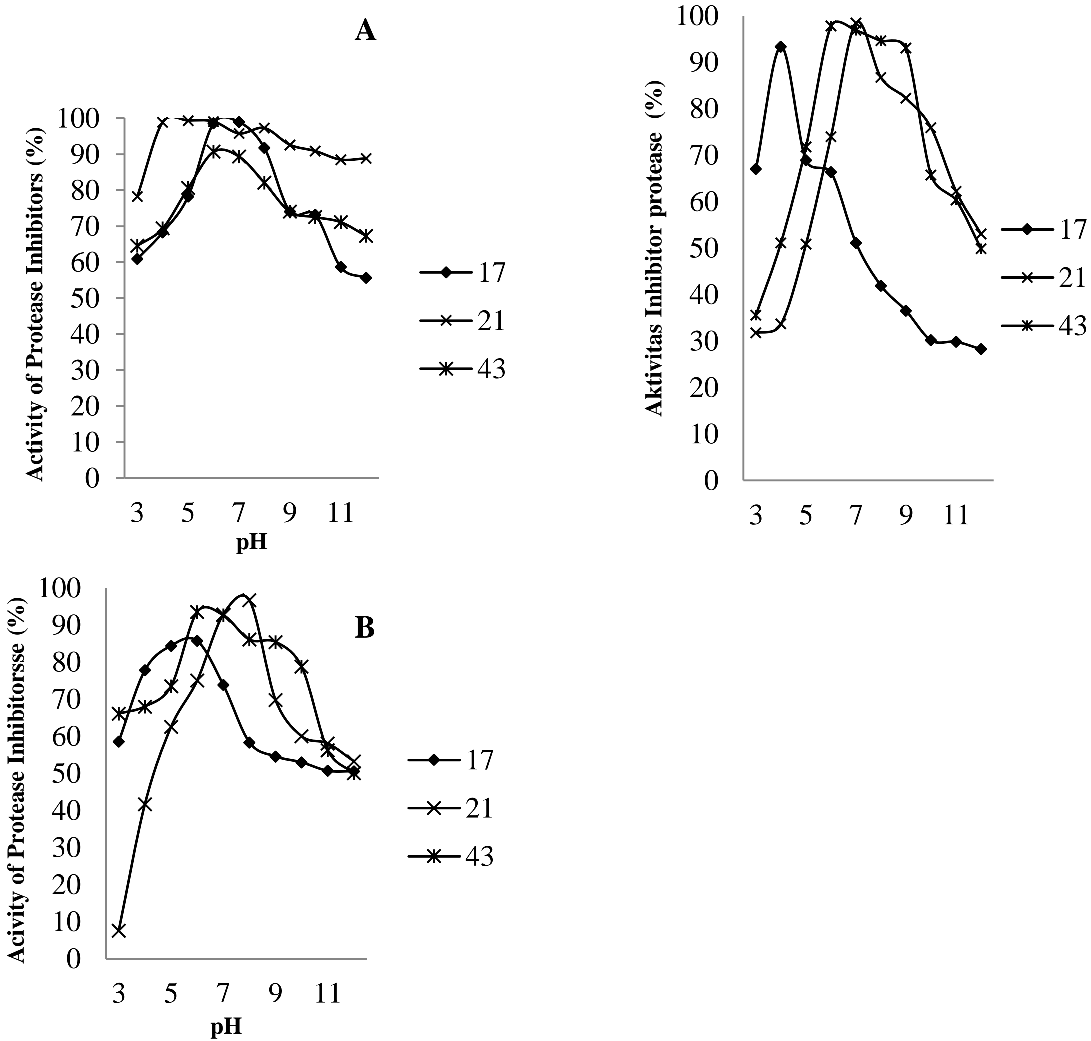


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Figure 5 Amplification of the encoding gene of 16S rRNA from three isolates potentially producing protease inhibitors.

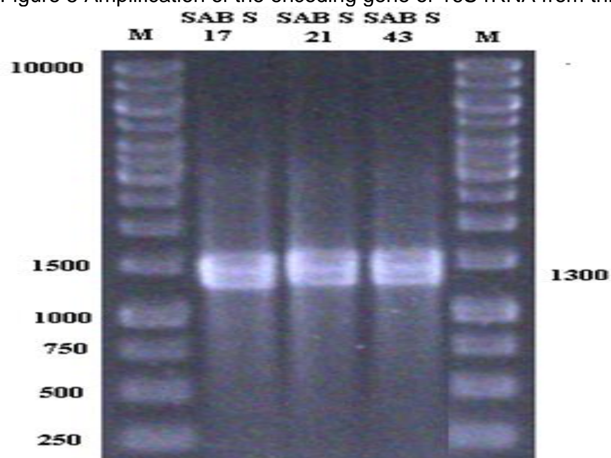
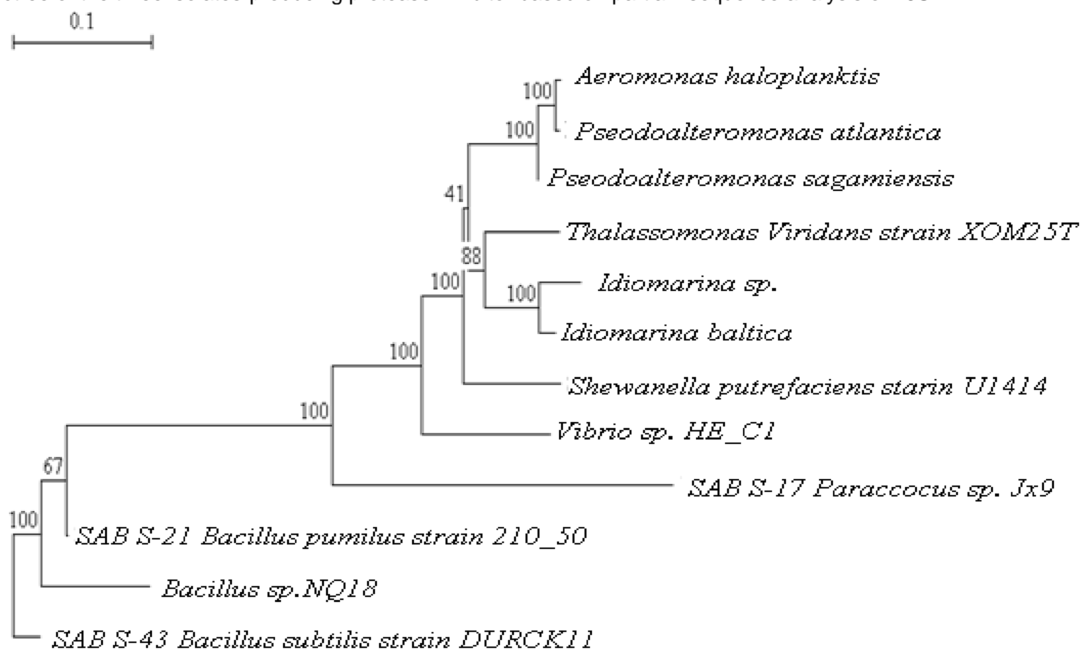


Figure 6. Phylogenetictree of the three isolates-producing protease inhibitor based on partial sequence analysis of 16S rRNA



DISCUSSION

The three isolates in this study were alleged to capable of producing protease inhibitors which inhibited protease activities fromthree species of pathogenic bacteria (*P. aeruginosa, S. aureus, and EPEC K11*) in degrading protein (skim milk). This evidence was characterized by the absence or reductionof protease zones around the colonies compared to controls, on medium containing protein (skim). The allegation was then confirmed by testing the activities of protease inhibitors using casein. The test showed that SAB S-17, SAB S-21, and SAB S-43 produced protease inhibitor activities at an incubation time of 20, 24 and 12 hours respectively. This is consistent with research conducted by Nurhayati *et al.*[11] which found that isolate associated with sponges produced protease inhibitors against protease from*S. aureus*, E. coli, and *P. aeruginosa* at an incubation times of 20, 24, and 12 hours, respectively. Similar results can be seen in *Serratia marcescens* which had the highest extracellular inhibitor activities at the 18th hour, and intracellular inhibitor activities at the 12 hours [12]. This incubation period for the protease inhibitor productionwas much shorter than other bacteria, such a*s Streptomyces rishiensis* (24-36 hours) with shorter incubation period, bacteria in our study will be more efficient and preferable for industrial scale than bacteria which need longer incubation period to produce protease inhibitors. Temperature and pH also affect the activity of protease inhibitors. The protease inhibitor has a maximum activity at a certaintemperature, its activity increases along with the increase in temperature until it reaches the optimum temperature. Afurther temperature

increase will cause its activity to decrease.In our study, the SAB S-17 isolate was capable of producing protease inhibitor activity at 60°C and optimum pH of 6 to the proteinase-K substrate. Some species such as *Clostridiumthermocellum*needs higher temperatures to produce protease inhibitors against Gram negative bacteria (at 70ºC and is optimum at pH 4).[13]

In our study, the SAB S-21 isolate produced protease inhibitor of which activities against the CEP increased very sharply fromthe temperature of 10ºC to 30ºC. This will make the SAB S-21 a very potential source or protease inhibitors since it already productive in much lower temperatures.

The optimum temperature range shown by the three isolates of 30ºC to 60ºC implies that the activitiesof protease inhibitors in inhibiting protease formation by pathogenic bacteria varies in the temperature range. Higher temperature, i.e, 70ºC decreased the activities, probably because the enzyme is denatured by the heat resulting in structural damage, so there is no inhibition or slight inhibition.

All protein inhibitors in this study showed optimum activities at acidic pH (pH 4-6) but poor activities at alkaline pH (pH 10). This was different from study which found that the activities of protease inhibitors was optimum at pH 8[2,14,15] this study, the range of pH for SAB S-21 was wide, from acid to alkaline pH, probably was influenced by variations in the substrate used and by the characteristics of bacterial isolates. The wide range of optimum pH will give more opportunities to apply for industrial purposes.

The phylogenetic tree of the three isolates capable of producing protease inhibitorcompounds (Figure 6) showed that the SAB S-43 isolate and SAB S-21 existed in one group together with *Bacillus sp.* NQ18. SAB S-21 was later identified as (*Bacillus pumilus* strain 210_50) which possessed protease inhibitor capability in addition to antifungalcharacteristics.[16]SAB S-43 was identified as *Bacillus subtilis strain* DURCK11*B. subtilis* is considered to be the best studied Gram-positive bacteria and model organism to study bacterial chromosome replication and cell differentiation. This bacterium is one of the bacteria in the production of enzymes that are secreted and used on an industrial scale by biotech companies, but use as a source of protease inhibitors has not been established.SAB S-17 (*Paracoccussp Jx9*) was an interesting species because its aerobic respiration system has already been used as a model for mitochondria functions and many other component functions such as cytochrome oxidase.[17]Quorum sensing and quorum sensing inhibition activities have been reported recently for this bacteria, and genomic analysis supports its potential use for novel therapeutic development.[18]*Shewanellaputrefaciens* strain U1414 was clustered apart from other reference bacteria, while other reference bacteria, *Idiomarinabaltica* strain OS145 and *Idiomarina sp.* YCWA10 were present in one group along with *Thalassomonasviridans* strain XOM25T, CECT 5083T. Other groups of *Pseudoalteromonasatlantica,* *P.sagamiensis*, and *Alteromonashaloplanktis* existed in one group.

This study successfully identified three species of bacteria capable of producing protease inhibitors with ashorter incubation period, a wide range of optimum pH and lower temperature of action, making these species are very ideal candidates for various biotechnological applications and purposes, especially in medicine and in pharmaceutical industries.

Thisstudy hadlimitations in the number of substrates tested, namely subtilisin, CEP, and Proteinase-K. Third, this study had not yet purified the protease inhibitors substances.Further studies were needed to characterize protease inhibitor compounds produced by these sponge *Jaspis*sp- assocoated bacteria, as well as to efficiently apply their use for the production or protease inhibitor in industrial scale.

CONCLUSION

The three isolates associated with sponge *Jaspis sp* werepotential sources for protease inhibitor compounds and identified as*Paracoccus sp.* Jx9, *Bacillus pumilu*s strain 210_50, and *Bacillus subtilis* strain DURCK11. The isolate withstrongest protease inhibitor activities against the three substrates was *Bacillus pumilu*s strain 210_50 with the highest activity at 12 and 24 hours. The optimum temperature range achieved by*Bacillus pumilu*s strain 210_50 in producing the highest protease inhibitor activity was 20ºC to 30ºC. The optimum pH for optimum production of protease inhibitors was widely ranged from acid to alkaline pH 4-8.

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