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RESEARCH ARTICLE

Exploration and molecular identification of proteolytic bacteria from rusip pacific oyster (*Crassostrea gigas*) as anticoagulant agent candidates

Muhammad Ardi Afriansyah [1], Sudarwin [1], Sri Sinto Dewi [1],

Gusti Dimas Refian Akbar [1]

[1]Medical Laboratory Technology Study Program, Universitas Muhammadiyah Semarang, Semarang, Indonesia

Correspondence:
M. Ardi Afriansyah
Medical Laboratory Technology Study Program, Universitas Muhammadiyah Semarang, Semarang – 50273, Indonesia

Email: afriansyah@unimus.ac.id

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Abstract

The marine symbiont *Staphylococcus epidermidis* strain CGF-6, a protease-producing bacterium, has been successfully isolated from Rusip Pacific Oyster (*Crassostrea gigas)*. *S. epidermidis* is a non-spore-forming, Gram-positive coccus commonly found in marine environments due to their ability to tolerate high salinity. The aim of this study was to identify proteolytic bacteria from Rusip fermented *C. gigas* as potential candidates for the development of anticoagulant agents. Bacterial isolation was performed through the fermentation process of Rusip. After seven days, bacterial colonies were purified three times using Nutrient Agar. The selection of proteolytic bacterial was conducted qualitatively using a skim milk agar medium. The bacterial isolates exhibiting the highest protease activity were identified through 16S rRNA gene sequencing using universal primers Bact 27F and UniB 1492R. Phylogenetic tree analysis, conducted with the MEGA X program, helped determine the relationships between species. Out of the 18 bacterial isolates obtained from the Rusip fermentation of *C. gigas*, three isolates (CGF-1, CGF-6, and CGF-11) exhibited hydrolysis zones around their colonies on skim milk agar, indicating protease activity. Among these, isolate CGF-6 showed the highest proteolytic index of 0.5 and was identified as *Staphylococcus epidermidis* strain CGF-6. *S. epidermidis* strain CGF-6 has the potential to serve as a valuable source of protease production for the development of anticoagulant agents. However, further studies, including enzyme characterisation, optimisation, and both *in vitro* and *in vivo* anticoagulant activity tests, are necessary to assess the efficacy and safety of this enzyme as a candidate for anticoagulant agents.

1. INTRODUCTION

Protease enzymes can be derived from various sources, including microorganisms, plants, and animals found in both marine and terrestrial ecosystems. Recent studies have shown promising results in exploring protease-producing marine microbes. Specifically, researchers have successfully isolated protease-producing bacteria from marine organisms such as sand sea cucumbers (1), brown algae (2), seaweed (3), and nudibranch (4). One noteworthy example is the Pacific oysters (*Crassostrea gigas*), a bivalve with a soft body protected by two shell valves. These oysters have a relatively high protein content. ranging from 50% to 56%. Due to their ecological and economic significance, oysters provide numerous benefits (5). They have extensive industrial applications, including functional foods, natural pharmaceuticals, and valuable bioactive compounds. The bioactive components found in Pacific oysters exhibit various activities, such as anticoagulant, antioxidant, anti-inflammatory, antimicrobial, anticancer, antihypertensive, and immunomodulatory effects (6). The abundance of these bioactive compounds, particularly proteins, along with other essential nutrients like minerals, glycogen, essential amino acids, and fatty acids, makes Pacific oysters a promising natural resource for cultivating proteolytic bacteria (7).

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Proteases obtained from marine environments generally exhibit greater activity compared to those sourced from terrestrial environments. The complexity of the marine environment, characterised by high salinity, high pressure, low temperature, and unique light conditions, contributes to the distinct characteristics of enzymes produced by marine and terrestrial microorganisms (8). Utilizing marine symbiotic microorganisms for protease enzyme production is advantageous due to the suitability of these complex environments for harsh industrial processes. This has led to the recent development of marine microbial enzyme technology, resulting in enzyme products that are used in pharmaceuticals, food additives, and fine chemicals (9). Furthermore, using microorganisms as sources of protease offers notable flexibility. Their ability to be propagated through colony breeding allows for large-scale and continuous production with relatively short production cycles. Additionally, the potential for genetic manipulation enables the optimisation of protease enzyme production (10).

Proteolytic bacteria typically belong to genera such as *Staphylococcus*, *Streptococcus*, *Bacillus*, *Pseudomonas*, and *Proteus*. These species are commonly found in marine environments, as they thrive in high-salinity conditions and possess inherent proteolytic capabilities. They produce protease enzymes extracellularly as secondary metabolites and are generally non-pathogenic (11). Proteases have widespread applications across various industries, especially those that utilize enzyme-based technologies. However, while employing broad-spectrum enzymes like proteases holds great potential, it comes with drawbacks, such as susceptibility to degradation and high production costs (12). Moreover, the clinical application of enzymes must adhere to regulatory requirements, including being free from toxic substances and maintaining high purity level. Numerous protease enzymes that serve as anticoagulants have received approval from the Food and Drug Administration (FDA). Nonetheless, there is an ongoing need for novel therapeutic enzymes to address emerging medical challenges (13).

The search for anticoagulant agents derived from natural sources remains an active area of research, driven by the need for safe and effective anticoagulants with minimal adverse effects on the human body. Currently available anticoagulants often require high dosages and may lead to potential complications. The growing acceptance of using protease-producing bacteria in industrial settings to develop eco-friendly enzymes has spurred increased interest in exploring bacterial strains capable of producing proteases with anticoagulant potential (14). Ongoing research indicates that proteolytic bacteria should be isolated from marine biota rich in bioactive compounds that act as anticoagulants. When selecting candidate sources of protease producers for anticoagulants, several criteria must be considered: the capacity to produce protease, stability, viability, and non-toxicity. Additionally, both *in vitro* and *in vivo* studies are crucial for assessing the anticoagulant activity of the produced proteases. These studies evaluate enzyme pathogenicity and determine the efficacy of potential anticoagulant agents (15). The use of marine organisms, such as the Pacific oyster, as a natural resource rich in microbial diversity has not been fully explored for this purpose. However, the potential for discovering protease-producing bacteria is promising, given that most marine microorganisms are non-pathogenic. Therefore, this research aims to isolate and identify proteolytic bacteria from rusip Pacific oysters as a potential source of protease production for developing anticoagulant agents.

2. MATERIALS AND METHODS

2.1. Rusip Fermentation and Bacterial Isolation

Sample of *C. gigas* were collected from Ngebum Beach, Kaliwungu, Kendal Regency, Central Java, Indonesia. The morphology and characteristics of the sample were checked based on the description of the species *Crassostrea gigas* refers to https://www.fisheries.noaa.gov/species/pacific-oyster. Fresh oyster samples were sterilized using alcohol 70% and sterile distilled water, then crushed using sterile mortar. Rusip fermentation using a mixture of 25% coarse salt (1.25 grams), and 10% palm sugar (0.5 grams), combined with 5 grams of crushed oyster meat. The mixture was put into a sterile closed container (anaerobic) and left for 7 days at a temperature of 25°C. A 1000µL aliquot of the fermentation product was mixed with sterile physiological NaCl (Cat. No. 3526099002, No Brand, Indonesia) at concentration ranging from 10^{-1} – 10^{-5} and homogenized. Then, 100µL of each dilution was spread onto Zobell Marine Agar (ZMA) (Cat. No. 2216, HiMedia, India) using a sterile spreader and incubated at 37°C for 24 hours. ZMA is a specific media for bacteria from marine environment. Colonies which appear with diverse morphological characteristics in the medium were then separated on Nutrient Agar (NA) plates (Cat No. M561, HiMedia, India) through three rounds of streaking to obtain pure colonies (2).

2.2. Morphological Identification

Microscopic identification was carried out using the Gram staining method (Cat. No. 77730-1KT-F, MERCK, USA). Bacterial colonies growing on the medium were stained using crystal violet, iodine, alcohol, and safraninD. Morphological identification was observed under a microscope with an 1000x total magnification using an oil immersion lens, focusing on shape, colour, arrangement and Gram characteristics. Macroscopic identification was based on the colony characteristics observed on the medium including shape, color, edge, elevation, and consistency (2).

2.3. Selection of Proteolytic Bacteria

To qualitatively assess protease activity, the pure isolate was cultured on 5% (w/v) Skim Milk Agar (SMA) (Cat. No. M763, HiMedia, India) and incubated at 37°C for 24-48 hours. The presence of a clear zone surrounding the colony indicated proteolytic activity. This hydrolysis zone suggests the bacteria possess proteolytic activity, which can be expressed as a proteolytic index. The determination of proteolytic index can be calculated using the following formula:

$$\text{Hidrolysis capacity} = \frac{\text{clear zone diameter} - \text{bacterial colony diameter}}{\text{clear zone diameter}} \quad \ldots\ldots (1)$$

The hydrolysis capacity index serves as a reference for assessing the presence of protease activity in millimeters and the bacteria's ability to produce protease (2).

2.4. Molecular Identification

2.4.1. 16S rRNA Identification

Bacterial DNA was extracted using the Quick-DNAMagbead Plus Kit (Zymo Research, D4082, US). The purity of the genomic DNA was assessed with a Nanodrop spectrophotometer (Thermo Scientific™). A total of 50 µL DNA sample was measured using Nanodrop spectrophotometer at 260 and 280 nm wavelengths to calculate the absorbance ratio of A260/280. The Identification of the 16S rRNA gene was conducted at PT. Genetika Science Indonesia. DNA amplification was performed using Thermal Cycler™ and universal primers Bact 27F (5' AGAGTTGATCCTGGCTCAG 3') and UniB 1492R (5' GGTTACCTTGTTACGACTT 3') (1). The amplification process employed MyTaq HS Red Mix, 2X (Bioline, BIO-25048, US). The PCR reaction consisted of 12.5 µL of MyTaq HS Red Mix (2X), 25 µL of double-distilled water (dd H_2O), and 50 µL of CGF-6 DNA with a concentration of 1.96 ng/µL. The PCR protocol was performed for 36 cycles, included denaturation at 95°C for 4 minutes, followed by annealing at 55°C for 35 seconds, and extension at 72°C for 45 seconds. PCR products (1400bp) were analysed using 1% TBE agarose gel electrophoresis, followed by visualization of amplicons under UV light at 312 nm.

2.4.2. Phylogenetic Tree Construction

The 16S rRNA gene was sequenced using the Sanger sequencing method. The DNA sequencing results were analysed using Base Assemble software, processed manually and compared with data from https://blast.ncbi.nlm.nih.gov/Blast.cgi through the BLAST program (Basic Local Alignment Search-Tool for Nucleotide) to compares sequences against GenBank database to find the closest matches based in similarity scores. Phylogenetic Tree analysis and design were conducted using the MEGA X program by Neighbor-joining method to determine the evolutionary relationship between the bacteria.

3. RESULTS AND DISCUSSION

3.1. Bacterial Exploration from Rusip Pacific Oyster

Pacific oysters (*Crassostrea gigas*) (Figure 1) have characteristics consistent with the literature (16), including an elongated "cupped" shaped shell with rough and slightly sharp edges, and a white interior. The bacterial isolation results from rusip (fermented Pacific oyster products) yielded 18 distinct bacterial isolates, which were successfully purified using Nutrient Agar medium (Figure 2).

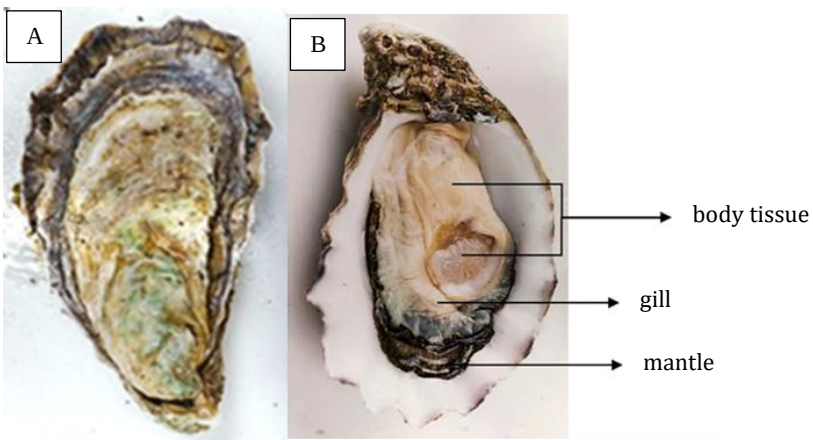

Figure 1. Pacific oysters (*Crassostrea gigas*). (A) Shells. (B) Inside the body.

Approximately 40% of the Pacific oyster's body consist of meat that is protected by the shell (Figure 1A). The shell is composed of about 95% calcium carbonate (C_aCO_3) and 5% shell protein. As a result, oyster shells are widely utilized in health products and environmentally friendly waste processing. In this study, the utilized body part of *Crassostrea gigas* was the whole tissue, including body tissue, gills, and mantle (Figure 1B). The body tissue of Pacific oyster contains bioactive compounds with various biological activities, such as antioxidants, anti-inflammatory, antimicrobial, antihypertensive, anticancer, and anticoagulants properties. The use of the entire Pacific oyster tissue is further supported by its high protein content, which can enhance the acquisition of proteolytic microorganisms through the fermentation process (6).

3.2. Proteolytic Bacterial Selection

Three bacterial isolates designated CGF-1, CGF-6, and CGF-11, were isolated from rusip Pacific oyster (*Crassostrea gigas)* and exhibited protease hydrolysis activity (Figure 3). Among these isolates, CGF-6 demonstrated the most significant protease hydrolysis activity, outperforming both CGF-1 and CGF-11. Proteolytic activity was qualitatively assessed on skim milk agar medium by measuring the size of the proteolytic zone created by the bacterial isolates on the screening medium. To produce secondary metabolites, including protease enzymes, bacteria require cultivation in a medium containing a protein substrate like skim milk, which contains casein that readily reacts with these enzymes (17).

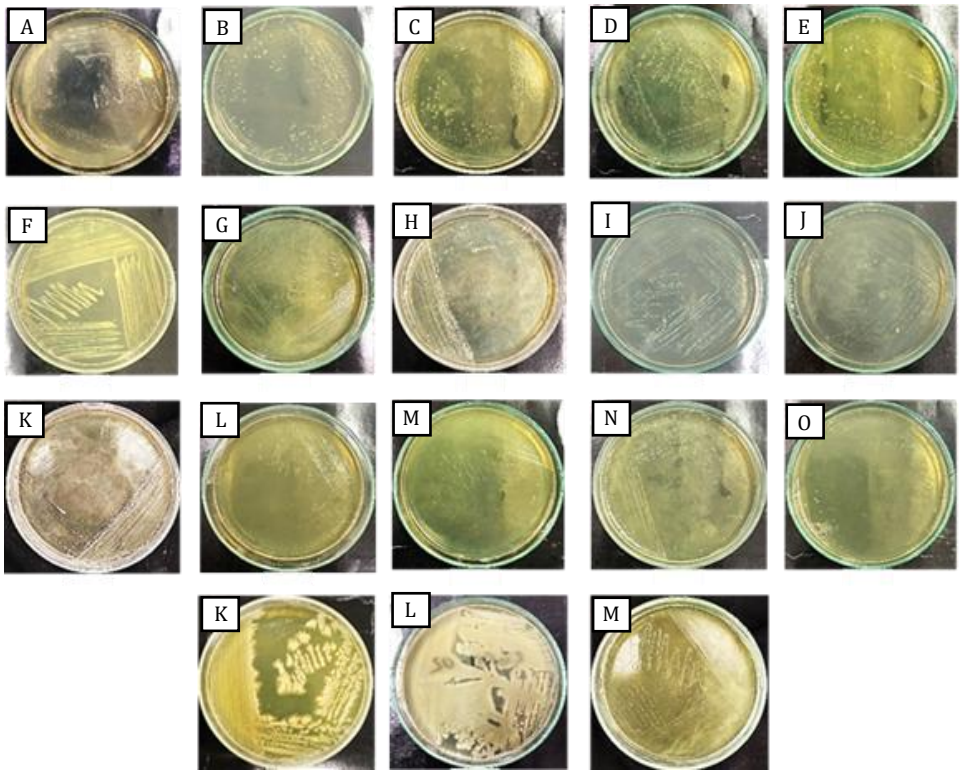

Figure 2. 18 pure bacterial isolates associated with rusip Pacific oysters (*C. gigas*). (A) CGF-1. (B) CGF-2. (C) CGF-3. (D) CGF-4. (E) CGF-5. (F) CGF-6. (G) CGF-7. (H) CGF-8. (I) CGF-9. (J) CGF-10. (K) CGF-11. (L) CGF-12. (M) CGF-13. (N) CGF-14. (O) CGF-15. (P) CGF-16. (Q) CGF-17. (R) CGF-18.

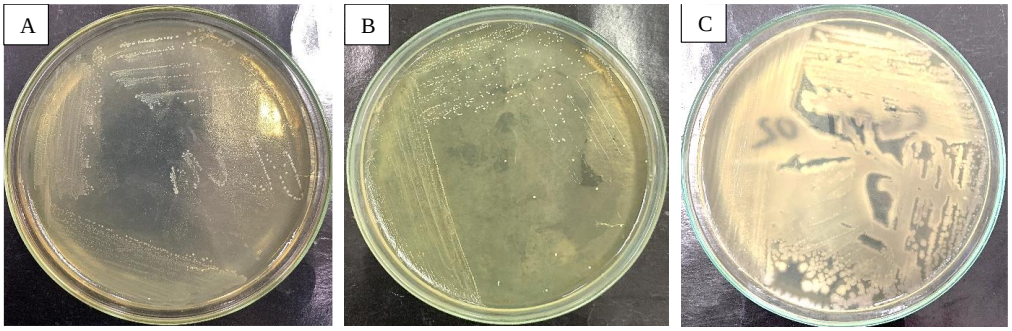

Figure 3. Three bacterial colonies used in this study. (A) CGF-1. (B) CGF-6. (C) CGF-11

Screening results indicated hydrolysis zones produced by CGF-1, CGF-6, and CGF-11, which exhibited proteolytic indices of 0.2, 0.5, and 0.4 cm, respectively (Table 1). All these three strains demonstrated the ability to inhibit casein within the medium. The presence of a proteolytic zone signifies the bacterium's capability to produce protease enzymes, as this zone results from the bacterial protease activity that cleaves peptide bonds in the casein substrate within the skim milk agar. Microbial proteases produced extracellularly play a crucial role in protein structural remodeling through peptide bond hydrolysis. These enzymes exhibit a variety of physicochemical and catalytic properties, including specific substrate-binding affinities (18). The objective of this test was to assess the bacteria's ability to produce protease enzymes, which is essential for identifying potential anticoagulant agents. Protease has extensive functions including as an anticoagulant. Protease can be categorized into alkaline, serine, and metalloprotease, with alkaline protease being the most widely used in industrial applications. Protease produced by microorganisms are often more efficient and cost-effective than those derived from animals and plants, as they can be scaled up, genetically manipulated, and produced consistently at lower costs (19).

Three proteolytic bacterial isolates (as detailed in Table 1) were obtained from fermented rusip Pacific oyster tissue, each exhibiting distinct characteristics. Isolates CGF-1 and CGF-6 displayed similar macroscopic traits, while CGF-11 showed distinct differences. Each colony, characterised by unique morphology, was purified through a minimum three rounds of sub culturing to achieve pure bacterial isolates.

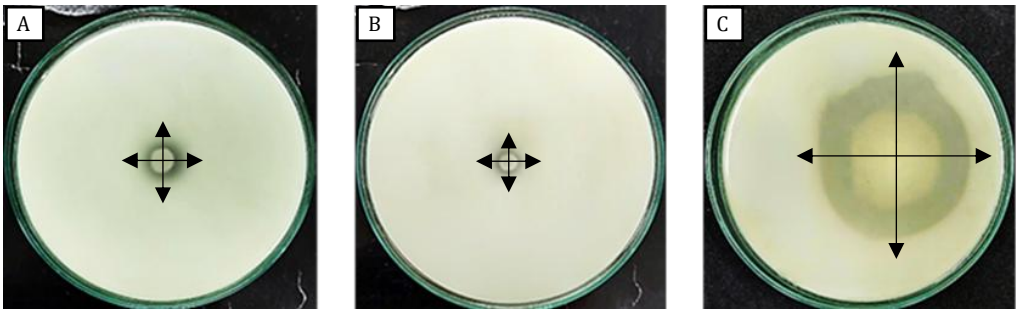

Figure 4. Bacteria that produce protease enzymes show the presence of a proteolytic zone in skim milk agar medium. The diameter of proteolytic zone: A. CGF-1= 0.2 cm, B. CGF-6= 0.5 cm, C. CGF-11= 0.4 cm.

Table 1. Morphological data of three proteolytic bacteria

Characteristic	Bacterial Isolate		
	CGF-1	CGF-6	CGF-11
Macroscopic			
Colony form	Punctiform	Punctiform	Irregular
Color	Transparent	Transparent	Cream
Edge	Entire	Entire	Undulate
Elevation	Convex	Convex	Flat
Consistency	Smooth	Smooth	Smooth
Microscopic			
Form	Coccus	Coccus	Bacillus
Color	Purple	Purple	Red
Gram type	Positive	Positive	Negative
Spore	Negative	Negative	Positive
Proteolytic index	0.2cm	0.5cm	0.4cm

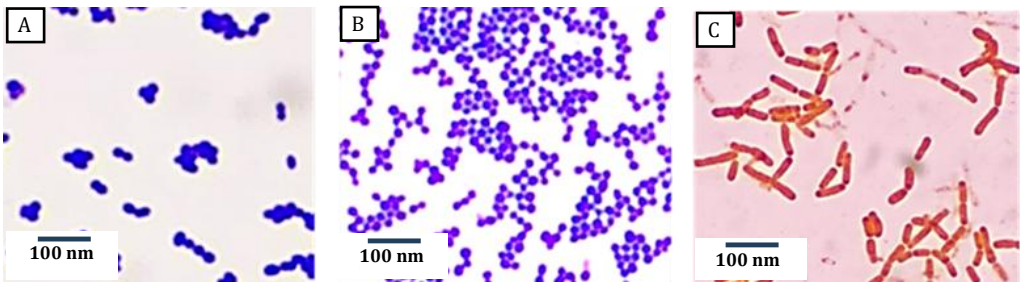

Figure 5. Gram morphology of three proteolytic isolates under the microscopes at 100x magnification. (A) CGF-1. (B) CGF-6. (C) CGF-011.

The morphological and biochemical characteristics of the three pure bacterial isolates were identified using Gram staining and microscopic observation at 100x magnification. The purpose of Gram staining is to classify bacteria into two groups: Gram-positive and Gram-negative. If the bacteria appear purple, they are classified as Gram-positive; if red, they are classified as Gram-negative (1). Isolates CGF-1 and CGF-6 were identified as Gram-positive cocci, while isolate CGF-11 was identified as a Gram-negative bacillus (Figure 5). Notably, CGF-1 and CGF-6 formed spores, whereas CGF-11 did not (Table 1). Spores serve as a defense mechanism for bacteria, enabling them to withstand extreme environmental conditions. Bacteria that can form spores are often difficult to eradicate because these spores protect them from exposure to disinfectants (20).

3.3. Molecular Identification

3.3.1. DNA Quantification Test

The basic principle of DNA extraction involves breaking down the cell walls and membranes to isolate the DNA contained in the nucleoid without damaging it (21). The purity of the isolated DNA was assessed using a NanoDrop™ Spectrophotometer, measuring the absorbance ratio of A260/280. Table 2 presents the purity and concentration levels of the genomic DNA isolates determined by absorbance analysis. Only isolate CGF-6 was identified in this study, as it exhibited the highest proteolytic activity, indicating its potential as a source for producing protease enzymes to develop anticoagulant agents.

DNA is considered pure when the absorbance ratio is 1.8 at λ260/280. Our results indicated a high level of DNA purity, with an absorbance of 1.96. A ratio between 1.8 and 2.0 signifies high purity. Ratios lower than 1.8 suggest protein contamination during DNA preparation, while ratios above 2.0 indicate RNA contamination. Such contamination can affect the accuracy and reliability of molecular analyses (22). Thus, the purity and concentration levels of DNA are critical for the success of molecular analysis procedures. The A260/280 absorbance ratio is a widely accepted and effective method for assessing DNA purity and concentration. Nucleic acids absorb maximally at 260 nm, while proteins absorb light more strongly at 280 nm (23).

3.3.2. Amplification of 16S rRNA Gene

Figure 6 demonstrates the amplification results of the 16s rRNA gene. Genomic DNA extracted from bacterial isolates served as the template for amplification using the PCR method. The PCR amplification results were subjected to electrophoresis on a 1% TBE agarose gel and visualized with a UV transilluminator, resulting in a distinct band approximately 1400 bp in size, as indicated by the DNA marker. Sequencing is conducted after confirming successful amplification to identify the bacterial species.

The purity and concentration of the extracted DNA significantly affect the visualization of the DNA band. The amplification of the 16S rRNA gene was performed using the primers Bact 27F and UniB 1492R. The 16S rRNA gene is a highly conserved region in bacterial genomes, although it undergoes gradual evolutionary changes. Analysing the 16S rRNA gene is crucial for determining bacterial phylogeny and taxonomy, serving as a universal genetic marker for bacterial identification. The quality of 16S rRNA gene amplification depends on the size of the amplified DNA fragment (24).

Table 2. Data on the purity and concentration of genetic DNA of the bacterial isolate CGF-6

Isolate	Concentration	$A_{260/280}$	Volume
CGF-6	99.3 ng/µl	1.96	50 µl

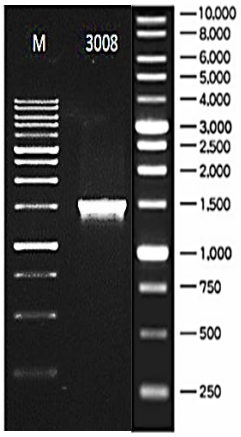

Figure 6. Amplification of CGF-6 DNA using Bact 27F- UniB 1492R primers.
M = 1k b DNA ladder (Cat. No. DL006, Geneaid, Taiwan); 3008 = CGF-6 bacterial isolate

3.3.3. BLAST Analysis

BLAST analysis refers to http://www.ncbi.nlm.nih.gov. The results of BLAST analysis for the CGF-6 isolate demonstrated a similarity to species in GenBank (Table 3). The CGF-6 isolate shows a homology value of 99.86% with *Staphylococcus epidermidis*. Based on this level of homology, the GGF-6 isolate is classified within the same species as *Staphylococcus epidermidis*, belonging to the genus *Staphylococcus*.

Typically, the homology percentage reflects identity at the genus level but can vary significantly at the species level. A homology level exceeding 97% generally indicates that two organisms belong to the same species, while levels between 93% and 97% suggests they belong to the same genus. Homology levels below 93% usually indicate family-level differences (25). However, it is important to note that a homology level below 70% does not necessarily suggest a new species, especially if there is a lack of data in GenBank, which is insufficient evidence to support such a claim (26).

3.3.4. Phylogenetic Tree Construction

As reported in Figure 7, it can be indicated that the bacterial isolate CGF-6 is closely related to *Staphylococcus epidermidis* strain, as they share a phylogenetic branch, clade, or genus. The same homology level (99.86%) was observed in various strains, including *S. epidermidis* strain G003 (accession number KX926554.1), *Staphylococcus* sp. DMS G06 (accession number KR709224.1), *S. capitis* subsp. capitis strain Ph 20A1 (accession number KT719989.1), *Staphylococcus* sp. strain ZG14-1 (accession number OQ981408.1), *S. epidermidis* strain SA (accession number OR342081.1), *S. epidermidis* strain HDS (accession number OR342084.1), *Staphylococcus* sp. strain UFLA01-930 (accession number KX555442.1), *Staphylococcus* strain SA-144 (accession number KY194740.1), *Staphylococcus* sp. strain 1910ICU161 (accession number MT225634.1), and *S. epidermidis* strain 1910ICU248 (accession number MT225635.1).

Table 3. Top 10 BLAST result

Strain	Species	Similarity	Accession Number
Staphylococcus epidermidis strain CGF-6	*Staphylococcus epidermidis* strain G003	99.86%	KX926554.1
	Staphylococcus sp. strain DMS G06	99.86%	KR709224.1
	Staphylococcus capitis subsp. *capitis* strain Ph-20A1	99.86%	KT719989.1
	Staphylococcus sp. strain ZG14-1	99.86%	OQ981408.1
	Staphylococcus epidermidis strain SA	99.86%	OR342081.1
	Staphylococcus epidermidis strain HDS	99.86%	OR342084.1
	Staphylococcus sp. strain UFLA01-930	99.86%	KX555442.1
	Staphylococcus strain SA-144	99.86%	KY194740.1
	Staphylococcus sp. strain 1910ICU161	99.86%	MT225634.1
	Staphylococcus epidermidis strain 1910ICU248	99.86%	MT225635.1

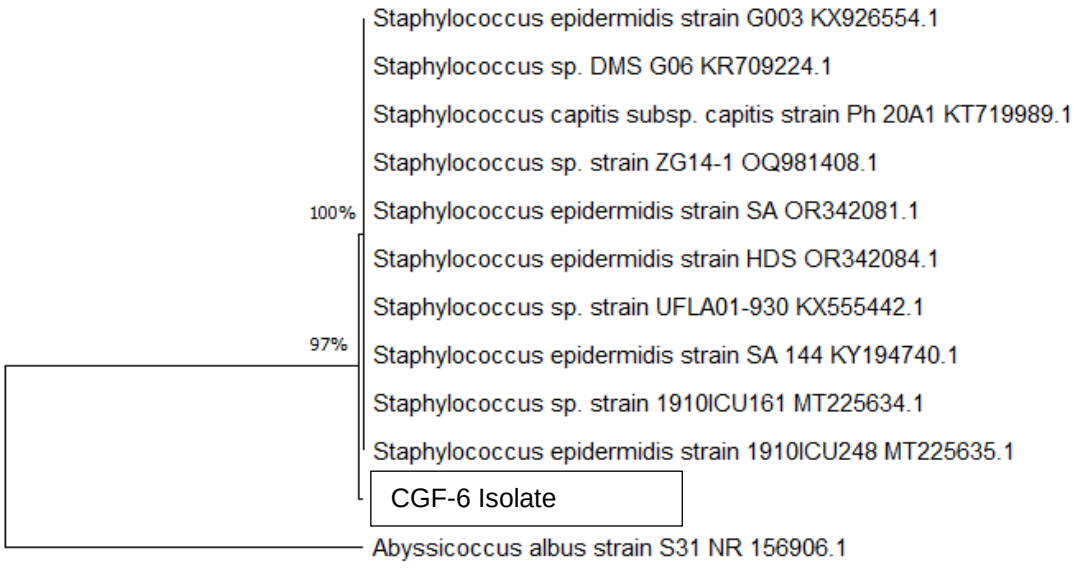

Figure 7. Phylogenetic tree of CGF-6 bacterial isolates

S. epidermidis is a Gram-positive, non-spore-forming coccus that belongs to the *Staphylococcus* genus. It is a common resident of human mucous membranes, particularly the skin and is recognised as part of the normal human skin microbiota. Although not inherently pathogenic, some strains exhibit significant halotolerant, allowing them to thrive in marine environments (27). Numerous studies have explored protease-producing microorganisms within marine biota, including sea cucumbers, brown algae, shrimp, seaweed, tuna, coral, and nudibranchs. Common isolates from these sources include bacteria from the genera *Staphylococcus*, *Bacillus*, and *Pseudomonas* (28). Previous studies reported on proteolytic bacteria isolated from rusip fermented sea cucumber tissue, identified as *S. hominis* strain HSFT-2 and *Bacillus cereus* strain HSFI-10, which exhibited proteolytic activity (1,28).

Furthermore, *Staphylococcus saprophyticus*, another protease-producing species of *Staphylococcus*, has been successfully isolated from marine sediments. The protease enzyme produced by this strain shows several notable characteristics, including a molecular weight of 28 kDa, remarkable thermostability (remaining stable at temperatures ranging from 10 to 80°C), and robust stability against a variety of chemical agents, such as surfactants, oxidizing agents, bleaching agents, and hydrophobic solvents. These combined properties suggest strong potential for industrial applications of this enzyme (29).

Protease is an enzyme that catalyses peptide bonds in protein structures. Using appropriate substrates can enhance the effectiveness of proteases in regulating physiological processes, including fibrinolysis. Fibrinolytic protease belongs to the serine protease group and is often produced by microorganisms. This type of protease has the same mechanism as plasmin (protease-like plasmin) in the body and is known to have fibrinolytic activity. Protease like plasmin is a fibrin clot-dissolving agent that directly works to dissolve fibrin in blood clots without activating plasminogen like the fibrinolysis process in the body (30).

Several studies have reported that proteases derived from marine microbes exhibit anticoagulant activity. Bacteria such as *S. hominis*, *S. aureus*, *Staphylococcus sciuri*, *S. saprophyticus*, *B. cereus*, *B. thuringiensis*, and *B. tequilensis* demonstrate anticoagulant properties by prolonging blood clotting times and lysing blood clots in vitro. Notably, novel Staphylokinase (SAK), a fibrin-specific plasminogen activator, produced by *Staphylococcus* species, has been identified and shows established anticoagulant activity. SAK has also been investigated in patients with myocardial infarction (31,32).

In our research, we identified a protease-producing bacterium, specifically the *S. epidermidis* strain CGF-6. As a member of the *Staphylococcus* genus, the strain may possess potential anticoagulant activity that has yet to be investigated. To assess its potential as an anticoagulant agent, we perform test such as the fibrin plate assay, thrombolytic assay, and clotting time assay. These tests can help evaluate the anticoagulant activity in vitro by measuring clotting time and the degradation of fibrin, which are crucial characteristic of effective anticoagulant agents (33).

The protease produced by *S. epidermidis* CGF-6 belongs to the serine protease family. Serine proteases are found in both prokaryotic and eukaryotic cells and play roles in cellular and physiological processes, including haemostasis, the blood clotting cascade, and fibrinolysis. Previous studies have demonstrated the involvement of serine proteases as anticoagulants and antiplatelets (34).

This study provides valuable insights into potential sources of proteolytic bacteria within rusip Pacific oysters. It serves as a reference point for identifying protease-producing bacteria with the potential to be developed as anticoagulant agents. However, it is important to note that this research represents the initial stages of exploring protease-producing bacteria, and the characterisation and testing of their anticoagulant activity have not yet been addressed. Future research will involve the extraction of protease enzymes from these bacteria, followed by purification steps utilizing precipitation and chromatography techniques. Enzyme characterisation will be conducted using zymography methods to gather comprehensive data on the activity of the protease enzymes.

Evaluating the efficacy of these proteases as anticoagulant agents requires both in vitro and in vivo testing. In vitro assays will include methodologies, such as fibrinolytic assays, clot formation and lysis assays (CloFAL), Euglobulin clot lysis assays, anti-platelet aggregation activity assays, and Thromboelastographic. In vivo activity assessments will include the D-dimer test, ferric chloride-induced thrombosis models, and rat grain flap models (35,36). These assays aim to evaluate the efficacy and potential pathogenicity of the enzyme candidates as anticoagulant agents. Ultimately, successful outcomes from these investigations could lead to the development of novel drugs and laboratory diagnostic reagents.

4. CONCLUSIONS

Eighteen bacterial isolates were successfully obtained from the fermentation product of rusip, a traditional dish made from Pacific oyster. Among these isolates, three were identified as proteolytic bacteria: CGF-1, CGF-6, and CGF-11. Notably, the CGF-6 isolate demonstrated the highest proteolytic index and was identified as a strain of *Staphylococcus epidermidis*, which designated as CGF-6. This isolate shows promise as a source for producing protease enzymes that could be applied in the development of anticoagulant agents. Further research is needed to purify and characterise these enzymes in order to optimise their activity. Additionally, in vitro and in vivo studies are essential to evaluate the anticoagulant activity and efficacy of potential agent candidates.

Author contributions: MAA: Conceptualization. M, SS: Drafting manuscript. MAA, SSD: Methodology. GDRA: Analysis data and documentation.

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Conflict of interest: The author declares no conflict of interest.

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