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Characterization of extracellular protease-producing bacteria isolated from the coastal environment of Bangladesh and optimization of parameters for enzyme production

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Abstract

Bangladesh's coastal soil and water harbor a diverse array of native microorganisms that can serve as a significant source of natural protease enzymes, with broad applications in biotechnology, food industries, and environmental sectors. The objective of this study was to isolate and identify native protease-producing bacteria from Cox's Bazar, a coastal region of Bangladesh, and to optimize the parameters for enzyme production. The bacteria found in the soil and water samples were initially assessed using the zone of inhibition technique and hemolytic test. Five of the isolated bacteria exhibited proteolytic activity and were found to be non-pathogenic. The isolates were subsequently identified through morphological, biochemical, molecular, and evolutionary analysis. Among the identified isolates (*Bacillus subtilis, Xenorhabdus nematophilia*, and *Enterobacter cloacae*), *B. subtilis* was determined to be the most productive bacterial species. A temperature of 35°C, pH 7, 36 h of incubation, and galactose as the carbon source, were the best conditions for protease production. The enzyme activity of *B. subtilis* was found to be 0.37 U/mL, compared to 0.56 U/mL for a commercial enzyme. These findings suggest that *B. subtilis* obtained from the coastal water of Bangladesh has the potential to serve as a local source for protease production, with applications in food processing, animal feed, dairy, meat, textiles, waste management, and pharmaceutical industries in Bangladesh.

Keywords: Proteolytic, *Bacillus subtilis*, Enzyme, Microorganism

1. Introduction

Proteases, or peptidases, are enzymes essential to all living organisms and possess considerable commercial value. They are ubiquitous entities found in plants, animals, and microbes, and play a key role in protein synthesis and their regulation. Proteases demonstrate precise selectivity in their actions, as each of them is specialized in breaking down specific polypeptide sequences of amino acids under optimal conditions in the environment [1]. The renewed interest in proteolytic enzymes stems not only from their essential function in cellular metabolic processes but also from their growing importance in a range of industrial applications [2]. Proteases represent a significant category of industrial enzymes, with broad applications in human food production. Proteases have plenty of uses beyond human nutrition, offering distinct benefits in the fish and livestock feed industry. Their capacity to break down proteins into simple peptides enhances digestibility and metabolism [3]. Thus, these enzymes are widely employed in dairy, bakery, animal and plant protein processing, as well as in meat, fish, and seafood processing. Additionally, proteases have practical applications in several industries including leather

production, textile manufacturing, waste management, agriculture, cosmetics, pharmaceuticals, and animal husbandry [4–7]. Notably, proteases also have potential applications in the preparation of therapeutic agents against fatal diseases like acquired immunodeficiency syndrome (AIDS) and cancer [1]. While commercially important proteases are derived from microbial, animal, and plant sources, microbial enzymes are preferred for industrial and biotechnological purposes due to their extracellular secretion, ease of cultivation, high yields, and shorter production cycles. Microorganisms play a crucial role in the synthesis of several enzymes, with proteases accounting for approximately 60% of the global enzyme market [1,4].

In Bangladesh, a heavy reliance on imported protease enzymes has emerged as a significant challenge for industries that depend on these biocatalysts. Imported enzymes not only increase production costs but also expose the domestic market to potential contamination problems [8]. Imported protease enzymes often undergo long-distance transportation and handling, which may compromise their quality due to their low stability and shelf life [9]. These factors not only influence the efficiency of industrial operations but also give rise to issues regarding the safety and reliability of the final products. To address these problems effectively, it is imperative to build indigenous production capabilities for protease enzymes in Bangladesh. Implementing these procedures would lead to both cost savings and enhancement of quality control and safety protocols, thereby creating a more sustainable and secure supply chain for the nation's enzyme-related industries.

To date, no attempt has been made in Bangladesh to identify and optimize the use of native microorganisms for protease enzyme production. This study aims to fill that gap by identifying native bacteria that can produce exogenous protease enzyme and optimizing production conditions. This initiative can help establish a local reservoir of industrially viable enzymes, with a particular focus on applications in animal feed and related sectors. Ultimately, the study seeks to offer a sustainable, locally sourced alternative to imported enzymes for use in various industrial processes.

2. Materials and methods

2.1 Sample collection

Soil and water samples were obtained from Curuskul, labeled as CW and CS, and Uttar Nuniachara union, labeled as UW and US, respectively, in the coastal region of Cox’s Bazar, Bangladesh. The soil samples were collected using gloved hands, promptly packed in polythene bags with sample information, and stored in an icebox. The water samples were collected directly into sample bottles, immediately sealed, and then kept in an icebox.

2.2 Isolation and Screening of Protease producing Bacteria

Bacteria from the samples were initially isolated using a ten-fold serial dilution method with normal saline, diluted up to 10^{-9} across nine test tubes. A 0.1 mL volume from each diluted sample was then spread onto Petri dishes and incubated at 37ºC for 24 hours (h). Single colonies were then isolated from the spread plates based on distinct morphological features, including size, shape, color, surface texture, opacity, and motility. Proteolytic bacterial strains were screened on agar plates supplemented with 1% NaCl, 5% casein, and nutrient media (NB). A volume of 5 µL of each broth culture was dropped onto a paper disc on the casein-agar plates, which were then incubated at 37°C for 24 h. After incubation, clear hydrolytic zone-forming bacterial strains were identified as protease-producing bacterial colonies. To determine their pathogenicity, a hemolytic test was conducted by streaking the selected bacteria on agar plates containing 5% sheep blood, followed by incubation at 35°C for 24 h.

2.3 Morphological and biochemical characterization of bacterial isolates

Morphological characteristics of all the isolates were assessed based on colony appearance, shape, color, and motility tests. The selected isolates were placed onto nutrient agar plates and cultured for 24 h at 35°C. After the incubation period, the colony was examined for its color, shape, surface, opacity, and size, followed by Gram staining. Isolated bacterial strains were examined for selective screening using biochemical tests, including the catalase test, oxidase test, methyl red (MR)Voges-Proskauer (VP) MR-VP test, citrate agar test, gelatinase test, urease test, starch hydrolysis test, and sugar fermentation test [10].

2.4 Molecular identification by MALDI-TOF MS and gene sequencing

For initial molecular identification of the selected isolates, at first Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) was used. For MALDI-MS analysis, pure isolates were mixed with an organic compound matrix, which absorbs energy, and a laser was used to ionize the sample. The

protonated ions were classified by mass-to-charge ratio (m/z) and quantified using time-of-flight (TOF) analyzers, resulting in a unique peptide mass fingerprint (PMF) generated for each analyte derived from the TOF data [11].

The Monarch® Genomic Deoxyribonucleic Acid (DNA) Purification Kit (New England Biolabs, USA) was used for the extraction and purification of genomic DNA from bacterial cells. To verify the purity of the isolated DNA products, a NanoDrop analysis was performed. Genomic DNA of the selected isolates was extracted and amplified using polymerase chain reaction (PCR). The PCR reaction mixture was prepared using 25.0 µl of PCR master mix, 2.5µl of bacterial 16S rRNA forward primer 27F (AGAGTTTGATCCTGGCTCAG), 2.5µl of reverse primer 1492R (CGGTTACCTTGTTACGACTT), 10 µl of DNA template, and nuclease free water up to 50 µl. After gel electrophoresis, the amplified PCR product was sent for gene sequencing. The resulting amplified genomic sequence was then uploaded to the National Center for Biotechnology Information (NCBI) database and analyzed using the NCBI Basic Local Alignment Search Tool (BLAST) to determine sequence similarity. After identifying the closest neighbors of the isolates, a phylogenetic tree was created using the Maximum Likelihood approach and Tamura-Nei model [12]. Evolutionary analyses were performed using MEGA11 software.

2.5 Crude enzyme optimization

The best isolate was selected for protease enzyme production based on clear zone diameter. The medium used for enzyme production contained the following ingredients: 2.0% casein, 1.0% dextrose, 1.0% peptone, 2.0% KH_2PO_4, 0.2% NaCl, and 0.002% $CaCl_2$. 1.0% of the selected isolate was added to 50 mL of production medium and then incubated at 37°C. After the samples were centrifuged at 10,000 rpm for 10 minutes, the supernatant was collected as crude enzyme for further investigation. To optimize the activity of the crude enzyme, it was subjected to different conditions, including temperature (20 to 45°C), pH (4 to 9), incubation period (12 to 72 h at 12h intervals), and various carbon sources (glucose, fructose, galactose, sucrose, maltose, mannose, and lactose). The activity of the crude protease enzyme was measured using the Lowry protein estimation method [13].

2.6 Partial purification of crude enzyme

NH_4SO_4 precipitation was used to purify crude enzymes produced under optimal conditions. A 75% NH_4SO_4 solution was added dropwise from a burette to the enzyme solution until precipitates began to form. The mixture was then centrifuged at 10,000 rpm for 15 minutes, and the supernatant was carefully discarded. The precipitate was collected using a series of washing steps to enhance activity: distilled water was added to the centrifuge tube, the tube was shaken to redissolve the precipitate, followed by centrifugation and recollection of the precipitate. Finally, the precipitate was dried in a freeze dryer before enzyme activity was assessed.

2.7 Determination of protease activity

After purification of the crude enzyme, the activity of the pure enzyme was assayed using a universal protease activity method with casein as the substrate [14]. To compare the activity, a commercial protease enzyme (Sigma P2143-5G) at three concentrations - 10, 20 and 30 µg/mL - was also tested in this assay. Enzyme solutions were mixed by rotation and incubated at 37°C for 10 minutes. The reaction mixture consisted of 1% (w/v) casein in 50 mM phosphate buffer (pH 7.0). To stop the reaction, 5 mL of the Trichloroacetic acid (TCA) reagent was added to each tube. A required volume of enzyme solution was then added to each tube to bring the total volume to 1 mL, including the blank, and the tubes were incubated at 37°C for 30 minutes. A 0.45 µm polyether sulfone syringe filter was used to filter each solution. 2 mL of the filtrate were transferred to a new tube, and 5 mL of Na_2CO_3 was added. Subsequently, Folin's reagent was added, which reacts primarily with free tyrosine, and the tubes were incubated at 37ºC for 30 minutes. A 0.45 µm polyether sulfone syringe filter was used again to filter 2 mL of each reaction mixture, and absorbance was measured at 660 nm. A separate L-tyrosine standard curve was prepared to quantify the amount of tyrosine released (Figure 4). Finally, protease activity was calculated using the following formula. One unit (U) of enzyme activity is defined as the amount of enzyme that releases 1 micromole (µmol) of tyrosine equivalents per minute under the assay conditions.

$$Units/mL\ enzyme = \frac{(\mu mol\ tyrosine\ equivalents\ released) \times (total\ volume\ of\ assay)}{(Volume\ of\ enzyme\ used) \times (time\ of\ assay) \times (volume\ used\ in\ colorimetric\ Determination)}$$

3. Results

3.1 Isolation of protease producing bacteria from coastal soil and water

In this study, 15 different bacterial isolates were obtained from soil and water samples collected from two different sites (Table 1). Of these, 10 demonstrated proteolytic activity, as evidenced by the formation of clear zones around their colonies on casein-agar media. Following a pathogenicity test, five isolates of proteolytic

bacteria (CW-2, CW-3, UW-3, UW-5, and US-3) were found to be non-pathogenic, exhibiting gamma hemolysis. The isolates showing the largest zones of inhibition on casein-agar media were selected for further screening (Figure 1).

Table 1 Primary screening of protease producing isolates based on the proteolytic activity and pathogenicity test.

Sample source	Bacterial isolates	Proteolytic test	Pathogenicity test	Clear zone diameter (mm)
Curuscul Union, Water	CW-1	Positive	α	-
	CW-2	Positive	γ	22
	CW-3	Positive	γ	16
	CW-4	Positive	β	-
	CW-5	Negative	β	-
	CW-6	Negative	α	-
Uttor Nuniachora, Water	UW-3	Positive	γ	12
	UW-5	Positive	γ	18
	UW-9	Negative	α	-
Curuscul Union, Soil	CS-1	Negative	α	-
	CS-2	Negative	β	-
	CS-3	Positive	α	-
	CS-4	Positive	β	-
Uttor Nuniachora, Soil	US-3	Positive	γ	15
	US-4	Positive	α	-

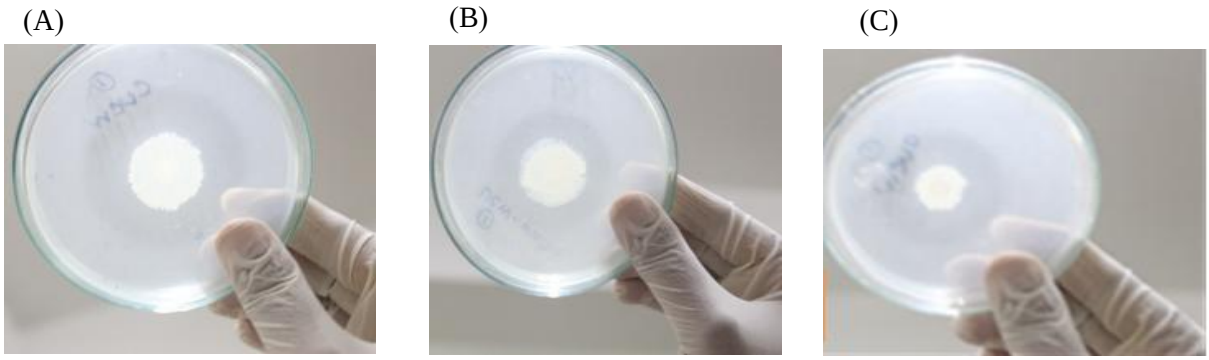

Figure 1 Proteolytic screening of non-hemolytic protease producing isolates (A) CW-2, (B) CW-3 and (C) US-3.

3.2 Morphological and biochemical screening of protease producing bacteria

Morphological and biochemical characteristics of the five isolates are presented in Table 2. The morphological traits of CW-2 and UW-5 were identical, while those of CW-3 and US-3 were quite similar. In contrast, UW-3 exhibited a uniquely colored colony. Each of the five isolates produced smooth, moving colonies. Biochemical characterization revealed that among the five isolates, two were gram (+) and three were gram (-). All five isolates were oxidase (-), while four were catalase (+) and one catalase negative (-). In other tests, two isolates were positive for the MR test, and three isolates were positive for the VP test. All isolates tested positive for gelatinase, indicating their ability to break down protein. In terms of sugar fermentation, all five isolates were positive for dextrose and glucose. For sucrose, four were positive and one was negative. Similarly, four were positive and one negative for starch hydrolysis. Regarding urease test, all isolates tested negative.

Table 2 Morphological and biochemical characterization of proteolytic and non-pathogenic isolates.

Characteristics	Bacterial isolates				
	CW-2	CW-3	UW-3	UW-5	US-3
Morphological					
Shape	Rod	Rod	Rod	Rod	Rod
Color	White	Off white	Greyish white	White	Off white
Surface	Smooth	Smooth	Smooth	Smooth	Smooth
Opacity	Opaque	Translucent	Opaque	Opaque	Translucent
Motility	Motile	Motile	Motile	Motile	Motile
Biochemical					
Gram Staining	+	-	-	+	-
Catalase	+	+	-	+	+
Oxidase	+	+	+	+	+
Methyl Red (MR)	-	+	-	-	+
Voges Proskauer (VP)	+	-	+	+	-
Gelatinase	+	+	+	+	+
Starch hydrolysis	+	+	-	+	+
Urease	-	-	-	-	-
Citrate agar	+	+	-	+	+
Sugar fermentation					
Sucrose	+	+	-	+	+
Galactose	-	+	-	-	+
Glucose	+	+	+	+	+
Dextrose	+	+	+	+	+

3.3 Molecular identification of potential protease producing bacteria

The protease producing isolates were identified through MALDI-TOF and 16S rRNA gene analysis and evolutionary analysis. From the BLAST (NCBI) result CW-2 isolates displayed 98.48% sequence similarity to *Bacillus subtilis* (Table 3 and Figure 2). The other two isolates UW-3 and US-3 were identified as *Xenorhabdus nematophilia* and *Enterobacter cloacae* with 96.54 and 98.91% sequence similarity respectively.

Table 3 Molecular identification of protease producing isolates.

Isolate code	MALDI-TOF	Identification (%)	Gene sequencing	Identification (%)
CW-2	*Bacillus*	99.45	*Bacillus Subtilis*	98.48
CW-3	*Enterobacter*	99.91	*Enterobacter cloacae*	98.91
UW-3	*Xenorhabdus*	99.54	*Xenorhabdus*	96.54
UW-5	*Bacillus sp.*	99.91	*Bacillus Subtilis*	98.91
US-3	*Enterobacter*	99.21	*Enterobacter cloacae*	97.21

3.4 Crude enzyme optimization

Among the isolates, CW-2 (*Bacillus subtilis)* was selected for purification and optimization studies, as it formed the largest clear zone (22 mm) on casein-agar media (Figure 1 and Table 1). Enzyme production media with different pH values, ranging from pH 4 to 9, were prepared, and the crude enzyme was separated and assayed using the Lowry method. The selected production media were used for extracellular protease production. Further physiochemical parameters (incubation time, temperature and sources of carbon) were optimized to maximize protease enzyme production. Crude enzyme from the media with pH 7 showed the highest activity (0.18 ± 0.03 U/mL) (Figure 3A), while 35°C was found to be the optimal temperature (0.29 ± 0.03 U/mL) (Figure 3B). The optimal incubation time for protease production by *Bacillus* sp. was 36 hours, showing maximum enzymatic activity (0.23 ± 0.03 U/mL). Enzyme production decreased significantly at both shorter and longer incubation times Figure 3c). At conditions deviating from these optimized parameters, enzyme activity was markedly lower. Among the seven carbohydrates tested, the media containing galactose exhibited the highest enzymatic activity (0.30 ± 0.04 U/mL) (Figure 3D).

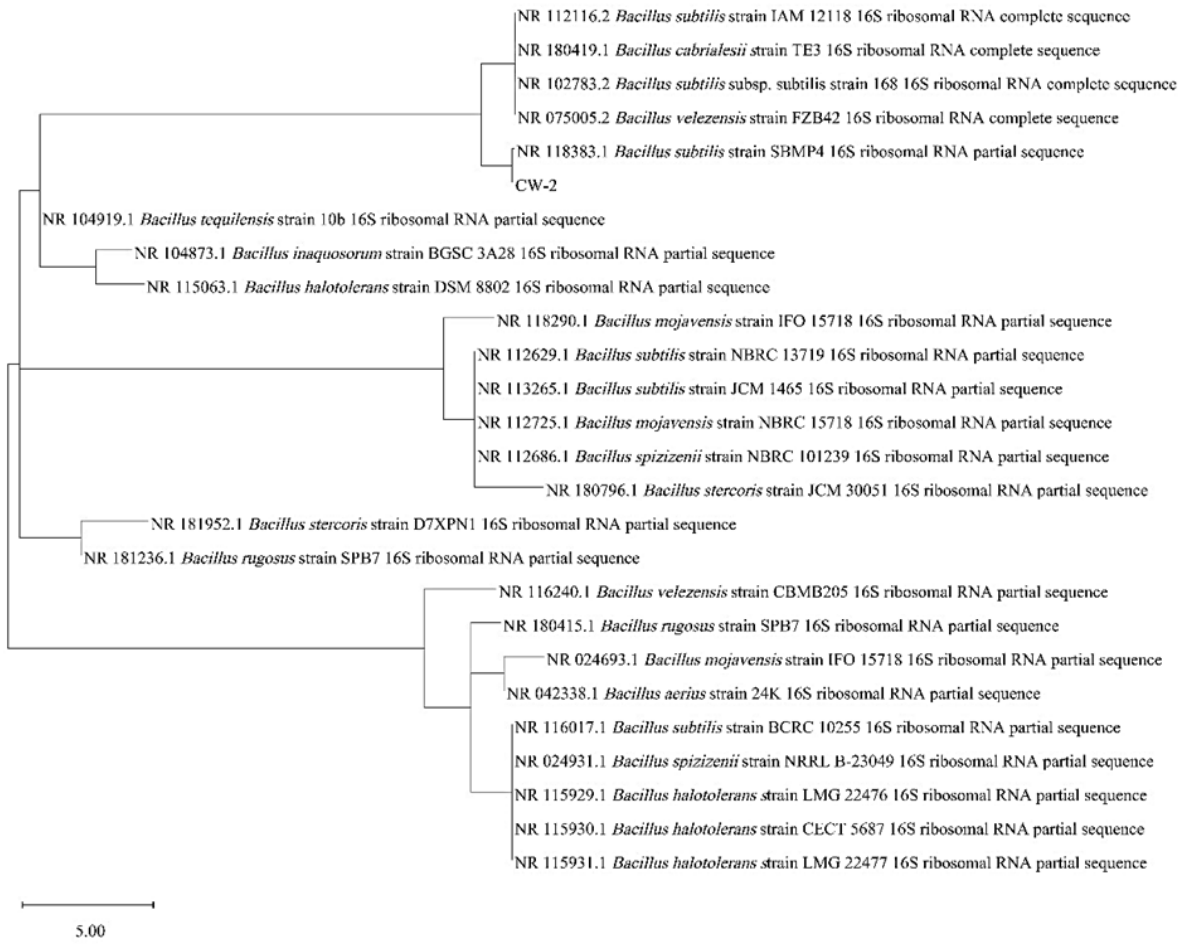

Figure 2 The phylogenic tree of *Bacillus subtilis* (CW-2) based on 16S rRNA gene sequences.

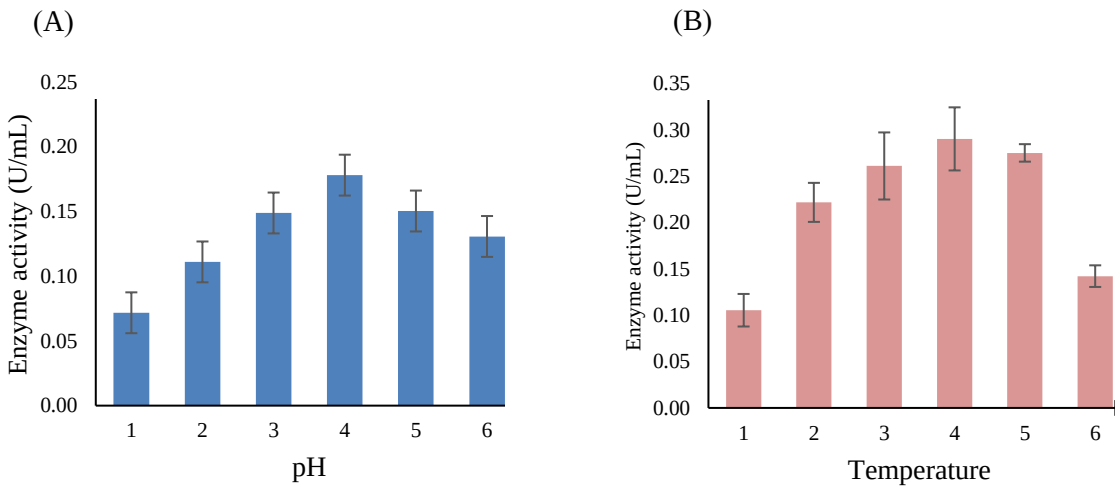

Figure 3 Effect of different physiological parameters on protease activity (A) pH; (B) Temperature; (C) Incubation time and (D) Carbon source.

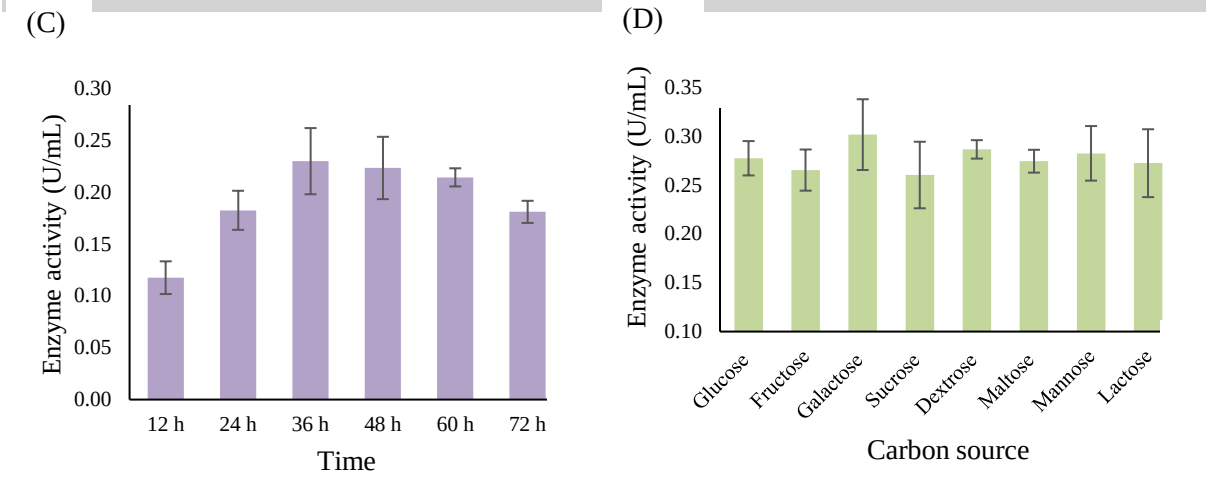

Figure 3 (CONT.) Effect of different physiological parameters on protease activity (A) pH; (B) Temperature; (C) Incubation time and (D) Carbon source.

3.5 Purification and enzyme assay

After optimization of physical and chemical parameters, mass production of the enzyme was carried out in shaker incubator at pH 7, 35°C, using galactose as the carbon source, with an incubation period of 36 hours. Absorbance values from the protease assay of the purified enzyme at different concentrations were compared to an L-tyrosine standard curve to correlate absorbance changes with the amount of tyrosine in micromoles released (Figure 4). Sigma's commercial protease exhibited an activity of 0.566 U/mL, while the enzyme from the native isolate CW-2 (*Bacillus subtilis*) demonstrated an activity of 0.379 U/mL - comparable to the commercial enzyme (Table 4). These findings suggest that the native enzyme has the potential to serve as a viable substitute for commercial proteases in various industrial sectors.

Table 4 Comparative assay of commercial and isolated bacterial enzyme.

Enzyme	Absorbance at OD_{600} for different concentrations of enzyme			Enzyme activity (U/mL)
	10 µg/mL	20 µg/mL	30 µg/mL	
Commercial enzyme	0.12	0.36	0.53	0.566
Bacillus sp. enzyme	-	0.16	0.42	0.379

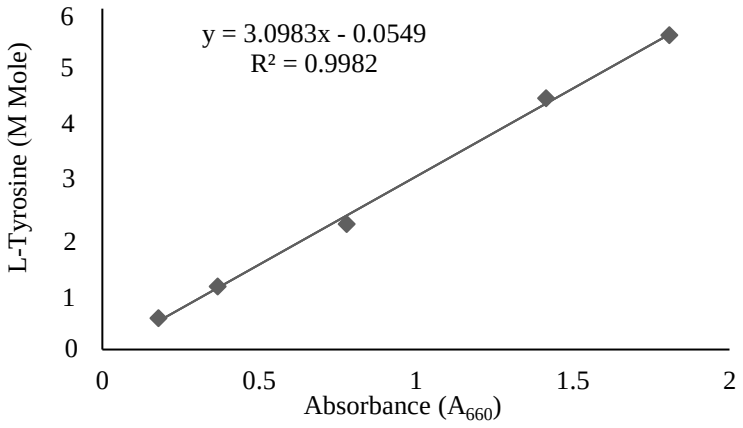

Figure 4 L-tyrosine standard curve.

4. Discussions

Microorganisms account for approximately two-thirds of the global commercial protease market [1]. Multiple studies have documented that the accumulation of organic compounds and high productivity contribute to significant bacterial diversity in the dynamic ecosystems of coastal regions. Fungi and bacteria found in coastal and marine environments are among the primary sources of microbial proteases [4,5]. In this context, the present

study aimed to isolate exogenous protease-producing bacteria from the indigenous coastal environment of Bangladesh and to optimize protease enzyme production from the selected strains. A total of 15 isolates were identified, of which five strains - four from coastal water and one from coastal soil - were determined to be non-pathogenic protease producers. All five isolates tested positive in the gelatinase assay, indicating their proteolytic activity. The biochemical test results of the isolates were compared with previously published data and are presented in Table 5. MALDI-TOF MS was used to identify all five isolates as belonging to the genera *Bacillus, Xenorhabdus*, and *Enterobacter*. These identifications were subsequently confirmed as *Bacillus subtilis, Xenorhabdus nematophilia*, and *Enterobacter cloacae* through DNA sequencing and phylogenetic analysis. Of these three species, *Bacillus subtilis* was found to be the most effective proteolytic bacterium.

Table 5 Comparison of biochemical characteristics of *Bacillus subtilis* (CW-2).

Name of the biochemical tests	Our Findings	Reference study[15]	Reference study[16]	Reference study[17]	Reference study[18]	Reference study[19]
Gram Staining	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	Not specified	+
Methyl Red (MR)	–	–	–	–	+	–
Voges Proskauer (VP)	+	+	+	+	+	+
Gelatinase	+	+	Not specified	+	+	+
Starch hydrolysis	+	+	+	+	+	+
Urease	–	–	–	–	–	–
Citrate agar	+	Not specified	Not specified	+	+	+
Hemolysis Test	Nonpathogenic	Nonpathogenic	Nonpathogenic	Nonpathogenic	Nonpathogenic	Nonpathogenic

Previous studies have demonstrated the widespread and effective use of bacterial isolates from *Bacillus* species for the production of extracellular proteases [5,14]. Various species of *Bacillus* bacteria - specifically *Bacillus pseudofirmus*, *B. proteolyticus*, *B. aquimaris*, *B. subtilis*, and *B. cereus* - have been recognized as producers of different types of proteases, including alkaline, neutral, and acidic proteases [6,7,20,21]. In another study, *B. subtilis* isolated from seawater was identified as a potential protease producer [5]. In coastal mudflats, Liu et al. (2023) identified predominant cultivated bacterial genera, including *Bacillus*, *Halobacillus*, and *Cytobacillus*, as significant producers of protease enzymes [22].

Among the different types of microbial proteases, those originating from *Bacillus* species are of particular importance, as they are recognized for their ability to generate efficient enzymes suitable for industrial uses [1]. Consistent with previous studies, the potential for extracellular enzyme production by *B. subtilis* was confirmed in this study, suggesting its applicability in various commercial sectors. Another isolate, *X. nematophilia*, is known to be pathogenic to nematodes; however, no pathogenicity has been reported in fish or humans. *Xenorhabdus* has been identified as a valuable source of protease, lipase and various bioactive compounds [23]. *Enterobacter cloacae* was also described as an effective protease producer in a study by Fajingbesi et al. (2018) [24].

The production of microbial protease enzymes is strongly influenced by culture conditions. Enzyme activity and the transport of compounds across the cell membrane are significantly affected by the pH and temperature of the culture medium. In this study, the highest activity of the crude enzyme was observed at pH 7 (0.18 ± 0.03 U/mL). According to Rupali (2015), protease obtained from *Bacillus* sp. exhibited its highest level of activity at a pH of 7.4 [25]. Similarly, Sahin et al. (2020) reported that *B. subtilis* achieved peak protease production at pH 7.5 [26]. Another study revealed that pH 7.5, within a broader range of 6-9, is ideal for protease synthesis under neutral or alkaline conditions, further validating these findings [27]. Most *Bacillus* species exhibit optimal protease synthesis within a pH range of 7.0-11.0 [28]. Additionally, in our study, crude protease enzyme activity measured at OD_{750} was highest (0.29 ± 0.03 U/mL) at 35°C. Patel et al. (2019) similarly reported that 35-40°C is the optimum temperature range for protease production by *Bacillus* sp. [27]. Several other studies have identified 37°C as the most suitable temperature for protease production by *Bacillus* sp. [15,25,26]. Some studies have also documented protease production under extreme conditions, including temperatures as high as 60ºC [31].

This study found that a 36-hour incubation period was the most effective for protease production, which is consistent with the findings of Pant et al. (2015) [29]. In contrast, another study reported that a 48-h incubation period yielded the highest level of protease production [25]. In a separate investigation, *B. subtilis* exhibited peak

protease production at both 24h and 48h [30]. Similarly, Sahin et al. (2020) observed that the highest level of protease production from *B. subtilis* occurred after 24h of fermentation [26]. These findings suggest that the optimal incubation time for maximum protease production typically ranges from 24 to 48 hours. In the present study, media containing galactose as the carbon source resulted in the highest enzymatic activity. Pant et al. (2015) also reported that the addition of galactose in the medium enhanced enzyme synthesis by 0.5% in *B. subtilis* compared to other carbon sources [29], supporting the current findings. Effective utilization of galactose for improved protease productivity in *B. subtilis* was also noted by Choi et al. (2024) [32]. However, other studies have identified glucose, xylose, and fructose as effective carbon sources for protease-producing bacteria [21,33]. According to Padmapriya et al. (2012), starch had the greatest influence on protease production by *B. subtilis* [19]. Variations in the optimal carbon source for protease production can depend on several factors, including the specific bacterial strain, culture conditions, and experimental design. Moreover, the majority of *B. subtilis* strains are considered highly metabolically versatile, given their capacity to utilize a wide range of carbon sources [34].

After partial purification, the activity of the enzyme produced under optimized conditions was found to be 0.37 U/mL, whereas the activity of the commercial Sigma enzyme was 0.56 U/mL. The protease production range observed by Deviga et al. (2022) in *B. subtilis* was between 0.42 U/mL and 0.74 U/mL [30], which is consistent with the findings of this study. Improved purification techniques, such as chromatography and electrophoresis, may further enhance the activity of purified enzymes. In one study, the activity of partially purified enzymes was reported to be between 35 and 38 U/mg [26]. Another investigation recorded a maximum enzyme activity of 20.74 U/mL for purified protease from *B. subtilis* [2]. The increased activity observed in partially purified protease, compared to directly extracted enzymes, can be attributed to concentration effects and the specific conditions employed during the purification process [35].

5. Conclusions

The coastal water and soil of Bangladesh were found to harbor protease-producing bacteria. Proteases are industrially important enzymes that enhance protein digestibility in food and feed processing, while also demonstrating broad utility in textile, pharmaceutical, and therapeutic applications. The isolate *B. subtilis* (CW-2) exhibited the highest protease activity, comparable to that of a commercial enzyme, when conditions were optimized to 35ºC for 36h at pH 7.0, using galactose as the carbon source. These locally isolated proteolytic cultures can be directly utilized for the cost-effective and rapid production of protease enzymes, thereby streamlining subsequent processing. Future studies might focus on whole-genome sequencing of the unique strain, as well as genetic and metabolic optimization, and the exploration of other native isolates to enhance the applicability of these microbial enzymes.

6. Acknowledgements

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7. Conflicts of interest

The authors declare that they have no conflict of interest.

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