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Protease Producing Bacteria and Activity in Gut of Tiger Shrimp (*Penaeus monodon*)

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ABSTRACT

Proteases, the group of enzyme with significant commercial value, was isolated from proteolytic bacteria available in gut of tiger shrimp (*Penaeus monodon*). The isolation of bacteria from the gut of the collected shrimp was performed by serial dilution and plating method. Six bacterial isolates were screened out on the basis of their formation of zone of casein hydrolysis on skim milk agar. Among the six isolates, three isolates (S₁, S₃ and S₅) were found gram positive and the rest three isolates (S₂, S₄ and S₆) were found gram negative. Protease activity of the isolates was determined both qualitatively and quantitatively. In qualitative plate assay, isolate S₁ exhibited the largest clear zone (30±1.13 mm) in skim milk agar and isolate S₅ exhibited the lowest (18±1.41 mm). Quantitative protease assay was performed by using azo-casein as substrate. Protease activity of isolates S₁, S₂, S₃, S₄, S₅ and S₆ were found 49.75±2.13, 60.50±1.97, 66.25±2.41, 56.25±1.69, 59.25±1.32 and 52.75±2.21 U mL⁻¹, respectively following incubation at 37°C in aerobic condition for 24 h. The effect of pH and NaCl concentration on the growth and protease production of the isolates were also studied by assaying protease activity at different pH range and NaCl concentrations. The isolates exhibited maximum protease production at varying pH and NaCl concentrations. The data showed potentiality of the bacterial isolates to be a useful source of industrial protease. Finally, the isolates were also tested against six standard antibiotics (Tetracycline, Nitrofurantoin, Erythromycin, Penicillin, Ciprofloxacin and Doxycycline) to observe their antibiotic sensitivity and the isolates S₅ and S₆ were found resistant to all of the six antibiotics and isolates S₃ and S₄ were found sensitive to all of the antibiotics.

Key words: Proteolytic bacteria, proteases enzyme, *Penaeus monodon*, antibiotics, pH, NaCl, qualitative and quantitative protease assay

INTRODUCTION

Enzymes are considered as a vital resource utilized by the food, chemical and allied industries to produce a wide range of biotechnology products and have already been recognized as valuable catalysts for various organic transformations and production of fine chemicals and pharmaceuticals (Gupta *et al.*, 2002a). The estimated values of the worldwide sales of industrial enzymes were

\$220 billion (Rao *et al.*, 2009). The worldwide increasing demand for enzymes due to their high-performance, environment benefits and their use in food industry for fermentation, food processing, amino-acids and specialty foods and rapid increase in world energy prices which has made enzyme related processing and product more cost effective. In case of fisheries sector it is used as fish skin removal, scale removal, roe production, membrane removal, seafood flavorings and pigments extraction. In Bangladesh this scenario is different as Bangladesh is basically a textile manufacturing country. Enzyme consumption in pharmaceuticals industry is negligible when compared to the consumption in textile industry. Bangladesh does not have any enzyme manufacturing company. It only has some formulators who use imported concentrated enzymes. Hence Bangladesh enzyme demand is being covered extensively from import. The major enzyme exporter countries include Singapore, Sri Lanka, China, Malaysia, Germany and Canada Japan and Finland. According to Bangladesh Bank the country has imported 16.3 million USD from July 2011 to May 2012 (Amin, 2012). The major enzymes that are utilized by the industries are lipases, proteases, amylase, xylanases, cellulases, pectinases and pullulanases etc. Proteolytic enzymes are found in all living organisms, playing an essential role in cell growth and differentiation. The extracellular proteases have commercial value and have been applied in multiple processes in various industrial sectors. It is one of the third largest groups of industrial enzymes and it accounts for about 60% of total worldwide sale in the market. Microorganisms are the most desired sources for enzyme production because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Kocher and Mishra, 2009). In general, microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals (Gupta *et al.*, 2002b). Based on their acid-base behavior, proteases are classified into three groups, such as acid, neutral and alkaline proteases. Alkaline (serine) proteases are active over a broad pH (7-12) and temperature (35-80°C) ranges, they are worldwide center of attraction for researchers. Alkaline proteases hold a major share of the enzyme market with two third shares in detergent industry alone (Anwar and Saleemuddin, 2000). The important protease producing bacteria are species of *Bacillus*, *Pseudomonas*, *Halomonas*, *Arthrobacter* and *Serratia* (Donaghy and Mackay, 1993). Alkaline proteases in the gut of fish have received much attention in recent years (Chong *et al.*, 2002; Fu *et al.*, 2005). If we can find out potential enzyme producing strains of microorganisms it will be helpful for our industry as well as for the economy. This study was therefore conducted to find out an easy and cost-effective method of isolating this important protease enzyme producing bacteria from the shrimp (*Penaeus monodon*) gut and analysis of the protease activity both qualitatively and quantitatively. The study was also focused on studying the effect of two important parameters (e.g., pH and NaCl concentration) on protease activity.

MATERIALS AND METHODS

Collection of shrimp: The shrimp sample was collected from the fish market (Kawran Bazar, Dhaka). Sample was kept in sterilized plastic bag aseptically. Samples washed with sterile Phosphate Buffer Saline (PBS) to remove sand, detritus as well as microorganisms attached to the surface of shrimp. Then the gut sample was collected aseptically from the shrimp and homogenized with ringer stock solution using Stomacher.

Isolation of bacteria from the gut of shrimp: Serial dilution (10^{-1} - 10^{-6}) of shrimp gut sample was performed by using sterile normal saline. The dilutions were plated on nutrient agar medium using spread plate method and incubated at 37°C for 24 h.

Screening of protease producing bacteria: The random individual colonies were picked separately from nutrient agar medium. The purified isolates were tested for activity using skim milk agar (peptone: 5 g L⁻¹, beef extract: 3 g L⁻¹, skim milk: 5 g L⁻¹ and agar: 15 g L⁻¹). A single colony of each strain was streaked on skim milk agar and incubated at 37°C for 24 h. Positive zone of clearance was observed due to hydrolysis casein. Among the isolates six bacterial strains were formed clear zone around the colonies.

Purification of the isolates: Six isolated organisms were purified through repeated subculture method. Streak plate methods were used for this purpose. Skim milk agar was used as media. When a plate yielded only one type of colony, the organisms were considered to be pure.

Presumptive identification of the bacterial isolates: Microscopic observation, different morphological and biochemical characteristics accompanied with colony characteristics were observed for the presumptive identification of bacterial isolates.

Enzyme extraction: Firstly isolates inoculated into nutrient broth media and incubated at 37°C and 120 rpm for 48 h in a shaking incubator. After 48 h 1 mL culture media were withdrawn in eppendorf tubes and subjected to centrifugation at 10,000 rpm for 10 min. Then the supernatant were collected and transferred to another tube using syringe filtration method. The prepared cell free extract can be used as enzyme source for protease assay.

Assay of proteolytic activity: Protease assay for all six isolates were performed both qualitatively and quantitatively. Qualitative analysis of protease enzyme was performed by agar well diffusion method. For quantitative analysis of enzyme activity azo-casein was used as a substrate.

Qualitative plate assay: Freshly prepared skim milk agar plates were punched aseptically using sterile cork borer to obtain wells. The wells were loaded with 75 µL of enzyme extract and kept in incubator at 37°C for 24 h. After 24 h of incubation clear zone were measured to determine the protease activity.

Protease assay: Protease activity of supernatant was determined with azo-casein (Sigma Co. St. Louis, Mo.) as substrate by a modified procedure described by Kreger and Lockwood (1981). The 400 µL of supernatant were taken in eppendorf tube. Then 1% azo-casein in 0.05 M tris HCl (pH 8.5) was mixed with supernatant. The mixture was incubated in water bath incubator at 37°C for 1 h. After incubated the mixture 135 µL of 35% TCA were added in a mixture to stop the reaction. Then the mixture was kept in ice for 15 min. The mixture was centrifuged at 13000 for 10 min. After centrifuged 750 µL of supernatant were collected and added 750 µL of 1 N NaOH solution in the supernatant with mixed well. Then the absorbance was read at 440 nm. The blank was prepared in the same manner except that 135 µL of 35% TCA was added before addition of azo-casein. One unit of protease activity is defined as the amount of enzyme that produces an increase in the absorbance of 0.01 under the above assay condition.

Effect of NaCl concentration on protease activity: Different concentrations (1.5-3.5%) of NaCl were added in a nutrient broth media. Then isolated organisms were inoculated in nutrient broth media. After that inoculating organism were kept in a shaking incubator for 24 h, 120 rpm at 37°C. After 24 h 1 mL culture media were taken in eppendorf tubes for centrifugation. The eppendorf tubes were centrifuged at 10,000 rpm for 10 min. Then supernatant (enzyme) were separated from bacterial cell. Then protease activity of supernatant was determined with azo-casein (Sigma Co. St. Louis. Mo.) as substrate by a modified procedure described by Kreger and Lockwood (1981).

Effect of pH on protease activity: Different concentrations (pH 4-9) of pH were used in a nutrient broth media. Electronic pH meter, highly concentrated H₂SO₄ and KOH were used for adjusting several pH ranges in a nutrient broth media. Then isolated organisms were inoculated in nutrient broth media. A “control” nutrient broth media were also kept in shaking incubator. After that inoculating organism were kept in a shaking incubator for 24 h, 120 rpm at 37°C. After 24 h 1 mL culture media were taken in eppendorf tubes for centrifugation. The eppendorf tubes were centrifuged at 10,000 rpm for 10 min. Then supernatant (enzyme) were separated from bacterial cell. Then protease activity of supernatant was determined with azo-casein (Sigma Co. St. Louis. Mo.) as substrate by a modified procedure described by Kreger and Lockwood (1981).

Antibiogram: Antibiotics that are commonly used during shrimp cultivation were selected for this study. Antibiotic sensitivity of the isolates was measured *in vitro* by employing the modified Kirby-Bauer method (Bauer *et al.*, 1966). The plates were examined and the diameters of the zone of complete inhibition were measured in mm. The zone of diameters for individual antimicrobial agents was translated into susceptible, intermediate and resistant categories by referring to an interpretation table (Barry *et al.*, 1985).

RESULTS

The study was performed to isolate, characterize and optimization of growth and enzymatic activity of proteolytic bacteria isolated from the gut of tiger shrimp (*Penaeus monodon*). Enrichment of samples, selective plating, microscopy, biochemical tests and different enzymatic assay was applied for this purpose.

Screening of protease producing bacteria: Six isolates were selected as protease producing bacteria due to their growth on skim milk agar plate showing zone of casein hydrolysis (Fig. 1). The isolates were hereby referred as S₁, S₂, S₃, S₄, S₅ and S₆.

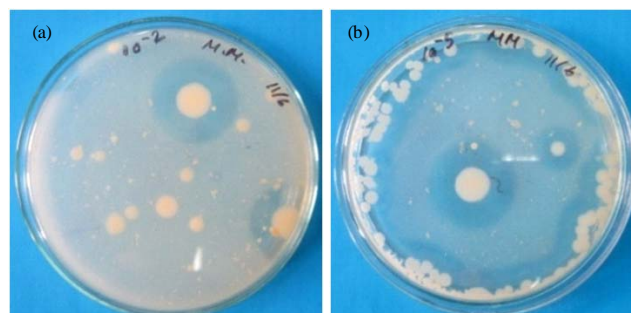


Fig. 1(a-b): Dilution plates of the samples

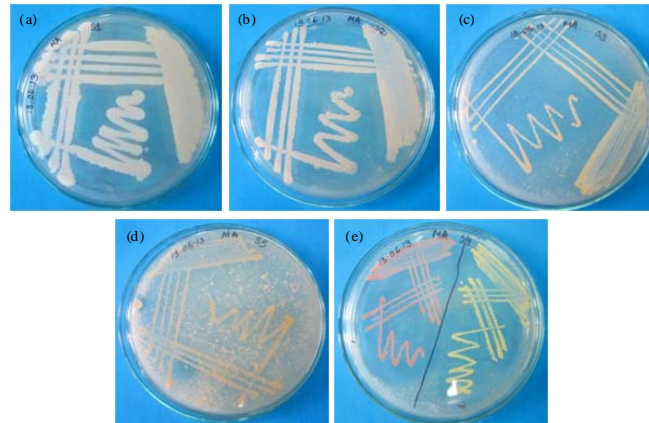


Fig. 2(a-e): Bacterial colonies on agar plates, (a) S₁ bacteria, (b) S₂ bacteria, (c) S₃ bacteria, (d) S₅ bacteria, (e) S₄ (Orange color) and S₆ (Yellow color) bacteria

Table 1: Colony characteristics of isolates bacteria on skim milk agar plates

Characteristic	Isolates					
	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆
Color	White	White	Off-white	Orange	Yellow	Yellow
Pigmentation	No	No	No	Orange	Yellow	Yellow
Size	Large	Medium	Small	Pinpoint	Medium	Pinpoint
Appearance	Shinny	Dull	Shinny	Shinny	Shinny	Shinny
Optical property	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Form	Circular	Circular	Circular	Circular	Irregular	Circular
Elevation	Flat	Umbonate	Convex	Convex	Raised	Convex
Margin	Undulate	Entire	Entire	Entire	Undulate	Entire
Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth

Table 2: Morphological characteristics of the isolates

Isolates	Size	Shape	Arrangement	Color	Gram-reaction
S ₁	Short	Rod	Chain forming	Purple	+ve
S ₂	Short	Rod	Chain/Diploid	Pink	-ve
S ₃	Short	Cocci	Cluster forming	Purple	+ve
S ₄	Short	Rod	Single	Pink	-ve
S ₅	Short	Cocci	Cluster forming	Purple	+ve
S ₆	Short	Rod	Free forming	Pink	-ve

Purification of the isolates: The isolates were streaked on skim milk agar. Following overnight incubation at 37°C, bacterial colonies were found on the agar plates and zone of casein hydrolysis also observed. Colony characteristics of the bacterial isolates are summarized in Table 1. Most of these isolates were white (Fig. 2a-c) and yellow (Fig. 2d and e), in color, circular form (Fig. 2a-c and e) and shinny appearance with opaque optical property (Fig. 2a and c-e). The texture of all these isolates was smooth and was convex elevated.

Presumptive identification of the bacterial isolates: Following gram staining procedure all of the six isolates were observed under microscope. The Fig. 3a-f showed the morphological characteristics of the isolates. In Table 2 morphological characteristics of the isolates are summarized. Most of these isolates were short size, rod shape and cluster forming arrangement. Three isolates such as S₂, S₄ and S₆ (Fig. 3b, d and f), could not retain the stain of

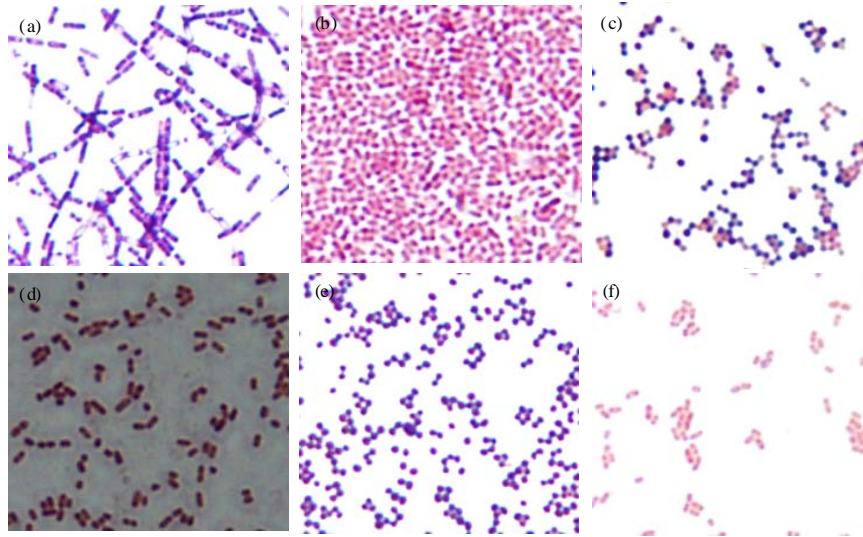


Fig. 3(a-f): Macroscopic view of six isolates, (a) S₁ bacteria, (b) S₂ bacteria, (c) S₃ bacteria, (d) S₄ bacteria, (e) S₅ bacteria and (f) S₆ bacteria

Table 3: Biochemical characteristics of the isolates

Characteristic Test	Isolates					
	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆
Oxidase	-ve	-ve	-ve	-ve	-ve	-ve
Catalase	-ve	-ve	-ve	-ve	-ve	-ve
KIA	S-red B-yellow	S-yellow B-yellow	S-red B-yellow	S-yellow B-yellow	S-yellow B-yellow	S-yellow B-yellow
Indole	-ve	+ve	-ve	-ve	-ve	-ve
MR	-ve	-ve	-ve	-ve	-ve	-ve
VP	-ve	-ve	-ve	-ve	-ve	-ve
Citrate	-ve	-ve	-ve	-ve	-ve	-ve
MIU	-ve	-ve	-ve	-ve	-ve	+ve
Lactose						
Dextrose	+ve	+ve	+ve	-ve	+ve	+ve
Sucrose	+ve	+ve	+ve	-ve	+ve	+ve
Gelatin hydrolysis	-ve	-ve	-ve	-ve	-ve	-ve
LIA	S-purple B-yellow	S-purple B-yellow	S-purple B-yellow	S-purple B-yellow	S-purple B-yellow	S-purple B-yellow
Starch hydrolysis	+ve	-ve	-ve	+ve	-ve	-ve

KIA: Kligler's iron agar, MR: Methyl red, VP: Voges-proskauer; MIU: Motility indole urease, LIA: Lysine iron agar, S: Slant, B: Butt

crystal violet because of the thin peptidoglycan layer in the periplasm of these isolates. Isolates S₁, S₃ and S₅ were gram positive (Fig. 3a, c and e).

In biochemical test of the isolates (Table 3), were unable to produced free oxygen as gas bubbles in catalase reaction and showed oxidase negative reaction by forming black color. S₁ and S₃ were alkaline slant (red) and acid butt (yellow) without gas production indicated that only glucose was used. S₂, S₄, S₅ and S₆ acid slant and acid butt indicated that lactose fermentation was occurred. Only S₂ isolate was indole positive. Six isolates were showed MR, VP and citrate negative reaction. Only of S₆ these isolates was motile. S₁ and S₃ of these isolates showed lactose positive. Six isolates were showed dextrose and sucrose fermentation. Six isolates were unable to gelatin hydrolysis. Six isolates were showed yellow butt means negative decarboxylation and purple slant means negative deamination. Only S₁ and S₄ of these isolates were able to hydrolysis of starch.

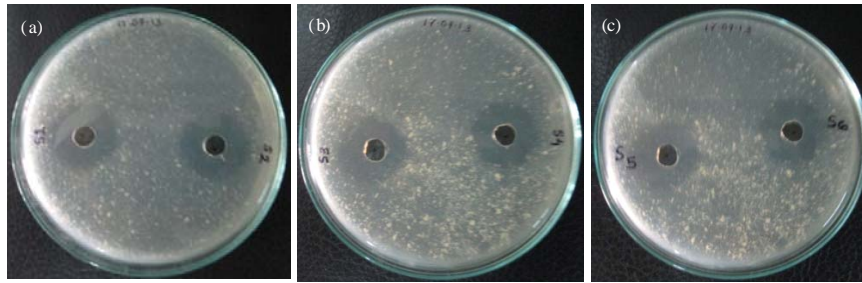


Fig. 4(a-c): Clear zone of protease activity, (a) Isolates S₁ and S₂, (b) Isolates S₃ and S₄, (c) Isolates S₅ and S₆

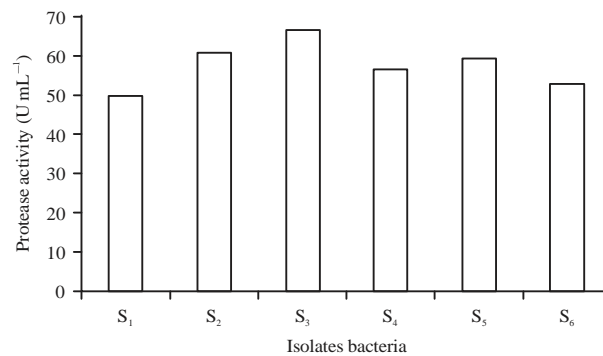


Fig. 5: Protease activity of isolates bacteria

Table 4: Results of plate assay

Isolates	Zone (including wells diameter) (mm)
S ₁	30±1.13
S ₂	29±1.21
S ₃	25±1.09
S ₄	23±0.09
S ₅	18±1.41
S ₆	29±0.08

Qualitative plate assay: Result of plate assay was estimated for 24 h (Table 4). Clear zone (Fig. 4a-c), was measured in diameter (mm) to get protease activity. S₁ of these isolates was showed highest clear zone 30±1.13 mm (Fig. 4a) and S₅ showed lowest clear zone 18±1.41 mm (Fig. 4c).

1% Azo-casein substrate assay: Proteolytic activity was observed by 1% azo-casein assay. Enzyme stability was also observed for different pH and NaCl concentration. Absorbance was measured at 440 nm in Spectrophotometer.

Enzyme assay of isolates: Protease activity of isolates bacteria fluctuate without treatment of NaCl (Fig. 5). Isolate S₃ was showed highest protease activity (66.25±2.41 U mL⁻¹) and S₁ showed lowest protease activity (49.75±2.13 U mL⁻¹).

Effect of NaCl concentration on protease activity of isolated bacteria: Protease activity of isolates bacteria fluctuate with various NaCl concentrations (Fig. 6). S₁ showed highest protease

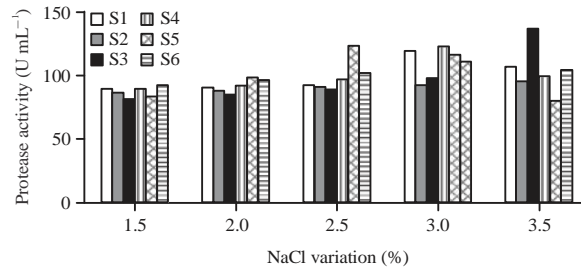


Fig. 6: Effect of NaCl on protease activity

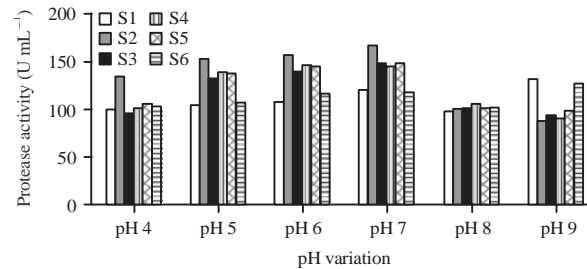


Fig. 7: Effect of pH on protease activity

activity ($121 \pm 1.21 \text{ U mL}^{-1}$) at 3% NaCl and lowest protease activity ($89.50 \pm 1.27 \text{ U mL}^{-1}$) at 1.5% NaCl condition. S_2 showed highest protease activity ($97 \pm 2.19 \text{ U mL}^{-1}$) at 3.5% NaCl and lowest protease activity ($87.50 \pm 2.34 \text{ U mL}^{-1}$) at 1.5% NaCl condition. S_3 showed highest protease activity ($137 \pm 2.61 \text{ U mL}^{-1}$) at 3.5% NaCl and lowest protease activity ($82.25 \pm 2.49 \text{ U mL}^{-1}$) at 1.5% NaCl condition. S_4 showed highest protease activity ($123.5 \pm 1.89 \text{ U mL}^{-1}$) at 3% NaCl and lowest protease activity ($90.25 \pm 1.67 \text{ U mL}^{-1}$) at 1.5% NaCl condition. S_5 showed highest protease activity ($123.5 \pm 2.33 \text{ U mL}^{-1}$) at 2.5% NaCl and lowest protease activity ($83.75 \pm 2.11 \text{ U mL}^{-1}$) at 1.5% NaCl condition. S_6 showed highest protease activity ($112.25 \pm 1.64 \text{ U mL}^{-1}$) at 3% NaCl and lowest protease activity ($93.5 \pm 2.31 \text{ U mL}^{-1}$) at 1.5% NaCl condition.

Effect of pH on protease activity of isolated bacteria: Protease activity of isolates bacteria fluctuate with various pH values (Fig. 7). S_1 showed highest protease activity ($132 \pm 1.82 \text{ U mL}^{-1}$) at pH 9 and lowest protease activity ($100.75 \pm 2.08 \text{ U mL}^{-1}$) at pH 4 condition. S_2 showed highest protease activity ($166.75 \pm 2.37 \text{ U mL}^{-1}$) at pH 7 and lowest protease activity ($87.50 \pm 2.02 \text{ U mL}^{-1}$) at pH 9 condition. S_3 showed highest protease activity ($148.75 \pm 1.81 \text{ U mL}^{-1}$) at pH 7 and lowest protease activity ($94.25 \pm 2.39 \text{ U mL}^{-1}$) at pH 9 condition. S_4 showed highest protease activity ($146.75 \pm 1.63 \text{ U mL}^{-1}$) at pH 6 and lowest protease activity ($90.25 \pm 1.89 \text{ U mL}^{-1}$) at pH 9 condition. S_5 showed highest protease activity ($147.75 \pm 2.91 \text{ U mL}^{-1}$) at pH 7 and lowest protease activity ($98 \pm 2.45 \text{ U mL}^{-1}$) at pH 9 condition. S_6 showed highest protease activity ($127.50 \pm 2.59 \text{ U mL}^{-1}$) at pH 9 and lowest protease activity ($102.25 \pm 1.96 \text{ U mL}^{-1}$) at pH 8 condition.

Antibiotic sensitivity patterns of the isolates: Antibiotic sensitivity against 6 standard antibiotics was performed for each isolates. Antibiotic sensitivity pattern are compiled in Table 5. S_1 and S_2 of these isolates showed both resistance and sensitive pattern. S_3 and S_4 of these isolates were showed sensitive all of the antibiotics. On the other hand S_5 and S_6 of these isolates showed resistance all of the antibiotics.

Table 5: Antibiotic sensitivity pattern of the isolates

Antibiotics	Isolates					
	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆
Tetracycline 30	R	S	S	S	R	R
Nitrofurantoin 300	R	S	S	S	R	R
Erythromycin 15	S	R	S	S	R	R
Penicillin G 10	R	S	S	S	R	R
Ciprofloxacin 5	S	R	S	S	R	R
Doxycycline 30	S	R	S	S	R	R

S: Sensitive and R: Resistance

DISCUSSION

In this study we evaluated protease producing ability of the isolates proteolytic bacteria from the shrimp gut. Proteolytic enzyme producers are helpful for the health of the ecosystems of this earth as these microbes decompose the dead and decaying animal or plant tissues in water or land. They can create pollution free environment and they are responsible for the recycling of nutrients (Gupta *et al.*, 2008). Shrimp feeds contain a high amount of protein and proteolytic bacteria are likely to be the bacterial groups that will govern the quality of water and sediment in shrimp ponds and have a major influence on shrimp health. Proteolytic bacteria in shrimp cultivation play a major role by improving water quality and controlling shrimp diseases (Rattanachauy *et al.*, 2007). Moreover many researchers reported that the *Pseudomonas* sp. is normal flora in shrimp ponds (Chythanya *et al.*, 2002). The intestinal tract of shrimp is a complex ecosystem that harbors a diverse bacterial community. This bacterial population of the gut confers many benefits to the intestinal physiology of the host. They exhibit a very important and diversified enzymatic potential. These bacteria are capable of producing proteolytic, amylolytic, cellulolytic, lipolytic and chitinolytic enzymes which is important for digestion of proteins, carbohydrates, cellulose, lipids and chitin (Bairagi *et al.*, 2002; Gutowska *et al.*, 2004). By a microscopic observation S₁, S₃ and S₅ was found gram positive and S₂, S₄ and S₆ gram negative. In qualitative plate assay protease from isolate S₁ showed highest activity (30±1.13 mm) and S₅ showed lowest activity (18±1.41 mm) on the skim milk agar plates. In quantitative assay protease activity of isolates S₁, S₂, S₃, S₄, S₅ and S₆ were showed 49.75±2.13, 60.50±1.97, 66.25±2.41, 56.25±1.69, 59.25±1.32 and 52.75±2.21 U mL⁻¹, respectively at 37°C in aerobic condition for 24 h. The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. The protease activities of the isolates were also affected by the pH. Increase in pH resulted in corresponding increase in protease production. The supplementation of sodium chloride was used as inducers for protease secretion. Increased the concentration of NaCl increased the protease production. It was earlier established that bacteria utilizes the sodium-driven solute transport systems for their survival and adaptation in high pH environments Isolate S₁ showed maximum protease activity (121±1.21 U mL⁻¹) on 3% NaCl concentration and maximum protease activity (132±1.82 U mL⁻¹) on pH 9. *Pseudomonas aeruginosa* showed that higher growth and maximum protease activity 344 U mL⁻¹ was observed at 3% NaCl (Kumar *et al.*, 2011). Isolate S₂ showed maximum protease activity (97±2.19 U mL⁻¹) on 3.5% NaCl concentration and maximum protease activity (166.75±2.37 U mL) on pH 7. Isolate S₃ showed maximum protease activity (137±2.61 U mL⁻¹) on 3.5% NaCl concentration and maximum protease activity (148.75±1.81 U mL⁻¹) on pH 7. Isolate S₄ showed maximum protease activity (123.5±1.89 U mL⁻¹) on 3% NaCl concentration and protease

activity (146.75 ± 1.63 U mL⁻¹) on pH 6. Isolate S₅ showed protease activity (123.5 ± 2.33 U mL⁻¹) on 2.5% NaCl concentration and protease activity (147.75 ± 2.91 U mL⁻¹) on pH 7. Isolate S₆ showed maximum protease activity (112.25 ± 1.64 U mL⁻¹) on 3% NaCl concentration and protease activity (127.50 ± 2.59 U mL⁻¹) on pH 9. *Pseudomonas aeruginosa* showed that higher growth and maximum protease activity 215 U mL⁻¹ was observed at pH 9 (Kumar *et al.*, 2011). Protease production was at maximum at pH 7 and 9 for *Bacillus* sp. and *Pseudomonas* sp. Optimum protease activity of 34 U mL⁻¹ for *Geobacillus* sp. (Amara *et al.*, 2009). Maximum protease production from the genus *Bacillus* was at pH 8 while at pH 10, the protease production was about 60% (Mahendran *et al.*, 2010). Most of the isolates were showed lowest protease activity at pH 4 and 1.5% NaCl condition. The lowest conditions for protease production were studied at pH 4 and 1.5% concentration (Kumar *et al.*, 2011). It was also reported that the growth of *Bacillus* sp. is regulated by sodium gradient (Bhunia *et al.*, 2012). Most of the isolates were showed highest protease activity at 3% NaCl concentration and pH 6-9 condition. The optimum conditions for protease production were at 37°C, pH 9 and 3% NaCl concentration (Kalaiarasi and Sunitha, 2009; Porro *et al.*, 2003). Antibiotic sensitivity of isolate S₁ showed sensitive on the Erythromycin, Ciprofloxacin and Doxycycline, resistance against the Tetracycline, Nitrofurantoin and Penicillin. S₂ bacteria showed sensitive on the Tetracycline, Nitrofurantoin and Penicillin, resistance against the Erythromycin, Ciprofloxacin and Doxycycline. S₃ and S₄ bacteria showed sensitive on the all experimental antibiotics. S₅ and S₆ bacteria showed resistance against all the experimental antibiotics.

CONCLUSION

The findings in this study provide evidence for protease producing ability of the isolated bacteria that were found from the gut of tiger shrimp (*Penaeus monodon*). From the gut of tiger shrimp six types of protease producing bacteria were isolated on the basis of formation of clear zone in skim milk agar plates. All isolates showed appreciable protease activity on different NaCl and pH variation. Bangladesh import large amount of protease enzyme from foreign countries. Isolates bacteria will be used as a protease enzyme source in Bangladesh. Bangladesh is an industrial country whose industry needs large amount of protease enzyme every day. Isolates bacteria will be supportive for Bangladeshi industry. Protease activity of the isolated bacteria highly showed on 2.5-3.5% NaCl concentration and pH 6-9. Therefore, bacterial isolates has potential that could be commercially exploited to assist in protein degradation in various industrial processes in Bangladesh and decrease dependency on imported protease enzyme.

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REFERENCES

- Amara, A.A., S.R. Salem and M.S.A. Shabeb, 2009. The possibility to use bacterial protease and lipase as biodetergent. *Global J. Biotechnol. Biochem.*, 4: 104-114.
- Amin, A.S.M.T., 2012. Enzyme innovation and its market. *Bangladesh Textile Today*, 1: 6-10.
- Anwar, A. and M. Saleemuddin, 2000. Alkaline protease from *Spilosoma obliqua*: Potential applications in bio-formulations. *Biotechnol. Applied Biochem.*, 31: 85-89.

- Bairagi, A., K.S. Ghosh, S.K. Sen and A.K. Ray, 2002. Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquacult. Int.*, 10: 109-121.
- Barry, A.L., R.N. Jones, C. Thornsberry, L.W. Ayers and R. Kundargi, 1985. Imipenem (N-Formimidoyl Thienamycin): *In vitro* antimicrobial activity and β -lactamase stability. *Diagn. Microbiol. Infect. Dis.*, 3: 93-104.
- Bauer, A.W., W.M. Kirby, J.C. Sherris and M. Turck, 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.*, 45: 493-496.
- Bhunia, B., B. Basak and A. Dey, 2012. A review on production of serine alkaline protease by *Bacillus* spp. *J. Biochem. Technol.*, 3: 448-457.
- Chong, A.S.C., R. Hashim, L. Chow-Yang and A.B. Ali, 2002. Partial characterization and activities of proteases from the digestive tract of discus fish (*Symphysodon aequifasciata*). *Aquaculture*, 203: 321-333.
- Chythanya, R., I. Karunasagar and I. Karunasagar, 2002. Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* I-2 strain. *Aquaculture*, 208: 1-10.
- Donaghy, J.A. and A.M. McKay, 1993. Production and properties of an alkaline protease by *Aureobasidium pullulans*. *J. Applied Bacteriol.*, 74: 662-666.
- Fu, X.Y., C.H. Xue, B.C. Miao, Z.J. Li, X. Gao and W.G. Yang, 2005. Characterization of proteases from the digestive tract of sea cucumber (*Stichopus japonicus*): High alkaline protease activity. *Aquaculture*, 246: 321-329.
- Gupta, A., B. Joseph, A. Mani and G. Thomas, 2008. Biosynthesis and properties of an extracellular thermostable serine alkaline protease from *Virgibacillus pantothenicus*. *World J. Microbiol. Biotechnol.*, 24: 237-243.
- Gupta, R., Q. Beg and P. Lorenz, 2002. Bacterial alkaline proteases: Molecular approaches and industrial applications. *Applied Microbiol. Biotechnol.*, 59: 15-32.
- Gupta, R., Q. Beg, S. Khan and B. Chauhan, 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Applied Microbiol. Biotechnol.*, 60: 381-395.
- Gutowska, M.A., J.C. Drazen and B.H. Robison, 2004. Digestive chitinolytic activity in marine fishes of Monterey Bay, California. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.*, 139: 351-358.
- Kalaiarasi, K. and P.U. Sunitha, 2009. Optimization of alkaline protease production from *Pseudomonas fluorescens* isolated from meat waste contaminated soil. *Afr. J. Biotechnol.*, 8: 7035-7041.
- Kocher, G.S. and S. Mishra, 2009. Immobilization of *Bacillus circulans* MTCC 7906 for enhanced production of alkaline protease under batch and packed bed fermentation conditions. *Internet J. Microbiol.*, Vol. 7, No. 2.
- Kreger, A. and D. Lockwood, 1981. Detection of extracellular toxin(s) produced by *Vibrio vulnificus*. *Infect. Immunity*, 33: 583-590.
- Kumar, R.S., D. Prabhu, T. Shankar, S. Sankaralingam and K.T.K. Anandapandian, 2011. Optimization of alkalophilic protease production by *Pseudomonas aeruginosa* isolated from the gut of *Penaeus monodon*. *World J. Fish Mar. Sci.*, 3: 371-375.
- Mahendran, S., S. Sankaralingam, T. Shankar and P. Vijayabaskar, 2010. Alkalophilic protease enzyme production from estuarine *Bacillus aquimaris*. *World J. Fish Mar. Sci.*, 2: 436-443.

- Porro, S.C., E. Mellado, C. Bertoido, Antranikian and A. Ventosa, 2003. Screening characterization of the protease produced by moderately halophilic bacterium *Pseudomonas* spp. strain p76. *Extremophiles*, 7: 221-228.
- Rao, C.S., T. Sathish, P. Ravichandra and R.S. Prakasham, 2009. Characterization of thermo- and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications. *Process Biochem.*, 44: 262-268.
- Rattanachuay, P., D. Kantachote and P. Sutinanalert, 2007. Selection of proteolytic bacteria with ability to inhibit *Vibrio harveyi* during white shrimp (*Litopenaeus vannamei*) cultivation. *Songklanakarin J. Sci. Technol.*, 29: 235-243.