Isolation and Characterization of Alkaline Proteases Producing Indigenous *Bacillus* sp. as a Source of Thrombolytic and Fibrinolytic Agents

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ABSTRACT: Fibrinolytic enzymes derived from bacteria can be effective as a potent, safe and cost-effective thrombolytic agent for the prevention and treatment of cardiovascular diseases. In this study, microorganisms were isolated from various soil samples and screened for their protease activity and protein concentration. Isolates with higher protease activity were subsequently tested for their thrombolytic and fibrinolytic activity. Of a total 37 isolates, 7 exhibited significant zone in SMA media (zone ratio above 1.3). Of these, 8 isolates showed increased protease activity with values ranging from 120 to 199 U/ml. The highest protease activity was observed for the isolate GST12 obtained from mung bean (green gram) soil. The freeze-dried concentrated crude enzymes were screened by *in vitro* clot lysis method. Six of these eight concentrated enzymes demonstrated clot lysis above 20%, whereas, enzymes from GST12 and GST21 exhibited the highest clot lysis activity (about 27%), which is comparable with standard drug streptokinase. These two enzymes are also strongly associated with fibrinolytic activity. Our findings identify 8 isolates having potential thrombolytic and fibrinolytic activities that belong to Gram-positive *Bacillus*.

Key words: Fibrinolytic enzymes, *Bacillus* sp., thrombolytic enzymes, thrombosis, alkaline protease.

INTRODUCTION

The term cardiovascular disease (CVD) defines a wide range of diseases affecting the heart and blood vessels, such as hypertension, coronary heart disease, cerebrovascular disease, heart failure and other heart diseases. These cardiovascular diseases have been the leading causes of global death over the past 15 years (World Health Organization, 2017). Intravascular thrombosis is a major precipitating factor in cardiovascular diseases, accounting for about 17.79 million deaths annually, representing 31.8% of the total mortality rate globally.[1-3] During the formation of thrombus (clot), the soluble plasma protein, fibrinogen, is converted into insoluble fibrin by an enzyme that forms a fibrin clot. The dissolution of this thrombus is dependent upon the action of an endogenous serine proteinase, plasmin, which is initially generated from its precursor zymogen, plasminogen, by enzymes known as plasminogen activators.[4] From previous studies, it has been found that the best approach to thrombolysis can be an intravenous injection of an enzyme capable of converting plasminogen to plasmin, to restore the blood flow to that area. In addition to the commonly available anticoagulant and antiplatelet drugs, thrombolytic enzyme therapies are being practiced widely now a days.[2,5]

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Based on different working mechanisms, the thrombolytic agents can be classified into two types; the first one is plasminogen activators, such as tissue-type plasminogen activator (t-PA)[6] and urokinase[7], and the second one is plasmin-like proteins, such as lumbrokinase from earthworm and fibrolase from snake venom, which can directly degrade the fibrin in blood clots, thereby dissolves the whole thrombi rapidly.[8-10] Despite their potential as treating agent, all available thrombolytic agents showed similar shortcomings including the need for large therapeutic doses, limited efficacy and specificity, allergic reactions, re-occlusion and bleeding complications.[11] As most of them are derived from bacterial sources or via recombinant DNA technology, they are costly as well.[12,13] Thus, a cheap and effective alternative is necessary, which will overcome these problems. Studies have confirmed that microbes can be a safe potential source for expressing these fibrinolytic enzymes. Different sources including traditional fermented foods[10], marine microorganisms[14], agro-industrial wastes as well as various soil samples[15,16] like feather decomposed soil[17] has been proven to contain microbes producing thrombolytic and fibrinolytic enzymes.

Bacilli is one of the largest groups with a great diversity of strains and are known to produce extracellular proteases during the post-exponential and stationary phases under most culture conditions.[18] So, in this study, several soil samples were collected to isolate and identify alkaline protease producing *Bacillus* species. Here, we took an attempt to identify the strains having the highest potential to produce proteases and screened the crude proteases to characterize thrombolytic activity using the fibrin plate method or by direct *in vitro* clot lysis method.

MATERIALS AND METHODS

Soil sample collection. Total 10 soil samples from rice field, jute field, garden, ash gourd field, mung bean/ green gram field, black gram field, chili field, bottle gourd field, aloe vera field, and slaughterhouse were collected and used as samples for isolation of *Bacillus* species.

Each soil sample was collected in an airtight plastic container with a clean stainless steel spoon. Samples were preserved in dry and cool condition until further processing.

Isolation and separation of spore former *Bacillus* from soil samples. Soil samples were dissolved in sterile 0.8% NaCl solution and treated at 80°C for 15 min in a water bath to kill most vegetative microbes. The supernatants were diluted by 10^6 times serial dilution. The serial dilution of the only slaughterhouse soil samples was done by 10^{10} times because it showed high number of colony forming units (cfu). After dilution, 100 µl of the supernatant was spread on Tryptic Soy Agar (TSA) medium plate and incubated at 37°C for overnight. From various bacterial colonies, pure cultures were obtained by sub-culturing on TSA plates. All isolates were subjected to Gram staining and, after Gram staining, Gram-positive rod or short rod-shaped bacteria were primarily considered ~~as~~to be *Bacillus* spp.

Screening of proteolytic bacteria on skim milk agar (SMA) plates. All the isolates of presumptive *Bacillus* bacteria were primarily screened for their protease activity through casein hydrolysis on skim milk agar (SMA) plates. Each isolate was inoculated on SMA plates with a sterilized needle and incubated overnight at 37°C. A clear zone on SMA plate indicates proteolytic enzyme.

Their zone ratio of casein hydrolysis was calculated as:

$$\text{Zone ratio of casein hydrolysis} = \frac{\text{Diameter of clear zone}}{\text{Diameter of the colony}}$$

Isolates with a zone ratio of casein hydrolysis higher than 1.3 were selected for further screening procedures.

Production of cell-free crude enzyme. Selected organisms from subculture plates were grown overnight in Tryptic soy broth (TSB) media. Then 2.5 ml of each of the seed culture (5% of the total volume of fermentation) of organisms was grown respectively in two different types of media- alkaline protease producing broth (APPB) and soybean-molasses media. The inoculated flask was placed in a thermostated orbital shaker for 48 hours at 30°C and

150 rpm. After 48 hours, the cultures were centrifuged at 6,000 rpm for 10 min. The cell-free supernatant was preserved as crude enzyme at 4°C and used for enzyme assay, protein estimation, clot lysis activity and other methods. For fibrinolytic activity assay, the crude enzymes are sterilized by syringe filters of pore size 0.22 µm and stored at -20°C.

Protease enzyme activity assay. Enzyme activity was determined using azocasein (Sigma, USA) as substrate by a modified procedure described by Kreger et al.[19] For each enzyme sample, one control and two tests were used. One unit of protease activity was determined as the amount of enzyme that produces an increase in absorbance of 0.01 at 440 nm under the above assay condition. The value obtained is expressed in U/ml.

Protease activity (U/ml) = Absorbance at 440 nm × dilution factor

Estimation of extracellular soluble protein. The extracellular soluble protein concentration was also determined by the Bradford method.[20] This was done using protein quantification kit-rapid from Sigma, USA. First, a standard curve was prepared by diluting BSA to 5 different concentrations and adding CBB solution to it. Here, distilled water was taken as control. The absorbance was measured at 595 nm. Using the same procedure, the absorbance of the samples was also observed to calculate the soluble protein concentration.

The amount of soluble protein was determined from that standard curve using the following equation and expressed as mg per ml of the test sample.

$y = mx$

Where, y = absorbance at 595 nm, x = protein concentration in mg/ml, m= slope of the standard curve

Determination of specific activity. Using the proteolytic activity value from enzyme assay and the value from the Bradford method of extracellular soluble protein, the specific activity was calculated as U/µg using the following equation:

$$\text{Specific activity (U/µg)} = \frac{\text{Protease activity (U/ml)}}{\text{Extracellular protein conc. (µg/ml)}}$$

Thrombolytic activity study by clot lysis method. The thrombolytic activity in terms of *in vitro* clot lysis was carried as reported earlier by Prasad et al.[21] Venous blood was drawn from healthy human volunteers and 500 µl of blood was transferred to each of the previously weighed Eppendorf tubes. It was then incubated in simulated body temperature in a heat controlled incubator and clot was formed. Serum was completely removed and each tube having clot was again weighed to determine the clot weight.

Clot weight = Weight of clot containing tube – weight of tube alone

Streptokinase served as positive control and PBS (or distilled water) served as a negative thrombolytic control. The difference obtained in weight taken before and after clot lysis was expressed as a percentage of clot lysis.

Fibrinolytic activity assay

Fibrinolytic activity assay by absorbance method. Fibrinolytic activity was estimated according to a modified protocol of that followed by Ningthoujam et al.[3] Streptokinase standard solution was used as a positive control (standard) and distilled water is used as a negative control in this method. Then 700 µl 50 mM Tris-HCl buffer (pH 8.0) was mixed with 200 µl 0.72% (w/v) fibrinogen solution and incubated at 37°C for 5 min. The rest of the things were added to it in proportion to the first ones. After incubation and centrifugation, the absorbance was measured at 280 nm. Fibrinolytic unit (FU) is calculated according to the following equation:

$$\text{Fibrinolytic Unit (FU)} = \frac{\text{Abs. of supernatant} - \text{abs. of blank} \times \text{dilution factor}}{0.001 \times 0.1 \times 60}$$

Fibrinolytic activity assay using plasminogen-rich fibrin plate method. In this method, qualitative and quantitative analysis of the fibrinolytic activity of the samples can be performed. It was done by a modified method of that following Balaraman and

Prabakaran,[22] which was modified from the original fibrin plate method of Astrup and Müllertz.[23] Fibrinogen solution (4.5 ml of 0.72%) in phosphate buffer saline (pH 8.0) was poured into a sterile petri dish of 5 cm diameter. Plasminogen (500 µl of 10 U/ml) solution in cold deionized water was added to it and mix well. Then 500 µl of 20 U/ml thrombin solution dissolved in 0.9% NaCl solution was added to it and mix well. Immediate clot formation occurs producing a semisolid layer. Samples and standards were applied by micropipette immediate below the upper surface of the clot layer to prevent overflowing of them. The plates were then incubated at 37°C for 90 min. Analysis of fibrinolytic activity was done by visual observation.

Phenotypic characterization

Morphological characterization. Isolates producing significant protease activity were subjected to morphological characterization. These isolates were freshly cultured on TSA media overnight for 24 hours at 37°C and their growth and colony characteristics were observed and recorded.

Biochemical characterization. The Gram-positive, rod-shaped, spore-forming *Bacilli* were selected for additional identification tests. Subsequent identification tests, including eosin methylene blue agar test, catalase test, glucose fermentation, and hydrogen sulfide gas production (KIA) test, citrate utilization test, starch hydrolysis test, hemolysis test, lecithinase and lipase test, susceptibility test to penicillin and mannitol hydrolysis tests were performed.

Storage and maintenance of isolates. Isolates were maintained by sub-culturing in TSA media and stored at -20^{o}C in 20% glycerol stock for further work.

RESULTS AND DISCUSSION

Presumption of *Bacillus* species. Ten soil samples from rice field, jute field, garden, ash gourd field, mung bean/ green gram field, black gram field, chili field, bottle gourd field, aloe vera field, and slaughterhouse were used as samples for isolation of *Bacillus* species. A total of thirty five distinctive colonies from the 10 soil samples were isolated and pure-cultured on TSA plates**.** Then these 35 isolates were Gram-stained and their shapes were recorded. Among them, 32 isolates were found in either rod-shaped or short shaped which were presumed as *Bacillus* species, whereas 3 isolates were round-shaped (Table 1). Further screening of activity and characterization were carried out using those 32 isolates.

Fermentation of isolates in differet media helped to find active proteolytic bacteria. The selected 32 isolates were inoculated in SMA plates with a sterilized needle. After overnight incubation at 37°C, 19 of these isolates produced clear zones in SMA plates. Among them, 17 isolates coded as GST03, GST05, GST06, GST07, GST09, GST10, GST11, GST12, GST13, GST14, GST16, GST21, GST25, GST26, GST32, GST33, and GST34 gave the zone ratio of casein hydrolysis above 1.3 (Table 1).

The 17 selected isolates were fermented using APPB media, enabling them to produce extracellular alkaline proteases in the culture media. After analyzing the data of these crude enzymes, it was found that the activity of the enzymes produced in this medium was not encouraging enough to proceed further. Only 8 isolates were selected from this fermentation, which could give promising results, and thus, fermentation media were changed.

Eight selected isoaltes from APPB media such as GST06, GST07, GST09, GST10, GST12, GST14, GST21, and GST34, were also fermented in soybean molasses media (SMM), where 5% culture were added in the new fermentation media to evulate their enzyme producing abilty.

Diffrent isolates produced different protein concentration and exhibited variable protease activity. The potease enzyme activity of the selected crude enzymes was recorded by azo-casein method in U/ml.[24] Bradford method[20] was used to determine the protein concentration of the crude enzymes in µg/ml and using the equation mentioned in the method section, specific protease activity of the crude

enzymes was calculated as U/µg (Table 2). Only GST06 and GST12 exhibited a specific protease activity higher than 5.

However, even after changing the fermentation media, the protease activity was not increased significantly to accept as a choice. So, the crude enzymes from fermentation in soybean-molasses media were freeze-dried to make them more concentrated followed by the addition of calculating amount of distilled water.

Table 1. Gram staining and proteolytic activity screening in Skim-milk agar plate of the isolates from different soil samples.

Soil source	Strain ID.	Gram staining		SMA screening		
		Gram reaction	Morphology	Clear zone diameter (cm)	Colony diameter (cm)	Zone ratio
	GST01	Positive	Rod shaped	-	-	-
Rice	GST02	Positive	Rod shaped	-	-	-
	GST03	Positive	Rod shaped	0.9	0.65	1.38
	GST04	Positive	Rod shaped	-	-	-
Jute	GST05	Positive	Rod shaped	0.6	0.4	1.5
	GST06	Positive	Short rod shaped	0.3	0.15	2
Garden	GST07	Positive	Short rod shaped	1.2	0.4	3
Ash gourd	GST08	Positive	Rod shaped	-	-	-
	GST09	Positive	Rod shaped	1	0.4	2.5
Mung bean	GST10	Positive	Rod shaped	0.95	0.35	2.71
	GST11	Positive	Rod shaped	3	1	3
	GST12	Positive	Rod shaped	0.6	0.25	2.4
Black gram	GST13	Positive	Rod shaped	0.5	0.35	1.43
	GST14	Positive	Rod shaped	0.75	0.35	2.14
	GST15	Positive	Rod shaped	-	-	-
	GST16	Positive	Rod shaped	0.8	0.6	1.33
Bottle gourd	GST17	Positive	Rod shaped	-	-	-
	GST18	Positive	Round shaped	-	-	-
	GST19	Positive	Rod shaped	-	-	-
	GST20	Positive	Rod shaped	-	-	-
	GST21	Positive	Short rod shaped	1.25	0.5	2.5
Chilli	GST22	Positive	Rod shaped	-	-	-
Aloevera	GST23	Positive	Rod shaped	0.8	0.65	1.23
	GST24	Positive	Rod shaped	1.15	1.05	1.1
	GST25	Positive	Rod shaped	0.4	0.2	2
	GST26	Positive	Rod shaped	0.2	0.15	1.33
	GST27	Positive	Rod shaped	-	-	-
	GST28	Positive	Rod round &shaped	-	-	-
	GST29	Positive	Rod round &shaped	-	-	-
	GST30	Positive	Rod shaped	-	-	-
	GST31	Positive	Round shaped	-	-	-
Slaughter - house	GST32	Positive	Rod & round shaped	1.075	0.675	1.6
	GST33	Positive	Rod shaped	0.85	0.55	1.55
	GST34	Positive	Rod Shaped	0.7	0.35	2
	GST35	Positive	Rod Shaped	-	-	-

Table 2. Protease activity, protein concentration and specific protease activity of crude enzymes obtained from isolates fermented in soybean-molasses media.

Isolates	Protease activity (U/ml)	Protein conc. (µg/ml)	Specific protease activity (U/µg)
GST06	1475	239	6.172
GST07	1406	304	4.625
GST09	1429	304	4.701
GST10	1424	369	3.860
GST12	1792	229	7.825
GST14	1443	339	4.257
GST21	1298	289	4.491
GST34	964	194	4.969

The protease enzyme activity of the concentrated crude enzymes from the fermentation of the 8 selected isolates (GST06, GST07, GST09, GST10, GST12, GST14, GST21, and GST34) in Soybean-molasses media were determined and recorded by also-casein method in U/ml (Table 3). Interestingly, concentrated crude enzymes showed a significant increase in protease activity. Among isolates, GST07, GST09, GST12, GST14 and GST21 showed protease activity more than 13,000 U/ml.

Crude enzymes of the isolates showed significant thrombolytic activity. Next, the clot lysis activity of the crude enzymes (100µl each) was done on human blood with streptokinase (30,000 IU/ml) as positive control and distilled water as a negative control. All concentrated crude enzymes showed significant clot lysis activity. Interestingly, GST12 and GST21 demonstrated an average clot lysis of 27%, which was equivalent to the streptokinase standard (Figure 1).

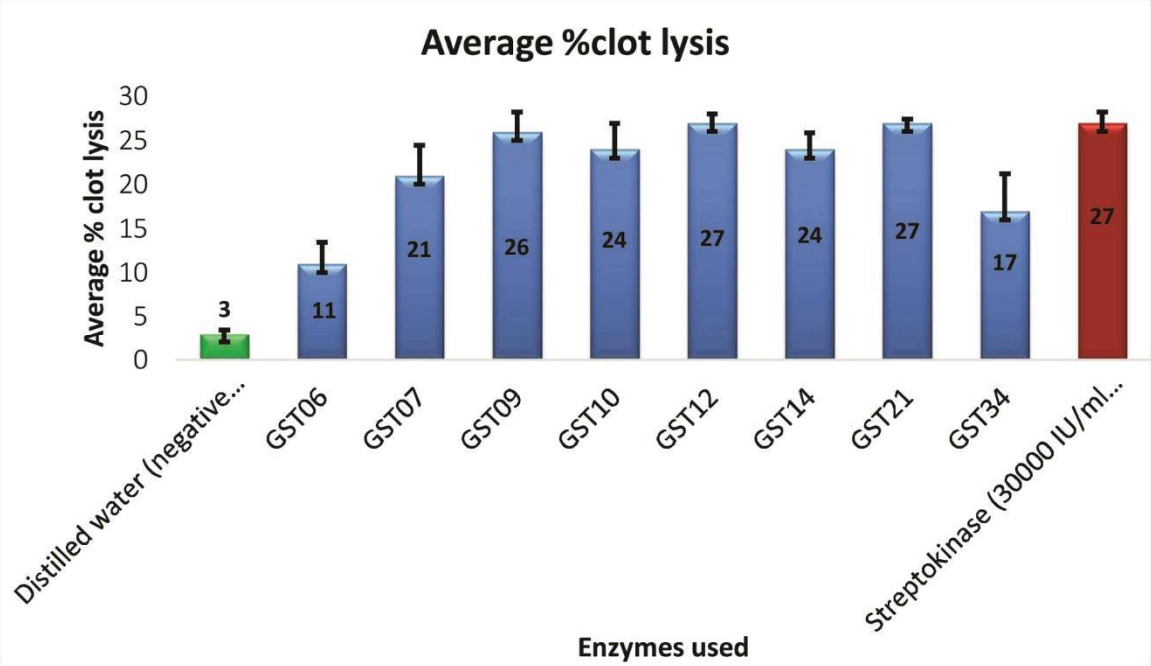

Figure 1. Clot lysis (%) of the concentrated crude enzymes of the isolates fermented in soybean-molasses media. Data are represented as mean ± SEM, n=3 replicates per group.

Crude enzymes exhibited fibrinolytic activity. To the fibrin solution produced by the reaction of fibrinogen and thrombin, all 8 of the crude enzymes of the isolates fermented in soybean-molasses media were added as samples and streptokinase 30,000 IU/ml solution was added as standard. All crude enzymes showed fairly strong clot lysis activity (Figure 2). However, the results obtained by this method may not be reliable because different molecules and salts may be present in the crude enzymes which can have a significant absorbance at 280 nm. On the other hand, positive control streptokinase was free from such ingredients.

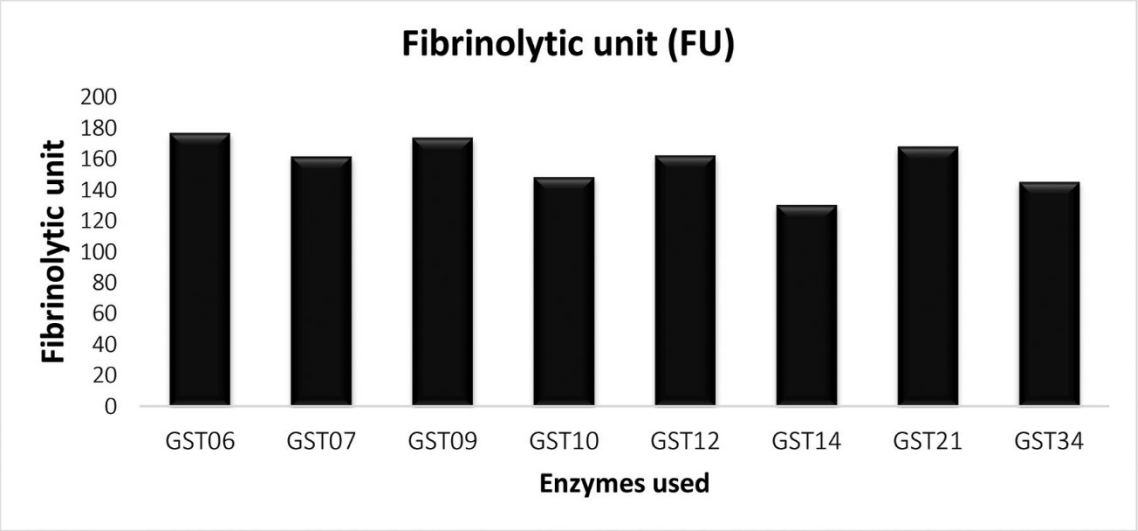

Figure 2. Fibrin assay of crude enzymes of isolates fermented in soybean-molasses media.

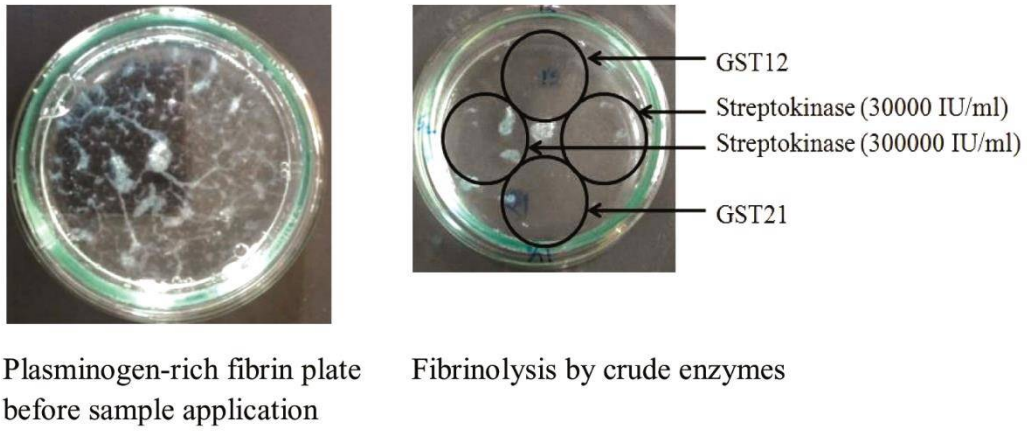

Figure 3. Fibrin plate assay of crude enzymes produced by ioslates GST12 and GST21. Streptokinase (30000 IU/ml) was used as positive control.

Following preparation of the plasminogen-rich fibrin plate, two selected concentrated crude enzymes from fermentation in soybean-molasses media, GST12 and GST21, were applied and incubated. Visual observation had showed that both enzymes have a faster and more comprehensive capacity for fibrinolytic activity compared to the standard (Figure 3). This leads to the conclusion that a new

thrombolytic protease may be isolated from these isolates. Summary of these strains is given in the table (Table 4).

Gram staining and morphological characterization of the isolates. Gram staining, microscopic observation and visual observation of colony characteristics were carried out and recorded. The observations were recorded and analyzed (Table 5).

Biochemical characterization. After a series of biochemical test, the following data (Table 6) were found about the selected bacterial strains. All the 8 isolates showed no growth in Eosin methylene blue (EMB) agar and all of them were catalase-positive

Table 3. Protease activity of the concentrated crude enzymes of the isolates fermented in soybean-molasses media.

Isolate		Dilution folid	Protease activity (U/ml)	Average protease activity (U/ml)
GST06	1.161	100	11610	11600
	1.159	100	11590	
GST07	1.317	100	13170	13115
	1.306	100	13060	
GST09	1.391	100	13910	13600
	1.329	100	13290	
GST10	1.014	100	10140	10400
	1.066	100	10660	
GST12	1.953	100	19530	19515
	1.950	100	19500	
GST14	1.532	100	15320	15435
	1.555	100	15550	
GST21	1.324	100	13240	13135
	1.303	100	13030	
GST34	0.754	100	7540	7770
	0.800	100	8000	

Table 4. Protease activity of crude and concentrated enzymes produced by GST12 and GST21 in different medium.

Activity	GST12	GST21
Zone ratio of casein hydrolysis in SMA media	2.4	2.5
Protease activity of crude enzymes in APPB media (U/ml)	199	191
Protease activity of crude enzymes in soybean-molasses media (U/ml)	1792	1298
Protease activity of concentrated crude enzymes in soybean-molasses media (U/ml)	19515	13135
Thrombolytic activity of concentrated crude enzymes in soybean-molasses media (%)	27	27
Fibrinolytic unit (by absorbance method)	162.667	168.333
Fibrinolytic activity in plasminogen-rich fibrin plate	Faster and clearer than standard	Faster and clearer than standard

Table 5. Morphological characterization of selected isolates.

Characteristics	GST06	GST07	GST09	GST10	GST12	GST14	GST21	GST34
Gram reaction	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Cell morphology	Short rod	Short rod	Rod	Rod	Rod	Rod	Short rod	Rod
Form	Irregular	Circular	Irregular	Circular	Circular	Irregular	Circular	Irregular
Pigmentation	Off-white	White	Off-white	White	Off-white	Off-white	White	White
Growth	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Margin	Undulate	Entire	Undulate	Entire	Entire	Undulate	Entire	Lobate
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Rough	Smooth	Smooth
Elevation	Flat	Raised	Flat	Raised	Flat	Crater form	Raised	Flat
Colony size	Small	Medium to large	Small to medium	Medium to large	Small	Medium	Small to medium	Small
Stickiness to surface	Less	More	Less	More	Less	More	More	Less
Stickiness to loop	Less	Less	More	Less	More	Less	More	Less
Removal from surface	Easy	Difficult	Easy	Moderate	Easy	Easy	Moderate	Easy

Table 6. Biochemical characterization of selected isolates.

Characteristics	GST06	GST07	GST09	GST10	GST12	GST14	GST21	GST34
Casein hydrolysis	+	+	+	+	+	+	+	+
Starch hydrolysis	-	+	+	+	-	+	+	+
Lipase	-	-	-	-	-	-	-	-
Lecithinase	-	-	-	-	-	-	-	-
Hemolysis	Beta-hemolytic	Gamma-hemolytic	Gamma-hemolytic	Alpha-hemolytic	Beta-hemolytic	Alpha-hemolytic	Alpha-hemolytic	Alpha-hemolytic
Catalase	+	+	+	+	+	+	+	+
Glucose fermentation	+	-	+	+	-	-	-	+
Lactose fermentation	-	-	+	-	-	-	-	+
H_2S production	-	-	-	-	-	-	-	-
Citrate utilization	+	-	+	-	+	-	-	-
Penicillin susceptibility	+	+	-	-	+	-	+/-	+/-
Mannitol fermentation	+	+	+	+	+	+	-	-

capable of starch hydrolysis, which indicated that they may be Gram-positive *Bacillus* species. No zone of egg precipitation in egg yolk emulsion (EYE) agar media for all the 8 isolates indicated that they belong to the genus *Bacillus.* Further biochemical characterization and genetic sequencing are necessary to identify the strains undoubtedly.

CONCLUSION

In this research, 35 different Gram positive spore formers were isolated from different agricultural and slaughterhouse soil samples, among which 17 isolates showed higher casein hydrolysis activity. Of these 17 isolates, 8 isolates with higher protease production in fermentation were assessed for thrombolytic and fibrinolytic activities. Although all the 8 isolates have substantial thrombolytic and fibrinolytic activities, 2 of these isolates are more potential candidates.

Morphological and biochemical characterization indicated that these 8 isolates may belong to the genus *Bacillus*. Further research is required for genetic modification(s) in order to obtain better production, activity and purification of thrombolytic and fibrinolytic enzymes from these isolates.

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Competing interest. Authors declare no conflict of interest.

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