Bacillus tequilensis ZMS-2: A novel source of alkaline protease with antimicrobial, anti-coagulant, fibrinolytic and dehairing potentials

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Abstract: Emerging resistance to existing antimicrobial agents is one of the growing concerns and a serious problem for public health globally. Currently available antimicrobial agents are potent and effective but surfacing resistance to these drugs has not been ruled out so far. Therefore, it is utmost important to explore new bioactive compounds from natural sources to meet future needs. The present study was designed to produce, optimize, characterize and evaluate antimicrobial, fibrinolytic and anti-coagulant potential of a new alkaline protease. Proteolytic strain from desert soils of Tharparkar, Pakistan was subjected to 16S rDNA sequencing and identified as *Bacillus tequilensis* ZMS-2(Genbank Accession No. MK101013). During submerged fermentation at 37ºC, maximum enzyme production (454 U/ml) was observed with 24h old inoculum. The best incubation time was 72h (544 U/ml), optimum inoculum size and pH was 10% at pH 8 with 494 and 506 U/ml, respectively. The best carbon source was starch (571 U/ml), while ideal substrate was wheat bran (536 U/ml). Optimal temperature and pH for proteolytic activity was 60ºC (420 U/ml) and 8 (332 U/ml). Alkaline protease showed antibacterial activity against *Staphylococcus aureus* (27mm), *Bacillus licheniformis* (20mm), *Klebsiella pneumoniae* (17mm) and *Escherichia coli* (15mm). The strain *B. tequilensis* ZMS-2 also exhibited anti-coagulant, fibrinolytic and dehairing potential suggesting application of its protease in various industries.

Keywords: Antimicrobial, *Bacillus tequilensis* ZMS-2, alkaline protease, anti-coagulant, fibrinolytic, dehairing.

INTRODUCTION

Antimicrobial resistance (AMR) is an alarming public health distress and a leading cause of ineffectiveness of antibiotics which increased the incidences of treatment failure leading to ten millions deaths worldwide (deKraker *et al.,* 2016). The World Health Organization (WHO) has already regarded AMR as a most serious issue to be resolved through joint global efforts on priority basis (WHO, 2016). Currently available antimicrobial agents have established efficacy but we cannot rule out the emergence of resistance to these drugs.

According to WHO, 31% of the world's mortality rate is due to cardiovascular diseases leading to the death of approximately 18 million people annually (Kim *et al.,* 2008).The main cause of myocardial infarction and other cardiovascular diseases is the formation of fibrin clot in blood vessels followed by thrombosis (Bode *et al.,* 1996).

Proteases are diversified group of hydrolytic enzymes involved in the regulation of metabolism as well as infectious diseases (Tersariol *et al.,* 2002). Their main function is to provide peptide nutrients to host by hydrolysis of polypeptides substrates. Due to their proteolytic potential, proteases contribute to pathogenesis by acting as virulence factors. Some authors even listed them the top virulence factors among other extracellular factors (Secades and Guijarro, 1999). Some bacterial proteases with antimicrobial potential include, *Xylariapsidii* KT30 protease antagonistic against *Bacillus subtilis* and *Staphylococcus aureus* (Taufik *et al.,* 2016*).* Similarly, antimicrobial potential of protease from marine *Bacillus subtilis* was reported against *Klebsiella pneumoniae, Micrococcus luteus, S. aureus, Bacillus pumilus* and *Arthrobacter sp.* (Rachanamol *et al.,* 2017).

Protease having the potential to degrade fibrin clot are grouped as fibrinolytic enzymes. Majority of the fibrinolytic proteases are serine proteases having higher substrate specificity to fibrin as compared to other broad specific proteases (Usharani and Muthuraj, 2018).The studies on the mechanism of action and structure-function relationship can further help to develop novel anticoagulant therapeutic agents of commercial importance (Kumar *et al.,* 2013).

Protease also have the potential to be used as dehairing agent during hide/skin processing in tanneries. Furthermore, it also improves waste water quality with no/less use of sodium sulfide and lime. Correlation between proteolytic activity and dehairing has been reported by using commercial proteases for dehairing of animal skin (Huang *et al.,* 2003).

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The review of literature revealed that until now, there is no study related to the application of alkaline protease from *Bacillus tequilensis* as antimicrobial, anti-coagulant and fibrinolytic agents. The purpose of current study was to isolate, optimize, characterize, purify and evaluate the antimicrobial, anti-coagulant and fibrinolytic potential of alkaline protease from thermophilic *Bacillus tequilensis* ZMS-2.

MATERIALS AND METHODS

Isolation and Screening of Microorganism

Soil samples from desert of Mithi, District Tharparkar, Sindh, Pakistan were serially diluted, inoculated on nutrient agar plates and incubated for 24-48h at 37°C. Isolated bacterial colonies were streaked on skim milk agar containing skim milk powder (10%) and agar (2%) to isolate producer strains.

Identification of microorganism

The colonial morphology, Gram reaction, microscopic characteristics, biochemical tests and 16S rDNA sequencing were used for the identification. The strain was subjected to colony PCR using16S rDNA universal primers (forward primer 5'- AGA GTT TGA TCC TGG CTC AG -3' and reverse primer 5'- CGG TTA CCT TGT TAC GAC TT -3'). PCR product was run on 1% agarose gel with 1Kb DNA ladder (MOLEQULE-ON) for visual confirmation. The product was sequenced at BGI sequence service China (Sanger and Coulson, 1975), refined and subjected to nucleotide BLAST (NCBI), followed by alignment using Clustal X. Phylogenetic tree was reconstructed using Kimura 2-parameter model by Maximum Likelihood method with 1000 bootstrap replicates, using Mega 7 software (Kimura, 1980).

Production and assay of alkaline protease

Growth medium was prepared by adding loopful of 24 h fresh culture to sterile broth containing 1% glucose, 0.5% skim milk powder, 0.1% KH_2PO_4, 0.1% $MgSO_4.7H_2O$, pH 7 and incubated on shaker (150rpm) for 24h at 37°C. The 10% (v/v) of 24h old inoculum was added to production medium containing 0.5% glucose; 1% skim milk powder; 0.1% peptone; 0.1% KH_2PO_4; 0.1% $MgSO_4.7H_2O$; 0.1% $CaCl_2.2H_2O$ and incubated on shaker for 120h. Sampling was done every 24h following centrifugation at 4000 rpm for 30 min to obtain cell-free filtrate. Proteolytic units were assessed by colorimetric method using azocasein as substrate (Caldas *et al.,* 2002).One unit of enzyme activity was defined as the amount of enzyme which yield an increase in absorbance of 0.001 at 440nm in 30min at 37ºC.

Optimization of protease production in submerged fermentation

Effect of incubation time on the production of proteolytic enzyme was examined by taking samples from production medium at constant interludes of 24h upto 120h. Effect of age of inoculum was studied by inoculating the same media with inocula of different ages (24, 48 and 72h), while size of inoculum was optimized using three different volumes of inocula (10, 15, and 20%). Effect of initial pH was identified using production media with different initial pH (7, 8, 9, 10, 11). Different substrates like wheat bran, rice bran, etc. were used in production medium to identify the ideal nitrogen source. Similarly, glucose was replaced with various carbon sources to identify the best carbon source for production of enzyme.

Characterization of crude protease

Optimum temperature was identified by incubating the assay reagents at temperatures (40ºC to 80ºC). Similarly, optimum pH was studied by dissolving azocasein in buffers of different pH (Citrate: 5, 6; Phosphate: 7; Tris-HCl: 8, Tris-NaOH: 9, 10, 11) and assayed for enzyme units. Various metal ions of 50mM (Na^+, K^+, Ca^{2+}, Fe^{2+}, Mg^{2+}) and detergents (4%w/v) solutions were added to the assay mixture to check their effect on enzymatic activity (Khajuria *et al.,* 2015).

Partial purification of protease

Cell free filtrate was subjected to ammonium sulfate precipitation by gradual addition of salt (10-70%). Pellet was recovered by centrifugation at 4000rpm for 30min and dissolved in equal volume of Tris-HCl buffer (pH 8).

Antimicrobial activity of Protease ZMS-2

The antibacterial potential of protease ZMS-2 was evaluated for crude (140 U/ml) and partially purified (470 U/ml) enzyme by measuring zone of inhibition using agar well diffusion method. The activity was tested against *Staphylococcus aureus, Bacillus licheniformis, Klebsiella pneumoniae,* and *Escherichia coli*. The 100 µL each of crude and partially purified protease was used while Tris-HCl buffer (pH 8) and Ceftriaxone preparations (10 µg/ml) were used as negative and positive control, respectively.

Anti-coagulant and fibrinolytic activity of Protease ZMS-2

Anti-coagulant activity of protease ZMS-2 (470 U/ml) was observed using method defined by Condrea *et al.,* 1983.Similarly, for fibrinolytic activity, fibrin clot was suspended in 3ml of Tris-HCl buffer (pH 8) having 470 units of enzyme and incubated at 37ºC. Hydrolysis of fibrin clot was observed every 30 min through naked eye and turbidity was examined using spectrophotometer (A600nm).

Dehairing potential of Protease ZMS-2

Pieces of goat skin were washed with detergent solutions and cut into 09 x 07 cm weighing 9.5 gm. Two pieces of skin were processed in flasks on shaker incubator (100 rpm) at 37 ºC with cell free filtrate (266 U/ml) in a ratio

of 1 ml of liquid per gram of skin. Processed skin fragments were observed under light microscope (10x) to compare epidermis and root bulb of treated and untreated skin (Huang *et al.,* 2003).

STATISTICAL ANALYSIS

All experiments were performed in duplicates and standard deviation of each experiment result was calculated using PRISM (version 5.01) software.

RESULTS

Isolation and identification of microorganism

The isolated strain was Gram positive, rod shaped, non motile and spore former with off white colonies (fig. 1 a-b). Biochemical characteristics included positive production of alpha-amylase and catalase enzyme while no oxidase and urease production was observed. Strain ZMS-2 exhibited 99% homology with the strains of *B. tequilensis.* Moreover, as per phylogenetic analysis, the designated strain was finaly identified as *Bacillus tequilensis* ZMS-2 (Genbank Accession # MK101013) (fig. 1 c-d).

Production optimization through submerged fermentation

During submerged fermentation, 422 U/ml were observed after 24 h of incubation which reached to 544 U/ml after 72h. However, enzyme units decreased to 484 U/ml after

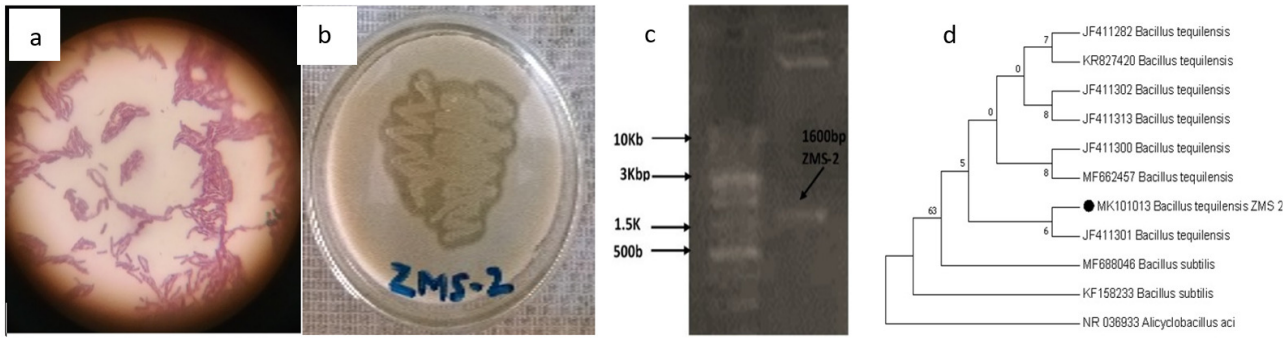

Fig. 1: Gram stain of *B. tequilensis* ZMS-2 (a), Proteolytic activity of *B. tequilensis* ZMS-2 on skim milk agar (b), Amplified 16S rRNA gene of 1600bp of strain ZMS-2 on 1% agarose gel (c), Phylogenetic tree reconstructed using Maximum Likelihood method, strain ZMS-2 clustered with identified *Bacillus tequilensis* strains, whereas the strain *Alicyclobacillus acidocaldarius* (NR036933) was used as out group (d).

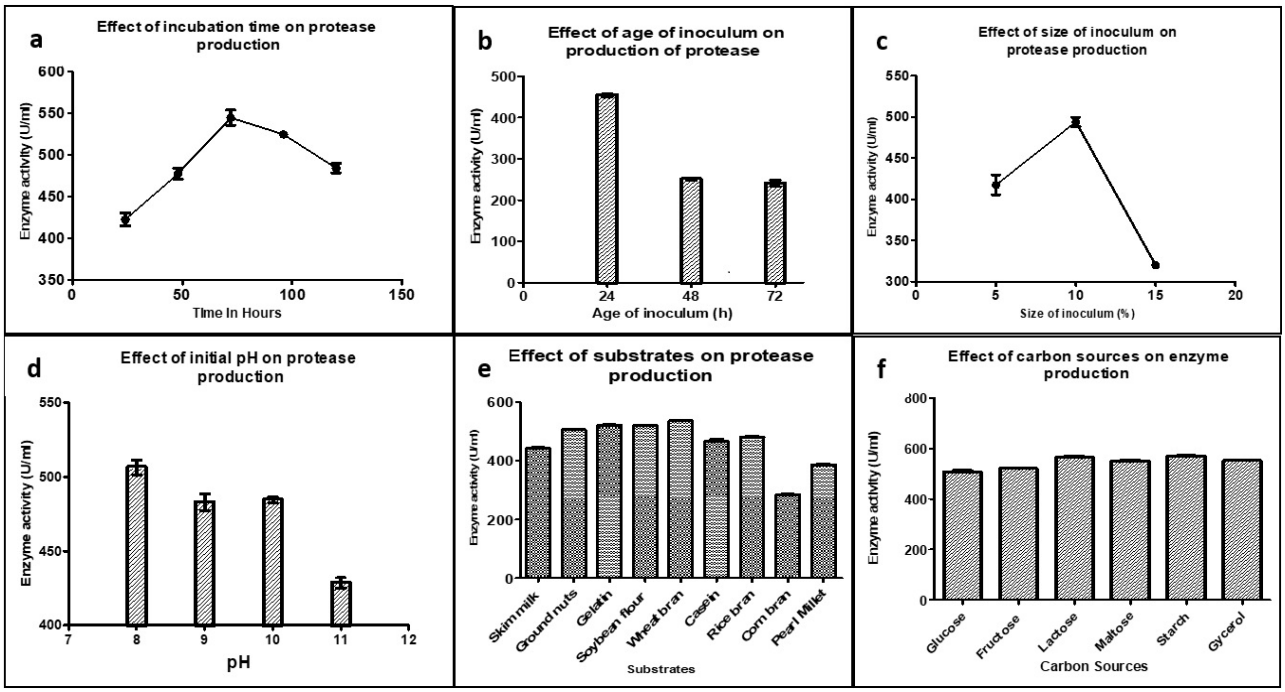

Fig. 2: Optimization of protease production: Effect of incubation time (a), Effect of age of inoculum (b) Effect of size of inoculum (c), Effect of initial pH (d), Effect of substrate (e), Effect of carbon sources (f).

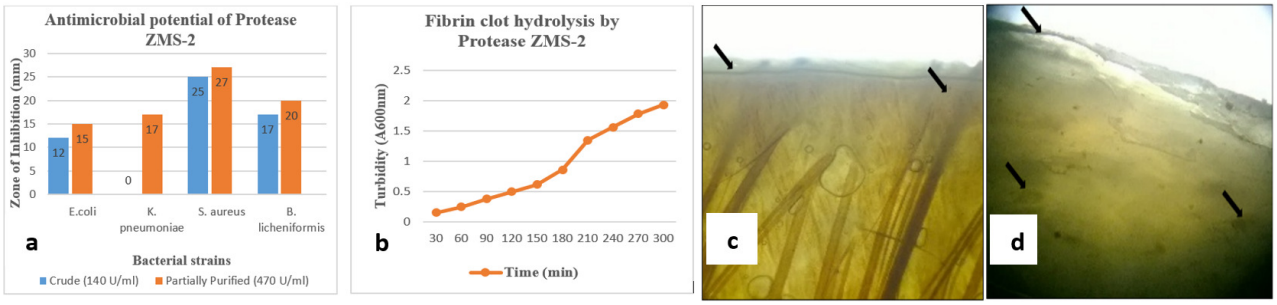

Fig. 3: Antimicrobial potential of Protease ZMS-2 (a), Fibrin clot hydrolysis by Protease ZMS-2 (b), Goat skin before enzyme treatment showing normal epidermis and hair bulb (c), Goat skin after enzyme treatment showing degraded epidermis and hair bulb (d).

120h (fig. 2-a). Optimum age of inoculum observed was 24 h where 454 U/ml were observed (fig. 2-b). Ideal size of inoculum investigated for *B. tequilensis* ZMS-2 was 10% (v/v) which produced 494U/ml of proteolytic enzyme (fig. 2-c). Maximum units (506U/ml) of proteolytic enzyme were observed at pH 8, however, minimal decrease in protease units was observed at high range of alkaline pH (fig. 2-d). Among substrates, wheat bran showed maximum activity (536U/ml) while minimum activity was observed with corn bran (284 U/ml) (fig. 2-e). Maximum units (571U/ml) were observed with starch as compared to other carbon sources (fig. 2-f).

Characterization of crude protease

Protease was found active at wide range of temperatures (40-70ºC), however, maximum proteolytic activity (420 U/ml) was observed at 60ºC, confirming its thermophilic nature. Proteolytic units of 332 U/ml and 323 U/ml were observed at pH 8 and 9, respectively. Crude protease lost 5.85% activity with addition of K^+32.21% with Ca^{2+}, 20.50% with Na^+, 30.02% with Fe^{2+} and 13.28% with Mg^{2+}. Furthermore, protease retained 55% activity with commercially available detergents i.e., Surf Excel, 38.5% with Ariel, 36.5% with Brite, 48.9% with Bonus Tri-star and 49.6% with Lemon Max. However, an increase of 11% in proteolytic activity was observed with Tween 80.

Partial purification of protease

A 3.5 fold increase in proteolytic units (crude 266, purified 938U/ml) were observed in partially purified protease and complete precipitation of enzyme was recorded at 70% saturation of ammonium sulfate.

Antimicrobial activity of protease ZMS-2

Protease ZMS-2 exhibited antibacterial activity against tested bacterial clinical isolates (fig. 3-a), including: *S. aureus* (27mm), *B. licheniformis* (20mm), *K. pneumoniae* (17mm) and *E. coli* (15mm),confirming its antibacterial potential.

Anti-coagulant and fibrinolytic activity of Protease ZMS-2

Protease ZMS-2 showed excellent anti-coagulant potential by increasing the plasma clotting time by 352sec as compared to control clotting time of 120 sec. Similarly, complete hydrolysis of fibrin clot was observed after 5 hours at 37°C (fig. 3-b), confirming the fibrinolytic potential of protease ZMS-2.

Dehairing potential of Protease ZMS-2

Protease ZMS-2 (266 U/ml) successfully dehaired goat skin after 14 h of treatment at 37ºC on rotary shaker at 100 rpm. In control sample, poor hair loosening was observed and it was difficult even to remove hair mechanically like plucking with forceps. Microscopic analysis of test samples confirmed complete recovery of hair and degradation of epidermis and hair bulb (fig. 3c-d).

DISCUSSION

Identification of bacterial strain using 16S rDNA sequencing is considered as gold standard as compared to conventional microbiological methods (Hugenholtz and Tyson, 2008). The indigenouly isolated strain ZMS-2 in the reconstructed phylogenetic tree of 16S rRNA gene, developed by Maximum Likelihood method using appropriate evolutionary model, was clustered with already identified *Bacillus tequilensis* strain.

Maximum units of extracellular protease were observed after 72 h of incubation with majority of substrate. However, production of high titer protease was observed in 48h when wheat bran was used as substrate. The time of production varies among different microorganisms which depends on the genetic potential, cultural and environmental conditions during fermentation (Bajaj and Sharma, 2011). In developing countries, the cost of enzyme is a major hurdle for its production and application at industrial level. The ability of a strain to produce maximum proteolytic units in minimum time is

highly desirable as it warranties the cost effective production of enzymes.

Optimum units of protease were observed with 24h old culture taking 10% v/v inoculum. In a small inoculum size, the ratio between surface area and volume remains too high. Similarly, high concentration of inoculum causes early depletion of nutrients and dissolved oxygen resulting a decrease in enzymatic yield (Rahman *et al.,* 2006). Ideal pH of medium for the production of enzyme observed was pH 8. The role of pH of production medium in maintaining homeostasis and regulation of cell physiology is very crucial. The cell growth and production of hydrolytic enzymes is highly reliant on the concentration of H^+ ions in the production medium (Kumar *et al.,* 2012). The use agro-based substrate can ensure the process economy leading to the cost effective production of enzyme. *Bacillus tequilensis* ZMS-2 effectively utilized wheat bran for the production of maximum units (536 U/ml) of protease as compared to other substrates. Gaur *et al.,* 2014, also reported wheat bran as an ideal substrate for protease production. Similarly, optimal protease units (571 U/ml) were observed with starch as compared to other carbon sources. The observed decline in proteolytic units with simple carbon sources is due to catabolite repression.

The thermophilic nature and activity at wide range of pH make present protease an ideal candidate for commercial applications. These qualities favor the stability and activity of enzyme during harsh industrial conditions of pH and temperature. Protease ZMS-2 was found active at wide range of temperature and pH having maximum activity at 60ºC and pH 8, confirming its thermophilic and alkaline nature. Inhibitory effect of metal ions was observed on proteolytic activity of protease ZMS-2. Metal ions can influence enzymatic activity by accepting or donating electrons, act themselves as electrophiles, can facade the effect of nucleophile and may bind with enzyme or direct enzyme substrate bonding. The compatibility of proteases with detergents and denaturants is highly anticipated for industrial applications Exposure to solvents and detergents trigger unfolding, structural changes and/or remove essential water layer from enzyme molecules (Khajuria *et al.,* 2015) Protease ZMS-2 was found compatible with commercial detergents, which is highly desirable for its applications in detergents, leather and other relevant industries.

The proteolytic potential of bacterial proteases can be well exploited as new targets for antimicrobial agents. Our study on newly isolated *B. tequilensis* ZMS-2 added valuable findings to this underexplored area as a step toward the development of bioactive peptide agents. The highest antimicrobial activity of protease was observed against *S. aureus* (27mm) which is a notorious Gram-positive bacterium involved in skin infections, respiratory diseases and food poisoning. *S. aureus* has acquired numerous strategies to develop resistance to almost all antibiotics (Kuroda *et al.,* 2001). The zone of inhibition observed with protease ZMS-2 against *K. pneumoniae* was 17mm. *K. pneumoniae* is a Gram-negative encapsulated pathogenic bacteria and the most common cause of hospital-acquired pneumonia accounting for 3 to 8% of all nosocomial bacterial infections (Jondle *et al.,* 2018). Similarly, protease also showed activity against *Escherichia coli* (15mm), *which* is a common Gram-negative human pathogen, causing urinary tract infections, bacteraemia and diarrhea (Kaper *et al.,* 2004) and *B. licheniformis* (20mm). Our findings also confirms the extended antimicrobial potential of bacterial protease over fungal protease reported previously (Rachanamol *et al.,* 2017).

Currently available fibrinolytic agents for clinical use are mostly plasminogen activators including urokinase, and bacterial plasminogen activator streptokinase. They have shortcomings like low fibrin specificity, are very costly and cause undesired side effects including gastrointestinal bleeding and allergic reactions (Mahajan *et al.,* 2012). Protease ZMS-2 exhibited excellent anti-coagulant and fibrinolytic potential in vitro which may be due to the proteolysis of blood clotting factors resulting the obliteration of thrombin generation and platelets aggregation. The main edge of fibrinolytic proteases over other plasminogen activators is their higher specificity and cost effectiveness.

Microscopic analysis of dehaired skin revealed degraded epidermis, hair bulb and complete recovery of hair, therefore, our study confirms that proteases are efficient and ecofriendly alternative for dehairing of animals skins. Furthermore, deharing potential and comparable results for structure of epidermis and hair bulb have previously been reported (Huang *et al.,* 2003; Macedo *et al.,* 2005). Further studies leading to the purification and understanding the mode of action are necessary for its use as potential pharmaceutical and other commercial product.

CONCLUSION

This study reports the isolation of *Bacillus tequilensis* strain for the first time in Pakistan. The newly isolated strain was concluded as a potent producer of thermophilic alkaline protease by utilizing various agro-based substrates. Protease ZMS-2 has shown antibacterial, anti-coagulant, fibrinolytic and dehairing potentials suggesting further studies for its use at industrial level.

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