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Detection of Proteases from *Bacillus* Species Isolated from Agricultural Soil

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ABSTRACT

Present research project was conducted to investigate the protease production by gram positive *Bacillus* species and to investigate their role in dehairing of animal hides. Proteases are widely employed as they have replaced the use of conventional chemical based dehairing methods as they are ecofriendly, easy to cultivate and have high productivity rate. Experimental work was conducted in September, 2022 to July, 2023. 25 agricultural soil samples were collected from different local areas of Haripur and subjected to isolation and purification of *Bacillus* strains using LB media. Out of 25 samples, n =19 samples showed growth on LB agar media. These samples were then screened for species confirmation, which was carried out by Culturing, Microscopy and Biochemical testing and further confirmation by MALDI-TOF. Cultural identification showed filamentous and irregular white colonies. Microscopic view showed gram positive purple rods. Biochemical tests were positive for catalase, Simmon citrate and indole test and are negative for urease, TSI and oxidase test. After MALDI-TOF out of n=19 samples, n=4 samples showed confirmation for *Bacillus* subtilis. These samples showed 99.9% similarity with *Bacillus* subtilis. The protease production of these selected strains was checked by performing protease assay using casein as a substrate. Specificity of the proteases was determined by optimum temperature (60°C) and pH (8.0). The results demonstrated that proteases exhibited stability at broad pH (8.0) and temperature (60°C). Furthermore, the potential applications of proteases in dehairing of animal hides was evaluated through enzymatic de hairing experiments using raw hides from livestock. The efficiency of proteases mediated dehairing was compared with traditional chemical and mechanical dehairing method. The findings of this study provide valuable insight into the diversity and potential of proteases produced by *Bacillus* species in agricultural soil and their applications in the dehairing process.

INTRODUCTION

Proteases are a group of enzymes that can hydrolyze peptide bond of proteins. Protease enzyme is present in almost all plants, animals and microorganisms. Proteases are the most dominant groups of enzymes that have many industrial applications because they are easily available and they have a fast growth rate. Proteases from a microbial origin are more preferable for industrial applications because they have more economic value as compare to proteases from animal or plant origin. Microbial proteases are easy to cultivate, they have high productivity rate and they are easy to be manipulated genetically. These enzymes have a great biotechnological importance in leather industry as they are used for de hairing and bating of animal hides. They are used as an alternative to various toxic chemicals which are used in other conventional methods of dehairing. Proteases can be obtained from a variety of sources. Some of them are plant proteases, animal proteases, Microbial proteases, Marine organisms and insect proteases.

Another method for production of proteases is recombinant DNA technology or enzyme engineering technique. This process involves modification of genes encoding proteases which enhance their production, stability, substrate specificity or any other desirable properties. Enzyme engineering techniques can be used to optimize protease production and create variants with improved characteristics for various industrial applications. These methods are used to produce bacteria, yeast or recombinant proteases on a large scale. These methods are used to obtain the highest expression level of alkaline microbial proteases. However, in recent years, the yield of alkaline proteases has failed to satisfy the industrial demands, which caused the problem of

lack of large scale industrial enzymes Researchers are more focused on the study of microbial proteases because they are immensely utilized in industries. These proteases have a lot of biotechnological applications because of their high yield, easy genetic manipulations, cost effectiveness and less time consumption. They account for two-third of microbial share around the globe. The classification of proteases is mainly based on their acidic and basic properties, on the basis of their functional group and on the position of peptide bond.

Keratin proteases also known as keratinases, those enzymes that are capable to degrade keratin protein. Keratin is a structural protein which is found in hair, feathers, nails and other epidermal structures. Microorganisms which produce keratinases are bacteria, fungi, actinomycetes, plants and insects. Keratinases play a major role in textile processing, waste management and biomedical research. Several microorganisms produce alkaline proteases such as *Bacillus*, *Pseudomonas* and *Aspergillus* species. They can also be derived from plant and marine organisms, but microbial sources are preferred because of ease of cultivation and high production yield. These alkaline proteases are extensively found in soil, water, and highly alkaline conditions. Alkaline proteases have different working activity and are widely employed in detergent manufacturing, leather processing, food processing and bioremediation. Acidic proteases are those proteases which exhibit optimum activity at acidic PH ranges. They play important role in various biological processes, they are involved in intracellular protein degradation, facilitating the turnover of proteins within cell. Neutral proteases also known as neutral peptidases are those proteases which show activity in neutral or very low alkaline PH. Their temperature tolerance range is also very low and they belong to the genus *Bacillus*. Neutral proteases have crucial function in biological systems. They play an important role in digestion of dietary proteins in small intestine where PH is close to neutral e.g. trypsin, chymotrypsin and elastases. Microbial proteases are of great importance because of their rapid production rate, stability to changing environmental conditions, high tolerance and relatively low cost. Some of the applications of microbial proteases are protein hydrolysis, food and feed industry, dairy industry, baking industry, meat tenderization, brewing industry, protein modification, waste management, biodegradation, composting, enzymatic waste treatment, waste valorization, detergent industry, photographic industry, pharmaceutical industry, silk degumming, leather industry and peptide synthesis.

The problem is that conventional dehairing process in the leather industry relies heavily on the use of chemical based agents, which pose significant environmental concerns. There is a need for alternative methods that can effectively remove hair from animal hides without compromising the quality of leather. The detection and characterization of proteases produced by *Bacillus* species isolated from agricultural soil offer a promising solution to this problem. Bacterial proteases play a significant role in leather industry by facilitating the degradation of collagen and other proteins found in raw hides, leading to improved leather processing, quality and yield. The identification and understanding of these proteases and characteristics of these *Bacillus* strains could potentially offer promising opportunities for the development of eco-friendly and cost-effective enzymatic treatments in the leather manufacturing sector.

Identification and characterization of proteases from *Bacillus* species can contribute to the development of more efficient and eco-friendly processes in these industries. Proteases isolated from agricultural soil for dehairing purpose offer significant advantages including sustainability, environmental friendliness and improved product quality. By exploring the proteases produced by *Bacillus* species isolated from agricultural soil, this research can provide an eco-friendly alternative for dehairing, reducing the reliance on chemical based methods. The utilization of proteases from *Bacillus* specie offers a greener and more sustainable approach to leather production.

MATERIAL AND METHODS

About 25 different samples of soil were collected from some local areas of Haripur district, Pakistan. These areas include Khanpur, Mang, Tofkiyan, Joulian, Jab, TIP, Hattar, Sikandarpur, Sarai Naimat Khan, Nartopa, Kaag, Darwaza, Beer, Mankarai and Pharhala. These samples were collected in sterile polythene bags and properly labelled. To ensure sterility, all of the equipments were autoclaved, and the samples were cultured in relevant media by providing relevant conditions to isolate the appropriate bacteria. Different types of biochemical tests (Simmon citrate test, Catalase test, Indole test, Methyl Red test, Urease test, TSI test, Oxidase test) and gram microscopy was performed for initial screening and to confirm the bacterial strain.

Preparation of Preservation Media

Confirmed isolated strains were preserved for the future use. Preservation media was prepared by adding 70% of distilled water to 30% of glycerol, then autoclaved. After this, 1ml of the media was taken in sterile Eppendorf tube and colony from a pure culture was added with the help of sterile loop. Then these tubes were placed in refrigerator and preserved at -20°C.

Confirmation of *Bacillus* Strains by using MALDI-TOF Technique

Matrix-assisted laser Desorption/ionization-time of flight is a powerful method for identifying bacteria. First, a small sample of bacteria is mixed with a special matrix,

which helps to create ions when hit by a laser. This matrix is then dried on a metal plate. The plate is then placed in a mass spectrometer, which is a machine that measures the masses of molecules. When the plate is exposed to a laser, a small portion of the bacterial sample turns into ions (charged particles). These ions travel through a tube and their "time of flight" is measured. The time it takes for each ion to reach the detector depends on its mass. The mass spectrum generated from this data serves as a unique fingerprint for the bacterial species. By comparing the spectrum to a database of known bacterial fingerprints, the specific bacterial species can be quickly and accurately identified.

Casein Test

To determine the production of proteases from *Bacillus* species, a proteases assay was performed by using casein as a substrate.

Preparation of Subculture

Before carrying out casein test, a subculture was prepared by inoculating a loopful of bacterial colony in 10ml of nutrient broth in a screw cap tube by using a sterile loop in a biosafety cabinet. Then it was grown in a psychotherm incubated shaker for 72 hours at 31ºC. Then culture was centrifuged for 10 minutes at 10,000 rpm at 4ºC. Then supernatant was collected and used as a crude enzyme sample.

Protease Assay

Then a protease assay was carried out for determining protease activity by using casein as a substrate. For this assay, first 1.5% casein was mixed in 0.05 M tris HCl. Then 500µl of crude enzyme sample and 500µL of 1.5% casein in 0.05 M tris HCl was taken in a screw cap tube. This mixture was then incubated in a water bath at 37ºC for 30 minutes. Then 3 ml of tri chloro acetic acid was added in each of the sample mixture to stop the reaction and sample was put on ice for 15 minutes. Solution was then centrifuged at 5000rpm for 5 minutes and supernatant was collected. In the 2ml of supernatant 2ml of 0.5 M NaOH solution was added and mixed well to make a final volume of 4 ml. In the same manner blank solution was also prepared except for that 3ml of tri chloro acetic acid was added before enzyme was added. At the end absorption was measured at 440nm.

Temperature Effect on Protease Activity

For determination of the effect of temperature on protease activity, 1% casein solution, 0.06 M $CaCl_2$ solution and 0.2 M Tris-HCl buffer (pH 8.0) were prepared. Then 1ml of 1% casein, 100µl of 0.06 M $CaCl_2$, 100µl of 0.2 M Tris-HCl buffer and 100µl of crude enzyme sample were mixed 24 in screw cap tubes and then incubated for 10 minutes at various temperatures (37^0, 40^o, 50^o, 60^o, 65^o) and absorbance was measured at 440nm after each incubation to determine the protease activity.

pH Effect on Protease Activity

To determine the effect of pH on protease activity, different buffers with different pH ranges were used. These are Acetate buffer, Sodium Phosphate buffer, Tris HCl buffer and Glycine NaOH buffer. All these buffers were used at 0.05 M concentrations.

Salts Effect on Protease Activity

To determine the effect of salts on protease activity, different salt solutions were prepared ($ZnSO_4$, $MgSO_2$, $CuSO_4$, NaCl, KCl). 200µl of enzyme solution, 400µl of 1% casein and 400µl of each salt were mixed, and spectrophotometry was carried out at 440nm to measure the effect of these salts on protease activity.

Temperature Effect on Bacterial Growth

For the effect of temperature on bacterial growth, the bacterial culture was inoculated in nutrient broth in sterile test tubes and incubated at different temperatures (25°, 30°, 40°, 50°, 60°, 70°, 80°) for 48 hours. After incubation, the growth profile of bacteria was checked by measuring the absorbance at 440 and 600nm.

Direct Dehairing Activity of Enzyme

To check the de hairing activity of enzyme, fresh goat skin was bought and cut into 4×4 grid before washing and it was incubated for 24 hours at 37°C in a sterile polythene bag. Then bacterial colonies were inoculated in nutrient broth and incubated at 37°C for 20 hours. Then they were centrifuged at 4000rpm for 8 minutes and supernatant was collected. This supernatant was added on already incubated goat skin. 1% sodium azide solution was also added in the supernatant so that no organism can grow. Then the de hairing capability of organisms was checked by slightly rubbing the goat skin. Nutrient broth was used as a negative control.

RESULTS AND DISCUSSION

Only 9 soil samples showed the growth of different types of bacteria on Luria Bertani agar media from selected 25 soil samples as shown in Table 1. Culture based identification of bacterial isolates were confirmed with gram microscopy, biochemical testing and MALDI-TOFF technique.

Table 1

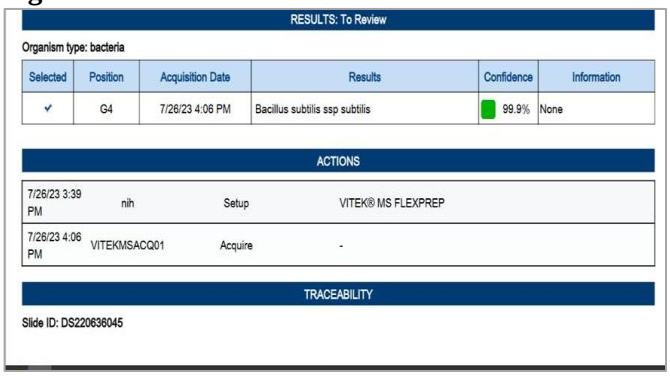
Interpretations of biochemical test of culture based identification

Sample Code	Colony Appearance	Gram Microscopy	Simmon Citrate	Catalase Test	Indole Test	MR Test	Urease Test	TSI Test	Oxidase Test
S2	Circular gray white colonies	GPR	+	+	+	+	-	-	-

S4	Gray white colonies	GPR	-	+	-	+	+	-	+
S9	Filamentous white colonies	GPR	+	+	+	+	-	-	-
S11	Straight fuzzy colonies	GPR	+	+	+	+	-	-	-
S13	Flat white colonies	GPR	+	+	+	+	-	-	-
S14	Straight gray white colonies	GPR	+	+	+	+	-	-	-
S15	Filamentous colonies	GPR	-	+	+	+	-	-	-
S18	White colonies	GPR	+	+	-	+	-	-	-
S21	White colonies	GPR	+	+	+	+	-	-	-

Confirmation of *Bacillus* strain by using Maldi Toff Technique

Figure 1



From the measured absorbance for each sample, proteolytic activity was calculated by using formula

Proteolytic activity = $Absorbance\ of\ sample - Absorbance\ of\ control\ Absorbance\ of\ standard\ solution \times 1\ T$

Where,

· Absorbance of control = 0.009

Control solution was prepared in the same manner except for that 3ml of tri chloro acetic acid was added before enzyme was added.

· Absorbance of standard solution = 0.003

Distilled water was used as standard solution

T = Time taken for spectrophotometry i.e 2 minutes

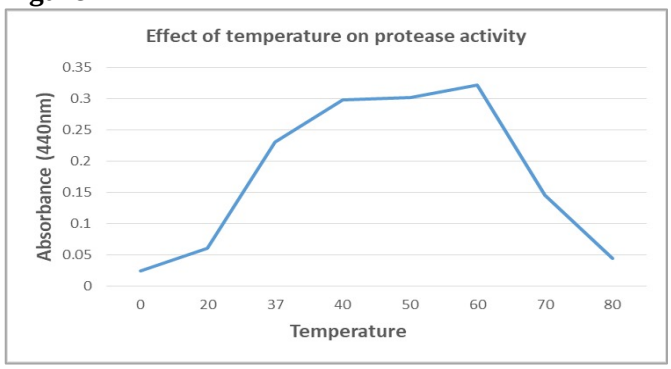
By protease assay, the proteolytic activity was found to be 22.8, 20.6, 19.6 and 24.8 for the samples which showed protease production by *Bacillus* subtilis strains. It was as high as 24.8 U/ml. These protease were mostly active at pH 8.0 and 60°C with casein as substrate. These proteases were also activated by MgSO4, CuSO4, ZnSO4 , NaCl, KCl.

Table 2

Measured absorbance and calculated protease activity

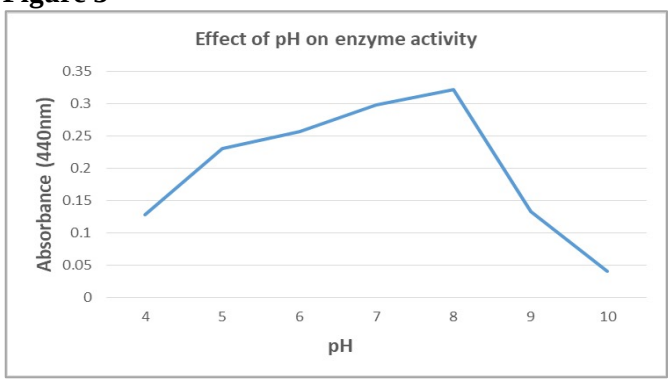
Samples	Absorbance (440nm)	Protease activity (U/ml)
S2	0.146	22.8
S4	0.133	20.6
S9	0.127	19.6
S11	0.158	24.8

Figure 2



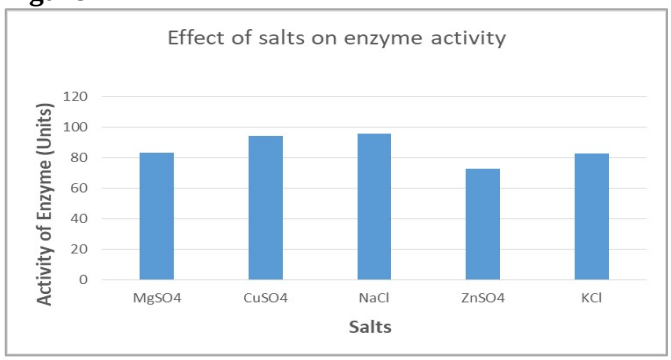
Proteases activity increases with increase in temperature and their activity is maximum at optimum temperature (600°C) and above that there is a certain decline in protease activity.

Figure 3



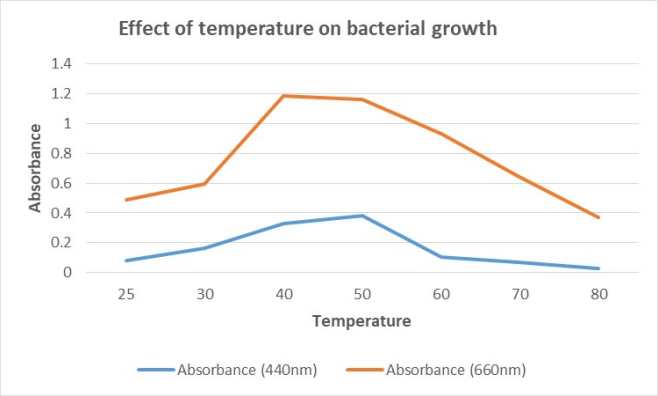
Proteases activity increases with increase in pH and their activity is maximum at optimum pH (8.0) and above that there is a certain decline in protease activity

Figure 4



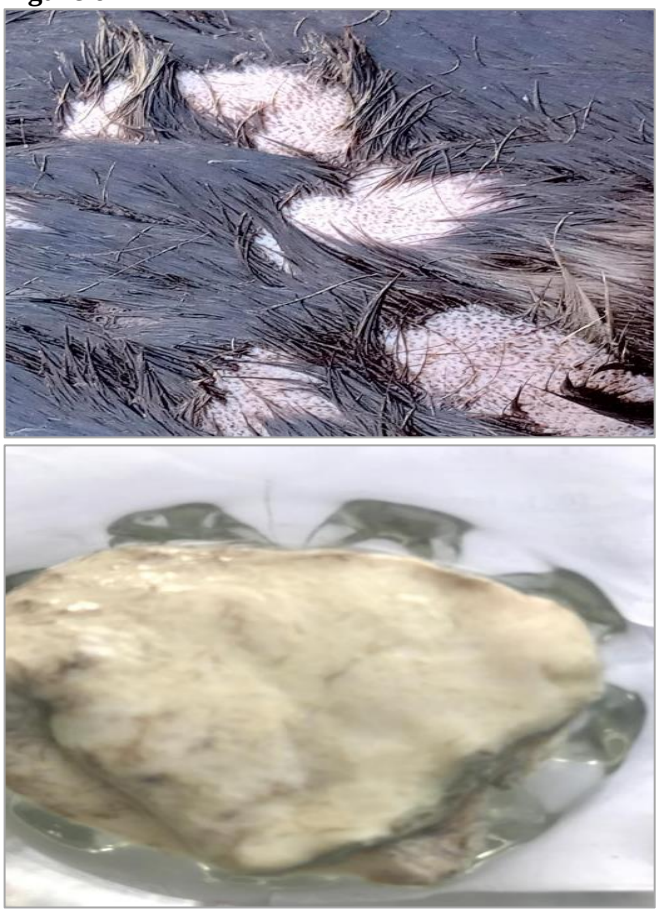
By protease assay, the effect of salts on enzyme activity was found to be 83.16, 94.5, 96.1, 73 and 82.8 U/ml, which showed that these proteases were activated by $MgSO_4$, $CuSO_4$, $ZnSO_4$, NaCl, KCl.

Figure 5



Bacterial growth increases with increase in temperature and also with the increase of wavelength. Once the optimum temperature is achieved, bacterial growth started to decline

Figure 6



A 4×4 piece of animal skin was divided into 9 equal sections. After incubating for 12 hours, hair were removed from 4 out of 9 sections, constituting 44% hair removal. Subsequently, following a 20 hour incubation, hair were removed from 8.5 out of 9 sections, resulting in 95% hair removal.

DISCUSSION

Bacillus species are most commonly found in agricultural soil and known to be prolific producers of various enzymes, including proteases. Through various experimental analyses, it was successfully identified that several *Bacillus* species exhibit proteases activity. The characterization of proteases is crucial as it provides valuable information about their potential applications in various industrial sectors, including agriculture and biotechnology. Biochemical testing and Molecular identification showed the detection of *Bacillus subtilis* which is isolated from agricultural soil. Initially, much study was not conducted on proteases because suitable proteases have not been found for dehairing activity. But this study shows that proteases could accomplish the whole process of dehairing and they are also helpful in leather industry. The results of this study demonstrated that *Bacillus* species are capable of producing proteases. *Bacillus* strains are known for their diverse enzymatic activities and many of them produce proteases to support their growth and survival. The presence of various proteolytic enzymes in *bacillus* specie has been reported indicating their potential for various industrial applications. Proteases produced by *Bacillus* species have shown promising enzymatic activity as they have the ability to breakdown collagen and they can also facilitate the dehairing process. This enzymatic treatment results in more soft and supple leather as compare to conventional chemical treatments. Furthermore, protease based dehairing processes are more likely to enhance the quality and uniformity of leather, which leads to improved final product formation. Proteases are ecofriendly alternative that can significantly reduce the environmental impact of leather processing Casein assay was performed which was easy, quick and simple assay which is used to assay proteases from different bacteria. The assay was carried out by setting up assay conditions, where the optimum temperature for enzyme activity was 60^{o}C and optimum pH was 8.5 which shows the alkaline nature of proteases. 60^{o}C was not the only optimum temperature for proteases as some of the proteases also show optimum activity in the range of 30°C - 80°C and optimum PH also fluctuates depending on the specie from which proteases are isolated. The dehairing was carried out by continuous incubation of animal skin ranging from 6-12 hours and dehairing was carried out at different time intervals because bacterial isolates produce moderate to high amount of enzyme for dehairing. After 6 hours of incubation 5-10% of hair removal has been observed by gentle rubbing. After 8 hours of incubation, 50-60% hair removal has been observed but complete dehairing was achieved after 12 hours of incubation. *Bacillus* derived proteases had gained significant attention in leather industry due to their effectiveness and environmental friendliness as an alternative to chemical dehairing

agents. The use of proteases not only result in better quality leather but also reduces environmental pollution associated with traditional chemical dehairing processes. The use of proteases in leather industry offers several advantages over traditional chemical based methods. Thermostable proteases have advantages in various fields due to their resilience against denaturation and enhanced activity at higher temperatures, which can lead to improved efficiency and cost-effectiveness in processes like protein degradation, food processing, and detergent production. To focus on the thermostability of proteases, which are naturally found in both mesophilic and thermophilic microorganisms. Noteworthy, they encompass a wide thermal activity range (40°C - 80°C), and facile adaptability to large scale production due to straight forward cultivation and maintenance protocols. *Bacillus* subtilis holds significance in the industrial manufacturing of thermophilic proteases. Research into growth conditions involving temperature and PH implies the likelihood of Bacillus subtilis being both alkaline and thermophilic. The detection and characterization of proteases from *Bacillus* species provided valuable insight into the enzymatic capabilities of microorganisms and their potential applications. The identified proteases- producing *Bacillus* strains could be further explored for developing bioformulations, eco-friendly agricultural practices and sustainable leather processing technologies.

CONCLUSION

The detection of proteases from *Bacillus* species highlights their potential in various biotechnological applications, particularly in the leather industry. The study confirms that *Bacillus* strains are efficient producers of proteolytic enzymes, which can be utilized as eco-friendly alternatives to conventional chemical-based dehairing processes. Microbial proteases offer a sustainable approach to removing hair from animal hides, reducing environmental pollution and minimizing the use of harmful chemicals such as sulfides. Further research on optimizing protease production and activity can enhance their efficiency in leather processing, contributing to a more sustainable and environmentally friendly tanning industry.

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