Research Article

Isolation, screening, characterization of proteolytic bacteria and production of protease with its potential applications

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Citation

Aneela Sharif, Zahida Nasreen and Sehrish Kalsoom. Isolation, screening, characterization of proteolytic bacteria and production of protease with its potential applications. Pure and Applied Biology. Vol. 9, Issue 4, pp2250-2271. <http://dx.doi.org/10.19045/bspab.2020.90240>

Received: 09/03/2020

Revised: 12/06/2020

Accepted: 16/06/2020

Online First: 07/07/2020

Abstract

The purpose of this study was production of protease enzyme by isolated bacteria and evaluation of their potential for various industrial applications. Two strains AN16 and AN13 exhibiting maximum proteolytic activity were used for further studies. The activity of crude enzymes was completely inhibited by phenyl-methyl-sulphobnly fluoride (PMSF) and ethylene diamine tetra-acetic acid (EDTA) had no such negative effect on activity. Enzymes were relatively stable in presence of surface active agents such as Triton X-100, Tween-80, Tween-20 and oxidizing agent H₂O₂. The enzymes exhibited maximum activity within pH range of 7-9. The optimum temperature was recorded to be 37°C. The relative activity of isolate AN13 protease in presence of metal ions Ca²⁺, Fe²⁺, Mn²⁺, Mg²⁺, Zn²⁺, Co²⁺, Hg²⁺, Na⁺ was as follow: 107%, 99%, 156%, 100%, 105%, 97%, 90%, 104% and 102%, 101%, 145%, 80%, 98%, 90%, 98%, 96% for AN16 protease, respectively. The degradation percentage of chicken feathers by crude protease was recorded to be 55.50% for AN16 followed by AN13 (39.79%). Study of dehairing activity of protease resulted in complete removal of hairs from hide. De-staining studies showed excellent proteinaceous stain removal efficiency. Antibacterial and antifungal activity of synthesized Silver nanoparticles (AgNPs) used against *E. coli* (25mm), *Salmonella typhus* (18mm) and fungal strain *Malassezia* (17mm) were also recorded. Removal of gelatin from waste x-ray films by enzyme extract showed a weight loss of about 6% (AN16) and 5% (AN13). Different low cost media were also tested to support the growth of these proteolytic bacteria exhibiting positive results.

Keywords: AgNP; Dehairing; Detergents additives; Feather degradation; Protease; Silver Recovery

Introduction

In recent years, there is tremendous rise in the usage of biological catalyst enzymes as industrial catalytic agents. Protease (synonymous as peptidase or proteinase) constitutes one of the largest category of enzymes, which are being used in different industrial sectors [1]. Proteases are enzyme

capable of catalyzing hydrolytic reactions in which protein molecules are degraded to peptides and amino acids [2, 3].

Protease comprises 60% of enzyme sales globally. Protease has wide applications in different industries such as laundry detergent additives, for synthesis of peptides, food processing, animal feedstuff production,

animal hide processing, in pharmaceutical industry, production of amino acids, as cleaning agents of contact lens, sewage treatment etc. [4, 5].

Proteases are produced by different microbes such as bacteria, yeasts, actinomycetes molds, and are also produced by different plants and animals [6]. Microorganisms are an attractive source of protease, due to the distinct advantages they offer over plant and animal protease [7].

Microbial protease offer several advantages therefore proteases of microbial origin are given preference then those produced by plants and animals [8, 9].

Large varieties of proteases are produced by microorganisms that are either intracellular or extracellular. Extracellular proteases are important for microbial cell because these are able to hydrolyze proteins in cell-free settings. Because of this property, extracellular proteases are valued in different industrial applications where they assist in protein degradation and thus are commercially important [10].

Globally, millions of tons of feathers are produced as a waste by-product by poultry sector annually [11] which contains about 90% keratin that offers high degree of recalcitrance [12].

Degradation of feather keratin by Bacterial strains producing keratinases is an alternate way to increase the nutritive worth of waste feathers and to avoid environmental pollution. There is increasing attentiveness toward proteases of microbial origin in different commercially important areas of biological sciences such as environment related studies, biomedicine and biotechnology [13].

Keratinase produced by bacteria which are a type of microbial proteases are of great interest due to their effectiveness against unsolvable keratinous waste. There has been extensive research to find out feather keratin hydrolysis potential of protease enzymes that

is a by-product of poultry industry being produced in large quantities [14].

It was stated 35% of the total enzymes produced by various microbes are utilized in detergent industry [15].

Alkaline proteases with keratin hydrolytic action also offer an efficient alternate for treatment of animal hide. Alkaline proteases are also used for recycling of waste x-ray films for recovery of silver. Alkaline proteases are being used for managing effluents from different food based industries and household wastes [16].

Alkaline proteases have high stability even in the existence of different metal ions, denaturing agents, surfactants, oxidants and detergents. Along with these features oxidant and pH stability of a protease are also most prominent features for their application in detergents and bleaches to improve the washing efficiency. High thermo-stability of alkaline proteases appears to facilitate washing. The use of proteases in detergents could be a possible solution for the problem of removing proteinaceous stains from fabrics [17].

Proteases that have been explored so far are not enough to meet current high industrial demands. Therefore, continuous efforts are being put forward by researchers to find a proteases having novel characteristics of industrial interest from different bacterial strains [18].

Nanotechnology encompassing production and usage of nanoparticles is a rising field having substantial potential for applications in large number of industrial sectors [19]. Silver nanoparticles (AgNPs) are widely acknowledged now-a-days due to their potential for application in many fields such as pharmaceutical industry, biomolecular detection and also can be used widely for their antimicrobial activities [20].

Gelatin is a protein that is present in animal collagen tissues that usually contain high proportion of glycine, proline and 4-

hydroxyproline amino acid residue. X-ray films are coated with emulsion layers made up of silver and gelatin, so there is possibility to degrade this gelatin coating by protease and to release and recover silver [21]. Silver is an important metal from industrial point of view and is being applied in different sectors such as x-rays and photographic films, silverwares and electronic devices. Recovery of silver from used X-ray films is desirable in order to conserve this valuable metal, being important also from environmental view point [22]. Silver is preferred for x rays and photographic films due to its high quality and light-sensitive properties for creating a photographic image. Burning the X-ray films for silver recovery can create environmental problems as well as health issues. On contrary, enzymes from microorganisms break the gelatin layer embedded with silver in films generating pollution free stripping [23].

Different types of agricultural left over materials such as sugarcane waste, wheat bran, rice bran, potato peel, green gram husk, oil cakes etc. are valuable for supporting growth and production purpose of different enzymes such as cellulase, insulinase, lipase, protease and so on. The agricultural waste by-products can be utilized as substrates for production of enzymes and is advantageous because these are inexpensive, their readily availability and their non-toxicity [24].

Therefore, this study focuses on isolation and screening of potential bacterial strains capable of producing proteases enzyme, utilization of agro-based waste substrates for enzyme production and usage of protease enzyme in various industrial applications. In this research work, soil samples were obtained from various locations of Mianwali District of Punjab, Pakistan for screening of proteolytic bacteria. More over extraction of proteases and their industrially valued applications are assessed.

Materials and methods

Sample collection

Poultry farms and alkaline soil were selected for collection of soil samples. These samples were collected by using sterile spatula in plastic bags and were carried to microbiology laboratory by maintaining aseptic conditions. For future use, these samples were stored in laboratory.

Isolation of Bacteria

The microorganisms were isolated by serial dilution and spread plate technique on the Casein Agar Media (CAM) containing casein 2%, peptone 0.5% and agar 1.5% [25]. In this method, 1.0 g soil sample was added to 100ml of distilled water for making a suspension of sample and mixed it well for 15 min. Suitable dilutions of the sample suspension were prepared and 0.1 ml was drawn from each dilution, spread onto the plates of CAM, using sterile glass spreader and incubation was done for 24 hours at 37°C for observation of bacterial growth [26]. The clear halo areas of casein hydrolysis were an indication of protease secretion [27]. Then, the protease producers were repeatedly sub cultured on Nutrient Agar Media (NAM) and CAM for pure culturing of bacterial isolates and were preserved on CAM slants at 4°C. Isolated strains were named as AN1, AN2, AN3, AN16.

Low cost media screening

Different agro-based waste materials (rice bran M1, wheat bran M2 [28], orange peel M3, green gram husk M4 [28] banana peel M5 and potato peel M6 [29] were collected from local markets, processed and grinded to obtain suitable particle size. These were then labeled and stored till further use. Cotton oil cakes MO1, Mustard oil cakes MO2 and MO3 (Cotton oil cakes +Mustard oil cakes) were also tested to check their ability of supporting the growth of proteolytic bacterial strains. All of the above mentioned different substrates were tested for growth of proteolytic bacteria. These substrates were

also used in different combinations in order to check which combination supports maximum growth.

Preparation of low cost substrate media

Different agro-industrial waste materials, viz. orange peel, green gram husk, rice bran, wheat bran, potato peel, banana peel, cotton oil cakes and mustard oil cakes were obtained and dried using sunlight. These completely dried waste materials were then grinded in a mixer grinder and stored for further use [30]. These agro-based substrates were then screened for the production of protease with little modifications [31]. 4g of each of the above mentioned substrate and along with agar were added in 100ml of distilled water in 250ml Erlenmeyer flasks. The cotton plugged flasks were autoclaved for 15 mins at 121°C and then were allowed to cool. The contents of the flasks were then poured into petri plates and allowed to solidify completely. All strains were then streaked onto the plates and incubated for 24 hours at 37°C.

All the strains were studied to determine colonial characteristics. Colonial characteristics of all 16 isolated proteolytic strains were noted down. Characteristics of

all strains were determined on CAM and also on Low cost media plates and were noted down.

Determination of proteolytic activity

The Proteolytic activity of all isolated strains was studied by using skim milk agar medium. The milk agar media was prepared according to [32] with little modification. This media was then autoclaved and poured in petri-plates (20ml/plate). After complete solidification of the media in plates, strains were streaked onto the surface of media (Fig. 1) and on separate milk agar plates wells of 5 mm diameter (4 wells per plate) were cut under sterile conditions. These wells were then inoculated with 10µL of nutrient broth culture media having 24-hour growth of selected strains. The plates were subjected to incubation at 37 °C for 24 hours. Protease production was examined by zone of clearance of opaque milk protein. The diameter of the halo or clear zone formed around the well was measured in order to quantify protease enzyme activity [33]. The high yielding protease strains were assessed by measuring the zone of hydrolysis on skimmed milk agar.

Figure 1. Proteolytic activity of isolated bacteria on skim milk agar media. Proteolytic activity was confirmed by appearance of clear zones around streaked strains

Production of crude protease enzyme extract

The inoculum from pure culture of each isolated proteolytic strain was inoculated into

100 mL of alkaline protease broth (0.5% glucose, 0.75% peptone, 0.25% yeast extract, 0.1% K₂HPO₄, 0.1% MgSO₄, 1.0% Na₂CO₃, 1.0% NaCl), pH adjusted to 9.0 and

incubation was done at 37°C [34]. The fermented alkaline protease broth was then subjected to centrifugation at 10,000 rpm for 10 mins. The obtained cell –free supernatant served as crude enzyme extract [35].

Proteases assay

The protease assay was carried out to measure protease activity as described by [27]. The total volume of reaction mixture was 600 µL and its components include 0.5% azocasein in 50 mM of Tris-HCl buffer and suitably diluted crude enzyme extract. The incubation of reaction mixture was carried out for a period of 1 hour at 37°C and then 0.5 mL of 10% trichloroacetic acid (TCA) was added to stop reaction. The un-reacted azocasein precipitate were separated by centrifugation, 1000 µL of supernatant was obtained and equal volume of 1.0 M NaOH was mixed. Spectrophotometer was used to read Absorbance at 440 nm. One unit of protease activity was defined as the amount of enzyme producing 1µg of tyrosine in 1 min under assay conditions. All protease assays were done in triplicate.

Characterization of protease

Surface active agents (surfactants) and enzymes inhibitors effect on activity of protease

The effects on enzymes activity under different surfactants as, Tween 20, 80, Triton X-100 (0.2% and 0.4% final concentration), oxidizing agents such as H₂O₂ and enzymes inhibitors including, phenyl-methyl-sulphobnnyl fluoride (PMSF) and ethylene diamine tetra-acetic acid (EDTA) were evaluated by incubation of enzymes for 10 min with each chemical at room temperature. The activity in the absence of inhibitors and surfactants (control) were referred 100% relative activity. The residual activities were studied and compared to controls.

Effects of metal ions on enzyme activity

CaCl₂, MgSO₄, ZnSO₄, FeSO₄, CoCl₂, HgCl₂, NaCl and MnSO₄ were added to reaction mixture at 1mM concentration to

study effect of metal ions on enzyme activity. Relative activity of enzyme were recorded at room temperature. The enzymes activity without metal ions under similar conditions was expressed as 100%.

Determination of thermostability of protease enzyme

The crude enzymes activity within a temperature range of 37°C to 50°C were assayed to determine effect of temperature on enzyme activities. The enzymes were preincubated for one hour in 40 mM sodium phosphate buffer to measure thermo stability of enzymes.

Effect of pH on enzyme activity

The crude enzymes activity was assayed was at pH values within range of 6 to 12 in sodium phosphate buffer (40 mM). The enzymes were preincubated for 1 hour at 37° C to measure activity under assay conditions.

Applications of bacterial protease enzyme extract

Inoculum for feather degradation studies

The growth medium for feather degradation studies was prepared [36]. 50ml of alkaline protease broth was prepared as described above, sterilized at 121°C for 15mins and inoculated with the isolate AN13 and AN1 strains. The incubation of broth was done for 24 hrs. at 37°C and used as inoculum.

Degradation of chicken feather

Degradation of chicken feather was carried out as described by [36]. The feathers were cut into small pieces of about 1cm long and added to the fermentation media in flasks. The flasks were then incubated at 37°C for 4 days. The resulting degradation of keratinous waste was determined in percentage (%) of weight loss after 4 days of incubation and absorbance of reaction mixture was determined after 4 and 8 days of incubation

Evaluation of degree of degradation (DD)

Degree of degradation was measured as per method stated by [36]. Feathers were weighed before and after degradation. The residual feathers after completion of 4 days

period of incubation were removed from reaction mixture and were washed and completely dried to evaluate degree of degradation by using the following formula:

$$DD (\%) = (TF - RF) \times 100 / TF$$

Where,

TF –Total Feather

And

RF –Residual Feather.

De-Hairing Activity of protease enzyme

Broth for protease enzyme production was prepared, inoculated with strains AN13 and AN16 and incubated for a period of 3 days [37]. The supernatant of alkaline protease broth was centrifugated at 10,000 rpm for 15 mins and it was used as crude enzyme for dehairing purpose.

Dehairing studies were done by adding the crude enzyme extract on detergent washed cow hide by dip method, to check for its ability to remove hairs. In this method, washed hide pieces were immersed in 10ml of crude enzyme extract in petri plates and then incubated at 37°C for 14 hours [38].

Proteinaceous stain removal studies

Detergent additive properties of bacterial proteases enzyme was studied on white cotton cloth pieces (10×10 cm) blemished with chocolate, and spicy food as per [39].

The following experimental groups were set to study stain removal efficiency of protease enzyme:

1. Flask with distilled water (100 ml) + stained cloth (stained with spicy food and chocolate).
2. Flask with distilled water (100 ml) + stained cloth (stained with spicy food and chocolate) + 1 ml Ariel detergent (10mg/ml).
3. Flask with distilled water (100 ml) + stained cloth (stained with spicy food and chocolate) + 1 ml Ariel detergent (10mg/ml) + 2 ml of enzyme extract.

All the flasks were then kept in incubator at 60°C for 15 minutes to check removal of proteinaceous stains. Untreated stained pieces of cloth were labelled as control.

Synthesis of silver nanoparticles by isolated strains

Isolated bacterial strains AN13 and AN16 were inoculated in 100 ml sterile nutrient broth in Erlenmeyer flask and incubated for 48 h at 37°C. Cultures were centrifugated at 10,000 rpm for 15 minutes to obtain supernatant for AgNPs synthesis. The supernatant was then utilized to study AgNPs production by adding it with AgNO₃ solution at 1mM final concentration [40].

For the synthesis of AgNPs by isolated strains, 5ml silver nitrate (AgNO₃) solution (1mM) was mixed with 10 ml of the supernatant and another solution without silver nitrate addition was labeled as control. The prepared solutions were then incubated at 30°C for a period of 24 hrs. All solutions were incubated under dark conditions to avoid any photochemical reactions. After 24 hours, the solution turned into brown from yellow solution. The AgNPs were purified by centrifugation at 10,000 rpm for 15 mins, supernatant and particles were collected for further use [41].

Characterization of nanoparticles

The color changes from yellow to dark brown color in reaction mixture evidenced the production of AgNPs and this was due to efficient reduction of the Ag ions by bacteria. The exact mechanism of silver ion's reduction and production of silver nanoparticles is still not clear, but it is believed that enzymes including nitrate reductase are the agents responsible for silver nanoparticles synthesis. The colored solution were further studied to measure the absorbance against distinct wave length for confirmation for AgNPs formation.

Formation of silver nanoparticles can be detected easily with the help of spectroscopy. So reaction mixture absorbance was determined at 400nm in order to measures optical density [41].

Antibacterial and Antifungal Activity of silver nanoparticles

Antibacterial activity and antifungal activity of prepared AgNPs were carried out by Well diffusion method against Gram negative bacteria *Escherichia coli*, *Salmonella typhus* and *Malassezia sp.* The bacterial cultures and fungal strains were brought into nutrient broth for antibacterial and antifungal assay. Wells of 5 mm diameter were cut on MacConkey Agar for *E. coli*, *Salmonella-Shigella*(SS) agar for *Salmonella typhus* by sterilized cork borer. Composition of the media used for fungi was: Peptone 2%, Glucose 1%, Yeast extract 0.2%, Ox Bilo 0.8%, Glycerol 1%, Tween 80 0.5%, Agar 1.5%. [42]. Then 1ml of broth cultures were evenly spread on solidified media. 5 μ l of synthesized AgNPs were transferred into the wells and then incubation of plates was carried out at 37°C for 24 hours and the inhibition zones were determined [43].

Clearing of waste X-Ray films and Hydrolysis of gelatin by crude enzyme extract

X-ray films were cleaned by washing with distilled water and wiped with cotton balls soaked with ethanol and were then kept in clean glass Petri plates. The washed films were dried in an oven at 40°C for 20 min. Waste x-ray films (3 x 3 cm pieces) were then subjected to incubation with 10ml of each culture supernatant of AN13 and AN16 so that the films were completely dipped in the solution at 40°C, pH 10 with continuous shaking. There was a gradual increase in turbidity of the reaction mixture (as the hydrolysis continued). Progress of hydrolysis was examined by observing turbidity and determining the absorbance at 400 nm [44].

Statistical analysis

All experiments were conducted three times and the results were stated as the mean. Means, standard deviations (SD) and standard error of mean (SEM) were analyzed using MS Excel. Single factor analysis of

variance (ANOVA) was carried out for some experimental data while remaining data was evaluated by t-test using MS Excel. Results were regarded statistically significant only when p-value was equal to or less than 0.05. All the data are presented graphically as mean $\pm$ S.D. of replicates. The error bars in the experiments indicate $\pm$ standard deviation.

Results and discussion

Isolation of bacteria

A total of 16 morphologically distinct colonies were isolated by using CAM from the collected poultry form soil samples and alkaline soil samples. 6 isolates from poultry form soil samples and 10 isolates from alkaline soil sample were isolated. All the isolated strains were streaked repeatedly on NAM and CAM for pure culturing. Pure cultured colonies were then preserved on CAM slants, covered with paraffin oil and stored till use at 4°C.

Screening of proteolytic activity of isolated strains

All isolates were studied for their proteolytic activity on skim milk agar plates and activity was expressed in mm diameter of zones of clearance. Protease activity was observed from zone of clearance (Fig. 1) formed around the colony on agar surface and also the diameter of zone of hydrolysis around wells was measured as described in (Table 1). Among 16 isolates, AN16 was identified as the most potential strain; since AN16 showed the maximum proteolytic activity having average halo zone of 34.75mm diameter followed by AN13 with clear zone of 31.5 mm and AN9 with zone diameter of 30.5 mm and AN11 of 30 mm (Fig. 2). These AN13 and AN16 strains showing largest zone of clearance were chosen for further studies. The results of the screening studies thus indicated that maximum zone of clearance and thereby maximum protease activity by AN9, AN11, AN13 and AN16 and all the isolated strains are significantly different in their proteolytic activity ($P < 0.05$).

Table 1. Average values of Halo Zone on skim milk agar after 24 hours of incubation

Strains	Diameter of zone of clearance (mm) Means $\pm$ SEM
AN1	28.25 $\pm$ 2.46
AN2	27.5 $\pm$ 1.44
AN3	22.25 $\pm$ 1.10
AN4	20 $\pm$ 0.81
AN5	18 $\pm$ 1.22
AN6	17.75 $\pm$ 1.31
AN7	22 $\pm$ 0.70
AN8	29.75 $\pm$ 1.93
AN9	30.5 $\pm$ 2.17
AN10	22 $\pm$ 1.77
AN11	30 $\pm$ 1.63
AN12	20.25 $\pm$ 3.09
AN13	31.5 $\pm$ 1.5
AN14	29.5 $\pm$ 3.01
AN15	26 $\pm$ 0.57
AN16	34.75 $\pm$ 0.85
Control	Nil

Values represent means of four replicates $\pm$ S.E.M

Figure 2. Zones of clearance of skim milk agar media (mean $\pm$ SD $n=4$). All the 16 isolated strains are significantly different from each other in their proteolytic activity ($P < 0.0001$)

Low cost substrates screening

Substrate serves as mean to support growth of bacterial strains. A desirable solid substrate can support good growth and maximum product formation. It has been reported that at industrial level, substrates

used for enzyme production for various applications account for 40 % of the total cost; therefore, new formulation of media with cost-effective components is necessary [45, 46]. Of all the substrate tested for growth, all the 8 substrates which include

green gram, rice bran, orange peel, banana peel, potato peel and wheat bran, mustard oil cakes and cotton oil cakes ideally supported the growth of proteolytic strains (Table 2). Cattle feed (oil cakes) and green gram husks

supported maximum growth hence it was further use to grow the isolated strains instead of using CAM. Morphological characters of colonies on low cost media were noted down.

Table 2. Growth of isolated strains on low cost media

Isolated Strains	Green gram (M4)	Rice Bran (M1)	Orange Peel (M3)	Banana peel (M5)	Potato peel (M6)	Wheat Bran (M2)	Mustard oil cakes (MO2)	Cotton oil cakes (MO1)
AN1	++	+	+	+	—	+	++	+
AN2	+	—	+	+	+	++	+	+
AN3	+	+	+	+	+	++	+	++
AN4	++	+	—	+	++	+	+	+
AN5	+	+	+	+	+	+	++	+
AN6	+	+	—	+	+	+	+	—
AN7	+	+	+	—	+	—	+	+
AN8	++	—	+	+	+	+	+	++
AN9	—	+	+	—	+	+	++	+
AN10	+	+	—	+	+	+	+	+
AN11	+	+	+	+	—	+	+	+
AN12	+	—	+	++	+	—	+	+
AN13	++	+	+	++	+	+	+	++
AN14	+	+	+	+	—	—	+	++
AN15	—	+	+	—	+	+	++	+
AN16	+	+	+	+	+	+	+	++

(—) represents no significant growth activity; (+) represents significant growth activity; (++) represents highly significant growth activity

Characterization studies

Effect of surfactants and inhibitors on protease activity

Different chemical compounds effect on activity of crude enzyme of isolated strains AN13 and AN16 showed variations (Table 3). Triton-X-100 increased activity of AN13 crude enzymes at 0.4% of final concentration, in contrast to Tween-80 negatively affected enzymes activity especially of AN16 crude enzymes. Tween-20 had no such negative effect on activity of AN13 crude protease and had slight negative effect on AN16 crude enzymes. The enzymes were relatively stable in presence of EDTA. Proteases activity was inhibited drastically under PSMF.

These results are in contrast to earlier reports where all used inhibitors such as EDTA,

Triton-X-100, Tween-80 negatively affected enzyme activities. Activity of protease was decreased 50% with EDTA and 43% with SDS [47].

Metal ions effect on enzyme activity

The enzyme activity in presence of different metal ions is described in (Table 4). The ion Ca^{2+} increased the protease activity thus making enzymes of both strains stable. In this context, the ions Mg^{2+} had no inhibitory effect on AN13 crude enzyme but negatively affected AN16 enzymes and Fe^{2+} had no such negative effect on activity. In contrast to this, Mn^{2+} remarkably increased enzyme activity (56%) which may be due to the fact the enzyme uses Mn^{2+} as a cofactor.

It has been reported that *Bacillus cereus* NC77 produced protease was activated in presence of Zn^{2+} , Mg^{2+} , Mn^{2+} , Ca^{2+} ,

Na²⁺ and Co²⁺ with 153, 134, 116, 128, 125 and 109 % increment in relative activities respectively [47]. The 145, 142, 115, 119,

136, and 133% of relative activity of protease by addition of Zn²⁺, Ca²⁺, Cu²⁺, Mn²⁺, Na⁺, and K⁺ have been reported [48].

Table 3. Effect of some chemical compounds on proteases activity

Chemical compounds	Final concentration	Residual activity of protease enzymes (%)	
		AN13	AN16
Control	-	100	100
Phenylmethanesulphonyl fluoride (PMSF)	0.2%	15	88
	0.4%	6	60
Ethylenediaminetetraacetic acid (EDTA)	0.2%	98	95
	0.4%	96	90
Triton X-100	0.2%	97	99
	0.4%	105	90
Tween-80	0.2%	96	85
	0.4%	90	80
Tween-20	0.2%	99	97
	0.4%	97	90
Urea	0.2%	108	90
	0.4%	99	88
H ₂ O ₂	0.2%	105	100
	0.4%	101	98

Table 4. Effect on different metal ions on activity of protease

Metal ions	Relative activity (%)	
	AN13	AN16
Ca ²⁺	107	102
Fe ²⁺	99	101
Mn ²⁺	156	145
Mg ²⁺	100	80
Zn ²⁺	105	98
Co ²⁺	97	90
Hg ²⁺	99	98
Na ⁺	101	96

Thermostability of enzyme activity

The thermostability of crude enzymes of strains AN13 and AN16 were studied and it was found that enzymes were active within the temperature range of 37°C to 50°C with maximum activity at 37°C (Fig. 3) thus making them suitable for their applications in industries. The crude enzymes activity under different temperatures has been reported earlier. The protease produced by strain

SD11 showed activity within range of 30 to 70°C and highest activity was recorded at 60°C. The relative activity at 50 and 60°C was 97% and 100% respectively.

Effect of pH on enzyme activity

The pH effect on enzyme activity was studied (Fig. 4) and it was found that enzymes showed maximum activities at 7- 9 pH thus making them desirable for future uses.

This type of results has already been reported. *Bacillus cereus* NC77 has been reported to have optimum pH 7.0 to 8.0 for enzyme production [47]. The SD11 Marine

Bacteria protease showed maximum activities at pH between 8.0–11.0 and 10.0 was recorded to be optimum pH [48].

Figure 3. Effect of temperature on crude protease activity of (a) AN13 and (b) AN16

Figure 4. Effect of pH on protease activity of (a) AN13 and (b) AN16

Degradation of Chicken feather and evaluation of degree of degradation (DD)

The crude enzyme extracts from AN13 and AN16 were used for analyzing the degree of chicken feather degradation. After 4 days of incubation, the maximum average degradation capacity was exhibited by AN16 and AN13 was 55.50% and 39.79% respectively (Table 5). Optical density was also measured at 400nm after 4 and 8 days (Fig. 5) of incubation by spectrophotometer.

This is the confirmation of earlier reports where maximum feather degradation capacity was exhibited by different bacterial strains as described by [35]. Degradation capability of the crude enzyme extract could be credited to microbial degradation of feather keratin which involves proteolysis and sulfitolysis. All experiments were performed in triplicates. Hydrolysis of keratin of feathers by bacterial protease preparation was also reported by [49].

Table 5. Average values of Degree of degradation DD (%) = (TF-RF) ×100/TF of chicken feathers after 4 days of incubation

Strains	Initial weight of feather (g)	Weight of residual feather (g) (After 4 days). Means ±S.E.M	Degradation capacity% (DD)
AN13	1	0.6020±9.4876*	39.79%
AN16	1	0.4449±11.4797*	55.50%
Control	1	1±0	0

Asterisk (*) represents statistical significance (*t*-test). P value was found to be statistically significant ($P<0.05$). The p value indicates the comparison of feather degradation activity of two strains as compared to control. This comparison is found to be statistically significant

Figure 5. Optical density of zero samples, after 4 and 8 days of incubation (means ±SD, $n=3$). P value was statistically significant ($P<0.05$)

According to [50], feather weight loss after degradation by *Bacillus licheniformes* was found to be 87.2% and about 49.4% weight loss by *Bacillus subtilis*. Similarly, it was studied that the Keratinase enzyme produced by *Streptomyces sp.* JRS 18 effectively degraded keratin present in chick feathers, thus helping in poultry waste management [13]. Degradation of chicken feathers by *Bacillus sp.* has also been stated by [51]. It was studied that keratinase produced by *Bacillus sp.* possess high feather degradation efficiency [52]. Similarly, [53] found that protease produced by bacterial strain *Bacillus amyloliquefaciens* has potential to degrade chicken feathers. *Bacillus sp.* was found to be able to cause 1% feather degradation within 7 days [54]. *Micrococcus sp.* and *Streptococcus sp.* showed keratin degradation activity as evident from decrease in the feathers weight from 0.05gm, to 0.03gm [55].

De-staining Activity

To study detergent additive properties of protease enzyme for improvement of washing performance of detergent, de-staining studies were carried out. After incubation, cloth pieces were washed with water and air dried. Rinsed cloth pieces were visually examined and it showed that protease enzyme significantly improved removal of stains. The addition of the crude enzyme extract in detergent (Ariel) considerably improved the removal of spicy food and chocolate stains within 15 minutes. The best results were observed when protease and detergent both were used together. The compatibility of bacterial along with detergents has also been reported by [56] where protease retained maximum activity even after treatment with detergent thus proving the constancy of protease in detergents. The protease enzyme produced removed the spicy food and chocolate stains

with great efficiency within the short time period of 15 minutes which indicates it's prospective to be used as detergent additive in laundry detergent industries. This is in accordance with earlier reported work of [57] on laundry detergent additive application of protease where protease enzyme was used in conjunction with detergent to improve proteinous stain removal from fabrics. The similar observations were also reported by [58, 59, 60]. Protease produced by *Bacillus subtilis* also showed high potential of removing the dye and blood stains from cloth. Blood stains were removed efficiently by alkaline protease that was used along with detergent within short period of time as compared to when only detergent was used for stain removal [61]. The protease obtained from *Streptomyces gulbargensis* completely removed proteinous stains within 20 min of incubation of cotton pieces of cloths [62]. Protease enzyme produced by *Bacillus subtilis* was found to be very efficient in removal of blood stain from cloth within 30 min so it can be used as bio-detergent [63]. In the same way, protease was able to retain its 50-76% of activity at 40°C in detergents such as surf excel, Ariel and Ghadi etc. and thus it is compatible to be used with commercial detergents for improving stain removal efficiency [23]. Thus it is proved that protease enzyme can be used in detergents. The *B. licheniformis* crude protease enzyme showed remarkable blood stain removal

efficiency and compatibility with different commercial detergents [64].

Dehairing of hide

Protease dehairing activity was carried out by dip method. After 14 hours of incubation, dehairing activity of enzyme obtained from isolated strain AN13 and AN16 was proved to be efficient in dip method. Adequate dehairing activity by dip method was observed. At the end of the incubation, the skin pieces were scraped gently with the fingers to loosen the hair. Complete skin depilation was observed after incubation of the skin with the partial purified enzyme produced by AN13 and AN16 (Fig.6) whereas, the hair on hide remain intact in control. Similar dehairing studies with bacterial protease were reported earlier by [65]. Dehairing of skin by protease produced by *Bacillus sp.* has also been demonstrated in a study by [66]. [66] also reported complete removal of hair from skin by simple scraping with fingers. [67] reported excellent hide dehairing ability of *Nocardia* *dassonvillei* NRC2aza.

Keratinase produced by *Bacillus thuringiensis* strain Bt407 caused swelling of root hairs and subsequent removal of hair from goat hide after 24 hours of incubation, so it can be used as depilatory agent in leather industry [68]. Some other bacterial strains having similar depilatory effect on hides are *Bacillus pumilus* CBS Alkaline Proteinase [69], *Bacillus cereus* MCM B-326 [70] and *Bacillus safensis* LAU 13 [71].

Figure 6. (a) Cow hides before incubation with protease enzyme extract of AN13 and AN16. (b) Cow hides after 24 hrs of incubation with protease enzyme extract of AN13 and AN16

Synthesis of silver nanoparticles

After 24h of incubation, AgNPs production was confirmed by visual examination of Culture supernatant changing in color from light yellow to brown (Fig. 7). Control (without AgNO₃) exhibited no evidence of color development in the reaction media when incubated for the same time period and under same conditions.

The AgNO₃-treated culture supernatant turned brown in color suggesting synthesis of AgNPs [72]. Thus it supported the fact that

color change is indication of AgNPs formation. Nitrate reductase, a NADPH-dependent enzyme is responsible for change in color due to silver metal ions reduction and thus is the main agent carrying synthesis of silver nanoparticles [73]. Silver ions underwent bio reduction with the help of reduction of specific enzymes leading to formation of silver metal ion aggregates which in turn form the desired silver nanoparticle [73, 74].

Figure 7. (a) Control solution for synthesis of silver nanoparticles by isolated bacterial strains. (b) Solution after synthesis of silver nanoparticles by isolated bacterial strains. Color changes indicate formation of nanoparticles

UV Spectroscopy

The confirmation of the AgNPs synthesis was examined by UV spectroscopic studies by drawing out aliquots of colloidal solution (after reaction completion) that were used for UV-spectroscopic observations. The reaction mixture showed absorbance at 400nm and thus confirmed the nanoparticles synthesis by using the culture supernatant. The UV absorbance is shown in (Fig. 8). Absorbance was measured after 24 hours of incubation. UV Absorbance of zero samples

was also measured. The OD absorption units for AN13 was at 2.213 and for AN16, it was 2.121 after 24 hours of incubation as compared to control with OD absorption units at 1.118 for AN13 and 1.012 for AN16 (Table 6).

The control (without AgNO₃) exhibited no signs of absorption at 400 nm. The samples exposed to the silver nitrate solution showed absorbance at around 400 nm. The occurrence of absorbance indicates the accumulation of the AgNPs in the solution.

Figure 8. Optical density of zero samples and after 24 hrs of incubation (means ±S.D., n=3)

Table 6. OD values of silver nanoparticles solution after 24 hrs of incubation

Strains	Optical Density of zero sample (Means ±S.E.M)	Optical Density after 24 hours (Means ±S.E.M)
AN13	1.121±0.052*	2.213±0.085**
AN16	1.127±0.052*	2.121±0.118**
Control	1.011±0.080	1.011±0.080

Values represent means of three replicates± S.E.M. The values are significantly different in comparison with corresponding control *P<0.001, **P<0.0001

Antibacterial and Antifungal activity of AgNPs solution

The antimicrobial activities of biologically synthesized AgNPs were tested against an isolated pathogenic gram negative bacteria and a fungal strain *Malassezia sp.* and results were recorded in (Table 7). The AgNPs showed strong antibacterial activity against Gram-negative bacteria, *Escherichia coli* and *Salmonella typhi*. The maximum inhibition zone was observed against *Escherichia coli* having a halo zone of 25 mm diameter and maximum halo zone produced against *Salmonella typhi* by the silver nanoparticles was 18 mm. The inhibition zone against isolated fungal spp. was found to be about 17mm in diameter (Fig. 9). No zone of inhibition was observed around the well filled with only 1 mM AgNO₃ (Control) confirming antibacterial activity of bio-synthesized silver nanoparticles. This is in confirmation with earlier reports where microbially synthesized AgNPS exhibited high antimicrobial activity. Silver

nanoparticles produced by *Bacillus sp.* and marine bacteria *Ocrobactrum anthropi* exhibited zone of inhibition measuring 13mm and 14mm against *Salmonella typhi*, 17 mm and 15mm against *Salmonella paratyphi* respectively while both AgNPs exhibited 15mm zone of inhibition against *Staphylococcus aureus* [75]. Antibacterial action of silver nanoparticles synthesized by *Streptococcus sp.* showed very strong inhibitory effect against *S. typhi* (40mm inhibitory zones) followed by *E.coli* (34mm zone of inhibition) [76]. Inhibitory effect of AgNPs against *E.coli* has also been described [77]. Silver nanoparticles are also been reported to be effective against important disease causing bacterial strains such as *E.coli*, *Salmonella typhimurium*, *Bacillus subtilis* and *Enterococcus faecalis* [78]. The general mechanism behind antibacterial properties of silver nanoparticles is the Ag ions from nanoparticles that are supposed to get attached with cell wall of bacteria having negative charge and further causing its lyses.

This in turn leads to denaturation of major proteins thus causing cell death [79]. The nanoparticles synthesis mechanism is considered to be reduction of metal ions, in this case Ag ions into silver nanoparticles by bacterially secreted enzymes such as nitrate reductase [19]. Similar process has been discovered in *Bacillus licheniformis* where nanoparticles synthesis by silver ions reduction was observed and enzyme responsible for it was found to be nitrate

reductase [80]. The syntheses of silver nanoparticles by *Bacillus* strain and by *Bacillus megaterium* have also been reported [81]. Antifungal properties of AgNPs have also been reported. It is shown that AgNPs have significant antifungal activity against plant pathogenic fungus *Rhizoctonia solani* [82]. Likewise, Silver nanoparticles can be effective as antifungal agent against *Aspergillus sp.* [83].

Table 7. Silver nanoparticles zones of inhibition against *E. Coli*, *Salmonella typhus* and a fungus *Malassezia spp.*

Strains	Halo zones Diameters (mm) (Means $\pm$ S.E.M)		
	<i>E. Coli</i>	<i>Salmonella typhus</i>	<i>Malassezia spp</i>
AN13	16.5 $\pm$ 3.30*	6.75 $\pm$ 1.43**	10.5 $\pm$ 0.64*
AN16	11 $\pm$ 1.41*	15.25 $\pm$ 1.37**	11.75 $\pm$ 2.78*
Control	2.12 $\pm$ 0.27	2.02 $\pm$ 0.40	1.62 $\pm$ 0.31

Values represent means of four replicates $\pm$ S.E.M. Values are significantly different in comparison with control (One way ANOVA). * represents p value (P<0.01). ** represents p value (p<0.001)

Figure 9. Silver nanoparticles (produced by AN13 and AN16) zone of inhibitions against *E. coli* *Salmonella typhus* and a fungal specie *Malassezia*. (Means $\pm$ S.D., n=4)

Gelatin hydrolysis and release of silver from Waste X-ray films

Incubation of waste X-ray films with proteolytic enzymes released gelatin bound with silver into the reaction solution and clean x-ray films were recovered. Protease crude extract of strain AN13 and AN16 were tested for their gelatin removal efficiency from X-ray films. The gelatin degradation capability of isolated strains AN13 and AN16 is shown in (Table 8). The weight loss after

treatment was around 5% (w/w) for AN13 and 6% for AN16.

The bacterial protease enzyme extract worked on the gelatin coating of x-ray film and thus caused release of the bound metallic silver into the solution. The increase in optical density of reaction mixture after 24 hrs. as compared to zero sample solution confirmed the release of silver due to hydrolysis of gelatin (Table 9). The solution was filtered to separate the black silver

released after hydrolysis by an ordinary filtration methods [40].

This is in confirmation with earlier reports where recovery of silver with degradation of gelation by protease produced by *Conidiobolus coronatus* and *Streptomyces avermectinus* was observed [84].

Protease produced by *Bacillus cereus* strain S8 has high potential to be applied for the silver recovery from waste X-ray films

through gelatin coatings [85]. Similarly, protease from *Bacillus subtilis* was found to be effective for silver recovery [86]. Proteolytic enzymes have also been reported to be involved in treatment of used X-ray film for silver recovery [87]. The *Bacillus licheniformis* crude protease showed breakdown of gelatin layer from X-ray films making it helpful in recovery of silver [64].

Table 8. Percentage (%) weight loss of treated X-Ray Film pieces

Strains	Initial weight (Before) in (g)	Residual weight (After treatment) in (g)	% weight loss
AN13	0.45	0.39±0.57*	5%
AN16	0.46	0.39±0.88*	6.3%
Control	0.45	0.44±0.005	Nil

Values represent replicate of three means ±S.E.M. Values are significantly different in comparison with control (One way ANOVA). * represents p value (P<0.0001)

Table 9. Optical density (OD) measured at 400 nm after 24 hours

Strains	Optical Density of zero sample (Means ± S.E.M)	Optical Density after 24 hours (Means ± S.E.M)
AN13	0.715±0.002*	0.8853±0.128**
AN16	0.751±0.035*	2.314±0.217**
Control	0.237± 0.136	0.237± 0.136

Values are significantly different in comparison with control (One way ANOVA). * represents p value (*P<0.005, **P< 0.0005)

Conclusion

The results of this study showed the significance of efficient protease producer strains AN13 and AN16. Thus AN13 and AN16 strain protease may find the applications in different industrial processes and biotechnological fields. Findings of this study supports that naturally available sources can be used for isolation of potent microbes and usage of agricultural by products for supporting growth and production of industrially important enzymes. The present study also revealed that the protease enzyme produced by AN13 and AN16 strain showed positive result in removal of chocolate and spicy food stain from the stained cloth. Therefore, this enzyme found significant application in

detergent industry. Protease enzyme produced by these strains has extreme chicken feather degradation activity so it can be employed for poultry feed production and in keratin waste management. This study also proved that protease enzyme of AN13 and AN16 can be used efficiently in leather industry for eco-friendly, efficient and low cost removal of hairs from hides. These strains also have potential to synthesize silver nanoparticles that can be used for their antibacterial and antifungal activity in pharmaceutical industry. They can also be used in silver recovery due to their ability to degrade gelatin in used x-ray films after which silver can be recovered from them. Hence, it is proved that protease enzyme produced by these isolated strains have

immense potential to be applied in various commercial industries.

Authors' contributions

Conceived and designed the experiments: Z Nasreen & A Sharif, Performed the experiments: A Sharif, Analyzed the data: A Sharif & Z Nasreen, Contributed materials/analysis/ tools: S Kalsoom & Z Nasreen, Wrote the paper: A Sharif.

Acknowledgments

Authors are thankful to University of Mianwali, Mianwali and District Diagnostic Lab, Mianwali for providing necessary facilities for conducting this research.

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