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Solid state fermentation of keratinolytic proteases production using *Bacillus* spp. isolated from hair and mud sample of traditional leather processing ponds in North Gondar, Ethiopia

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Abstract
The objective of the present study was, production of keratinolytic protease (s) through solid state fermentation using bacteria isolated from traditional leather processing stagnant ponds (Ponds are in use for last several years). Samples were collected from four different locations of Sebaha, North Gondar and isolated casein proteolytic bacteria. Partial purification enzymes was carried out using 80% saturated ammonium sulfate. Morphological and biochemical techniques were used to characterize the bacteria. Four isolated *Bacillus* species from two different regions showed highest proteolytic activity ranging from 26.7 U/ml (*Bacillus* strain Hs-3), 23 U/ml (*Bacillus* strain ms-1), 21.1 U/ml (*Bacillus* strain ms-2), 17.1 U/ml (*Bacillus* strain Hs-1). The optimum pH for protease production and stability of *Bacillus* species were 7 and 8 respectively. The optimum temperature for isolates ms-1 and ms-2 was found to be 37 °C, whereas for isolate Hs-1 and Hs-3, was at 30 °C. Maximum enzyme activity was observed at 0.2M NaCl. The optimum production time was 48 hours, *Bacillus spp.* grown best in wheat bran and rice bran carbon sources and at 1:3 ratio of media to moisture content showed highest enzymatic activity. Proteolytic activity of crude enzyme tested with and without traditional fruit extract (*Lagenaria abyssinica*) were compared and tested on various substrates. Complete dehairing of cattle hide after 24h of incubation and complete removal of blood stains was only observed with *Bacillus spp.* Hs-3 crude enzyme along with fruit juice. The present study suggests that, the two isolates should be further characterized and optimized for pure enzyme production.

Keywords: *Bacillus spp.,* crude enzyme, de-hairing, leather industry, keratinolytic protease

Introduction
Enzymes are also important for thousands of metabolic processes that sustain life (Robinson, 2015). While enzymes are definite for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell are responsible for which metabolic pathways occur in that body parts of living things. Organisms are also differentially enriched in sets of enzymes to compartmentalize function within the cell. Enzymes are applied in various fields, including technical use, food manufacturing, animal nutrition, cosmetics, medication, and as tools for research and development. At present, almost thousands enzymes are known (Robinson, 2015). Among enzymes that play great role for biochemical reactions, proteases play significant catalytic roles for metabolism of proteins (Jisha *et al.*, 2013) [10-16].
Proteases are hydrolytic enzymes found in every organism to carry out important physiological functions. These include: cell division, regulating protein turnover, activation of zymogenic performance, blood clotting, lysis of blood clot, processing and transport of secretary proteins across membrane, nutrition, regulation of gene expression and virulence factors. Proteases differ in their specific activities, substrate specificities, pH and temperature optima and stability, active site, and catalytic mechanisms. All these features contributed in diversifying their classification and practical applications in industries involving protein hydrolysis (Jisha *et al.*, 2013) [10-16].
Proteases represent one of the most important groups of industrial enzymes, because of their widespread use in detergents and dairy industry and industrial sales of protease are estimated at more than $350 million annually (Kumar *et al*., 2012) [27]. Proteases account for 65% of the global industrial enzyme market (Cherry and Fidantsef, 2003). The proteases of industrial importance are obtained from animals, plants and microorganisms. The proteolytic enzymes

hydrolyse the peptide links of proteins and peptides to form smaller subunits of amino acid**s** and are produced both extracellularly as well as intracellularly (Gajju *et al*., 1996; Kumar *et al*., 2003). The proteases play an important role in a wide range of industrial processes *viz*., baking, brewing, detergents, leather processing, pharmaceuticals, meat tenderization, cosmetics and medical diagnosis (Bhalla *et al*., 1999; Gupta *et al*., 2002; Kumar *et al*., 2003; Kumar and Bhalla, 2005; Najafi *et al*., 2005).

Microbial proteases are among the most important, extensively studied groups since the Development of enzymology and currently they are further divided as acidophilic, Neutralophilic and alkaliphilic. Neutralophilic and alkaliphilic microbial alkaline proteases possess a considerable industrial potential due to their biochemical diversity and stability at extreme pH environments, respectively (Moon *et al.,* 1994) [32]. However, the demanding industrial conditions for technological applications and cost of protease production required continuous exercise for search of new microbial resources. Enzyme cost is also the most critical factor limiting wide use of protease for different applications. A large part of this cost is accounted for the production cost of the enzyme. Therefore, reduction in the production cost of enzymes could greatly reduce the cost of the enzyme. In submerged fermentation up to 40% of the total production cost of enzymes is due to the cost of the growth substrate (Enshasy *et al.,* 2008) [14]. In this regard, SSF which uses cheap agricultural residues have enormous potential in reducing enzyme production cost. So, studies on protease that are produced in SSF by microorganisms are scarce in literature. As a result, it is of great importance to pursue such studies. This type of fermentation process also does not require highly caliber equipment and energy for agitation to provide oxygen (Iqbal *et al.*, 2011).

This study was focused on production of proteases through SSF using cheap substrate. To produce protease in such a way, isolation of protease producing bacteria using standard methods is significant. Once protease producing bacteria obtained, their enzyme activities has to be characterize in order to evaluate the capacity of the enzyme for industrial application. In this study, the sources of potential bacteria for production of protease were stagnant pond used de-haring of leather. Ethiopians have basic traditional leather processing knowledge using stagnant water as microbial source for de-hairing purpose. Here bacteria associated with stagnant water may serve as protease source to de-hair leather.

Materials and Methods

Isolation of keratinolytic protease producing microbes

Samples were collected from hair of processing leather kept for couple of days and from bottom mud of the sixteen different traditional leather processing stagnant ponds of Sabaha, North Gondar. Collected samples were transferred to sterile labelled tubes and stored at 4°C until used.

Keratinolytic protease producing microorganisms were isolated using a medium containing wheat bran 10g, K^2HPO^4 0.1g, $MgSO_4.7H_2O$ 0.02g, $CaCl_2$ 0.01g and casein 1.0g in a 250 ml Erlenmeyer flask and 30 ml of water added to make final bran to moisture ratio was 1:3 and thoroughly mixed, and autoclaved at 121 ºC for 15 min. Then media was inoculated with 3 ml of collected sample suspension and incubated at 37°C for 5 days (Sharma *et al.,* 2017).

1g of the above fermented sample was suspended in 5 ml of sterilized distilled water in a glass tube and kept in shaker at 121 rpm for 30 minutes at room temperature. This suspension was serially diluted (10^{-1} to 10^{-12}) and spread on a sterile nutrient agar plates and incubated for 24h. Individual colonies were isolated and screened for keratinolytic protease production.

Morphological characterization of the bacterial isolates

Macroscopic and microscopic characterization of isolates

After 24h of incubation on nutrient agar, colonies were observed for configuration, margin, elevation, opacity, pigment and cell shape (Duncan, 2005) [12], Gram staining (Harley and Prescott, 2002) [23], endospore staining and motility of microorganisms were studied microscopically.

Motility test

Bacterial motility was observed on casein containing semisolid nutrient agar. Highly motile organisms were spread throughout the tube and growth of non-motile organisms observe along the stab line only (Ali *et al.,* 2017) [5].

Endospore staining

Endospore staining was carried out by preparing heat fixed smears from a 24h old bacterial culture on clean microscopic slides. The slides were then covered with Malachite green and placed in a beaker that had been kept in a boiling water bath for 3 to 5 minutes to allow the dye to penetrate the endospore. After counterstaining the vegetative cells with Safranin solution, the bacteria were observed using a standard microscope (Harley and Prescott, 2002) [23].

Biochemical characterization of isolates

A loop-full of sample from an overnight culture was streaked on to nutrient agar plate and incubated for 24h at 37°C and the culture were used for different biochemical tests. Presence or absence of changes in the media was recorded as positive and negative, respectively, and the results were interpreted using Berge's Manual of Determinative Bacteriology (Holt *et al.*, 1994) [25].

Catalase test: Thick emulsions of each bacteria isolates were prepared on a clean slide. Three drops of 3% hydrogen peroxide were added on each of the slides. Formation of bubbles was observed as positive result (Adetunji *et al*., 2012).

Starch hydrolysis test

This test was carried out by dividing starch agar plate into four equal sectors using a marker. After labeling the organism's name, the test organisms were spot inoculated and incubated for 24 h (Harley and Prescott, 2002) [23]. Zone of hydrolysis of starch was detected as a brownish clear zone in a blue black background after flooding the starch agar plate with Iodine solution. The presence of zone of hydrolysis on the plate indicated the ability of the test organism to metabolize starch.

Urea hydrolysis test

Urease test was carried out by preparing urea broth containing phenol red as pH indicator. After inoculating the broth with the test isolate and incubating the culture for 24h, color change of the broth from red to pink was observed and recorded as a positive result for urease test (Harley and Prescott, 2002) [23].

Carbohydrate fermentation test

Carbohydrate fermentation patterns of microorganisms were

studied using media containing different carbohydrates such as glucose, lactose, galactose, D-xylose, mannitol and cellulose as carbon source and phenol red as the pH indicator. The experiment involved both control and experimental groups. The fermentation of carbohydrates were observed for a color change from red to yellow after 24h of incubation (Harley and Prescott, 2002) [23].

Gas production using triple sugar iron test (TSI)

Gas production was detected using TSI agar slants which are prepared from a mixture of agar, a pH-sensitive dye (phenol red), 1% lactose, 1% sucrose, 0.1% glucose, sodium thiosulfate and ferrous sulfate (Harley and Prescott, 2002; Sharma, 2007) [45]. The bacterial isolate to be studied were inoculated both by streaking on slant and stabbing the butt. After incubating the inoculated TSI agar slant tubes for 24 hours, presence of H_2 S, color change on the slant and in the butt were observed and interpreted according to Sharma (2007) [45]. Production of H_2 S was indicated by the blackening of the TSI medium.

Seed culture medium

For enzyme production, bacterial cells from a 24h culture were inoculated into 100 ml Erlenmeyer flasks containing 50 ml of sterile inoculation medium containing glucose, $CaCl_2$, K_2HPO_4 and $MgSO_4$ and casein. The composition of the inoculum medium was the same as that of the medium described for culture maintenance. The cultures were grown at 37ºC for 24h, 2% (v/v) of the culture was used to inoculate the production medium (Ghaemi *et al*., 2007).

Screening for keratinolytic protease production

Bacterial colonies were screened for keratinolytic protease production using casein-yeast extract peptone (CYP) agar medium and the plates were incubated at 37°C for 48h. Colonies with halo zone were considered as positive for proteolytic activity and these colonies were isolated and repeated till single uniform colonies were obtained Gessesse A. *e t a l*., 2011) [20].

To screen bacterial colonies for high keratinolytic protease activity, bacteria inoculated into 30 ml glass with 5 ml of keratinolytic protease production medium and incubated 16h at 121 rpm at room temperature and 5 % (v/v) of the 16h inoculum was inoculated in to 50 ml of keratinolytic protease production medium in 250 ml Erlenmeyer flask and incubated at room temperature for 5 days under constant shaking at 121 rpm. Five ml of the fermented broth was taken and centrifuged at 6000 rpm for 5 min and the cell free supernatant was used as enzyme source.

Optimization of the growth conditions for production of keratinolytic protease

Effect of fermentation time on the production of keratinolytic protease: To determine the time for maximum production of keratinolytic protease, the culture in the medium containing wheat bran, peptone, yeast extracts, casein, $CaCl_2$, K_2HPO_4 and $MgSO_4$ was incubated at 37°C for 24-72h and the keratinolytic protease activity was determined at 12h intervals. Thus 2 ml of culture broth was collected after each interval and keratinolytic protease activity was determined (Serin*et al*., 2012).

Effect of temperature on the production of keratinolytic protease

The optimum temperature for keratinolytic protease production was determined by incubating the culture at different temperatures (i.e. 25, 30, 37, 40, 45 and 50°C), for 48h. At the end of incubation period, the cell free culture filtrate was tested for keratinolytic protease activity (Muthu and Christudhas, 2012) [34].

Effect of pH on the production of keratinolytic protease

The effect of pH on the production of keratinolytic protease was investigated by adjusting the pH of the growth medium to pH 5.0, 6.0, 7.0, 8.0, and 9.0 and incubating at 37°C for 48h.

Effect of carbon source on the production of keratinolytic protease: Glucose, rice bran, wheat bran, and sucrose were used as carbon sources. The cultures were incubated at 37°C for 48h (Akcan, 2012) [2-44].

Effect of different hair source on the production of keratinolytic protease: Cow skin, Goat skin, Human hair and Feathers were used as carbon sources. Human hair was previously washed with distilled water. Feathers were washed with 0.1% (v/v) Triton X-100 and distilled water and then cut into small pieces to enhance the contact surface and the other substrates were not pretreated (Syed *et al*. 2009)

Effect of nitrogen source on the production of keratinolytic protease: Two different sources of nitrogen, viz. organic nitrogen and inorganic nitrogen were tested for their potentials to enhance keratinolytic protease production. The production medium was initially supplemented with different organic nitrogen sources such as yeast extract, peptone, casein, each at 1% (w/v) and inorganic nitrogen sources such as, $(NH_4)_2SO_4$, and NH_4Cl at 1% (w/v) were tested after incubating the culture for 48h (Akcan, 2012) [2-44].

Effect of NaCl concentration on the production of keratinolytic protease: NaCl was added at various concentrations, i.e. 0.0, 0.2, 0.4, 0.6 and 0.8M, into the keratinolytic protease production medium and crude enzyme activity was checked after 48h of incubation (Agrawa *et al*., 2012) [4].

Effect of moisture level on keratinolytic protease production: The effect of moisture level on keratinolytic protease production from the bacterial isolates (1% inoculum) were determined by adding moistening medium to wheat bran at level of 1:2, 1:3, 1:4 and 1:5 (w/v). SSF medium were incubated at 37°C and the crude enzymes were harvested after 48h of fermentation time using centrifugation at 10,000 rpm for 6 min. The activity of the crude enzymes was determined.

Effect of inoculums size on keratinolytic protease production: The effect of inoculum size on protease production from the selected bacterial isolates were checked by inoculating the SSF medium (wheat bran to moistening medium at 1:3 ratio) with inoculum size of 5%, 10%, 15% and 20%. After incubating the medium at 37°C for 48h, crude enzymes were harvested and enzyme activity was checked.

Solid State Fermentation (SSF)

SSF medium with (g/g): wheat bran 10; K_2HPO_4 0.1; $MgSO_4.7H_2O$ 0.02; $CaCl_2$ 0.01; and casein 1.0 (What is the pH of the medium or it is a standard medium used) was prepared in a 250 ml Erlenmeyer flask and the solid substrate moistened in 1:3 ratio and incubated at 37°C for 5 days. Then, keratinolytic protease harvested by soaking the fermented

solid with ten volumes of distilled water per gram solid substrate (wheat bran), under shaking at 121rpm for 30 minutes at room temperature. At the end of the extraction, the suspension was hand squeezed through a double layered muslin cloth and the particulate materials clarified by centrifugation at 10,000 rpm for 5 minutes. Recovery efficiency was calculated from the crude supernatant keratinolytic protease activity by dividing total activity of at each squeezing stage to the overall keratinolytic protease activity at three stages (Roussos *et al*., 1992).

Characterization of keratinolytic protease

Casein hydrolysis test

Keratinolytic protease bacterial colonies were inoculated on nutrient agar containing 1% casein (w/v) and incubated at 37ºC for 24 h. Casein hydrolysis was visualized by applying 30% trichloroacetic acid on the agar surface. A transparent halo around the bacterial growth was considered as being a positive reaction (Vishwannatha *et al.,* 2010).

Determination of the keratinolytic protease activity of selected isolates

Casein -TCA method

Hydrolysis on casein agar test were cultured at 37°C for 48h on media consisting of (g/l): wheat bran (1.0), peptone (10.0), yeast extract (0.2), $CaCl_2$ (0.1), K_2HPO_4 (0.5) and $MgSO_4$ (0.1). The culture broth was centrifuged at 10000 rpm for 15 min at 4°C and used as enzyme source for quantitative studies. Keratinolytic protease activity was determined using casein as a substrate as described by Hema and Shiny (2012) [24]. The reaction mixture contained a total volume of 2 ml which in turn was composed of 1 ml of 1% (w/v) casein in 50 mM sodium phosphate buffer (pH 7) and 1 ml enzyme solution. After 20 min of incubation at 37°C, the reaction was terminated by adding 2 ml of 10% (w/v)) trichloroacetic acid (TCA) and again incubated at 37°C for 20 min. After separation of the un-reacted casein precipitate by centrifugation at 10000 rpm for 15 min, 0.5 ml of clear supernatant was mixed with 0.5 ml of 1N Folin-Ciocalteau's phenol reagent. After incubation for 20 min at 37°C, absorbance was measured at 660 nm against a reagent blank. One unit of protease activity is defined as the amount of enzyme that releases 1 µg amino acid equivalent to tyrosine per min under the standard assay conditions (Hema and Shiny, 2012, Sevinc and Demirkan, 2011). As a reference to keratinolytic protease enzyme activity, tyrosine standard curve was generated using an appropriate amount of tyrosine diluted in water. The suitably diluted samples (0.1 – 1.5 mg/ml) were treated similar to the experimental enzyme catalyzed reaction mixture and then were measured using a spectrophotometer at a wavelength of 660 nm (Hema and Shiny, 2012).

Units/ml = µ mole of tyrosine x reaction volume

Sample volume X reaction time X volume assay (Source: Folin and Ciocalteu, 1929)

Effect of pH on the activity and stability of keratinolytic protease

The crude keratinolytic protease was incubated at different pH values of 5, 6, 7, 8, 9, 10 and 11 with phosphate buffer (pH 7.0). The effect on the activity was studied by incubating for 20 min and determining the remaining activity following the standard keratinolytic protease assay procedures described above. The effect on the stability was studied by pre-incubating for 12h and determined the enzyme activity (Ovievera *et al*., 2010).

Effect of temperature on the activity and stability of keratinolytic protease

This experiment was performed by incubating keratinolytic protease at different temperatures viz.: 30, 40, 50 60, 70, 80 and 90ºC. The effect on the activity was studied by incubating for 20 min and determined the enzyme activity. The effect on the stability was studied by pre-incubating for 12h and determined the enzyme activity.

Partial purification of crude enzyme

Partially purified enzymes were obtained by ammonium sulfate precipitation and dialysis using membrane tube (Saxena and Singh, 2011) [43]. Ammonium sulfate powder was added slowly to the crude enzymes until reached 80% saturation and crude enzymes were allowed to precipitate for 60 min with gentle mixing at room temperature. The precipitates were recovered by centrifugation at 12,000 rpm for 20 min at room temperature. The precipitates recovered from ammonium sulfate precipitation were dissolved in 0.1 M phosphate buffer (pH 7) for 4h. Using membrane dialysis tube, the precipitates obtained from ammonium sulfate precipitation were dialyzed overnight against the same buffer and re-centrifuged. Finally, the supernatants were used as partially purified enzymes for further study. Enzyme activity was determined at each step.

Molecular weight determination of partially purified keratinolytic protease using sodium doedcyl sulfate polyacrylamide gel electrophoresis

Molecular weights of the partially purified keratinolytic protease were determined by performing Sodium Doedcyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with 10% polyacrylamide gel by following the method described by (Annamalai *et al.,* 2011). First, 80 ml 10% resolving gel solution and 20 ml 4% stacking gel solution were prepared. In the study, 1.5 mm gel thickness was prepared and allowed to cast in a vertical plate system (8x7 cm). After casting the gel, samples (purified enzyme solution) prepared with equal volume of 2x loading buffer along with standard protein marker (6X Tris protein 100kDA) were loaded into electrophoretic wells. Upper and lower tanks were filled by tank buffer (pH 8.3) and electrophoresis was done at room temperature using a constant current of 120 mA for 4h. After completing running of the samples, the gel was disassembled. Then, the gel was stained in a solution containing 0.25% Coomassie Blue R-250 in 50% Methanol, 10% Glacial acetic acid and 40% H_2O and distained in solution containing 5% Methanol, 7.5% Glacial acetic acid and 87.5% H_2O at room temperature for 4 and 12h, respectively. Finally, the distained gel was visualized through gel documentation system and the molecular weights of the enzymes were determined by comparing with size of standard protein marker (10-100kDA).

Test for enzymatic dehairing of cow hide and feather degradation

Three sets of cow hides were washed with distilled water and cut into 15x20 cm pieces. Control was treated with distilled water, the second piece was treated with enzyme solution alone and the third piece was treated with enzyme along with traditional fruit for dehairing (Entelya) and fruit for softening

(gull fruit) at pH 7.0 and incubated under constant shaking at 121 rpm at room temperature and skin pieces were examined at 12 hours and 24 hours and noted down the percentage dehairing and amount of scud formation. Similar treatments were carried out for percentage feather degradation.

Test for enzymatic degradation of blood stains

Blood drop was taken on cloth and allowed to dry and blood stain was treated with either distilled water/ enzyme/ detergent/ enzyme along with gulo/ enzyme along with gulo and Entelya at room temperature for five minutes. Then washed with tap water and noted the percentage blood stain removal.

Data Analysis

All the experiements were performed in triplicates and data was tabulated and ANOVA test was performed using SPSS (version 20.0) statistical software at 95 significance level ($P \leq 0.05$).

Results and Discussion

Isolation, screening and selection of keratinolytic protease producing bacterial isolates:

Initially 215 colonies were isolated from 16 different samples and out of that, 145 colonies (67.4%) were keratinolytic protease positives (Table1). The isolates showed great variation in clear zone of hydrolysis on casein agar plates ranging from minimum 1mm to the maximum 20mm. Our results was in good agreement with earlier studies reported clear zone of casein hydrolysis ranging from 1-20mm (Akpomie *et al*., 2012) [3] (Ogbonnaya and Odiase, 2012) [41].

Selection of the best keratinolytic protease producing bacteria

Among the total of 145 positive isolates, 11 isolates with relatively large clear zone of hydrolysis were selected for further investigation. The selection of potent bacteria was done by corresponding the isolates with each other in terms of both their diameter of clear zone of hydrolysis and their keratinolytic protease activities (Table1). The results showed that the isolates with higher clear zone of hydrolysis also give higher keratinolytic protease production (Table1). This step resulted in selection of four potentially potent isolates, named as ms-1 and ms-2 from mud, Hs-1 and Hs-3 from hair.

Table 1: Screening of the 11 keratinolytic protease producing isolates

Sample source	Positive isolates	Zone of clearance (mm)	Protease activity after 48 hours (U/ml)
	ms-1	11	4.23
	ms-2	15.5	13.68
Mud sample	ms-3	13	8.1
	ms-4	09	2.4
	ms-5	10	1.65
	Hs-1	17	13.44
	Hs-2	12	10.0
Hair sample	Hs-3	14	15.5
	Hs-4	13	11.7
	Hs-5	11	12.1
	Hs-6	11	10.5

Phenotypic characterization of the bacterial isolates

Several bacterial strains producing high keratinolytic protease were identified. Bacterial cells were observed under light microscopy after Gram's and endospore staining. Physiological and biochemical characteristics were studied using Berge's Manuals of Systematic Bacteriology. The isolates ms-1, ms-2, Hs-1 and Hs-3 were identified as spore-forming bacterial species, were catalase positive and could grow under aerobic conditions confirmed as the genus *Bacillus*.

Effect of culture conditions on keratinolytic protease production under solid state fermentation

The effect of temperature, pH, moisture level, carbon sources, nitrogen sources, NaCl concentration and inoculums size on keratinolytic protease production from selected isolates were determined.

Effect of carbon source, nitrogen source and moisture ratio on the production of keratinolytic protease

Among the various carbon sources used for keratinolytic protease production, complex carbon sources like wheat bran and rice bran were found to be the best and easily available substrates. Wheat bran showed maximum enzyme production even better than glucose for isolates ms-1(10.8U/ml) ms-2 (13 U/ml), Hs-1 (13.75 U/ml) and Hs-3(17.18 U/ml) (Fig 1). The effect of moisture level on enzyme production was determined by growing the bacterial isolates on wheat bran supplemented with moistening medium at different ratios (w/v). In all isolates, maximum keratinolytic protease activity was shown at moisture content 1:3 (8.9 U/ml, 11.5 U/ml, 11U/ml and 13.75 U/ml for isolates ms-1, ms-2, Hs-1 and Hs-3 respectively). The outcome of various nitrogen sources (organic and inorganic nitrogen sources) on keratinolytic protease production of the four selected isolates (i.e. ms-1, ms-2, Hs-1 and Hs-3) was examined. It was observed that the growth medium containing casein yielded highest activity in all isolates, 23 U/ml, 21.1 U/ml, 17.1 U/ml and 26.7 U/ml, for ms-1, ms-2, Hs-1 and Hs-3 respectively. This was followed by peptone, yeast extract, ammonium sulphate and ammonium chloride (Fig.1). Within the enzymes of the isolates, significant difference in keratinolytic protease activity were shown except Hs-3 ($P< 0.05$). Based on this study, organic nitrogen sources (casein, yeast extract and peptone) were support better for keratinolytic protease production compared to inorganic nitrogen sources (ammonium sulphate and ammonium chloride). This maximum keratinolytic protease production by casein, peptone and yeast extract might be due to the presence of high nutritional amino acids and compatible nitrogen source in these organic nitrogen sources. In the other, least production of keratinolytic protease was observed in SSF medium supplemented with, ammonium sulphate and ammonium chloride respectively. Therefore, casein was selected as substrate for further optimization.

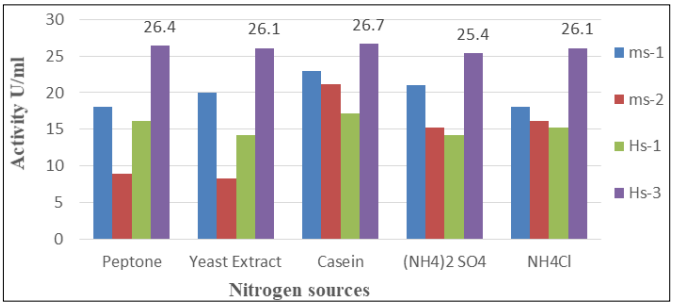


Fig (A)

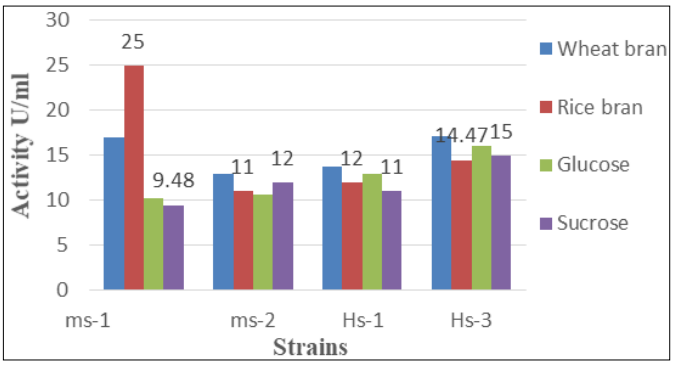


Fig (B)

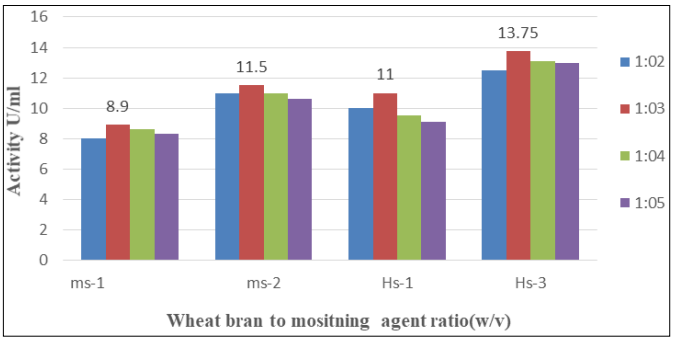


Fig 1(A, B, C): Effect of nitrogen, carbon sources and moisture ratio on keratinolytic protease production. (n=? P=>0.05)

This might be due to the inability of the bacteria isolates to utilize these nitrogen sources in degrading these nitrogen sources into utilizable forms, which was in agreement with results reported by Shyam *et al*., 2013. Similarly, Niadu and Devi (2005) [35] also reported repressing ability of inorganic nitrogen sources in the *Bacillus* isolate K-30. These authors suggested inability of the *Bacillus* isolates to utilize inorganic nitrogen sources.

Microbial growth medium for enzyme production at industrial scale takes about 30-40% production cost Enshasy *et al*., 2008. By using wheat bran alone, appreciable amount of keratinolytic protease production can be achieved, implying presence of enough nutrients in wheat bran that support the growth of the isolate and keratinolytic protease production. This observation was in agreement with previous studies which suggested that larger amount of enzyme was synthesized when carbon sources were poorly utilized for growth purposes Tambekar and Tambekar, 2013. The keratinolytic protease activity within the enzymes of the isolates were not shown significant difference (P>0.05) except ms-1. Among the several factors that are essential for microbial growth and enzyme production under solid-state fermentation, moisture level is one of the most critical factor Pandey *et al.*, 2000; Mrudula *et al*.(2011) [33].

In the present study, in all isolates, high enzyme activity was obtained when the substrate to moisture ratio maintained at 1:3. In all isolates, any further increase or decrease of moisture ratio from the optimum (1:3) resulted in a slight decline of enzyme production. This slight reduction of enzyme yields at low moisture level might be due to clumping of solid particles, reduction in solubility of the nutrients of the substrate, low degree of swelling and higher water tension Mrudula *et al*., 2011. The low enzyme activity at high moisture level (at 1:5) might be due decreased oxygen availability and steric hindrance of the growth of the isolates by reduction in porosity of the wheat bran Mrudula *et al*., 2011. Different studies showed difference in optimization of moisture content for the production of keratinolytic protease. (Paul et al. 2014) reported that 1:3 moisture content as an optimum moisture ratio for enzyme production from *Bacillus* species, which was in agreement with the present study. On the other hand, Salwa *et al*., 2012 were reported that the optimum moisture ratio for enzyme production from *Bacillus cereus* and *Bacillus* species were 1:2 and 1:2.5, respectively. These reports demonstrated slightly lower moisture ratio for maximum enzyme production compared to the result of present study. This might be due to the difference in the nature of the solid substrates used for fermentation. The keratinolytic protease activity within the enzymes of the isolates were not shown significant difference (P>0.05).

Effect of initial pH, time and temperature on the production of keratinolytic protease

The optimum pH for keratinolytic protease production for the four isolates was 7.0 although the enzyme was active in the pH range of 7- 11 (Fig. 2). At pH 7, the keratinolytic protease activities for ms-1, ms-2, Hs-1 and Hs-3 were 22 u/ml, 25 u/ml, 22 u/ml and 26.44 u/ml respectively. However, previous studies shown that the optimum pH range for keratinolytic protease production was usually between 7 and 9 (Al-Shehri and Mostafa, 2004; Qadar *et al*., 2009; Sevinc and Demirkan, 2011; Josephine *et al*., 2012) [26].

In the present study the optimum keratinolytic protease production time for the four isolates was found to be 48 hours corresponding to keratinolytic protease activity of 6.4 U/ml for ms-1, 22.6 U/ml for ms-2, 20 U/ml for Hs-1 and 24 U/ml for Hs-3 (Fig. 2). After 72 hours of incubation time, no further increase in keratinolytic protease production; and no pronounced drop in keratinolytic protease production was observed. This might be due to the decrease in microbial growth associated with the depletion of available nutrient, loss of moisture content, production of toxic metabolites and autolysis of produced keratinolytic protease (Sumantha *et al*., 2006) [48].

The optimum temperature for isolates ms-1 and ms-2 was found to be 37°C, corresponding to keratinolytic protease activities of 16.2 U/ml and 13 U/ml, respectively. Whereas for isolate Hs-1 and Hs-3 the maximum activities 20 and 23 were obtained at 30°C.

However, considerable decreases in activity were observed with further increase in temperature beyond the maximum for the respective isolates (Fig.2). It might be due the fact that at high temperature, the growth of the bacteria was hindered.

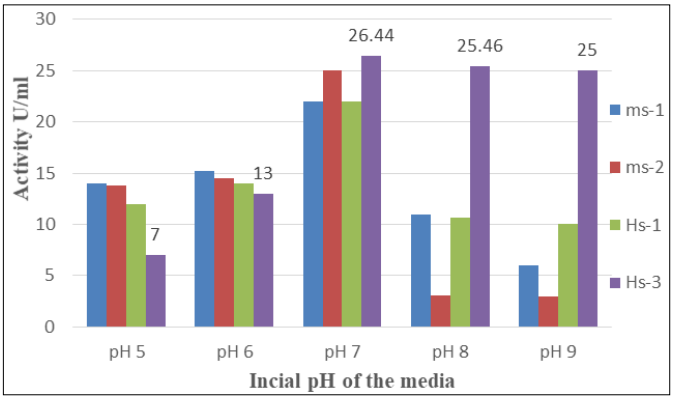


Fig (A)

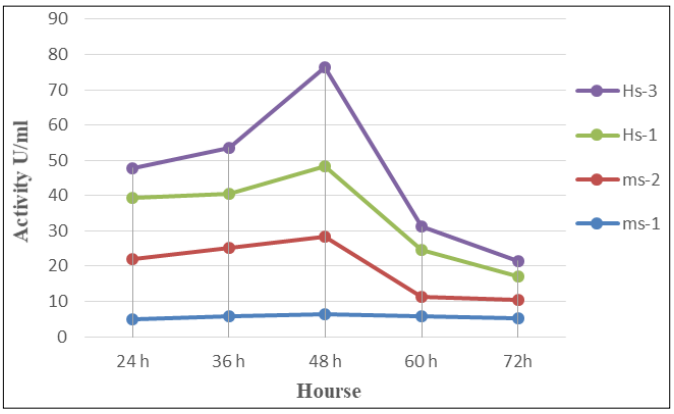


Fig (B)

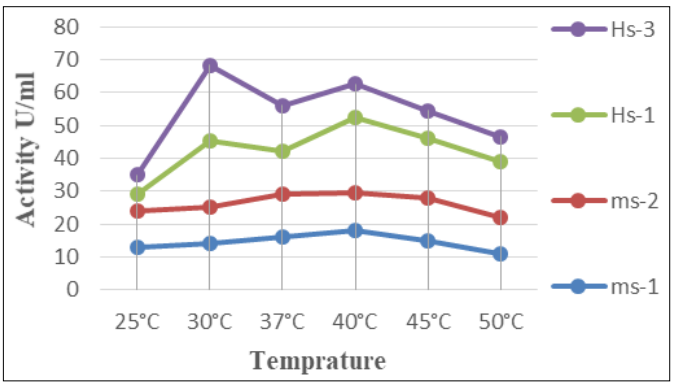


Fig 2 (A, B, C): The effect of initial pH, time and temperature of the media on keratinolytic protease production (n=16; P=>0.05)

Supplementary increase in initial pH values resulted in the decrement of keratinolytic protease production. This might be the isolates prefer neutral pH for optimum growth Gangadharan *et al*. (2006) [18]. Normally, *Bacillus spp*. prefer neutral or slightly alkaline between 6.8 and 7.2 pH for keratinolytic protease production at the initial stage of fermentation Benjamin *et al.* (2013) [10]. For bacteria isolated from meso philic environments, reports from earlier studies revealed that an optimum pH for keratinolytic protease production was pH 7 Meenakshi *et al.* (2009) [31] Ashwini *et al.* (2011) [8] Siva Kumar *et al*. (2012) [27]. The keratinolytic protease activity within the enzymes of the isolates were shown significant difference ($P< 0.05$). These results are in accordance with observations made by Dirhams, (1987), Gessesse, (1997) [21] and Qadar *et al.* (2009) [38], where maximum enzyme production was observed during continuous growth of the culture at the late exponential phase and early stationary phase of the growth and thereafter number of viable cells decreased due to depletion of readily available carbon sources and other nutrients. The keratinolytic protease activity within the enzymes of the isolates were shown significant difference ($P< 0.05$). According to the report of Aiba *et al*. (1983) [1] high temperature may inactivate the expression of the gene responsible for the synthesis of protease enzyme. At relatively low temperature (< 25°C), protease production was very low due to the reason at low temperature bacterial growth was relatively slow (Christiansson *et al*, 1989). Several reports indicate that maximum keratinolytic protease production was achieved at 35-40°C for certain *Bacillus* spp. Qadar *et al*. (2009) [38] Kumara *et al.* (2012) [27] Josephine *et al*., 2012. These results suggest that isolates ms-1, ms-2, Hs-1 and Hs-3 belong to the meso philic keratinolytic protease group. The keratinolytic protease activity within the enzymes of the isolates were not shown significant difference ($P>0.05$) except ms-2.

Effect of inoculum size and NaCl concentrations on keratinolytic protease production

The size of inoculum plays an important role in the production of high keratinolytic protease (Saxena and Singh, 2011; Shyam *et al*., 2013) [46]. In the present study, 10% was found to be an optimum inoculum size for keratinolytic protease production in all isolates (i.e 9.1 U/ml, 18.5 U/ml, 13.1 U/ml and 27 U/ml for isolates ms-1, ms-2, Hs-1 and Hs-3 respectively).

Various NaCl concentrations (i.e. 0, 0.2, 0.4, 0.6, 0.8M) were used to determine optimum level required for the production of keratinolytic protease by the four selected isolates (i.e. ms-1, ms-2, Hs-2 and Hs-3).

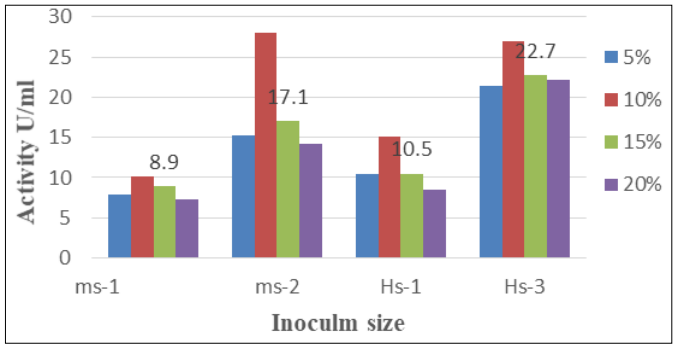


Fig (A)

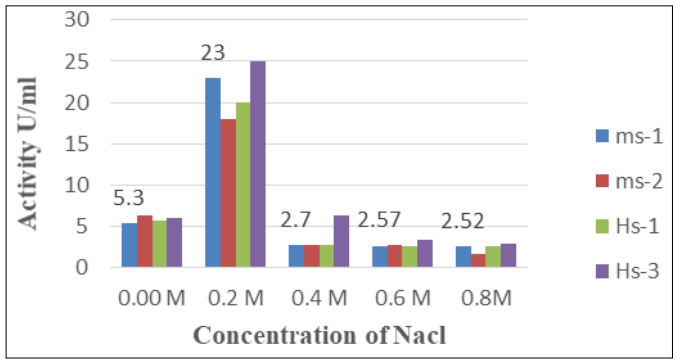


Fig 3 (A, B): The effect of inoculums size and NaCl concentrations on keratinolytic protease production (n=16; P=>0.05)

According to this study, inoculum size higher or lower than 10% has been shown to decrease keratinolytic protease production. The decreased keratinolytic protease yield at lower inoculum size might be due to the need of longer time by the bacteria isolates to grow to an optimum number to utilize the substrate and form the desired product. On the other hand, the low keratinolytic protease production at higher inoculums size (>20%) might be due to the stressful

conditions created by the microbial cells such as depletion of nutrients, pH fluctuation, change in availability of oxygen and competition to limited resources, which were in line with the results reported by Kumar *et a*. (2010) and Shyam *et al.*, 2013) [46]. The keratinolytic protease activity within the enzymes of the isolates were not shown significant difference (P>0.05) except Ms-2. It was observed that the growth medium containing 0.2M NaCl yielded the maximum activity in all isolates (23 U/ml, 18 U/ml, 20 U/ml and 25 U/ml for. ms-1, ms-2, Hs-1 and Hs-3 respectively) (Fig. 3). This was followed by 0.00M of NaCl for isolates of ms-1 and ms-2 which resulted in activities of 5.3 U/ml and 6.2 U/ml, respectively. Whereas for isolate Hs-1 and Hs-3 is 5.7, 6 U/ml respectively. Similar report has been reported in alkali-tolerant *Bacillus patagoniensis* Oliver *et al.*, 2006, and Halophilic and alkaliphilic bacterial isolates showed activity at 4M NaCl Patel *et al*., 2006) [37]. The keratinolytic protease activity within the enzymes of the isolates were shown significant difference (P< 0.05).

Characterization of keratinolytic protease

Effect of pH on the activities and stability of keratinolytic proteases of the selected isolates

Effect of pH on the activity of keratinolytic protease was studied by incubating the reaction mixture at pH values ranging from 5 to 11 and a temperature of 37°C for 20 min. The highest keratinolytic protease activity in ms-2, Hs-1and Hs-3 was shown at pH 7.0 whereas in ms-1 the highest activity was recorded at pH 9 (Fig.4). The effect of pH on enzyme stability was examined by incubating the reaction mixture at pH values ranging from 5.0 to 11.0 and a temperature 37°C for 12 hours with casein in sodium phosphate buffer. The results showed that the stability of keratinolytic protease was higher at pH values ranging from 7.0 to 10.0 than at lower pH values exhibiting maximum stability at pH 8.0 in ms-1, ms-2, Hs-1; and Hs-3 (Fig. 4).

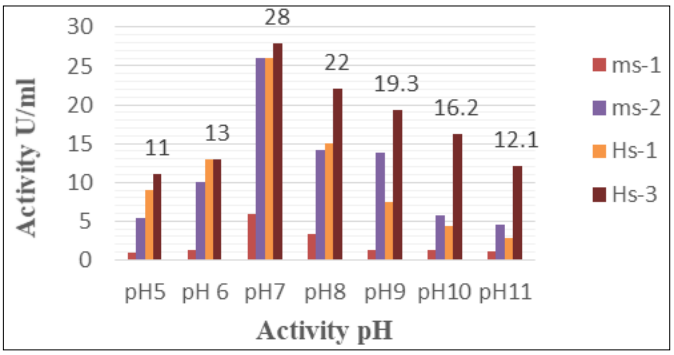


Fig (A)

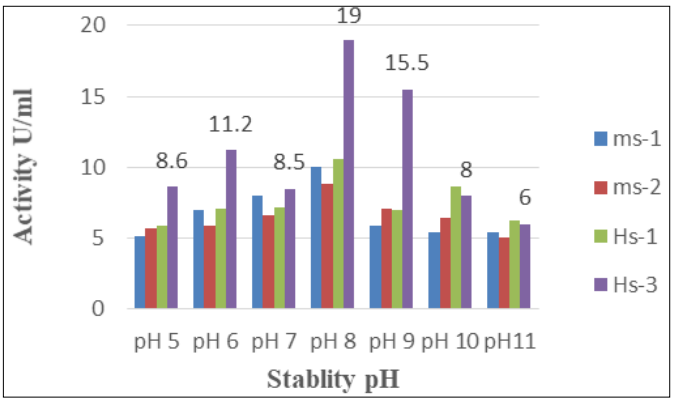


Fig 4 (A, B): Effect of pH on the activity and stability of keratinolytic protease (n=16; P=>0.05)

The optimum pH of the enzymes was 7.0 with more than 75% enzyme activity. This suggests the enzymes would be useful in processes that require a wide pH range from slight acidic to alkaline medium. The results of the present study were in line with activity of keratinolytic protease produced from *Bacillus cereus* at wide range of pH and maximum activity at pH 7.0 (Mrudula *et al*., 2011) [33]. Moreover, it was in agreement with the activity of the earlier reported keratinolytic protease produced from *Bacillus* species, which was in the range of pH 6-8 with an optimum activity at pH range of 7-8 (Salwa *et al.*, 2012) [42]. Also, relative lowest keratinolytic protease activity of isolates ms-1 (1.0 U/ml) and ms-2 (5.4 U/ml) were observed at pH 5. The keratinolytic protease activity within the enzymes of the isolates were shown significant difference (P< 0.05). These findings suggest that the keratinolytic proteases of the four isolates belonged to the alkaline protease class. In agreement with this, the optimum pH for stability of alkaline proteases from *Bacillus spp*. has been previously reported in various studies as lying between 9.0 and 11.0 (Deng *et al.*, 2010; Kumara *et al.*, 2012) [27].The keratinolytic protease activity within the enzymes of the isolates were shown significant difference (P< 0.05).

Effect of temperature on the activities and stability of keratinolytic proteases of the selected isolates

Effect of temperature on the activity of keratinolytic protease was studied, by incubating the culture filtrate with the substrate at temperatures ranging from 25 to 90ºC and at optimum pH for 20 min. The highest keratinolytic protease activity for isolates ms-1 and ms-2 was recorded at 40ºC, whereas for Hs-1 and Hs-3 it was 50ºC (Fig. 5). Some earlier reports had also indicated varying optimum temperatures in the range of 30-90 °C (Beg and Gupta 2003). The effect of temperature on the stability of keratinolytic proteases was also measured by pre-incubating them at the optimum pH for 12 hours. As shown in Fig. 5, the enzyme is active at temperatures between 30 and 90°C, with a highest stability obtained when held at 50°C for all strains for 12 hours.

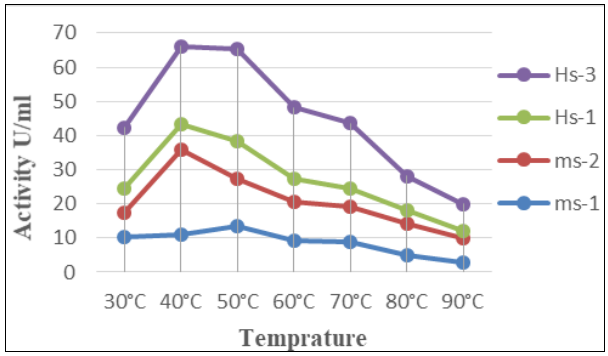


Fig (A)

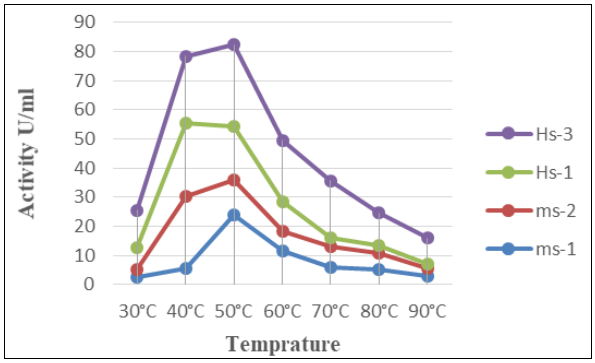


Fig 5 (A, B): Effect of incubation temperature on the activity and stability of keratinolytic proteases (n=16; P=>0.05)

The results of this study clearly indicate that the optimum temperature of proteolytic activity is in the range of the optimum temperature of enzyme production. The keratinolytic protease activity within the enzymes of the isolates were shown significant difference ($P< 0.05$). From the previous reports in stability of enzymes (Al-Shehri and Mostafa, 2004) [6-15], the protease activity was relatively stable at temperatures ranging from 50-65°C and 85.2% of the activity was retained after incubation at 60°C. The stability of protease enzyme could be due to the organisms' genetic adaptability to carry out their biological activities at higher temperatures (Brock, 2012). The keratinolytic protease activity within the enzymes of the isolates were shown significant difference ($P< 0.05$).

Partial purification of crude enzymes for hair removal

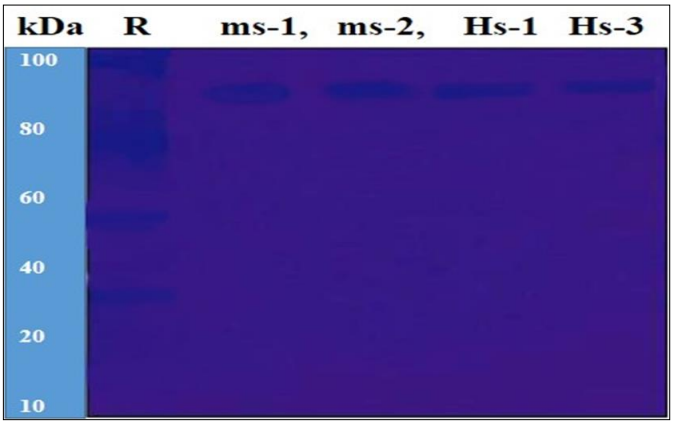
The crude enzymes produced from the selected bacterial isolates were partially purified by ammonium sulfate precipitation at 80% saturation level and dialysis using phosphate buffer. After precipitating the crude enzymes of isolates ms-1, ms-2, Hs-1 and Hs-3 by adding ammonium sulfate, purity of the enzymes were increased by 2.4, 2.6 and 2.5 folds, respectively. Moreover, after dialysis, purity of the enzymes of the isolates ms-1, ms-2, Hs-1 and Hs-3 were increased by 2.8, 2.9, and 3.0 folds, respectively (Table 2).

Table 2: Partial purification of keratinolytic Protease produced from selected bacterial isolates (n=16; P=>0.05)

Bacterial Isolates	Purification steps	Total volume (ml)	Enzyme Activity (U/ml)	Final Purification (folds)
ms-1	Crude	50	7.8	1.0
	(NH3)2SO4 Precipitation	20	14.8	2.0
	Dialysis	9	20.5	2.7
ms-2	Crude	50	12.2	1.0
	(NH3)2SO4 Precipitation	21	29.5	2.42
	Dialysis	8	33.4	2.7
Hs-1	Crude	50	7.5	1.0
	(NH3)2SO4 Precipitation	18	18.5	2.5
	Dialysis	11	36.5	4.9
	Crude	50	8.3	1.0
Hs-3	(NH3)2SO4 Precipitation	25	15.8	1.9
	Dialysis	10	42.2	5.1

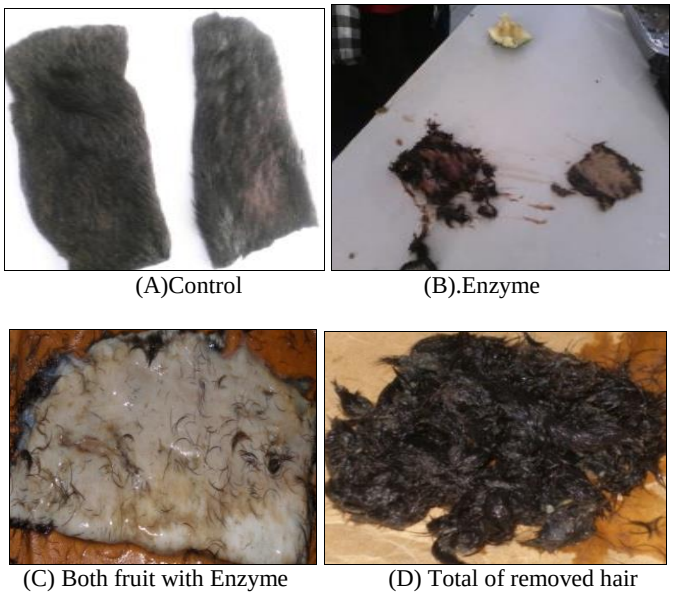
Molecular weight determination of partially purified keratinolytic protease using SDS PAGE

Molecular weights of the partially purified Keratinolytic protease produced from the bacterial isolates were determined using SDS PAGE. In this study, 6X Tries protein (10-100kDA) was used as reference marker protein. The partially purified enzymes had a single protein band on the SDS PAGE. In relation to migration of the reference marker protein, the approximate molecular weights of the partially purified enzymes were found to be 80 kDa (Figure14).



Enzymatic evaluations of cow hide de-haring

To evaluate the potential use of this enzyme as a hide depilating agent in leather industries, pair of cow hide was taken and added to 250 ml flask containing enzyme, enzyme and fruit Entelya (*Lagenaria abyssinica*) and gulo (*Ricinus communis* L.) At a time from each pair taken and hair removal trail was done. As shown in Fig. 6 below, complete de-haring of the enzyme treated skin was achieved in 24 hours, at room temperature, with 27.2 U/ml cow hide resulting pelt (hide) of natural pore (grain) on dehaired surface.



(A)Control (B).Enzyme

(C) Both fruit with Enzyme (D) Total of removed hair

Fig 6: Example of cow hair de-haring performance analysis test of Hs-3. (A) Control with distilled water, B. Enzyme only, C. Enzyme with Entelya. (n=16; P=>0.05)

Results of enzymatic cow hide de-haring showed successful use of the enzyme as a de-hairing agent. Complete de-hairing of hide with fruit was achieved at 12 hours. Because of specificity to hydrolysen on collagen protein part at hair roots in hide, keratinolytic proteases are very important in shortening hide de-haring time and in production of high quality full gain leather having natural hair pores on the surface (Siva Subramanian *et al.*, 2008) [47]. Cow hide usually treated with de-hairing chemicals in adrum for 24 hours (Thanikaivelan *et al*., 2004) [50]. Shortening of de-haring time has been also reported, 20 hours for *Aspergilus flavus* protease by Malathi & Chakraborty, (1991) [30], and 9 hours for keratinases of *Bacillus subtilis* S14 by Macedo *et al.*,

(2005). Thus, keratinolytic protease has a potential to substitute environmentally objectionable de-haring chemicals for hide/skin de-haring in leather industries and for production of quality leather products.

Enzymatic feather degrading process

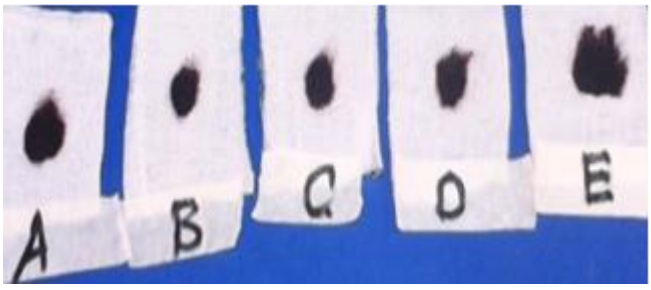
To evaluate the potential use of this enzyme as a feather depilating agent in poultry industries, pair of hen feather was taken and added to 20 ml test tube containing enzyme, enzyme and fruit Entelya (*Lagenaria abyssinica*) and gulo (*Ricinus communis* L.) at a time from each pair taken and feather degradation trail was done. Complete feather degradation of the enzyme treated feather was achieved in 24 hours, at room temperature, with 27.2 U/ml hen feather resulting break natural shapes on feathers.

Quantitative determinations of enzyme for de-haring processes

The quantitative determination of the all selected strain enzymes wear effective until serial dilution three with pieces of the two fruits. The de-haring process and feather degrading wear effective up to three batches with effective de-haring process and degrading process but the detergent application was for two batches only.

Evaluations of washing performance of Hs-3 strain's Enzyme

In order to evaluate the performance of Hs-3 in terms of ability to remove harsh stains, namely those caused by oils or human blood, several pieces of stained cotton cloth were incubated at different conditions (Fig. 7). The findings from these assays revealed that the blood stain removal levels achieved with the use of Hs-3 with the fruit were more effective than the ones obtained with detergent, or detergents alone. In fact, Hs-3 facilitated the release of proteinaceous materials in a much easier way than the commercialized SB 309 protease. Furthermore, the combination of Hs-3 and the detergents detergent resulted in complete stain removal (Fig. 7). In fact, a similar study has previously reported on the usefulness of alkaline proteases from *Spilosoma obliqua*, *B. brevis* (Banerjee *et al.*, 1999) in the assistance of blood stain removal from cotton cloth both in the presence and absence of detergents, but, in terms of reported results, the Hs-3 enzyme was more effective.



A. Before wash with enzyme

B. After wash with enzyme

Fig 7: Example of washing performance analysis test of Hs-3. Stained cloth pieces with blood. (A) Control stained cloth pieces washed with distilled water, B. Detergent (7mg/ml). C. Enzyme only, (D) Enzyme with gollo and (E). Enzyme with Entelya. (n=16; P=>0.05)

Quantitative determinations of enzyme for de-haring processes

The quantitative determination of the all selected strain enzymes wear effective until serial dilution three with pieces of the two fruits. The de-haring process and feather degrading wear effective up to three batches with effective de-haring process and degrading process but the detergent application was for two batches only.

Conclusion

The aim of this study was to isolate potentially potent keratinolytic protease producing bacteria (Bacillus spp.) from two different sample sources (mud and hair from several traditional leather processing pond) and to optimize their cultivation condition for maximum keratinolytic protease production. Keratinolytic proteases are one of the most important groups of industrial enzymes with considerable application in the animal feed processing, leather industry, medical activity, beverage industry and others sectors. From a total of 215 pure bacterial colonies, 145 (67.4%) were found as keratinolytic protease positive, out of the 145 keratinolytic protease positives, following selection criteria on the basis of their clear zone diameter on milk agar plate, ms-1 and ms-2 mud sample collected, Hs-1 and Hs-3 hair sample collected from Seveha. Based on the results of different morphological, physiological and biochemical tests done, these isolates were found to be members of the genus *Bacillus spp*.

The effect of different physical and chemical parameters; incubation period (24, 36, 48, 60 and 72); temperature (25, 30, 37, 40, 45, and 50°C); initial pH of media (5, 6, 7, 8 and 9); different carbon sources (Wheat bran, rice bran, glucose and sucrose) and from hair (Human hair, cow skin hair, goat skin hair and feather); organic and inorganic nitrogen sources (Casein, peptone, yeast extract, ammonium sulphate and ammonium chloride); inoculums size (5%, 10%, 15% and 20% v/v); NaCl concentration (0.00, 0.2, 0.4, 0.6, and 0.8M) and moisture level (1:2, 1:3, 1:4 and 1:5 v/w) on keratinolytic protease production by these isolates were studied. The potential of the crude enzyme harvested from these isolates were also evaluated for hair removal from a piece of skin.

Time courses of keratinolytic protease production in all isolates indicates that the production increases as time increases up to the optimum time of incubation and decline after wards. The maximum keratinolytic protease was harvested after 48 hrs in all isolates. The effect of different temperatures show as the incubation temperature increases production increases up to the optimum temperature, but beyond the optimum production decreased. Isolates ms-1 and Hs-1 produce maximum keratinolytic protease at 40°C, whereas 30°C was optimum for isolate ms-2 and Hs-3. All isolates produced maximum keratinolytic protease at pH 7 when compared to some slight acidic and alkaline ph.

Isolate ms-2, Hs-1 and Hs-3 gave maximum keratinolytic protease in medium supplemented with wheat bran whereas isolate ms-1 gave high keratinolytic protease in the present of rice bran. Isolate ms-1, ms-2, Hs-1 and Hs-3 gave maximum keratinolytic protease in medium supplemented with cow skin hair gave high keratinolytic protease. The effect of nitrogen sources indicated that in all isolates organic nitrogen sources resulted in maximum keratinolytic protease production as compared to inorganic nitrogen sources. In isolate ms-1, ms-2, Hs-1 and Hs-3 maximum keratinolytic protease was obtained in a medium containing casein.

The production curve of effect of different size of inoculums on keratinolytic protease production revealed that

keratinolytic protease production increased when the percent of inoculums increased up to the optimum and decreased beyond the optimum size. In all isolates maximum keratinolytic protease were harvested in 10% v/v inoculums. The effect of moisture level on keratinolytic protease production indicated that keratinolytic protease production increased with increased bran to moistening agent till optimum decreased beyond the optimum and all isolates give maximum protease at 1:3 v/w bran to moisture ratio. On the other hand, production of keratinolytic protease is also influenced by the concentration of NaCl on the growth media. The optimum NaCl concentration was found to be 0.2 M for all four isolates.

Although many potent isolates are on market for enzyme production, scientists prefer studying new isolates because they could be alternative for commercial use in many aspects. Many studies showed that researches will continue to isolate alternative strains for production of enzymes as well as keratinolytic proteases. The isolated new source of keratinolytic protease producing bacteria, from the soil and water samples that are collected from traditional leather processing ponds might be an alternative source for the potential industrial applications.

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