Laundry Detergent Compatibility and Dehairing Efficiency of Alkaline Thermostable Protease Produced from *Aspergillus terreus* under Solid-state Fermentation

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Abstract: *Aspergillus terreus* was chosen for production of alkaline protease using solid-state fermentation (SSF). The maximum enzyme yield reached about 34.87 U/mg protein after optimization of fermentation parameters. The produced alkaline protease was purified by precipitation with iso-propanol and then purified through gel filtration and ion exchange column chromatography with a yield of 53.58% and 5.09-fold purification. The enzyme has shown to have a molecular weight of 35 kDa. Optimal pH and temperature for the enzyme activity were 9.5 and 50°C respectively. The highest activity was reported towards casein, with an apparent Km value of 6.66 mg/mL and Vₘₐₓ was 30 U/mL. The enzyme activity was greatly repressed by phenylmethylsulfonyl fluoride (PMSF). Sodium dodecyl sulfate (SDS) caused activation in enzyme activity. The enzyme retained about 83.8, 70.6, 74.5, 76.4 and 66.4% of its original activity after incubation with Aerial, Leader, Oxi, Persil and Tide, respectively for 8 h at 60°C. Adding of the enzyme in detergents improved the cleansing performance to the blood stains and suggested to be used as a detergent additive. Our outcomes showed that protease could be used as environment green-approach in dehairing process.

Key words: detergent compatibility, dehairing efficiency, alkaline protease, purification, *Aspergillus terreus*, wheat straw

1 Introduction

Protease enzymes (EC 3.4.21-24) are fit for hydrolyzing the bonds between amino acids of protein molecule and they are the major industrial enzymes, which represented 60% of the complete synthetically produced enzymes. Alkaline proteases of microbial origin have impressive industrial intensity because of their biochemical decent variety and enormous applications in detergents, pharmaceutical, nourishment, dairy and leather industries.

Fungi are the most microbes producing proteases with many advantages, since they are normally GRAS (generally regarded as safe) strains and their enzymes are secreted outside the cell which easily recover the enzyme.

Despite the fact that fungi are significant wellsprings of alkaline proteases, studies on their protease production are confined for the reason that the fungi prefer acidic and neutral pH values. Thus, it is of prime interest to search for extremophilic fungi and optimize their growth conditions to maximize their enzyme production for their novel and versatile applications. Alkaline proteases are essentially utilized in detergent industry and they should not be in pure structure. Nevertheless, proteases that are utilized in medical applications must have a high level of purity.

Thermostable enzymes are those that have high activity and hold an incredible part of this action when present under relatively high temperatures. Expanded thermostability is a significant impact for the reasonableness of an enzyme in industrial applications. By applying inhibition studies we can deduce the nature of enzyme as well as the characteristics of its active side and its cofactor requirements.

Nowadays, the use of enzyme (protease)-based detergents is preferred over the conventional synthetic ones in view of their cleaning properties, better performance at

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lower washing temperature and the attenuation of environmental pollution.

Alkaline proteases are used in dehairing and bating stages of leather manufacturing process. The efficiency of alkaline protease in dehairing process is attributed to that alkaline media allow the tumescence of hair roots and the subsequent attack of protease on the hair follicle protein allows soft hair removal. In the present study, we tried to develop a cost-effective medium, based on cheap and locally available raw materials, optimize the culture condition for maximal production of alkaline protease from Aspergillus terreus, purify and characterize an alkaline protease of A. terreus. As well as to find out its appropriateness in laundry detergents and leather industry as dehairing agent.

2 Materials and Methods

2.1 Chemicals

Folin reagent, corn steep liquor and soya bean meal were obtained from Sigma-Aldrich (St. Louis, Mo). Cane and beet molasses were obtained from Hawamdiah and Kafr-El-Sheikh sugar factories, respectively, Egyptol was obtained from clean way Company, Cairo, Egypt. Crude whey was obtained from Kingdom of Saudi Arabia and Sharm Sheikh sugar factories, respectively. All the chemicals were of analytical grade.

2.2 Isolation and identification of thermophilic fungi

Fungi used in this work were isolated from different soil samples obtained from Kingdom of Saudi Arabia and Sharm El-Sheikh, Egypt. The direct plate procedure was utilized for fungal isolation. Yeast extract soluble starch agar (YpsS) medium was used throughout isolation studies. The medium was having the following compositions (g/L): starch, 15.0; magnesium sulphate, 1.0; dipotassium hydrogen phosphate, 1.0; and yeast extract 3.0. The samples were incubated at 45°C for 10 days. The purified isolates were identified according to their morphological and colonial characteristics. All cultures were cultivated on YpsS agar slants every 8 weeks and incubated for the seventh day at 45°C and subsequently stored at 4°C as stock.

2.3 Screening of alkaline protease producers

Obtained fungi were evaluated for alkaline protease production using plate assay method with minor modifications. The medium containing (g/L) glycerol 5, casein 20, yeast extract 3, NaCl 5 and agar 20, initial pH 9.0 and incubated at 45°C for seven days. The plates were then stained with coomassie brilliant blue pursued by destaining to measure the radius of hydrolysis zone due to the action of alkaline protease.

2.4 Solid-State Fermentation (SSF)

Various agro-industrial residues such as wheat bran, wheat straw, rice bran, ground nut shell, soya bean meal, corn cobs and sugar cane bagasse, were evaluated for their potential as substrate in SSF for alkaline protease production. SSF was carried out by taking 5.0 g of substrate in 250 mL Erlenmeyer flask, moistening it with 5.0 mL of salt solution containing 0.1% dipotassium hydrogen phosphate, 0.5% magnesium sulphate, 0.5% sodium chloride and 0.004% ferrous sulphate. All the flasks were adjusted to pH 9.0 with 0.1 N NaOH, autoclaved, inoculated with one mL of spore suspension of Aspergillus terreus containing about 10⁷ spores/mL and incubated at 45°C for 5 days.

2.5 Protease assay

Proteolytic activity was examined by the method embraced by Keay et al. with minor adjustments. One milliliter of enzyme was blended with 0.8 mL 1% of casein solution in 0.2 M Tris-HCl buffer pH 9. The mix was kept at 40°C for 30 min. The reaction was finished by the adding 2 mL 0.4 M trichloroacetic acid, incubated for 20 min at 40°C and filtered. At that point 1 mL from the filtrate with 5 mL 0.4 M Na₂CO₃ and 1 mL Folin phenol reagent were combined together. The blend was incubated at 37°C for 20 min and the final concentration was monitored at 660 nm. One unit of enzyme activity was defined as the amount of enzyme wanted to discharge one μmol tyrosine under typical assay conditions. The whole assay was done in triplicates.

2.6 Keratinase assay

Keratinase activity was resolved by the technique detailed by Nickerson et al. with some alternations. The reaction blend comprised of 1mL enzyme solution, 10 mg of keratin in 0.2 M Tris-HCl buffer pH 9, blend was incubated under agitation using mixing bar for 1 h at 40°C, at that point the reaction was terminated by heating up the mixture and filtered through filter paper. One unit of enzyme activity was defined as the amount of enzyme creating 1 μg of keratin/min.

2.7 Lipase assay

Lipase activity was estimated by hydrolysis of p-nitrophenyl palmitate (p-NPP) with some modifications. One milliliter of enzyme solution was added to 0.2 M Tris-HCl buffer pH 9 containing 0.2% (w/v) sodium deoxycholate and 0.1% (w/v) Arabic gum in a last volume of 3.0 mL. This blend was incubated for 5 minutes at 30°C. p-NPP (0.3 mM final concentration) was added to the mixture, shaken, enabling the reaction to forward for 3 minutes. Lipase activity was determined by the rate of p-nitrophenol production (p-NP), estimated at 405 nm. One unit of enzyme activity is defined as the amount of enzyme forming 1 μmol of p-NP min⁻¹.
2.8 Protein determination

The protein content of the enzyme preparations was measured by the method of Lowry et al. All determinations were achieved in triplicates and the mean values are presented.

2.9 Optimization of alkaline protease production parameters

The SSF was also optimized through various parameters including moisture content of substrate (58, 71.5, 78.3, 82 and 85%), incubation period (2, 3, 4, 5, 6 and 7 days), incubation temperature (30, 35, 40, 45, 50, 55 and 60°C), size of inoculum (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL of spores suspension) and initial pH of basal medium (8.0, 8.5, 9.0, 9.5, 10.0, 10.5 and 11.0). The effect of supplementation of the basal medium with different by-products (molasses, beet molasses and corn steep liquor) as additives (5%, v/v) and effect of different concentrations of whey (5, 10, 15, 20, 25, and 30%) were also ascertained.

2.10 Treatment of Cane and Beet molasses (CM&BM)

Two hundred milliliter molasses was diluted to one liter by distilled water, autoclaved, filtered to abstract the deposited mud and the filtrate was kept in refrigerator. Molasses and whey were analyzed for carbohydrates, nitrogen content and metal ions by the Services Central Lab, National Research Centre Cairo-Egypt.

2.11 Enzyme purification

Enzyme purification was begun by precipitation of 500 mL of crude enzyme preparation (CEP) with (NH₄)₂SO₄ and organic solvents, i.e., acetone, ethanol and iso-propanol. After standing overnight at 4°C, the precipitates were gathered by centrifugation at 12,000 g for 15 min, dissolved in 2 mL 0.2 M Tris-HCl buffer (pH 9). Protein precipitates containing vast majority of the enzyme activity were then fractionated on Sephadex G-100 column (1.5 × 45 cm). After elution active fractions were loaded on DEAE-cellulose column. Column was eluted with gradient of 0-0.8M NaCl prepared in the corresponding buffer at a flow rate of 20 mL/h and 5 mL fractions were gathered. This enzyme was lyophilized for further examinations.

2.12 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was completed by Laemmli, utilizing 10% polyacrylamide. Proteins were detected by Coomassie Brilliant Blue R250 stain.

2.13 Determination of kinetic parameters

Optimum temperature of the enzyme activity was determined by incubating enzyme at different temperatures extending from 30 to 80°C in 0.2 M Tris-HCl buffer (pH 9.0). Thermostability of the enzyme was monitored at various temperatures for different periods. The optimum pH of the protease activity was investigated by measurements at 50°C in different buffers covering the pH range of 7.0-12.0. The pH stability of the enzyme was determined by measuring its remaining activity for different periods with different pH values. Substrate specificity of alkaline protease with different protein substrates (1%) of bovine serum albumin, egg albumin, casein and gelatin was tested by blending the purified enzyme and substrates in 0.2 M glycine-NaOH buffer (pH 9.5) and incubating for 30 min at 50°C. Enzyme was treated by various concentrations of casein and different kinetic parameters were calculated. Michaelis constant (Km) and maximum velocity (Vmax) of enzyme activity were calculated by linear regression from Lineweaver-Burk plot. Impact of different metal ions and a few inhibitors on activity of the purified alkaline protease at 5 mM final concentration, for example, ethylenediaminetetra acetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) and iodoacetate.

2.14 Compatibility of alkaline protease with detergent components

The remaining activity of alkaline protease was assessed in the existence of various detergent components. The surfactants utilized were Sodium dodecyl sulfate (SDS), triton X-100 and tween-80, tween 20 whereas the oxidizing and bleaching agents were hydrogen peroxide and sodium perborate, respectively. 1.9 mL of (1.0 & 5.0%) surfactant/oxidizing agent was mixed with 0.1 mL of crude enzyme (34.87 U/mg protein) in 0.1 M glycine-NaOH buffer (pH 9.5) and preincubated for 30 min at 45°C then the remaining activity of protease was estimated.

2.15 Compatibility of alkaline protease with local laundry detergents

Aerial, Leader, Oxi, Persil, Savo and Tide were utilized as local laundry cleansers. Detergents were diluted to a concentration of 7.0 mg/mL to mimic washing condition. The diluted detergent was heated at 95°C for 10 min to inactivate the endogenous enzymes found in it. A reaction mixture comprising 0.3 mL of crude enzyme (34.87 U/mg protein) and 5.7 mL detergent was preincubated at 28, 60 and 90°C. Then the residual activity was evaluated at 0, 0.5, 1, 2, 4, 8, 16 h. The enzyme activity without detergent was taken as control 100%.

2.16 Cleansing potential of the alkaline protease

The destaining power of alkaline protease for disposing of blood stains from cloth pieces was examined by the method of Najafi et al. with minor alternations. The small white cloth pieces (4 × 4 cm) were stained with blood for 15 min and then dried for 5 min at 80°C. One piece of dried cloth was soaked in 20 mL tap water and 1 mL of detergent (0.7%, w/v). Another piece was soaked in 20 mL tap water,
1 mL of detergent (0.7%, w/v) and 3 mL of crude alkaline protease (34.87 U/mg protein). The soaked pieces were incubated at 50°C for 40 min, then the cloth pieces were rinsed washed with tap water for 2 min and then exposed to visual estimation. The unstained cloth was considered the control.

2.17 Preparation of hides for enzyme treatments (British Leather Confederation, BLC method)
A fresh fleshed cow hide was preserved by NaCl 25% (w/v) then washed with an Egyptol and cut into 10 × 5 cm² pieces (30 g weight). Two pieces were mixed in shaken flask at (4 rpm) with crude protease solution (3:1, w/v) at 28°C ± 2 for 6−36 h. Lime and Na₂S were used instead of the enzyme solution for comparison. The unhairy leather was analyzed by visual evaluation, physical measurements and scanning electron microscope (SEM) measurements.

2.18 Physical measurements
The hides (chemically or enzymatically treated) were rated for some mechanical properties as tensile strength (N/mm²) and elongation (%) by Lab of Tanning and Leather Technology, National Research Center (NRC), Cairo, Egypt. Higher values indicate superior properties.

2.19 Scanning electron microscope (SEM) analysis
Enzymatic and sulphide-lime unhairy hides were examined at Services Central Lab (NRC) by SEM (JEOL, made in Japan, maximum magnification 622.50 ×, maximum resolution power 0.2 nm/line) Applying 100 × magnifications, the surface holes, the grain surface, and the opening up of fiber bundle were observed. The clean grain surface elucidates no grain injury due to hide unhairing process.

2.20 Statistical analysis
The obtained data were statistically analyzed with SPSS (Scientific Package for Scientific Social Studies, version 20), in which the equations of the hypothesis tests, including the mean, standard deviation, T-statistics value and probabilities (p) were used. Results were considered highly significant, significant, or non-significant, where p ≤ 0.01, p ≤ 0.05 and p > 0.05, and represented by HS, S and NS, respectively.

3 Results
3.1 Screening of fungal isolates for alkaline protease production
Twelve fungal isolates were investigated for their capability to produce alkaline protease belonged to ten genera, namely, Asbsidia, Aspergillus, Chaetomium, Emericella, Humicola, Penicillium, Rhizopus, Thermomces and Trichoderma by using plate assay method.

Among the twelve isolates, Aspergillus terreus, Thermo-
myces lanuginosus, Aspergillus fumigatus, Humicola insolens, Emericella nidulans and Chaetomium thermophi-
le recorded the highest proteolytic activity. The most potent fungus to produce alkaline protease was A. terreus exhibited a diameter of clear zone of (16.0 ± 0.11 mm).

3.2 Screening of different agro-industrial residues for alkaline protease production under SSF
Various agro-industrial residues were tested as substrate to produce alkaline protease from A. terreus (Table 1). Wheat bran was found to be the most potential substrate for the alkaline protease production using giving maximum specific enzyme activity 17.26 ± 0.43 U/mg protein followed by rice bran 11.21 ± 0.15 U/mg protein. While Soya bean meal recorded the lowest specific enzyme activity 3.21 ± 0.25 U/mg protein.

3.3 Optimization of fermentation parameters for maximum alkaline protease production
The production of alkaline protease is influenced by various physical parameters including initial moisture content, incubation period, incubation temperature, size of the substrate than 71.5 %. Results showed that the maximal alkaline protease production was obtained at initial moisture content of wheat bran of 71.5% (20.82 ± 0.14 U/ mg protein, Table 1). An increase in the moisture content of the substrate than 71.5% resulted in a decline in alkaline protease yield.

3.4 Effect of initial moisture content on alkaline protease production by A. terreus under SSF
Gradual initial moisture contents were used to investigate the best moisture content for maximum protease production by A. terreus. Results showed that the maximal production of alkaline protease was obtained at initial moisture content of wheat bran of 71.5% (20.82 ± 0.14 U/mg protein, Table 1). An increase in the moisture content of the substrate than 71.5% resulted in a decline in alkaline protease yield.

3.5 Effect of different incubation periods on alkaline protease production by A. terreus under SSF
Experimental results showed that the maximum alkaline protease activity was obtained after six days and the activity decreased after this time of incubation (22.5 ± 0.44 U/mg protein, Table 1).

3.6 Effect of incubation temperature on alkaline protease production by A. terreus under SSF
With respect to the influence of incubation temperature on enzyme production Table 1 showed that maximum enzyme activity of 22.5 ± 0.11 U/mg protein was obtained by A. terreus at 45°C.
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Table 1  Impact of various parameters on alkaline protease production by from Aspergillus terreus.

| agro-industrial residues | Wheat bran | Wheat straw | Rice bran | ground nut shell | Soya bean meal | Corn cobs | Sugar cane bagasse |
|--------------------------|------------|-------------|-----------|------------------|---------------|-----------|-------------------|
| Specific Enzyme activity (U/mg) | 17.26 ± 0.43 | 8.1 ± 0.09 HS | 11.21 ± 0.15 HS | 8.1 ± 0.30 NS | 3.21 ± 0.25 HS | 9.93 ± 0.32 HS | 10.05 ± 0.54 S |
| Initial moisture content (%) | 58 | 71.5 | 78.3 | 82 | 85 |
| Specific Enzyme activity (U/mg) | 17.24 ± 0.39 HS | 20.82 ± 0.14 | 14.3 ± 0.38 NS | 10.74 ± 0.28 HS | 8.2 ± 0.29 S |
| Incubation period day | 2 | 3 | 4 | 5 | 6 | 7 |
| Specific Enzyme activity (U/mg) | 7.32 ± 0.25 HS | 13.54 ± 1.02 HS | 18.82 ± 0.44 HS | 20.82 ± 0.48 S | 22.5 ± 0.44 | 9.33 ± 0.33 HS |
| Incubation temperature °C | 12.54 ± 1.02 S | 17.82 ± 0.25 HS | 19.72 ± 0.38 S | 22.5 ± 0.11 | 20.12 ± 0.07 HS | 16.13 ± 0.29 S | 18.28 ± 0.18 S |
| Inoculum size ml | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
| Specific Enzyme activity (U/mg) | 17.94 ± 0.08 HS | 22.5 ± 0.19 HS | 23.23 ± 0.10 S | 25.55 ± 0.20 | 21.00 ± 0.21 HS | 18.28 ± 0.18 S |
| Initial pH | 8 | 8.5 | 9 | 9.5 | 10 | 10.5 | 11 |
| Specific Enzyme activity (U/mg) | 20.5 ± 0.46 NS | 21.3 ± 0.31 HS | 25.55 ± 0.09 HS | 28.14 ± 0.20 S | 30.28 ± 0.37 HS | 25.74 ± 0.17 S | 11.38 ± 1.34 NS |
| additives 5% (v/v) Whey | Cane molasses | Beet molasses | Corn steep liquor |
| Specific Enzyme activity (U/mg) | 32.15 ± 0.37 | 30.8 ± 0.62 S | 21.66 ± 0.11 HS | 25.92 ± 1.14 HS |
| Whey concentration (%) | 5 | 10 | 15 | 20 | 25 | 30 |
| Specific Enzyme activity (U/mg) | 32.15 ± 0.27 S | 34.87 ± 0.82 | 31.2 ± 0.59 HS | 30.87 ± 0.10 S | 31.94 ± 0.99 S | 31.22 ± 0.34 S |

3.7 Effect of inoculums size on alkaline protease production by A. terreus under SSF

The inoculum size is a significant factor, which determines the production of biomass. The maximum enzymatic activity was gained when the culture medium was inoculated with 2 mL of inoculums containing approximately 2 × 10⁷ spores/(25.5 ± 0.20 U/mg protein, Table 1). With an additional increase in inoculums size, enzyme productivity decreased.

3.8 Effect of initial pH of basal medium on alkaline protease production by A. terreus under SSF

In order to evaluate the consequence of initial pH of media on the alkaline protease production, fungal cultures were grown at different pH (8-11). Our results (Table 1) showed that the maximum protease production was recorded at pH 10 (30.82 ± 0.37 U/mg protein, Table 1).

3.9 Effect of different additives on alkaline protease production by A. terreus under SSF

As shown in Table 1, several by-products (whey, cane molasses, beet molasses and corn steep liquor) have been added as additives to enhance protease production. The addition of whey to optimized salt basal medium by (5%, v/v) resulted in maximum alkaline protease production by A. terreus (32.15 ± 0.37 U/mg protein) followed by cane molasses (30.8 ± 0.62 U/mg protein). In addition, increase the concentration of whey to 10%, v/v resulted in increasing the enzyme productivity (0.3487 U/mg protein).

3.10 Determination of mixture of alkaline enzymes in the crude enzyme preparation of A. terreus

As shown in Fig. 1 the crude enzyme preparation of A. terreus showed a variety of alkaline enzyme activities, it recorded 34.87, 7.15, 8.23 U/mg protein for alkaline protease, keratinase and lipase, respectively.

3.11 Purification

A summary of purification process for the produced al-
Table 2  Summary of purification of alkaline protease from *A. terreus*.

| Purification step  | Total activity (U) | Total protein (mg) | Specific activity (U/mg protein) | Yield (%) | Purification fold |
|-------------------|--------------------|--------------------|----------------------------------|-----------|------------------|
| CEP               | 9940               | 285                | 34.87                            | 100.0     | 1.00             |
| CFP: iso-propanol (2:1, v/v) | 7698               | 124                | 62.01                            | 67.38     | 1.78             |
| Sephadex G-100    | 5993               | 81                 | 73.98                            | 60.29     | 2.12             |
| DEAE-Cellulose    | 5326               | 30                 | 177.53                           | 53.58     | 5.09             |

3.12 Determination of enzyme purity

The molecular weight of the protease was found in Table 2. The results reveal that precipitation of proteins from the crude enzyme preparation (CEP) by isopropanol (2:1 v/v) provided a fraction that has the highest total enzyme activity. Completion of enzyme purification was achieved by gel filtration through Sephadex G-100 and ion-exchange chromatography with DEAE-cellulose. The highest activity of protease was found in fractions 16–22 in gel filtration step and in the fraction 19–25 ion-exchange chromatography with a yield of 53.58% and 5.09-fold purification.

3.13 Effect of temperature on enzyme activity and stability

Optimum activity of *A. terreus* alkaline protease in its purified state was recorded at 50°C and decreasing the activity above this degree (Fig. 3a). Concerning to the thermostability of the purified enzyme (Fig. 3b), the purified enzyme retained 100% of its original activity at 30–50°C after incubation for complete hour. It also retained about 82% of this activity at 60°C, 66% at 70°C and 40% at 80°C after incubation for complete hour.

3.14 Effect of pH on enzyme activity and stability

The results (Fig. 3c) reveal that the purified enzyme showed a great activity at broad alkaline pH values with the maximum at pH 9.5. The enzyme had a good stability on the alkaline side. It retained full activity after 60 min of incubation at pH 8.5 and about 86% of the original activity was restored at pH 9.5 and retained about 74% of the original activity after 60 min of incubation at pH 10.5 (Fig. 3d).

3.15 Substrate specificity of *A. terreus* alkaline protease

Results shown in Table 3 reveal that the enzyme had a relative activity towards various protein substrates. The enzyme expressed its greatest activity towards casein (28 ± 0.21 U/mL) and the lowest against bovine serum albumin (11.67 ± 0.31 U/mL). From the Lineweaver-Burk plot, the Michaelis constant (Km) and maximum protease activity (Vmax) was calculated to be 6.66 mg/mL and 30 U/mL, respectively at concentration of 10 mg/mL of casein (data not shown).

3.16 Effect of different metal ions and inhibitors on alkaline protease activity

The effect of some metal ions on alkaline protease activity was tested in Table 4. The results showed that K⁺ acts as potent activator, where the relative enzyme activity was significantly increased to 133%. Moreover, Ca²⁺, Mn²⁺ and Zn²⁺ had moderate stimulatory effect on enzyme activity which recorded 105.11, 111.32 and 116.11% respectively. But, the enzyme activity was suppressed in the presence of Co²⁺, Cu²⁺, Hg²⁺. The effects of various enzyme inhibitors on the activity of *A. terreus* alkaline protease are also evaluated. The enzyme activity was greatly inhibited by PMSF.
where the residual activity of purified protease recorded 22.65%. Moreover, the enzyme activity was slightly suppressed in the presence of EDTA and iodoacetate where the residual activity recorded 75.89 and 82.76% respectively.

3.17 The impact of surfactants and oxidizing agents on alkaline protease

The impact of various surfactants and oxidants agents at different concentrations on alkaline protease activity was studied (Fig. 4). The results showed that SDS caused activation in enzyme activity where the enzyme activity was significantly increased to 120 and 105% of the original activity at a final concentration of 1 and 5%, respectively. Other surfactants such as Triton X-100 and Tween-20, Tween 80 caused slightly decline in the alkaline protease activity at their high concentrations. While the enzyme retained about 80 and 66% of its original activity with 5% of H2O2 and NaHBO4, respectively.

### Table 3

| Substrate               | Protease activity (U/ml) |
|-------------------------|--------------------------|
| Bovine serum albumin    | 11.67 ± 0.31             |
| Egg albumin             | 17.55 ± 0.11             |
| Casein                  | 28 ± 0.21                |
| Gelatin                 | 20.67 ± 0.45             |

3.18 Compatibility of alkaline protease with local laundry detergents

The alkaline protease was 100% stable and compatible until 2 hours at 60°C with all the tested detergents except leader with retention of 84.4% of its original activity. And the enzyme retained about 83.8, 70.6, 74.5, 76.4 and 66.4% of its original activity after incubation with Aerial, Leader,
Fig. 4 Stability of the alkaline protease of *A. terreus* with various detergent components.

Fig. 5 Compatibility of alkaline protease with local laundry detergents at 60°C.

Oxi, Persil and Tide, respectively for 8 h at 60°C (Fig. 5). Moreover, the enzyme retained about 70.5, 60.6, 66.4, 64.6 and 60.3% of its original activity with Aerial, Leader, Oxi, Persil and Tide, respectively for 8 h at 28°C (data not shown). Also, the enzyme retained about 48.4, 47.6, 40.3, 42.5 and 43.6% of its original activity with Aerial, Leader, Oxi, Persil and Tide, respectively for 8 h at 90°C (data not shown).

3.19 Cleansing potential of the alkaline protease as a detergent additive

Results showed that the protease-leader (Non enzymatic commercial detergent) mixture had the ability to totally remove blood stain from the cloth piece (Fig. 6).

3.20 Dehairing capability of alkaline protease of *A. terreus*

The process of cow-hide unhairing by utilizing a crude alkaline protease compared to the conventional chemical techniques using 3% (w/v) Na₂S and 4% (w/v) Ca(OH)₂ was
achieved, then the unhaired skins were exposed to visual assessment, physical measurements and SEM investigation. It has been noticed the simplicity of removing hair from treated samples with crude alkaline protease by A. terreus was probable after 34h while the conventional chemical method needed about 26 h. Moreover, hand valuation of the unhaired hide samples for various organoleptic properties, such as fullness, softness, grain softness and tightness was carried out and demonstrated organoleptic properties of the unhaired specimen by crude protease were more competent than those treated by chemical method. The results obtained in Table 5 indicated that all the measured physical parameter as tensile strength at yield, tensile strength at rupture and elongation at yield for crude protease-treated sample is higher than those treated by chemical methods except elongation at rupture which recorded high value with chemical treatment.

3.21 Scanning electron microscope (SEM) examination
The results demonstrated that the enzymatic unhaired hide only displayed a smooth surface without any deposition of chemicals or grain damage and showed that opening up the fiber bundles was comparable (Figs. 7a and 7b).

4 Discussion
The industrial demand of proteases with novel and better properties continues to stimulate the researchers in this area so efficiency of various fungal isolates was
screened for production of alkaline protease and the most potant fungus for maximum production was *A. terreus*. Similar results recorded different species of *Aspergillus* for alkaline protease production\(^1\)\(^{21-25}\).

Solid state fermentation (SSF) has several potential advantages including low capital cost, low energy expenditure, less expensive downstream processing, low waste water output, higher concentration of the products and potential higher volumetric productivity\(^26\). Wheat bran was considered as the universal substrate among various substrates because it acts as a complete nutritious feed for microorganisms having unique mechanical properties as structure retention and lack of particle agglomeration\(^27\). Moreover, the biochemical structure of wheat bran shows that it includes various soluble sugars like galactose, glucose, xylose, arabinose, etc. which help in the initiation of microbial growth and replication\(^28\). Several reports described wheat bran as effective substrate for protease production\(^1\)\(^{23}\). Therefore, wheat bran was selected as the optimal solid substance for subsequent cultural optimization to enhance the protease productivity by *A. terreus*.

No distinct medium has been acknowledged for the greatest yield of alkaline proteases. Every microorganism has its own physiological processes for greatest protease production. So different physio-chemical parameters were utilized to improve the enzyme synthesizing.

Initial moisture content is a serious factor in SSF since the moisture of the medium determines the rate of growth and product yield\(^29\). Maximal production of alkaline protease was obtained at initial moisture content of 71.5\%\(^30\). Similar result was obtained by Malathi and Chakraborty\(^23\) who recorded initial moisture content of 66\% for maximum protease production from *A. flavus*. Lower moisture levels decrease the nutrients solubility of substrates, a higher water tension and lower degree of substrate swelling\(^30\). Moreover, higher moisture contents were reported to reduce porosity, increase of stickiness, reduction in gas volume, lowered gas exchange and enhanced formation of aerial mycelium\(^31\).

Incubation period is one of the most important factors in enzyme production. Maximum enzyme production could be obtained only after a specific incubation period that allows the fungal culture to grow at a steady state and to be long enough to permit a moderate amount of product to be formed and within longer incubation periods, the enzyme activity decreased which might be due to shortage of nutrients, or change in initial pH of medium\(^32\).

The best incubation period for maximal alkaline protease production was 6 days. The same incubation period was reported by Ali\(^33\).

The temperature of the process is a parameter that influences the reaction rates and the cellular metabolism\(^34\). Similar results were obtained by Hossain *et al.*\(^35\) who recorded the same temperature (45°C) for protease production from *A. flavus* AP2. Higher incubation temperature was reported by Murthy and Naidu\(^36\) for *A. oryzae* protease at 60°C.

Inoculum size is also a serious agent that affects the production of metabolites under SSF conditions. This can be traced back to nutrient restrictions, self inhibiting effect of accumulated spores and the produced non-volatile substances\(^36\). The maximum productivity was achieved with 2 mL of spore suspension. The decrease that was seen with larger inoculums sizes could be due to the shortage of nutrients available for the larger biomass and faster growth of the culture\(^7\).

Initial pH of the culture affects the enzymatic processes and the transport of several components by the cell membrane\(^38\). The maximal productivity was reported at pH 10 and this finding was in agree with one that recorded the same initial pH of protease from *Aspergillus* sp.\(^20\)\(^24\).

The supplementation of the cultures of *A. terreus* with different by products was also investigated. The induction effect of whey on protease production was also recorded for *A. terreus*\(^33\)\(^39\). Analysis of crude whey showed the following percentage: carbohydrates (3.69\%), nitrogenous substances (1.48\%) and minerals (%) Na 0.98, K 0.76, Ca 1.00, Mg 0.17, Fe 0.05, Mn (0.015) and Zn (0.045). Moreover, the crude enzyme preparation of *A. terreus* showed a significant activity for different alkaline enzymes beside protease such as keratinase and lipase and this may enhance the crude preparation efficiency in destaining and dehairing applications.

Precipitation is the most ordinarily method for recovery of protein from crude biological mixture and also performs both purification and concentration step\(^40\). The produced enzyme was best precipitated with iso-propanol (2:1 v/v). The same precipitating agent was previously found convenient for maximal precipitation of alkaline protease\(^3\)\(^40\).

The appearance of protease as a single band has molecular weight of 35KD ensures the homogeneity and purity of the enzyme. This was in complete accord with that from *A. oryzae*\(^3\)\(^40\).

The purified protease from *A. terreus* exhibited maximum activity at 50°C, and at higher temperatures, a gradual decrease in enzyme activity was observed. This can be explained by the effect of temperature in increasing the reaction velocity and also affects the rate of enzyme destruction, producing a gradual fall in the concentration of active enzyme. Our results were in complete accordance with that recorded for protease of *A. clavatus* by Hajji *et al.*\(^42\) and *Trichoderma harzianum* by Savitha *et al.*\(^43\).

Stability of the alkaline protease was attributed to their genetic adaptability to carry out their biological activity at higher temperatures\(^44\). The purified alkaline protease of *A. terreus* was found to have good activity at alkaline value with the maximum at pH 9.5. These results are in agreement with the previous findings on another protease from...
The activity and of alkaline protease in the presence of SDS is important because SDS-stable enzymes are not generally available. Coincident with our results SDS caused activation in activity of another protease. Tween 80 caused slightly decrease in the alkaline protease activity. These results are in agreement with the previous findings on other protease from Bacillus cereus which showed high stability with tween 80 and alkaline protease from A. terreus which showed slightly decrease in activity with H2O2. A good detergent protease must be stable and compatible with surfactant, bleaches and oxidizing agents found in detergent formulation.

Our results showed that the alkaline protease has high stability and compatibility with the most common laundry detergent at high degree of temperatures. Also it shows a considerable stability at room temperature. Therefore, we can say that alkaline protease produced from A. terreus is being promising additive components for local laundry detergents. Stability and compatibility of the alkaline protease of Aspergillus species with detergents has been previously recorded.

The addition of proteases to detergents considerably increases the detergent cleaning performance and decreasing the consumption of surface active substances, thereby decreasing the pollution load. Usefulness of alkaline proteases in the facilitation of blood stains removal from cotton cloth was also reported by Devi et al. and Niyonzi-Ma and More.

The potency of using enzymes in leather processing is very high because their high functional and selective catalytic properties, and their high stability in wide pH and temperature range. Enzymatic dehauling may be attributed to the role of protease in degrading non-collagenous constituents of the skin and elimination non-fibrillar proteins. SEM analysis indicate that enzyme-treated sample showed a clean surface indicating no grain damage because the enzyme only digest around the hair roots, resulting in complete removal of hair. Also, the extent of opening up of fiber bundles was more comparable in contrast to that of conventional chemical. Similar reports were previously recorded that the proteolytic enzymes were the most suitable agents in dehairing process.

5 Conclusion

Based on our study, we concluded that the optimized wheat bran amended by whey (20%, v/v) were proved to be the most suitable substrates for maximum alkaline protease production in solid state fermentation by Aspergillus terreus. Various parameters including moisture content of substrate, incubation period, incubation temperature, size of inoculum and initial pH were optimized to obtain the highest enzyme yield. The purified enzyme was stable at a broad range of alkaline pH and high temperature. The enzyme showed high affinity for various protein substrates, stability and compatibility with surfactants and local powder detergents exhibiting a good washing performance. Dehairing capacity of alkaline protease for animal hide without chemical support was demonstrated and it could be exploited as an eco-friendly dehairing agent in leather industry. Similar results reported the potentiality of protease in removing hide hairs.

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