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# Statistical Optimization of Alkaline Protease Enzyme Produced by *Bacillus subtilis* MH266414 and its Application in Different Industries

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**A B S T R A C T**

Proteases are an important class of enzymes with numerous industrial applications. Under submerged cultivation conditions, the *Bacillus subtilis* MH266414 generated alkaline protease. In order to maximise the factors such as pH, incubation period, and temperature, response surface methodology-based central composite design (CCD) was utilised. The quadratic model was shown to be reasonably adjusted with the experimental data by the CCD design. To assess the changes in the response surface and to understand the connection between the culture conditions and the enzyme yield, statistics-based contour and 3-D plots were created. In this investigation, a highest yield of protease activity of 63.0725 U/mL were obtained after optimization. The crude enzyme was purified using ammonium sulphate precipitation and DEAE Sephadex column chromatography. The fraction obtained from the column shows a specific activity of 3512U/mg$^{-1}$. The isolated protease also gives better result of washing and dehairing results. This result implies that this purified enzyme from *Bacillus subtilis* MH266414 can be used as biotechnological tool for various industries.

## Introduction

Proteases are representatives of worldwide enzymes sales and are important biotechnological enzymes which have a broad range of application in different industrial process and manufacturing products. It also has application in the field of pharmaceutical and food industries due to their hydrolytic mode of nature (Razzaq *et al.*, 2019). Due to this increasing demand of this enzyme, proteases from animals and plants are insufficient to fulfil the industrial demand. So it is necessary to produce this enzyme in a low cost and an easy manner (Kumar *et al.*, 2005). Microbial proteases are known as essential family of proteases which has advantage over animal and plant proteases, because it can be cultured in large

within a shorter period of time by optimizing the culture conditions in a laboratory set up. The capacity of the microorganism to tolerate harsh environmental condition makes them ideal source for making enzymes with high stability which are active in extreme environmental conditions (Gurung *et al.,* 2013).

One of the most significant groups of microbial protease producers are bacteria with the genus *Bacillus* being the most well-known producer. Several *Bacillus* sps produces more than 20g/L of protein due to their high capability of protein secretion (Harwood and Cranenburgh, 2008). Most of the *Bacillus* sps secrete alkaline and neutral proteases which is promising for the industrial use of enzymes (Rehman *et al.,* 2017). Alkaline proteases are generally synthesized by submerged fermentation. Several methods have been developed for the production of *Bacillus* proteases such as optimization of the fermentation parameters. Fermentation media optimization is a very essential factor because the enzyme production and the growth of the organism in the media are strongly influenced by the nutritional factors like carbon source and Nitrogen source. Besides these nutritional factors several growth factors is important in this biological process. So the media components and culture conditions needed to be optimized.

Most microbial enzymes are extracellular in nature which is directly excreted into the production media, which is removed usually by a process of centrifugation or filtration, leaving the crude enzyme in the form of supernatant. By salting out the culture supernatant is concentrated.

Ammonium sulphate is typically used as the precipitation agent as it reduces solubility of the desired proteins (Nassar *et al.,* 2015). The enzyme activities increasing too many fold at 70% saturation. Among the *Bacillus* sps, *Bacillus subtilis* is considered as the leading producer of alkaline proteases at commercial level (Glimenz *et al.,* 2000).

There has been an extensive studies focused on microbial alkaline protease from *Bacillus* due to their enhanced applications of these enzymes including synthesis of bioactive peptides, food processing, dehairing of animal skin as well as detergent formulation for the removal of blood stains from fabrics. Therefore the present studies investigate the production, purification and application of the alkaline protease from *Bacillus subtilis* MH266414 and statistical optimization of the fermentation condition for its enhanced production.

## Materials and Methods

### Inoculum collection and Culturing

Slant cultures of *Bacillus subtilis* MH266414 were obtained from Centre for bioscience and Nano science Research, Coimbatore, Tamil Nadu. The culture was then sub cultured onto Nutrient agar plates for further studies.

### Protease enzyme assay

Protease enzyme activity was checked using the culture supernatant collected by centrifuging the production media inoculated with *Bacillus subtilis* MH266414which was comprised of Caesin 5.5g; $KH_2PO_4$-1g, $MgSO_4.7H_2O$-0.3g; $FeSO_4$-0.2g; $ZnSO_4$-0.2g; $CaCO_3$-1g with maintain a pH-9.0. enzyme activity was performed according to the method described by Cupp-Enyard (2008). 0.65% casein prepared in 50mM potassium phosphate buffer (pH7.5) serves as the substrate. 5ml of the substrate solution was added to the reaction tube containing crude protease enzyme which was then kept at 37°C for 10 minutes. Reaction was terminated with the addition of 20% ice cold Trichloro Acetic acid in both reaction and the blank tube. Precipitated proteins the reaction tube was removed by centrifugation. 2.5ml of 0.5M alkaline Na2CO3 was added to both the tubes after centrifugation followed by the addition of 1ml of Folin–Ciolcaltea reagent and kept for 30 minutes. The absorbance was measured at 660nm against the

blank sample. One unit of protease activity was defined as the amount of enzyme required to produce an absorbance at 660nm that corresponds to 1µg of tyrosine per minute liberated under specific conditions of the assay (Hameed *et al.,* 1996).

## Optimization of culture conditions for protease production using Response surface methodology (RSM)

The experiment is a widely used statistical technique that may be used to construct and optimize the experimental process. It involves selecting the best experimental design and estimating the impact of multiple variables simultaneously as well as their individual effects. Statistical tool Design Expert (7.1.5 version) was used to create the Response Surface technique and CCD. Using the isolate *Bacillus subtilis* MH266414 three variables such as pH, incubation time, and temperature were optimized for increased protease production. The components were divided into two levels based on CCD: 1, for low level, and +1, for high level. The process parameters were optimized over the course of 30 runs, with six replications at the centre point to measure the pure experimental uncertainty variance (Ellaiah *et al.,* 2002). The coefficient of determination ($R^2$), analysis of variance (ANOVA), and response plots were used to assess the outcomes. The most popular second-order polynomial equation was created using RSM to fit the experimental results and define the most significant model terms:

$$Y = \beta_0 \Sigma \beta_i X_i + \Sigma \beta_i X_i \beta_{ij} + \Sigma X_i X_j,$$

Where $\beta_0$, $\beta_i$, and $\beta_{ij}$ are constant regression coefficients of the model; $X_i$ and $X_j$ are independent variables; and $Y$ is the predicted response.

## Purification of protease enzyme

*B.subtilis* was grown in optimized culture media with pH 9.0 containing 1% glucose; 1% yeast extract; Casein 5.5g/L; $KH_2PO_4$-1g/L, $MgSO_4$. $7H_2O$-0.3g/L; $FeSO_4$-0.2g/L; $ZnSO_4$-0.2g/L; $CaCO_3$-1g/L which was incubated at 55°C. The culture supernatant was used for the purification process of the enzyme. The supernatant was precipitated with 10-100% ammonium sulphate at 4°C as per the standard chart. The resultant precipitate was collected by centrifugation at 10,000 rpm for 10 minutes and was dissolved in minimal amount of phosphate buffer with pH 7.0. These solutions were then loaded to DEAE-Sephadex A-50 column which was pre-equilibrated with the same phosphate buffer. The fractions were collected with a flow rate of 5ml/h and the absorbance of protein was measured by spectrophotometrically at 280nm and also determined the specific activity by assay method.

## Application of purified protease enzyme

## Removal of blood stain from cloth

The efficacy of purified protease in blood stain removal was studied on white cloth piece of 5×5 in size which was stained with blood and dried for an hour. After drying the stained cloths were treated with 5ml of purified enzyme and were incubated at 40°C for 30 minutes. Untreated cloths without the addition of enzyme serve as the control. After incubation the cloth piece were washed in tap water and was visually examined to evaluate the effect of protease enzyme in blood stain removal (Nadeem *et al.,* 2008).

## Dehairing of goat hides

Dehairing of goat hides using purified protease enzyme was done using paint method (Briki *et al.*, 2016). The goat skin were collected from slaughter house which was pre-soaked in water to remove the dirt and blood clots and allowed to dry to remove excess of water and initial weight of the skin was noted. Both enzymatic and commercial method of dehairing was compared in this study. Commercial dehairing was initiated with 10% lime and 3 % sulphide or with 10 % commercial protease. Enzymatic method was done using purified protease enzyme with varying concentration ranging from 1 - 6 % made with distilled water. Both enzyme and

commercial chemical was applied on the flesh side of the Goat skin and stored at different incubation temperature and time. Effect of commercial chemical, different purified protease concentration, incubation time and temperature were analysed by the extent of dehairing after scrapping with a blunted knife.

## Calculation

$$\text{Percentage of Dehairing} = \frac{\text{Initial weight of the skin before dehairing} - \text{Final weight of the skin after dehairing}}{\text{Initial weight of the skin before dehairing}} \times 100$$

## Destaining of dye from cloth

The use of proteases as a detergent ingredient was tested on a piece of white cotton material (5 $cm^2$) dyed with methylene blue and Orange –G dye and dried at room temperature for overnight. 20ml of commercial detergent (10mg/ml) was heated at 70°C to deactivate the enzyme present in it. Then 2ml of crude protease and purified enzyme sample was added to each 10ml of deactivated commercial detergent. Cloth pieces were taken in three different petriplates. One plate was taken as the control which containing stained cloth and 10ml distilled water. Second plate containing crude enzyme and detergent mixture. Third plate was added with Purified enzyme and detergent mixture solution. All the plates were incubated for overnight at room temperature. After incubation, cloth pieces were taken out washed with water. Visual examination was conducted and difference was recorded.

## Results and Discussion

In the area of fermentation biotechnology, producing proteases with commercial value by using suitable organism and inexpensive growth medium has been a notable accomplishment. All microorganisms have the ability of producing proteases, and many different bacterial species have been shown to produce alkaline proteases. The *Bacillus* species are the most major and particular producers of alkaline proteases among the diverse bacterial group (Priest, 1977; Ward, 1985). Detergents, leather processing, as well as the treatment of tannery waste all offer significant industrial possibilities for alkaline proteases. The present study deals with the optimization of the culture conditions for protease production and the purification of the protease enzyme. The amount of protease enzyme produced in the culture media was analysed using standard tyrosine method. The protease activity was calculated based on microgram of tyrosine that was released under standard assay conditions. Sample were analysed by standard protease assay method after 24 hour fermentation at a pH of 9 and a temperature of 37°C. As a result, the sample that was taken had a protease activity of 17.2 U/ml.

## Optimization of culture conditions for protease production using Response surface methodology (RSM)

The main goal of RSM, a mathematical and statistical technique used to analyse various parameters and create the best growth medium for *B. subtilis*, is to optimize the response, which is affected by various kinds of parameters and is effective and valuable (Beg and Gupta, 2003). The CCD was used to determine the optimized conditions for increased protease enzyme synthesis. The CCD experiment setup and response results were given in Table.1. Multiple regression analysis was used to determine a polynomial coefficient for each term in the equation. Table.2 provides a summary of the analysis of variance (ANOVA) for the response surface quadratic model. The *F* value was utilized to determine whether equation had statistical significance. The *F* value in this study, 16.169, suggested that the model was very significant. The model's good fit could also be demonstrated by the *P* value (Probe value>*F*, 0.0001), and there was only a 0.0001 probability that the model's *F* value could have been caused by noise. The model's good fit was demonstrated by the high determination coefficient ($R^2$ = 0.94), which also suggested that the variable components were to

blame for the 94% sample variation in PA. A high significance of the model was shown by the value of the adjusted determination coefficient, which was likewise high (adjusted $R^2$ =0.88). Figure.1 shows the 3D graphical representation. The findings show a strong relationship between pH, incubation time, and temperature for protease synthesis. Using CCD, the optimum amounts were discovered. The maximal protease activity predicted by the model was 63.0725 U/mL. The equation for the generated regression connection is as follows:

$$Y = 38,553 + 9,24\ X_1 + 3,37\ X_2 + 3,716\ X_3 + 2,99\ X_4 + 2,576\ X_1X_2$$

Where Y is the enzyme production whereas$X_1$, $X_2$, and $X_3$, are indicating the temperature, pH and incubation period respectively. The improvement of fermentation conditions for the synthesis of protease enzyme demonstrated an increase in the rate of production economics and is a desirable technological advancement.

## Purification of protease enzyme

Purification process of the alkaline protease produced by *Bacillus subtilis* MH266414 was carried out by ammonium sulphate precipitation method. At 70% ammonium sulphate saturation maximum protease enzyme activity was observed. Another set of researchers isolated alkaline proteases and found that the maximum specific activity was at 80% ammonium sulphate saturation (Mary *et al.*, 2017). These findings were consistent with those of the current investigation. The active fractions were pooled and applied directly on a DEAE-Sepharose column. The fraction obtained from the column shows a specific activity of 3512U/$mg^{-1}$.

## Application of purified protease enzyme

Complete blood stain removal from cotton material was demonstrated using partially purified protease (Fig.2), although only partially purified enzyme removed the stain, while distilled water alone faded it. According to these findings, the enzyme is stable in detergent and could be used as a detergent additive. Because of their better cleansing properties and efficacy at low washing temperatures, thermo stable alkaline proteases are good candidates for use in detergents. Giri *et al.,* (2011) isolated and described a detergent-compatible alkaline protease from *B. subtilis* VSG-4, which improved the cleaning efficacy of commercial detergents. The results presented here are consistent with those of other *Bacillus* species proteases that have demonstrated stability in the presence of various surfactants (Singh *et al.*, 2012).

On fresh goat skin, the protease isolated from *Bacillus subtilis* MH266414 was tested for dehairing effectiveness. Protease was dissolved in various concentrations (1, 2, 3, 4, 5, and 6%) and formed into a paste. The paste was applied to the flesh side of the skin and folded for 6-18 hours for dehairing. Within 18 hours of incubation, the protease generated by *Bacillus subtilis* MH266414 successfully dehaired goat hides. Control samples showed colour changes and swelling. This could be owing to the fact that the control sample contains higher lime and sulphide. Similarly, Thanikaivelan *et al.,* (2004) observed that lime enhances goat skin dehairing in a cost-effective manner. Increased exposure duration causes tiny holes in the skin. Wang *et al.,* (2007) achieved comparable results. A selective proteolytic action occurred in the root hair which initiates dehairing in a shorter period of time. The effect of protease dehairing on goat skin was studied using scanning electron microscopy (Fig.4). The results showed that enzyme-treated skin was totally dehaired when compared to chemical-treated skin.

To strengthen the use of protease as an eco-friendly detergent addition, a staining test was conducted. By carrying out an experiment in triplicate at room temperature for overnight, the findings were verified. Using detergent, crude and purified enzyme, both methylene blue and orange- G dye were eliminated from the cotton piece.

**Table.1** The CCD design of experiment and protease activity yield

| S. No. | Incubation time | Temperature | Effect of pH | Protease activity (U/ml) |
|---|---|---|---|---|
| **1** | 1 | 0.5 | 0.5 | 21.767 |
| **2** | 3 | 0.5 | 0.5 | 32.514 |
| **3** | 1 | 1.5 | 0.5 | 22.336 |
| **4** | 3 | 1.5 | 0.5 | 48.768 |
| **5** | 1 | 0.5 | 1.5 | 36.928 |
| **6** | 3 | 0.5 | 1.5 | 45.327 |
| **7** | 1 | 1.5 | 1.5 | 37.754 |
| **8** | 3 | 1.5 | 1.5 | 49.791 |
| **9** | 1 | 0.5 | 0.5 | 21.291 |
| **10** | 3 | 0.5 | 0.5 | 40.855 |
| **11** | 1 | 1.5 | 0.5 | 33.913 |
| **12** | 3 | 1.5 | 0.5 | 57.849 |
| **13** | 1 | 0.5 | 1.5 | 36.113 |
| **14** | 3 | 0.5 | 1.5 | 47.092 |
| **15** | 1 | 1.5 | 1.5 | 33.7969 |
| **16** | 3 | 1.5 | 1.5 | 63.0725 |
| **17** | 0 | 1 | 1 | 19.3324 |
| **18** | 4 | 1 | 1 | 59.1449 |
| **19** | 2 | 0 | 1 | 37.6667 |
| **20** | 2 | 2 | 1 | 45.0290 |
| **21** | 2 | 1 | 0 | 27.0000 |
| **22** | 2 | 1 | 2 | 35.9275 |
| **23** | 2 | 1 | 1 | 25.2609 |
| **24** | 2 | 1 | 1 | 41.3623 |
| **25** | 2 | 1 | 1 | 35.8841 |
| **26** | 2 | 1 | 1 | 38.1594 |
| **27** | 2 | 1 | 1 | 43.6232 |
| **28** | 2 | 1 | 1 | 38.6667 |
| **29** | 2 | 1 | 1 | 36.0580 |
| **30** | 2 | 1 | 1 | 38.9275 |

**Fig.1** Three dimensional representations of the response surfaces generated by the model that illustrated of Incubation time, Temperature and pH.

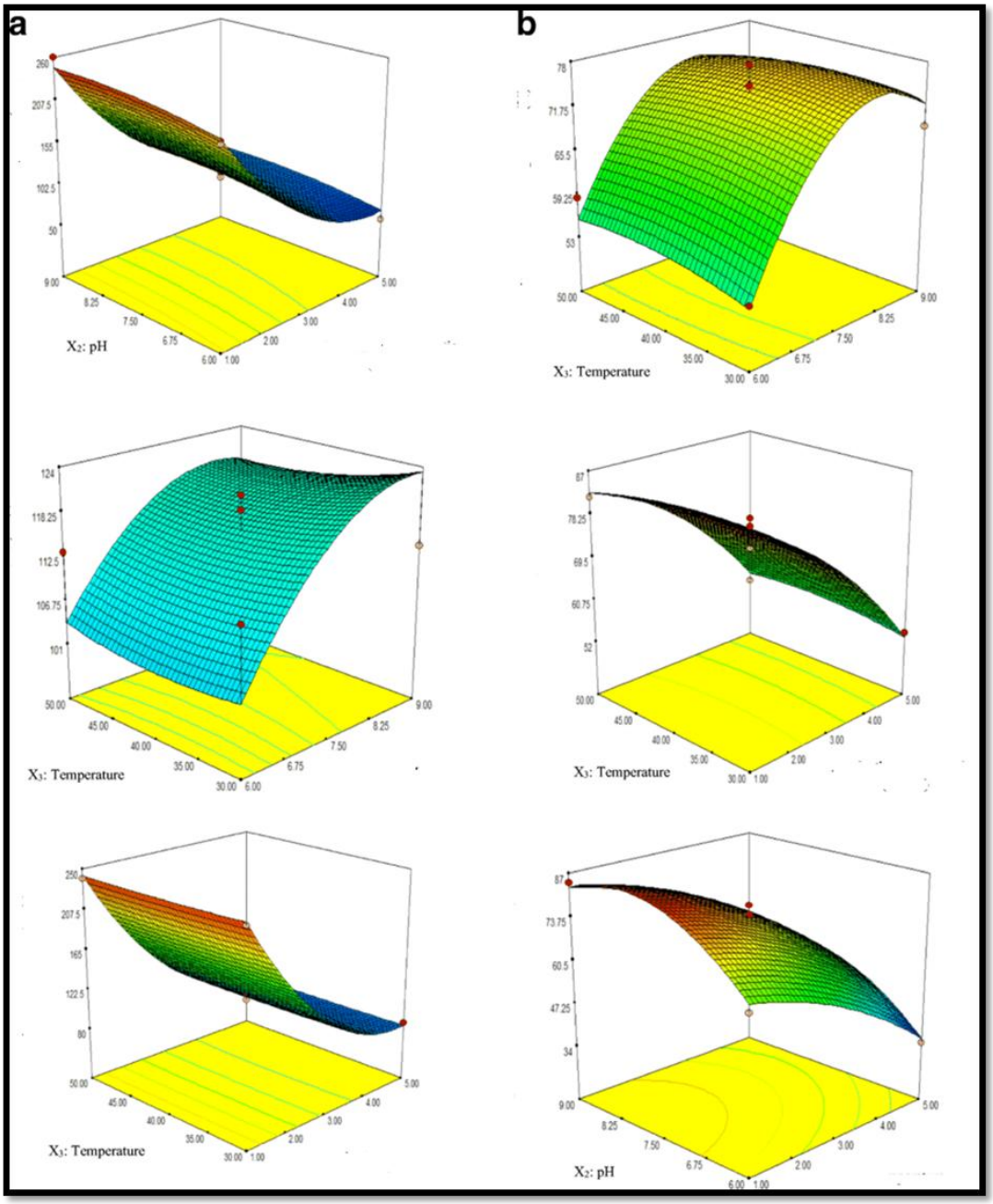

**Table.2** ANOVA for response surface quadratic model

| Source | Sum of Squares | Df | Mean Square | Value *F* | Value *P* Prob > F | |
|---|---|---|---|---|---|---|
| **Model** | 34354.56 | 15 | 321.873 | 17.89 | < 0.0001 | Significant |
| **$X_1$ Incubation time** | 2034.984 | 1 | 2034.984 | 141.938 | < 0.0001 | |
| **$X_2$ Temperature** | 267.460 | 1 | 267.460 | 18.655 | 0.0006 | |
| **$X_3$ Effect of pH** | 325.879 | 1 | 325.879 | 22.730 | 0.0002 | |
| **$X_1^2$** | 12.256 | 1 | 12.256 | 0.855 | 0.369 | |
| **$X_2^2$** | 39.217 | 1 | 39.217 | 2.735 | 0.119 | |
| **$X_3^2$** | 44.608 | 1 | 44.608 | 3.111 | 0.098 | |
| **Residual** | 215.058 | 15 | 14.337 | - | - | |
| **Lack of fit** | 175.694 | 10 | 17.569 | 2.232 | 0.1944 | |
| **Pure error** | 39.363 | 5 | 7.873 | - | - | |
| **CoR total** | 3460.450 | 29 | - | - | - | |

**Fig.2** Removal of blood stain removal in untreated cloth (A), Stain removal in Protease enzyme *Bacillus subtilis* MH266414 treated cloth (B)

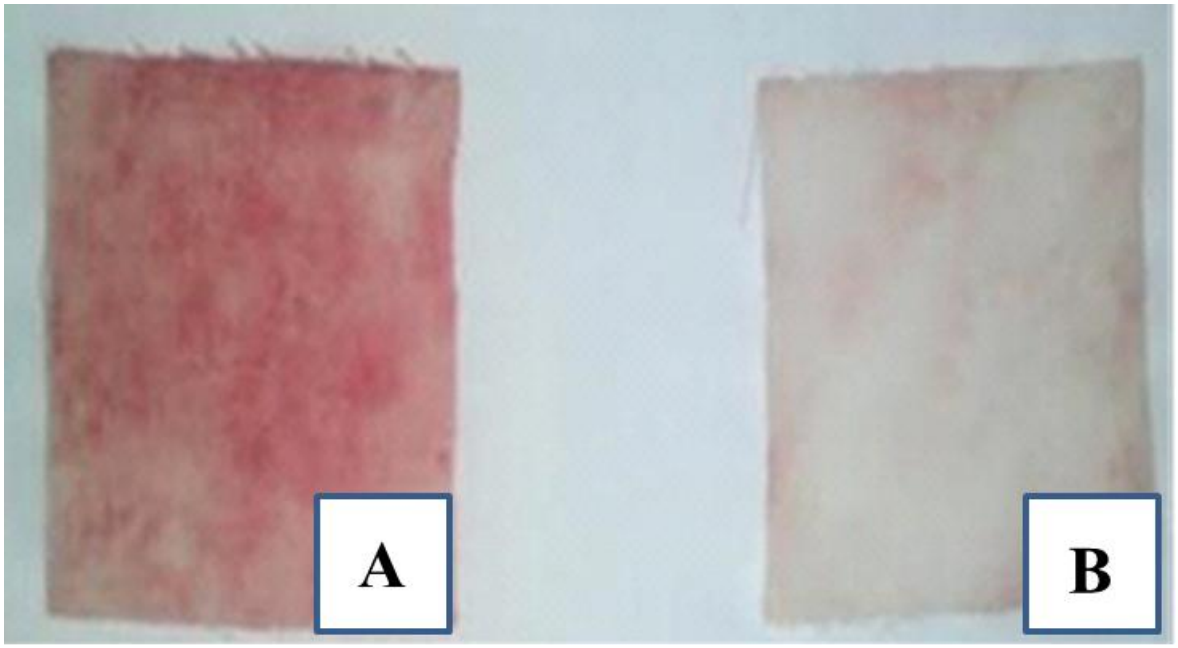

**Fig.3** Before dehairing process (A), Dehairing with Protease enzyme (B) from *Bacillus subtilis* MH266414.

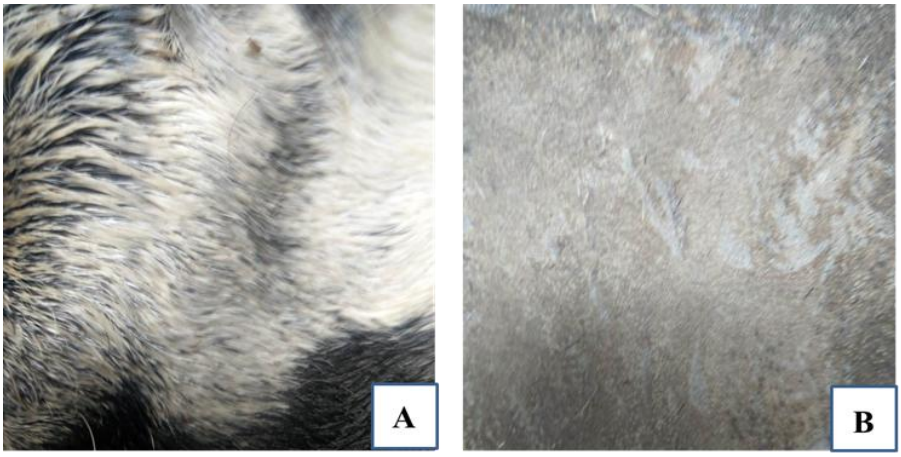


**Fig.4** Scanning electron micrograph of the Goat skin. A- Chemical treated skin; B- Enzyme treated skin

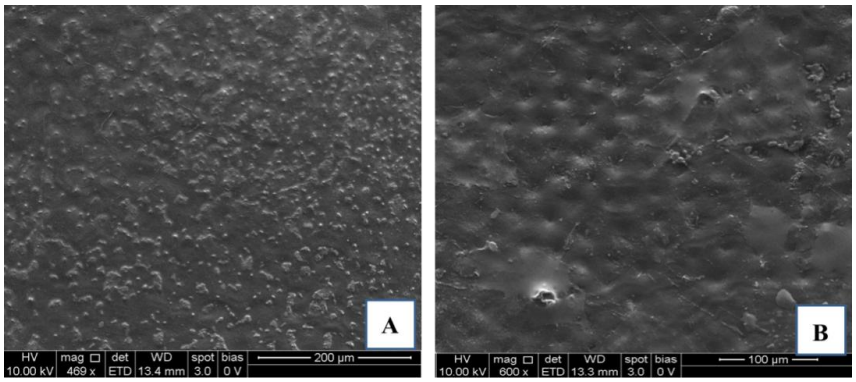


**Fig.5** Destaining effect of alkaline protease enzyme. A-cloth stained with Orange-G dye; B- Stained cloth treated with mixture of Crude protease and deactivated detergent; C- Cloth treated with mixture of purified protease enzyme and deactivated detergent; D-cloth stained with Methylene blue dye; E-Stained cloth treated with mixture of Crude protease and deactivated detergent; F- Cloth treated with mixture of purified protease enzyme and deactivated detergent.

When detergent and purified protease were applied together, the dye stain was completely removed. On the other hand, washing a piece of cotton with crude protease produced the mild dye traces depicted in Fig.5.

These findings corroborate some earlier research on the stability and compatibility of protease with commercially available detergents carried out by Maurer (2004) and Bezawada *et al.*, (2011) to assess the efficiency of various proteases. The current study concluded that the bacterial isolate produces protease under alkaline culture conditions, and that several parameters strongly affect protease development and synthesis. The findings of this study on several aspects will be relevant in the future generation of protease by these microorganisms.

Proteases produced by *Bacillus subtilis* are by far the most important class of enzymes used in industry. The findings provided here are consistent with the literature, as various *Bacillus subtilis* strains are known to be good alkaline protease producers and are frequently employed in the detergent sector and leather industry.

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