

## Protease Production and Enzymatic Soaking of Salt-Preserved Buffalo Hides for Leather Processing

### SHORT COMMUNICATION

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### Abstract

Response surface methodological (RSM) optimization of protease by *Pseudomonas aeruginosa* MCM B327, increased 1.3-fold activity with 1% inoculum having cell density of  $27.57 \times 10^9$  cells mL<sup>-1</sup> at pH 7, 30°C and 72 h of incubation. Protease enzyme recovered from *P. aeruginosa* showed characteristic activities against diverse proteins of hide. Enzyme was found to be active with substrates e.g. casein, Bovine serum albumin, gelatin, elastin, haemoglobin but inactive against keratin and collagen. During leather manufacturing, non-collagenase and non-keratinase activities have advantageous in a quality leather and hair saving process, respectively. Increased proteolytic enzyme concentration (0.1-0.5%) in soaking process showed increased water penetration because of hydrolysis of albumin and elastin proteins as indicated by opened fibers in histopathological sections. These findings suggest, protease secreted by *P. aeruginosa* may have application in soaking operation of leather processing for minimizing harmful dehairing chemicals and processing time.

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### INTRODUCTION

Proteases can be obtained from different sources such as bacteria, fungi, or certain insects. In order to obtain commercially viable yields of protease it is essential to optimize cultivation media for the growth of organism and production of protease. Proteases have several applications in detergent, pharmaceuticals, foods, beverages and leather industries. Various statistical methods such as response surface methodology (RSM), central composite design (CCD), artificial neural network (ANN) models were used for optimization of protease production by many researchers (Puri et al. 2002; Dutta et al. 2005; Rao et al. 2006; Saxena and Singh, 2010).

Leather sector has offered unique advantages in terms of raw material resources, manufacturing capacity, expertise and knowledge base employment and export potential (Luthra, 2006). Leather is a byproduct of meat industry and is prepared from heterogeneous fibrous mass of animal skins/ hides. It has been reported that chemical composition of skins and hides vary from species to species as well as their locations (Muthian et al. 1968). Animal skin/ hides are biological matrix significantly loaded with approx. 33 % protein and approx. 2% of fat or triglycerides (Sharphouse, 1983).

Due to advancement in meat industries and meat demand, animal skin production was more and needed to preserve it. Traditionally, common salt (30-40%) is used for skin/ hides preservations.

Leather is a stabilized collagen product and has different leather processing steps are shown in Figure 1. Beam-house operations are very important to produce high quality leather. Traditional chemical leather processing generates huge amount of environmental pollutions (Thanikaivelan et al. 2004; Kanagraj et al. 2006). Whereas, enzyme biocatalysts were found to be effective in soaking, dehairing, bating and degreasing operations of environmental friendly leather processing.

Soaking is the removal of globular proteins, dirt, dung, blood and salts. Protease produced from *Aspergillus flavus*, *A. parasiticus*, *A. oryzae*, and *Bacillus subtilis* were reported for soaking of animal skins. Some amylolytic enzymes from *A. awamori* were also showing soaking application (Kamini et al., 1999). A soaking method using proteolytic enzymes and carbohydrases in the pH range of 5.5 to 10 has been described by Kamini et al. (1999). Soaking enzymes are available in market but in literature very limited reports are available. Thus, this paper focuses on the production of bacterial protease

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and its use in soaking of buffalo hide for quality leather and its substrate specific characterization.

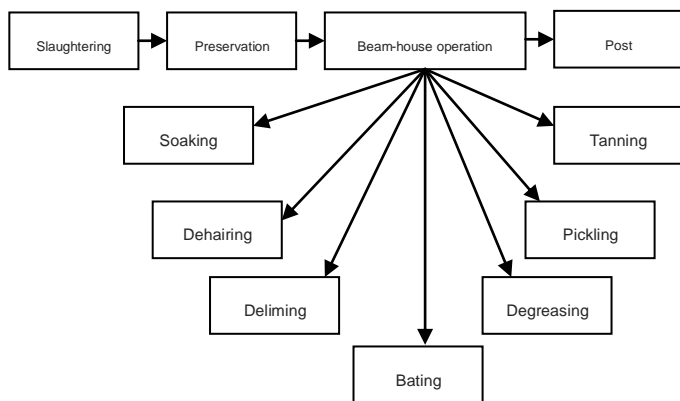


Figure 1. Leather processing steps.

## MATERIALS AND METHODS

### Chemicals and Substrates

All chemicals and reagents of analytical grade were used. All types of substrates, like casein, BSA, gelatin, elastin-orcein, collagen, and keratin azure were purchased from Sigma Chemicals (St. Louis, MO, USA). The buffalo hide was obtained from Local Municipal Corporation Slaughterhouse (Pune, India).

### Factorial Design for Protease Production

Our previous publication showed soybean meal-tryptone (ST) as best medium (Zambare et al., 2011). In this study the optimum concentrations of soybean meal and tryptone were obtained by non-linear factorial design and RSM. The experiment of different run was conducted in 250 mL Erlenmeyer flasks containing 100 mL of ST medium inoculated with the freshly prepared 1% v/v inoculums (24 h grown in Nutrient broth) and incubated for 72 h with 150 rpm at 30°C. After cultivation, the cell-free supernatant was obtained by centrifugation at 10,000 rpm and the extracellular protease activity (caseinolytic) of the fermented broth was determined. In the next stage RSM was used to study the interactive effects of two variables, i.e. soybean meal and tryptone for improving the protease activity. Each factor in the factorial design was studied at four

different concentrations (0.5- 2.0%). The experiment was conducted in duplicate. This design is represented by second-order polynomial regression model, Equation (1) for the predicted activity of the enzyme as:

$$\text{Predicted activity} = \beta_0 + \beta_1X + \beta_2Y + \beta_3XY + \beta_4XX + \beta_5YY \quad (1)$$

Where, X- coded variable for soybean meal, Y coded variable for tryptone and  $\beta_0, \beta_1, \beta_2, \beta_3, \beta_4, \beta_5$  are coefficients for their respective variables.

### Enzyme Preparation and Partial Purification

The protease was produced in ST medium (pH 7.0) at 30°C under shake culture condition (150 rpm) with 1% inoculum. The cell growth was measured at 600 nm. Cell free supernatants were analyzed for enzyme activity at 3 h interval up to 96 h. The cell free supernatant (CFS) was obtained by centrifugation and the proteolytic activity was measured on different substrates. The enzyme from CFS was partially purified by ammonium sulphate precipitation at 60% saturation followed by overnight dialysis against Tris-HCl buffer (pH 8) under cold condition at 4°C. This precipitate was used for substrate specificities and soaking application of leather processing at laboratory scale.

### Enzyme Assays

#### Protease

Protease activity was measured using caseinolytic assay (Zambare et al. 2011a). The culture supernatant (1 mL) was incubated in 4 mL of 0.625% casein (Tris-HCl buffer, pH 8.0) at 35°C for 30 min. The reaction was stopped by the addition of 5 mL of trichloroacetic acid (5%) and the casein hydrolysis product was measured by modified Folin-Ciocalteu method, against inactive enzyme at 660nm. A standard graph was generated using standard tyrosine of 10–50  $\mu\text{g mL}^{-1}$ . One unit (U) of protease activity was defined as the amount of enzyme, which liberated 1  $\mu\text{g}$  tyrosine per min at 35°C.

#### Albuminase

The albuminase activity was determined according to protease assay as described above with Bovine Serum Albumin (BSA) as a substrate. One unit (U) of albuminase activity was defined as the amount of enzyme, which liberated 1  $\mu\text{g}$  tyrosine per min at 35°C.

### Elastase

Elastin-orcein assay was used to determine elastase activity using method described by Sachar et al. (1995). Twenty mg of elastin-orcein substrate was incubated with 0.5 mL of suitably diluted enzyme in 20 mL of Tris-HCl buffer (pH 8.0) at 37°C for 20 min. 2.5 mL of 0.7 M phosphate buffer of pH 7.0 was added. The mixture was centrifuged and the absorbance of the supernatant was read at 578nm. One unit of activity was defined as the amount of enzyme hydrolyzes 1 mg of substrate per min at 37°C.

### Keratinase

Keratinase activity was determined using keratin azure as substrate (Takami et al. 1990). The cell free supernatant (1 mL) was mixed with 4 mg keratin azure in 1 mL of Tris-HCl buffer (pH 8.0) incubated at 37°C for 60 min. The reaction was stopped by centrifugation. The absorbance of supernatant was read at 595nm. One unit of activity was defined as the amount of enzyme, which increased absorbance by 0.001 under standard assay conditions.

### Collagenase

The collagenase activity on collagen substrate was determined as described by Woessner (1961). 2 mL of collagen solution (1 mg mL<sup>-1</sup>) was prepared in Tris-HCl, pH 8.0, to that 0.5 mL of suitably diluted enzyme was added and incubated at 37°C for 1 h. The reaction mixture was filtered through Whatmann filter No. 1. One mL of 0.05M chloramine-T was added to the filtrate and incubated at room temperature for 20 min. To this 1.0 mL of 3.15M perchloric acid was added. After 5 minute, 1.0 mL of freshly prepared 20% solution of *p*-dimethyl amino benzaldehyde was added, and incubated at 60°C for 30 min. The pink color developed was read at 557nm against blank containing distilled water instead of enzyme solution. A standard graph was generated using standard hydroxyproline solutions of 1 - 10 µgmL<sup>-1</sup>. One unit of activity was defined as the amount of enzyme, which liberated 1µg hydroxyproline per min at 37°C.

### Hemoglobin hydrolysis

Hemoglobin hydrolysis assay was determined using method described by Sarath et al. (1996). Five milliliter haemoglobin substrate (2%) was incubated with 1 ml of enzyme solution at 35°C for 20 min. The reaction was stopped by addition of 10 mL, 5% trichloroacetic acid and kept for 10 min at same temperature. The assay mixture was centrifuged and the supernatant was read

at 280nm against inactive enzyme as blank. One haemoglobin hydrolysis unit (HHU) was defined as the amount of enzyme, which increase the absorbance at 280nm under assay conditions.

### Enzymatic Soaking

Salt preserved buffalo hide cut into pieces of approximate 35 gm (10cm x 10cm size) and was used for enzymatic soaking studies. Soaking was carried out in 300% water (with 0.05% benzoic acid as preservative) and different concentrations of protease from *P. aeruginosa* (0.1, 0.3 and 0.5% w/w of hide) with only water as a control. This soaking experiment was performed at room temperature. All pieces were observed periodically (up to 18h) for its looseness, water holding capacity and hair loosening. The soaked pieces were dehaired with conventional chemical dehairing with 10% lime, 2.5% sodium sulphide and 20% water (Raju et al., 1996). Dehaired pelts were washed and fixed in 10% (v/v) formaldehyde for histopathological analysis.

### Enzymatic Soaking

The pelts were dehydrated with 80, 95 and 100% (v/v) of alcohol gradients followed by xylene treatment, and embedded in paraffin. Longitudinal sections (L.S.) of hide embedded in paraffin wax were obtained using a microtome. The sections were fixed on slides using starch paste containing thymol, which acts as a preservative. The sections were stained with Harris's haematoxylin stain followed by 0.5% (v/v) HCl and diluted ammonia (John and Merriline, 2002). The slides were observed microscopically (Labophot 2 microscope, Japan) for epidermis, hair root follicles, and corium (collagen layer).

## RESULTS AND DISCUSSIONS

### Factorial Design for Protease Production

Protease production using single factor is time consuming process and since last decade many reports are on protease production optimization using statistical experimental designs (Puri et al. 2002; Chauhan and Gupta, 2004). The experimental factorial design showed that both variables had significant effect on protease production. The observed response showed maximum protease activity of 389.42 UmL<sup>-1</sup> with 0.5% soybean meal and 1.5% tryptone which was in good agreement

with predicted activity 373.76 U mL<sup>-1</sup> (Table 1). Overall Reddy et al. (2008) reported such protease production optimization by RSM.

From multiple linear regressions, the coefficients of the respective variables were calculated. The predicted response equation as shown below was obtained by adding the coefficients, concentrations of the variables in Equation 2.

$$\text{Protease activity} = 113.836 + 117.291X + 271.734Y - 34.491XY - 26.060XX - 77.305YY \quad (2)$$

The larger the magnitudes of t-value smaller the p-value, the more significant is the corresponding coefficient. The significance level was tested by one-way ANOVA and found to be dependent upon tryptone concentration. The significance was <0.05 because the observed F value was

higher than the theoretical F value. The Fisher F-test with a very low probability value demonstrated a very high significance for the regression model (Table 2). The goodness of fit of the model was checked by the determination coefficient (R<sup>2</sup>). In this case, the value of the determination coefficient, R<sup>2</sup>= 0.684, indicates that only 31.6% of the total variations are not explained by the model. The value of adjusted determination coefficient, Ad. R<sup>2</sup>= 0.525, is moderate, which indicates a moderate significance (p value < 0.05) of the model (Adinarayana and Ellaiah, 2002). A higher value of the correlation coefficient, R=0.827, signifies an excellent correlation between the independent variables. At the same time a relatively lower value of the coefficient of variation, CV=17.3, indicates improved precision and reliability of the conducted experiments (Adinarayana and Ellaiah, 2002).

Table 1. Observed responses and predicted values of protease activity.

| Run | Concentrations (%) |              | Protease activity (U mL <sup>-1</sup> ) |                    | Residual standard deviation |
|-----|--------------------|--------------|---|--------------------|-----------------------------|
|     | Tryptone           | Soybean meal | Observed response                       | Predicted response |                             |
| 1   | 0.5                | 0.5          | 243.3                                   | 273.885            | -1.154                      |
| 2   | 0.5                | 1.0          | 331.04                                  | 343.150            | -0.457                      |
| 3   | 0.5                | 1.5          | 389.42                                  | 373.763            | 0.591                       |
| 4   | 0.5                | 2.0          | 370.19                                  | 365.724            | 0.169                       |
| 5   | 1.0                | 0.5          | 354.22                                  | 304.362            | 1.881                       |
| 6   | 1.0                | 1.0          | 376.88                                  | 365.005            | 0.448                       |
| 7   | 1.0                | 1.5          | 386.16                                  | 386.995            | -0.032                      |
| 8   | 1.0                | 2.0          | 377.16                                  | 370.333            | 0.258                       |
| 9   | 1.5                | 0.5          | 320.05                                  | 321.810            | -0.066                      |
| 10  | 1.5                | 1.0          | 362.63                                  | 373.830            | -0.423                      |
| 11  | 1.5                | 1.5          | 370.87                                  | 387.198            | -0.616                      |
| 12  | 1.5                | 2.0          | 323.48                                  | 361.913            | -1.45                       |
| 13  | 2.0                | 0.5          | 309.05                                  | 326.228            | -0.648                      |
| 14  | 2.0                | 1.0          | 380.06                                  | 369.625            | 0.394                       |
| 15  | 2.0                | 1.5          | 376.88                                  | 374.370            | 0.095                       |
| 16  | 2.0                | 2.0          | 367.27                                  | 340.462            | 1.012                       |

Different % concentrations of soybean meal and tryptone affected the protease activity (Figure 2). It was observed that the enzyme production increased with increase in

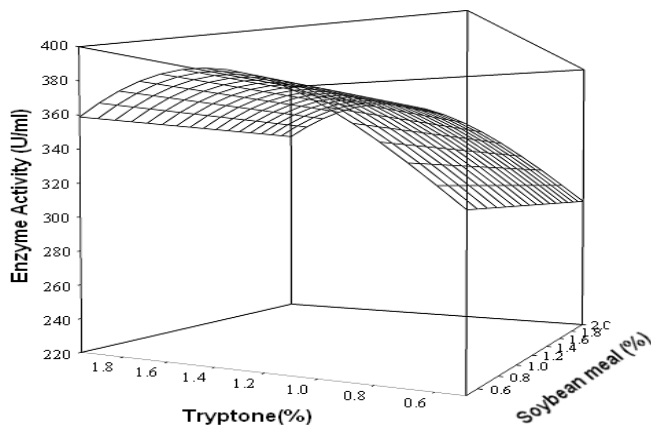
tryptone concentration. On the other hand increase in soybean meal concentration had not increased the enzyme production. This suggested that, the enzyme

production is induced when the medium is supplemented with tryptone.

**Table 2.** Analysis of variance (ANOVA) for factorial design.

| SOV        | SS       | DF | MS       | F value | p value |
|------------|----------|----|----------|---------|---------|
| Regression | 15172.37 | 5  | 3034.476 | 4.321   | 0.024*  |
| Residual   | 7022.38  | 10 | 702.239  |         |         |
| Total      | 22194.76 | 15 |          |         |         |

SOV=Source of variance, SS=Sum of squares, DF=Degree of freedom, MS=Mean square,  $R^2=0.684$ ,  $R=0.827$ , Adjusted  $R^2=0.525$ , \*Significant at  $<0.05$



**Figure 2.** Three-dimensional graph of interaction of soybean meal and tryptone.

Ammonium sulphate precipitated protease from *P. aeruginosa* obtained was studied for the ability to hydrolyze different skin proteins with artificial substrates. Protease from *P. aeruginosa* showed highest activity against albuminous substrates followed by gelatin, elastin and haemoglobin. Albumin and haemoglobin hydrolysis showing the degradation of albuminous and blood protein adhered to the hide during slaughtering. From leather industry point of view present protease was found to be inactive against keratin and collagen substrates (Table 3). Non-keratinase activity showed hair saving and recovered hair may have application as a byproduct for production of synthetic fibers, biogas production, foaming agent for fire extinguishers, while hydrolyzed hair is used as agricultural fertilizer, soil conditioner, compost, additive in chrome tanning or retanning processes, animal/poultry feed and also for production of cosmetics, pharmaceuticals and amino acid like cysteine (Thanikaivelan et al. 2004). Leather is nothing but a collagen and non collagenolytic protease activity

produced undamaged and high quality leather with higher strength.

**Table 3.** Enzyme activities against different hide proteins.

| Protein substrates | Enzyme activity (U mL <sup>-1</sup> ) ± SD |
|--------------------|--|
| Casein             | 2427 ± 201                                 |
| BSA                | 773 ± 102                                  |
| Gelatin            | 698 ± 52                                   |
| Elastic-orcein     | 8 ± 0.95                                   |
| Haemoglobin        | 8 ± 0.33                                   |
| Keratin azure      | 00   |
| Collagen           | 00   |

### Enzymatic Soaking

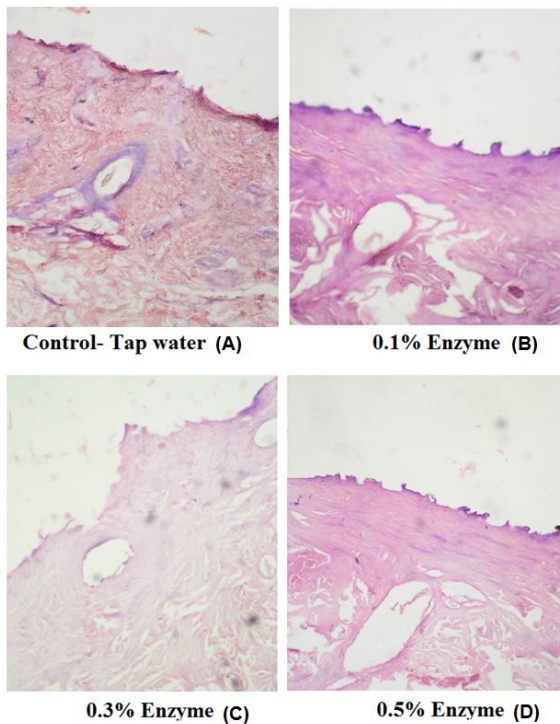
Dry hide and skin require longer period of soaking for complete rehydration. To minimize the soaking time, enzymes have an important role. The hide pieces were soaked in water bath with different enzyme concentrations and were observed periodically for its water holding capacity as well as the softening of the hide. Table 4, is showing increase in % moisture content of the buffalo hide as the enzyme concentration increased from 0.1-0.5%. This increase in % moisture was due to the hydrolysis of the proteins especially globular proteins present in hide. The complete softening of buffalo hide was observed at 16-18 h. Similarly, soaking time of 20 h was observed with 1% acid proteinase from *Rhizopus rhizopodiformis* and sodium bisulphite (Kamini et al., 1999). Puvankrishnan and Dhar (1988) reported that a period of 36-48 h was necessary to wet back dry hides. Three commercial bacterial alkaline protease preparations were also studied for soaking of salted cow hides (Kamini et al., 1999). Thus, use of hydrolytic enzymes (protease, amylase) results in a decrease in soaking time, clean pelt and hydrolysis of unwanted proteins.

**Table 4.** Effect of enzyme concentrations on soaking.

| Soaking conditions | % Moisture |
|--------------------|------------|
| Tap water          | 29.61      |
| Enzyme 0.1%        | 57.26      |
| Enzyme 0.3%        | 67.24      |
| Enzyme 0.5%        | 84.37      |

Soaked pieces were dehaired using lime-sulphide method and the L.S. of dehaired pelts are shown in Figure 3. Enzymatic soaking allows the loosening of scud, initiate

opening of the fiber. As per earlier reports it also improved the softness and elasticity of rabbit skin and increased the area of fur (Puvankrishnan and Dhar, 1988). The soaked hide pieces showed empty follicles whereas the control piece showed disintegrated hair follicles. Thus, soaking help for dehairing operation of leather processing which reduces the need for dehairing chemicals by 30-60% (Nielsen, 2006).



**Figure 3.** Micrographs of dehairing pelts soaked with A: tap water-control, B: 0.1% enzyme and C: 0.3% enzyme, D-0.5% enzyme of *P. aeruginosa* MCM B-327 (A showed disintegrated hair follicles, B to D showed empty follicles and well opened collagen fibers).

## CONCLUSION

The protease from *P. aeruginosa* has a property to hydrolyze unnecessary skin proteins for the production of collagen stabilized leather. Application of enzyme during soaking reduced the soaking time, promoted hair loosening, and assisted dehairing process by minimizing the use of chemicals.

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