Indian Journal of Exprimental Biology
Vol. 51, April 2013, pp. 336-342

Statistical medium optimization of an alkaline protease from *Pseudomonas aeruginosa* MTCC 10501, its characterization and application in leather processing

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Received 23 July 2012; revised 26 December 2012

Proteases are shown to have greener mode of application in leather processing for dehairing of goat skins and cow hides. Production of protease by submerged fermentation with potent activity is reported using a new isolate *P. aeruginosa* MTCC 10501. The production parameters were optimized by statistical methods such as Plackett-Burman and response surface methodology. The optimized production medium contained (g/L); tryptone, 2.5; yeast extract, 3.0; skim milk 30.0; dextrose 1.0; inoculum concentration 4%: initial *p*H 6.0; incubation temperature 30 °C and optimum production at 48 h with protease activity of 7.6 U/mL. The protease had the following characteristics: *p*H optima, 9.0; temperature optima 50 °C; *p*H stability between 5.0-10.0 and temperature stability between 10-40 °C. The protease was observed to have high potential for dehairing of goat skins in the pre- tanning process comparable to that of the chemical process as evidenced by histology. The method offers cleaner processing using enzyme only instead of toxic chemicals in the pre-tanning process of leather manufacture.

Keywords: Dehairing, Protease, *Pseudomonas aeruginosa*, Response surface methodology

Although, proteolytic enzymes are produced and are in continuous usage, they are not sufficient enough to meet the growing demands in the world market for different applications[1]. Enzymatic dehairing of hides and skins is suggested as an environmentally friendly alternative to the conventional chemical process which causes pollution to the environment[2,3]. Conventionally, lime and sodium sulphide are used for dehairing of skin and hides. Hence, rationalization of the dehairing process by systemic use of proteases in place of lime and sulphide becomes an issue of primary importance in leather processing[4]. This ultimately leads to a substantial reduction of effluent load and toxicity, in addition to improvement in leather quality[4].

Protease production by microorganism is greatly influenced by media components, especially carbon and nitrogen sources and physical factors such as temperature, *p*H, incubation time, agitation and inoculum density[5]. *Pseudomonas* species have been exploited for their ability to produce enzymes for the dehairing process[6,7]. A potent protease producing bacteria was isolated from slaughter house soil and later identified as *Pseudomonas aeruginosa* MTCC 10501. In this study, statistical media optimization for production of protease from *Pseudomonas aeruginosa* using tools such as Plackett-Burman and response surface methodology (RSM), characterization and potential for dehairing of goat skins are reported.

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Materials and Methods

Materials—All the organic media components mentioned were sourced from HiMedia, India. Inorganic chemicals and Hammerstein casein (Biochemical grade) were purchased from SD Fine Chemicals. The bacterial isolate, identified by CSIR-Institute of Microbial Technology, Chandigarh as *Pseudomonas aeruginosa* MTCC 10501 isolated from slaughter house soil, Puliyanthope, Chennai, India was chosen for protease production. All the experiments were carried out in triplicate.

Statistical optimization of protease production—The culture medium used in this work for the protease production contained (g/L) tryptone (5); yeast extract (2.5); dextrose (1.0); skim milk powder (28); initial *p*H 6.0; inoculum concentration 4% and maintained at 30 °C for 48 h in a shaking incubator (120 rpm). After 48 h of growth, the cells were harvested at 8000 rpm

for 10 min using centrifuge and the supernatant thus obtained was used as crude enzyme for further studies. The bacterial isolate was maintained in the skim milk agar slants comprising the above components.

Media optimization for protease production was carried out using Plackett-Burman design and RSM. Plackett-Burman is a two level screening design, which is well suited for detecting significant factors in a fermentation process. Preliminary studies were carried out by elimination method to identify the crucial nutrient factors from the basal growth medium for higher protease production (unpublished data). The selected nutrient factors were included in the experimental Plackett-Burman design and the various levels of the experimental parameters are summarized in Table 1. Further optimization was carried out by Response Surface Methodology (RSM). Statistical analysis of the model was performed using the 'Design Expert' software package (Version 6.0.11, Stat-Ease Inc., Minneapolis, USA). The significant factors were further subjected to central composite design, a tool of RSM.

Protease assay—Protease assay was carried out with slight modification[8]. Hammerstein casein (1.9 mL of 1%), biochemical grade, prepared in 0.1 *M* carbonate buffer (*p*H 9.5) was taken in test tubes and the tubes were preincubated at 40 ℃ for 10 min in a water bath. Then, 0.1 mL of suitably diluted cell free broth containing protease was added to the substrate solution and the tubes were incubated in a water bath for 10 min at 40 °C The reaction was stopped by addition of 3.0 mL of 5% trichloroacetic acid and the tubes were kept at ambient temperature (25-30 °C) for 15 min. The precipitate was then filtered using Whatmann No.1 filter paper and the absorbance of the filterate was determined at 280 nm using UV-VIS spectrophotometer. One unit was defined as the liberation of 1 mg tyrosine from casein as substrate per mL of enzyme in 10 min.

Enzyme characteristics—The crude protease was subjected to 80% ammonium sulphate precipitation and redissolved in 0.1 *M* carbonate buffer, *p*H 9.5. Partially purified (80%) ammonium sulphate saturated lyophilized enzyme (250 U/g) was used for characterization studies. To determine the optimum *p*H for the protease activity, 1.9 mL of 1% casein solution was prepared using 0.1 *M* of different buffers, such as carbonate-bicarbonate buffer (*p*H 9.5-10.5), Glycine-NaOH buffer (*p*H 9.0) and phosphate buffer (*p*H 7.0-8.0). Suitably diluted enzyme (0.1 mL) was added to the mixture, kept at 40 °C for 10 min and protease activity was estimated. Similarly, 1% bovine hemoglobin solution was prepared using acetate buffer (*p*H 4.0-5.0) and phosphate buffer (*p*H 6.0) and the protease activity was measured since casein cannot be solubilized below *p*H 7.0.

To examine the *p*H stability of protease, 10 mL aliquots of protease were buffered with an equal volume of 0.1 *M* buffer of different *p*H values (4.0- 10.5) and incubated at 30 °C for 24 h. After the incubation period, aliquots were taken and the relative activity was measured by the standard procedure.

To determine the optimum temperature for protease activity, 0.1 mL of the suitably diluted enzyme was added to 1.9 mL of 1% casein solution prepared using carbonate buffer, *p*H 9.0 and the assay mixture was incubated at various temperatures (10-70 °C) for 10 min. Then, the protease activity was determined. To study the temperature stability, 5.0 mL of 0.1 *M* carbonate buffer, *p*H 9.0 was mixed with 5.0 ml of protease and the solution was incubated at different temperatures (10-70 °C) for 4 h. After the incubation period, the relative activity was measured. Collagenase[9] and keratinase[10] were assayed.

Dehairing studies and histological analysis—Dehairing experiments and histological staining were carried out as per Sivasubramaniam *et al.*[4]. Partially purified protease enzyme from *P. aeruginosa* MTCC 10501 having an activity of 250 Units/g was used in this study for dehairing of goat skins. For dehairing experiments, three different dehairing experiments were carried out viz., conventional lime-sulfide process, enzyme-assisted process using SPIC enzyme and protease only process. Two wet-salted goat skins, each weighing approximately 2.5 kg, were initially soaked in water for 8 h employing a float of 300% water. Prior to dehairing step, all the soaked skins were cut along the backbone line into two halves namely right and left and numbered. For the conventional process, 10% lime and 2% sodium sulfide was mixed with 10% water and made as paste was applied on the flesh side, left for 18 h at ambient temperature (32 °C) and dehaired using conventional beam and knife method. Similarly, 1.5% SPIC enzyme, 5% lime, 0.5% sodium sulfide and 10% water was mixed and made as paste for enzyme-assisted process. For experimental group, dip method was adopted for goat skin, wherein the soaked skins were suspended in dehairing float comprising 2% enzyme, 100% water with *p*H 9.0 adjusted using

0.2% sodium carbonate. The float was left for 18 h and dehaired. All the percentages were based on soaked weight. For histological studies, samples of 1.0 cm^2 were cut from identical official sampling potions of the corresponding dehaired pelts of skins, washed thoroughly and fixed in formal saline (prepared by adding 0.9 g sodium chloride in 100 mL of 10% formaldehyde solution). Samples were then dehydrated with ethanol series. After embedding in paraffin block, sections of 6 µm thickness were obtained using microtome and were stained using hematoxylin and eosin to examine the histological features.

Results and Discussion

Statistical optimization of protease production— The bacterial isolate *P. aeruginosa* MTCC 10501 isolated in the laboratory was subjected to media optimization by statistical design methods such as Placektt-Burman design using seven factors (Table 1) and this resulted in identifying four critical factors viz. trypsin, yeast extract, skim milk and time (Table 2). Dextrose served as a carbon source and hence it was included in the subsequent design at a fixed concentration of 1.0 g/L. Similarly, inoculum level was kept constant. Optimization was carried out using a five level (-α, -1, 0, +1, +α) four factorial central composite design (CCD) for the production and this resulted in optimum protease activity of 7.6 U/mL in the optimized medium of trypsin 2.5 g/L; Yeast extract 3.0 g/L; skim milk 30 g/L; time 48 h with other factors remaining constant (Table 3). The adequacy of the model and its fitness were evaluated by ANOVA (Table 4) and the regression coefficient (R^2) of quadratic model was calculated (Table 5). The ANOVA (Table 4) for the quadratic model was highly significant with an F value of 18.6 as shown by Fishers's F test, along with a very low probability value (P<0.0001) which was significant at 95% confidence interval. At the same time, a relatively lower value of coefficient of variation (CV= 15.1) indicated a better precision and reliability of the experiments carried out. The determination coefficient (R^2) of the model was 0.9526 indicating that 95.26% of variability in the response could be accounted by the model. The final predictive equation was as follows:

Protease activity (U/ml) = 3.78-1.59*A+0.27*B-0.64*D-0.29*A*B+0.37*A*D-0.41*C*D-0.38*A^2+0.42*B^2-0.38*D^2

From the model, it is clear that the linear coefficients A, B, D, quadratic term A^2, B^2, $D^2$and cross product term AB, AD, CD were highly significant (Table 5). The contour graph for the interaction between the factors AB, AD and CD are shown in Fig. 1(a-c). The interaction studies between factors A and B show that by decreasing the tryptone (A) and increasing the yeast extract concentration (B),

Table 2—Analysis of variance (ANOVA) for the selected Plackett-Burman design

Factors	Estimate	*P*-value
A-Tryptone	-0.581	0.118
B- Yeast extract	-0.558	0.111
C-Dextrose	0.560	0.148
D- Skim milk	-0.535	0.135
E- Inoculum	0.572	0.152
F-*p*H	0.802	0.212
G- Time	0.938	0.030

Table 1—Plackett-Burman design of experimental runs for protease production

Expt.run	A-tryptone (g/L)	B- yeast extract (g/L)	C- dextrose (g/L)	D- skim milk (g/L)	E- inoculum (%)	F- *p*H	G – Time (h)	Protease activity (U/mL)
1	7.5(+)	2.0(-)	0.5(-)	20.0(-)	5.0(+)	6.0(-)	72(+)	8.0
2	2.5(-)	3.0(+)	0.5(-)	36.0(+)	5.0(+)	6.0(-)	72(+)	8.0
3	2.5(-)	3.0(+)	1.5(+)	20.0(-)	5.0(+)	8.0(+)	24(-)	7.3
4	7.5(-)	3.0(+)	0.5(-)	36.0(+)	5.0(+)	8.0(+)	24(-)	1.0
5	2.5(-)	3.0(+)	1.5(+)	36.0(+)	3.0(-)	6.0(-)	24(-)	5.7
6	7.5(+)	3.0(+)	0.5(-)	20.0(-)	3.0(-)	8.0(+)	24(-)	6.2
7	2.5(-)	2.0(-)	0.5(-)	36.0(+)	3.0(-)	8.0(+)	72(+)	6.8
8	7.5(+)	2.0(-)	1.5(+)	36.0(+)	3.0(-)	8.0(+)	72(+)	7.0
9	2.5(-)	2.0(-)	0.5(-)	20.0(-)	3.0(-)	6.0(-)	24(-)	5.9
10	7.5(+)	3.0(+)	1.5(+)	20.0(-)	3.0(-)	6.0(-)	72(+)	6.1
11	7.5(+)	2.0(-)	1.5(+)	36.0(+)	5.0(+)	6.0(-)	24(-)	5.8
12	2.5(-)	2.0(-)	1.5(+)	20.0(-)	5.0(+)	8.0(+)	24(-)	7.2

Table 3—Design matrix of central composite design for production of protease in terms of actual factors

Run no.	A:Tryptone (g/L)	B:Yeast extract (g/L)	C: Skim milk (g/L)	D: Time (h)	Protease activity (U/mL)	
					Observed response	Predicted response
1	12.5	2.0	10.0	48	1.57	1.62
2	12.5	2.0	30.0	96	1.52	1.87
3	12.5	3.0	10.0	96	1.74	1.73
4	12.5	3.0	30.0	48	2.68	3.33
5	2.5	2.0	10.0	96	5.13	4.31
6	2.5	3.0	10.0	48	7.09	6.57
7	2.5	3.0	30.0	96	4.64	4.42
8	7.5	2.5	20.0	72	3.97	4.17
9	2.5	2.0	30.0	48	6.17	6.01
10	7.5	2.5	20.0	72	3.67	4.17
11	2.5	3.0	30.0	48	7.63	7.48
12	12.5	3.0	10.0	48	1.75	1.65
13	12.5	2.0	10.0	96	1.87	2.09
14	7.5	2.5	20.0	72	4.12	3.84
15	12.5	2.0	30.0	48	2.80	2.40
16	2.5	2.0	30.0	96	3.18	3.34
17	12.5	2.0	10.0	48	4.29	4.69
18	2.5	3.0	10.0	96	4.03	4.50
19	12.5	3.0	30.0	96	1.43	1.11
20	7.5	2.5	20.0	72	3.84	3.84
21	7.5	2.5	34.1	72	3.39	3.42
22	7.5	2.5	20.0	72	3.59	3.35
23	7.5	2.5	5.8	72	2.73	2.92
24	7.5	2.5	20.0	72	3.97	3.35
25	7.5	1.8	20.0	72	3.70	3.81
26	14.6	2.5	20.0	72	0.67	0.31
27	0.4	2.5	20.0	72	4.26	4.83
28	7.5	3.2	20.0	72	4.48	4.59
29	7.5	2.5	20.0	38	3.37	3.50
30	7.5	2.5	20.0	106	1.58	1.67

Table 4—Analysis of variance for quadratic model

Source	Sum of Squares	df	Mean Square	F Value	*P*-value Prob>F
Model	73.18	14	5.23	18.67	<0.0001
Tryptone (A)	50.88	1.0	50.89	181.74	<0.0001*
Yeast extract (B)	1.54	1.0	1.55	5.53	0.0351*
Skim milk (C)	0.61	1.0	0.62	2.20	0.1620
Time (D)	8.4	1.0	8.40	30.02	0.0001*
AB	1.42	1.0	1.43	5.09	0.0419*
AC	0.01	1.0	0.01	0.04	0.8448
AD	2.23	1.0	2.23	7.97	0.0144*
BC	0.06	1.0	0.06	0.21	0.6519
BD	1.09	1.0	1.09	3.89	0.0701
CD	2.7	1.0	2.70	9.66	0.0083*
A^2	1.36	1.0	1.37	4.88	0.0456*
B^2	1.62	1.0	1.63	5.81	0.0315*
C^2	0.07	1.0	0.07	0.26	0.6191
D^2	1.31	1.0	1.32	4.70	0.0494*
Residual	3.64	13	0.28		

Lack of fit:0.0712 (not significant)
*Significant
R^2 = 0.9526 CV= 15.1

maximum protease activity was observed. Similarly, by lowering the tryptone concentration (A) and time (D) and by increasing the skim milk concentration (C) and reducing the time (D), maximum protease activity was observed.

For partial purification of protease, the supernatant of 48 h grown culture was used as crude source of enzyme. Protease from the culture media was partially purified by the ammonium sulfate precipitation at 80% saturation level.

Effect of pH on the activity and the stability of protease—Studies on protease activity as a function of *p*H using casein as a substrate showed that the enzyme was active in the range of 7.0 to 10.0 and it exhibited optimum activity at *p*H 9.0 indicating that it was an alkaline protease. There was a gradual reduction in activity in the acidic *p*H when hemoglobin was used as substrate (Fig. 2). This is similar to a report wherein protease produced by *Bacillus cereus* MCM B-326 showed optimum activity at *p*H 9.0 and any further variation of the reaction mixture caused reduction in catalytic activity[11]. With assumption that potential application might need about 24 h operational conditions, under varying *p*H, the protease was incubated at different *p*H for 24 h and its relative activity was analyzed. It was observed that the enzyme had remarkable stability in *p*H range of 5.0-10.0 with optimal stability observed at *p*H 6.0 (Fig. 2).

Table 5—Coefficients of quadratic model for optimization of protease production

Coefficient	Value	Standard error	*t*-value	*P*-value
β (A-A)	-1.59	0.12	13.25	0.0001*
B(B-B)	+0.27	0.12	2.25	0.0351*
B(D-D)	-0.64	0.12	5.33	0.0001*
β(AB)	-0.29	0.13	2.23	0.0419*
B(AD)	+0.37	0.13	2.84	0.0144*
B(CD)	-0.41	0.13	3.15	0.0083*
β (A^2)	-0.38	0.18	2.11	0.0456*
B (B^2)	+0.42	0.18	2.33	0.0315*
β (D^2)	-0.38	0.18	2.11	0.0494*

A=Tryptone; B=Yeast extract; C=Skim milk; D=Time
*Significant

Effect of temperature on the activity and the stability of protease—The effect of temperature on protease activity showed that the enzyme was active at a broad range of temperature (30-70 °C) and maximum activity was observed at 50 °C (Fig. 3) whereas maximum protease activity was reported at 70 ℃ from *Bacillus circulans*[12]. The enzyme exhibited thermal stability up to 50 °C showing that the enzyme was stable at high temperature. The enzyme was found to be highly stable in the temperature range of 10-40 °C and reduction in thermal stability was observed from 50 °C onwards (Fig. 3). There are a few reports of protease from *P. aeruginosa* with characteristic features. Optimal activity at *p*H 7.0 and temperature optima at 30 °C[6], 55 °C[11] and 70 °C[12] are reported.

Collagenase and keratinase assay—The collagenase and keratinase assays were performed and the assay result indicates that there is no

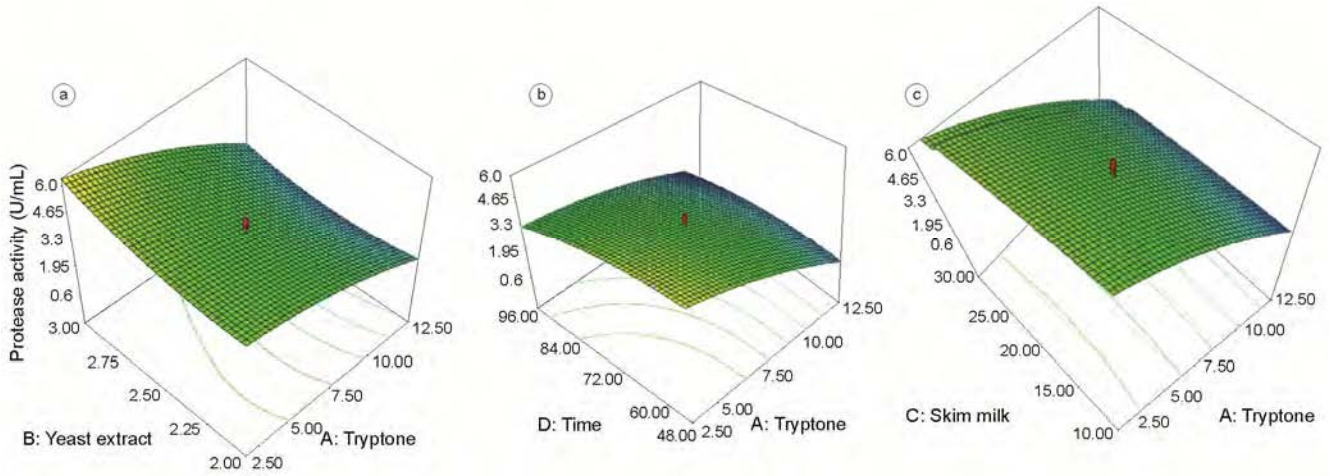

Fig. 1—Cumulative interaction between (a) tryptone and yeast extract; (b) tryptone and time, and (c) skim milk and time

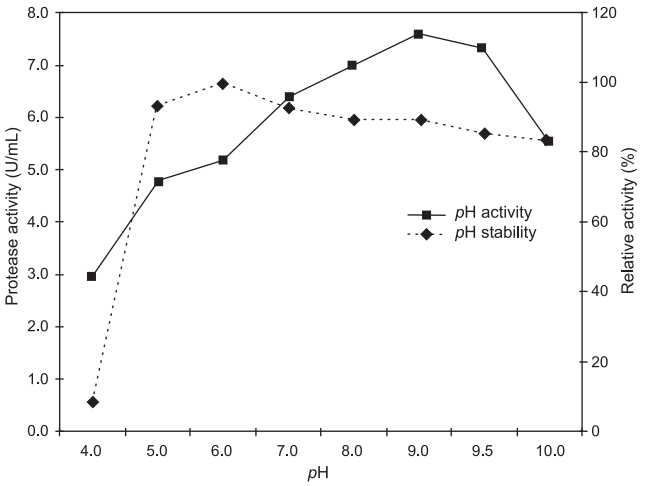

Fig. 2—Effect of *p*H on protease activity and stability

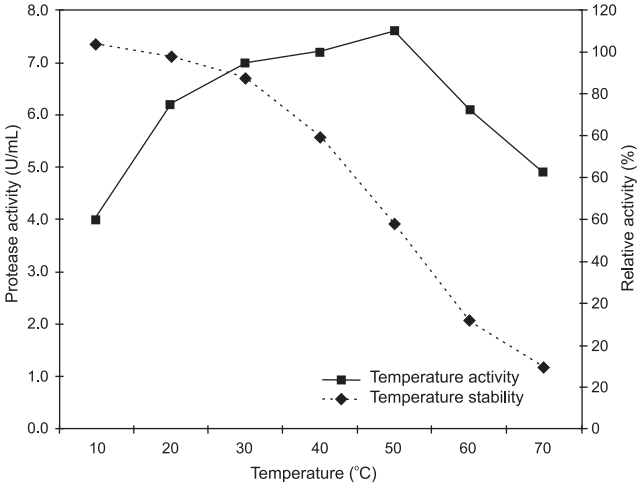

Fig. 3—Effect of temperature on protease activity and stability

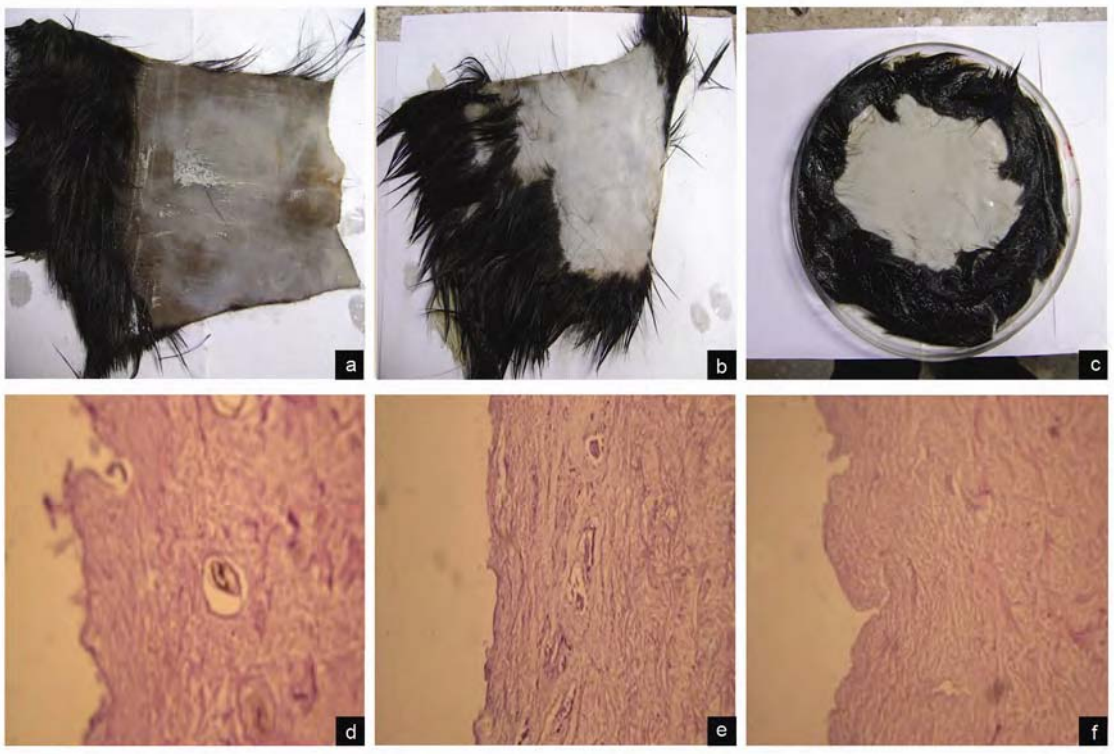

Fig. 4—Dehaired pelt following (a) conventional method, (b) enzyme assisted method, (c) experimental protease; histological staining of (d) conventional dehaired pelt, (e) enzyme assisted dehaired pelt, and (f) enzymatic dehaired pelt.

collagenase as well as keratinase activity in the enzyme preparation. The results on collagenase and keratinase activity demonstrate that the enzyme preparation is free from collagenase and keratinase and this product is suitable for use in dehairing experiment.

Dehairing studies and histological analysis—The dehaired pelts obtained by conventional method, SPIC protease assisted with sodium sulphide and alkaline protease alone from this study were compared. Visual observation substantiated with histological analysis Fig. 4 (a-e). In conventional treated pelt, incomplete hair removal was observed with presence of hair shaft and follicles (Fig. 4 a and d) whereas in enzyme assisted dehaired pelt, moderate hair removal with retention of hair shaft and follicles (Fig. 4 b, and e)

was noticed. In experimental dehaired pelts from protease treated goat skin total absence of fine hairs and epidermis (Fig. 4 c and f) were observed.

The present study is similar to a report on statistical optimization of alkaline protease production by response surface methodology[15]. Enzyme based products as biocatalysts in place of chemicals is currently being explored in many areas of leather processing[4,11,16,17]. Similarly, application of an alkaline protease for dehairing of goat skin using 1% enzyme as well as 0.5% enzyme in presence of 1% sulfide for complete dehairing at 18 h is reported[17]. Sulfide-free dehairing using 3-6% enzyme resulting in partial dehairing and complete dehairing using 6% enzyme in presence of 0.5% sodium sulphide is reported[7]. Sodium sulphide generates pollution load[1] which was totally avoided with the use of 2% enzyme process alone in the present study.

Conclusions

In this study, statistical medium optimization of potent protease production as well as application for dehairing of goat skin is reported. It could be concluded that bacterial protease with characteristic features such as *p*H optima at 9.0 and *p*H stability in the range of 5.0-10.0 could be effectively used for dehairing of goat skins. Histological examination of enzymatically dehaired skin sample showed better quality dehaired pelt compared to conventional and enzyme assisted dehaired pelt.

Acknowledgement

The authors thank Dr. A B Mandal, Director, CSIR-CLRI, Chennai, India for his kind permission to publish the work.

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