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A novel ZMS-2 Protease: Evaluation of production dynamics through Response Surface Methodology and application in leather tannery.

Abstract

Proteolytic enzymes are the most versatile and commercially viable group of enzymes comprising over 65% share in the global enzyme market amongst which alkaline proteases have extensive applications in detergent and leather industry. Current study was designed to assess the potential of an alkaline serine protease from *Bacillus subtilis* ZMS-2 as a bating agent. Initially, the production parameters were investigated through Response Surface Methodology (RSM) using Plackett-Burman Design, which identified substrate, agitation speed and incubation temperature as the most significant factors. The optimal levels of these factors were determined through the Box-Behnken experimental analysis as 0.436% substrate concentration, 36.5 °C incubation temperature and 56 rpm agitation speed. The statistical optimization experiments increased the volumetric production of enzyme by 3.94 times (2246 U mL^{-1}) than the initial titer (571 U mL^{-1}). The enzyme was partially purified and characterized as metal ions and detergent compatible serine protease having optimum activity at pH 8 and 60 °C. During the pilot-scale application as a bating agent, the enzyme (340 U mL^{-1}) successfully removed the hair roots and other unwanted proteins from goat skins as observed during scudding and confirmed through Scanning Electron Microscopy. The processed skins displayed enhanced porosity, thumb impression, smoothness and pliability. These findings provide a strong basis for the use of this protease as an efficient and eco-friendly alternative for bating of animal skins in leather tanneries.

Keywords: *Bacillus subtilis ZMS-2; serine protease; Response Surface Methodology; bating.*

1. Introduction

Proteolytic enzymes are bioactive macromolecules which constitute indispensable parts of life on earth including microorganisms, animals and plants. Microorganisms are generally preferred for production of commercially important enzymes due to their ease of genetic modification, rapid growth and limited cultivation space (Shaikh et al., 2019). Proteases have extended applications in industries such as silk degumming, leather processing, peptide synthesis, feed and food production, detergent manufacturing, photography and waste management, thus having a global market share of over 65% (Barzkar et al., 2020; Gupta et al., 2002).

Alkaline proteases constitute the largest group having optimal activity at neutral to alkaline pH, and are either serine or metallo proteases (Sharma et al., 2017). Serine proteases are specialized enzyme possessing the serine residue in their catalytic active sites (Khan et al., 2021). The compatibility with metal ions, commercial detergents, denaturants and the attributes of withstanding to the change in pH and temperature makes an enzyme ideal for its commercial applications.

The production of microbial proteases in submerged fermentation is prejudiced by numerous physico-chemical factors including carbon/nitrogen sources, salts, incubation time, size and age of inoculum, pH, agitation and incubation temperature (Khan et al., 2019; Shaikh et al., 2019). The use of Response Surface Methodology for the identification of significant parameter influencing the production dynamics of hydrolytic enzymes is highly desirable and reported in multiple studies (JayaKumar et al., 2021; Bhavikatti et al., 2020; Ejaz et al., 2019).

During the chemical dehairing, lime and sulfide act like shaving blades leaving behind the intact hair roots. Bating is an important pre-tanning step where hair roots and other unwanted proteins are removed from the dehaired skin as well as to split the fiber into fibril (Kanagaraj et al.,

2015). Efficient bating results into high porosity and thumb impression, smoothness, pliability of the processed skin.

Current study reports production optimization, characterization and pilot-scale application of ZMS-2 protease in leather tannery.

2. Materials and methods

2.1. Materials

Fresh goat skins for bating studies were acquired from local slaughterhouse at Gol market, Karachi, Pakistan. The commercial bating enzyme (Resopan RN®) was purchased from Shafi Reso Chemicals (SRC), Lahore, Pakistan. The components of growth medium, chemicals for dehairing and azocasein/assay reagents used in this study were purchased from Oxoid Ltd. and Sigma-Aldrich, respectively.

2.2. Producer strain

Bacillus subtilis strain ZMS-2 (Khan et al., 2022) previously isolated from desert soils was revived on nutrient agar for succeeding studies.

2.3. Inoculum preparation

Inoculum was prepared by adding a loopful of 24h culture into medium containing; 1% glucose; 0.5% wheat bran; 0.1% $CaCl_2$; 0.1% KH_2PO_4; 0.1% $MgSO_4.7H_2O$ and incubated for 24h at 37 °C.

2.4. Initial production medium

The 24 h old inoculum (10%) of strain *B. subtilis* ZMS-2 was added to production medium containing 0.5% starch; 1% wheat bran; 0.1% peptone; 0.1% KH_2PO_4; 0.1% $MgSO_4.7H_2O$ and incubated for 72h at 37 °C (Khan et al., 2019). After completion of fermentation process, the

cell free filtrate was obtained by centrifugation at 8000 rpm for 15 min at 4 °C. Proteolytic units in the cell free filtrate were assessed by UV-Visible spectrophotometer using azocasein as substrate as reported by Caldas et al. (2002).

One unit of enzyme activity was defined as the amount of enzyme which yields an increase in absorbance of 0.001 optical density (OD) at 440 nm in 30 min at 37 ℃.

2.5. Statistical optimization of protease production

2.5.1 Plackett-Burman Design (PBD) experiments

PBD was applied eight variables to identify the most significant variables affecting the production of protease at two levels (Table 1). The experiments were conducted by adding inoculum (5% or 10%) into media supplemented with 0.5% or 1% substrate. The pH of the medium was 6 or 8 while media contained 0.5% or 1% starch. The medium was incubated at 37 °C or 45 °C for 24 or 72 h with (150 rpm) or without agitation. Based on first order polynomial equation, 20 experiments were performed. Post incubation, cell-free filtrate was harvested and assayed for protease activity which was taken as response.

2.5.2. Box-Behnken Design (BBD) experiments

In BBD, each significant variable was studied at three levels i.e., substrates concentration (0, 0.5 or 1%); agitation (0, 75 or 150 rpm) and temperature (35, 37.5 or 40°C) resulted into a combination of 15 experiments. The non-significant factors including starch concentration (0.5%), pH 8, inoculum (10%) and incubation time (72 h) were kept constant. The response function representing the activity of protease was partitioned into linear, quadratic, and interactive components. After the analysis of BBD, response optimizer was run and experiment was performed in order to get optimized values of parameters.

2.5.3. Statistical analysis

The experimental responses were evaluated using two-way analysis of variance (ANOVA) and results were produced for the independent variables. The linear, quadratic and interaction regression coefficient of each entity in the model were resolved. Using the confidence level of 95% and f-value at a probability (P) of 0.05, the significance of all entities in the polynomial was statistically investigated and all coefficients were figured out using Minitab version 17.0.

2.6. Partial purification

The cell-free filtrate (1000mL) having 2246 U mL^{-1} was subjected to ammonium sulfate precipitation up to 70% saturation level at 4°C on magnetic stirrer for partial purification. Precipitates were recovered through centrifugation at 8000 rpm for 15 min at 4°C and re-suspended in an equal volume of 50mM Tris-HCl buffer (pH 8) followed by overnight dialysis to remove excessive salts. The proteolytic units of partially purified protease were estimated by performing protease assay as mentioned in section 2.4 and stored at -20 °C till further use.

2.7. Biochemical characterizations

The partially purified enzyme was diluted by adding 2 mL of enzyme into 10 mL 50mM Tris-HCl buffer before the characterization. The optimum pH and temperature for proteolytic activity was identified by assaying the enzyme at varied temperatures (20-80 °C) and pH (4-11). Similarly, the effects of metal ions and inhibitors were evaluated by adding 50μl of respective 5mM solution to the assay reagents (Table 4). Furthermore, the effect of commercial detergents on the proteolytic activity was also evaluated using 1% detergent solution (Mechri et al. 2019).

2.8. Pilot-scale application of protease as bating agent

After optimized production of protease a series of trials was conducted to check its application in leather processing as a bating agent at tannery section of Leather Research Centre, PCSIR,

Karachi, Pakistan. Experimental rotary drums (Diameter: 45 cm, Width: 30 cm, capacity 50L) were used to carry out bating process employing dip method having a speed of 20-30 rpm. The proteolytic units of enzyme were adjusted using 50mM Tris- HCl buffer to that of a commercial enzyme Resopan RN® (340 U mL^{-1}) before the pilot-scale bating studies. Four goat skins were processed through conventional dehairing method using 4% Na_2S and 6% $Ca(OH)_2$. After dehairing, liming and de-liming, skins were subjected to bating by adding 2% w/w of ammonium sulfate followed by addition of 2% w/w of both Resopan RN® bat (as per manufacturer's recommendations) and protease of the present study into separate experimental rotary drums and run for 60-90min.

2.9. Topological analysis using scanning electron microscope (SEM)

Skin pieces from the enzymatically processed skin were dried in oven at 40-50°C for 4-5h, coated with 250A° thick gold using Ion Sputtering Device (JEC-1500, Jeol, Japan) and examined by Analytical SEM (JSM-6380, Jeol, Japan). The microscopy imageries were recorded at different magnifications ranging from 100-400x at a voltage of 10 kV.

3. Results

The optimization strategy used in this study by employing PBD revealed titers of the protease from 72 to 605 U mL^{-1} by varying experimental conditions (Table 1), yet substrate concentration, agitation and temperature appeared as significant factors after considering Pareto chart (Fig. 1) and p values (Table 2).

Consequently, the significant factors were further evaluated by BBD keeping non-substantial variables constant. The ANOVA analysis of the calculated enzyme units from the proposed 15 experiments runs (Table 3) revealed that the model for protease production was significant, giving R2 value of 86.81%.

Correlation analysis among the factors was performed in order to determine the interaction of factors and their collective effect on response. The interaction between the factors affecting the production of protease was examined contrary to two independent variables and the optimum values were computed by contour plots (Fig. 2). Increase in temperature along with increase in substrate concentration had significant effect on alkaline serine protease production which showed the directly proportional relationship between temperature and substrate concentration (Fig. 2).

After analyzing the data set and comparable responses, Minitab software proposed an experimental design comprised of a single run based on the previous results of BBD. A maximum of protease titer of 2254.06 units response was predicted under optimum conditions i.e., substrate concentration 0.426 %, agitation 56 rpm and temperature 36.5 °C (Fig. 3). The results revealed the experimental values 2246 U mL^{-1} that were comparable with the predicted values confirming the accuracy of RSM to predict the optimum levels of factors.

During partial purification, the enzyme was completely salted out at 70% ammonium sulfate saturation with 1.87-fold increase in proteolytic activity (4200 U mL^{-1}). The enzyme has showed maximum activity at 60 °C and pH 8 (Fig.4a, 4b), completely lost its proteolytic activity when assayed in the presence of 5 mM PMSF and displayed compatibility with metal ions and detergents (Table 4).

The enzyme exhibited a comparable bating potential to that of commercial bat as analyzed by the scudding process effectively removing the hair roots and other unwanted non-structural protein from the skin (Fig. 5b). Furthermore, observation of porosity, thumb impression, smoothness, and pliability of the processed skin also confirmed the effective bating property of protease. The

SEM analysis of the processed skin displayed smooth morphology, better structure and degraded epidermis as well as degraded hair bulb (Fig. 5c-d).

4. Discussion

One of major hindrances in the commercial application of enzyme is its cost, therefore, enzyme yield is improved either through genetic modification of the strain or through process optimization (Zafar et al. 2021).

Here, the significant factors as revealed by the PBD found to have been reported earlier for carbohydrolases (Rashid et al., 2020). For instance, the balance between C/N sources and substrate in production medium affect the growth rate as well as the production of enzymes (Ejaz et al., 2019; Jayakumar et al., 2021). Similarly, some studies have reported the concentration of tryptone and agitation as significant variable for the production of hydrolytic enzymes (Bhavikatti et al., 2020; Rashid et al., 2020). Moreover, majority of the microbes produce maximum titers of enzymes at their optimal growth temperature as studied by Sohail et al. (2009) and Ejaz et al. (2019).

The BBD potentially consider the quadratic effects hence providing a precise value of each significant factor (Rashid et al., 2020). The analysis of calculated units of each experiment using ANOVA resulted that the model for the enzyme production was significant, having R^2 value of 86.81%. As per Sood et al. (2019), the model was found to be a complete fit as the R^2 ranged between 81.3 and 96.4% which showed suitable fitting of the model. The analysis of optimal values by contour plots revealed that the enzyme production was highly influenced by an increase in temperature as well as substrate concentration (Fig. 2).

The proposed single experimental run predicted 2254.06 U mL^{-1} by the software at specified condition; the results revealed the experimental values 2246 U mL^{-1} being comparable with the

predicted values. This further confirms the accuracy of RSM to describe the optimum levels of factors. Furthermore, the statistical model not only resulted in a 3.94-fold increase in the production titer but also minimized the use of substrate for production further ensuring its cost-effectiveness which is essentially important for the scale up at industrial level (Jaya Kumar et al., 2021).

ZMS-2 protease displayed optimum activity at 60 °C and pH 8-9, thus retained its catalytic potential at broad range of pH and temperature. During the interaction with PMSF the enzyme lost its activity which categorizes it as serine protease (Mechri et al., 2019). These characteristics of temperature, pH, metal ions and detergents compatibility suggests this protease an ideal candidate for industrial applications. Further studies on structural elucidation are required to understand the underlying mechanism of tolerance to metal ions, temperature and pH.

ZMS-2 protease (2% w/w) efficiently removed the hair roots and unwanted non-structural proteins (globulin, albumin, elastin, etc.) from the goat skin without affecting the main structural protein collagen; the data is in coherence with a previous study (Al Mamun et al., 2015). Furthermore, not all proteolytic enzymes are applicable for dehairing/bating purpose due to their collageno-lytic activity (Huang et al., 2003). Therefore, those enzymes having efficient keratinolytic and minimal collageno-lytic potentials are ideal for tanneries (Zambare et al., 2007). During the process of dehairing/bating, proteases selectively degrade the soft keratin tissues inside the hair follicle, thereby pulling out the intact hairs without disturbing the tensile strength of the processed leather (Thanikaivelan et al., 2004). This removal of hair at the level of roots is very important step during the leather processing which provide the ideal smoothness and pliability to the processed skin as displayed by this enzyme.

The skins processed using ZMS-2 protease as bating agent showed smooth texture, degraded epidermis and hair bulb which is essentially important for pulling out the hair along with root (Fig. 5c). The surface topology of skins when observed at high resolution (400 x) also indicated that the enzyme was highly specific towards the keratin and had no adverse effect on the collagen structure of the skin (Fig. 5d) (Zambare et al., 2007). These findings provide a strong basis for the use of alkaline serine protease from *B. subtilis* ZMS-2 as an efficient, ecofriendly and cost-effective alternative for the bating of animal skins on industrial scale.

5. Conclusion

Current study reports the optimization of physico-chemical parameters for the enhanced production of a thermophilic alkaline serine protease from *B. subtilis* ZMS-2. The initial production yield (571 U mL^{-1}) was improved using statistical tools to 2246 U mL^{-1} with a 3.94-fold increase in activity. The result of ANOVA and second-order model revealed that the effects of casein, incubation temperature and agitation at various levels were significant for protease production. The enzyme was identified as metal ions and detergent compatible serine protease with an optimal activity at 60 °C and pH 8. The enzyme showed successful pilot-scale application as a bating agent with promising effects on leather pelt. Furthermore, scale-up production is highly desirable for its use as bating agent in leather industry.

Declaration of interest

There is no conflict of interests to declare.

Acknowledgment

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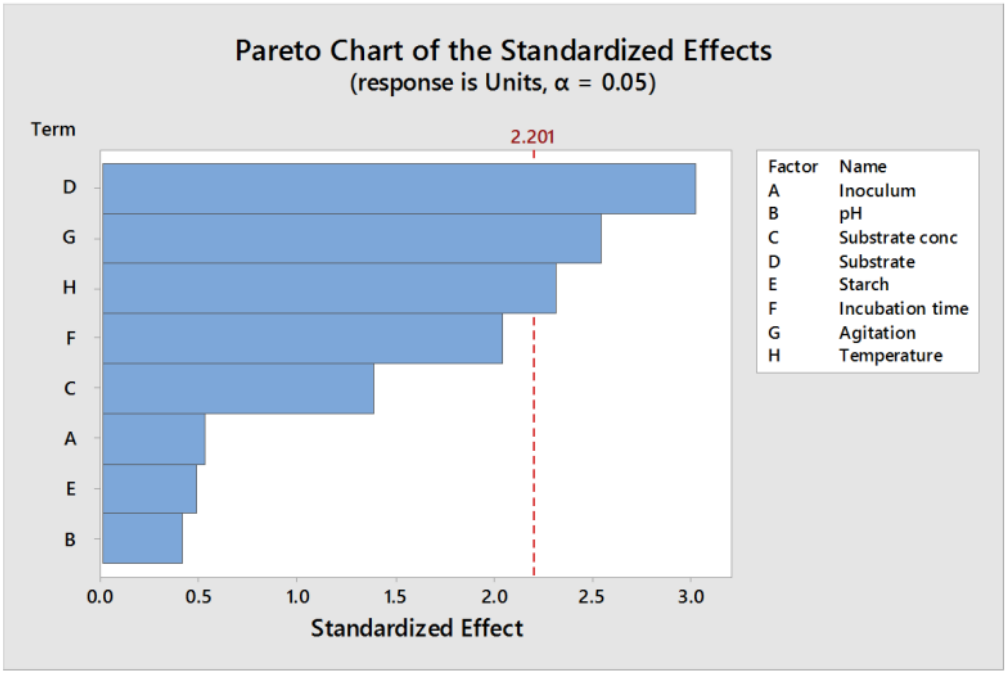


Fig. 1. Pareto charts showing the effect of factors on alkaline serine protease production.

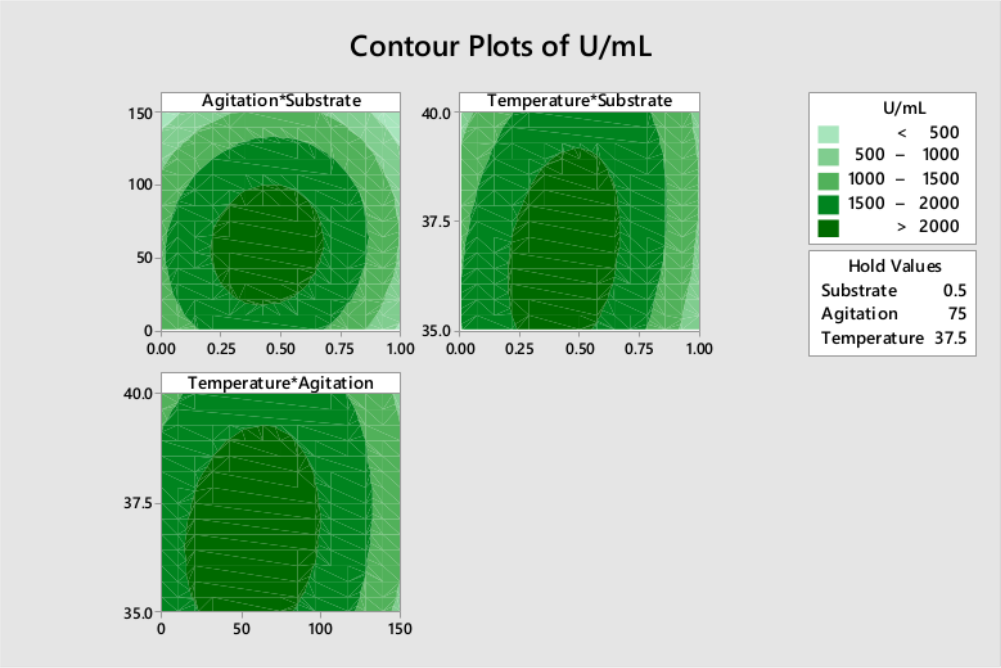


Fig. 2. Contour plots showing interaction between a) agitation and temperature b) temperature and substrate c) temperature and agitation.

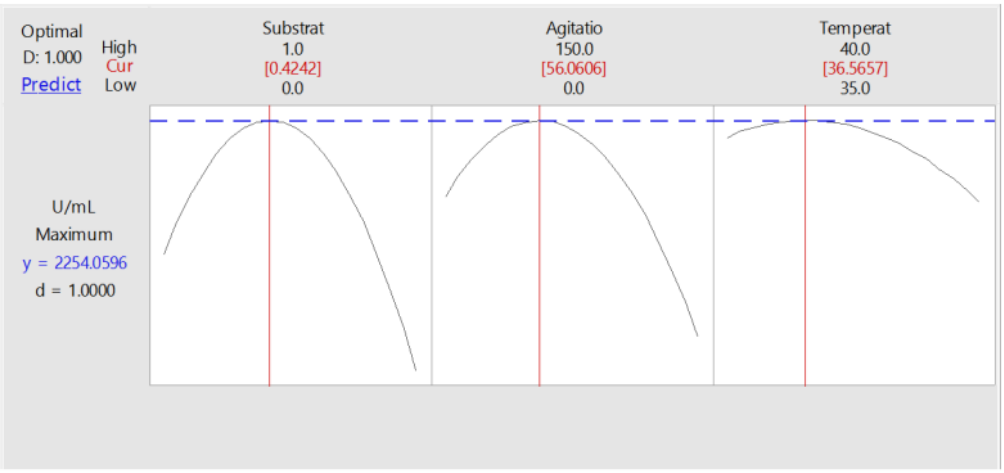


Fig. 3. Multiple response prediction showing the maximum production of alkaline serine protease as 2254.06 U mL^{-1}

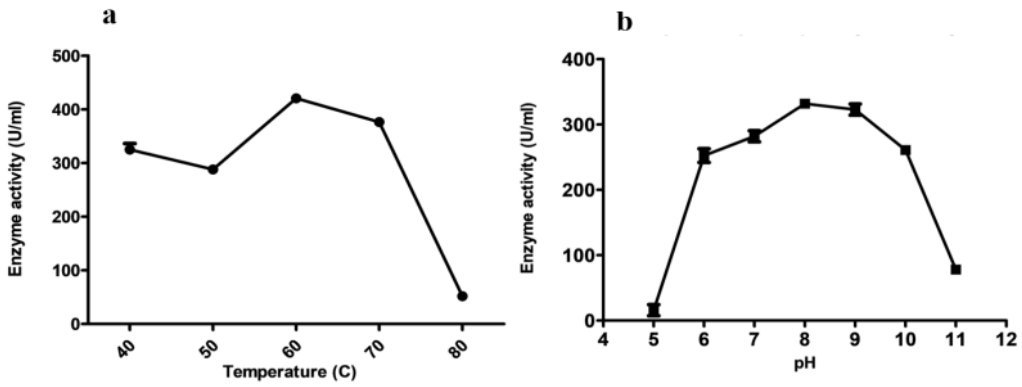
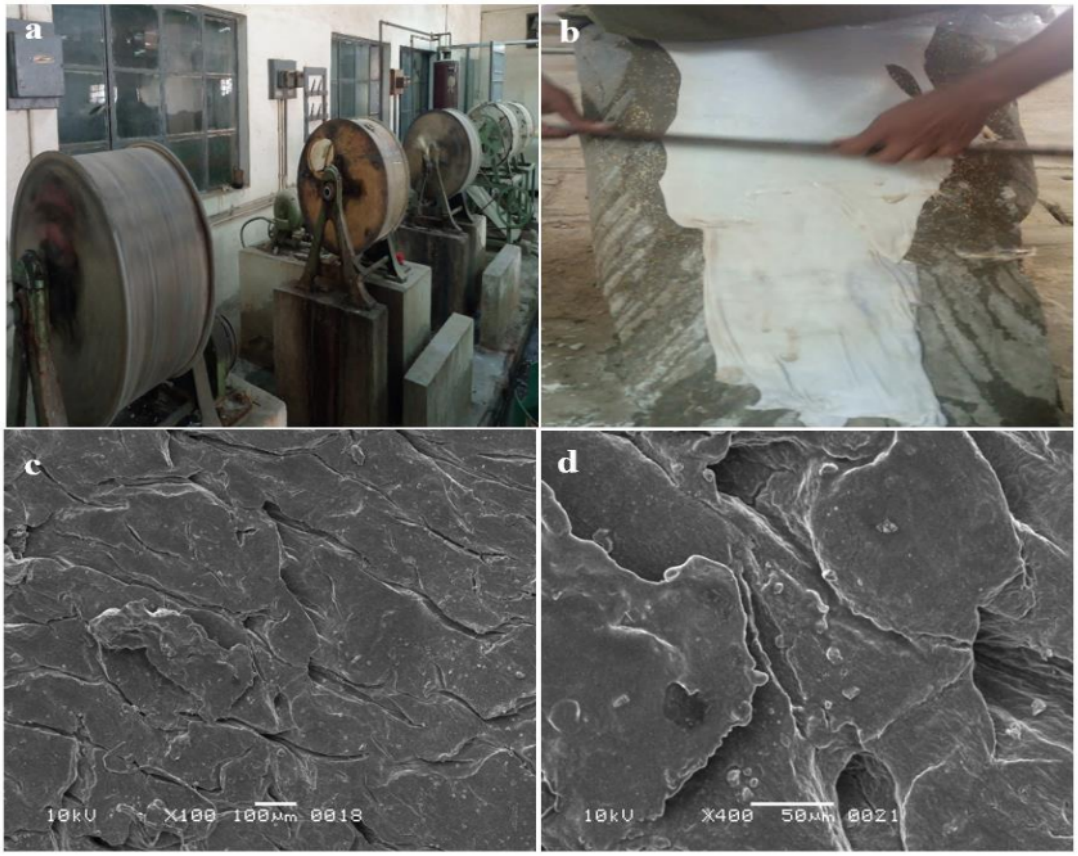


Fig.4. Effect of temperature (a) temperature and (b) pH on the activity of alkaline serine protease



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335 Fig. 5. (a)Tannery facility at Leather Research Center, PCSIR, Karachi, (b) Scudding of skin
336 after bating showing removal of hair roots and unwanted proteins, SEM images of processed
337 skin at (c) 100x and (d) 400x.

Table 1. Plackett-Burman Design with experimental response for the screening of significant factors influencing the alkaline serine protease production.

Run Order	Inoculum (%)	pH	Substrate concentration (%)	Substrate	Starch concentration (%)	Incubation time (h)	Agitation	Temperature (°C)	Units* (U mL^{-1})
1	10	6	1	WB	0.5	24	With	37	181
2	10	8	0.5	WB	1	24	With	37	110
3	5	8	1	C	1	72	With	37	605
4	5	6	1	WB	0.5	72	without	37	338
5	10	6	0.5	WB	1	24	without	45	174
6	10	8	0.5	C	1	72	With	45	203
7	10	8	1	C	0.5	72	without	37	1192
8	10	8	1	WB	0.5	24	without	45	292.5
9	5	8	1	WB	1	24	With	45	72
10	10	6	1	WB	1	72	With	37	289.5
11	5	8	0.5	WB	1	72	without	37	410
12	10	6	1	C	1	72	without	45	421
13	5	8	0.5	WB	0.5	72	without	45	182
14	5	6	1	C	1	24	without	45	495.5
15	5	6	0.5	WB	0.5	72	With	45	254
16	5	6	0.5	C	1	24	without	37	504
17	10	6	0.5	C	0.5	72	With	45	364.5
18	10	8	0.5	C	0.5	24	without	37	445.5
19	5	8	1	C	0.5	24	With	45	118.5
20	5	6	0.5	C	0.5	24	With	37	290

*The values represent average of triplicate with insignificant standard deviation.

WB = Wheat Bran, C = Casein

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348 Table 2. Regression analysis of Plackett-Burman Design for alkaline serine protease production.

Term	Main effect	Coefficient	T-Value	P-Value
Constant		347.1	9.01	0.000
Inoculum	40.4	20.2	0.52	0.611
pH	31.9	16.0	0.41	0.687
Substrate conc	106.8	53.4	1.39	0.193
Substrate	-233.6	-116.8	-3.03	0.011
Starch	-37.4	-18.7	-0.49	0.637
Incubation time	157.6	78.8	2.04	0.066
Agitation	196.7	98.3	2.55	0.027
Temperature	-178.8	-89.4	-2.32	0.041

349

350 Table 3. Box-Behnken Design for alkaline serine protease production by *B. subtilis* ZMS-2.

Run Order	Substrate (gm)	Agitation (rpm)	Temperature (°C)	Units*
1	0	0	37.5	714.0
2	1	0	37.5	99.0
3	0	150	37.5	513.0
4	1	150	37.5	402.7
5	0	75	35	1629.3
6	1	75	35	735.3
7	0	75	40	667.0
8	1	75	40	712.3
9	0.5	0	35	1938.7
10	0.5	150	35	412.3
11	0.5	0	40	1759.0
12	0.5	150	40	713.3
13	0.5	75	37.5	2129.3
14	0.5	75	37.5	2210.0
15	0.5	75	37.5	2199.3

351 *The values represent average of triplicate with insignificant standard deviation.

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353 Table 4. Effect of inhibitors, metal ions and detergents proteolytic activity.

Inhibitors/metal ions/detergents	Concentration	Residual activity (%)
Control	-	100 ± 4.32
PMSF	5mM	0 ± 2.41
EDTA	5mM	68 ± 3.52
K^+	5mM	95 ± 1.65
Ca^+	5mM	74 ± 2.78
Na^+	5mM	85 ± 3.13
Fe^{2+}	5mM	76 ± 3.61
Mg^{2+}	5mM	90 ± 4.26
Surf Excel	1%	65 ± 2.72
Ariel	1%	61 ± 3.37
Bonus Tri-Star	1%	72 ± 1.81
Lemon Max	1%	76 ± 2.52
Brite	1%	74 ± 2.93
Tween 80	1%	124 ± 2.18

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