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Alkaline protease production using eggshells and membrane-based substrates: Process modeling, optimization, and evaluation of detergent potency

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Abstract

This study focused on the use and eggshells and associated membranes (ESM) as a substrate for producing alkaline protease (AlkP). Furthermore, the study was performed to optimize the production process and check the feasibility of using the enzyme as a detergent. *Bacillus mojavensis* was used as a biological agent for fermentative enzyme production. The process was optimized with four factors at three levels using response surface methodology (RSM) coupled with the Box–Behnken design. Through RSM, optimal parameters for the production of AlkP were found to be the following: pH 9.08, temperature 39.74 °C, and ESM 197.8 g/l or 19.78% (w/v) with an incubation time of 48 h. Thereafter, the obtained enzyme was partially characterized. It retained more than 93.97% of its original activity in the pH range between 8 and 10, with optimal activity at pH 10. The minimum residual activity of AlkP was 39.6% of its original activity at pH 6.0. AlkP was found to be active at temperatures between 30 and 80 °C, but maximal at 60 °C. Residual activities were found to be 67.4%, 71.7%, 75.7%, 78.7%, and 78.7% in solutions with NaCl concentrations of 1.0, 1.5, 2, 2.5, and 3.0 M, respectively, at pH 8.0. A detergent compatibility study revealed that the obtained AlkP is quite effective as a detergent.

Keywords: Alkaline protease (AlkP), Eggshell and membrane (ESM), Detergent, RSM, Activity, Stability

1. Introduction

Numerous chemical transformations using inorganic catalysts have economic, energetic, and environmental disadvantages. However, all these drawbacks can be virtually eliminated using biocatalysts and/or industrial enzymes. Biocatalysts offer green and hygienic chemical processes and are evolving as an alternative to chemical technology [1]. Enzymatic conversion has recently received attention. It is becoming an attractive alternative to the use of inorganic catalysts. Proteases are vital to all forms of life. Among the various origins of protease, microbial sources are the most significant and broadly studied enzymes. They have an alkaline optimal pH range that has been extensively exploited in various industrial processes [2]. Of the various protease–producing microbes, alkaliphilic and neutralophilic microorganism possess high industrial potential on the basis of their stability over a wide pH range and biochemical diversity [3]. Nevertheless, ever-increasing demand for alkaline protease (AlkP) for various technological applications and the high cost of alkaline protease production have prompted a search for new microbial resources [4]. The genus, *Bacillus*, is an important source of alkaline proteases for industrial processes. It is the most used genera for commercial for alkaline protease production [1]. For economical utilization, wastes should be recycled, reused, and channeled toward the production of value-added products. Ethiopia is rich in agricultural residues, including poultry wastes such as eggshells [5]. Earlier studies reported that $CaCO_3$ significantly induces enzyme production [6]. Eggshells and their membranes (ESM) are a nonedible waste that is largely discarded. It is an important source of numerous bioactive compounds that can be obtained by effective separation [7]. Disposal of eggshells and their underlying membrane wastes produces materials that are abrasive, foul-smelling, and polluting [6, 8]. Approximately 136 million eggs are produced annually in Ethiopia. Egg shells and their membranes are normally disposed of in landfills [9, 10]. A study has suggested the ecosphere can be improved by effectively recycling chicken eggshells. This diminishes the requirements for solid waste management and provides for a beneficial raw material that can be used in the production of AlkP [11]. In Ethiopia, research on enzyme production is in the developing stage. At present, no enzyme production is done. The current study focuses on using an ESM-based substrate for AlkP production and its process optimization.

2. Materials and methods

2.1 Bacterial strain

Bacillus mojavensis was procured from the Bioengineering Laboratory at the Ethiopian Biodiversity Institute (EBI), Ethiopia. Stock cultures were carried until use in 2 mL sample tubes containing 50% (v/v) glycerol at –70 °C. To stimulate cultures, strains were inoculated into 20 mL of a medium comprised of (g/l): peptone (7.5), glucose (5), NaCl (0.5), and

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Table 1 Levels of four independent variables

Variables (Factors)	Units	Level		
		Maximum (+1)	Medium (0)	Minimum (-1)
pH	pH	10	9	8
Temperature	°C	45	37.5	30
ESM	% (w/v)	25	20	15
Incubation time	hours	72	54	36

$MgSO_4.7H_2O$ (0.1) with its pH adjusted to 8.0 using Tris-HCl buffer in a 200 ml conical flask. The inoculated seed medium was incubated at 37 °C for 24 h in a shaking incubator (Thermo Shaker Incubator; Shaker Incubator Lab; Model: DW-SI-1102C) operating at 180 rpm for 14 h [12, 13].

2.2 Substrate collection, preprocessing, and characterization

Eggshells were collected from a local market and bakeries in Addis Ababa. This city lies at an elevation of 2,355 meters (7,726 ft.) located at 9°1′48″N 38°44′24″E (9.03000°N 38.74000°E). To remove impurities and contaminants, such as insects and dirt, the ESM was washed using distilled water and allowed to dry under sunlight at 35–40 °C for a day. The ESM was finely ground using an analytical mill (Laval Lab Equipment, Canada; Model: Knife Mill Blender Pulverisette 11). Then, the resulting powder was sieved to collect 100 µm diameter and smaller particles using standard ASTM sieves for the fermentation process. Additionally, the ground ESM sample was subjected to proximate analysis and Fourier transform infrared (FTIR) characterization.

2.3 Inoculum preparation and production of crude protease

The inoculum for protease production was grown from a loopful of *B. mojavensis* culture in 30 mL of an activated seed culture that was composed of (g/l): bacteriological peptone (7.5), glucose (5), NaCl (0.5), and $MgSO_4.7H_2O$ (0.1) with its pH adjusted to 9.0 using Tris-HCl buffer in a 200 mL conical flask. The inoculated seed medium was incubated at 37 °C for 24 h at 180 rpm in a shaking incubator (Stuart, UK). This 24 h old culture was used as a seed culture and added to the working medium at room temperature [12]. A crude alkaline protease production medium was comprised of the components of Horikoshi-I alkaline medium [14] as follows (g/l): D-glucose (10 g), peptone (5 g), yeast extract (5 g), KH_2PO_4 (1 g), $MgSO_4$ $7H_2O$ (0.2 g), Na_2CO_3 (5 g), and distilled water (1000 ml) [15]. Glucose and sodium carbonate were separately autoclaved and then mixed with the sterile medium. Then, the culture was centrifuged at 3000 rpm (Biosan, Model: LMC300) to remove cells and insoluble materials. The culture supernatant was then used as a source of crude enzyme.

2.4 Enzyme activity assay

Protease activity was determined using casein as a substrate [16, 17]. Casein (200 g/l or 2% w/v) and a glycine–NaOH buffer at pH 10.0 (100 mM) were prepared and mixed using a vortex to give a final concentration of 100 g/l or 1% casein and 50 mM glycine–NaOH (pH 10.0) buffer. Then, the reaction mixture containing 500 µl of the above-prepared substrate was added to each of three clean Eppendorf tubes. Two duplicate tubes contained enzyme while the other was a blank containing no enzyme. To the Eppendorf tubes, 500 µl of a crude enzyme was added and the tubes were incubated at 50 °C for 30 min in a water bath. Then, 1000 µl of 10% trichloroacetic acid (TCA) was added to stop the reaction. Thereafter, 500 µl of crude enzyme was added to the enzyme blank. This reaction mixture was incubated at room temperature for 10 min to allow undigested casein to precipitate. After that, the reaction mixtures were centrifuged at 10,000 rpm for 5 min. Then, 1000 µl of the clear supernatant from each reaction mixture was transferred to the respective clean and labeled test tubes into which 2.5 ml of a 0.5 M sodium carbonate solution was added. After that, 500 µl of 1:10 diluted Folin–Ciocalteau's phenol reagent was added to the solution and thoroughly mixed using a vortex followed by incubation in a dark room for 25 min. Optical density was determined using a JENWAY 6300 UV/Vis at 660 nm. Enzyme activity was calculated against an enzyme blank. One unit of alkaline protease is the amount of enzyme that releases 1 µg of amino acid equivalent as tyrosine per min under standard assay conditions [17].

2.5 Box–Behnken design

From published reports, the process parameters for this research were selected as initial pH values of 8.0, 9.0, and 10.0, temperatures of 30, 37.5, and 45 °C [18], incubation times of 36, 54, 72 h, and ESM concentrations of 150g/l, 200g/l, and 250g/l (15%, 20%, and 25% (w/v)) [7] (Table 1). Then, Box–Behnken design (BBD) was applied for the design of the experiments. On the basis of the number of factors and levels required, 29 experimental runs were conducted for process optimization aided by Design-Expert, Version 7.0.0 (Stat-Ease, Minneapolis, MN). Analysis of variance (ANOVA) was used to check the adequacy of the design. The model applied to fit the results of the three-level design is shown as equation (1).

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \varepsilon \quad (1)$$

where X_1, X_2,…, X_n are the input factors that influence the response Y; n is the number of variables, b_0 is a constant, b_{ii} (i = 1, 2,…n) are quadratic coefficients, b_{ij} (i = 1, 2,…, n; j = 1, 2,…, n) are the interaction coefficients, and ε is random error. Prior to protease production, a bacterial inoculum from the seed broth was grown on nutrient agar and skim milk agar media plates incubated for 24 h to check the viability of cultures. The cultures were carried on agar slants.

2.6 Physiochemical characterization of alkaline protease

2.6.1 The influence of pH on enzyme activity and stability

The influence of pH on the activity of alkaline protease was examined by assaying the enzyme at various pH values. The buffers used were KH_2PO_4 and K_2HPO_4 (pH 6.0–7.0), Tris-HCl (pH 7.5–8.5), glycine–NaOH (pH 9.0–10), and Na_2PO_4 and NaOH (pH 10.5–12). All the buffers were used at a concentration of 50 mM using casein as a substrate. The enzyme assay procedure is as given in Section 2.4, and relative activity was calculated. The pH stability of ESM alkaline protease was examined by measuring it at pH 8.0 and 10, which is a requirement of many industrial applications. The influence of pH on the stability of the protease was determined according to the method of Gessesse et al. [17]. Crude enzyme samples were blended with various buffers and incubated at 40 °C for 1 h. Residual activity was calculated using casein in glycine–NaOH (pH 10.0) and Tris-HCl (pH 8.0) as substrates employing a

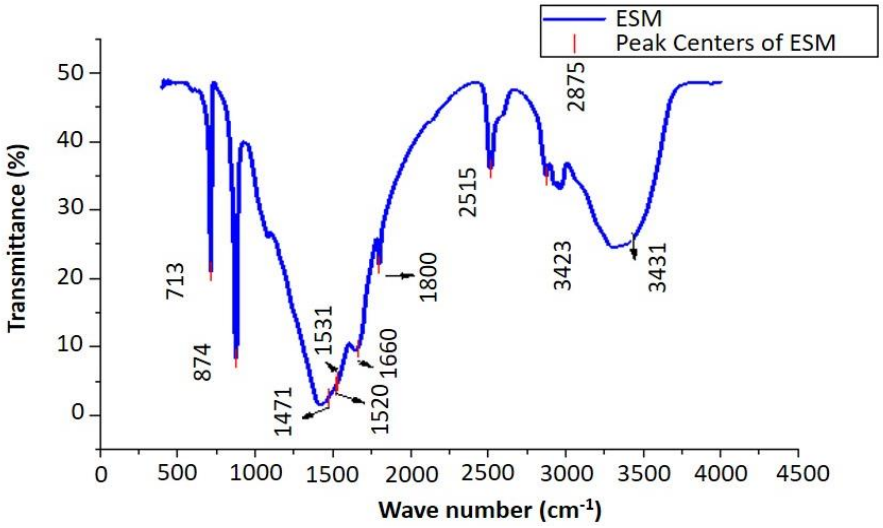

Figure 1 Fourier transform infrared (FTIR) spectrum analysis of ESM

standard protease assay procedure. The same buffers for different pH values were used and their concentrations were kept at 50 mM as those in the above discussion of enzyme activity determination. The highest enzyme activity at a particular pH was taken as reference (i.e., 100%). The percent residual activity was calculated as the ratio between the activity determined at a specified pH to its maximum observed activity.

2.6.2 Effect of temperature on the enzyme activity and stability

The temperature profile of protease activity was determined by incubating the enzyme at various temperatures from 30 to 85 °C at 5 °C intervals in a water bath using 1% casein in 50 mM Tris-HCl buffer as a substrate (pH 8.0). Then, the enzyme activity was determined following the standard protease assay procedure described above. The relative active activity was calculated to determine the optimal temperature for proteolysis. Thermal stability of the enzyme was determined according to Gessesse et al. [17]. The enzyme was appropriately diluted with a glycine-NaOH buffer (pH 10.0, 0.1 M). The effect of temperature on the stability of ESM alkaline protease was investigated by incubating the enzyme assay mixture for 1 h at 40, 45, 50, 55 and 60 °C before the assay in different Eppendorf or centrifuge tubes. The assay was done by taking five-aliquot samples at 10 min intervals. Thereafter, enzyme samples were withdrawn every 10 min and stored at 4 °C. Then, their residual activity was determined using casein as a substrate following the standard protease assay procedure above. In this case, the highest enzyme activity at a particular temperature was taken as reference (i.e., 100%).

2.6.3 The effect of sodium chloride on enzyme activity and stability

The ionic strength of the alkaline protease was determined by adding 0 to 3 M NaCl (1 ml at 0.5 M intervals) to the reaction mixture followed by the standard protease assay at pH 10.0. Relative activity was determined by taking the sample with the largest activity as 100%. The stability of the alkaline protease in various concentrations of sodium chloride was examined at 0.5 M intervals in the range of 0 to 3 M NaCl according to the modified method of Devi et al. [19]. First, the required concentration of NaCl was prepared by dissolving an appropriate amount of this salt in a 100 mM glycine-NaOH buffer (pH 10.0). Then, equal volumes of various NaCl concentrations in buffers and enzyme were mixed in clean tubes and incubated at 60 °C for 1 h. Then, the mixture was withdrawn and the standard protease assay done. The enzyme not treated with NaCl but incubated at a similar condition was taken as having 100% activity.

2.7 Study on detergent compatibility

The washing performance of the purified protease was compared with commercially available laundry detergents such as Ariel and Largo (from the Repi Detergent Factory) as per the procedure of El-Hassayeb and Abdel Aziz [20] and Rai et al. [21] with the following changes. Appropriately diluted concentrations of protease (final concentrations ranging from 25% to 75%) were mixed with a fixed concentration of Ariel powder (7 mg/ml to stimulate washing conditions) prior to mixing. The Ariel mixture was heated to eliminate the indigenous protease. Then, the wash performance of the crude protease was assessed by the blood stain removal test on cotton fabrics, as reported by Rai et al. [21], with the following changes. First, white cotton cloth was cut into 4×5 cm^2 pieces. All the pieces were stained with 1.0 ml of fresh goat blood (obtained from a slaughterhouse) and then allowed to dry at 40 °C overnight. Flasks containing these samples were kept at 25, 37, and 45 °C for 10 min followed by removal of the cloth pieces, and the leftover washes were used to assess the blood quantity (hemoglobin) removed.

3. Results and discussion

3.1 Characterization of ESM

The moisture content, ash value, and volatile matter were examined and their compositions were found to be 0.99%, 45.87%, 2.6%, respectively. The fixed-carbon content of eggshell was calculated by subtracting the percentages of moisture, volatile matter, and ash from sample (Figure 1) and it was found to be 50.54%. Moisture has a negative effect on the environment, causing odor. It is favorable to propagate microorganisms due to its ash content, and fixed carbon content, minerals (Ca, Mg, K) and trace minerals that support grown.

From the FTIR analysis, it can be seen that a marked peak ascribed to carbonate CO_3^{2-} at 874 and 875 cm^{-1} appears in both spectra (Figure 1). Hariharan et al. [22] reported a similar absorption peak at 875 cm^{-1} for egg shell and membrane powder. The appearance of these bands confirms the presence of carbonate. Spectra of elements such as Mg and Fe had a transmittance value between the wavenumbers of 1052 and 1520. The FTIR result showed the presence of minerals that can fulfill the nutritional requirement of *B. mojavensis*. Therefore, ESM can be used as a substrate as well as an inducer for the production of AlkP due to the presence of Ca, carbonates and trace minerals.

Table 2 Experimental runs from BBD using AlkP production as a response

Run	Std	Factor 1	Factor 2	Factor 3	Factor 4	AlkP Production	
		A: pH	B: Temperature (°C)	C: ESM % (w/v)	D: Incubation Time (h)	Experimental result	Predicted result
1	25	9	37.5	20	54	211.097	210.50
2	1	8	30	20	54	108.038	105.41
3	24	9	45	20	72	73.3611	75.78
4	16	9	45	25	54	86.4903	84.33
5	27	9	37.5	20	54	214.103	210.50
6	6	9	37.5	25	36	133.716	135.97
7	20	10	37.5	25	54	112.555	115.21
8	9	8	37.5	20	36	115.651	118.32
9	23	9	30	20	72	92.4903	95.47
10	12	10	37.5	20	72	131.103	128.29
11	3	8	45	20	54	85.635	83.19
12	26	9	37.5	20	54	209.103	210.50
13	28	9	37.5	20	54	211.103	210.50
14	15	9	30	25	54	102.329	99.81
15	21	9	30	20	36	107.522	105.29
16	13	9	30	15	54	114.651	116.67
17	18	10	37.5	15	54	137.555	135.29
18	4	10	45	20	54	89.7805	92.36
19	2	10	30	20	54	120.281	112.67
20	5	9	37.5	15	36	74.6192	77.25
21	7	9	37.5	15	72	141.103	138.49
22	17	8	37.5	15	54	110.522	108.06
23	14	9	45	15	54	77.2321	79.61
24	11	8	37.5	20	72	87.635	90.04
25	22	9	45	20	36	75.2321	72.45
26	8	9	37.5	20	72	70.6192	67.93
27	10	10	37.5	20	36	109.038	106.50
28	29	9	37.5	20	54	207.103	210.58
29	19	8	37.5	20	54	113.555	116.01

3.2 Statistical analysis of the experimental results

The data obtained and listed in Table 2 were analyzed using Design-Expert, Version 7.0, to assess the effects of pH, temperature, ESM concentration, and incubation time. The dependent variable used as a response parameter was the production of AlkP. All experiments were conducted in a randomized manner to reduce the effect of unexpected variability in the observed response due to extraneous factors.

3.3 Development of a model equation and model adequacy check

The relationship between controllable experimental factors (pH, temperature, ESM, incubation time) was assessed using ANOVA, while the response (AlkP production) and the interactions of the dependent variables were established. They are given by the quadratic regression model (Equation 2) in the form of coded factors.

$$\begin{aligned}\text{AlkP Activity} = {} & +210.50 + 6.61 \times \text{A} - 13.13 \times \text{B} - 3.03 \\ & \times \text{C} - 2.02 \times \text{A} \times \text{B} - 7.01 \times \text{A} \times \text{C} \\ & + 12.52 \times \text{A} \times \text{D} + 5.40 \times \text{B} \times \text{C} + 3.29 \\ & \times \text{B} \times \text{D} + 32.4 \times \text{C} \times \text{D}\end{aligned} \quad (2)$$

where A is the pH, B is the temperature, C is the concentration of ESM, and D is the incubation time.

The fitness of the model was checked using *p*-values, F-values and regression coefficients (Table 3). The model F-value, 328.54, indicates that the model is significant. There is only a 0.01% chance that a model F-value this large could occur due to noise. Values of Prob > F less than 0.0500 suggest that model terms are significant. The contribution of each source terms for the model equation can be seen in Table 3.

Clearly, the model is significant as it has a low *p*-value, 0.0001, and a high R^2 value, 0.9960. In general, the model is acceptable as the R^2 value showed that the model could account for 99.6% of the variation of the experiment [23]. In this case, A, B, C, AC, AD, BC, CD, A^2, B^2, C^2, D^2 are significant model terms. The *p*-value greater than 0.0001 suggest the model terms are not significant. Therefore, discounting the insignificant model terms helps in model reduction, which in turn improves model adequacy. The lack of fit F-value, 2.56, suggests the lack of fit is not significant compared to the pure error. There is an 18.82% chance that a lack of fit F-value this large could occur because of noise. Nonsignificant lack of fit is good, so then, it is significant in the quadratic model. A graph of the actual response vs. the predicted response values helps to identify values or group of values that are not easily predicted by the model (Figure 2). A 45° line should split the data points evenly. Therefore, the model can easily predict the data points. If the model is correct and the assumptions are met, the residuals will be structureless. In particular, they will be unrelated to any other variable, including the predicted response. An easy check can be done by plotting the residuals vs. the fitted (predicted) values. A plot of the residuals vs. increasing predicted response values tests the assumption of constant variance. It shows random scatter indicating that there is no need for any change to minimize error.

3.4 Interaction effects of the factors

There is a combined effect of pH and temperature on the production of alkaline protease at most of the design points of the two factors. The maximum alkaline protease activity was attained at 37 °C and pH 9.0. At a temperature of 30 °C and pH values of 8 and 10, optimal production of AlkP was observed. However, at

Table 3 Summary of the ANOVA and parameter estimates for model coefficients of BBD

Source	Sum of Squares	df	Mean Square	F-value	*p*-value Prob > F	Remark
Model	58237.48	12	4853.12	328.54	< 0.0001	Significant
A: pH	523.73	1	523.73	35.46	< 0.0001	
B: Temperature	2069.29	1	2069.29	140.09	< 0.0001	
C: ESM	110.52	1	110.52	7.48	0.0147	
D:Incubation time	31.58	1	31.58	2.14	0.1631	
AC	196.46	1	196.46	13.3	0.0022	
AD	627.03	1	627.03	42.45	< 0.0001	
BC	116.43	1	116.43	7.88	0.0126	
CD	4197.78	1	4197.78	284.18	< 0.0001	
A^2	12009.54	1	12009.54	813.01	< 0.0001	
B^2	28742.68	1	28742.68	1945.8	< 0.0001	
C^2	15465.5	1	15465.5	1046.97	< 0.0001	
D^2	20843.08	1	20843.08	1411.02	< 0.0001	
Residual	236.35	16	14.77			
Lack of fit	209.15	12	17.43	2.56	0.1882	Not significant
Pure error	27.19	4	6.8			
Cor. Total	58473.83	28				

Std = 3.84; CV = 3.16%; R^2 = 0.996; Adj-R^2 = 0.992; Pre-R^2 = 0.98; Adeq precision = 55.4

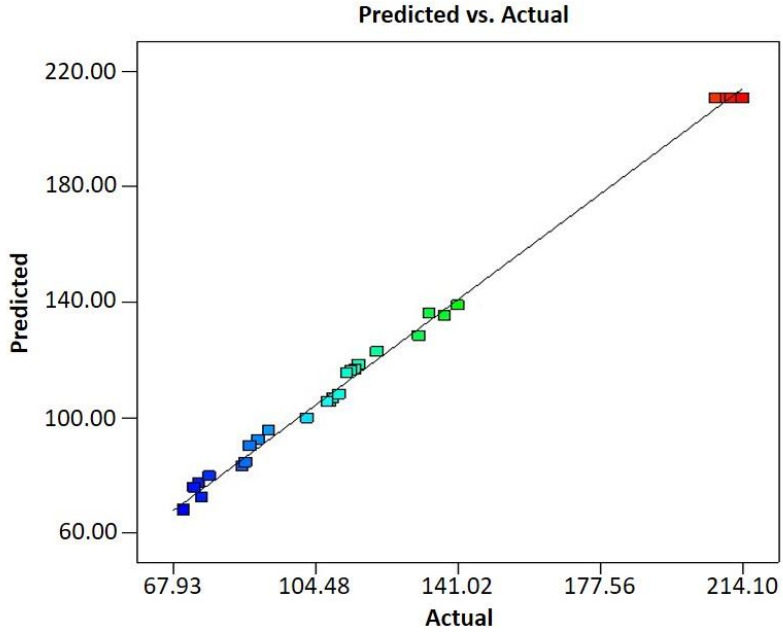

Figure 2 Predicted vs. actual values

45 °C and pH 10, AlkP production was minimal (Figure 3(a)). Figure 3(b) shows the interaction effects of ESM concentration and the medium pH. As can be seen clearly from Figure 3(b) for both factors, AlkP production is maximal at mid-level and near mid-level pH values. Incubation time and pH interaction show a significant effect on protease production. Figure 3(c) presents a concave curve, which shows that at the mid-point, the enzyme production is maximal. Maximal temperature and ESM interaction effects were observed at the mid-point of the design factor levels, whereas at the extreme point of interaction, a low level of production was observed (Figure 3(d)). As the incubation time and temperature increased, a substantial incremental increase in the protease production was achieved until their average levels, but further increases in both factors resulted in decreased protease production (Figure 3(e)). The effect of interactions on the concentration of ESM and incubation time for fermentation was positive. A significant increase in enzyme production was observed from the lower level to the mid-point for both factors (Figure 3(f)). Optimal production was attained at a 20% (200g/l) ESM concentration and 48 h incubation time. When values increased from the average or mid-point, AlkP production decreased.

3.5 Optimization of process parameters

To find the ideal solution, numerical optimization was done for all the given solutions to determine which were best optimized. The best solution for a particular goal may not be acceptable due to cost, efficiency, or practicality. Desirability ranges from 0 to 1 for any given response. The program combines individual desirability into a single number and then looks for the greatest overall desirability. A value of 1 shows an ideal case, while 0 indicates a solution that falls outside of desirable limits. The numerically optimized desirability was 0.9819, which is near the ideal case and indicates that the responses fall in the desirable limit. The optimum point was at 36.74 °C, pH 9.08, ESM 19.78% (197.8 g/l), and an incubation time of 48 h (Table 4). Hence, the maximum production or activity was attained at order 27 or Run 5 (at the middle level of the factor levels). The points are closer to the maximum production parameters, but with some optimized parameters. Optimization of fermentation process parameters was done to establish favorable conditions for growing the microorganism and thereby minimize unutilized media components at the end of fermentation. No particular medium has been defined for the maximal alkaline protease production from

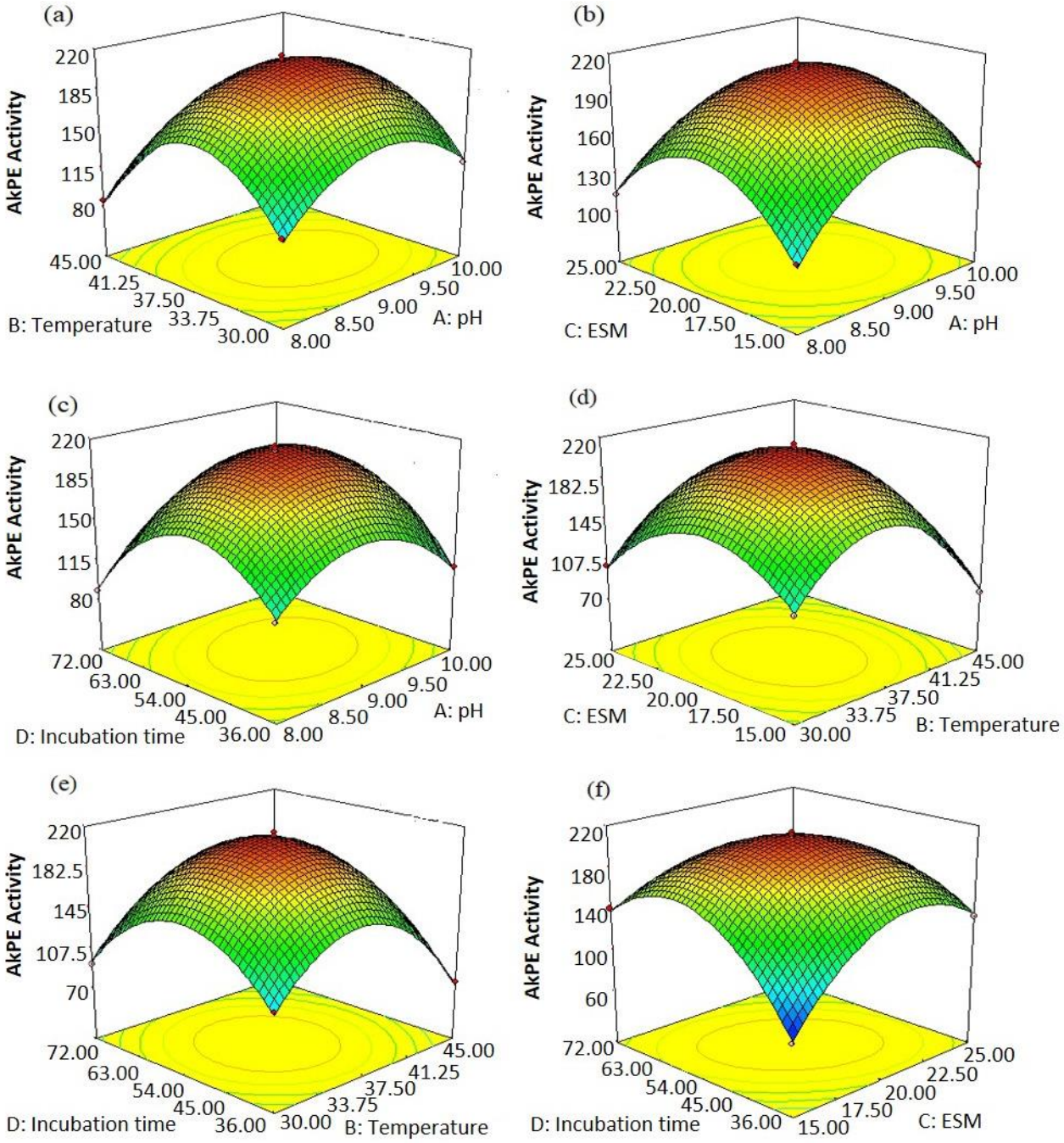

Figure 3 The interactive effects on protease production, (a) temperature vs. pH, (b) ESM concentration vs. pH, (c) incubation time vs. pH, (d) ESM concentration vs. temperature, (e) incubation time vs. temperature, (f) incubation time vs. ESM concentration

Table 4 Optimal point with its desirability for combined effects

Number	pH	Temperature	ESM	Incubation Time	AlkP Activity	Desirability
1	9.08	36.74	19.78	48 h	211.5076	0.982

various microbial sources, including *B. mojavensis*. Each organism or strain has its own particular requirements for maximal enzyme production [24, 25]. Using ESM as a substrate induces the production of AlkP to a greater degree than with no ESM [11]. Maximal alkaline protease activity with no ESM (0% ESM) was 136.37 U/ml, while with ESM, it was 214 U/ml. Using ESM as a substrate led to higher enzyme production.

3.6 Validation of the model

The model fit is accurate and reliable for predicting the alkaline protease production using ESM while varying operating parameters. On the basis of the second-order models, numerical optimization was done to maximize the AlkP production using Design-Expert, Version 7.0. The optimal values of the parameters are shown in Table 4. They include a pH of 9.08, a temperature of 36.74 ºC, concentration of ESM 19.78%, and an incubation time of 48 h. The analysis shows 211.51 U/ml of AlkP was produced with a diserability of 0.9819, which shows strong model performance.

3.7 Physicochemical characterization of crude alkaline protease

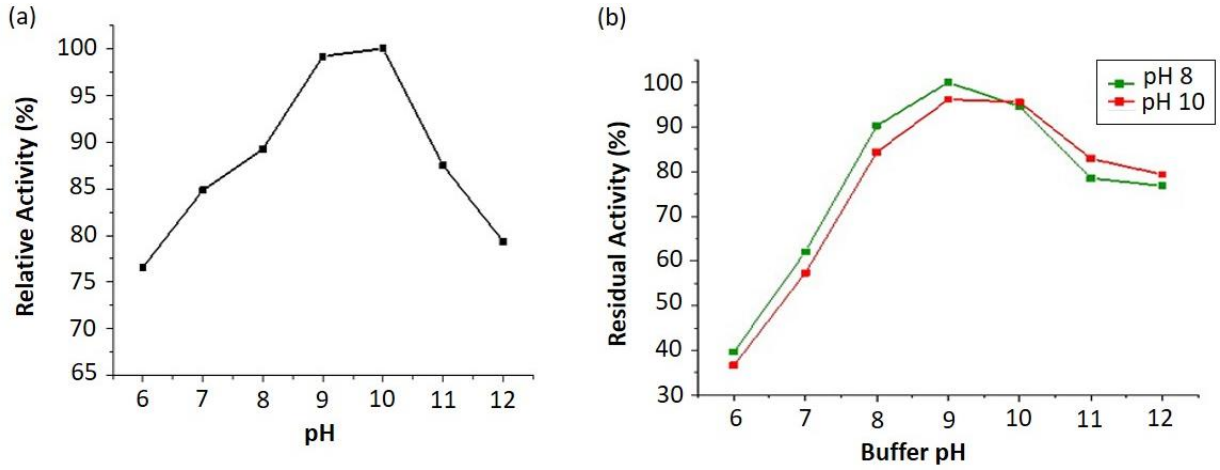

Figure 4 pH profile with relative activity (a), and pH stability profile (b) of ESM alkaline protease

3.7.1 Effect of pH on activity and stability

AlkP was active over a broad pH range of 6.0–12.0 (Figure 4). Additionally, it showed 87.495% retained activity in this pH range. Interestingly, 93.97% of its original activity was maintained in buffers at pH values ranging from 8 to 10. Optimal activity was found at pH 10, as indicated by the peak in Figure 4. The activity of AlkP was found to progressively increase with pH in the range from 6.0 to 10.0. However, above pH 10.0, enzymatic activity dropped sharply. AlkP showed 89.24%, 99.16%, and 87.49% maximal activity at pH 8.0, 9.0, and 11.0, respectively. Minimal relative activity was observed at pH 6.0 (76.57%) and pH 12.0 (79.37%). When the assay reaction mixture was varied, it showed an increase from pH 6 to 8. Then enzyme activity became stable. Maximal enzyme activity was observed between pH values of 8 and 10. Within this stability zone, more than 94% of the residual activity was retained. Above pH 10.0, activity slightly decreased. At pH 12, it fell to 80%. Overall activity was more than 80%. The original retained activities at pH values of 8, 9, and 10 were 86%, 100%, and 99%, respectively. When the assay mixture was at pH 8, the stability profile showed an increase from pH 6 to 8. A stability zone existed from pH 8–10 with more than 95% retained residual activity. Above pH 10.0, activity started to decrease and fell to 76%. Its minimal residual activity, 39.6% of its original activity, was observed at pH 6.0. As can be seen in the stability profile, AlkP in both assay buffers is stable between pH 8 and 10. Most industrial processes work at alkaline pH values, pH 8 to 10, and this AlkP fits these requirements. The pH effect on activity and stability showed that more than 93.97% of its original activity was retained using buffers with pH values ranging from 8 to 10, with the optimal activity at pH 10.0. AlkP was active over a broad pH range, 6.0–12.0, with 87.495% of its activity retained. More than 93.97% of its original activity was retained using buffers with pH values ranging from 8 to 10, with the optimal activity at pH 10.0, as indicated by the peak in Figure 4. This result is in agreement with the studies of Gessesse et al. [17] and Kumar et al. [26]. The activity of AlkP is found to progressively increase with the pH from 6.0 to 10.0. However, above pH 10.0, it drops sharply. AlkP showed 89.24%, 99.16%, and 87.49% of its maximal activity at pH 8.0, 9.0, and 10.0, respectively. Minimum relative activity was recorded at pH 6.0 (76.57%) and 12.0 (79.37%). Most industrial processes are alkaline with a pH range between 8 and 10. These results indicate that the AlkP of the current study can be used in detergents as well as industrial processes, but will not function in an acidic environment. The assay reaction mixture shows incremental activity increases from pH 6 to 8. Then it is stable. Maximal enzyme activity is obtained between pH 8 and 10. Within the stability zone, more than 94% of residual activity is retained. Above pH 10.0, it slightly decreases. This result is similar to that reported by Vadlamani et al. [27]. As shown in Figure 4(b), the stability profile, AlkP is stable between pH 8 and 10 in both assay buffers. According to Ramnani et al., this enzyme was active over a wide pH range (7–12.0) and optimal at pH 10 [28]. The enzyme used in this study was found to have a similar pH stability to commercially important detergent enzymes [27, 29]. As shown in Figure 4, AlkP in both assay buffers shows stability between pH 8 and 10. A similar report showed the highest enzymatic activity over a pH range of 8.0-10.0 [30].

3.7.2 Effect of temperature on activity and stability

With the standard protease assay protocol, the relative activity of the acquired protease was determined over the temperature range of 30–70 °C. ESM alkaline protease was found to be very active at all tested temperatures between 30 and 80 °C, as shown in Figure 5. Maximal activity was observed at 60 °C (Figure 5). The protease activity progressively increased with temperature from 30 to 60 °C and then gradually declined above the optimal temperature. Within the temperature range of 40–70 °C, ESM alkaline protease retained more than 74% of its maximal activity. A stability investigation of the protease with respect to temperature was done at 40, 45, 50, 55, and 60 °C. The thermal stability of ESM alkaline protease was tested at pH 10.0. Thermal stability profiles for relative activity and residual activity are shown in Figure 5(b). The enzyme showed maximal thermal stability at 10–40 min with 96% and 97% residual activity when assayed at 40 and 45 °C, respectively, after 1 h of incubation. Relatively higher thermal stability of the ESM alkaline protease was obtained at temperatures of 40 and 50 °C, 99% and 98% of residual activity, respectively (Figure 5(b)). The ESM alkaline protease was found to be very active at all temperatures tested between 30 and 80 °C, with maximal activity at 60 °C. The enzyme produced from *Bacillus* species showed maximal activity (100%) at 50 °C [13]. Alkaline protease isolated from *Bacillus* showed the highest enzyme activity of 126.77 ± 1.73 U/ml at 55 °C [14]. This protease showed a maximal thermal stability at 10-40 min, 96% and 97% residual activity at 40 and 45 °C, respectively, after 1 h of incubation. After a 40-min incubation at assay temperatures of 40, 45, 50, 55, and 60 °C, the residual thermal stability sharply dropped by 86%, 85%, 65%, 49% and 39%. Above 50 °C, it was inactivated after a 1 h incubation. Similarly, Gessesse et al. [17] reported that incubating at higher temperatures for more than 1 h denatures the enzyme. Maximal activity was attained at pH 10 and 55–60 °C [14]. Relatively higher thermal stability of ESM alkaline protease was obtained at 40 and 50 °C, 99% and 98% of residual activity, respectively. A similar result was reported by Vadlamani et al.

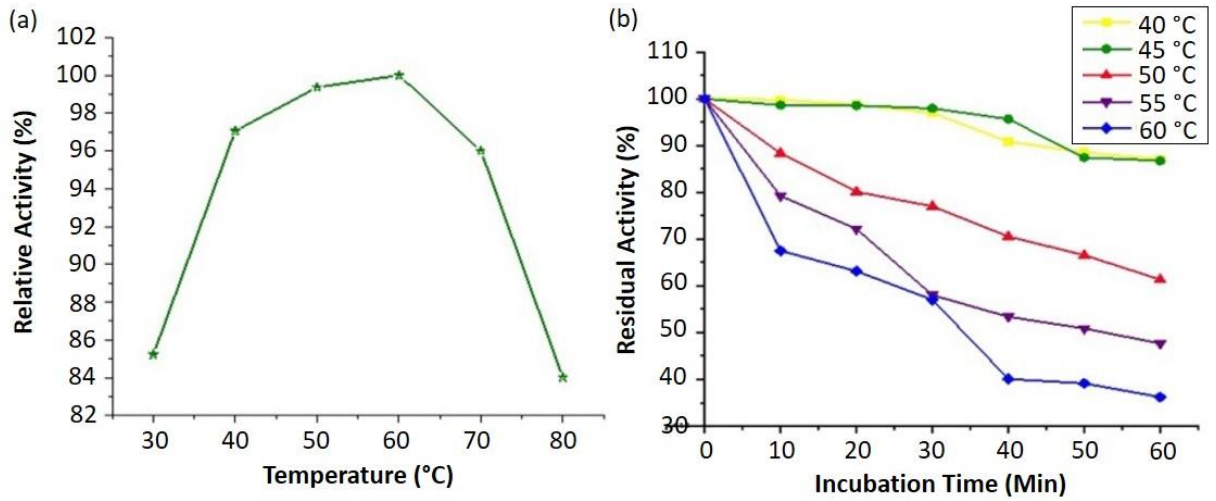

Figure 5 Temperature profile (a), and thermal stability profile (b) of ESM alkaline protease

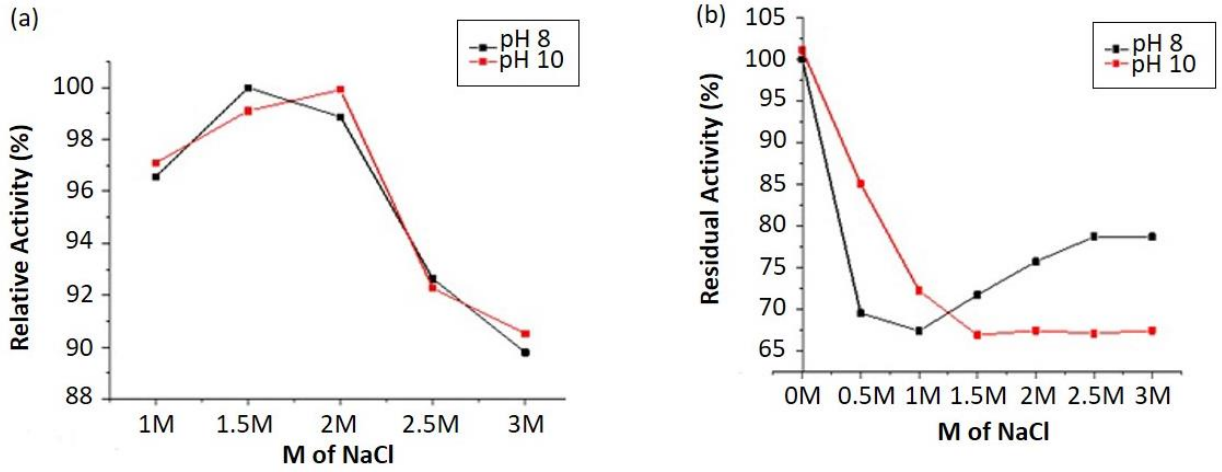

Figure 6 The effect of NaCl concentration on enzyme activity (a) and stability (b)

[27]. Some reports of crude protease showed substantial activity over the temperature range of 40˚C to 65˚C, with complete inactivation at 80˚C [6, 12, 21, 31].

3.7.3 Effect of sodium chloride on activity and stability

The performance of the enzyme was investigated at various sodium chloride concentrations. Maximal alkaline protease activity was obtained at 1.5 and 2 M NaCl at pH 8.0 (98.87%) and 10.0 (99.25%). Between NaCl concentrations of 1 and 2.0 M, the enzyme showed more than 97% optimal activity (at pH 8 and 10). Even at the highest concentration of NaCl, 3 M, ESM alkaline protease showed 89% and 90% of the relative optimum activity (at pH 8 and 10). Yet over both tested pH ranges, increases in the NaCl concentration beyond 1.5 M at pH 8 or 2 M at pH 10 led to a reduction in activity. Additionally, at all studied NaCl concentrations, comparatively higher relative activities were observed. At each tested NaCl concentration, pH 10 stability sharply decreased between 0.5 and 1.0 M, with 70.9% residual activity retained. However, for pH 8, the enzyme was comparably stable in 0.5 and 1.0 M NaCl concentrations with a residual activity of 68.5%. As shown in Figure 6(b), when the NaCl concentration was increased from 1 to 3 M with a 1 h incubation at pH 10, the residual activity decreased and attained a minimal value of 56%, whereas the optimum stability was attained between in a NaCl concentration range of 1 and 3.0 M. At pH 8, the residual activity increased with the NaCl concentration. It was observed that the residual activity values at 1.0, 1.5, 2, 2.5, and 3.0 M were 67.4%, 71.7%, 75.7%, 78.7%, and 78.7%, respectively. From Figure 6(b), it can be concluded that ESM alkaline protease is more stable over a NaCl concentration range of 1.0–3.0 M at pH 10. The results agreed with the observations of Marathe et al. [14]. At pH 8, despite an increase in residual activity between 1.0 and 3.0 M, enzyme activity was not stable because of fluctuations. A relatively higher residual activity was observed at pH 10.0 than 8.0. At 1–2 M NaCl, an optimum activity of > 97% was recorded at pH values 8 and 10. This result is in agreement with Vadlamani et al. [27] who showed that between NaCl concentrations 0 and 3 M, i.e., 2 M, maximal activity was achieved. Optimal stability was attained at concentrations between 1 and 3.0 M NaCl. At pH 8, the residual activities increased as the NaCl concentration was increased from 1.0, 1.5, 2, 2.5, and 3 M. The salt tolerance of *B. mojavensis* protease was examined at levels greater than 2 M NaCl and its relative activity was found to decrease. This result is similar to that reported by Vadlamani et al. [27]. Thus, maximal activity was found at pH 8 with 1.5 M NaCl and at pH 10 with 2 M NaCl. From this, it is possible conclude that the stability profile and proteolytic efficiency of an assay mixture differs at pH 8 and 10. Alkaline protease from *Bacillus* showed a tolerance to NaCl [14, 32].

3.8 Investigation of blood stain removal

The stain removal potential of the enzyme was determined. Various concentrations of the enzyme were used to treat bloodstains. A remarkable degradation of blood is clearly observed in the images of Figure 7. This alkaline protease has

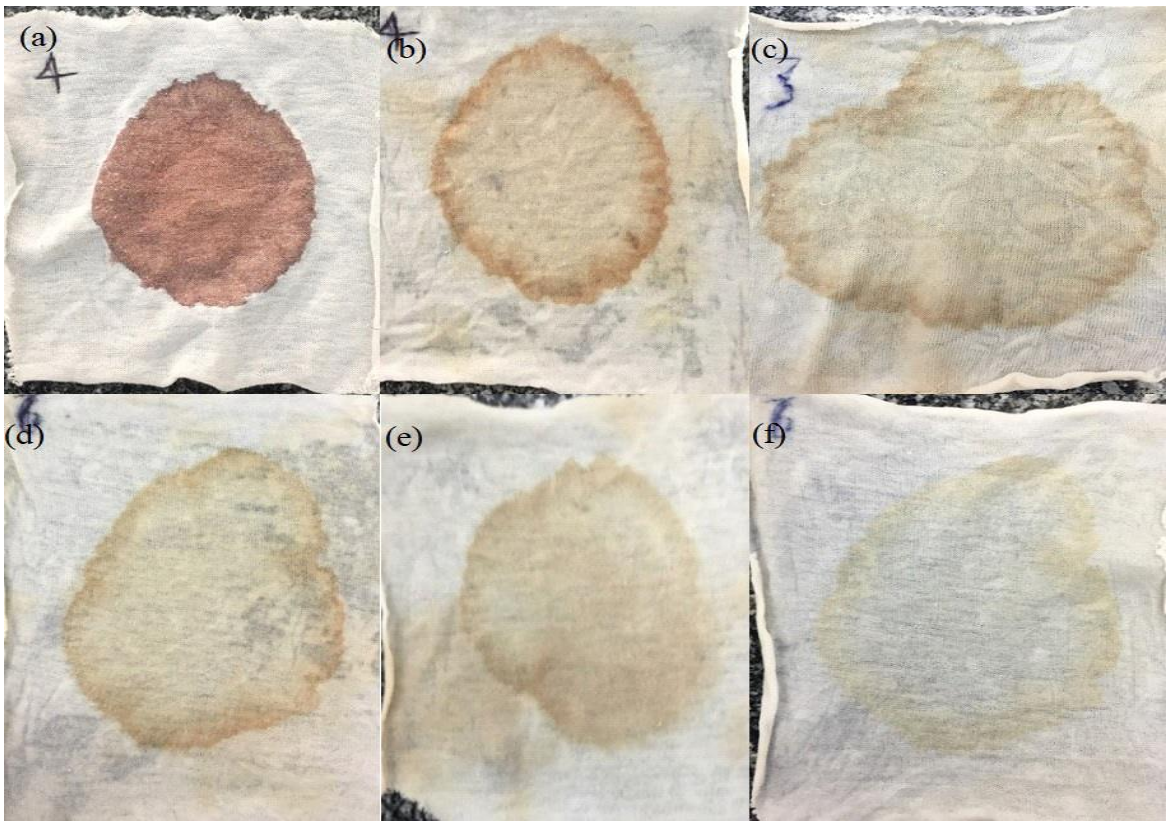

Figure 7 Bloodstain removal: (a) bloodstain, treated with (b) tap water, (c) 100% detergent, (d) detergent (25% enzyme), (e) detergent (50% enzyme), and (f) detergent (75% enzyme)

potential applications in detergents for bloodstain removal. The Ariel detergent shows a higher enzyme activity for ESM alkaline protease and it is effective in removing blood stains. These results are in agreement with the observation of Haddar et al. [33]. A previous study by Biosci et al. [32] showed the effectiveness of alkaline protease produced by *Bacillus* species on blood stain removal. Hence, the present study reports increased alkaline protease production by ESM under optimized cultural conditions for applications as a textile detergent, with a promising role in the leather industry.

4. Conclusions

Media formulated using eggshell waste for the production of alkaline proteases are cost-effective. The acquired enzyme is highly active at an optimum temperature of 60 °C with between 10 and 40 min of incubation. It retains more than 97% of its original activity at pH 11. It is also active over broad pH and temperature ranges. AlkP in both assay buffers shows stability between pH values of 8 and 10, where most of the industrial alkaline processes work. Thus, the AlkP enzyme produced in the current study fits these requirements. The enzyme is highly effective for stain removal and is stable in the presence of detergents. These properties infer its suitability as an additive in detergent formulations. This enzyme could have great potential in the leather industry to remove hair with an environmentally friendly approach. However, further experimentation is required.

5. References

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