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Full Length Research Paper

Hydrolysis of proteinaceous tannery solid waste for the production of extracellular acidic protease by *Selenomonas ruminantium*

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The objective of this study was to produce protease from *Selenomonas ruminantium* using animal fleshing (ANFL), an untanned tannery solid waste as the sole protein source. Optimization of the minimal medium composition for the production of protease was carried out by a statistical approach using response surface methodology (RSM) which includes the variables such as NH_4Cl, K_2HPO_4, KH_2PO_4 and NaCl. The isolate was found to produce maximum protease at pH 6 and at a temperature of about 40°C. Protease was purified 56 fold with a total yield of 28.14%. The enzyme was found to be monomeric having a molecular weight around 53 kDa. The purified enzyme was stable at a pH of about 4 revealing its acid protease nature and was also found to be stable up to 40°C. The enzyme was activated by divalent cations like Ca^{2+} and Mg^{2+} and inhibited by dithiothreitol (DTT), where the latter suggested its cysteine protease nature. The enzyme had good stability in the presence of non-ionic surfactants like tween 20, tween 40, tween 80 and triton X100 and also in the presence of solvents like methanol, ethanol and isopropanol. These characteristics reveal the potential of the enzyme for different industrial applications.

Key words: Acid protease, animal fleshing, optimization, response surface methodology (RSM), *Selenomonas ruminantium*.

INTRODUCTION

The leather manufacturing industries generate huge quantities of by-products and solid wastes far exceeding the quantum of finished leather (Maire and Lipsett, 1980). In India, approximately 150,000 tons of offal comprising raw hide trimmings, limed animal fleshing, green animal fleshing, hide splits and chrome shavings are available which are either utilized or underutilized, thus posing a solid waste disposal problem in tanneries (Rao et al., 1994). Animal fleshing (ANFL) is one of the major solid wastes that are generated during pretanning operations of leather processing. It contains the highest protein content (50 to 60%), which is currently being wasted (Ravindran et al., 2008; Ganesh Kumar et al., 2008). ANFL discharged from the tannery industries are processed by chemical or thermal process, which is an energy intensive and time consuming process (Bajza and Markovic, 1999). An alternative ecofriendly method is enzyme production from solid wastes through anaerobic fermentation. This process can be carried out at low chemical and energy input. Furthermore, the enzyme obtained can be used as suitable biocatalyst for industrial and laboratory purposes (Bajza and Vrcek, 2001). Hydrolytic enzymes like proteases and lipases represent the class of enzymes, which occupy a pivotal position with respect to their physiological roles as well as their commercial applications. Proteases constitute at least

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Abbreviations: ANFL, Animal fleshing; **RSM,** response surface methodology; **DTT,** dithiothreitol.

Table 1. Experimental Range of Variables for the Central Composite Design.

Variable	Coded symbol	Range of variable (g/l)		
		Low (-1)	Mid (0)	High (+1)
NH_4Cl	A	0.25	0.5	0.75
K_2HPO_4	B	0.5	0.75	1
KH_2PO_4	C	0.25	0.5	0.75
NaCl	D	0.5	1	1.5

65% of the total industrial enzyme market (Rao et al., 1998). They are used for various industrial applications, such as laundry detergents, leather preparation, protein recovery or solubilization and organic synthesis (Anwar and Saleemuddin, 1998). Also, proteolytic enzymes have many physiological functions, ranging from generalized protein digestion to more specific regulated processes (Kaminishi et al., 1994). The proteinaceous solid waste of ANFL was hydrolysed in anaerobic process through enzymes by *Selenomonas ruminantium* (Ravindran and Sekaran, 2010). Acid proteases find immense applications in pharmaceutical industry (Sjodahl et al., 2002). Attempts have been made to produce protease enzyme, using various substrates such as “shrimp” and “crab” shell powder (Spelzini et al., 2005), “soybean” meal (Wang et al., 2005), “nug” meal (Joo and chang, 2005), “pigeon pea” wastes (Gessesse, 1997), “feather” meal (Johnvesly et al., 2002) and “fish” wastes (Roberta et al., 2006) for the production of protease. However, reports on the production of protease using ANFL as the substrate are very scanty and the reports were observed in the production of proteases from *Pseudomonas* (Ganesh Kumar et al., 2008a) and *Synergistes* (Ganesh Kumar et al., 2008b) and fermentation preservation (Bhaskar et al., 2007) and acidogenic fermentation using *Clostridium* sp. (Ganesh Kumar et al., 2008c). It is a well-known fact that the extracellular protease production in microorganisms is greatly influenced by media components (Sumant Puri et al., 2002). 30 to 40% of the production cost of industrial enzymes is estimated to be the cost of the growth medium (Han-Seung et al., 2002). To develop a bioprocess for industrial purpose, it is important to optimize highly significant factors affecting this process. The ‘one-at-a-time strategy’ of improving fermentation conditions is the most frequently used operation in biotechnology to obtain maximum cell density; high yields of the desired metabolic product, or enzyme levels in the microbial system. This approach is not only time consuming, but also ignores the combined interactions between physicochemical parameters (Haaland, 1989). On the contrary, the RSM, which includes factorial design and regression analysis, helps in evaluating the effective factors and building models to study interaction and select optimum conditions of variables for a desirable response (Haaland, 1989; De Coninck et al., 2000). The present study deals with the production, purification and characterization of the acid cysteine protease produced using ANFL as a sole carbon source by the isolated strain *S. ruminantium* under anaerobic fermentation process.

MATERIALS AND METHODS

Substrate preparation

Limed ANFL, a predominant solid waste emanated from the tanneries during leather manufacture was collected from Central Leather Research Institute, Chennai and was treated with ammonia solution (25% v/v) for 3 to 4 h to remove the adhered calcium salts on ANFL. The pH of the delimed fleshing was then adjusted to 7.0±0.2 by suspending it in water. The fleshing was further washed in running water. The ANFL particles of 0.25 cm were stored at 4°C until the start of the experiments.

Organisms and culture conditions

Stock cultures of *S. ruminantium* HM000123 cultured in anaerobic basal broth served as starter cultures (Ravindran and Sekaran, 2010). The medium was adjusted to pH 6 and N_2 gas was flushed into the medium three times applying the vacuum alternately prior to being autoclaved.

Optimization of protease production by response surface methodology

The response surface statistical design was used to optimize the cost effective minimal medium and evaluate the main effects, interaction effects and quadratic effects of the variables namely ammonium chloride (A), dipotassium hydrogen orthophosphate (B), potassium dihydrogen orthophosphate (C) and sodium chloride (D) which were selected based on the results obtained by the classical method. Also, ANFL (1 g/100ml of minimal medium) was used as a constant factor, which serves as the substrate for protease production by the isolated strain. A five-level four-factorial central composite rotatable design (CCRD) was used for exploring response surfaces and constructing second order polynomial models with an α value of ± 1.414. The levels of the four independent and dependent variables for the CCRD design-generated experimental runs used in the present study are given in Table 1. The relationship between the variation of the response, Y_c (protease activity, U/ml) and the variation of factors A to D are represented by a second-order mathematical model using the equation:

$$Y_c = \beta_o+\beta_1X_1+\beta_2X_2+ \beta_3X_3+ \beta_4X_4 \text{ (intercept and main effects)}$$
$$+ \beta_{11}X_1^2+\beta_{22}X_2^2+ \beta_{33}X_3^2+ \beta_{44}X_4^2 \text{ (interactions)}$$
$$+ \beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{14}X_1X_4+ \beta_{23}X_2X_3+ \beta_{24}X_2X_4+ \beta_{34}X_3X_4 \text{ (Quadratic effects)}$$

Where, Y_c is the measured response associated with each factor level combination; β_o is the intercept; β_1 to β_{34} are the regression

coefficients which represented the linear quadratic and interactive effects of X1, X2, X3, and X4 factors on the response, respectively; and X1, X2, X3 and X4 are the independent variables corresponding to the factors A, B, C and D. The treatment combinations of CCRD were allocated in three blocks, and each block had 10 runs. The first two blocks each had eight factorial points and two center points. The last block had eight axial points and two center points. Thus, in total, the experimental setup consisted of 30 trials, and the value of the dependent response was the mean of triplicates. Statistical analysis of the model was performed using the 'Design Expert' software package (Version 6.0.11, Stat-Ease Inc., Minneapolis, USA).

Effect of pH and temperature on protease production

The effect of pH on protease production from *S. ruminantium* under study was determined by inoculating the organism in the fermentation minimal media optimized from RSM of different pH (3 to 8). The optimum pH was concluded by albuminolytic activity in the pH range of 3 to 6 and caseinolytic activity in the pH range of 7 to 8. Similarly, the effect of temperature on protease production was studied by inoculating the organism in optimized fermentation minimal media (pH-6) set at different temperatures (20 to 60°C).

Experimental design for culture conditions

The anaerobic batch fermentation experiments were conducted in 100 ml minimal medium with a composition of (g/l) NaCl, 0.5; NH_4Cl, 0.25; K_2HPO_4, 1.0; and KH_2PO_4, 0.75 which was optimized from response surface methodology with 1 g ANFL (size of 0.25 cm) as substrate. The trace element solution of 1 ml, containing composition of (g/l) $MgSO_4$ (0.49); $FeSO_4$ (0.055); $CoCl_2$ (0.028); $MnCl_2$ (0.019); $CaCl_2$ (0.147); and $NH_4Mo_7O_{24}$ (0.123) was added. All chemicals for the medium and different solutions used in this work were from Merck's company (Merck Specialities Pvt. Ltd, Mumbai, Maharashtra, India). The media was autoclaved at 120°C at 15 psi for 15 min and prior to being autoclaved the pH was adjusted to 6 and nitrogen gas was flushed into the medium as described before. Fermentations were carried out by seeding inoculums (5% v/v) and incubation (without agitation) at 37°C. All experiments were carried out in triplicates.

Determination of protease activity

Enzyme preparations were routinely assayed by incubation with (1%) bovine serum albumin in sodium acetate buffer (50 mM, pH 4.0) at 37°C for 10 min. The reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid and the mixture was kept at room temperature for 10 min followed by filtration through Whatman filter paper No. 1. The absorbance of the filtrate was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute under optimized conditions (Germaine et al., 1978).

Enzyme purification

The cell free culture supernatant was precipitated using solid ammonium sulphate from 50 to 80% of saturation. The resulting precipitate was centrifuged at 6,000 x g for 20 min and the pellet was dissolved in a minimum volume of 100 mM citrate buffer, pH 4 and dialyzed against repeated changes of the same buffer 24 h, at 4°C. The dialyzed enzyme was then further purified by ion exchange chromatography (DEAE cellulose) followed by gel filtration chromatography (Sephadex G100 column). The protease fractions were collected, assayed for protein content, enzymatic activity and lyophilized.

SDS-polyacrylamide gel electrophoresis and zymographic assay

SDS-PAGE was carried out for the determination of molecular mass and purity of the protease in a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate at 4ºC according to the method of Laemmli (1970), after heating the samples at 75°C for 4 min. A ready to use molecular marker was used as a standard. Zymographic analysis was performed in polyacrylamide gel slabs containing SDS and gelatin (0.1%) as co-polymerized substrate. Following SDS-PAGE and zymogram, the patterns of proteins were stained with Coomassie brilliant blue (CBB) R-250 (0.1% w/v) in methanol, acetic acid and water with a ratio of 4:1:5, respectively. The above solution without CBB R-250 was used as destaining solution.

Amino acid analysis

The protein was hydrolyzed with 6N HCl for 24 h at 110°C in an evacuated sealed tube. The hydrolysate was filtered through Whatman No.1 filter paper and the filtrate was evaporated to dryness in a flash evaporator and the amino acid composition was analyzed on model C18 column in Agilent model 1100 HPLC analyzer (Ganesh Kumar et al., 2008b).

Characterization of purified protease

Effect of pH and temperature on protease activity and stability

The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures from 20 to 60°C. The thermostability of the enzyme was measured after pre-incubation of the enzyme for 15 and 30 min at various temperatures. The effect of pH on protease activity was determined by incubating the reaction mixture at pH values ranging from 3.0 to 8.0, in the following buffer systems: 100 mM Citrate buffer (pH 3.0 to 6.0) and 100 mM Phosphate buffer (pH 7.0 to 8.0). To check the pH stability, the enzyme solution was mixed with equal amount of each buffer solution and after incubation for 1 h at 40°C; protease activity was measured under standard assay conditions.

Effect of protease inhibitors and metal ions on activity of the purified protease

The effect of inhibitors on the protease activity was examined after the protease had been pre-incubated with inhibitor for 30 min at room temperature, and the residual activity was determined by standard assay method. For this assay, 1 and 5 mM concentrations of inhibitors such as phenyl methyl sulphonyl fluoride (PMSF), ethylene diamine tetra acetic acid (EDTA), dithiothreitol (DTT) and pepstatin A were used. All chemicals for the medium and different solutions used in this work were from HiMedia company (HiMedia Laboratories Pvt. Ltd, Mumbai, Maharashtra, India). For the determination of the influence of metal ions, the same procedure was used for the metal ions (1 and 5 mM) of $MgCl_2$, $MnSO_4$, $CaCl_2$, $CuSO_4$ and $ZnSO_4$. The level of effect of metal ions on the purified enzyme was expressed as a percentage of the relative activity.

Effect of surfactants and solvents on protease activity

The enzyme was incubated with 1% concentration of non-ionic

Table 2. Central composite rotatable design (CCRD) matrix of independent variables and the corresponding experimental and predicted values of protease production by *Selenomonas ruminantium*.

Run	Medium components (g/l)				Protease activity (U/ml)	
	NH_4Cl	K_2HPO_4	KH_2PO_4	NaCl	Experimental	Predicted
1	0.25	1	0.75	1.5	377.23	385.661
2	0.75	1	0.25	1.5	246.46	258.696
3	0.25	0.5	0.75	0.5	282.93	271.846
4	0.75	1	0.75	0.5	281.56	300.95
5	0.5	0.75	0.5	1	341.23	328.415
6	0.25	0.5	0.25	1.5	324.92	306.682
7	0.5	0.75	0.5	1	307.23	328.415
8	0.25	1	0.25	0.5	302.76	315.737
9	0.75	0.5	0.25	0.5	270.46	263.181
10	0.75	0.5	0.75	1.5	379.69	367.865
11	0.5	0.75	0.5	1	341.53	328.201
12	0.75	1	0.25	0.5	238.46	241.933
13	0.25	0.5	0.25	0.5	226.76	251.524
14	0.5	0.75	0.5	1	317	328.201
15	0.25	1	0.25	1.5	302.3	297.269
16	0.75	0.5	0.25	1.5	337	355.354
17	0.25	0.5	0.75	1.5	340.3	337.359
18	0.25	1	0.75	0.5	413.38	395.558
19	0.75	1	0.75	1.5	352.3	328.068
20	0.75	0.5	0.75	0.5	258.92	264.483
21	0.5	1.10355	0.5	1	322.61	316.646
22	0.85355	0.75	0.5	1	302.46	292.07
23	0.5	0.75	0.5	1	334.3	325.777
24	0.5	0.75	0.5	0.29289	340.46	319.957
25	0.5	0.75	0.85355	1	306.76	331.868
26	0.5	0.39645	0.5	1	294.92	297.516
27	0.5	0.75	0.5	1.70711	361	378.135
28	0.14645	0.75	0.5	1	317.53	324.552
29	0.5	0.75	0.5	1	316.76	325.777
30	0.5	0.75	0.14645	1	296.92	268.444

surfactant like triton X100, tween 20, tween 40 and tween 80, and also with 1 mM concentration of anionic and cationic surfactants like sodium dodecyl sulphate and cetyl trimethyl ammonium bromide, respectively for 1 h at 50°C. The procedure was followed for solvents (10%) like ethanol, methanol (polar protic solvents), acetone, acetonitrile (polar aprotic solvents), toluene and diethyl ether (non-polar solvents) respectively. The relative activity was determined under standard assay condition.

Scanning electron microscopy

To observe the difference between the unhydrolyzed ANFL and hydrolyzed ANFL, scanning electron microscopy observations were performed for the ANFL samples which were fixed in 2%w/v glutaraldehyde for 2 h followed by washing with saline solutions. Then the samples were dehydrated in 30 to 100% water/ethanol series and air-dried. The dried samples were coated with 120 to 130 mm gold-in-argon medium using a scanning electron microscope (JSM-5600 LV, Jeol, Japan) at 20 kV, accelerating with an electron beam of voltage 5 to 6 nm.

RESULTS AND DISCUSSION

CCRD model fitting and ANOVA

A five-level four-factorial design was used to determine the variation of the responses (Y_c) due to the effect of the four independent critical variables or due to the interaction between them along with ANFL (1 g/100ml of minimal medium) as a constant factor that serves as the substrate for protease production. The experimental model having a total of 30 runs, which are allocated in three blocks each having 10 runs and the results of increase in protease production by *S. ruminantium* is given in Table 2. The coefficients and their statistical significances (ANOVA) are given in Table 3. The adequacy of the model and fitness were evaluated by ANOVA and regression coefficients. The ANOVA for the quadratic model was highly significant with an *F* value of 6.16 as shown by

Table 3. ANOVA for response surface quadratic model Analysis for production of protease

Source	Sum of squares	Df	Mean square	*F* value	P value (prob. > *F*)
Block	192.576	2	96.2882		
Model	44925.1	14	3208.94	6.16166	0.0011
A	2603.32	1	2603.32	4.99877	0.0435[a]
B	902.953	1	902.953	1.73381	0.2107[b]
C	9925.37	1	9925.37	19.0583	0.0008[a]
D	8351.62	1	8351.62	16.0364	0.0015[a]
AB	7561.75	1	7561.75	14.5197	0.0022[a]
AC	340.103	1	340.103	0.65305	0.4336[b]
AD	1379.16	1	1379.16	2.64821	0.1276[b]
BC	3228.74	1	3228.74	6.19968	0.0271[a]
BD	5657.99	1	5657.99	10.8642	0.0058[a]
CD	114.363	1	114.363	0.21959	0.6471[b]
A^2	692.506	1	692.506	1.32972	0.2696[b]
B^2	793.475	1	793.475	1.5236	0.2389[b]
C^2	1490.13	1	1490.13	2.86128	0.1146[b]
D^2	1229.04	1	1229.04	2.35994	0.1485[b]
Residual	6770.28	13	520.791		
Lack of Fit	5737.6	10	573.76	1.6668	0.3705
Pure Error	1032.69	3	344.229		
Cor Total	51888	29			

R^2 = 0.8690; cv = 7.25%; [a], significant ; [b], not significant.

Fisher's (*F*) test, along with a very low probability value (P model>F=0.001). The confidence that the regression equations would predict the observed values better than the mean for Y_c was 86.9% and the coefficient of determination (R^2) of the model was 0.8690. A *P* value of ≤ 0.0500 for any factor in the analysis of variance (ANOVA) indicates significant effect of the corresponding factors on the response, that is, protease activity (U/ml). At the same time, relatively lower value of coefficient of variation (CV=7.25%) indicated a better precision and reliability of the experiments carried out. The larger the *F* value and smaller the P value, the more significant is the corresponding coefficient. Analysis of the factorial design showed a high degree of fitting between predicted and experimental values, which is very good for goodness of fit showing that the model suitably represented the real relationship among the selected factors. The model equation for protease activity obtained is as follows:

$$Y_c = 327.46 - 11.48\,(A) + 6.76\,(B) + 22.42\,(C) + 20.56\,(D) - 21.91\,(AB) - 4.64\,(AC) + 9.36\,(AD) + 14.32\,(BC) - 18.95\,(BD) + 2.69\,(CD) - 8.73\,(A^2) - 9.34\,(B^2) - 12.81\,(C^2) + 11.63\,(D^2).$$

The polynomial equation represents the quantitative effect of process variable (A, B, C and D) and their interactions on the response Y_c. The values of the coefficients A, B, C and D are related to the effect of these variables on the response Y_c. Coefficients with more than 1 factor term and those with higher order terms represent interaction terms and quadratic relationship, respectively. A positive value represents an effect that favors the optimization, while a negative value indicates an antagonistic effect.

The relationship between the dependent and independent variables was further elucidated using response surface plots and contour plots. Figure 1a depicts the cumulative effects of ammonium chloride and dipotassium hydrogen phosphate when all the other factors were kept constant at their optimum levels. Protease activity was found to be gradually enhanced with an increasing concentration of dipotassium hydrogen phosphate from 0.3 upto 1 g/l and further increase in concentration decreased the protease activity. Likewise, the protease activity was maximum in low levels of ammonium chloride (0.25 g/l), whereas higher concentration of ammonium chloride decreased the protease production. The protease activity was found to be more than 350 U/ml in the culture medium containing 0.25 g/l of ammonium chloride and 1 g/l of dipotassium hydrogen phosphate.

The interactive effects of potassium dihydrogen phosphate and ammonium chloride are shown in Figure 1b. The culture medium having a concentration of 0.25 g/l of ammonium chloride and 0.75 g/l of potassium dihydrogen phosphate showed maximum protease production, showing an activity more than 345 U/ml. The protease activity increased with increasing concentration of potassium dihydrogen phosphate up to 0.75 g/l and then decreased

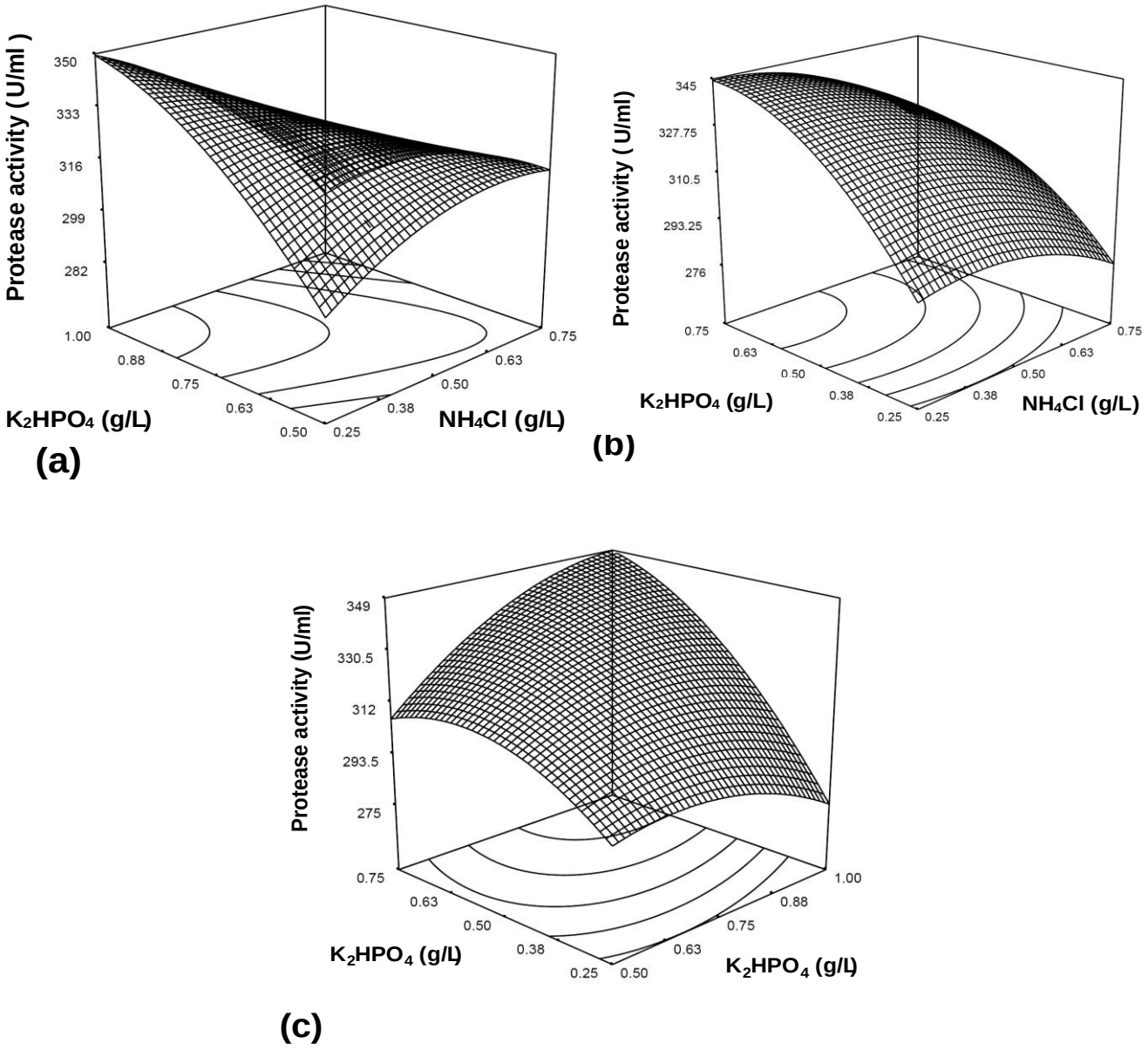

Figure 1. Response surface plot showing interactive effect of variables on protease production. a, K_2HPO_4 and NH4Cl; b, KH_2PO4 and NH_4Cl; c, K_2HPO_4 and KH_2PO_4..

with further increase in concentration.

The response surface plot shown in Figure 1c depicts the interactive effect of potassium dihydrogen phosphate and dipotassium hydrogen phosphate. The results obtained showed that the maximum protease yield was observed in the culture medium having 0.75 g/l of potassium dihydrogen phosphate and 1 g/l of dipotassium hydrogen phosphate when all the other factors were kept constant at their optimum levels yielding an activity more than 350 U/ml. At the end of the CCRD modelling for media optimization for protease production, it was concluded that the production medium with a composition of (g/l) NaCl (0.5), NH_4Cl (0.25). K_2HPO_4 (1.0), and KH_2PO_4 (0.75) was found to highly enhance the protease production. Roberta et al. (2006) also suggested that RSM proved to be a powerful tool in optimizing metallo-protease production by *Microbacterium* sp.

Effect of pH and temperature on protease production

Microorganisms are sensitive to the changes in the hydrogen ion concentration of their environment (Sharmin et al., 2005). The effect of medium pH on the production of protease (Figure 2) by the isolated strain *S. ruminantium* using ANFL as substrate revealed that the optimized

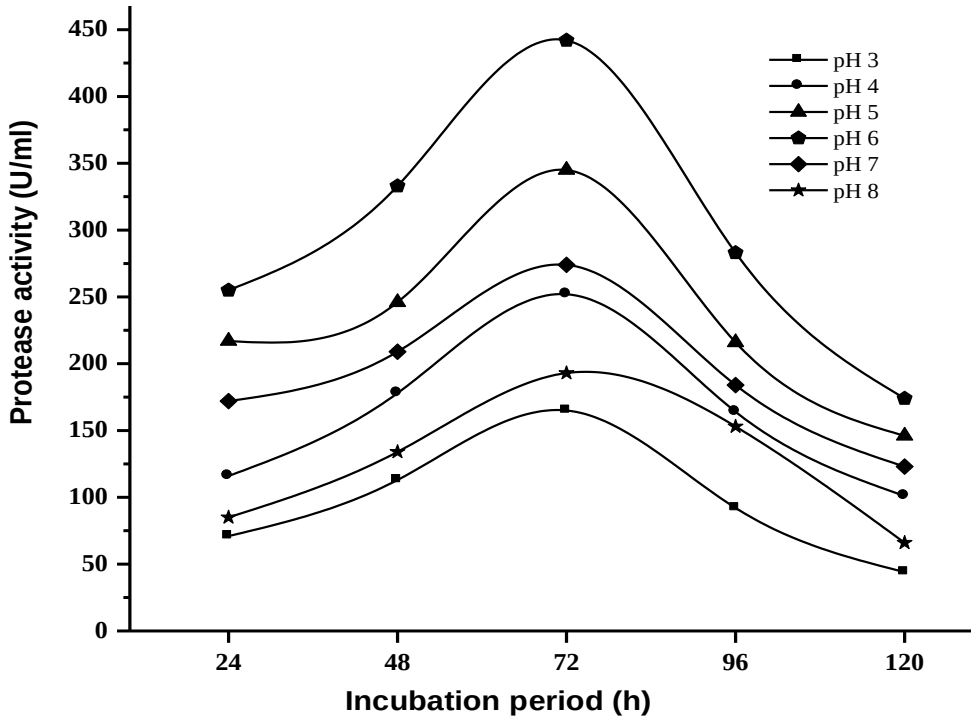

Figure 2. Effect of medium pH and incubation period on protease production.

medium with pH 6 was suitable for maximum production of protease while harvested at 72 h of incubation showing an activity of 442 U/ml. Similarly, the protease activity was further enhanced when the organism was grown in the optimized medium with adjusted pH 6 for 72 h and incubated at a temperature of 40°C (Figure 3). The protease activity was enhanced up to 462 U/ml when incubated at the above optimized conditions. The results coincides with the observations obtained from the production of protease from *Synergistes* sp. (Ganesh Kumar et al., 2008b).

Purification of protease enzyme

In the present study, protease enzyme from the isolated strain *S. ruminantium* was purified from cell free culture supernatant by ammonium sulphate precipitation, followed by dialysis, ion-exchange chromatography and gel filtration. Table 4 summarizes the purification steps employed and their results. Enzyme was purified starting from the culture filtrate with a yield of 28.14%. The homogeneity and molecular weight of the purified protease was then estimated by SDS page using protein markers of known molecular weight.

SDS page of purified protease from *Selenomonas ruminantium*

SDS PAGE results revealed that the enzymatic peak from the sephadex G-100 column chromatography yielded single band, testifying its homogeneity. The purified enzyme was homogeneous, as seen by a single protein both in SDS–PAGE and zymogram analysis. The apparent molecular mass of the purified enzyme was estimated to be around 53 kDa by SDS–PAGE and zymogram analysis (Figure 4). These results indicate that the purified enzyme in the present study is a monomeric enzyme.

Amino acid composition of protease

The amino acid composition in mol percent of the purified protease was: Glutamic acid, 26.4±1.8; aspartic acid, 16.4±1.1; serine, 4.4±0.3; histidine, 3.6±0.3; glycine, 6.2±0.43; threonine, 4.8±0.40; alanine, 12±0.84; arginine, 6.1±0.42; tyrosine, 3.8±0.33; valine, 1.8±0.12; methionine, 3±0.21; phenyl alanine, 2.6±0.18; isoleucine, 2±0.14; leucine, 3.4±0.23; lysine, 0.5±0.03; cysteine, 3.0±0.3.

Characterization of purified protease

Influence of pH and temperature on protease activity and stability

The pH is the most important factor, which markedly influence enzyme activity. Extremely high and low pH values generally leads to complete loss of activity for

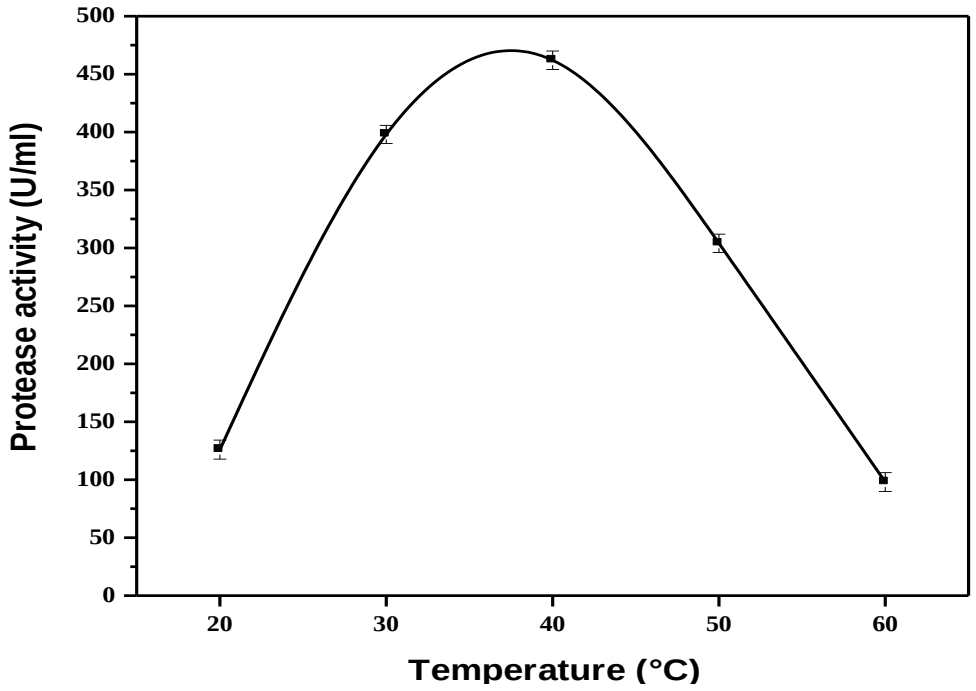

Figure 3. Effect of incubation temperature on protease production.

Table 4. Purification of extracellular acid protease.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude	41000	9760	4.2	100	1
Ammonium sulphate precipitation	34400	1286	26.74	83.9	6.36
Dialysis	28650	1135	25.24	69.87	6
Ion-exchange chromatography (DEAE)	12520	153	81.83	30.53	19.48
Sephadex G-100	11540	49	235.51	28.14	56.07

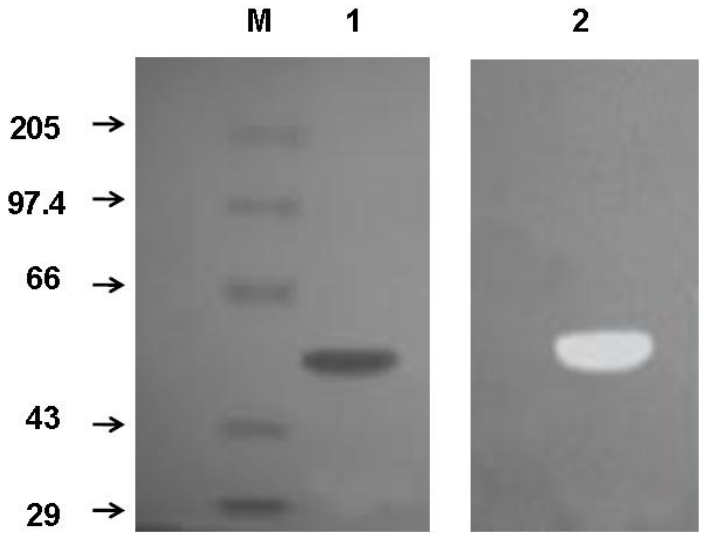

Figure 4. SDS PAGE and zymogram. M, marker; lane 1, purified protease; lane 2, zymogram of purified protease.

most enzymes (Shweta et al., 2010). The effect of pH on enzyme activity in the pH ranging from 3.0 to 8.0 (Figure 5) showed that optimal activity occurred in acidic pH (pH 4.0). Similarly, the pH stability of the enzyme determined by measurement of the relative activity after incubation at various pH revealed that the enzyme was also found to be stable in pH 4, which leads to the confirmation that the purified enzyme is an acid protease. Similar results were observed in protease production by *Saccharomyces lipolytica* and *Mucor pusillus* (Tetsuji and Ogrydziak, 1983; Somkuti and Babel, 1968), which had the optimum pH for protease activity to be around 4. The activity declined with further increase or decrease in the pH. Temperature is also a critical factor for maximum enzyme activity and it is a prerequisite for industrial enzymes to be active and stable at higher temperature (Kalpana devi et al., 2008). The enzyme stability analyzes at varied temperatures (Figure 6) showed that the enzyme is a mesophilic protease that remains active at a temperature of about 40°C. Enzyme became inactive at temperature

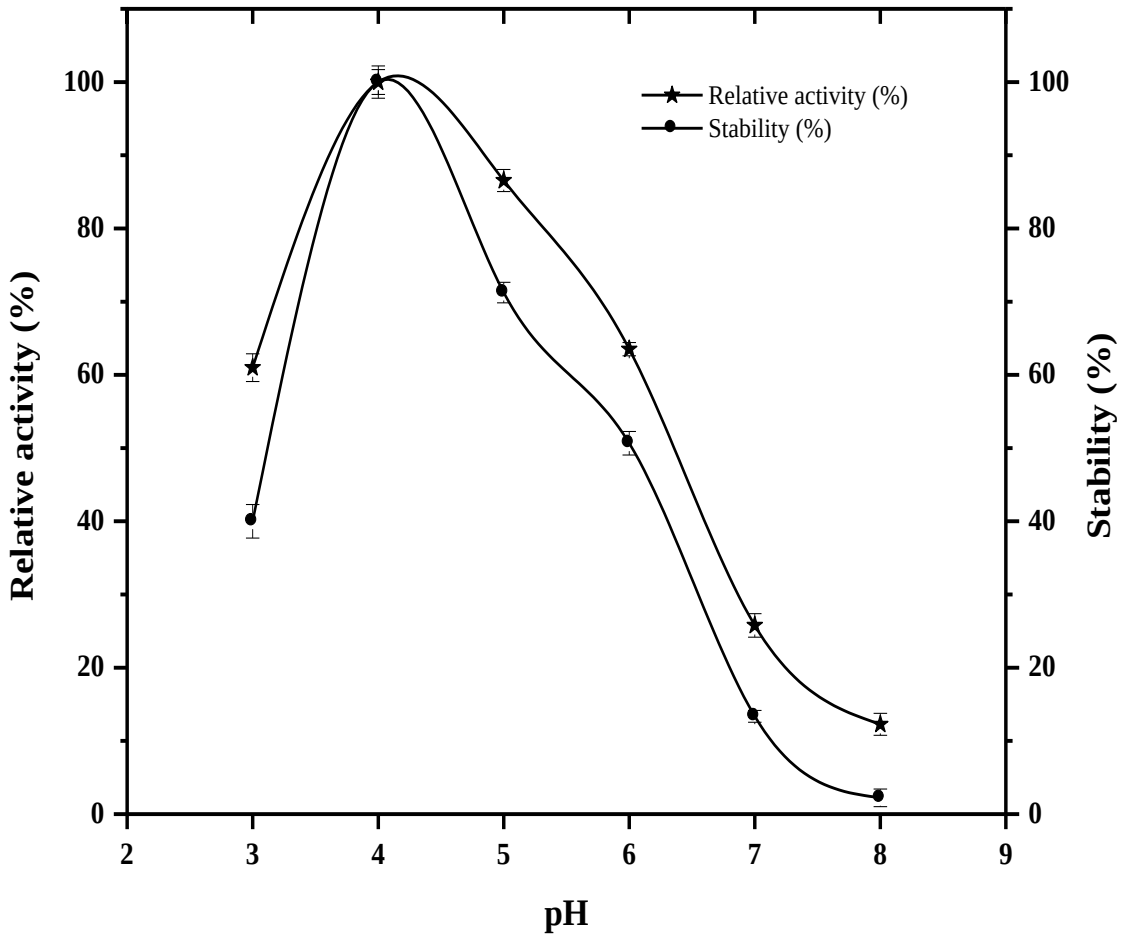

Figure 5. Effect of pH on protease activity and stability.

above 40°C; this proves instability of conformational structures at higher temperatures. Hence, the enzyme was regarded as a mesophilic acid protease. This result was similar to the enzyme isolated from *Bacillus latrosporus* (Usharani et al., 2010).

Effect of inhibitors and metal ions on protease activity

The relative activity of the purified enzyme in the presence of protease inhibitors are depicted in Figure 7. The observation revealed that the inhibitor DTT, a reducing agent had the most significant inhibitory effect over the enzyme activity with a relative activity below 30%. The inhibitory effect of the other protease inhibitors like EDTA, EGTA, PMSF and pepstatin A on the enzyme activity is negligible. The result reveals that the enzyme could be a cysteine protease. There are also reports on the production of cysteine protease from *Staphylococcus epidermidis* (Olesky et al., 2004; Dubin et al., 2001). When assayed in the presence of divalent cations, Ca^{2+} was found to be an activator of the enzyme stability whereas Mg^{2+} enhanced the activity to a lesser amount and other cations inhibited the activity of the purified enzyme (Figure 8). This result coincides with the protease obtained from *Candida olea* (Nelson and Young, 1987).

Effect of surfactants and solvents on protease activity

The purified protease was found to be resistant to denaturation by non-ionic surfactants like tween 20, tween 40, tween 80 and triton x100. But, the activity of the enzyme declined while incubated with SDS and CTAB revealing the instability of the enzyme in the presence of anionic and cationic surfactants (Figure 9). This result coincides with the alkaline serine protease obtained from *Serratia* sp. SYBC H, which was found to be stable in the presence of tween 80, glycerol and triton ×100 (Li et al., 2010). The purified protease was found to be quite stable in solvents like methanol, ethanol and isopropanol, whereas other solvents showed inhibitory action over the protease activity (Figure 10). Similar results were observed in the stability studies of protease

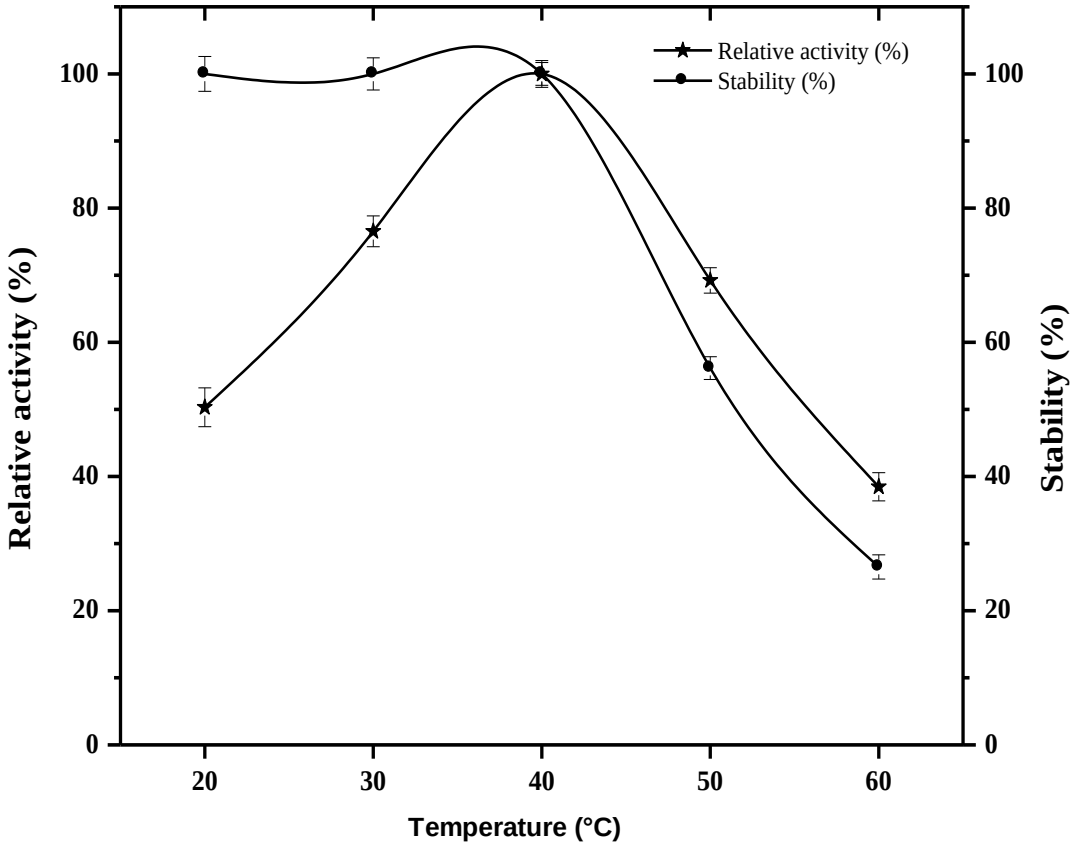

Figure 6. Effect of temperature on protease activity and stability.

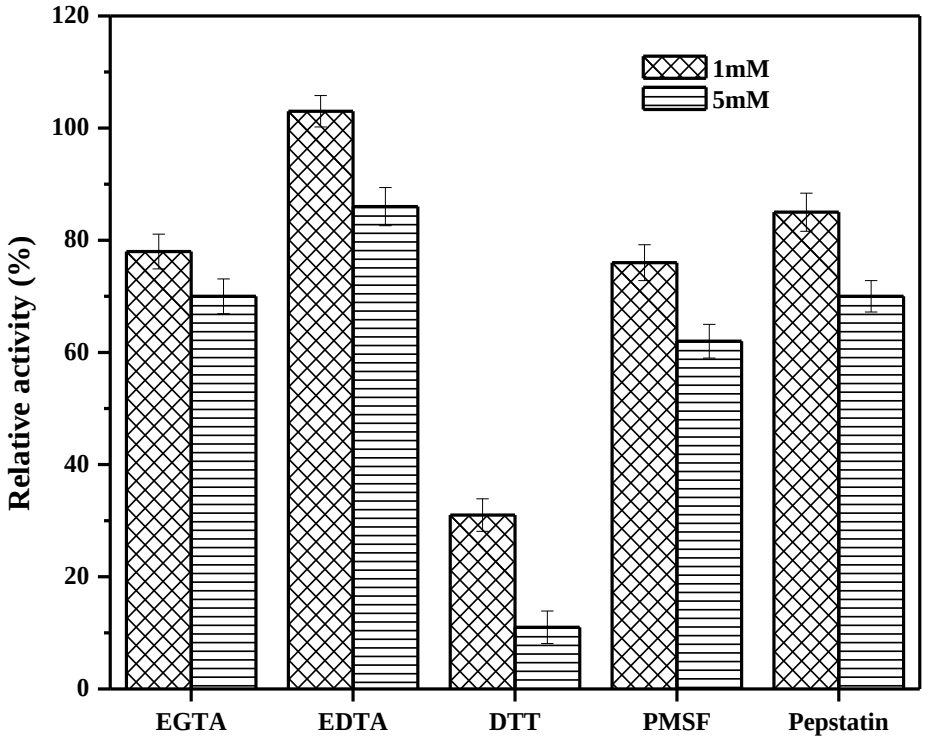

Figure 7. Effect of inhibitors on protease activity. EDTA, Ethylene diamine tetra acetic acid; DTT, dithiothreitol ; PMSF, phenyl methyl sulphonyl fluoride

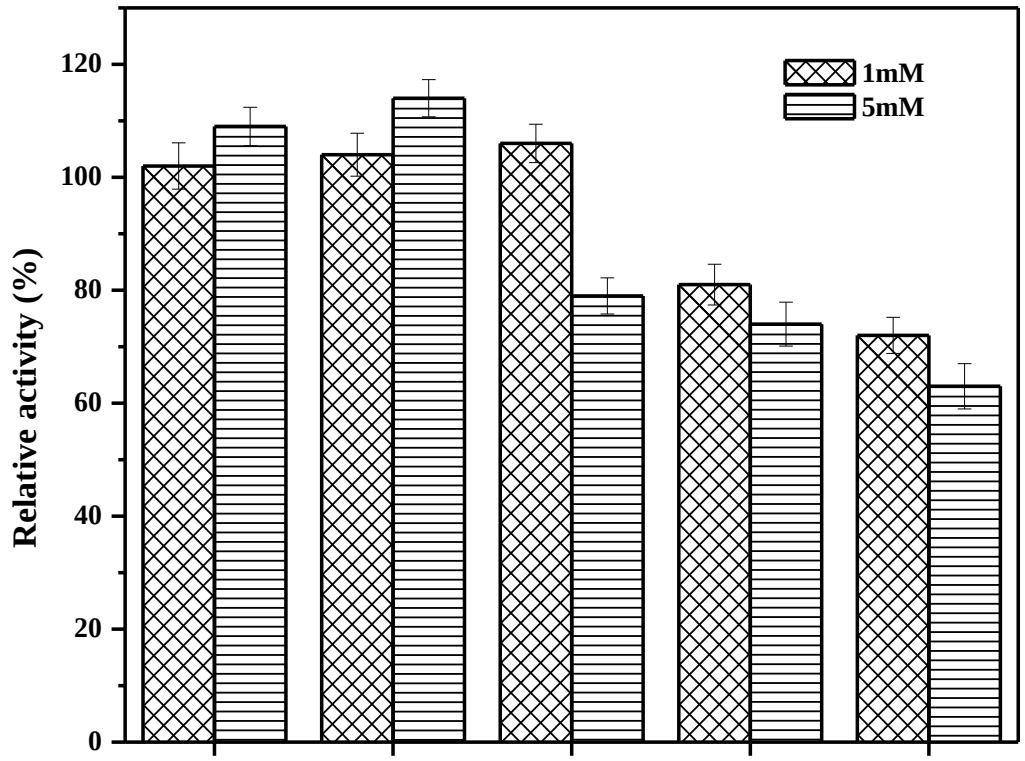

Figure 8. Effect of metal ions on protease activity.

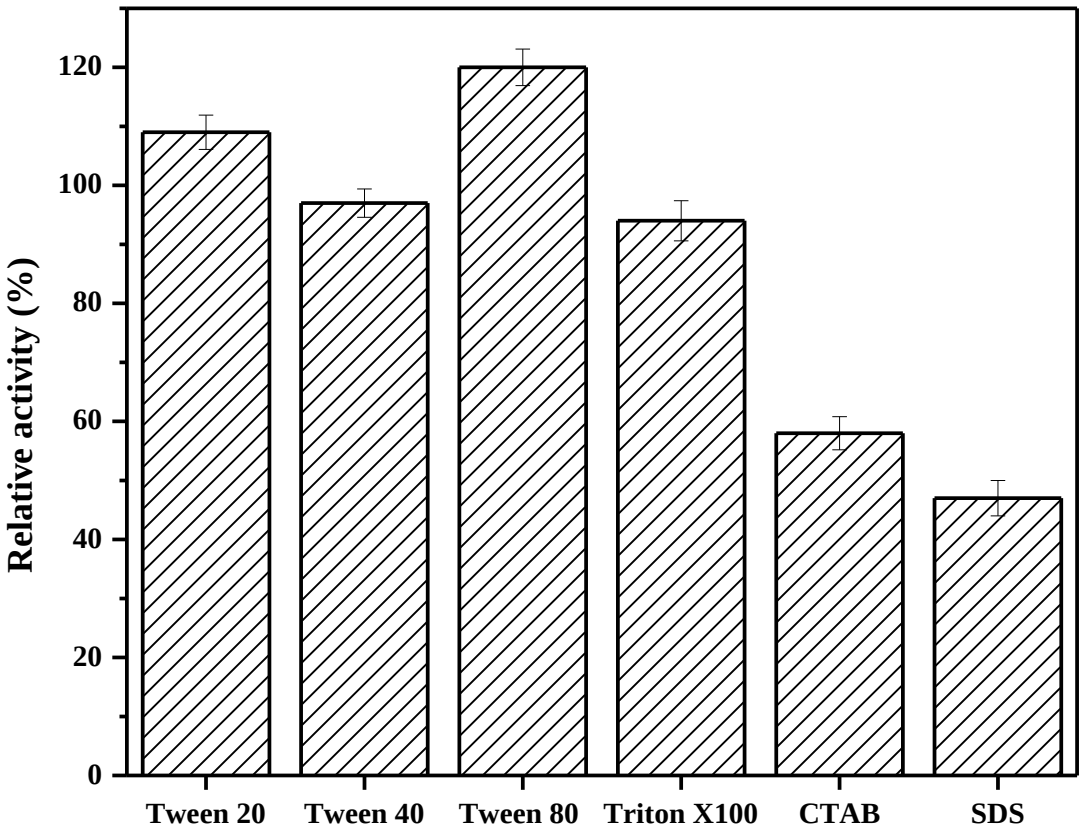

Figure 9. Effect of surfactants on protease activity.

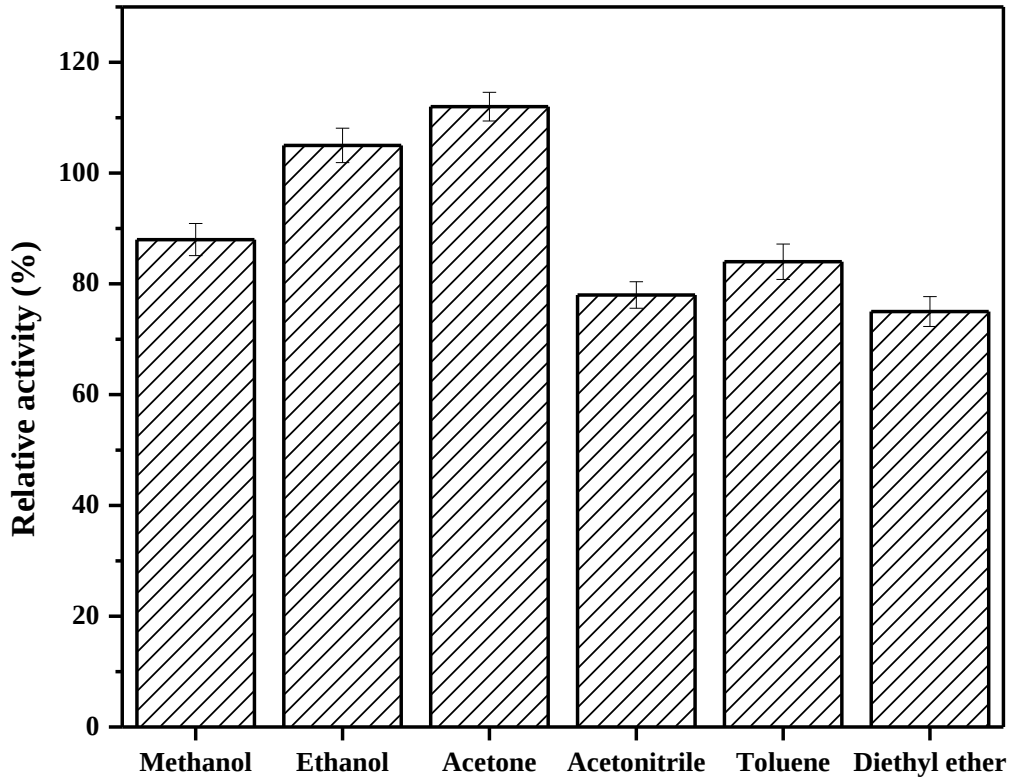

Figure 10. Effect of solvents on protease activity.

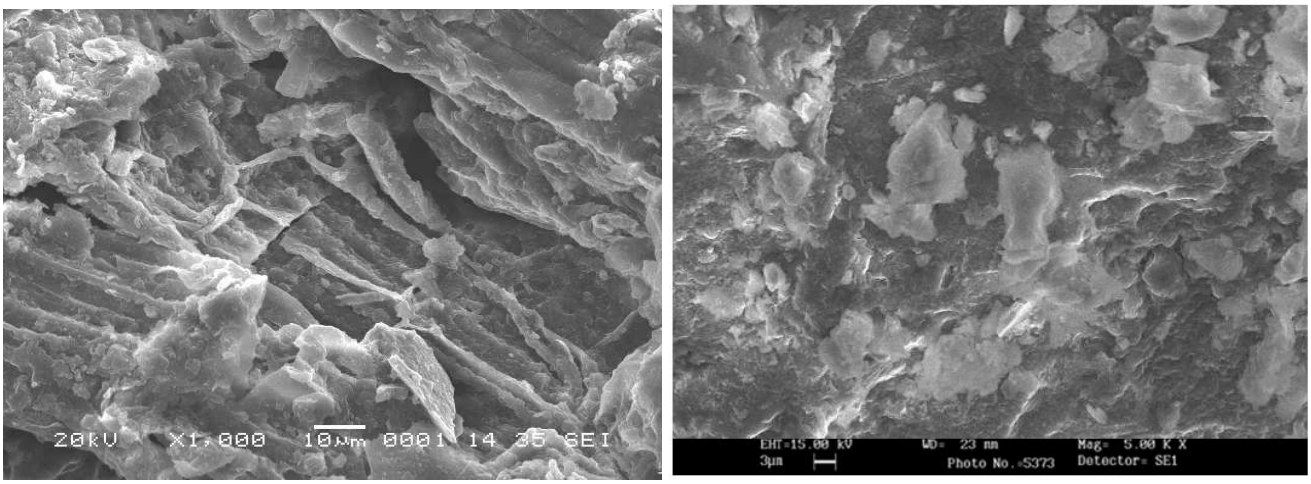

Figure 11. SEM image of (a) unhydrolyzed (before fermentation) and (b) hydrolyzed (after fermentation) ANFL.

from *Bacillus cereus* (Basma et al., 2003).

Scanning electron microscopy (SEM) for the confirmation of hydrolysis of ANFL

The surface morphologies of the unhydrolyzed and hydrolyzed ANFL are depicted in Figures 11a and b, respectively. The unhydrolyzed ANFL sample allowed the visualization of obvious fibril networks with a rough membranous structure and fine distribution of clustered tissue fibers. Whereas, the SEM image of hydrolyzed ANFL sample depicted the detachment and porosity of the fibril networks due to protein solubilization with comparably

smooth membranous structure. The rigidity of the fibril networks in unhydrolyzed ANFL sample when compared to the less rigid nature and detachment of the protein fibrils in hydrolyzed sample, presents the modulation of the ANFL morphology due to enzymatic degradation and these results corroborate with the findings of Ganesh Kumar et al. (2008).

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