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Statistical assessment of stability and compatibility of protease extracted from thermo tolerant *Bacillus licheniformis* BWU-1

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ABSTRACT

Proteolytic enzymes particularly proteases, have become an important and essential part of the industrial processes including pharmaceuticals, food products and laundry detergents. The studied physico-chemical parameters revealed that the optimum pH and temperature was 10 and at 50°C. Detergents such as Ariel, Tide, Rin, Ghadi, Surf excel, Nirma and Sassa possesses Proteolytic enzymes, mostly extracted by members belongs from the genus *Bacillus.* Present investigation, describes the compatibility of protease produced by the thermotolerant *Bacillus licheniformis* BWU-1 with commercial laundry detergents. Stability studies indicated that this enzyme retained about 178% and 143% of its maximum activity after 6 h at 45°C in presence of $CaCO_3$ and $MgSO_4$ respectively. A stronger inhibitory effect was observed in presence of $FeSO_4$ and $HgCl_2$ resulted in loss of enzyme activity. A lesser amount of inhibitory effect was observed at 1-2% triton X-100, 0.1-0.2 % SDS, 1-2% H2O2 and 1-4% EDTA all they showed significant p-value (<0.05) while remaining all the concentrations were inhibited the enzyme activity. After supplementation of Ca^{+2}, the protease retained more than 90 and 100 % of its activity after 1 h incubation at 50°C in presence of Rin and Surf excel respectively.

Introduction

The biosynthesis of proteolytic enzymes by microorganisms is having a scientific as well as practical importance. The genus *Bacillus licheniformis* BWU-1 played a significant role to produce such enzyme. Proteolytic enzymes are ubiquitous in occurrence and are essential for cell growth and differentiation. Proteases execute a large variety of functions and have significant in biotechnological applications (Dixon and Web,1964, Fox, *et al.,* 1991, Gupta, *et al.,* 2002). They signify one of the three largest groups of industrial enzymes and pivotal applications in detergents, leather, food, pharmaceutical industries, silver recovery and bioremediation processes (Gupta et al., 2002, Pastor et al., 2001, Tunga et al., 2003). The involvement of proteases in the life cycle of disease causing organisms has led them to become a potential target for developing therapeutic agents against grave diseases, such as cancer and AIDS (Rao et al., 1998). Thermostable proteases are advantageous in commercial applications because higher processing temperature can be employed, resulting in faster reaction rates, increase in the solubility of nongaseous reactants and products and reduced incidence of microbial contamination by mesophilic organisms (Beg et al., 2003, Ward, 1985). Enzymes occupy an important role in analytical biochemistry and many investigations are requiring their quantitation. Present investigation was aimed to statistical assessment of stability and compatibility of the protease by using various parameters.

Materials and Methods

Enzyme production media

A 4.0 ml inoculum was transferred in protease producing broth containing (g/lit); 5 Peptone, 10 Mannitol, 0.3 $CaCO_3$, 5 NH_4NO_3, 2.0 KH_2PO_4, 0.5 $MgSO_4$, 0.36 $ZnSO_4$. Adjust the pH by 0.1 N NaOH up to 9.0. The inoculated medium was placed in an orbital shaker at 45°C for 18 hrs and 150 rpm. Samples were withdrawn and centrifuged at 14,000 g for 15 min at 4°C. The obtained supernatant used as a source of crude enzyme.

Enzyme assay

Protease activity was measured by using casein as substrate (Huang et al., 2006). A mixture of 400 µl casein solution (2 % w/v in 50 mM Phosphate buffer pH 7.0) and 100 µl extracted enzyme were added in each tube and incubated for 10 min at 50°C. The reaction terminated by addition of 1ml trichloroacetic acid (10 % v/v). The mixture allowed to centrifuge at 14,000 g for 20 min and supernatant (1ml) was removed carefully. Tyrosine/tryptophan content was determined by using Lowery method. The blank was prepared by adding 1ml of TCA before addition of an enzyme. One unit of protease activity (U) is defined as the amount of enzyme that hydrolyzed casein to liberate one µmole tyrosine per min under the above condition (Norazizah *et al.*, 2005).

Effect of hydrogen Ion concentration on activity and stability of protease

The optimum pH was determined with casein 1% (w/v) as substrate dissolved in different buffers (citrate phosphate, pH 4-5, phosphate buffer, pH 6-8, and glycine NaOH, pH 9-11). The effect of pH on enzyme stability was determined by pre-incubating the enzyme without substrate at different pH values (4-11) for 24 h at room temperature and measuring the residual activity at 45ºC. (Wellingta *et al.,* 2004, Wellingta *et al.*, 2006).

Influence of temperature on protease activity and its stability

The effect of temperature on the enzyme activity was determined by performing the standard assay procedure at pH

7.0 within a temperature range from 30 to 100ºC. Thermostability was determined by incubation of crude enzyme at temperatures ranging from 30-100ºC for 6h in a constant-temperature incubator and water bath. After treatment the residual enzyme activities were assayed (Banerjee et al., 1999, Beg and Gupta, 2003, Camila et al., 2007).

Effect of enzyme activators

The effect of different enzyme activators on protease activity was determined by the addition of the corresponding ion at a final concentration of 0.5, 1.0 and 2.0 mM to the reaction mixture, and assayed under above standard conditions. The enzyme assay was carried out in the presence of KCl, $CaCO_3$, $MgSO_4$, $FeSO_4$, $ZnCl_2$, $MnSO_4$, $HgCl_2$, NaCl and $CuCl_2$. (Wellingta et al., 2004 and Wellingta et al., 2006)

Effect of surfactants and inhibitors

The effect of different surfactants and oxidizing agents on alkaline protease stability was studied by measuring the enzyme activity in the presence of these agents. The alkaline protease was incubated with different concentrations of surfactants like Tween 80, Triton X-100, sodium dodecyl sulphate (SDS) and oxidizing agents (Inhibitors) like H_2O_2 and EDTA for 1 h at 40 °C. The residual activity was then measured according to the standard assay conditions (Wellingta et al., 2006).

Compatibility with various commercial detergents

The ammonium sulfate-precipitated enzyme preparation was used for the detergent compatibility studies. The detergent brands used were Ariel, Tide, Rin, Ghadi, Surf excel, Nirma and Sassa. They were diluted in deionized water to a final concentration of 7 mgL^1 to simulate washing conditions. The enzyme in the detergent was deactivated by heating at 100ºC for 10 min. After that, a protease concentration of 0.06 mg/ml was added in solution and incubated at 50ºC for 1 hr. Aliquots (0.5 mL) were taken at different time intervals and the residual activity determined at 50ºC and compared with the control sample incubated at 50ºC without any detergent (Banerjee et al., 1999, Wellingta et al., 2006).

Effect of CaCO3 on protease activity on commercial detergent

The detergents were diluted in distilled water to a final concentration of 7 mg/ml. A protease concentration 0.06 mg/ml was incubated at 50°C in detergent in the presence of $CaCO_3$ (1 mM). At 15 min interval a sample (1 ml) was removed and the residual activity determined at 50°C and compared with the control sample incubated at 50°C with no detergents (Wellingta et al., 2006).

Statistical procedures

The value of mean and standard deviation were calculated. One way ANOVA was used to test the effect of different parameters on protease activity. All statistical analysis performed by using Minitab-15 software and Microsoft excel spreadsheet (Chap, 2003, Gurumani, 2009)

Result

Effect of hydrogen ion on protease activity and stability

The Protease activity was assayed at different pH ranging from 4 to 11. Enzyme activity increased with pH within the range 9 to 11.

A reduction of enzyme activity was observed at values 4 to 7. The optimum pH of this protease was 10. At pH 5 only 0.63 U/mg maximum activity was obtained where as at increased pH is directly proportional to the protease activity up to pH 10. At pH 11 activity of enzyme was decline of about 0.56 U/mg.

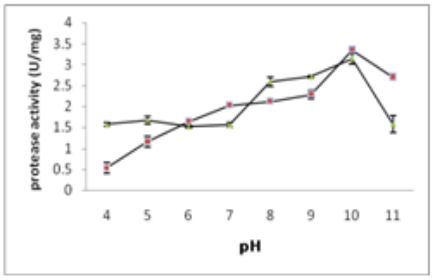

Fig. 2. Optimium pH (■) and stability (●) of protease produced by *B.licheniformis BWU-1.* Specific activity is expressed as percentage of the maximum

The enzyme was incubated at 45°C for 24 hrs, at pH values of 6 and 7 the enzyme activity was increased of about 0.12 and 0.47 U/mg respectively. While at pH 10 and 11 the enzyme activity were decreased about 0.21 and 1.12 U/mg respectively

Effect of temperature on protease activity and stability

Temperature plays an important role in activation and denaturation of enzyme. The protease activity was analyzed at different temperatures ranging from 30 to 100°C at constant pH 10 (Fig. 3). The obtained surface plot showed the maximum activity achieved at 60°C with a sharp decline percent activity before and after this value. The enzyme activity was increased with temperature at 50°C.

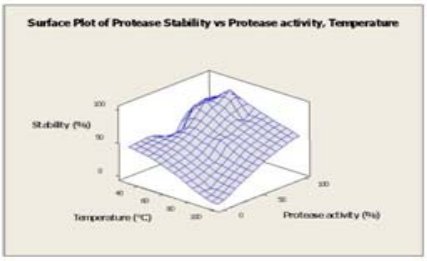

Fig. 3.Surface plot showed protease activity and its stability at various temperature

The thermostability of the protease was analyzed by incubating the enzyme without substrate at various temperatures within 30 to 100°C for 6 hrs. Thermostability profile indicated that the enzyme was stable at 50°C for 6h, while at 60°C it loose 21% its original activity. At temperature 70, 80 and 90°C the enzyme activity were drastically retarded and it was about 44 %, 80 % and 97% respectively.

Effect of enzyme activators on protease activity

The effect of different metal ions on protease activity was determined by regression analysis. The applied metal ion, such as $CaCO_3$, $MgSO_4$ and $MnSO_4$ at 1 mM concentration enhances the rate of reaction while KCL, $ZnCl_2$ and $HgCl_2$ showed inhibitory action against protease. The correlation in between 0.5, 1.0 and 2.0 mM concentration of metal ions was analyzed by regression equation and ANOVA. The obtained data showed 0.000 of its p-value indicates the used concentration of metal ions was significant. The result showed that the coefficient of determination (R^2) was 98.5 % and R^2 adj was 98.0 % which ensured satisfactory adjustment of the quadratic model to the experimental data (Table 2). The residual plot for 1.0 mM metal ion was proved by normal probability plot, versus fit and histogram. The regression equation confirms the obtained value of

Table 2: Regression equation and ANOVA

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The regression equation is
1.0 = 8.99 + 0.279 0.5 + 0.743 2.0


Predictor     Coef  SE Coef      T      P
Constant     8.991    6.296   1.43  0.203
0.5         0.2788   0.1707   1.63  0.153
2.0         0.7430   0.1581   4.70  0.003


S = 7.12505   R-Sq = 98.5%   R-Sq(adj) = 98.0%


Analysis of Variance (ANOVA)

Source           DF     SS     MS       F      P
Regression        2  20347  10173  200.40  0.000
Residual Error    6    305     51
Total             8  20652
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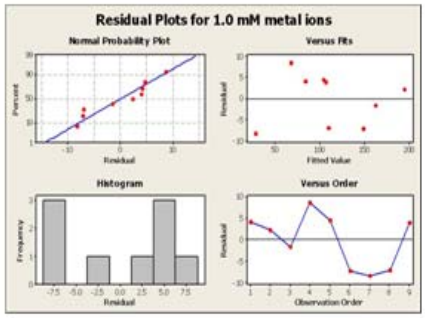


Fig 4: Showed a residual plots for 1.0 mM metal ions

Effect of inhibitors and some surfactants

Statistical analysis of the activity of enzyme in presence of surfactants and inhibitors showed in table 2. The enzyme solution was mixed with triton X-100, SDS, H_2O_2 and EDTA a metalloprotease inhibitor, and pre-incubated at 50°C for 15, 30 and 60 minutes, and then protease activity was determined as described earlier. The obtained results were recorded and were showed in the form of significance level.

Table 3: Effect of different surfactants and inhibitors on protease activity

Surfactant & Inhibitor	PROTEASE ACTIVITY (U/mg)		
	15 min	30 min	60 min
Triton X-100 1 %	2.64 (0.013)*	2.41 (0.015)*	1.35 (0.188)
Triton X-100 2 %	2.47 (0.007)*	1.29 (0.199)	0.62 (0.347)
Triton X-100 3 %	2.25 (0.037)*	1.07 (0.245)	0.45 (0.387)
Triton X-100 4 %	1.35 (0.188)	0.73 (0.321)	0.28 (0.429)
Triton X-100 5 %	1.07 (0.245)	0.34 (0.415)	0.06 (0.484)
SDS 0.1 %	2.47 (0.007)*	2.08 (0.061)	1.07 (0.245)
SDS 0.2 %	2.47 (0.007)*	2.13 (0.054)	1.35 (0.188)
SDS 0.3 %	2.02 (0.071)	1.91 (0.088)	1.07 (0.245)
SDS 0.4 %	1.52 (0.155)	1.07 (0.245)	0.51 (0.37)
SDS 0.5 %	1.18 (0.22)	0.56 (0.36)	0.06 (0.484)
H2O2 1%	2.47 (0.007)*	1.07 (0.245)	0.51 (0.37)
H2O2 2 %	2.41 (0.015)*	0.96 (0.26)	0.28 (0.42)
H2O2 3%	1.78 (0.109)	0.56 (0.36)	0.28 (0.42)
H2O2 4%	1.18 (0.22)	0.34 (0.41)	0.06 (0.484)
H2O2 5%	0.56 (0.36)	0.17 (0.47)	0.06 (0.484)
EDTA 1 mM	2.30 (0.03)*	1.74 (0.11)	1.52 (0.15)
EDTA 2mM	2.47 (0.007)*	2.02 (0.071)	1.35 (0.188)
EDTA 3mM	3.09 (0.057)*	2.69 (0.019)*	1.91 (0.08)
EDTA 5mM	2.13 (0.05)*	1.35 (0.188)	1.01 (0.25)
EDTA 10 mM	1.12 (0.23)	0.51 (0.37)	0.45 (0.38)

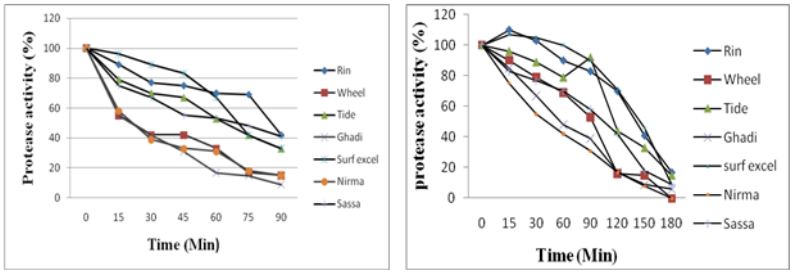
Significance level $p<0.05$, the numerical present in the bracket indicates p-values and * indicates the significant data

The used surfactant such as triton x-100 at 1%, 2% and 3 % accepts a null hypothesis hence it can be stable up to 15min where as at 30 and 60min it rejects the null hypothesis. At 15 min, 0.1 and 0.2 % SDS they were showed a p-value, 0.007 of the both concentration where as remaining had inhibitory action against the enzyme activity. In addition, except the 1 and 2 % H_2O_2 at 15 min all were showed a lethal effect on the enzyme and it determine by p-value. The used inhibitor, such as EDTA at 1,

2, 3 and 5 mM concentration has been noted a valuable p-value; shortly it accepts the null hypothesis.

Compatibility with various commercial detergents

The various detergents were used to check protease activity such as Rin, Wheel, Tide, Ghadi, Surf excel, Nirma and Sassa. Protease from thermotolerant *Bacillus licheniformis BWU-1* retained more than 77% and 89%, of its activity at 30 min incubation at 50°C in the presence of commercial detergent brands Rin and Surf excel respectively (Fig. 4a). After supplementation of optimized enzyme activator such as Ca^{+2}, the enzyme in the Rin and Surf excel was stable after 60 min at 50°C, retaining more than 90 and 100 % of its activity respectively (Fig 4b). This enzyme retained more than 50% activity with most of the detergent used in the present investigation up to 90 min by using Rin, Wheel, Tide, Surf excel and Sassa.



(a) (b)

Fig 5: (a) Compatibility of protease activity form *B. licheniformis* BWU-1with commercial detergents (b) Compatibility of protease in presence of 1 mM $CaCO_3$+enzyme+detergent. (100 % of enzyme activity 2.97 U/mgProtein)

Discussion

The obtained results of characterization were analyzed by applying a statistical method. The optimum pH for protease activity was at 10 pH. According to Wellingta et al., 2006 reported to protease S was active, are a very broad pH range and elevated activity were detectable at pH 10 in presence of metal ions. In contrast protease N and B retained relatively little activity above pH 9.0 (Wellingta et al., 2004). Thermostability profile indicated that the enzyme was stable at 50°C for 6h, while at 60°C it loose 21% its original activity. At temperature 70, 80 and 90°C the enzyme activity were drastically retarded and it was about 44 %, 80 % and 97% respectively. Such results were obtained during investigation by Wellingta (Wellingta et al., 2004). The temperature ranging from 70, 80 and 90° C were retard the protease activity up to 47 %, 76% and 97% respectively (Bhosale et al., 1995, Norazizah et al., 2005, Wellingta et al., 2004). The used EDTA concentration against the enzyme activity was noted significant value at 1, 2, 3 and 5 mM concentration. This finding was similar to that of Tunga et al, who reported that the protease from *Aspergillus parasiticus* was not inhibited by 5.0 mM EDTA. In *Bacillus licheniformis* ATCC 21415, the protease was inhibited by 20 mM EDTA and lost about 62% of its original activity (Tunga et al., 2003). The used surfactant was actively involved in retarding the protease activity (Wellingta et al., 2006). According to obtained results, presence of Ca^{+2} has been involved to increase the stability of the protease for longer time (Banerjee et al., 1999). Bhosale et al., reported that protease preparation from *Conidiobolus coronatus* showed compatibility at 50ºC, in the presence of 25 mM CaCl2 in a variety of commercial detergents and retained 16% activity in Revel, 11.4% activity in Ariel and 6.6% activity in Wheel (Bhosale et al., 1995, Wellingta et al., 2006).

Conclusion

In conclusive remarks, *Bacillus licheniformis* BWU-1 was found to show its potential to produce proteolytic enzymes with prospective use in industries. Such enzymes are efficiently worked in presence of metal ions. However, the extracted enzyme may be metalloprotease due to elevated activity in presence of $CaCO_3$. The protease showed a wide range of stability against pH, temperature as well as metal ions. The compatibility of the enzyme in different detergents shows that, it may be useful in various detergent preparations.

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