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Influence of Enzyme Activators on Alkaline Protease from Thermo-Tolerant *Bacillus licheniformis* JX849145

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Abstract: ***Proteolytic enzymes particularly proteases, have become an important and essential part of the industrial processes including pharmaceuticals, food products, laundry and detergents. Metal ions are involved in many enzyme catalyzed reaction. Hence prior to this, supplementation of ten different metal ions improved substantially the growth of B. licheniformis JX849145 and protease yield. Among ten metal ions, the Ca^{2+} (3 mM), Mg^{2+} (2 mM) and Zn^{2+} (2 mM) exhibits maximum protease production. The presence of K^{+}, Na^{+}, $Fe^{2+}$and Cu^{2+} ions in the medium resulted in significant reduction in both growth and production of protease. The integrated effect of metal ions was also investigated by Plackett-Burman design to find the mutual interaction of process variables. During growth of the cell, metal ions are required for stability of the cell at different physico chemical conditions. Therefore in present investigation the three optimized metal ions such as Mg^{2+}, Ca^{2+} and Zn^{2+} were used to find the superior combination at variable concentration. Earlier findings showed that the Metal ions such as Zn^{2+} often have a structural role, while notably Ca^{2+} is necessary for regulation of the activity. The designed Plackett Burman runs exhibit, 1.20 U/mg protease activity at 2 mM concentration of metal ion (Mg^{2+}, Ca^{2+} and Zn^{2+}). The correlation in between 0.5, 1.0 and 2.0 mM concentration of metal ions was analyzed by regression equation and analysis of variance (ANOVA). The obtained statistics showed 0.000 of its p value, it 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. Alkaline protease obtained from B. licheniformis JX849145 has properties such as optimum temperature at 50°C, optimal pH 10, stability towards metal ions and actively involved with commercial detergents that make this enzyme potentially useful for various industrial applications.***

Keywords: *Bacillus* strain, Enzyme activators and Protease.

1. Introduction

Enzymes are the one of major groups of products in biotechnology business. *Bacillus licheniformis* is a ubiquitous bacterium thought to be of importance in the environment as a contributor to nutrient cycling due to the production of protease and amylase enzymes (Wellingta et al, 2004). Proteases are the one of the most significant industrial enzymes accounting for nearly 65% of the total worldwide enzyme sales. Proteases are well-known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in the food, pharmaceutical, diagnostics, bioethanol production, fine chemical industries, leather, bioremediation process, waste processing industries and to remove sericine coating from raw silk fiber to achieve improved luster and softness (Palmer, 2004). Alkaline proteases produced by *Bacillus* species are of great importance in detergent industries due to their high thermal and pH stability (Norazizah et al, 2005 & Karl-Heinz Maurer, 2004).

2. Methods and Materials

2.1 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 was added in each tube and incubated for 10 min at 50°C. The reaction terminated by addition of 1ml trichloroacetic acid (TCA) (10% v/v). The mixture allowed to centrifuge at 14,000 g for 20 min and 1 ml supernatant 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 assay condition (Norazizah et al, 2005).

2.2 Determination of Total Protein Content

The total protein contents of the samples were determined according to the method described by Lowry (Lowry et al, 1951); the protein standard used was BSA (Merck). Protein standard solution, in the range of 0.5 to 5 mg/ml was prepared in triplicate to obtain a standard curve. Samples (cell-free supernatant) were diluted to 1 ml with distilled water so that the protein content would be within the range of the standards. Alkaline copper sulphate reagent (5 ml) was added to each tube and mixed well. The solutions were kept at room temperature for 10 minutes followed by the addition of 0.5 ml Folin & Ciocalteu's Phenol reagent (Merck) working solution. Each tube was rapidly mixed, and incubated in dark for 30 minutes. Absorbance of the samples was measured spectrophotometrically at 570 nm using UV/Vis spectrophotometer (Systronic- model 119) (Lowry et al, 1951).

2.3 Influence of Enzyme activators (metal ions) on protease production:

The effect of different metal ions on protease production was determined by the addition of the corresponding ion at a final concentration of 1.0, 2.0, 3.0 and 5.0 mM to the

production medium, and incubated at 45°C for 18 h. The enzyme production was carried out in the presence of KCl, CaCl2, MgSO4, FeSO4, CoCl2, ZnCl2, BaCl2, CuSO4 and NaCl. The extracted protease was assayed under standard conditions (Chandi & Subramanyam, 2004).

2.4 Optimization of divalent Enzyme activator (metal ion) concentrations by Plackett-Burman design:

The statistical method was used to select the effective concentration of different variables. In the present investigation, A-MgSO4, B-CaCO3 and C-ZnSO4 metal ions were used. Taking these variables in to consideration, a Plackett-Burman design was adopted for optimizing protease production from *B. licheniformis* JX849145. The statistical software Minitab-15 was used to analyze the experimental design. The minimum and maximum ranges of variables investigated and the complete experimental plan with respect to the actual value of the response is listed in Table 3.1. The protease production flask was maintained at 45°C for 18 h having 9 pH. Each experiment was conducted in triplicate and the mean protease activity was determined (Beg et al, 2003).

2.5 Effect of metal ions on protease activity

The effect of different metal ions 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, CaCO3, MgSO4, FeSO4, ZnCl2, MnSO4, HgCl2, NaCl and CuCl2. (Wellingta et al, 2004 & Wellingta et al, 2006).

3. Result and Discussion

3.1 Effect of Metal Ions on Protease Production

Supplementation of culture medium with metal cations improved substantially the protease production of *Bacillus licheniformis* JX849145 (Figure 1)

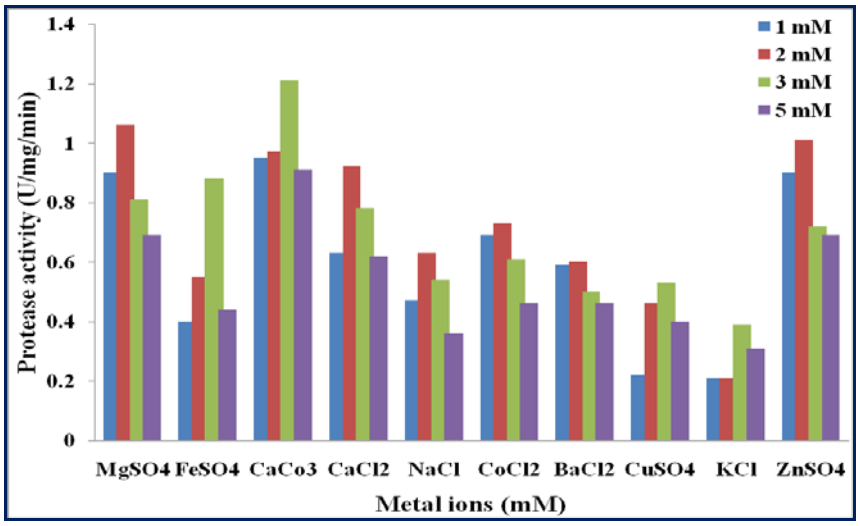

Figure 1: Effect of different metal ions (mM) on protease production by *B. licheniformis* JX849145

These results are in agreement with the earlier findings, which showed enhancement of protease production in presence of metal ions (Adinarayana et al, 2003 & Thangam, 2002). The stability of protease was improved in presence of metal ions (Paliwal et al, 1994). The highest level of protease production was observed in presence of Ca^{2+} it was 1.21 U/mg/min after 18 h incubation. According to Michael and John, the presence of chloride ions reduces the growth of organisms. Interestingly the results obtained during investigation revealed the presence of $CaCO_3$ enhances the growth as well as production of protease whereas $CaCl_2$ reduces the activity.

The protease activity was enthused in presence of Ca^{2+} ions in the medium. These results suggest that these metal ions impart thermal stability and plays vital role in maintaining the active conformation of the enzyme (Manachini et al, 1988). Addition, of Ca^{2+}, Mg^{2+} and Zn^{2+} resulted in high protease production (Table 1). Metal ions such as Zn^{2+} often have a structural role, while notably Ca^{2+} are necessary for regulation of the activity (Michael & John, 2006). Among the heavy metal ions tested Fe^{2+}, Cu^{2+}, K^{+} caused decrease in the production at 1mM while at 3mM concentration of Fe^{2+}, Cu^{2+}, and K^{+} could enhance the protease production in terms of their activity. This observation was confirmed by previous studies, which suggested inhibitory action in presence of Cu^{2+}, k^{+} and Fe^{2+} on proteases production (Norazizah et al, 2005). Even though effects of the different concentrations of metal cations on protease production vary such as Na^{+}, Co^{2+} and Ba^{+}, their presence in the culture medium improved the growth of *B. licheniformis* JX849145 while more or less specify its protease activity.

Table 1: Effect of metal ions (mM) on protease production by *B. licheniformis* JX849145.

Metal ions (mM)	Residual activity (570 nm)				Protein content (mg/ml)				Amino acid Content (µg/ml)				Protease activity (U/mg/min)			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
$MgSO_4$	0.921	1.129	1.021	0.623	2.0	2.45	2.2	1.3	1.8	2.6	1.8	0.9	0.90	1.06	0.81	0.69
$FeSO_4$	0.231	0.424	0.567	0.212	0.5	0.9	1.25	0.45	0.2	0.5	1.1	0.2	0.40	0.55	0.88	0.44
$CaCO_3$	0.928	1.329	1.521	1.102	2.0	2.9	3.3	2.4	1.9	2.8	4.0	2.2	0.95	0.97	1.21	0.91
$CaCl_2$	0.872	0.898	0.939	0.667	1.9	1.95	2.05	1.45	1.2	1.8	1.6	0.9	0.63	0.92	0.78	0.62
NaCl	0.691	0.792	0.503	0.497	1.5	1.73	1.1	1.1	0.7	1.1	0.6	0.4	0.47	0.63	0.54	0.36
$CoCl_2$	0.723	0.683	0.630	0.597	1.6	1.5	1.3	1.3	1.1	1.1	0.8	0.6	0.69	0.73	0.61	0.46
$BaCl_2$	0.841	0.993	1.090	0.812	1.85	2.15	2.4	1.75	1.1	1.3	1.2	0.8	0.59	0.60	0.50	0.46
$CuSO_4$	0.212	0.294	0.339	0.129	0.45	0.65	0.75	0.25	0.1	0.3	0.4	0.1	0.22	0.46	0.53	0.40
KCl	0.431	0.440	0.469	0.437	0.95	0.95	1.02	0.95	0.2	0.2	0.4	0.3	0.21	0.21	0.39	0.31
$ZnSO_4$	0.929	1.212	1.003	0.859	2.0	2.65	2.2	1.87	1.8	2.7	1.6	1.3	0.90	1.01	0.72	0.69

A: 1 mM, B: 2 mM, C: 3 mM and D: 5 mM

3.2 Optimization of divalent metal ion concentrations by Plackett-Burman design

As Plackett-Burman design is preliminary step to find the mutual interaction of process variables, therefore during investigation, three different metal ions such as $MgSO_4$, $CaCO_3$ and $ZnSO_4$ were used to find the superior combination at variable concentration. These selected metal ions have showed the maximum protease production during study. Hence there may be a possibility that integrated metal ions may leads to protease production. It was analyzed by Plackett-Burman design. The obtained response from design showed the effective combination for protease production was 2 mM of $MgSO_4$, 3mM of $CaCO_3$ and 2mM of $ZnSO_4$ resulting in 1.32 U/mg/min protease activity (Table 2).

Table 2: The Plackett-Burman design for three variables with actual value along with the observed protease activity.

Concentration (mM)			Response
$MgSO_4$	$CaCO_3$	$ZnSO_4$	protease activity (U/mg/min)
2	2	3	0.97
3	2	2	0.86
2	2	2	1.20
3	3	3	0.98
3	2	3	1.03
3	3	2	1.02
2	3	3	0.68
2	3	2	1.32
3	2	3	0.93

3.3 Effect of metal ions on protease activity

Storage stability is a prominent factor for commercialization of an enzyme. With the view of increasing the storage stability, the effect of various metal ions on crude protease was investigated. The effect of different metal ions on protease is shown in Table 2. A stronger inhibitory effect was observed in the presence of K^+, Cu^{2+} and Zn^{2+}.

Among all the metal ions used in the present investigation the metal like Hg^{2+} declines the protease activity at 0.5, 1.0 and 2.0mM concentrations as it reduces 69, 74 and 73% of its original activity respectively assayed under standard conditions. The protease production by *Brevibacillus brevis* was also inhibited by Hg^{2+}, Zn^{2+} and Cu^{2+} (Banerjee et al, 1999).

Table 2: Effect of metal ion concentrations on protease activity.

Metal ions (mM)	Protease activity (U/mg/min)			Std. deviation			Percent activity (%)		
	0.5 (mM)	1.0 (mM)	2.0 (mM)	0.5 (mM)	1.0 (mM)	2.0 (mM)	0.5 (mM)	1.0 (mM)	2.0 (mM)
KCL	5.15	5.45	5.2	0.01	0.03	0.03	85	66	83
$CaCO_3$	6.05	8.2	5.7	0.02	0.07	0.03	100	100	90
$MgSO_4$	5.9	5.1	5.3	0.07	0.07	0.03	97	62	84
$FeSO_4$	5.3	5.05	5.8	0.07	0.00	0.03	87	61	92
$ZnCl_2$	5.5	5.9	5.45	0.25	0.03	0.00	90	72	87
$MnSO_4$	5.15	4.0	6.3	0.28	0.00	0.07	85	49	100
$HgCl_2$	1.89	2.15	1.69	0.04	0.00	0.00	31	26	27
NaCl	5.7	5.6	5.05	0.07	0.02	0.02	94	68	80
$CuSO_4$	4.5	5.05	5.2	0.07	0.03	0.03	74	61	83

The inhibitory effect of heavy metal ions is well documented in the literature. It is known that the ions mercury, cadmium and lead react with the protein thiol groups (converting them to mercaptides), as well as with histidine and tryptophan residues (Wellingta et al, 2004). Moreover, by action of silver and mercury, the disulphide bonds were found to be hydrolytically degraded (Kumar et al, 1999).

Figure 2, showed at 0.5 and 1.0 mM concentration of Ca^{2+} demonstrate 100% of protease activity and at 2.0 mM concentration of Mn^{2+} showed highest residual activity.

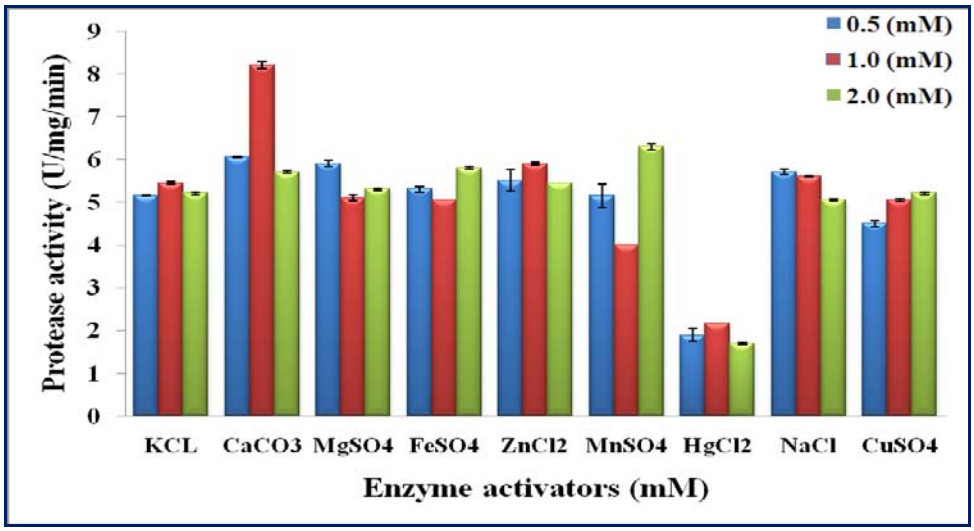

Figure 2: Effect of enzyme activators on protease activity with its standard deviation.

These results suggest that used metal ions apparently protect the enzyme against thermal denaturation and played a vital role in maintaining the active conformation of enzyme at higher temperatures (Beg & Gupta, 2003). The effect of Mn^{2+} on the activity of protease was also observed by Wellingta et al, (2004). This phenomenon indicates that the enzyme requires metal ions as cofactors. The obtained results correlate with the observations of Kunamneni et al (2003) who found that presence of Mg^{2+}, Ca^{2+} and Mn^{2+} increases the enzyme activity by 16%, 35% and 8%, respectively, while Hg^{2+} reduced the protease activity by 7% and has also been reported by Takeda et al, (2000).

The increased rate of proteolysis by proteases at high temperatures is one of the factors responsible for the rapid thermal inactivation of these enzymes (Ghorbel et al., 2003). Most alkaline proteases have been reported to be significantly stabilized by the addition of metal ions at higher temperatures (Ghorbel et al., 2003 & Singh et al., 2001). The improvement in protease thermostability against thermal inactivation in the presence of Ca^{2+} may be explained by the strengthening of interactions inside protein molecules and by binding of Ca^{2+} to autolysis sites (Ghorbel et al., 2003). The present protease was affected positively by the presence of Ca^{2+} ions, at temperatures 45ºC.

Statistical method was applied to check the effect of metal ions against 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 $HgCl_2$ reduce the protease activity. The correlation between 0.5, 1.0 and 2.0 mM concentration of metal ions was analyzed by regression equation and ANOVA (Table 3). The obtained data showed 0.000 of its p value indicates the used concentration of metal ions was significant.

Table 3 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			

S = 7.12505 R^2 = 98.5% R^2 (adj) = 98.0%

The result showed that, 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. The obtained coefficient values showed the designed model was significant.

At conclusive remark, the thermotolerant *B. licheniformis* JX849145 produces a class of thermoalkaline metalloprotease which can be useful for various industrial applications.

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