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## Protease Production from UV Mutated *Bacillus subtilis*

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### Abstract

UV mutation of the strain has significant contribution to enhance the yield of protease enzyme from *Bacillus subtilis* bacteria under the cultivation conditions in submerged fermentation. The fermentation medium used for the production of protease composed of carbon sources 1%, organic 1% or inorganic nitrogen sources 0.5%,  $K_2HPO_4$  0.2%,  $CaCl_2$  0.04% and  $MgSO_4$  0.02% by mutated *Bacillus subtilis* G-4 under the optimum parameters which are important to induce the mutated strain to produce high units of the protease, which were temperature 37.5 °C, pH 9, inoculum size 3% v/v, glucose 1% as carbon source and peptone 1% as nitrogen source were give the maximum  $455.25 \pm 1.66$  units of protease. The results of stability studies revealed that protease of *B. subtilis* G-4 was stable over a broad range of temperature (30 to 60 °C) and pH (8 to 12). However, maximum activity (155.45U/ml) was observed at temperature 50°C and pH 10. These characteristics render its potential use in detergent industries for detergent formulation.

**Key words :** Protease, UV mutation, *B. subtilis*, Parameters

### Introduction

Proteases are one of the industrially most important enzymes. Proteases constitute a large and complex group of enzyme which differ in properties such as specificity, active site and catalytic mechanism, pH, temperature optima and stability profile (Sandya *et al.*, 2004). Proteases are wide spread in nature such as plants, animals and microorganisms (Rao *et al.*, 1998). Microbial proteases can be produced from bacteria, fungi and yeast through submerged and solid-state fermentation (Kumar and Takagi, 1999, (Anwar and Saleemuddin, 2001) and (Haki and Rakshits, 2003). The success of microbial proteases in food and other biotechnological systems could be attributed to the broad biochemical diversity of the microorganisms, to the genetic manipulation of the organisms and the improvement of the techniques in the enzyme production, purification and characterization. Alkaline proteases have wide use in industrial processes such as food, leather, pharmaceutical and detergent formulation and for cleaning of membranes used in protein ultra filtration (Kumar and Takagi, 1999) and (Dayanandan *et al.*, 2003). Protease is one of the most important industrial enzymes occupying nearly 60% of the enzyme sales (Beg *et al.*, 2003; Adinarayan *et al.*, 2003). It is produced mainly by many members belonging to genus *Bacilli* especially, *B. licheniformis*; *B. horikoshii*, *B. sphaericus* (Mehrotra *et al.*, 1999).

Now a days, detergent industries are mainly focused on alkaline protease for its use in all types of laundry detergents

and in automatic dishwashing detergents to degrade proteinaceous stains (Maurer, 2004). The cost of the enzyme is a major issue in enzyme production and their applications in various industrial processes. Therefore utilization of cheap industrial waste has significant impact on enzyme utilization. The present study was conducted to improve bacterial strain through mutation and optimized the conditions such as pH, temperature, size of inoculum and growth medium to enhance enzyme yield to make the process of production cost effective.

### Materials and Methods

#### Microorganism

The parent *Bacillus subtilis* was obtained from Microbiology Lab, Food and Biotechnology Research Centre, PCSIR Labs Complex Lahore. The *Bacillus subtilis* was grown on nutrient agar slant (Oxoid) for 24 hr at 37°C. The culture was then preserved at 4°C and shifting to new slants after 25 days in order to keep viable. The pH of the medium was adjusted at 9 with 1N HCl / NaOH.

#### Bacterial suspension

For suspension preparation, 10 mL of sterile distilled water was added in 24 hour old *Bacillus subtilis* slant and cells were scraped with inoculating loop in Laminar Air Hood,

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(LABNICO). This bacterial suspension was used for UV treatment. The same procedure was used for inoculum preparation of mutated strain and bacterial suspension was used  $108^{-10}$  cfu/mL.

### Mutagenesis by ultraviolet irradiation

In six test tubes with the aforesaid bacterial suspension were taken and one of them (the control) was kept aside in dark and the rest five were given UV radiation (2600 Å) for time periods varying from 5 minutes to 25 minutes. A 0.1 mL of the UV treated bacterial suspensions was then inoculated in 20 mL Petri plates containing nutrient agar. These were incubated for 48 hr at 37°C for colony formation and colonies were counted under colony counter. The screening of the mutant was performed in 20 ml of Petri plates 1% skim milk and 2% agar. After the incubation of 48 hr at 37 °C, the plates were flooded with TCA solution for 5 minutes and the transparent circular zones around the colonies in an opaque white background detected protease secretion. Mutants for hyper-production of protease were detected visually by the intensity of the zones.

### Fermentation of growth medium

The medium used for the production of protease was composed of carbon sources 1%, organic 1% or inorganic nitrogen sources 0.5% ,  $K_2HPO_4$  0.2 % ,  $CaCl_2$  0.04% and  $MgSO_4$  0.02 % . The pH 9 of the medium was adjusted with 1N HCl / NaOH before sterilization at 121°C for 15 min. Three percent (v/v) of 24 h old inoculum suspension was transferred to 50 mL of growth medium in 250 ml Erlenmeyer flask. The inoculated fermentation medium was incubated in water bath shaker (Eyla, Japan) at 150 rpm at 37.5 °C for the 48 hr. After that fermented broth was centrifuged at 4 °C for 10 minutes at 10,000 rpm to get the clear solution.

### Parameters

Various process parameters such as effect of inoculum size (1 to 6 %), various temperatures ranges (25 to 45 °C) and different initial pH (6 to 12) were studied to optimize the level of each parameter for maximum protease production by *Bacillus subtilis* G-4.

### Effect of Nitrogen Sources

The importance of the nitrogen for the microorganism is very crucial and the various sources organic nitrogen such as corn steep liquor, peptone, urea and yeast extract at rate of

1%, where as inorganic nitrogen sources like  $NaNO_3$ ,  $NH_4H_2PO_4$ ,  $NH_4Cl$ ,  $(NH_4)_2HPO_4$ ,  $NH_4NO_3$ , and  $(NH_4)_2SO_4$  were added at rate of 0.5% in medium for the synthesis of enzyme by *Bacillus subtilis* G-4.

### Effect of Carbon Sources

The various carbon sources have different effect on the enzyme production by different microorganism and evaluated the effect of each carbon sources such as glucose, sucrose; fructose, maltose, xylose, sorbitol and galactose at rate of 1% in medium used for protease production.

### Protease activity

Protease activity was determined by a method of (Yang and Huang, 1994). The reaction mixture containing 2 mL of 1 % casein solution in 0.05 M glycine-NaOH buffer (pH=11) and 1 mL of enzyme solution were incubated at 60 °C for 15 min and the reaction was then stopped with the addition of 3 mL of 10 % trichloroacetic acid. After 10 min the entire mixture was centrifuged at 9000rpm for 10 min at 4 °C and absorbance of the liberated tyrosine was measured with respect to the blank at 280 nm. One proteolytic unit (U) was defined as the amount of the enzyme that releases 1 µg of tyrosine per min under assay conditions.

### Stability study

Stability studied of alkaline protease in the presence and absences of metal ions were conducted to find enzyme commercial exploitation.

### Effect of Metal ions on the thermo stability

The thermostability of protease was studied by incubating the enzyme in water bath (Eyla, Japan) at different temperature ranges 30 to 70° C for 1 h in the absence or presence of  $Ca^{2+}$ ,  $Cu^{2+}$  and  $Mg^{2+}$  ions at concentration of 5 mM. After the treatment the enzyme activity was measured according to the standard assay.

### Effect of Metal ions on the pH stability and activity of Enzyme

The pH stability of enzyme was observed at pH 7- 14 for 8 h at 40° C in the presence or absence of  $Ca^{2+}$ ,  $Cu^{2+}$  and  $Mg^{2+}$  at the concentration of 5 mM. Various pH value were adjusted with sodium phosphate buffer (pH 6-7), Tris-HCl buffer (pH 8-9) and glycine NaOH buffer (pH 10-12). After the treatment the enzyme activity was measured according to the standard assay.

**Result and Discussion**

**Improvement of Strain by mutation**

The successful development of the various strains required in the fermentation industry can be improved by mutation. The productivity of the parent strain *Bacillus subtilis* under investigation was improved by UV mutation. The survival data obtained on UV treatment for varying time periods are presented in Table I.

**Table I: Survival rate for *Bacillus subtilis* on UV treatment**

UV light Exposure (minutes)	Number of colony appear	Survival rate (%)
0	3000	100
5	21	0.83
10	12	0.4
15	8	0.26
20	4	0.13
25	1	0.03

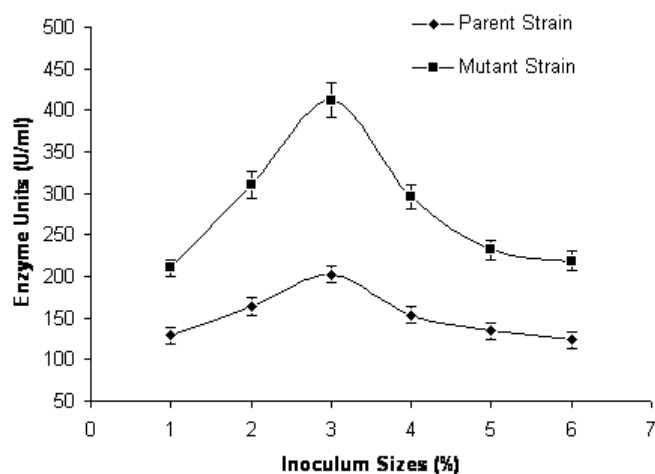
Survival rate (%) was calculated as follows:

For a treatment time (t) Survival (%) =  $100 \times (\text{Colony count obtained for time (t)}) / 3000$

UV treatment for 10 minutes resulted in a strain designated as *Bacillus subtilis*. It was observed that morphologically both parent and mutant strains looked alike. On studying the enzyme production on fermentation medium containing rice polish, it was found that the mutant was active giving approximately about 2 fold increases in protease production over the parent strain (i.e. parent strain gave  $235.23 \pm 1.86$  U/ml whereas  $455.25 \pm 1.76$  U/ml). It was reported that improvement of strain by UV exposure is very useful for the protease production (Gupta *et al.*, 2002).

**Effect of Inoculum size**

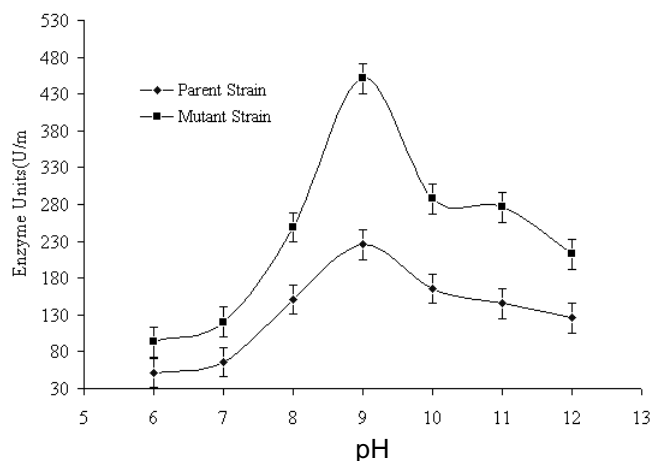
It is generally necessary to optimized age and size of the inoculum, because low density gives insufficient biomass and high density produces too much biomass and resulting depleting of nutrients necessary for protease fermentation. Different inoculum sizes were used and 3% inoculum of mutated *Bacillus subtilis* G-4 gave ( $412.31 \pm 1.45$  U/ml) units of protease (Fig. 1). The researchers were reported that inoculum size has crucial effect in fermentation process through microorganisms (Mangat *et al.*, 1998 ; Hornbeak *et al.*, 2004) Safey *et al.*, (2004) optimized the 24 hr aged inoculum and size  $1.0 \text{ cell/mL}$  ( $7.0 \times 10^3$ ) for the production of protease by *Bacillus subtilis*.



**Fig. 1: Effect of different inoculum size on protease production by parent and mutagenized strains of *Bacillus subtilis* G-4 in submerged fermentation. Bars indicate the SD among triplicates**

**Effect of Initial pH**

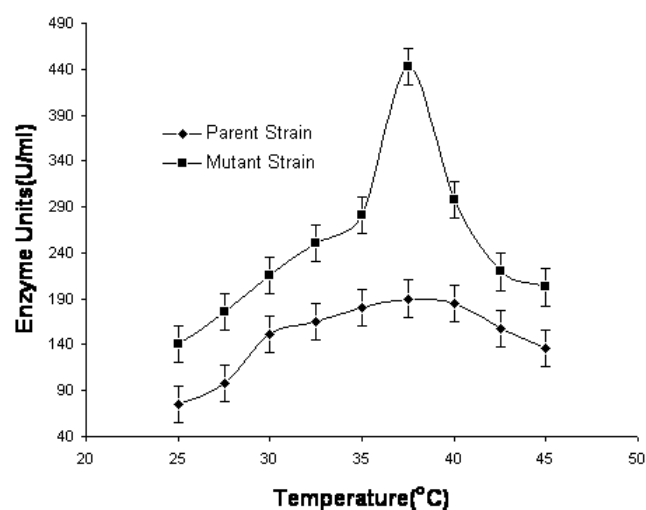
The metabolic activities of the microorganisms were very sensitive for pH variation. The maximum protease activity ( $451.12 \pm 1.85$  U/ml) was found at pH 9 by *Bacillus subtilis* G-4, however a further change in pH decreases the enzyme yield (Fig. 2). Similar finding was observed by (Ul-haq *et al.*, (2006), that maximum protease is obtained at pH 9 for *B.subtilis* IH-72 in a bioreactor. Ali *et al.*, (1998) has prescribed that optimum pH have important role in enzyme yield. It was reported that microorganisms exhibit more than one pH optimum for growth depending on the growth conditions, particularly, metal ions and temperature (Arai *et al.*, 2003).



**Fig. 2: Effect of various initial pH of the medium on protease production by parent and mutagenized strains of *Bacillus subtilis* G-4 in submerged fermentation. Bars indicate the SD among triplicates**

### Effect of Temperature

The maximum protease units  $442.43 \pm 1.75$  U/ml were observed at  $37.5^\circ\text{C}$  (Fig. 3). The same finding was reported by (Banerjee *et al.*, 1999), worked with *Bacillus brevis* in shake flask and found that protease enzyme show the maximum activity at  $37^\circ\text{C}$ . The worker were observed that maximum proteinase production was observed at  $37^\circ\text{C}$ , however, proteinase production was not affected by temperature within the range studied ( $7-45^\circ\text{C}$ ). The researcher (Joo *et al.*, 2003) was reported that  $45^\circ\text{C}$  is optimum temperature for protease production with the *Bacillus horikoshii*. The worker were investigated that production of proteases by *Streptococcus suis* serotype 2 was required optimum temperature ranged from  $25$  to  $42^\circ\text{C}$  (Wery *et al.* 2003; Jobin and Grenier, 2003).



**Fig. 3:** Effect of different incubation temperatures on protease production by parent and mutagenized strains of *Bacillus subtilis* G-4 in submerged fermentation. Bars indicate the SD among triplicates

### Effect of Nitrogen Sources

The macro and micronutrients are very important for the growth of microorganism. The importance of the nitrogen for the microorganism is very crucial. The various organic nitrogen sources such as corn steep liquor, peptone, urea and yeast extract were added at a rate of 1%, where as inorganic nitrogen sources like  $\text{NaNO}_3$ ,  $\text{NH}_4\text{H}_2\text{PO}_4$ ,  $\text{NH}_4\text{Cl}$ ,  $(\text{NH}_4)_2\text{HPO}_4$ ,  $\text{NH}_4\text{NO}_3$  and  $(\text{NH}_4)_2\text{SO}_4$  were added at a rate of 0.5% in medium for the synthesis of enzyme. Among the

organic nitrogen peptone, corn steep liquor and yeast extract were contributed to induce the protease but the peptone was best enhancer in this study (Table II). The inorganic nitrogen sources were less enhancer of protease as comparative to organic. The maximum protease units were observed in harvesting batch which was supplemented with  $(\text{NH}_4)_2\text{SO}_4$ . The researcher were reported that among various complex nitrogen sources, yeast extract and casamino acid were also found the most suitable sources for alkaline protease production in earlier investigations (Prakasham *et al.*, 2006; Rahman *et al.*, 2005). The worker was reported that organic nitrogen sources were better for enzyme production than inorganic ones (Feng *et al.*, 2001). Nadeem *et al.*, (2008) was reported that inorganic nitrogen sources, especially ammonium salts, inhibited the growth and protease production by *B. licheniformis* N-2.

**Table II:** Effect of Various organic and inorganic nitrogen sources on the protease production by *B. subtilis* G-4 in submerged fermentation at  $37.5^\circ\text{C}$  for 48 hour with agitation 150 rpm.

Organic nitrogen	Enzyme production (U/mL)	
	Parent strain	Mutant strain
Control	$155.34 \pm 1.12$	$224.34 \pm 1.18$
Corn steep Liquor	$185.56 \pm 1.21$	$275.86 \pm 1.31$
Peptone	$225.67 \pm 1.41$	$425.97 \pm 1.51$
Urea	$85.58 \pm 1.01$	$155.48 \pm 1.01$
Yeast extract	$125.69 \pm 1.14$	$215.24 \pm 1.21$
Beef extract	$105.47 \pm 1.21$	$194.43 \pm 1.25$
Inorganic nitrogen	Enzyme production (U/mL)	
	Parent strain	Mutant strain
Control	$155.34 \pm 1.12$	$224.34 \pm 1.18$
$\text{NaNO}_3$	$125.46 \pm 1.01$	$214.94 \pm 1.16$
$\text{NH}_4\text{H}_2\text{PO}_4$	$95.57 \pm 1.12$	$182.34 \pm 1.08$
$\text{NH}_4\text{Cl}$	$125.58 \pm 1.21$	$193.39 \pm 1.12$
$(\text{NH}_4)_2\text{HPO}_4$	$92.69 \pm 1.11$	$169.74 \pm 1.15$
$\text{NH}_4\text{NO}_3$	$107.97 \pm 1.21$	$202.34 \pm 1.28$
$(\text{NH}_4)_2\text{SO}_4$	$175.56 \pm 1.23$	$278.38 \pm 1.36$

Values are the average of three replicates.  $\pm$  denotes the standard deviation among triplicates.

### Effect of Carbon Sources

The various carbon sources have different effect on the enzyme production by different microorganism. The effect

of different carbon sources such as glucose, sucrose, fructose, maltose, xylose, sorbitol and galactose were studied at rate of 1% in medium and results were indicated that glucose was best protease enzyme enhancer in *Bacillus subtilis* G-4 (Table III). The similar findings were observed by many other worker that glucose has significant effect on protease production (Nadeem *et al.*, 2008; He *et al.*, 2003; Shafee *et al.*, 2005). Some other researchers were also found a considerable increase in alkaline protease production by *Bacillus sp.* with glucose as a carbon source compared to control (without external carbon source) (Adinarayana *et al.*, 2003; Prakasham *et al.*, 2006).

**Table III: Effect of Various Carbon sources on the protease production by *B. subtilis* G-4 in submerged fermentation at 37.5°C for 48 hour with agitation 150 rpm.**

Carbon sources	Enzyme production (U/mL)	
	Parent strain	Mutant strain
Control	145.42 ± 1.18	208.98 ± 1.23
Glucose	185.74 ± 1.32	324.24 ± 1.38
Sucrose	135.16 ± 1.11	234.74 ± 1.26
Fructose	85.57 ± 1.02	142.34 ± 1.08
Maltose	92.48 ± 1.01	153.34 ± 1.12
Xlose	93.69 ± 1.15	165.44 ± 1.17
Sorbitol	75.47 ± 1.08	143.64 ± 1.12
Galactose	78.56 ± 1.03	138.28 ± 1.21

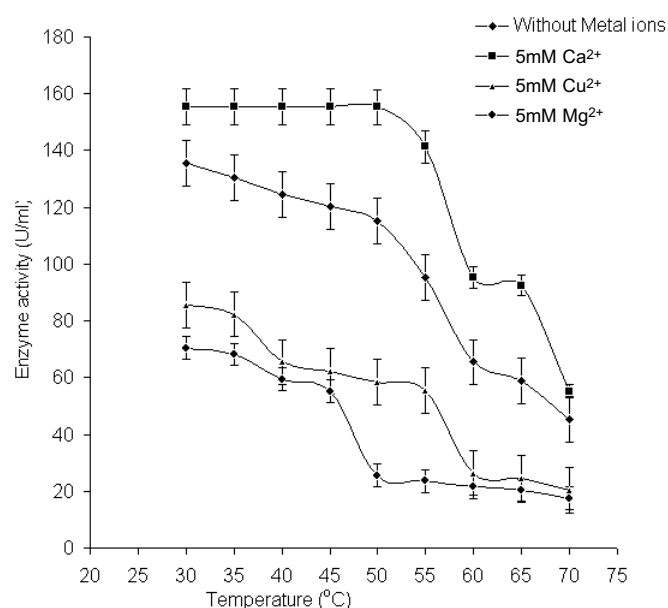
Values are the average of three replicates. ± denotes the standard deviation among triplicates.

### Stability study

#### Effect of Metal ions the thermo stability

The thermo stability of alkaline protease was examined by measuring the residual activity at 40 °C after incubation of the enzyme without substrate for various temperatures ranging from 30 to 70 °C in the presence of Ca<sup>2+</sup>, Cu<sup>2+</sup> and Mg<sup>2+</sup> ions and along with substrate for 30 min at 40 °C (Fig. 4). The enzyme was observed to be stable up to 50 °C and above this temperature its activity was decreased. The enzyme showed its 100% activity at 50°C and 61 % at 60 °C in the presence of metal ions. Johnvesly *et al.*, (2002) has found that 70 °C was optimum temperature for protease activity which was produced from thermoalkaliphilic *Bacillus sp.* JB-99. Lee *et al.*, (2002) has reported that, the optimum tem-

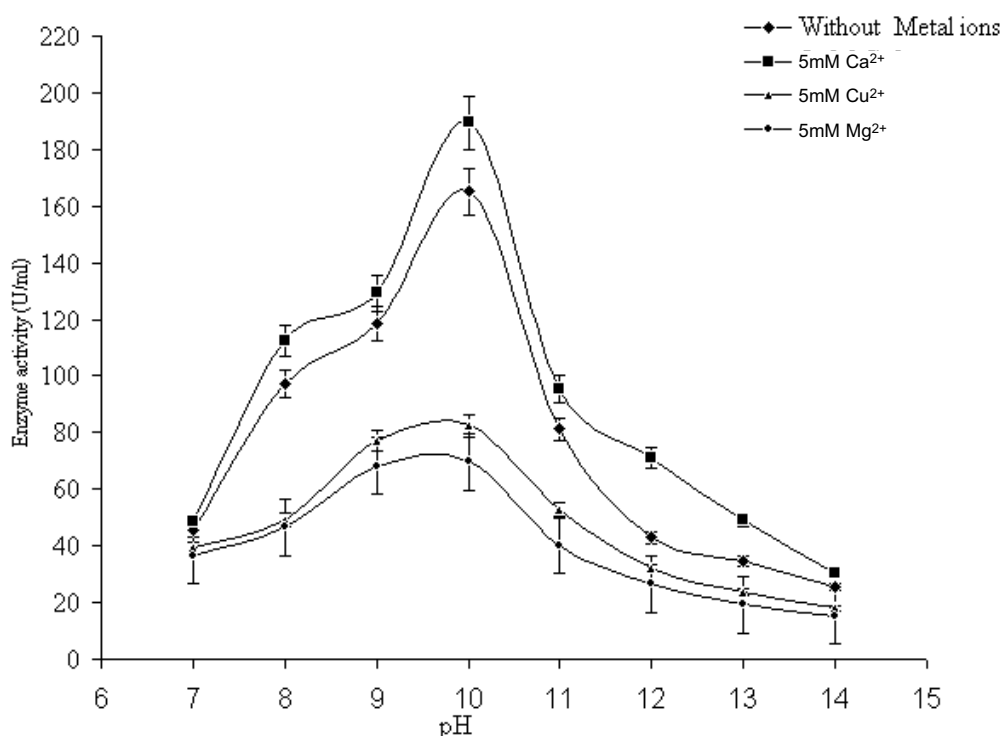
perature of protease has ranged from 40 to 50 °C. Other workers have observed that highest activity of extracellular alkaline protease produced from the alkalophilic bacterium *Alcaligenes faecalis* was exhibited at 55°C (Berla and Suseela, 2002). Ammar *et al.*, (2003) has reported that, the optimum temperature for thermostable purified protease enzyme was 55 °C. In the finding of Nadeem (Nadeem *et al.*, 2008) it was indicated that 5mM Ca<sup>2+</sup> increased the stability on alkaline protease.



**Fig. 4: Effect of different metal ions on the thermostability of protease produced by mutagenized strains of *Bacillus subtilis* G-4 in submerged fermentation. Bars indicate the SD among triplicates**

#### Effect of pH on the stability

These results of pH stability studied showed that enzyme was stable at pH 10 and lost 50 % of its residual activity at pH 11 (Fig. 5). The pH level is one of the factors that affect not only structure of enzymes but all proteins. The pH values beyond the range of 8-11 could alter the three-dimensional structure of alkaline protease by disturbing the electrostatic interactions among the charged amino acids, resulting in loss of enzyme activity. Similar results were reported by (Balassa *et al.*, 2000), who found 60 % proteolytic retention at pH 10 in the presence of 5 mM Ca<sup>2+</sup> ions. It was reported that the optimal pH for purified extracellular alkaline protease produced



**Fig . 5: Effect of different metal ions on the pH stability of protease produced by mutagenized strains of *Bacillus subtilis* G-4 in submerged fermentation. Bars indicate the SD among triplicates**

from the alkalophilic bacterium *Alcaligenes faecalis* was 9.0 (Berla and Suseela, 2002). Lee, *et al.*, (2002) has reported that, the optimum pH of purified protease was pH 8. Nadeem *et al.*, (2008) reported that 5mM Ca<sup>2+</sup> increased the stability of alkaline protease.

The results of stability studied showed that alkaline protease produced by *Bacillus subtilis* G-4 was stable over range of temperature and pH (8 to 11) in the presence of metal ions. The properties indicated that it can be used as a potential ingredient in detergent formulation.

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