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Original Research Article

Strain Improvement by UV Mutagenesis for Protease Overproduction from *Bacillus subtilis* E6-5 and Nutritional Optimization

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ABSTRACT

The purpose of the present study was to enhance the protease production of the parental type *Bacillus subtilis* E6-5 by UV irradiation. The parental type was subjected to UV irradiation at different distances (5-15 cm) for different time intervals (1-120 min). After each treatment, total 400 mutants were obtained. The mutants were screened on skim milk agar plates for the selection of best proteolytic mutant. Among mutants, the mutant MET39, which obtained at 15 cm distance and irradiation time 5 min of exposure, was selected as best mutant produced 1.5 fold more enzyme over the parent strain. The effects of nutritional factors (various carbon, nitrogen sources and metal ions) on the protease production from MET39 mutant strain were studied. The best carbon source was found as glycerol. Among the inorganic nitrogen sources, the highest enzyme production was obtained in the presence of tryptone. The metal ions did not indicate significant effect on enzyme production. In order to enhance the yield, new modified medium was obtained by combining the best carbon and nitrogen sources. In this medium, enzyme yield was enhanced 88% compared to basal medium. MET39 mutant strain might have a great potential for protease production at industrial scale.

Keywords: Protease, *Bacillus*, UV, Mutant, Nutritional Factors, Optimization

INTRODUCTION

Proteases, or proteolytic enzymes is the term for enzymes that decompose proteins by hydrolyzing their peptide bonds. Protease is one of the most important industrial enzymes due to having some biotechnological interests. It account for about 60% of the total worldwide sale of enzymes, and is widely used in several industries that include leather processing, meat processing, dairy, preparation of organic fertilizer, silk industry, and also for the recovery of silver from used X-ray films (Javed *et al.* 2013, Chellappan *et al.* 2006). It have reported that many microorganisms are capable of producing proteases, such as *Aspergillus oryzae* (Belmessikh *et al.* 2013), *Aureobasidium pullulans* (Chi *et al.* 2007), *Serratia marcescens* (Bach *et al.* 2012) and *Bacillus subtilis* (Auther *et al.* 2012, Contesini *et al.* 2018). A large proportion of commercial proteases are produced by *Bacillus* strains (Kumar *et al.* 1999, Gupta *et al.* 2002). Among these, *Bacillus subtilis* is the most important group in the enzyme industry (Boominadhan *et al.* 2009). In recent years, attempts have been made for the overproduction of microbial enzyme by induced mutagenesis (Singh *et al.* 2011).

Mutagenesis is generally induced via physical, chemicals and other radioactive emitting substances. Physical and chemical mutagens are promising and are used for screening of high yielding strains (Sreeju *et al.* 2011). Physical mutagen like UV irradiation and chemical mutagens are more overwhelming due to their simplicity and low cost procedure as compared to DNA recombinant technology (Munazzah *et al.* 2012). Ultraviolet radiation (UV) is one of the well - known and most commonly used mutagen (Basavaraju *et al.* 2014). It gives a high proposition of pyrimidine dimmers and includes all types of base pair substitutions (Rani *et al.* 2012). UV- light has also been reported to be mutagenic in variety of organisms (Singh *et al.* 2011). The correlation between the quantity of energy absorbed by DNA and the observed biological effects are illustrated inthe wavelength region between 254 and 320 nm (Prabhakaran *et al.* 2000).

It is universally used to induce genetically improved strains. (Sanchez *et al.* 1992). Mutation is the basic tool to improve the efficiency of microbe for alkaline protease production (Wang *et al.* 2007, Nadeem *et al.* 2010). For example, protease activity was increased by about 4-fold in *Bacillus stearothermophilus* by chemical mutagenesis (Zamost *et al.* 1990). *B. subtilis* S1-4 was selected, which exhibited 2.5-fold higher extracellular caseinolytic activity than did the parental strain. Wayne and Belinda (2003), Wang *et al.* (2016) revealed that *B. anthracis* spores may be three to four times more resistance to UV than spores of commonly used strains of *B. subtilis*.

On the other hand, it is a well-known fact that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources (McKeller and Cholette 1984, Jamel *et al.* 2011). Because, enzyme formation is largely dependent on the condition of growth of the culture and composition of nutrient medium.

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The present study describes enhanced protease production by the bacterium *Bacillus subtilis* E6-5 was improved through mutation by radiations (UV). The hyper-producer strain of protease was selected, and the optimization of the nutritional requirements for enzyme production were carried out.

MATERIALS AND METHODS

Organism and culture maintenance

In this study, *Bacillus subtilis E6*, which was isolated in our previous study, was used for mutagenesis. It was maintained on nutrient agar medium containing (g L-1); nutrient broth 8.0, NaCl 8.0, agar 20.0, pH 7.0. The strain was stored at 4°C.

Preparation of cell suspension

A loopful culture of *Bacillus subtilis E6-5* was transferred into 30 mL sterilized nutrient broth, and incubated at 37 °C for 18 h with agitation speed of 150 rpm. 10 mL of the inoculated broth was centrifuged at 10.000 rpm at 4 °C to get the cells in pellet form. The cells were re-suspended into the 10 mL sterilized normal saline (0.9 %) to prepare the cell suspension containing approximately 10^{9-11} cells mL-1.

UV mutagenesis

Mutagenesis of *Bacillus subtilis* E6-5 was carried out using different exposure times and radiation intensities. Various serial dilutions of pre-incubation period the culture (10^{-1} to 10^{-9}) of cell suspension were prepared in sterilized saline, and 1 mL of 10^{-9} dilution was inoculated on to skim milk agar plates. For adaptation to the reproduction environment, the petri dishes were incubated during the periods of 6, 8, 10 hours at 37°C. These plates were irradiated with UV irradiations at different distances (5, 10 and 15 cm) for different time intervals (from 1 to 120 min). A 30-w germicidal lamp at 254 nm (VL-130.G, Vilber, Germany) provided the UV irradiation. UV studies were carried out in the dark to prevent photoreactivation. After UV exposure experiments the plates were incubated for 24h at 37°C, and the appearing number of colonies on each plate was counted. Each colony was assumed to be formed from a single spore. Proteolytic activities of mutants were detected on the basis of appearance of clear zones around the bacterial colonies. The diameters of the clear halo-zone were measured by millimeter ruler. The strain showing the largest protease zone was selected, and used in further studies.

The lethality rate was evaluated based on the following equation (Zong *et al.* 2012);

$$\text{Lethality rate} = (U\text{-}T)/U \times 100\%$$

where U is the total colony count of the sample without treatment, and T is the total colony count after treatment with UV.

Protease production

Production of protease was performed in the medium containing (% w v-1) glucose-0.1, peptone-1, yeast extract-0.02, $MgSO_4$-0.01, $CaCl_2$-0.01, K_2HPO_4-0.05 (pH 7.0) (Qadar *et al.* 2009). Glucose was sterilized and added separately to the flasks. The precultures were cultivated in Nutrient Broth medium (0.8% w v-1) for 18 h. Then, overnight cultures with optical density of 0.3 at 600nm were inoculated at 1% in enzyme production media (150 mL in 500 mL erlenmeyer flasks) and incubated at 37 °C for 18, 24, 28, 32, 48 and 56 h in a shaking incubator at 150 rpm. At the end of each period, the cultures were centrifuged (6000 rpm, 10 min) and the supernatants were used for the determination of proteolytic activity. Bacterial biomass was determined by measuring optical density at 600 nm.

Enzyme activity

Total protease activity was measured using casein as substrate by the modification of the Anson Method (Keay and Wildi 1970). 1 mL of the culture supernatant was mixed with 1 mL 0.05 M phosphate buffer-0.1 M NaOH (pH 7.0 adjusted with phosphoric acid) containing 2% casein, and incubated for 10 min at 37 °C. The reaction was stopped by adding 2 mL 0.4 M Trichloroacetic acid. After 20 min stand at 37°C, the precipitate was removed by centrifugation at 6000 rpm for 10 min. 1mL of the supernatant was treated with 5 mL 0.4 M $NaCO_3$ and 1 mL of diluted Folin–Ciocalteu reagent (1:3). After 20 min of waiting in the dark at room temperature the optical density of the sample was measured at 660 nm. A standard curve was generated using solutions of 0–60 µg mL-1 tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg mL-1 tyrosine under the experimental conditions used.

Effect of nutritional factors on protease production

Various carbon sources such as fructose, sucrose, maltose, glycerol, potato starch, corn starch, wheat bran and wheat starch evaluated for their effect on protease production by replacing glucose in the basal medium (0.1% w v-1).

Organic nitrogen sources chosen for the study were tryptone, meat extract, skim milk, and corn step liquor by replacing peptone and yeast extract (total 1.02 % w v-1); and inorganic nitrogen sources were NH_4NO_3, KNO_3, $(NH_4)_2SO_4$, $NaNO_3$ and NH_4Cl_2. These nitrogen sources were used to replace the organic and inorganic source available in the basal medium.

The culture medium was supplemented with the following metal salts such as $LiSO_4$, $FeSO_4$, KCl, NaCl, $MnSO_4$ and $ZnSO_4$ replaced with $MgSO_4$ and $CaCl_2$ (total 0.02% w v-1). The each flask was inoculated with 1% inoculum, and incubated at 37 °C. Protease activity and biomass were tested.

A new medium including the best conditions with nutritional factors for protease production were improved, and the mutant was grown in this modified medium for 28 h. Protease yield was recorded, and compared with basal medium.

Results are means of three independent determinations.

RESULTS

Selection of best-proteolytic mutant

In the present study, it was determined that the 10 h of adaptation period to the medium was suitable. A parental strain of *Bacillus subtilis* E6-5 was exposed to UV irradiations from 1 to 120 min. Colony development was not observed fo long exposure times of UV. The lethality rate 90% was observed after exposure of 5 min and 15 cm distance. From UV treated plates, total 400 mutants were obtained. Among mutants one showed high proteolytic activity (12 mm), more than 100 mutants showed moderate activity (10-9 mm), more than 100 mutants showed weak activity (6-8 mm), (Table 1). *Bacillus subtilis* mutant MET39 was found as the highest zone of clearance (12 mm) comparable to parental type strain (8 mm) (Figure 1).

Table 1. Protease zone diameters of mutants after UV exposure.

Distance (cm)	**Time (min)**	**Adaptation Period (hour)**					
		6		8		10	
		Number of Mutant Colonies	**Zone Diameter**	**Number of Mutant Colonies**	**Zone Diameter**	**Number of Mutant Colonies**	**Zone Diameter**
5	1	>20	8	>20	8	>20	8
	5	>20	10	10	9	10	9
	10	>20	6	4	8	4	8
	15	17	6	1	7	1	7
	20	10	7	1	6	1	6
	30	colony was not observed		colony was not observed		colony was not observed	
10	1	14	10	15	7	>20	8
	5	4	7	6	10	18	10
	10	2	10	3	10	15	9
	15	2	8	1	10	1	8
	20	1	9	1	9	1	6
	30	colony was not observed		colony was not observed		colony was not observed	
15	1	10	10	>50	8	35	6
	5	1	10	>20	7	15	9
	10	1	7	8	8	**5**	**12**
	15	1	6	7	8	6	10
	20	1	6	1	9	12	10
	30	colony was not observed		colony was not observed		colony was not observed	

*Parental type: Zone Diameter 8 mm.

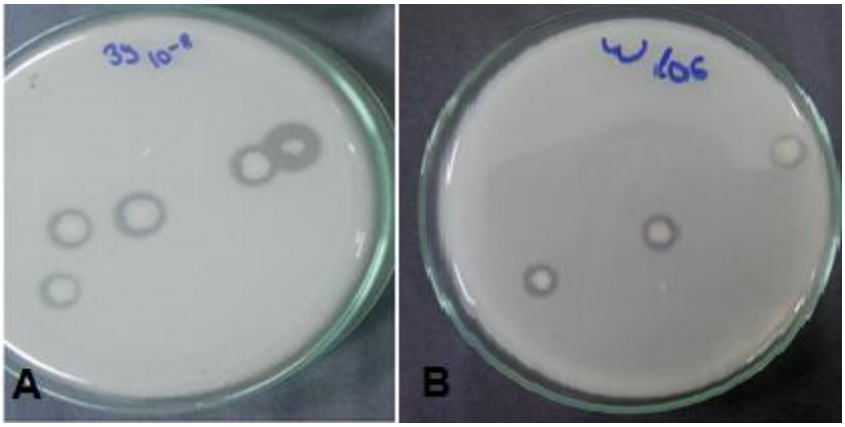

Figure 1. Protease zone diameters of mutant MET39 (A) and parental type (B) on skim milk agar medium.

Protease activity of mutant

Protease activity of mutant strain MET39 activity was checked for quantitative test in liquid media. The protease activity of parental type (67 IU mL-1) was attained after 28 h, while for the mutant MET39 was 80 IU mL-1 (Table 2). Maximum enzyme production was achieved in the middle of the logarithmic phase of parental type and mutant strain. Biomass was slightly increased. Further studies was continued with this hour.

Table 2. Enzyme activity and biomass of parental strain and mutant MET39 during different production period of times (18-56h). Values are shown as means of triplicates.

Time	E6-5 (parental type)		MET39	
	IU mL -1	**OD_{600}**	**IU mL -1**	**OD_{600}**
18	48	0,2	50	0,27
24	64	0,26	65	0,36
28	**67**	**0,32**	**80**	**0,42**
32	65	0,4	59	0,48
48	29	0,68	31	0,6
52	23	0,67	27	0,58
56	9	0,65	25	0,56

Optimization of nutritional factors

The nutritonal factors (various carbon, nitrogen sources and metal ions) were optimized to obtain high levels of protease by MET39 mutant. When glucose e in basal medium was replaced by various carbon sources, glycerol was the best source for protease production (Figure 2). The protease production was affected by carbon sources in the order: glycerol >wheat bran > sucrose (control) > fructose > wheat starch=corn starch=maltose>potato starch. Patato starch (52 IU mL-1) caused reduced protease synthesis. The increase in enzyme production was almost parallel to the growth rate for all carbon sources (48 h). The bacterial growth was affected by carbon sources in the order: Wheat bran> glycerol> fructose > sucrose (control) >wheat starch=corn starch>maltose>potato starch. Glycerol (114 IU mL-1) increased the production of protease by 42,5% when compared to basal medium with glucose (80 U mL-1). The enzyme production and bacterial growth were not parallel. Bacterial growth was low, while enzyme yield was higher in the presence of glycerol.

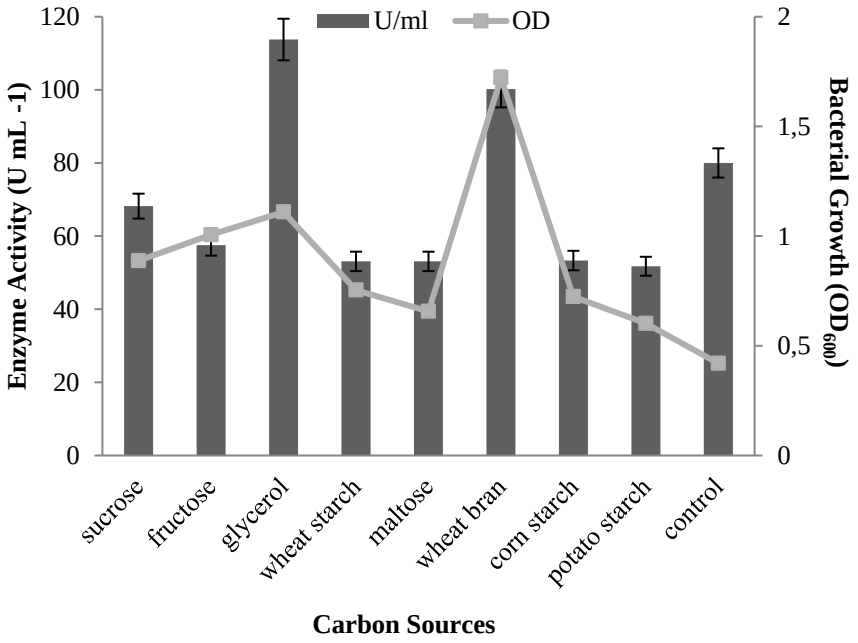

Figure 2. Effect of carbon sources on protease production by MET39. Results are means of three independent determinations. Bars correspond to standard deviation.

Effects of various organic and inorganic nitrogen sources on production of protease were investigated (Figure 3). Results obtained showed that the best nitrogen source for protease production was tryptone (348 IU mL-1) at 48 h and enzyme yield was 4,4 times increased compare to control medium (80 IU mL-1). Corn steep liquor, yeast and meat extracts are also effected for enzyme productions (Figure 3). But, bacterial growth was not parallel with enzyme production, only tryptone was noted as the same.

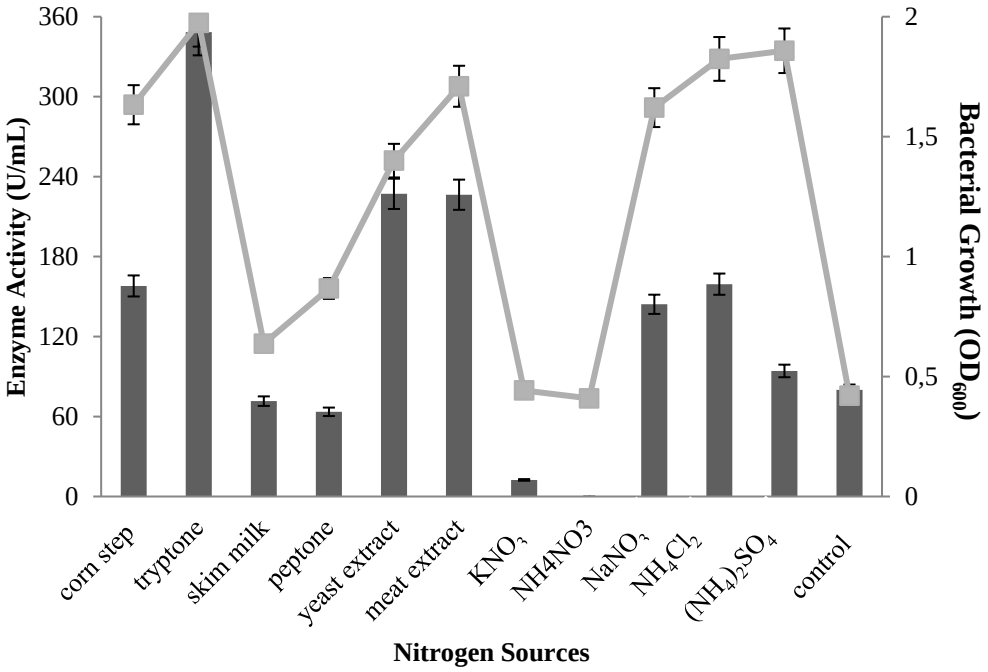

Figure 3. Effect of organic and inorganic nitrogen sources on protease production by MET39. Results are means of three independent determinations. Bars correspond to standard deviation.

Maximum protease production in organic nitrogen sources were tryptone> yeast extract> meat extract> corn step liquor> control> skim milk>peptone; for inorganic nitrogen sources were NH_4Cl_2 > $NaNO_3$ > $(NH_4)_2SO_4$ > KNO_3. Among inorganic nitrogen sources NH_4Cl_2 (159 IU mL-1) was best than others. But $(NH_4)_2SO_4$ and KNO_3 drastically inhibited protease production. The increase in enzyme production was not parallel to the bacterial growth rate for inorganic nitrogen sources.

Metal ions in media are an important factor that affects enzyme production due to act as inducers. Effects of each metal ion sources seperately on production of protease were investigated. In basal medium as a control Ca^{2+} and Mg^{2+} ions were combined. However metal ions did not promote protease production compare to control. In the presence of only $MgSO_4$ (78 IU mL-1) enzyme yield was almost same with control (80 IU mL-1). But, there was no positive effect of others salts. The metal ions in basal medium (control) showed better enzyme production than all tested metal ions (Figure 4).

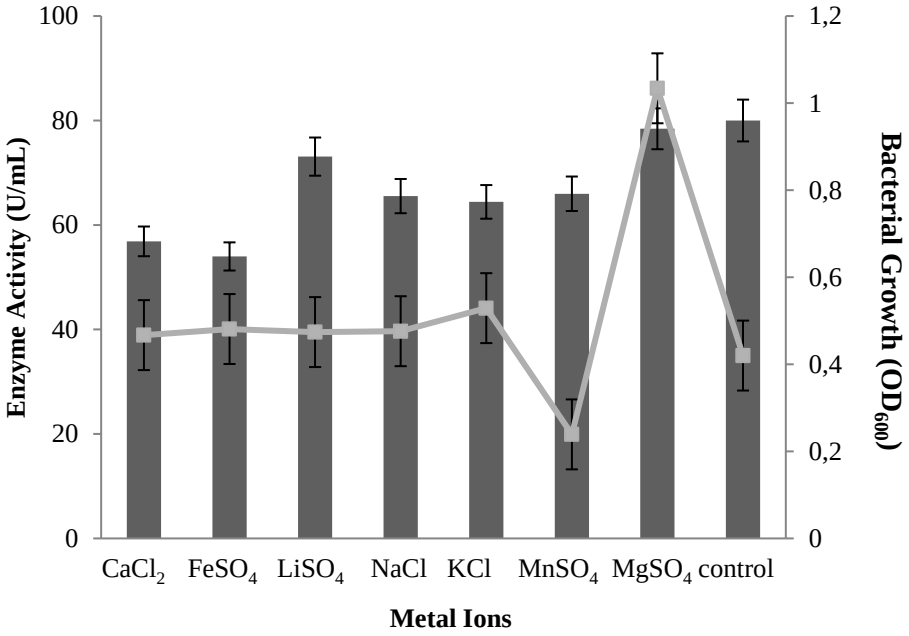

Figure 4. Effect of metal ions on protease production on protease production by MET39. Results are means of three independent determinations. Bars correspond to standard deviation.

In this study, nutritional factors were optimized for enhanced production of protease by mutant MET39 strain. Modified medium was established by selecting the best carbon, nitrogen and metal ions regarding to the results obtained. Glycerol as the best carbon source, tryptone as the nitrogen source and $MgSO_4$ + $CaCl_2$ as the metal ions were used in the modified medium. By optimizing the nutritional factors of protease production from MET39 strain was enhanced 88% enzyme yield as compared to basal medium (control). Growht was also increased (Figure 5).

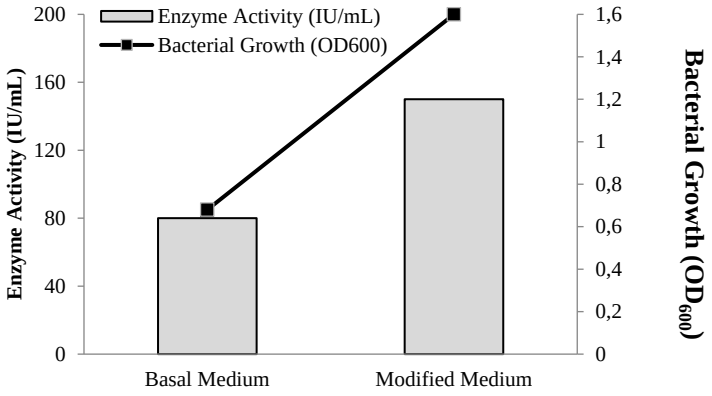

Figure 5. Protease activity and bacterial growth in modified and basal medium.

DISCUSSION

Mutation and screening of industrially useful microorganisms are very important for the successful development of various strains which are required in the fermentation industry (Dutta and Bannerjee 2006). Rao *et al.* (1998) emphasize that mutagenesis plays an important role in increasing protease yield by conventional methods or by recombinant DNA technology. It has been reported that *Bacillus subtilis* exhibits thousands of fold increases in enzyme activity through mutagenesis. Therefore, the parental strain *Bacillus subtilis* E6-5 was treated with UV irradiation to improve its productivity. In the case of UV irradiation treatment 10 % survival rate was obtained after 5 minutes with 15 cm distance. 400 *Bacillus* sp. mutants were obtained from UV exposure of *Bacillus subtilis* E6-5. Between these mutants one mutant has highest zone diameter of protease, and identified as *Bacillus subtilis* MET39. Solaiman *et al.* (2005) reported that the best mutant strains of *B. pumilus* was obtained at 20 min period of UV exposure time with distance of 5 cm. Sanchez *et al.* (1992) found that germinated spores of *B. megaterium* requires less UV exposure than *B. subtilis* spores for the formation of thymine dimers. Wang *et al.* (2016) obtained the *Bacillus subtilis* S1-4 mutant with UV mutation of 24 hours. In another study of Nadeem *et al.* (2010) the main strain of *Bacillus pumilus* was exposed to UV in the range of 30-120 minutes. A minimum survival rate of 1.95% was observed after exposure to UV radiation at 254 nm for 120 minutes. In this study 1,2 fold increase was observed in protease production on agar from mutant *Bacillus subtilis* MET39 compare to parental type. treated *Bacillus licheniformis* N-2 with the combination of UV, NTG (N-methyl-N-nitro-N-nitrosoguanidine) and MMS (methyl methane sulfonate) and obtained 9 protease positive mutants, and the proteolytic mutant strain showed 1.4 times higher protease activity than the parental strain. It has been reported by Shikha and Darmwal (2007) that *Bacillus pantotheneticus* has a 1.4-fold increase in alkaline production relative to the parental type.

Enzyme formation is largely dependent on the condition of growth of the culture and composition of nutrient medium. The present investigation was aimed at optimization of medium components which have been predicted to play a significant role in enhancing the production of protease. The environmental factors are showing great influence in the growth of the organisms and the production of enzymes. The present study was mainly focused on the production of protease form mutant MET39 by optimizing various nutritional factors such as carbon and nitrogen sources and also metal ions. The addition of carbon source in the form of either monosaccharides or polysaccharides could influence the production of enzyme (Sudharshan *et al.* 2007).

In this study, the medium containing glycerol as carbon source increased the protease activity 42% compare to the control. Usharani and Muthuraj (2010) reported *Bacillus laterosporus* to be capable of utilizing a wide range of carbon sources; the best carbon sources for protease secretion were soluble starch, trisodium citrate, citric acid and glycerol. The other findings were observed by some other workers that glucose has significant effect on protease production (Nadeem *et al.* 2008, Shafee *et al.* 2005). In another study, lactose was found to be an ideal carbon source among the selected carbon sources that yielded maximum biomass of 160 mg mL-1 while glycerol 129.3 mg mL-1.

The best nitrogen source was found to be tryptone, and enzyme production was increased approximately 4,4 times fold compare to basal medium (control). Additionally, enzyme activity was increased almost 2.8 times fold with yeast extract and meat extract. As inorganic nitrogen source NH_4Cl_2 was found 99% increase. This was

followed with $(NH_4)_2SO_4$ as 80%. This finding is in agreement with findings of Naidu and Devi (2005) who found that the best nitrogen source for protease production by *Bacillus* sp. was tryptone while beef extract and yeast extract was comparable. On the other hand Wang and Hsu (2005) reported that tryptone was found to increase protease production for a *Bacillus* strain who found out that casein and peptone were better nitrogen sources for protease production by *Prevotella ruminicola*. Raju and Divakar (2013) reported that they obtained 2 times more protease producing mutants in their mutation studies with UV radiation, ethyl methane sulphonate (EMS) and ethidium bromide with *Bacillus cereus*, and thatmutants with mutant carbon sources, such as *Bacillus cereus* GD55, peptone as an organic nitrogen source and NH_4NO_3 as an inorganic nitrogen source. Mabrouk *et al.* (1999) reported the highest level of protease production in the presence of lactose and glucose. On the other hand, *Prevotella ruminicola* (Wang and Hsu 2005) and *Pseudomonas aeruginosa* (Rahman *et al.* 2007) were found to produce the best protease in the presence of casein, peptone and yeast extract. Dutta *et al.* (2006) found that the mutant *Pseudomonas* sp. has the maximum activity from RAJR 044 and showed best protease activity in the presence of dextrose, maltose, ammonium sulphate and potassium.

On the other hand, metal ions in media are an important factor that affects enzyme production due to act as inducers. The effects of some metal ions on protease activities were investigated. But, in present studies showed that metal ion addition to the fermentation medium does not effect in enzyme activity. When $MgSO_4$ was used alone in the basal medium, the enzyme activity was found as same as control. Mrudula *et al.* (2012) has been reported the similar result with $MgSO_4$.

In the study, the efficiency of this mutant was further optimized by optimizing the breeding environment. A new modified medium was formed by using glycerol as a carbon source, tryptone as a nitrogen source and $MgSO_4 + CaCl_2$ as a metal ion. In this new modified medium, mutant strain MET39 showed 88% more protease production than the parental type strain.

Mutants showed different responses to UV radiation for alkaline protease production and these variations are more probably due to the differences induced in their genetic background. It is suggested that the increase in enzyme productivity might result from damage of genes located on plasmids which have a negative influence on the chromosomal alkaline protease production genes i.e. repression as reported by Solaiman *et al.* (2003). Both our results and those of other reserachers have been shown that the protease production patways of *Bacillus* strains and its mutans and other bacteria were very different.

Biotechnology are using for developing new effective enzymes with improved properties. Here, we reported that improvement of strain by UV exposure is very useful for the protease production. These mutant may have wide industrial application.

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