



## Induction of Alkaline Protease Production by *Bacillus* Mutants Through U.V. Irradiation

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### ABSTRACT

Proteases are well known enzymes for their wide range application in food industry, detergent industry and pharmaceuticals industry. They are also widely used in leather industry for dehairing and bating of hides as an alternative for toxic chemicals which in turn hamper the environment. In the present investigation bacteria were isolated from the leather industry soil sample. A total of 8 bacteria were isolated from soil by serial dilution method on gelatin agar medium. Among eight, three *Bacillus* sp. showing highest skim milk hydrolysis were selected for the production of alkaline protease and were named as isolate - 3, isolate - 5 and isolate - 7. The effect of pH, temperature, incubation period, carbon sources and nitrogen sources on production of alkaline protease was investigated by one factor-at-a-time method. The enzyme activity was observed maximum at pH 8 and temperature 37°C for all *Bacillus* isolates. Among the three *Bacillus* isolates, *Bacillus* isolate - 7 had maximum enzyme activity than other two isolates. *Bacillus* isolate - 7 exhibited the highest alkaline protease activity (3.32U) on submerged fermentation. The wild strain *Bacillus* isolate 7 was subjected to strain improvement through U.V irradiation. Induction of mutation in *Bacillus* strain was carried out by 0, 5, 10, 15 and 20 min exposure times of U.V irradiation. The wild type exhibited 3.45U protease activity where as mutant *Bacillus* isolate 7 showed 4.78 U protease activities.

**Keywords:** Alkaline protease, *Bacillus*, Mutagenesis, U.V Radiation.

### INTRODUCTION

Alkaline proteases are of great interest because of their high proteolytic activity and stability under alkaline conditions.<sup>1,2</sup> These enzymes find applications in detergents, feather processes, food processing, silk gumming, pharmaceuticals, bioremediation, biosynthesis and biotransformation.<sup>3</sup> Furthermore, fibrous proteins such as feather, hair are abundantly available in nature as waste. This waste can be converted into useful biomass, protein concentrate or amino acids using proteases from certain microorganisms. Proteases in detergent formulations facilitate washing efficiency of detergents by releasing proteinaceous stains. The majority of commercial alkaline proteases are produced by bacteria, especially *Bacillus* sp. and *Streptomyces*.<sup>4</sup>

Strain improvement is an essential part of process development for fermentation products. Improved strains can be achieved by inducing genetic variation in the natural strain and subsequent screening. Ultraviolet radiation is one of the well known and most commonly used mutagen. It is universally used to induce genetically improved strains. The aim of this study was to examine the effect of UV irradiation on alkaline protease production and induction of overproducing *Bacillus* strains. The promising strains in this study may be used in various economic industrial applications and for successive genetic improvement as well.

### MATERIALS AND METHODS

#### Isolation of protease producing microorganisms

Soil samples were collected from leather industry, at Common Effluent Treatment Plant, Varadayapalam and transported to laboratory under sterile conditions. Bacteria were isolated from soil by serial dilution technique on nutrient agar medium. 1g of soil sample was taken and was serially diluted up to 10<sup>-7</sup> dilution. 0.1ml aliquots of 10<sup>-4</sup>, 10<sup>-5</sup>, 10<sup>-6</sup> dilutions was spread onto the medium and incubated at room temperature for 24hr. A total of 8 bacterial isolates from enriched samples were streaked on skim milk agar medium containing pH-8 and incubated at 37°C for 24hr. After 24hr of incubation, plates were flooded with 1% tannic acid.

Isolates having a higher ratio of clearing zone to colony size were grown in liquid broth and the amount of protease production was determined from culture filtrate. The isolate which showed higher protease activity was selected and maintained on nutrient agar slants and sub cultured every fifteenth day. Selected isolates were identified based on morphological and biochemical characteristics.

#### Alkaline Protease Production medium

For the production of protease enzyme, three isolates were inoculated into the production medium. The culture production medium used in this work for protease production (g/l) contained Glucose - 15g, Urea - 20g, KH<sub>2</sub>PO<sub>4</sub> - 2g, K<sub>2</sub>HPO<sub>4</sub> - 2g, MgSO<sub>4</sub>.7H<sub>2</sub>O - 1g, CaCl<sub>2</sub> - 1g and casein - 1g maintained at 37°C for 24 to 72hr in a shaking



incubator (140rpm). At the end of each fermentation period, the whole fermentation broth was centrifuged at 2000rpm at 4°C for 15min, and the clear supernatant was used as crude enzyme preparation.

### Alkaline protease activity assay

Alkaline protease activity was determined by applying the method given by Meyers and Ahearn<sup>5</sup> with some modifications. According to this procedure 0.5 ml of Glycine NaOH buffer (0.2M, pH - 10) was added to 0.5 ml of appropriately diluted enzyme and was incubated with 1 ml of 1% casein solution (prepared in glycine NaOH buffer, pH - 10) for 15 min at 60°C. The reaction was stopped by the addition of 4ml of 5% (v/v) trichloro acetic acid. The contents were centrifuged after 1hr at 3000x g for 10 min and the filtrate was used for measuring protease activity on the basis of color change. 5 ml of 0.4 M Na<sub>2</sub>CO<sub>3</sub> solution was added to 1 ml of the filtrate and was kept for 10min. 0.5 ml of Folin-Ciocalteu's Phenol reagent of 1:1 dilution was added and kept in dark for 30 minutes at room temperature. The optical densities of the solutions were read against the sample blanks at 660nm using spectrophotometer. One alkaline protease unit (U) was equivalent to the amount of enzyme required to release 1mg/ml/min of tyrosine under the defined assay conditions.

### International units (IU)

One protease unit was defined as the amount, of enzyme that released 1 µg of tyrosine per ml per minute under the above assay conditions.

### Optimization of culture conditions for enzyme production

The cultural conditions (pH, temperature, incubation period and different sources of C and N) were optimized for maximum enzyme production using yeast extract casein medium<sup>6</sup> containing (g/L) glucose (10), casein (5), yeast extract (5), KH<sub>2</sub>PO<sub>4</sub> (2), and Na<sub>2</sub>CO<sub>3</sub> (2). The medium was incubated for 48-72 hr on incubator shaker.

### Effect of pH on alkaline protease production

The effect of pH on alkaline protease production from bacterial isolates under study was determined by growing each species in fermentation media having different pH using appropriate buffers, Tris HCl buffer (pH 8.0 - 11.0). Alkaline protease production was measured and monitored at 6 hr interval.

### Effect of temperature on alkaline protease production

The effect of temperature on alkaline protease production was studied by growing each bacterial isolate in fermentation media at different temperatures (30, 37, 40, 45°C). Alkaline protease production was monitored at 6hr intervals over a 72hr fermentation period through assay of alkaline protease activity.

### Effect of incubation period

Alkaline protease production and alkaline protease activity was measured and monitored at 24hr interval over 96hr fermentation period.

### Effect of different carbon sources on alkaline protease activity

For evaluation of effect of carbon source on enzyme production in culture media, 5 different carbon sources including dextrose, lactose, maltose, sucrose were added (10 g/L) to a basic culture medium.

### Effect of Different Nitrogen Sources on alkaline protease activity

For evaluation of effect of nitrogen source on enzyme production in culture media, Yeast extract, Peptone, Beet extract, soya bean were added (5 g/L) to a basic culture medium.

### UV Mutagenesis

Ultraviolet (UV) irradiation as physical mutagenic agent was used for over production of alkaline protease. Mutagenesis of bacterial strains was carried out according to Justin *et al.*<sup>7</sup> using different exposure times and radiation intensities i.e., distances from irradiation sources. Bacterial cells were grown in LB broth medium overnight with shaking at 37°C. A sample of each culture (ca. 10<sup>6</sup> cells/ml) was centrifuged, resuspended in saline solution (0.9% NaCl). Two ml of bacterial suspension was placed into 15 cm diameter glass Petri dishes at a distance of 15cm in dark and irradiated for 0, 5, 10, 15 and 20 min with gentle agitation, the suspension was exposed at distances of 5 and 10 cm for 10 min. A 30-w germicidal lamp T8 (260 nm) provided the UV irradiation. Portions of 0.5 ml of suitable dilutions of bacterial strain was spread in five LB plates and incubated at 37°C for 24hr. Colonies developed after incubation were counted and transplanted onto slants for further studies. The survival percentages were estimated for each treatment.

### Isolation of alkaline protease over producing mutants and enzyme assay

For isolation of overproducing mutants after UV irradiation, developed colonies were inoculated onto LB agar medium plus 1% skim milk under alkaline conditions and incubated at 37°C for 40 hr, the assayed for enzyme production according to Smith *et al.*<sup>8</sup> Depending upon the zone of clearance, mutants with high activity were selected on the basis of the relative growth production (C/G) of the bacterial colonies. Three measurements were used to determine the enzyme assay,

- 1) Enzyme production defined as lyses zone area, mm<sup>2</sup> (C).
- 2) Colony growth area, mm<sup>2</sup> (G), and
- 3) Relative growth production (C/G).



The enzyme activity of superior alkaline protease producing mutants was assayed according to the method of Udandi Boominadhan *et al.*<sup>9</sup> using GYP medium (Glucose yeast extract peptone medium). One unit of the enzyme activity is defined as amount of enzyme producing 1 $\mu$ g of tyrosine under standard assay conditions and expressed as unit ml<sup>-1</sup> of enzyme.

## RESULTS

### Isolation of protease producing microorganisms

In the present study, 8 bacterial isolates were isolated on nutrient agar medium (Figure 1) and was screened for alkaline protease production by inoculating them in skim milk agar medium (Figure 2) Physical and biochemical test showed that the organisms were *Bacillus* sp. Among which three *Bacilli* sp. showing maximum skim milk hydrolysis were selected for the production of alkaline protease and were named as isolate - 3, isolate - 5 and isolate -7.



Figure 1: Isolation of protease producing microorganisms



Figure 2: Skim milk agar hydrolysis by *Bacillus* isolates

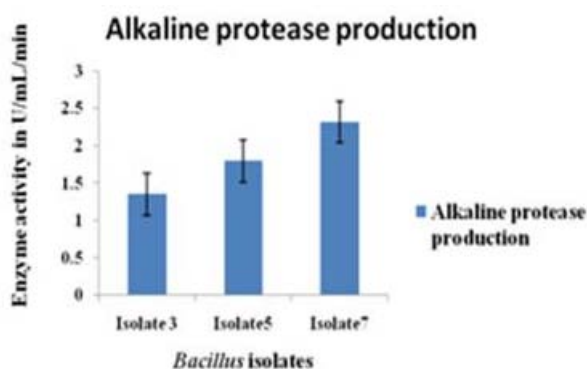


Figure 3: Alkaline protease production Each value is an average of 3 replicate samples.  $\pm$  Standard error.

### Enzyme Assay

Alkaline protease production by selected three *Bacilli*, *Bacillus* isolate -3, *Bacillus* isolate - 5 and *Bacillus* isolate - 7 was determined. Among the three isolates, *Bacillus* isolate-7 had maximum enzyme activity than other two isolates. *Bacillus* isolate-7 exhibited the highest alkaline protease activity (3.32U) on submerged fermentation (Figure 3).

### Optimization of culture conditions for enzyme production

The production of alkaline protease enzyme was affected with pH change of the medium. The alkaline protease enzyme activity was found maximum at pH 8.0 (1.56 U). The enzyme activity was decreased slowly above pH 8 and at pH 7 (Figure 4). The production of alkaline protease enzyme was highest at 37 $^{\circ}$ C (1.92U) for *Bacillus* isolate-5, the alkaline protease production decreased significantly at 40 $^{\circ}$ C (Figure 5). The enzyme activity was increased from 2<sup>nd</sup> day of incubation to 4<sup>th</sup> day and it is optimum (1.8U) on 72hr of fermentation at regular temperature 37 $^{\circ}$ C, pH 8.0 and it is gradually decreased on 96hr of incubation (Figure 6). The study of alkaline protease production with various carbon sources in the medium showed that the enzyme production was more with glucose (3.32U) (Figure 7). The remaining isolates exhibited less enzyme activity than *Bacillus* isolate 7 in medium supplemented with different carbon sources. The selected *Bacillus* isolate 7 grown well with all nitrogen sources tested. Casein was the best among all nitrogen sources tested with 3.37U alkaline protease activities (Figure 8). The remaining isolates exhibited less enzyme activity than in *Bacillus* isolate 7 medium supplemented with different nitrogen sources.

### Detection, isolation of UV mutants and their alkaline protease activity

The wild strain *Bacillus* isolate 7 was subjected to strain improvement (Figure 9). The numbers of survivals from each exposure are represented in Table 1 The wild type exhibited 3.45U protease activity where as mutant *Bacillus* isolate 7 showed 4.78U protease activities (Table 2 and 3). *Bacillus* mutant isolate7 showed 28% more protease production than the wild type strain.

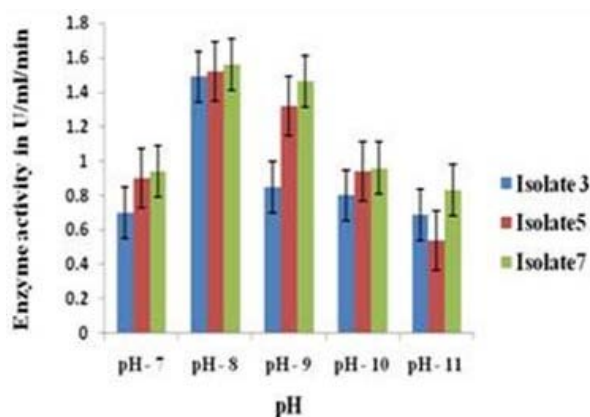
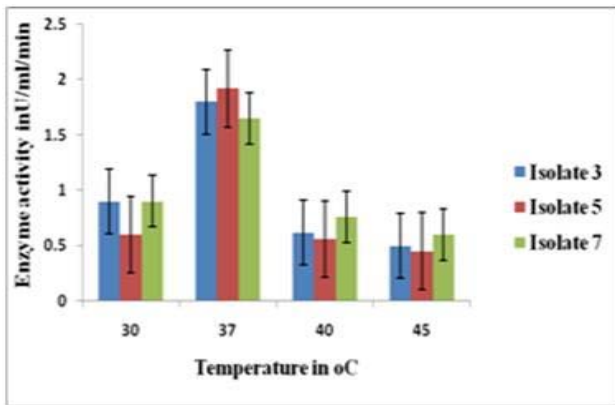
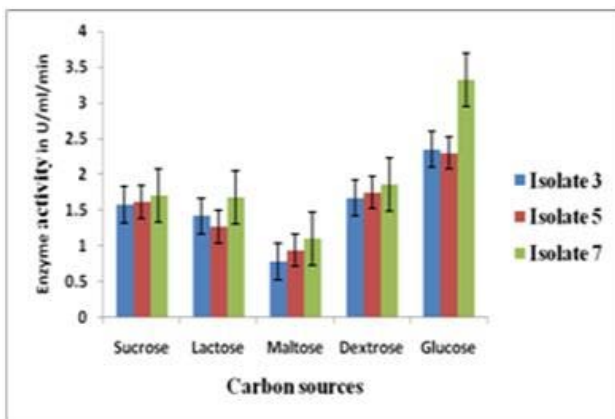


Figure 4: Effect of pH on alkaline protease production

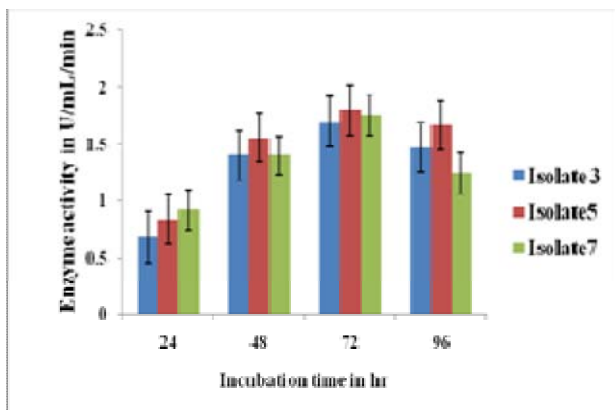


**Figure 5:** Effect of Temperature on alkaline protease production



Each value is an average of 3 replicate samples. ± Standard error.

**Figure 6:** Effect of incubation period on alkaline protease production



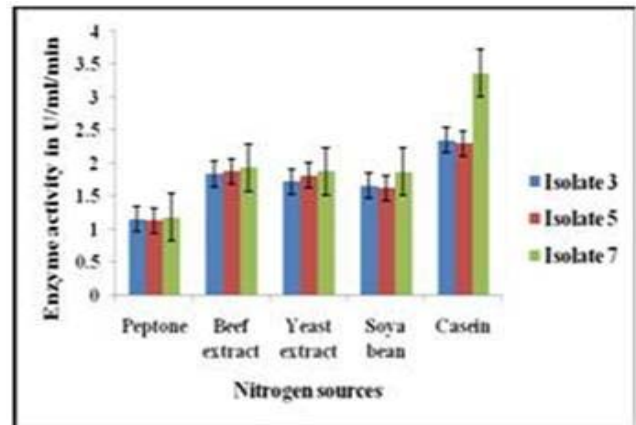
Each value is an average of 3 replicate samples. ± Standard error.

**Figure 7:** Effect of different carbon sources on alkaline protease production

**DISCUSSION**

Alkaline protease producing bacteria were isolated from leather industry soil and identified as *Bacillus* isolates. The identified bacterial isolates were plated on the skim milk agar plates at pH 8 and incubated at 37°C for 24hr. Clear zone of skim milk hydrolysis was observed after addition of 1% tannic acid. Among the three isolates, *Bacillus* isolate 7 had maximum enzyme activity than other two isolates. Maximum growth and maximum enzyme

production was observed at 72hr when cultured in casein containing medium (pH 8) under shaking condition (~150 rpm) at 37°C. In order to determine the influence of pH and temperature the production medium was adjusted to various levels of pH. The organism was found to grow over a wide range of pH and temperatures. *Bacillus* isolate 7 showed enzyme production at pH 8.0. Al-Shehri *et al.*<sup>10</sup> have reported the protease production by *B. licheniformis* at pH 8.0. Majority of *Bacillus* sp. showed growth and enzyme production under alkaline conditions. Several alkaline proteases have been purified and characterized from many *Bacillus* strains.<sup>11, 12</sup>



Each value is an average of 3 replicate samples. ± Standard error.

**Figure 8:** Effect of Different Nitrogen Sources on alkaline protease production

**Table 1:** Survival percent of *Bacillus* isolate 7 after UV exposure

Irradiation time (min)	Number of cells/ml after irradiation	Survival Percent	Kill percent
0 (control)	15.2 x 10 <sup>5</sup>	100	0
5	150 x 10 <sup>4</sup>	60.75	39.25
10	180 x 10 <sup>3</sup>	7.19	81.51
15	500 x 10 <sup>3</sup>	3.59	90.63
20	400 x 10 <sup>2</sup>	2.33	97.37

**Table 2:** The alkaline protease activity of the wild and mutant *Bacillus* isolates 7

Bacterial Type	Enzyme activity (U/ml/min)
Wild type <i>Bacillus</i> isolate 7	3.45
Mutant <i>Bacillus</i> isolate 7	4.78

**Table 3:** The alkaline protease production (C/G) of the *Bacillus* isolates 7 Mutant

Mutant	G	C	C/G
<i>Bacilli</i> isolate 7	25.4	658.34	25.91

G: Colony growth area (mm<sup>2</sup>); C: Lyses zone area (mm<sup>2</sup>)

The optimum temperature for alkaline protease production by *Bacillus* isolates was found to be 37°C (although it grows and produces alkaline protease in the range of 30 to 40°C). Protease production was found to



be highest at 72hr incubation period. The addition of carbon source in the form of either monosaccharide's or polysaccharides could influence the production of enzyme.<sup>13</sup> Among the carbon sources, glucose was found to support protease production. The similar findings were observed by many other workers that glucose has significant effect on protease production.<sup>14,15</sup> The best nitrogen source for the *Bacillus* isolates was casein for protease production. This finding is in agreement with findings of Wang and Hsu<sup>16</sup> who found out that casein and peptone were better nitrogen sources for protease production by *Prevotella ruminicola*. Some of the researchers 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.<sup>17,18</sup> The wild type *Bacillus* isolate 7 exhibited 3.45U protease activity where as mutant *Bacillus* isolate 7 showed 4.78U protease activities. Mutation can play an important role in improving the yield of alkaline protease with excellent dehairing effect.<sup>19</sup> 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.*<sup>20</sup> In the present study mutated *Bacillus* strains showed 28% more protease production than the wild type strain. It was reported that improvement of strain by UV exposure is very useful for the protease production<sup>21</sup>. Djamel *et al.*<sup>22</sup> reported hyper-producing acid protease UV mutant (S08M4) that produced 1400U/ml in liquid fermentation. UV mutation was also used for production of alkaline protease from *Bacillus* sp.<sup>23</sup> *Bacillus* strain was engineered for improvement of fibrinolytic enzyme yield through the rounds of protoplast fusion (genome shuffling).<sup>24</sup> The applications in detergent industries require their improved stability at elevated temperatures and pH and compatibility with various oxidants and metal ions.<sup>25,26</sup>



**Figure 9:** Alkaline protease activities of *Bacillus* isolate -7 mutants on skim milk agar medium

## CONCLUSION

The results obtained confirmed that UV-mutagenesis technique is an important tool in *Bacillus* improvement for increasing production of alkaline protease enzyme.

Thus, the selected *Bacillus* mutant has potential in biotechnological applications.

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