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# Enhancement Production of Fibrinolytic Enzymes from Some Bacillus Strains by UV Mutagenesis

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**Abstract:** Bacillus strains are the most promising enzyme producing microorganisms and are important source for extracellular enzymes. Fibrinolytic enzymes are recognized as impotent to dissolve fibrin clots to treat heart attack patients. Bacillus strains which produce such enzyme are considered as potent sources in microbial biotechnology. Improvement production of enzymes of Bacillus strains by UV mutagenesis has been found an efficient procedure. In this work, 4 isolated and identified bacillus strains preliminary screened for their proteolysis enzyme activity. UV mutation was done at different periods of times, 15 single colonies were obtained. Mutant bacterial strains were assayed Qualitatively and quantitatively for its production of fibrinolytic enzymes, results proved that increasing in enzyme activity by most mutants than their original wild type strains. All the strains enhanced enzyme activity and production of fibrinolytic enzymes after UV mutation comparing with the wild strains.

**Key words:** Bacillus · Proteolytic Enzyme · UV mutation

## INTRODUCTION

Fibrinolytic enzymes are enzyme used to dissolve fibrin clots, therefore cardiovascular diseases such as heart attack and stroke can be treated. Cardiovascular diseases (CVDs) are the one of the leading causes of death worldwide due to the formation of fibrin clot in the unbroken blood vessel walls accumulation wall or accumulation in it. Production of thrombolytic agents were carried out from many organisms, like, microorganisms and animals but microorganisms are identified as the potent sources of it due to the large quantity of production using fermentation methods [1]. Enzymes that produced from microbes serve as a safe and used in insufficiency of human enzymes. These enzymes are recognized as preferred source of industrial enzymes because in a short period, they can be produced in large quantities. They also have shorter generation times and by using genetic manipulation techniques on bacterial cells can increase the enzyme production [2]. Fibrinolytic enzymes in the bacteria dissolve the fibrin clots and are evidently used as thrombolytic agents. These enzymes have been detected in various bacteria, the most important among which is the genus *Bacillus*. Improvement of microbial strain by either conventional mutagenesis (UV or chemical exposure) or recombinant DNA technology can generate mutants exhibiting higher peptidase production in microorganisms. In bacteria, serine and metallo-proteases are the principal classes of proteases found in several species such as *Bacillus subtilis, Bacillus amyloliquefaciens, Pseudomonas sp., Lysobacter enzymogenes* and *Escherichia coli* [1]. The enzyme assay method for alkaline protease production from *Bacillus licheniformis NCIM-2042* was optimized [4], they also suggested that the enzyme is a serine alkaline protease. The efficiency of

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*Bacillus* alkaline protease enzyme production proved to be correlated with various levels of plasmid curing [5, 6]. Classical mutation and/or selection techniques, together with cloning and protein engineering strategies, have been exploited to develop *Bacillus* alkaline protease enzyme productions [7]. UV irradiation was used to induce genetically improved strains. Ultraviolet radiation is one of the earliest known and most commonly used as mutagen. It has differing effects on DNA, RNA and other molecules, such as enzymes, within the cell. DNA and RNA are the most vulnerable to UV damage due to their unique function and because of their highly complex structure and large size [8]. Determination of the effect of ultraviolet radiation alkaline protease production in *Bacillus pumilus* and *Bacillus alvei* was done [9]. They proved that the effect of UV was decreased by increasing the time of exposure while it increased by decreasing the distance of exposure. Results also revealed that the enzyme activity assayed under submerged conditions was more efficient than that obtained by the relative growth production (C/G) method because there is not always a correlation between zone diameter and ability to produce enzyme in submerged culture. Mutation induction will be done on the best available strain(s) to obtain mutants with high efficiency for enzyme production. Transformation change in alkaline protease gene expression after plasmid transfer technique. Up to seven and half times alkaline protease activity was found in transformants comparing with their recipient strain production. Protoplasts fusion is prepared by treating bacteria with a lytic enzyme such as lysozyme that removes the cell wall. Because of this treatment, the cell content would be enclosed only by the cell membrane [10]. The protoplasts have to be preserved in a hypertonic medium for their osmotic stability and survival. Then, in the presence of a fusogenic agent such as polyethylene glycol (PEG), protoplasts are induced to fuse and form transient hybrids. During this hybrid state, the genomes may re-assort and genetic recombination can occur [11]. So far, an increasing number of recombinant strains have been formed. Improvement the production of alkaline protease in *Bacillus* strains through protoplast technique was studied [12]. They found that all obtained fusants showed higher efficiencies in enzyme productivity where up to five times activity was found in fusants comparing with their parental strains production. The aim of this study is screening fibrinolytic enzymes production in some bacillus strains and study the effect of UV irradiation in fibrinolytic enzyme production with the best producing strain.

## MATERIALS AND METHODS

**Bacterial Strains and Cultivation:** Bacillus strains was isolated and identified by 16S rDNA sequences. Bacillus strains were cultured in Luria-Bertani broth medium (LB) containing (in g/L): peptone, 10.0; yeast extract, 5.0; NaCl, 5.0; agar 20 with pH adjusted to 7.2. Four bacterial strains were used in this study for fibrinolytic enzyme activity, 2 *Bacillus Subtilis*, *Bacillus thuringiensis* and *Bacillus licheniformis.*

**Spectrophotometric Screening of Fibrinolytic Enzyme Production by Bacterial Strains:** Spectrophotometric measurements of Fibrinolytic enzyme were carried out by Xiao *et al.* [13]. Proteolytic activity was assayed using casein as reaction substrate [13]. Reaction mixture was prepared by mixing 1 mL enzyme sample with 1 mL of 2% (w/v) casein in 20 mM phosphate buffer (pH 7.2). Following incubation at 55°C for 10 min, 2 mL of ice-cold 0.4 M trichloroacetic acid was added and then immersed in ice water for 20 min. After centrifugation at 10000×g for 10 min, the supernatant was mixed with 1ml Folin-Phenol reagent (0.33 M) and 5 mL Na2CO3 (0.4 M) solution. The mixture was incubated at 40°C for 20 min and the optical density of the produced color was measured at 660 nm using spectrophotometer.

**Fibrinolytic Enzyme Activity by Inhibition Zone Method:** Inhibition zone method was carried out according to Hassanein *et al.* [14], collected samples were plated onto casein agar plates containing (g/l): bacteriological agar 15, casein 5, peptone 5 and yeast extract 1. Plates were then incubated 24 h at 37°C. A clear zone gave an indication of proteolysis. Different colonies from the plates were purified through repeated streaking on fresh agar plates. Purified colonies were finally streaked onto fibrin agar plates composed of (g/l): fibrin 5, ammonium sulfate 2, CaCl2 1, K2HPO4 0.1, KH2PO4 0.1, MgSO47H2O 0.2 and agar 18, pH 8.0. Isolates, which formed a clear zone around their colonies, were selected.

**UV Irradiation:** UV irradiation method was done to *Bacillus subtilis* A strain, the bacterial liquid cultured were exposed to 280 nm short wavelengths UV light from 20 cm. Irradiation times were at 15, 30, 45, 60, 75, 105,120 min.

**Mutant's Selection:** After UV irradiation, 100 UL from each exposing time were spread over LB plates. All the plates were incubated 24 hr in dark environment at 37°C.

Then, only one colony appeared after 15 and 30 and 60 min, two colonies appeared after 45, 75, 105 and 120 min. and 3 colonies at 90 min appeared. Appeared and selected colonies re-streaked over LB plates.

**Screening of Enzyme Production after UV Irradiation:** Selected colonies from each exposing time was assayed for enzyme activity as described above [13], by spectrophotometer measurement and by disk inhibition methods as described by Hassanein *et al.* [14].

## RESULTS

Fibrinolytic enzymes assayed at 660 nm of all used strains are listed in (Table 1), *Bacillus licheniformis* strain was 0.104, *Bacillus thuringiensis* was 0.089, *Bacillus Subtilis* A was 0.160 and *Bacillus Subtilis* B was 0.089, the best strain was *Bacillus Subtilis* A, followed by *Bacillus licheniformis*, *Bacillus Subtilis* B and *Bacillus thuringiensis* respectively. (Fig. 1), also represents bacterial samples plated over casein agar plates, results also indicated that *Bacillus Subtilis* A produced good inhibition zone over casein agar plates.

Table 1: Fibrinolytic enzyme assay produced by studied bacillus strains

| No | Strains | Absorbance at 660 nm |
|---|---|---|
| 1 | *Bacillus licheniformis* | 0.104 |
| 2 | *Bacillus thuringiensis* | 0.069 |
| 3 | *Bacillus Subtilis* A | 0.160 |
| 4 | *Bacillus Subtilis* B | 0.089 |

**Spectrophotometric Screening of Fibrinolytic Enzyme Production after UV irradiation:** UV mutation of *Bacillus Subtilis* A was done, after irradiation period at 15, 30, 45, 60, 75, 105,120 min respectively. All the plates were incubated 24 hr in dark environment at 37°C. 15 colonies produced after irradiation periods, one colony produced after incubation after 15, 30 and 60 min, two colonies appeared after 45, 75, 105 and 120 min. and 3 colonies at 90 min. Results shown that all produced colonies were high efficiency fibrinolytic enzyme than the wild type strain (Table 2). The best produced mutant colony after 90 min. exposure time 90 A and 90 C followed by 75 A, 90 B, 45A and 45B respectively. All other mutant colonies were between 0.375 to 595 enzyme activities. The lowest mutant production was after 120 min.

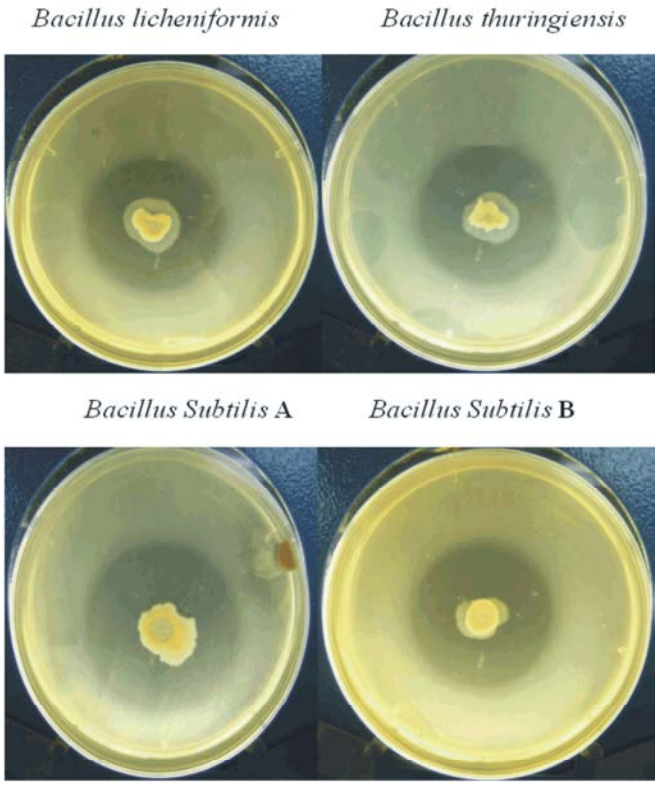


Fig. 1: Inhibition zone produced by Bacillus strains onto casein agar plates
Bacillus licheniformis (0.104), Bacillus thuringiensis (0.089), Bacillus Subtilis A (0.160) and Bacillus Subtilis B (0.089) inhibition zones.

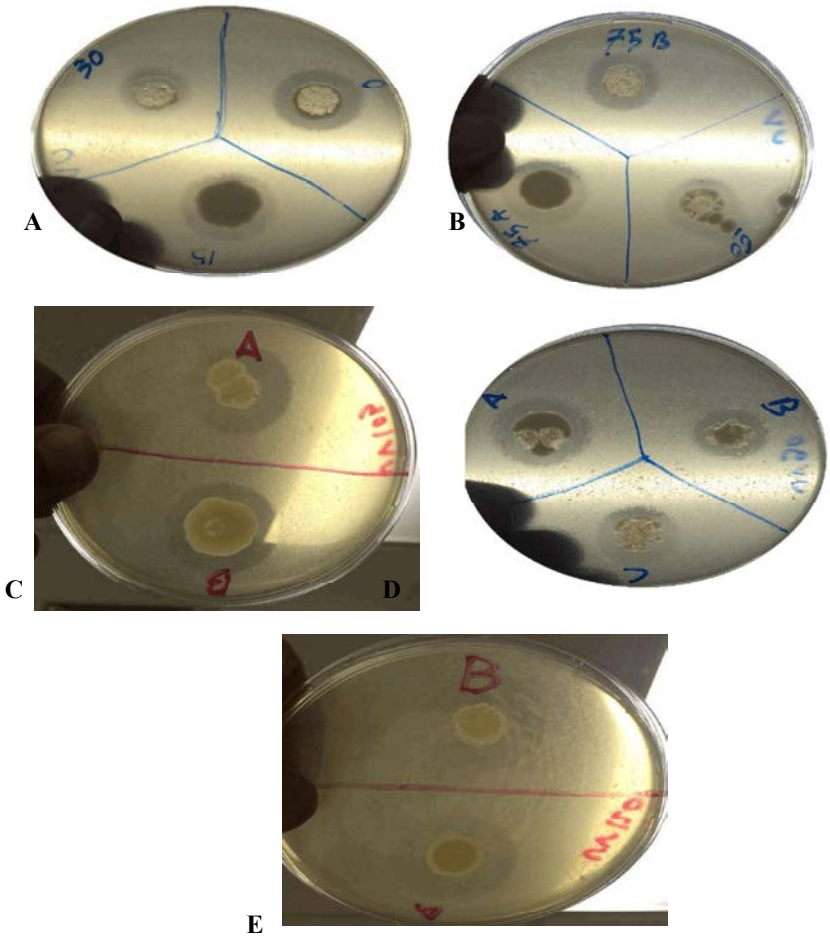


Fig. 2: Fibrinolytic activities produced by Mutants produced {plate **A** mutant colonies produced after 0, 15 and 30 min. (only one colony produced), Plate **B** mutant colonies produced after 60 and 75 min. (one colony produced after 60 min., two colonies after 75), Plate **C** three mutants produced after 90 min., Plate **D** two mutant colonies produced after 105 min., Plate **E** two mutant colonies produced after 120 min.}.

Table 2: Fibrinolytic enzyme assay produced by mutants produced from *Bacillus Subtilis* A after UV mutation

| No. | Mutants produced after each UV exposure time (min.) * | Absorbance at (660)nm |
|---|---|---|
| 1 | 0 | 0.160 |
| 2 | 15 | 0.534 |
| 3 | 30 | 0.542 |
| 4 | 45A | 0.650 |
| 5 | 45B | 0.608 |
| 6 | 60 | 0.574 |
| 7 | 75A | 0.667 |
| 8 | 75B | 0.595 |
| 9 | 90A | 0.731 |
| 10 | 90B | 0.646 |
| 11 | 90C | 0.692 |
| 12 | 105A | 0.581 |
| 13 | 105B | 0.503 |
| 14 | 120A | 0.489 |
| 15 | 120B | 0.375 |

* mutant colonies produced after each UV exposure time.

Table 3: Inhibition zone produced by selected mutants over casein agar plates

| No. | Mutants produced after each UV exposure time (min.) * | Zone (cm) |
|---|---|---|
| 1 | 0 | 0.7 |
| 2 | 15 | 0.6 |
| 3 | 30 | 0.6 |
| 4 | 45A | 0.9 |
| 5 | 45B | 0.8 |
| 6 | 60 | 0.7 |
| 7 | 75A | 1 |
| 8 | 75B | 0.8 |
| 9 | 90A | 1.4 |
| 10 | 90B | 0.9 |
| 11 | 90C | 1.2 |
| 12 | 105A | 0.7 |
| 13 | 105B | 0.5 |
| 14 | 120A | 0.5 |
| 15 | 120B | 0.2 |

*mutant colonies produced after each UV exposure time.

**Fibrinolytic Enzyme Screening after UV Irradiation on Casein Agar Plates:** Selected colonies after each exposing time of UV irradiation was assayed for enzyme activity by assaying inhibition zone produced over casein agar plates. Results reveals that the produced *Bacillus Subtilis* wild type strain displayed the least inhibition zone than other selected mutants as indicated in (Fig. 2 & Table 3). The most promising mutant strain was *Bacillus Subtilis* A after 90 min. exposure time followed by 90°C 75 A, 90 B, 45A and 45B respectively, these results are identical with the spectrophotometric method.

## DISCUSSION

The fibrinolytic enzyme-producing microbes have been showed important different major pharmaceutical and industrial applications [15-16]. Fibrinolytic enzyme was isolated from *Bacillus Subtilis* strains [17-18]. For better efficiency, random mutagenesis is an applicable technique for improvement and increasing the bacteria strains for fibrinolytic enzyme productivity [19-20].

In the present study wild type of, *Bacillus licheniformis*, *Bacillus thuringiensis*, *Bacillus Subtilis* A & B were screened for their production of fibrinolytic enzyme using Spectrophotometric Screening after growth of strains in liquid culture and on solid casein agar plates. All bacillus strains were differed in its fibrinolytic enzyme. The best producing strain was *Bacillus Subtilis* A. This *Bacillus Subtilis* A was subjected to UV irradiation (because it was the best producer strain) at irradiation period 15, 30, 45, 60, 75, 105,120 min. respectively, Survived UV mutants selected after each time were assayed for fibrinolytic enzyme by the same methods illustrated before. The highest enzyme production was *Bacillus Subtilis* A after 90 min. exposure time followed by 90 C 75 A, 90 B, 45A and 45B respectively. These results are in-agreement with the work of Vijayaraghavan *et al.* [21-22], Sangkharak *et al.* [23] and Sanchez *et al.* [24] on their work on bacillus strains after UV Irradiation and selection. All workers with the most universally and commonly used UV mutation have shown that, the Ultraviolet radiation with bacillus strains producing enzymes are useful and induce genetically improved strains [25-27]. In this research, fibrinolytic enzymes from produced mutant strain are promising for their fibrinolytic enzyme and these findings are useful for improvement of enzymes used in industrial applications.

## CONCLUSIONS

In this investigation, UV irradiation increased fibrinolytic enzyme production several times than their original *Bacillus Subtilis* wild type strain. These results increase enzyme production and reduce the cost used in other improvement methodologies such as cloning strategies. The given results also, are useful to be applied to other bacillus producing enzymes. This study concluded that the mutation techniques and may be culture optimization can result in faster and better overproduction of such bacillus enzymes.

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