Bacillus Cereus GD 55 Strain Improvement by Physical and Chemical Mutagenesis for Enhanced Production of Fibrinolytic Protease

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Abstract

This work has been undertaken to enhance the production of industrially important fibrinolytic protease by subjecting indigenous fibrinolytic protease producing *Bacillus cereus* to strain improvement by random mutagenesis using ultra-violet (UV) irradiation, ethyl methane sulfonate (EMS) and ethidium bromide treatment. Mutants were screened on the basis of enzyme assay by spectrophotometer using folin's phenol reagent. Ethyl methane sulfonate (EMS) and ethidium bromide treated *Bacillus cereus* GD 55 was proved to be the best for optimum production of fibrinolytic protease. The effect of different production parameters such as carbon source, inoculum sizes, pH, temperature, nitrogen source (inorganic and organic) and incubation time on fibrinolytic protease production by the mutated bacterial strain was studied. The enzyme production was assayed in submerged fermentation (SmF) condition. The maximum fibrinolytic protease production was observed with fructose 1% (18.60 ± 0.62 U/ml), inoculum size level 2% (22.10 ± 0.80 U/ml), pH 8.0 (28.65 ± 0.41 U/ml), temperature 35°C (28.68 ± 0.19 U/ml), NH_4NO_3 1% (34.24 ± 0.12 U/ml), peptone 1% (35.68 ± 0.27 U/ml) and incubation time 48 hours (38.92 ± 0.56 U/ml) in the production medium. EMS&EB-15 mutant strains were found to produce 2-4 fold more enzyme. Thus these findings have more impact on enzyme economy for biotechnological applications of microbial fibrinolytic proteases.

Key words: strain improvement, *Bacillus cereus*, submerged fermentation, fibrinolytic protease, optimum production

Introduction

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation [1]. The extracellular proteases are commercial value and find multiple applications in various industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers [2]. Of these, strains of *Bacillus sp*. dominate the industrial sector [3]. Fibrinolytic protease is well known as a sub class of protease, which has an ability to degrade fibrin [4-8]. Blood clots (fibrin) are formed from fibrinogen by thrombin (EC 3.4.21.5) and are lysed by plasmin (EC 3.4.21.7), which is activated from plasminogen by tissue plasminogen activator [9]. Deposition of fibrin in blood vessels normally increases thrombosis, resulting in myocardial infarction and other cardiovascular diseases [10-11].

Many microorganisms like species of *Pseudomonas* [12], *staphylococcus* [13], *Alteromonas* [14], *Coryneform bacteria* [15], *Penicillium* [16], *Asperigillus* [17-19], *Fusarium* [20-21], *Trichotecium* [22], *Actinomyces* [23-24], *streptomyces* [25-26], *Escherichia coli* [27] and *Bacillus* [28-34] have been evaluated for the production of fibrinolytic enzymes. Microbes serve as the preferred source of fibrinolytic proteases because of their rapid growth, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties. However, high cost and low yields of fibrinolytic proteases have been the main problems for its industrial production. Therefore, there is a great need to develop new strains with inexpensive mutagens that provides a high fibrinolytic protease yield.

For industrial use enzyme must be produced at low cost and should be reusable and reproducible. To achieve this many techniques have been developed for strain improvement. Strain improvement is usually done by mutating the microorganism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, ɣ-rays, UV rays, etc., and chemical mutagens such as NTG, EMS, EtBr, etc. [35]. There are a great number of literatures reported to use the strain improvement process for producing various industrial enzymes like lipase, chitinase, cellulase, glucoamylase and protease [36-38]. But there was no report available on mutation studies of *Bacillus cereus* for fibrinolytic protease production.

The present study was mainly focused on the strain improvement of *Bacillus cereus* through mutation and optimizing various parameters such as carbon sources, inorganic nitrogen sources, organic nitrogen sources, pH, temperature, substrate concentration, inoculums concentration and incubation time for enhanced production of fibrinolytic protease.

Materials and Methods

Chemicals

All chemicals and reagents of analytical grade were used in this research and mostly purchased from sigma USA. All the experiments were conducted in triplicate and the mean values are considered.

Microorganism and Inoculum preparation

The bacterial strain isolated from soils of various regions in Bangalore, India was mutated by physical and chemical mutagens, identified at Institute of Microbial Technology (IMTECH) Chandigarh, India as *Bacillus cereus* GD55. Stock cultures were maintained in nutrient broth medium with 70% glycerol, cultures were preserved at -20^{o}C [39]. The inoculum was prepared by transferring a loopful of stock culture (*Bacillus cereus* GD55) to a certain volume (100ml) of sterile nutrient broth stock medium, then incubated it overnight at 35^{o}C on a rotary shaker with 200 rpm, before being used to inoculation [40]. A stock suspension was prepared and adjusted to 7×10^{3} cell/ml^{-1}.

Strain improvement techniques

Physical (Radiation) mutagenesis by UV lamp

Four ml of the spore suspension containing 1×10^{7} spores/ml was pipette aseptically into sterile petridish of 80 mm diameter having a flat bottom. The exposure of spore suspension to UV light was carried at distance of 30 cm away from UV lamp (15W, 2537A°). The exposure times were 30-90 min [41]. Each UV exposed spore suspension was stored in dark overnight to avoid photo reactivation, then was serially diluted in saline and plated on fibrin agar medium. The plates were incubated for 48 hours at 35°C and the number of colonies in each plate was counted. Each colony was assumed to be formed from a single spore. Mutants for hyper production of fibrinolytic protease were detected visually by the intensity of zones.

Chemical mutagenesis

Ethidium bromide (EtBr) mutagenesis

Four ml of the spore suspension containing 1×10^{7} spores/ml was pipette aseptically into 15 ml of ethyl methane sulfonate solution and incubated at 35°C. The sample (2 ml) of this solution was taken at intervals of 30-270 min and centrifuged immediately at 10,000 rpm for 5 min at 35°C [42]. The supernatant was decanted and the cell pellet obtained was re suspended in 5 ml saline to stop the reaction. The washed cell suspension was serially diluted in saline and plated on fibrin agar medium. The plates were incubated for 48 hours at 35°C and the

number of colonies in each plate was counted. Each colony was assumed to be formed from a single spore. Mutants for hyper production of fibrinolytic protease were detected visually by the intensity of zones.

Ethyl methane sulfonate (EMS) mutagenesis

Four ml of the spore suspension containing 1×10^7 spores/ml was pipette aseptically into 15 ml of ethyl methane sulfonate solution and incubated at 35°C. The sample (2 ml) of this solution was taken at intervals of 30-270 min and centrifuged immediately at 10,000 rpm for 5 min at 35°C [42]. The supernatant was decanted and the cell pellet obtained was re suspended in 5 ml saline to stop the reaction. The washed cell suspension was serially diluted in saline and plated on fibrin agar medium. The plates were incubated for 48 hours at 35°C and the number of colonies in each plate was counted. Each colony was assumed to be formed from a single spore. Mutants for hyper production of fibrinolytic protease were detected visually by the intensity of zones.

Ethyl methane sulfonate (EMS) and Ethidium bromide (EtBr) mutagenesis

Four ml of the spore suspension containing 1×10^7 spores/ml was pipette aseptically into 15 ml of ethyl methane sulfonate and ethidium bromide solution and incubated at 35°C. The sample (2 ml) of this solution was taken at intervals of 30-270 min and centrifuged immediately at 10,000 rpm for 5 min at 35°C [43]. The supernatant was decanted and the cell pellet obtained was re suspended in 5 ml saline to stop the reaction. The washed cell suspension was serially diluted in saline and plated on fibrin agar medium. The plates were incubated for 48 hours at 35°C and the number of colonies in each plate was counted. Each colony was assumed to be formed from a single spore. Mutants for hyper production of fibrinolytic protease were detected visually by the intensity of zones.

Production of Fibrinolytic protease

Shake flask fermentation

Hyper fibrinolytic protease producing mutant strains of ultra-violet rays, ethyl methane sulfonate and ethidium bromide were inoculated separately in fermentation medium. The medium used for the production of fibrinolytic protease was composed of fructose (1%), peptone (1%), K_2HPO_4 0.2%, $CaC1_2$ 0.04% and $MgSO_4$ 0.02%. The pH 8 of the medium was adjusted with 1N HC1/NaOH. Two percent (v/v) of 24h old inoculum suspension was transferred to 50 mL of growth medium in 250 mL Erlenmeyer flasks. These flasks were then placed in the rotary incubator shaker (Lab top) rotating at 200rpm for 48 hours at 35°C. After the completion of fermentation the whole fermentation broth was centrifuged at 10,000 rpm at 4^oC for 10 minutes and the clear supernatant (crude enzyme) was used for the estimation of fibrinolytic protease. All the experiments were run parallel in triplicate.

Medium Optimization for Fibrinolytic Protease Production

Effect of Various Carbon Sources on Fibrinolytic Protease Production

To identify the suitable carbon sources for fibrinolytic protease production by the mutant *Bacillus cereus* GD55 the following different carbon sources were tested such as glucose, sucrose, fructose, maltose, arabinose, starch and mannose with sample concentration of 1% in the optimized production medium at 35°C [44].

Effect of Various Inoculum Concentrations on Fibrinolytic Protease Production

The fibrinolytic protease production by the selected experimental microorganism was determined by adding bacterium at different inoculum's concentrations such as 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5.0 % to test its ability to induce fibrinolytic protease production in the production medium [45].

Effect of Various pH on Fibrinolytic Protease Production

The effect of pH for fibrinolytic protease production was determined by culturing the bacterium in the production media with different pH. The experiment was carried out individually at various pH 3, 4, 5, 6, 7, 8, 9, 10 and 11. The enzyme assay was carried out after 42 hours of incubation at 35°C [46].

Effect of Various Temperatures on Fibrinolytic Protease Production

Temperature is an important role for the production of fibrinolytic protease. The effect of temperature on fibrinolytic protease production was studied by the incubating the culture media at various temperatures 10, 20, 30, 35, 40, 50, 60 and 70°C along with arbitrary control at 35°C [47].

Effect of Various Organic and Inorganic Nitrogen Sources on Fibrinolytic Protease Production

The fibrinolytic protease production by the selected bacterium was also optimized by supplementing different inorganic and organic nitrogen sources individually at the concentration of 1% such as KNO_3, NH_4C1, $(NH_4)_2HPO_4$, NH_4NO_3, $(NH4)_2SO_4$, casein, yeast extract, peptone, serine, Histidine and Aspartate [48].

Effect of Various Incubation Times on Fibrinolytic Protease Production

The fibrinolytic protease production by the selected experimental microorganism was determined by optimizing the media by adding the bacteria. The experiment was carried out individually at various incubation times such as 24, 48, 72, 96 and 120 hours. The enzyme assay was carried out individually after 48 hours of incubation [49].

Fibrinolytic Protease Assay

Fibrinolytic protease activity was carried out according to the method described by Greenberg 1957. The reaction mixture contained 8 mg bovine fibrin, 500µl enzyme in Phosphate buffer (0.05mM, pH 7.8) in a total volume of 1mL. This mixture was incubated for 30 min at 37^{o}C in a water bath. The reaction was stopped by adding 0.5mL of 15% cold tri chloroacetic acid (TCA). The mixture was centrifuged at 3,000 rpm for 10 min to remove precipitated fibrin. To 0.5mL of acid soluble filtrate 2.5mL of 0.3 N sodium hydroxide and 2.9% (w/v) sodium carbonate was added, followed by 0.75mL of Folin's phenol reagent. The mixture was incubated for 25 min at room temperature and the color developed was read at 650 nm [50]. The above said procedure was followed with heat killed enzyme and kept as blank. One unit of enzyme activity was calculated as the amount of enzyme which releases 1µg of tyrosine per minute under the specified reaction conditions.

Results and Discussion

Improvement of Strain by Physical (UV Radiation) mutagenesis

Data of table 1A shows the production of fibrinolytic protease by UV treated strains of *Bacillus cereus*. The parental strains of *Bacillus cereus* was subjected to UV treatment for different time intervals i.e. from 30-90 min. Thirty mutant strains of *Bacillus cereus* were isolated on the basis of bigger zone of fibrin hydrolysis in the petriplates. These strains were screened for fibrinolytic protease production in shake flask. The isolates were sub grouped into G-I (8-11 U/ml/min), G-II (11.1-13 U/ml/min) and G-III (more than 13.0 U/ml/min) according to their enzyme productivity (Table 1B). Of all the isolates investigated, maximum enzyme production (14.60 ± 1.15 U/ml/min) was obtained by *Bacillus cereus* No.17 which was selected after 70 minutes of UV treatment. The production of enzyme following the growth of the organism was found to be highly significant than other mutant derivatives. But this mutant was not stable. It was due to the reason that the mutant produced by UV irradiations had undergone back mutations when they were exposed to light [51].

Improvement of Strain by Ethidium bromide (EtBr) mutagenesis

The mutant strains of *Bacillus cereus* was selected after the treatment of parental strain with ethidium bromide (Table 2A). The wild strain of *Bacillus cereus* was subjected to ethidium bromide treatment for different time intervals i.e 30-270 min. Twenty one mutant strains of *Bacillus cereus* were selected on the basis of large zone of fibrin plate hydrolysis. Further screening of the strains for fibrinolytic protease production was done in shake flask. The selected strains were sub grouped into G-I (10.0-12.0 U/ml/min), G-II (12.1-14.0 U/ml/min) and G-III (more than 14.0 U/ml/min) according to their enzyme productivity (Table 2B). Of all the isolates investigated, maximum enzyme production (14.72 ± 1.54 U/ml/min) was obtained by *Bacillus cereus* mutant No.13. Fibrinolytic protease production was found to be highest by *Bacillus cereus* No 13. But after sub culturing this mutant reverted to its parental enzyme production.

Improvement of Strain by Ethyl methane sulfonate (EMS) mutagenesis

The strains of *Bacillus cereus* were screened after the chemical treatment with EMS (Table 3A). The parental strains of *Bacillus cereus* was subjected to EMS treatment for 30-270 min. Only six mutant strains of *Bacillus*

cereus were isolated on the basis of bigger zone of fibrin hydrolysis in the petriplates. These strains were screened for fibrinolytic protease production in shake flask. The isolates were sub grouped into G-I (12.0-14.0 U/ml/min) and G-II (more than 14.0 U/ml/min) according to their enzyme productivity (Table 3B). Of all the isolates investigated, maximum enzyme production (14.78 ± 0.67 U/ml/min) was obtained by *Bacillus cereus* No.4. This mutant showed improvement in the production of the enzyme but after subculturing it reverted to its parental enzyme production. It was due to the reason that the DNA of the mutant strains repaired their damaged part during its replication [52].

Improvement of Strain by Ethyl methane sulfonate (EMS) and Ethidium bromide (EtBr) mutagenesis

Chemical mutagens like ethidium bromide and ethyl methane sulfonate gave a stable and viable mutant for production of fibrinolytic protease. The effects of alternate treatment of different chemical mutagens are stronger than single mutagen. In the double mutagen studies the parental strain of *Bacillus cereus* was subjected to the alternate treatment of ethyl methane sulfonate and ethidium bromide for 30-270 min. (Table 4A). Twenty mutant strains of *Bacillus cereus* were selected on the basis of large zone of fibrin plate hydrolysis. Further screening of the strains for fibrinolytic protease production was done in shake flask. The selected strains were sub grouped into G-I (12.0-13.0 U/ml/min), G-II (13.1-14.0 U/ml/min) and G-III (more than 14.0 U/ml/min) according to their enzyme productivity (Table 4B). Of all the isolates investigated, maximum enzyme production (14.92 ± 0.83 U/ml/min) was obtained by *Bacillus cereus* mutant No.15. It remained a stable mutant after multiple culture cycles. It was due to the relationship between mutation rate and the amount of dose to the bacteria. The productivity increased was more than two fold than the parental strain from 7.46- 14.84 U/ml/min. This enhancement occurred due to increase in gene copy number and amplification of the DNA region [53].

Effect of Carbon Sources on Fibrinolytic Protease Production

The addition of various carbon sources in the form of either monosaccharide or polysaccharides may influence the production of fibrinolytic protease. Table 5 shows the effect of carbon sources on fibrinolytic protease production after 48 hours of incubation period at 35°C. In our present study, the influence of fructose was more (18.60 ± 0.62 U/ml) than the other carbon sources tested. Glucose was the second best supplementary carbon source (17.84 ± 0.10 U/ml). Mannose gave the lowest fibrinolytic protease activity (12.98 ± 0.10 U/ml). Different carbon sources have varied influence on the production of extracellular enzymes especially protease strains. These results are similar to the findings of Sivakumar who observed that glucose and fructose enhances the production of protease [54].

Effect of Various Inoculum Sizes on Fibrinolytic Protease Production

It is generally necessary to optimize age and size of the inoculums, because low density gives insufficient biomass and high density produces too much biomass and resulting depleting of nutrients necessary for fibrinolytic protease production. The researchers were reported that inoculums size has crucial effect in fermentation process through microorganisms [55]. In the present study, the initial inoculum level has played an important role in fibrinolytic protease production by mutant *Bacillus cereus*. The maximum fibrinolytic protease specific activity was registered at the 2% (22.10 ± 0.80 U/ml) of inoculum level. On the other hand, the minimum amount of fibrinolytic protease production was observed at 5% of (10.42 ± 0.20 U/ml) inoculums level (Table 6).

Effect of pH on Fibrinolytic Protease Production

Physical factors are important in any fermentation for optimization of biochemical production. The important physical factors that determine the bioprocess are pH, temperature, aeration and agitation. Table 7 shows the effect of various pH on fibrinolytic protease production after 48 hours of incubation period at 35°C. The maximum fibrinolytic protease production was observed at pH 8.0 (28.65 ± 0.41 U/ml) and minimum amount of fibrinolytic protease production was recorded at pH 3 (4.18 ± 0.29 U/ml). The enzyme production decreased which might be due to growth reduction and enzyme inactivation or suppression of cell viability. Low temperature values may reduce the metabolism of the microorganism and consequently, the enzyme synthesis.

Effect of Temperature on Fibrinolytic Protease Production

Table 8 shows the effect of various temperatures on fibrinolytic protease production. The maximum fibrinolytic protease production was obtained at 35°C (28.68 ± 0.19 U/ml). Followed by this, 30°C temperature (26.84 ± 0.18 U/ml) was the second best temperature on fibrinolytic protease production. Above this temperature level, the fibrinolytic protease production decreased, because the metabolic activities of microbes are very much

responding to temperature change. On the other hand, the minimum amount of fibrinolytic protease production was observed at temperature 70°C (4.62 ± 0.70 U/ml).

Effect of Inorganic Nitrogen Sources on Fibrinolytic Protease Production

In the present study, NH_4NO_3 was found to be the most suitable inorganic nitrogen source for *Bacillus cereus.* Table 9 shows the effect of different kinds of inorganic nitrogen sources on fibrinolytic protease production after 48 hours of incubation period at 35°C. The maximum amount of enzyme production was observed in NH_4NO_3 (34.24 ± 0.12 U/ml) supplemented medium and minimum amount of fibrinolytic protease production was observed in $(NH_4)_2 HPO_4$ (18.98 ± 0.12 U/ml) supplemented medium.

Effect of Organic Nitrogen Sources on Fibrinolytic Protease Production

The nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth of the organism and the production. The nature of the compound and the concentration that we used might stimulate or down modulate the production of enzymes. Table 10 shows the effect of different kinds of organic nitrogen sources on fibrinolytic protease production after 48 hours of incubation period at 35°C. The maximum amount of fibrinolytic protease production was observed in peptone (35.68 ± 0.27 U/ml) with supplemented medium and minimum enzyme activity was observed in yeast extract (28.30 ± 0.20 U/ml). In the present study experiment on the effect of supplementary nitrogen sources on fibrinolytic protease production under flask fermentation, showed that peptone was found to be a better nitrogen source for this isolate. Peptone is the best nitrogen source for fibrinolytic protease production, probably due to its high content in minerals, vitamins, coenzymes and nitrogen components.

Effect of Incubation Time on Fibrinolytic Protease Production

The effect of incubation time on fibrinolytic protease production showed that 48 hours was the optimum duration for maximum fibrinolytic protease activity. Table 11 illustrates the effect of different incubation times on fibrinolytic protease production. The maximum amount of fibrinolytic protease production was observed with 48 hours incubation time (38.92 ± 0.56 U/ml). Above this period the fibrinolytic protease activity started to decrease. The minimum amount of fibrinolytic protease production was obtained with 120 hours incubation (10.96 ± 0.12 U/ml). This is because, the cells may reach the decline phase and displayed low fibrinolytic protease synthesis.

Conclusion

The search for promising strains of fibrinolytic protease producers is a continuous process. Finally, from the above results it was concluded that *Bacillus cereus GD55* mutant strains were developed by using EtBr followed by EMS treatment remained as a stable mutant after multiple culture cycles. EMS&EB-15 mutant showed 2 fold higher fibrinolytic protease production than wild strain in flask fermentation. Thus the selected mutant has potential in minimizing the cost of enzyme for its biotechnological applications.

Acknowledgments

The authors would like to thank the chairman ACHARYA INSTITUTES Sri B.PREMNATH REDDY for providing laboratory facilities and supporting this research work.

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Table 1a: Mutants selected after the treatment of Ultraviolet radiations for Fibrinolytic Protease Production

Mutant No	Exposure time (minutes)	Fibrinolytic Protease production (U/ml/min)
1	30	10.12 ± 0.46
2	35	10.48 ± 0.76
3	35	10.92 ± 1.27
4	40	11.53 ± 1.40
5	50	11.96 ± 1.27
6	50	10.18 ± 0.46
7	50	11.10 ± 0.29
8	50	11.86 ± 0.45
9	55	11.48 ± 0.83
10	55	12.60 ± 0.23
11	60	13.10 ± 1.42
12	60	12.98 ± 0.55
13	60	12.61 ± 0.81
14	65	13.12 ± 1.61
15	65	10.84 ± 1.42
16	70	10.90 ± 0.42
17	70	14.60 ± 1.15
18	70	13.10 ± 1.41
19	70	11.46 ± 0.36
20	75	10.58 ± 0.29
21	80	11.10 ± 0.64
22	80	13.82 ± 0.25
23	80	12.81 ± 0.52
24	85	12.62 ± 1.14
25	85	11.90 ± 1.27
26	85	10.86 ± 1.40
27	90	10.12 ± 1.14
28	90	9.20 ± 1.14
29	90	8.60 ± 0.45
30	90	8.21 ± 0.29

Table 1b: Range of mutants selected after the treatment of Ultraviolet radiations for Fibrinolytic Protease Production

Subgrouping of mutants	Number of mutants	Range of Fibrinolytic protease (U/ml/min)
GI	12	8-11
GII	13	11.1-13

GIII	5	More than 13

Each value is an average of three replicates ± denotes standard deviation among replicates.

Initial pH 8.0
Incubation period 48 h
Incubation temperature 35 ± 2°C.

Table 2a: Mutants selected after the treatment of Ethidium bromide for Fibrinolytic Protease Production

Mutant No	**Exposure time (minutes)**	**Fibrinolytic Protease production (U/ml/min)**
1	35	10.68 ± 1.46
2	40	11.64 ± 0.30
3	40	11.82 ± 0.25
4	50	10.81 ± 1.27
5	50	12.51 ± 1.08
6	50	11.98 ± 1.14
7	50	12.10 ± 1.46
8	55	13.18 ± 0.26
9	55	11.16 ± 0.64
10	55	10.98 ± 0.36
11	60	10.64 ± 1.61
12	60	13.60 ± 1.27
13	**60**	**14.72 ± 1.54**
14	60	13.98 ± 0.20
15	65	12.84 ± 0.21
16	65	12.96 ± 1.91
17	65	12.30 ± 0.92
18	65	10.94 ± 0.55
19	70	13.91 ± 0.36
20	70	14.16 ± 0.79
21	70	12.48 ± 0.40

Subgrouping of mutants	**Number of mutants**	**Range of Fibrinolytic protease (U/ml/min)**
GI	9	10-12
GII	10	12.1-14
GIII	2	More than 14

Each value is an average of three replicates ± denotes standard deviation among replicates.

Initial pH 8.0
Incubation period 48 h
Incubation temperature 35 ± 2°C.

Table 2b: Range of mutants selected after the treatment of Ethidium bromide for Fibrinolytic Protease Production

Table 3a: Mutants selected after the treatment of EMS for Fibrinolytic Protease Production

Mutant No	Exposure time (minutes)	Fibrinolytic Protease production (U/ml/min)
1	40	12.98 ± 1.99
2	45	13.64 ± 1.40
3	50	13.91 ± 0.94
4	**60**	**14.78 ± 0.67**
5	60	14.62 ± 0.88
6	65	13.58 ± 1.32

Table 3b: Range of mutants selected after the treatment of EMS for Fibrinolytic Protease Production

Subgrouping of mutants	Number of mutants	Range of Fibrinolytic protease (U/ml/min)
GI	4	12-14
GII	2	More than 14

Each value is an average of three replicates ± denotes standard deviation among replicates.

Initial pH 8.0
Incubation period 48 h
Incubation temperature 35 ± 2°C.

Table 4a: Mutants selected after alternate treatment of Ethidium bromide and EMS for Fibrinolytic Protease Production

Mutant No	Exposure time (minutes)	Fibrinolytic Protease production (U/ml/min)
1	35	12.20 ± 0.84
2	40	13.18 ± 1.20
3	40	12.96 ± 1.34
4	50	12.64 ± 0.88
5	50	13.81 ± 1.62
6	50	14.58 ± 0.55
7	50	13.62 ± 0.83
8	55	14.72 ± 0.46
9	55	14.28 ± 0.30
10	55	13.64 ± 0.72
11	60	14.10 ± 1.27
12	60	13.98 ± 1.08
13	60	12.90 ± 0.55
14	60	13.24 ± 1.42
15	**65**	**14.92 ± 0.83**
16	65	12.75 ± 0.93
17	65	14.81 ± 0.52
18	65	13.62 ± 1.50
19	70	14.86 ± 0.88
20	70	13.54 ± 0.89

Table 4b: Range of mutants selected after alternate treatment of Ethidium bromide and EMS for Fibrinolytic Protease Production

Subgrouping of mutants	Number of mutants	Range of Fibrinolytic protease (U/ml/min)
GI	5	12-13
GII	8	13.1-14
GIII	7	More than 14

Each value is an average of three replicates ± denotes standard deviation among replicates.

Initial pH 8.0
Incubation period 48 h
Incubation temperature 35 ± 2°C.

Table 5: Effect of various Carbon sources on Fibrinolytic Protease Production

Carbon Source (1%)	Specific Activity (U/ml)
Glucose	17.84 ± 0.10
Sucrose	16.18 ± 0.15
Fructose	**18.60 ± 0.62**
Maltose	16.56 ± 0.25
Arabinose	17.12 ± 0.28
Starch	15.10 ± 0.16
Mannose	12.98 ± 0.10

Table 6: Effect of various Inoculum sizes on Fibrinolytic Protease Production

Inoculum sizes (%)	Specific Activity (U/ml)
0.5	18.20 ± 0.28
1.0	20.21 ± 0.18
1.5	21.60 ± 0.10
2.0	**22.10 ± 0.80**
3.0	19.98 ± 0.36
4.0	18.96 ± 0.58
5.0	10.42 ± 0.20

Table 7: Effect of various pHs on Fibrinolytic Protease Production

pH	Specific Activity (U/ml)
3	4.18 ± 0.29
4	6.62 ± 0.24
5	10.18 ± 0.21
6	18.58 ± 0.23
7	26.48 ± 0.17
8	**28.65 ± 0.41**
9	19.20 ± 0.23
10	10.60 ± 0.17
11	8.62 ± 0.25

Table 8: Effect of various temperatures on Fibrinolytic Protease Production

Temperature (^{0}C)	Specific Activity (U/ml)
10	6.82 ± 0.26
20	10.91 ± 0.13
30	26.84 ± 0.18
35	**28.68 ± 0.19**
40	27.12 ± 0.12
50	16.96 ± 0.15
60	8.58 ± 0.19
70	4.62 ± 0.70

Table 9: Effect of various Inorganic nitrogen sources on Fibrinolytic Protease Production

Nitrogen Source (1%)	Specific Activity (U/ml)
KNO_3	21.20 ± 0.10
NH_4C1	30.64 ± 0.10
$(NH_4)_2$ HPO_4	18.98 ± 0.12
NH_4NO_3	**34.24 ± 0.12**
$(NH_4)_2SO_4$	19.71 ± 0.29

Table 10: Effect of various Organic nitrogen sources on Fibrinolytic Protease Production

Organic nitrogen source (1%)	**Specific Activity (U/ml)**
Casein	32.50 ± 0.22
Yeast extract	28.30 ± 0.20
Peptone	**35.68 ± 0.27**
Serine	34.98 ± 0.20
Histidine	34.62 ± 0.17
Aspartate	30.46 ± 0.36

Table 11: Effect of various Incubation times on Fibrinolytic Protease Production

Incubation Time(hrs)	**Specific Activity (U/ml)**
24	36.64 ± 0.12
48	**38.92 ± 0.56**
72	30.18 ± 0.11
96	22.96 ± 0.98
120	10.96 ± 0.12