World Applied Sciences Journal 37 (2): 135-139, 2019
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2019.135.139

EMS Mutation Improve the Fibrinolytic Enzyme Activity in *Bacillus subtilis* Strain

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Abstract: Fibrinolytic enzymes (mainly proteases) are capable of digesting fibrin clot and they are used to treat some cardiovascular diseases, which are the leading cause of morbidity and mortality all over the world. Some bacterial strains are important source for proteases, which used to treat blood clots. A variety of bacterial strains is used for proteases production especially bacillus strains. This study was aimed to evaluate some chosen bacillus bacterial strains for their fibrinolytic enzyme production. The best producer *Bacillus subtilis* A was selected and mutated by EMS. Results showed that, the mutants obtained were screened for their fibrinolytic activity were able to produce the fibrinolytic activity more than its original wild type in growth medium. The results proved that mutation by EMS was effective as mutagenic agents with *Bacillus subtilis* for enhanced production of fibrinolytic enzyme. *In vitro* assays proved the produced fibrinolytic enzyme by *Bacillus subtilis* is highly promising and could be used in pharmaceutical industry as thrombolytic agent.

Key words: Mutation · Fibrinolytic enzyme · EMS · Chemical mutagenesis

INTRODUCTION

Microorganisms harbor proteases, which are used to treat blood clotting. These purified proteases are fibrinolytic enzymes that used to digest fibrin [1]. Proteases are large groups of hydrolytic enzymes, which degrade peptide bonds in proteins converting them to free amino acids [2]. Investigation of microbes that produce microbial fibrinolytic enzyme is important in treating intravascular thrombosis that cause cardiovascular diseases and possesses significant medical applications [3]. Purified enzymes from microbial source are used in such field. *Bacillus subtilis*, *Bacillus pumilus* [4-5] and *Pseudomonas aeruginosa* [6-7] are the major bacterial species, which contribute to proteases. Actinomyces such as *Streptomyces sp.* and *Saccharothrix xinjiangensi* [8], fungi like *Aspergillus ochraceus, Fusarium oxysporum*, *Penicillium chrysogenum* and *Rhizopus chinensis* [9-10] and algae as *Codiumlatum*, *Codiumdivaricatum* and *Codiumintricatum* [11]. *Mucor subtilissimu,* which produce a novel protease that has fibrinolytic activity with high degree of purity and can be used economically as a good source for new therapeutic agents to treat thrombosis [12]. Increase the production of fibrinolytic enzyme by Ethyl Methyl Sulfonate (EMS) used for increase these enzymes production and also for stability of strains [13]. Several works proved that Ethyl Methane Sulfonate random mutagenesis and screening of mutated microorganisms strains can develop high yield of fibrinolytic activity as indicated by several investigators [14-17].

The present study aimed to screen some bacillus strains for its productivity of fibrinolytic mutates and select the best producer *Bacillus subtilis* strain, these strains were mutated by EMS mutagen.

MARTIALS AND METHODS

Bacterial Strains: Four bacterial strains were used in this study for fibrinolytic enzyme activity, it were *Bacillus Subtilis* A, *Bacillus Subtilis* B, *Bacillus thuringiensis* and *Bacillus licheniformis*. The strains were identified as

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a strain related to Bacillus at the species level based on morphological and 16S rDNA sequences. It was grown for 48 h at 37°C on 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. A loopful was inoculated into liquid medium. After overnight incubation cultivation, Proteolytic activity was assayed in each used wild type strain.

EMS Mutagenesis and Mutant Selection: The culture suspension of *Bacillus subtilis* A was prepared as described before [18]. 5 ml of sterile solution of Ethyl methyl sulphonate (EMS) (200 µg/ml) was added to 5 ml of cell suspension culture. After the reaction mixture was proceeded, at each interval time of 15, 30, 45, 60, 75, 90, 105 and 120 minutes, cells were re-suspended immediately in sterile phosphate buffer (pH 7). Centrifugation was done at 5, 000 rpm for 10 min and the supernatant was discarded. Cell pellet was washed twice with sterile dd H2O water for removal of EMS traces and re-suspended in 10 ml of saline to stop the reaction. A total of 14 colonies were selected and were similarly tested for fibrinolytic enzyme productivity. Mutants produced after each time interval were screened in screening medium.

Screening of Fibrinolytic Enzyme Production by Bacterial Strains Before and after EMS: Screening of fibrinolytic enzyme was carried out by different methods, diffusion assay and spectrophotometric analysis as described before [19-20].

Fibrinolytic Assays in Fibrin Plate: Fibrin plate was used for Quantitative analyses of fibrinolytic activity following the method described [21]. Fibrin plate was prepared by pouring the solution composed of 0.75% fibrinogen in 10 mM Tris-HCl buffer (pH 7.2), 1.0% agarose and 42 U/mL thrombin into the petri dish [20]. The plate was left for an hour at room temperature to form fibrin clot. Fifteen microliters of the bacterial extract was carefully loaded onto filter disk on the plate for wild type strain and drop (10µL) of liquid culture for screening of mutants, then the plate was incubated at 37°C. Fibrinolytic activity was estimated by measuring the diameter of the lytic circle around each disk and each drop.

Spectrophotometric Measurement of Enzyme Activity: Casein reaction as a substrate was used to assay proteolytic activity of wild type and mutant strains [22, 20]. The 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) and incubated 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. centrifugation was done at 10000×g for 10 min. and 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.

RESULTS

The bacillus strains used in this study were screened for proteolytic enzyme activity using Fibrinolytic assays in fibrin plate. In the fibrin plate method fibrinolytic activity was estimated by measuring the diameter of the lytic circle around the disk as shown in Fig. (1). A clear zone around each colony was detected by each used strain. Results showed that each used strain was capable of proteolysis fibrin, but the best producer strain was *Bacillus subtilis* A, it produced clear and big zone over fibrin plate

EMS Mutagenesis and Screening of Produced Mutants: The suspension of *Bacillus subtilis* A was exposed to 200 µg/ml of EMS for different time intervals at 15, 30, 45, 60, 75, 90, 105 and 120 minutes respectively. A total of 14 colonies were selected from the plates and they were tested for fibrinolytic enzyme productivity as indicated in Tables 1 & 2 and Fig. 2.

One colony was chosen after each 15, 30 and 75 min. respectively, two chosen colonies after 45, 60, 90, 105 and 120. These produced colonies were assayed for its production of Fibrinolytic activity by the fibrin plate method and spectrophometric to assay proteolytic activity of mutant strains. Results indicated that all produced colonies were enhanced its activity of proteolytic enzyme production.

In the fibrin plates as indicated in Fig. 2, Fibrinolytic activity was estimated by measuring the diameter of the lytic circle around the well as indicated in Table 1. The best activity of mutants produced after 120 min. (120 C, 120 B and 120 A) respectively. Followed by the colony produced after 75min.

In spectrophometric assay of proteolytic activity by produced mutant strains are indicated in Table 2. Results shown that the best producer strain also were after 120 min. (120 C, 120 B and 120 A) respectively and showed maximum enzyme production. Followed by the colony produced after 75min. followed by other mutants after 120 min. of EMS treatment.

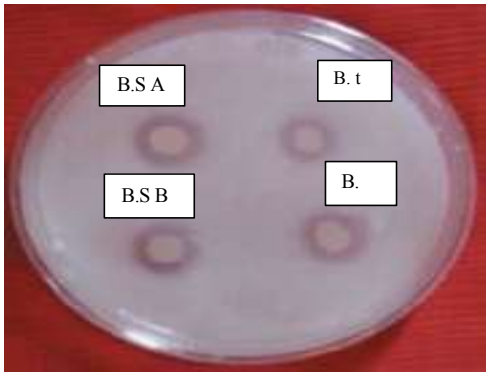

Fig. 1: Fibrinolytic activity in fibrin plate by the original wild type strains. *Bacillus subtilis* A (B.S A), *Bacillus subtilis* B (B.S B), *Bacillus thuringiensis* (B.T) and *Bacillus licheniformis* (B.L)

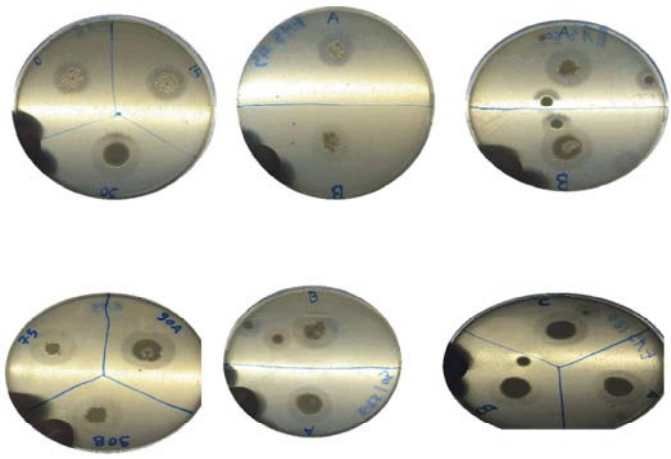

Fig. 2: Proteolytic enzyme activity produced by produced mutants after each EMS exposure time

Table 1: Inhibition zone diameter produced by each selected mutant colony

No.	Mutant detected after each treatment time (min.)	Inhibition zone (cm)
1	15	0.7
2	30	0.6
3	45A	0.8
4	45B	0.6
5	60A	0.7
6	60B	0.8
7	75	1
8	90A	0.9
9	90B	0.9
10	105A	0.9
11	105B	0.8
12	120A	1.1
13	120B	1.2
14	120C	1.3

Table 2: Spectrophometric assay of produced mutant strains

No.	Mutant detected after each treatment time (min.)	Absorbance at (660) nm.
1	15	0.561
2	30	0.648
3	45A	0.640
4	45B	0.529
5	60A	0.654
6	60B	0.641
7	75	0.679
8	90A	0.651
9	90B	0.628
10	105A	0.619
11	105B	0.682
12	120A	0.694
13	120B	0.705
14	120C	0.715

Results indicated that successful of mutation and enhancement fibrinloytic enzyme by EMS mutant in comparison with wild type strains as indicated in Figure 2 and Tables 1 and 2.

DISCUSSION

The wild strain used *Bacillus* strains isolated and identified earlier were screened for its fibrinolytic enzyme, the best strain was *Bacillus subtilis* A, on the basis of

zone of hydrolysis in medium as shown in results. Improvement of this strain with chemical mutagens (EMS) at different exposure time intervals was also done. The colony morphology of the EMS mutant strain was differed on the wild type strain it was smooth, convex and round. The selected mutants show an increase in its proteolytic enzyme. The obtained mutants after 120 minutes were hyper producer mutants in comparison to all other produced mutants and wild type strain showed maximum enzyme production depending on its zone of hydrolysis and production of protease by spectrophotometer, the present findings are similar to earlier work [23-26].

CONCLUSIONS

In recent years a great attention towards microbial fibrinolytic enzymes as these enzymes have specificity and less toxicity. In this investigation, chemical mutagen was done by Ethyl methyl sulphonate (EMS) and the fibrinolytic enzyme production increased several times than their original *Bacillus Subtilis* wild type strain. These results increase enzyme production and also reduce the cost used in other improvement methodologies such as cloning strategies. The given results also, are useful to apply to other bacillus producing enzymes. This study concluded that the mutation techniques result is faster and better for overproduction of such bacillus enzymes.

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