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Enhanced Production of Fibrinolytic Enzyme by *Bacillus* sp. Isolated from Vietnamese Traditional Fermented Soybean (*Tuong ban*) using Ultraviolet Irradiation and Chemical Mutation

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ABSTRACT

Forty-eight out of sixty-eight bacterial strains isolated from traditional fermented soybean samples (Tuong Ban) showed moderate to high fibrinolytic enzyme activity. *Bacillus* sp. HY6 had the highest fibrinolytic enzyme activity of 80.4 ± 1.2 FU/mL. The strain was selected for improving fibrinolytic activity via UV irradiation and chemically induced mutagenesis using Ethidium Bromide (EtBr) and Ethyl Methane Sulfonate (EMS). After UV-irradiation, the HY6-derived mutant, designated as strain U5_90.5, exhibited the highest fibrinolytic enzyme activity of 127 ± 5 FU/mL, about 1.6 times higher than that of the wild type HY6 strain. Strain U5_90.5 after UV mutation was subsequently subject to chemically induced mutation by EtBr and EMS. This double mutation resulted in strain ES4 which has the highest fibrinolytic enzyme activity of 404 ± 4 FU/mL. The enzyme production capability of the strain was stable after 10 generations which renders the strain a potential fibrinolytic enzyme producing strain for commercial applications. This result indicated that the *Bacillus* sp. ES4 could be a potential commercialized fibrinolytic enzyme producing bacterium.

Introduction

Cardiovascular diseases (CVD) are the leading global causes of death which accounted for approximately 18.6 and 20 million deaths in 2019 and 2020, respectively. The accumulation of fibrin in the blood vessels forms a thrombus that blocks the blood vessels, leading to myocardial infarction and other cardiovascular diseases (Peng *et al.,* 2005). Urokinase and tissue-type plasminogen activator (t-PA) have widely been used in thrombolytic therapy, but these agents have some

undesirable side effects and are expensive (Wang *et al.,* 2008). A potent fibrinolytic enzyme (nattokinase) was previously isolated from natto, a Japanese traditional fermented food (Sumi *et al.,* 1987), and has been proved as an ideal fibrinolytic agent which can be taken orally not only to dissolve blood clots but also to prevent and modulate the clotting process (Kwon *et al.,* 2011).

Fibrinolytic enzyme from microbes has attracted the attention of many researchers because of their potential as a thrombolytic agent. During the past decades, fibrinolytic enzymes have been identified in several microorganisms, especially from the genus *Bacillus*. Fermented foods have been considered the primary source for isolating bacterial strains which can excrete fibrinolytic enzymes. These fermented foods include Japanese Natto (Fujita *et al.,* 1993; Lucy *et al.,* 2019), Korean Chungkook-jiang soy sauce (Kim *et al.,* 1996), and Chinese Douchi (Peng *et al.,* 2003; Wang *et al.,* 2006; Hu *et al.,* 2019), Thua nao from Northern Thailand (Chantawannakul *et al.,* 2002), Korean Doen-jang (Choi *et al.,* 2005; Yao *et al.,* 2017), Korean Cheonggukjang (Jeong *et al.,* 2007), Korean Meju (Jo *et al.,* 2011), Indonesian fermented foods (Yanti *et al.,* 2018; Afifah *et al.,* 2014), traditional Vietnamese soybean-fermented products (Huy *et al.,* 2016), fermented soybean foods in Southeast Asia (Inatsu *et al.,* 2002), fermented soybean foods of North-East India (Thokchom *et al.,* 2014; Sharma *et al.,* 2020), Asian traditional fermented shrimp paste (Hua *et al.,* 2008). In addition to fermented foods, a few studies have reported fibrinolytic strains isolated from soil (Obeid *et al.,* 2015; Dubey *et al.,* 2011; Singh *et al.,* 2018; Ju *et al.,* 2019), aquatic environments (Agrebi *et al.,* 2009; Farraj *et al.,* 2020), and plant root (Ahamed *et al.,* 2022). However, microbial strains isolated from fermented products showed higher fibrinolytic enzyme activity compared with strains isolated from other sources, therefore the fermented foods are considered the main source for isolating bacterial strains capable of excreting fibrinolytic enzymes. In Vietnam, there have also been several studies to isolate microorganisms with fibrinolytic activity from traditional fermentation products such as fermented shrimp paste, shell waste of brackish water shrimp, soybean-fermented food samples, and traditional fermented soybean food which had fibrinolytic enzyme activity of 2.43-2.95 FU/mL, 75 U/mg protein, 29.7 - 77.9 FU/g, and 84 U/mg protein, respectively (Anh *et al.,* 2015; Thu *et al.,* 2020; Huy *et al.,* 2016; Minh *et al.,* 2022). In Vietnam, the traditional fermented soybean product is mainly Tuong (soybean paste) which is traditionally used as a dipping paste. Tuong is made from fermented soybeans originating in Ban village, Hung Yen province and therefore is named Tuong Ban. Nowadays, some provinces in the North and Central regions of Vietnam also make their own following the traditional methods for daily use. Being rich in protein content, tuong is enriched with bacteria that can produce extracellular protease during a fermentation period. Therefore, traditionally fermented soybean products from households are attractive sources of isolates with protease and fibrinolytic enzyme activity.

Hundreds of wildtype fibrinolytic bacterial strains have been obtained, however, their fibrinolytic enzyme activity and yield were not sufficiently high to be used in commercial application as therapeutic agents and functional food formulation. Thus, there is a need to establish novel strains which can overproduce fibrinolytic enzymes with high enzymatic activity. The strain improvement has been carried out using techniques such as mutagenesis by physical mutagens (UV, X-, ɤ-rays) and chemical mutagens (EtBr, EMS, etc.), because of their simplicity and low-cost procedure as compared to DNA recombinant technology. Random mutagenesis is universally used to induce genetically improved strains. This is a cost-effective method for obtaining an improved strain for industrial purposes (Sanghavi *et al.,* 2020).

Several mutants of the genus *Bacillus* were developed for the enhanced production of fibrinolytic enzymes using these techniques such as mutants of *B. subtilis* strain LD-8547 using UV, NTG (N-methyl-N'-nitroso-N-nitrosoguanidine), and ɤ-radiation (Wang *et al.,* 2008); *B. cereus* strain GD 55 by random mutagenesis using UV, EMS, and

EtBr (Raju and Divakar, 2013), *B. subtilis* S1-4 mutagenesis by combining UV and NTG treatment (Wang *et al.,* 2016), *Bacillus* sp. strain APR-4 through systemic mutagenesis using UV, EMS and EtBr (Thakhur *et al.,* 2017). These results showed that mutants increased 1.2 - 2.5-fold higher fibrinolytic protease production than parental strains (Wang *et al.,* 2008; Raju and Divakar, 2013; Wang *et al.,* 2016; Thakhur *et al.,* 2017).

To the best of our knowledge, there has not been research on the improvement of *Bacillus* sp. strain isolated from Vietnamese traditional fermented food (Tuong Ban) via mutagenesis for overproduction of fibrinolytic enzyme. In this study, we aimed to select *Bacillus* sp. strains from Tuong Ban for high fibrinolytic enzyme activity. The obtained strain with high fibrinolytic enzyme activity was subject to mutagenesis by physical (UV irradiation) and chemical (EtBr and EMS) mutagens to obtain a mutated strain with higher fibrinolytic enzyme activity than the wildtype strain.

Materials and Methods

Tuong Ban samples used in this study were collected from the Northern and Central provinces of Vietnam: Hung Yen (HY); Hai Duong (HD); Hai Phong (HP); Yen Bai (YB); Nghe An (NA); Bac Ninh (BN); Ha Noi (HN) and PhuTho (PT).

The Luria-Bertani (LB) broth composed of peptone 10 g/L; yeast extract 5 g/L; NaCl 10 g/L; pH 7.0 ~ 7.2, sterilized at 121^0C for 20 min, 1.5% (w/v) agar was added for solid medium (Ju *et al.,* 2019). Fermentation medium used to determine the activity of isolated bacterial strains with fibrinolytic enzyme activity (GY) composed of glucose 10g/L; yeast extract 50g/L; K_2HPO_4 1g/L; $MgSO_4$ 0.5g/L; pH 7.0. The fermentation medium to determine the activity of mutant strains with fibrinolytic enzyme activity (GYP) was modified from GY medium, the formula of this medium was as followed: glucose 10 g/L; yeast extract 5 g/L; peptone 5 g/L; NaCl 5 g/L; $MgSO_4$ 0.25 g/L; $CaCl_2$0.5 g/L (Roja *et al.,* 2012).
Culture media were purchased from Himedia (India) and Oxoid (England). Chemicals for analysis of analytical grade were purchased from Merck (Germany), Sigma (Germany), MP Biomedicals (France) and Wako (Japan). All the experiments were conducted in triplicate and the mean values were calculated and presented.

Screening of fibrinolytic production bacteria

Tuong Ban samples (10 g) were homogenized in 90 mL of 0.9% NaCl solution and subsequently held at 80°C for 20 min. Samples were serially diluted to a final dilution of 10^{-5} and then 100 µl of the diluted suspensions were spread onto LB agar plates and then incubated at 37°C (Wang *et al.,* 2008). After 24 hours, the colonies on LB agar plate were purified by streaking them onto fresh LB agar. Isolated colonies were streaked on LB agar amended with 1% skim milk and incubated at 37°C for 24h. The colonies with a clear zone of skim milk hydrolysis were selected and subject to testing for fibrinolytic enzyme activity. They were incubated in 50 mL GY medium in 250mL flasks at 37^0C for 24h while shaking at 150 rpm. Crude enzyme preparation was obtained by centrifuging the broth culture at 10,000 rpm for 10 min at 4^0C and then measuring for fibrinolytic enzyme activity.

Identification of the isolate

The bacterial strain which has the highest fibrinolytic enzyme activity was identified via 16S rRNA gene sequencing. The genomic DNA of the selected isolate was prepared as described previously (Nguyen *et al.,* 2007). Its 16S rRNA gene was amplified using the primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT-3'). The PCR reaction was carried out in a final volume of 20 µL containing 10 µL PCR Master mix 2x Promega Taq DNA Polymerase, 1 µL primer 10 µM 27F, 1 µL primer 10 µM 1492R, 1 µL of the DNA template, and 7 µL deionized water. The PCR was performed according to the following program: 2 min denaturation at 94^oC, followed by 30 cycles of 1 min denaturation at 94^oC; 1 min annealing at 50^oC; 1 min extension at 72^oC; and a final extension step of 2 min at 72^oC. The 16S rRNA gene was sequenced.

The sequences analysis was carried out on an ABI Prism, 3100-Avant Genetic Analyzer (Hitachi, Japan). The gene sequences were aligned with published sequences in Genbank database using the basic local alignment search tool (BLAST). The partial 16S rRNA nucleotide sequence of the selected strain has been deposited in the GenBank database.

Fibrinolytic enzyme activity assay

The fibrinolytic enzyme activity was determined according to the method described by Lin *et al.,* (2015) with some modifications as follows. The reaction mixture which contained 0.15 mL of 4 g/L fibrin solution (pH 7.4), 0.42 mL of 0.1 M Tris-HCl buffer (containing 0.01M $CaCl_2$, pH 7.4), and 30 µL crude enzyme preparation at the appropriate dilution was incubated at 37ºC for 30 minutes. The enzymatic reaction was stopped by adding 0.3 mL of 1.5 M trichloroacetic acid. The reaction mixture was then subject to centrifugation after which supernatant was collected and its absorbance was measured at 275 nm. One unit of the fibrinolytic enzyme activity was defined as the amount of enzyme that gave an increase in absorbance at 275 nm equivalent to 1 µg of tyrosine per minute at 37°C.

Enzyme activity was calculated using the following formula:

Fibrinolytic enzyme activity (FU/mL) = [(ODs – ODc)/(a x t x Ve)] × Vt x D

Wherein: ODs: optical density value of the experimental sample;

ODc: optical density value of the control;

Vt: total volume of solution (mL);

D: enzyme dilution coefficient;
t: reaction time (min);

a: constant (0.01);
Ve: enzyme volume (mL).

Preparation of cell suspension for mutation

The selected strain was grown in 50 mL LB medium in 250 mL Erlenmeyer flask at 37°C for 12 h. After that, 10 ml of the culture was centrifuged at 10,000 rpm for 10 min at 4°C.

The supernatant was removed, and the pellet was washed three times with sterile distilled water. Finally, the cell mass was resuspended in sterile distilled water to an approximate cell density of 1×10^{7} CFU/mL and used in later experiments.

Physical mutagenesis by UV Irradiation

The isolate with the highest fibrinolytic enzyme activity was used for physical mutagenesis by UV radiation according to the method described by Thakur *et al.,* (2017) and Raju and Divakar (2013) with some modifications. The bacterial strain was mutated by using a UV germicidal lamp (15 W, 254 nm) to enhance the production of the fibrinolytic enzyme. Cell suspension (2 mL) was transferred to a sterile petri plate and then irradiated in a UV chamber while keeping the distance between the UV lamp and petri plate fixed at 20 cm.

The exposure times were 30, 60, 90, 120, 150, and 180 min. Each UV exposed suspension was spread on LB agar medium plates (add 1% skim milk). The plates were incubated for 24 h at 37°C. Mutant strains for hyperproduction of the fibrinolytic enzyme were detected visually by the size of skim milk hydrolysis zones and picked up. Mutant strains were assayed quantitatively for fibrinolytic enzyme activity.

The mutant strain for the highest enzyme activity at the first UV-treatment time continued to be mutated for the second time. This process is done until the fibrinolytic enzyme activity of the mutant strain did not increase.

Mutagenesis by EtBr and/or EMS

Bacterial strain after mutagenesis by UV irradiation with the highest fibrinolytic enzyme activity was

selected for chemical mutation. Cell suspension (6ml) was transferred to a sterile 15mL tube, then EtBr or EMS or both chemicals was added at nominal concentrations of 50, 100, 150, 200, and 250 µg/mL and kept for different durations, i.e., 30, 60, 90, 120 and 150 min (Thakur *et al.,* 2017).

The chemical mutation was carried out at room temperature. After that, 1ml of the sample was withdrawn and centrifuged at 10,000 rpm for 10 min at 4°C. The cell pellet was washed three times with saline solution to remove mutagen from the sample before being resuspended in sterile distilled water. The resulting mutated cells were propagated by spreading on LB agar plates (with 1% skim milk) and incubated at 37°C for 24 h. The mutant strains were selected and grown on a modified GYP medium to determine fibrinolytic enzyme activity.

Determination of the stability of mutant strains

The mutant strain with the highest fibrinolytic enzyme activity was tested for its strain stability over generations according to the method described by Jun *et al.,* (2009) with some modifications.

Strains after mutating by UV light and chemicals for improving their high fibrinolytic enzyme activity were selected to determine the stability of the strain's fibrinolytic enzyme activity over 10 successive generations of culture on LB agar medium.

Every generation, the mutant strains were fermented on a modified GYP medium in 50mL of 250mL flask at 37°C with shaking at 150 rpm for 24 h. The crude enzyme was obtained by centrifuging the broth culture at 10,000 rpm for 10 min at 4^{0}C and then measured for fibrinolytic enzyme activity.

Results and Discussion

Screening of fibrinolytic enzyme producing bacteria

A total of 68 bacterial strains were isolated from Tuong Ban produced locally in eight Northern Vietnamese provinces. There was no obvious relation between geographical locations where the traditional fermented soybean pastes were produced and the fibrinolytic enzyme activities of the isolates. The high number of fibrinolytic enzyme producing strains isolated from Hung Yen is likely because more craft villages are producing traditional fermented soybean pastes in Hung Yen than in any other province in Vietnam. Forty-eight isolates showed fibrinolytic enzyme activity. Among the collection of thirteen strains that showed high fibrinolytic enzyme activity ranging from 20.2 ± 4.6 FU/ml to 49.2 ± 1.2 FU/ml (Fig 1), the strain HY6 exhibited the highest fibrinolytic enzyme activity (49.2 ± 1.2 FU/ml) and was subject to further study to improve fibrinolytic enzyme activity. The 16S rRNA gene sequence of HY6 showed the highest identity with that of *B. amyloliquefaciens* strain CHL4 (100%), *Bacillus* sp. strain HGUP 323 (100%), or *B. subtilis* strain 6B8 (100%). It was therefore named *Bacillus* sp. strain HY6 and the corresponding 16S rRNA gene sequence was submitted to GenBank under the accession number ON186099.

Bacillus spp. has been known to produce valuable extracellular enzymes including fibrinolytic enzymes. *Bacillus amyloliquefaciens* strain LSSE-62 which was isolated from Chinese soybean paste produced fibrinolytic enzyme with the activity of 39.28 FU/g under the optimal condition using chickpeas-based media (Wei *et al.,* 2011). Four strains of *B. amyloliquefaciens* isolated from Miso and Green Chili soybean-fermented products in the South of Vietnam had the fibrinolytic enzyme activities of 29.7 - 77.9 FU/g under solid-state fermentation using soybean oil cake powder (Huy *et al.,* 2016). *B. amyloliquefaciens* strain RSB34 isolated from doenjang purchased at a local market in Jinju, Republic of Korea, showed the highest fibrinolytic activity of 83.23 U/mL (Yao *et al.,* 2017). *B. subtilis* strain DC27 isolated from Douchi, a traditional Chinese fermented soybean food had the highest fibrinolytic enzyme activity of 163.1IU/mg protein (Hu *et al.,* 2019). *B. subtilis* strain TH19 isolated from Vietnamese traditional fermented soybean food had the protease activity of

84 U/mg protein (Minh *et al.,* 2022). Thus, our isolate produced the fibrinolytic enzyme comparable to those of published strains isolated from the traditional fermented soybean products.

Improvement of strain by UV mutagenesis

The strain HY6 was subject to successive UV irradiation, the results are shown in Fig. 2. After the first UV irradiation, the fibrinolytic enzyme activity of the mutated strains did not increase compared to the wildtype strain HY6 (i.e., 49.2 ± 1.2 FU/mL), regardless of irradiation duration. However, the wildtype strain HY6 significantly gained higher enzyme production after 3 to 5 times of UV exposure. Notably, after the 3rd mutation within 60 min, the fibrinolytic enzyme activity of the mutated strain, designated as U3_60.3, reached the highest value of 104 ± 3.2 FU/mL. This strain was subject to further UV irradiation, but its fibrinolytic enzyme activity was not significantly enhanced after the 3rd UV mutation, having its fibrinolytic enzyme activity values of 95.2 ± 5.6 FU/mL and 114 ± 8 FU/mL after the fourth and fifth UV irradiation within 90 min, respectively. Overall, UV irradiation increased fibrin hydrolysis activities in the wildtype strain of *Bacillus* sp. HY6. After 5 times of treatment with UV light, the fibrinolytic enzyme activity of the mutant strain, designated as U5_90.5, increased 2.3-fold compared to the wild-type strain, from 49.2 ± 1.2 FU/mL to 114 ± 8 FU/mL. The fibrinolytic enzyme activity of the original isolate was relatively higher than the UV mutated strains in published studies. Sher *et al.,* 2012 reported that a mutant of *B. subtilis* strain G-4 increased its protease production by approximately 2-fold over the wild strain using UV treatment in 10 min. Similarly, strain improvement by UV mutagenesis was observed in *B. subtilis* S1-4 mutant (Wang *et al.,* 2016). Demirkan *et al.,* 2018 obtained the *B. subtilis* E6-5 mutant by UV irradiation at a 15 cm distance and irradiation time of 5 min resulting in an increase in protease activity of 1.5-fold compared to the parent strain. However, mutagenesis with only UV irradiation does not improve sufficiently protease or fibrinolytic enzyme production to be able to be applied in large-scale enzyme production.

Improvement of strain by EtBr or EMS or both mutagenesis

The UV mutated strain U5_90.5 was selected for further strain improvement by treating with either EtBr or EMS or both chemicals. Thirty-five mutant strains derived from the UV-mutated strain U5_90.5 exhibiting a large hydrolysis zone on skim milk plate were selected for cultivation on a liquid medium to determine fibrinolytic enzyme activity.

Eight EtBr mutated strains showed substantially higher fibrinolytic enzyme activity compared to that of strain U5_90.5, ranging from 117.6 ± 5.6 FU/mL to 452 ± 4 FU/mL. Notably, two mutant strains which showed the highest fibrinolytic enzyme activity were E1 (452 ± 4 FU/mL) and E5 (440 ± 8 FU/mL). They were obtained after 30 min of exposure to 50 µg/mL and 150 µg/mL of EtBr, respectively (Fig. 3). The fibrinolytic enzyme producing mutants decreased with increasing EtBr concentration or exposure time.

Using similar selection criteria, thirty-eight mutated strains using EMS treatment were selected for subsequent screening for fibrin hydrolysis activities and twenty-eight strains showed higher fibrinolytic enzyme activity than that of strain U5_90.5. The enzyme activity of these EMS mutated strains increased from 128 ± 8 FU/mL to 456 ± 8 FU/mL. A mutant with the highest fibrinolytic enzyme activity was S33 (456 ± 8 FU/mL). It was obtained after 150 min of exposure to 100 µg/mL of ESM. Two mutant strains had fibrinolytic enzyme activity remarkedly higher than 350 FU/mL, i.e., S9 (364 ± 4 FU/mL) and S37 (376 ± 0 FU/mL) (Fig. 4).

Fig.1 Fibrinolytic enzyme activities of bacteria isolated from Tuong Ban samples collected fromHung Yen (HY); Hai Duong (HD); Hai Phong (HP); Yen Bai (YB); Nghe An (NA); Bac Ninh (BN); Ha Noi (HN) and Phu Tho (PT).

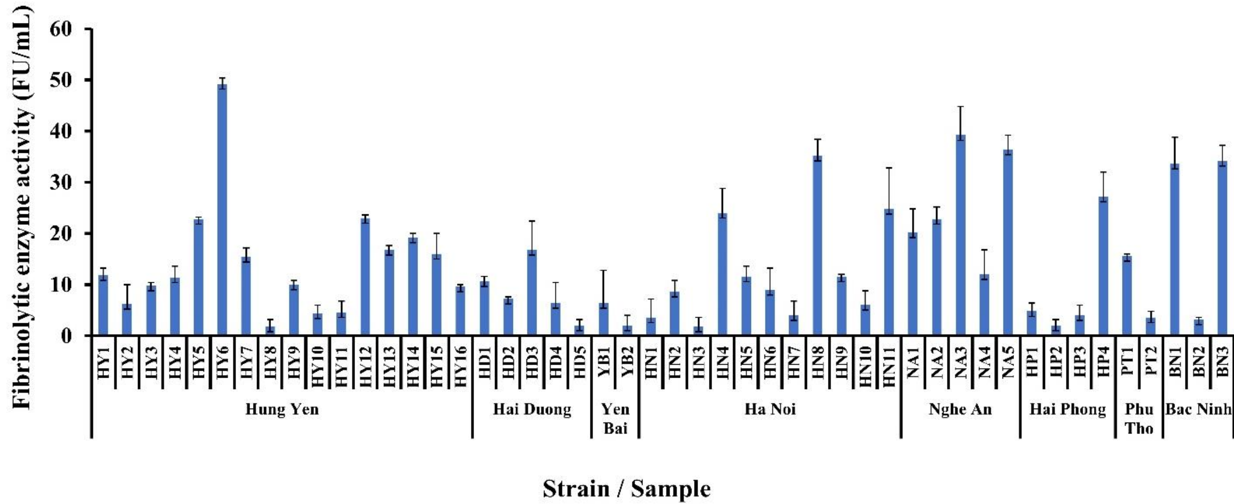


Fig.2 Fibrinolytic enzyme activities of mutants after UV irradiations. The mutants were obtained after 30, 60, 90, 120, 150, and 180 min exposure to UV lights. The number of irradiation treatments was denoted by Ui (i: 1-5).

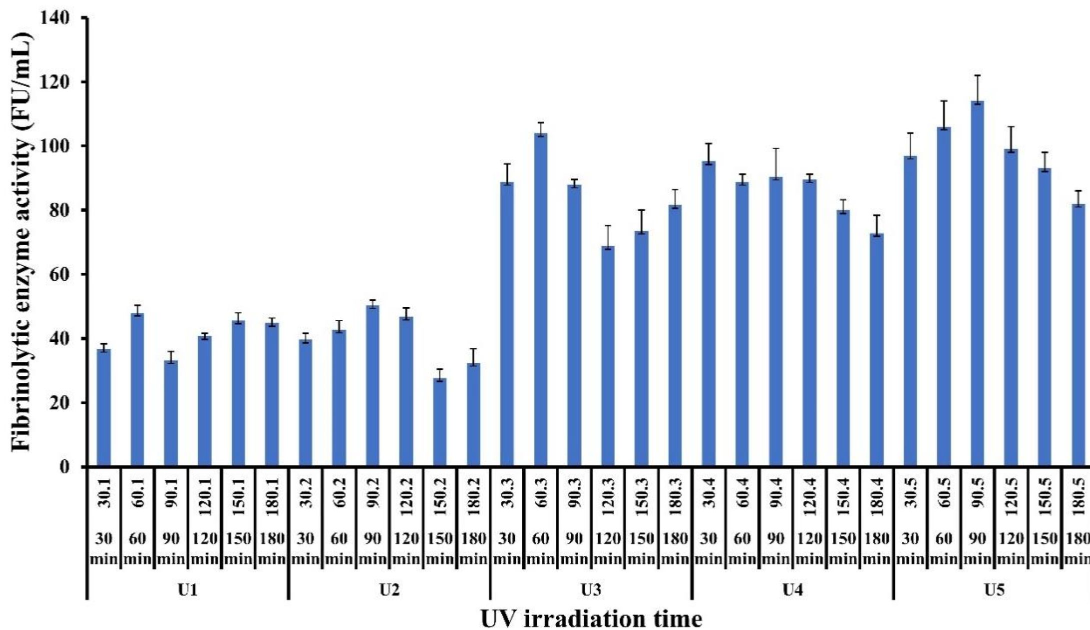


Fig.3 Fibrinolytic enzyme activities of mutants after treatment with EtBr. The mutants were obtained using EtBr at concentrations of 50, 100, 150, 200, and 250 µg/mL for different durations from 30, 60, 90, 120, to 150 min. The mutants were designated as E1 to E35.

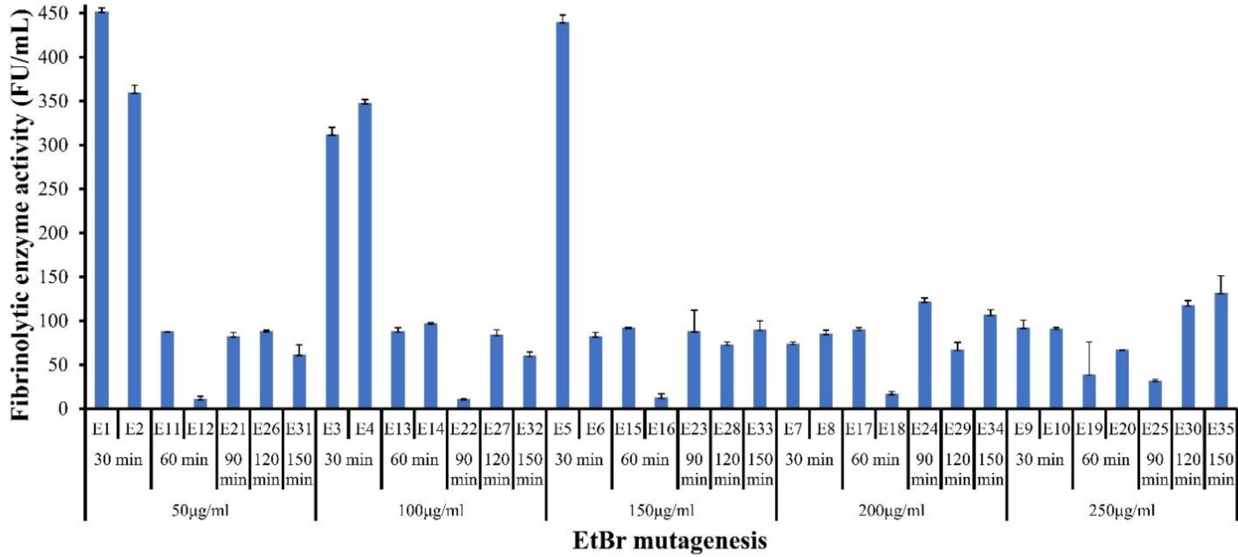


Fig.4 Fibrinolytic enzyme activities of mutants after treatment with EMS. The mutants were obtained using EMS at concentrations of 50, 100, 150, 200, and 250 µg/mL for different durations from 30, 60, 90, 120, to 150 min. The mutants were designatedas S1 to S38.

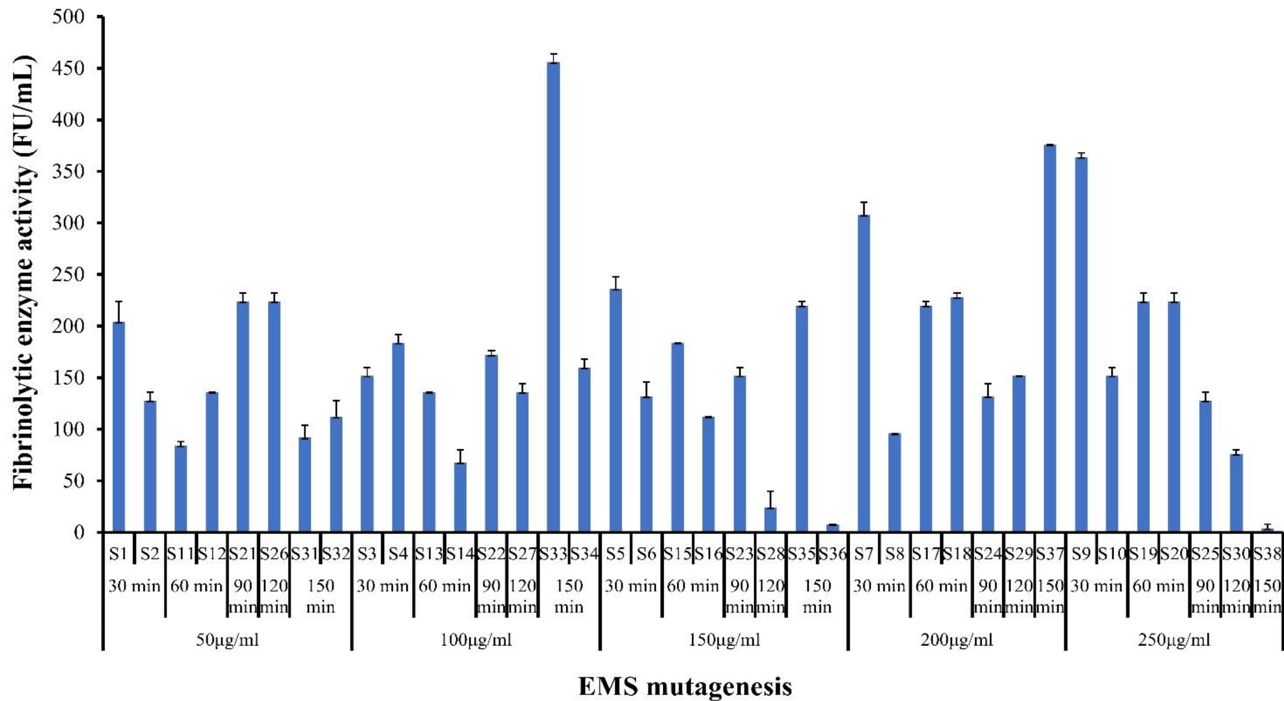


Fig.5 Fibrinolytic enzyme activities of mutants after treatment with both EtBr and EMS. The mutants were obtained using EtBr and EMS (at 1:1 ratio)at concentrations of 50, 100, 150, 200, and 250 µg/mL for different durations from 30, 60, 90, 120, to 150 min. The mutants were designated as ES1 to ES33.

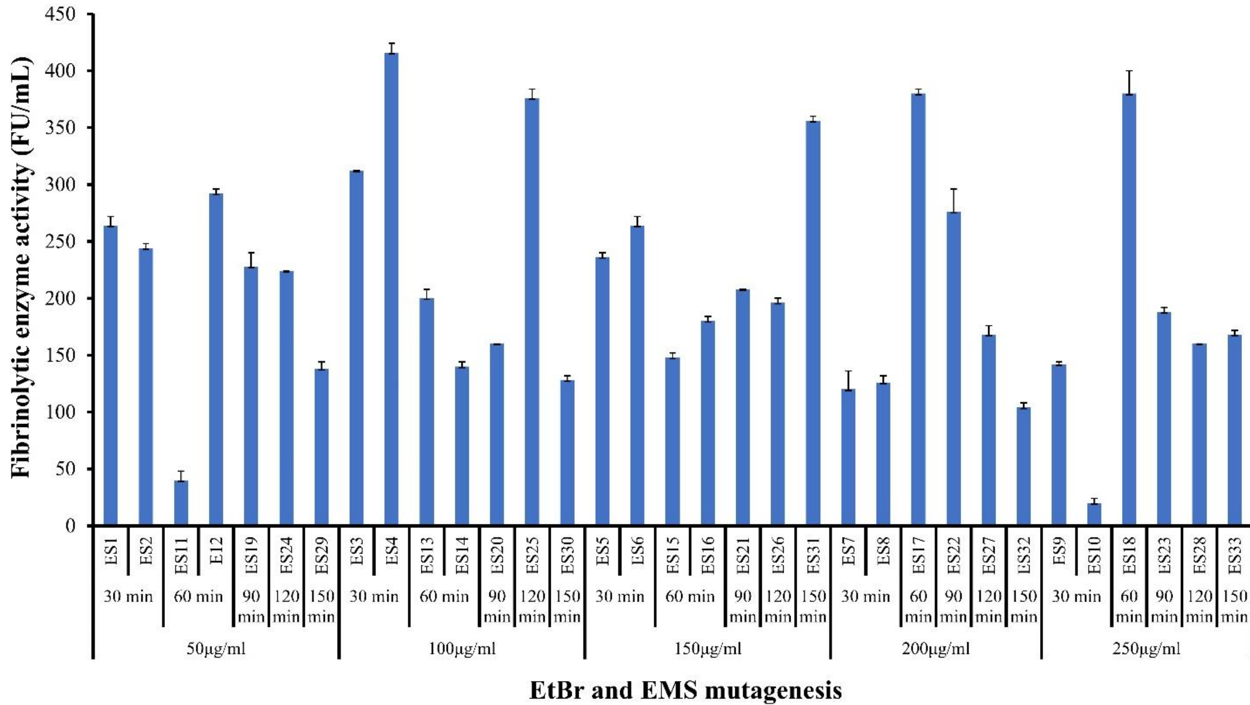
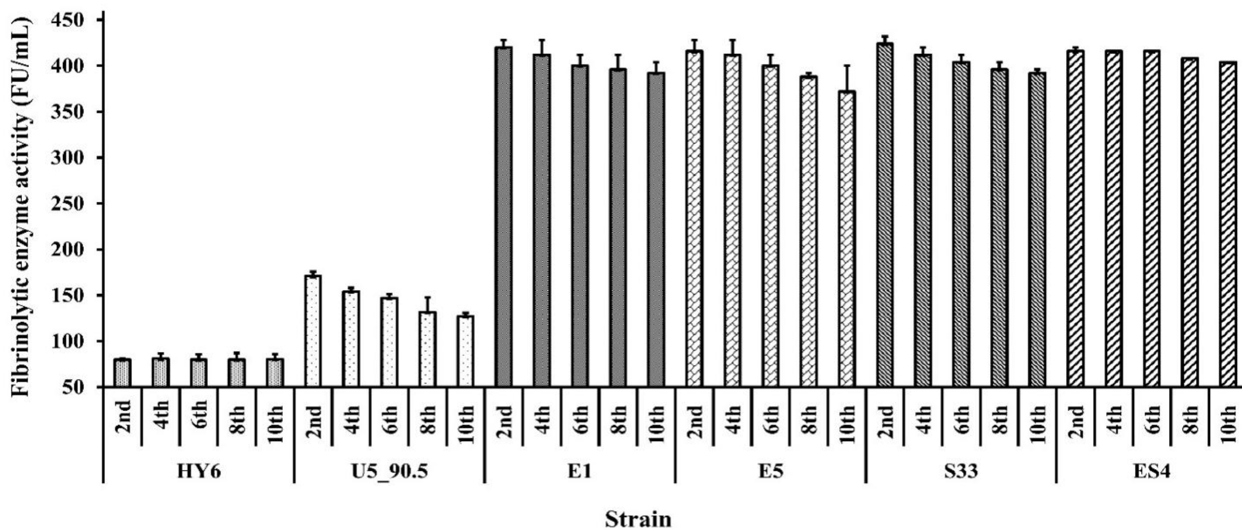


Fig.6 The stability of fibrinolytic enzyme activity of mutants for 10 generations. The fibrinolytic enzyme activity of the wild-type strain (HY6), the mutant using UV irradiation (U5_90.5), the mutants using EtBr (E1 and E5), the mutant using EMS (S33), the mutant using both EtBr and EMS (ES4) were determined for 10 generations.



Among thirty-three mutated strains using both EtBr and EMS treatment were selected, and thirty mutant strains had higher fibrinolytic enzyme activity than that of the derived strain U5_90.5, the enzyme activity of strains increased from 120 ± 16 FU/mL to 416 ± 8 FU/mL. Five mutant strains showed fibrinolytic enzyme activity higher than 350 FU/mL were ES4 (416 ± 8 FU/mL), ES17 (380 ± 4 FU/mL), ES18 (380 ± 8 FU/mL), ES25 (376 ± 8 FU/mL), and ES31 (356 ± 4 FU/mL) (Fig. 5). There was no significant difference in the fibrinolytic enzyme activity of these mutants. In addition, the number of mutants with high fibrinolytic enzyme was increased using both EtBr and EMS treatment compared with treating with either agent.

The results showed that the fibrinolytic enzyme activity of the mutants exposed to EtBr or/and EMS was enhanced and observed an increase in enzyme activity by 3.5 - 4-fold and 8 - 9-fold compared to that of the mutants using UV treatment and the wild-type strain, respectively. Chemical mutagenesis appeared to be more efficient for improving fibrinolytic enzyme production. Mutagenesis using EMS was found to be more effective than UV irradiation in the case of *Streptomyces venezuelae* for enhancing the production of fibrinolytic enzyme (Bhavani *et al.,* 2012). *B. cereus* GD55 mutant strain was developed by using EtBr followed by EMS treatment resulting in 2-fold higher fibrinolytic protease production than the wildtype strain (Raju and Divaka, 2013).

However, the combination of multiple mutagenic treatments may give better results. Wang *et al.,* (2008) reported the enhanced production of fibrinolytic enzyme from *B. subtilis* strain LD-8547 obtained by successive use of UV, NTG, and ɤ-radiation. When combining UV and NTG, the mutant of *B. subtilis* strain S1-4 exhibited 2.5-fold higher extracellular caseinolytic activity than did the wild-type strain (Wang *et al.,* 2016). Enhancement of protease production of some *Bacillus* spp. by physical and chemical mutagenesis resulted in an increase in productivity by 2-3-fold than the parental strains (Soliman *et al.,* 2016). Combinational exposure of wild strain to UV and EMS gave a better mutant with higher fibrinolytic enzyme activity than using either method (Gopinath *et al.,* 2020).

Fibrinolytic enzyme production stability by mutant strains

Strains HY6 and U5_90.5 showed higher activity of fibrinolytic enzyme on GYP medium than on GY medium. The enzyme activity of the wild-type strain HY6 increased from 49.2 ± 1.2 FU/mL on GY medium (Fig. 1) to 80.4 ± 1.2 FU/mL on GYP medium (Fig. 6), while fibrinolytic enzyme activity of the mutant strain U5_90.5 increased from 114. ± 8 FU/mL (Fig. 2) to 177.8 ± 9.8 FU/mL (Fig.6). Fibrinolytic enzyme activity of the wildtype strain HY6 isolated from traditionally fermented soybean was significantly improved by mutation with UV irradiation combined with EtBr and/or EMS which resulted in four mutant strains with fibrinolytic enzyme activity higher than 380 FU/mL, i.e., E1 (452 ± 4 FU/mL), E5 (440 ± 8 FU/mL), S33 (456 ± 8 FU/mL), and ES4 (416 ± 8 FU/mL). The mutant strains and the wild-type HY6 strain were examined for fibrinolytic enzyme production stability for over ten successive generations. The fibrinolytic enzyme activity of the mutant by UV treatment (U5_90.5) was unstable, and it decreases by 29% after 10 generations. While the activity of mutants by combinate UV with EtBr (E1 and E5) or EMS (S33) was reduced by about 12% or 8%, respectively, after 10 generations (Fig. 6). The mutated strain ES4 did not decrease its fibrinolytic enzyme production significantly over ten generations. The fibrinolytic enzyme activity of the strain ES4 reached 404 ± 4 FU/mL and was the highest among others after 10 generations. The productivity of the mutant strain ES4 was increased about five-fold compared to the wildtype strain HY6 on GYP medium. It was shown that the high fibrinolytic enzyme production by *Bacillus* sp. ES4 is stable and has the potential for use as a production strain in fermentation. The stability of *B. subtilis* LD-8547 mutant had been reported after four generations (Wang *et al.,* 2008). The mutation was found to improve the stability of

the *Streptomyces venezuela* using UV and EMS (Bhavani *et al.,* 2012). *Bacillus* sp. CAMB 10358 was treated with NTG, and two mutants were observed to be stable (Hussain *et al.,* 2019).

The fibrinolytic enzyme exhibits supreme antithrombotic properties in both treatment and prevention which render the enzyme a potential candidate for commercial application. The search for fibrinolytic enzyme producing bacteria and improvement of their enzyme production is essential for applications. In this study, fibrin hydrolyzing *Bacillus* sp. strain HY6 was isolated from traditional fermented soybean in the northern provinces of Vietnam. Fibrinolytic enzyme activity of this strain was significantly improved by UV irradiation combined with EtBr and EMS treatment. The mutated strain ES4 showed its fibrinolytic enzyme production stability for over 10 generations of which its activity reached 404 ± 4 FU/mL. Strain ES4 could be a potential commercialized fibrinolytic enzyme producing bacterium. Further study should be focused on the fermentation strategy to improve fibrinolytic enzyme production by this strain.

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