ISOLATION AND SCREENING OF *Bacillus* sp. PRODUCING FIBRINOLYTIC ENZYME WITH LOW CULTURE BROTH VISCOSITY

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SUMMARY

Cardiovascular diseases (CVDs) are the leading cause of deaths worldwide. Prevention and treatment of CVDs can differ by the type of condition but generally includes a healthy lifestyle (diet, exercise) and medication regulating blood pressure and cholesterol. In this context, fibrinolytic enzymes naturally produced by *Bacillus* genus are a potential agent for fighting against CVDs thanks to its blood clot breaking ability. However, the presence of poly-γ-glutamic acid (γ-PGA), a by-product of fermentation, leads to several limiting factors for enzyme downstream processing. This research concentrated on isolating and screening *Bacillus* strains producing fibrinolytic enzymes with low culture viscosity. From 25 samples of Vietnamese traditional fermented sauce (*Tuong-ban*), soil, seawater, and commercial natto samples, 73 isolates producing protease were selected. Among them, 40 isolates were able to produce fibrinolytic enzyme ranging from 6.4 to 98.5 FU/mL. Considering the culture viscosity during submersion cultivation, the strain TVY.04 was selected because of the highest fibrinolytic enzyme (98.5 FU/mL) and the lowest viscosity (3.87 cP). This was then identified as *Bacillus amyloliquefaciens* using 16S rRNA sequence. SDS-PAGE and TLC analyses suggested that *B. amyloliquefaciens* TVY.04 is a nattokinase- and γ-PGA producing strain.

Keywords: Isolation, screening, Bacillus. sp, fibrinolytic enzymes, traditional fermented soybean, viscosity, γ-PGA.

INTRODUCTION

Cardiovascular diseases (CVDs) are defined by the World Health Organization as a group of disorders of heart and blood vessels. They include stroke and heart diseases, which are considered the leading cause of death worldwide. Due to the important effects of CVDs on people's quality of life, there have been many successful studies in finding ways to prevent and treat these diseases. CVDs are treated by the extensive use of tissue-type plasminogen activator (t-PA), urokinase, and streptokinase, among other fibrinolytic agents. However, they are expensive and cause side effects such as allergic reactions, bleeding complications, and short half-lives. Therefore, the safety and efficacy of fibrinolytic enzymes from microorganisms, especially nattokinase (NK), have attracted much attention in recent years.

In 1987, nattokinase was first discovered in natto, a traditional Japanese fermented soybean food. Other fermented foods known as sources of fibrinolytic enzymes and fibrinolytic producing strains were Tofuyo (Japan); Chungkook-Jang soy sauce, Meju soybean paste, Doenjang (Korea); Douchi (China); and Thua Nao (Thailand) (Mine *et al.*, 2005). *Tuong-ban* is a Vietnamese traditional fermented sauce, that which has appeared in Vietnam since the 19th century and is widely used until now. The *Tuong-ban* making process included two fermentation steps of the main ingredients, sticky rice and soybean, by fungi and bacteria, respectively. Therefore, *Tuong-ban* is a potential source to isolate *Bacillus* producing fibrinolytic enzymes, thanks to the natural soybean fermentation step. Previously, Huy *et al.* (2016) isolated four strains of *Bacillus amyloliquefaciens* from commercial *Tuong-ban* sauces, and the highest fibrinolytic activity among all isolates reached 77.95 FU/g. More recently, Bui *et al.* (2022) reported *Bacillus* sp. isolated from *Tuong-ban*, which represented fibrinolytic activity of 49.2 FU/mL. Besides, soil and seawater were also used as sources for isolating strains producing fibrinolytic enzymes, but with significantly lower activities compared to isolates from soybean fermented foods. From published studies, fibrinolytic enzymes were successively discovered from different species of bacteria, namely, *Streptococcus, Paenibacillus, Pseudomonas*, and *Bacillus*. In addition, the most potential bacteria producing fibrinolytic enzymes were *Bacillus subtilis* and *Bacillus amyloliquefaciens* (Peng *et al.*, 2005).

Poly-γ-glutamic acid (γ-PGA) is a positively charged polypeptide composed of D/L-glutamic acid units linked by γ-amide bonds. Possessing several attractive properties, such as being water soluble, biodegradable, biocompatible, non-toxic, and non-immunogenic, γ-PGA can be widely used as a green and friendly biomaterial. However, for enzyme production, γ-PGA is an undesirable by-product. The presence of γ-PGA causes an increase in viscosity of the fermentation broth, reduces enzyme yield, and alleviates foaming, thereby hindering the downstream processing of the enzyme. Several *Bacillus* species, especially *B. subtilis* and *B. amyloliquefaciens* were known as glutamic acid-independent strains producing γ-PGA (Ogunleye *et al.*, 2015).

Therefore, both fibrinolytic activity and γ-PGA accumulation were important criteria when screening enzyme production strains.

From these above contexts, this work focused on isolating *Bacillus* strains producing high fibrinolytic activity in parallel with low γ-PGA accumulation during submerged fermentation. Various natural samples from Vietnamese biodiversity (soil, seawater, *Tuong-ban*) and some commercial Japanese natto products will be used as isolation sources.

MATERIALS AND METHODS

Materials

Sample collection for isolation

Eleven Vietnamese soy fermented samples were collected from different households that traditionally produce *Tuong-ban* in Bac Ninh (TBN, TBN2, TT, TTL, TTS), Ha Noi (THD, TDA), Hung Yen (THY, THY2), Nghe An (TNA), and Vinh Yen (TVY). Eleven soil samples were collected at different positions and depths in Da Nang (SDN), Cuc Phuong (SCP2, SCP3, SCP4, SCP5, SCP6, SCP7), Sapa (SSP10, SSP30), Tam Dao (STD), Thanh Hoa (STH). Two samples of commercialized Natto from Dashi Tappurigohan (CND) and Okame Takano (CNO) were purchased from Aeon Mall Long Bien in Ha Noi. One seawater sample was collected from Ghenh Da Dia, Phu Yen (SBG).

Medium composition

The skim-milk agar (SMA) plate consisting of (% w/v) peptone 1%, yeast extract 0.5%, NaCl 1%, agar 1.8%, and skimmed milk 1% was used for isolation and screening of protease-producing strains. The Luria-Bertani broth (LB) contains (%w/v): peptone 1%; yeast extract 0.5%; NaCl 1%, pH 7.0~7.2. The fermentation medium (GPY) includes (% w/v): glucose 1%, peptone 1%, yeast extract 1%, NaCl 0.5%, K_2HPO_4 0.1%, $MgSO_4$ 0.05%, and $CaCl_2$ 0.1%.

Methods

Isolation and screening of bacterial strains

The isolation and screening was performed according to Bui *et al.* (2022) with minor modification. One milliliter (liquid sample) or one gram (solid sample) was mixed with 9 mL sterilized NaCl 0.9% (w/v) and heated to 80°C for 20 min. The supernatant was then diluted in sterilized NaCl 0.9% (w/v) and spread onto the SMA plates. After 24 h of incubation at 37°C, colonies with clear halo (indicating skimmed milk degradation zone) were selected and isolated by streaking on fresh SMA plates repeatedly. One single colony from the SMA plate was inoculated into 5 mL of LB medium at 37°C, 150 rpm for 16 h. The seed culture broth was transferred to 10 mL of GPY medium at the initial $OD_{600\ nm}$ value of 0.2 and then incubated at 37°C, 150 rpm for 24 h. Culture broth was centrifuged at 10000 rpm for 10 min, and the supernatant was used for measurement of fibrinolytic activity.

Enzyme assay

The fibrinolytic activity was measured according to Liu *et al.* (2005) with fibrin 4 g/L as substrate. One unit of the fibrinolytic 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. The standard curve was $y = 0.0091x$ ($R^2 = 0.999$) with y is the absorbance at 275nm, x is the concentration of tyrosine (µg/ml).

Viscosity analysis

The viscosity of the cell-free culture was determined by a Brookfield DV2T Viscometer (Brookfield, Middleboro, MA, USA) using an LV spindle at room temperature, 100 rpm. After 1 min of torque stabilization, suspension viscosity was calculated as the average value for 30 seconds at the end of the step.

SDS – PAGE analysis

Electrophoresis analysis was carried out using 12% polyacrylamide for separating gel and 4% polyacrylamide for stacking gel (Laemmli, 1970). A fixed volume (15 µL) of samples was loaded, and the electrophoresis was carried out at 15 mA. Protein bands were visualized by staining with Coomassie brilliant blue R 250. Gangnam stain protein ladder (Amersham Biosciences CAT.NO.24052) was used as standard.

Identification of bacterial strain

A strain was identified on the basis of 16S rRNA gene sequence analysis. The 16S rRNA gene of the isolate was amplified using the corresponding 16S primer pairs: 27F and 1429R. PCR products were checked by 0.8% agarose gel electrophoresis with the DNA marker LADDER, DirectloadTM, 1 KB (0.5–10 kb) (Sigma-Aldrich) before being sequenced (1st BASE DNA Sequencing Services). The sequences were then analyzed and compared on a gene bank (https://blast.ncbi.nlm.nih.gov/) to identify the bacterial strain.

Analysis of exo-polysaccharide (EPS)

Samples (cell-free culture broth) were treated according to Goto and Kunioka (1992), and then deproteinized using the Sevag method (Fang *et al.*, 2013). The deproteinized EPS was hydrolyzed by 6M HCl at 110°C for 1 h, then thin layer chromatography (TLC Silica gel 60 F_{254}) was performed using mobile phase n-butanol: acetic acid: H_2O (3:1:1. v/v/v) to analyze its main components (Song *et al.*, 2019). L-glutamic acid (Sigma-Aldrich) was used as standard.

RESULTS AND DISCUSSIONS

Isolation of protease producing bacteria

Fibrinolytic enzyme is a sub-class of proteases, and consequently it is able to degrade skimmed milk. Therefore, protease producing strains were selected based on the formation of a clear skimmed milk degradation zone on SMA plates. Hence, a total of 73 protease producing isolates were obtained: 41 isolates from *Tuong-ban*, 23 isolates from soil, 7 isolates from Natto, and 2 isolates from seawater. The morphologies of colonies isolated from samples were milky, milky white, or pale yellow with elevated or flat surfaces that were smooth or wrinkled. In addition, these colonies had rounded, undulated, and serrated edges; and they were either mucinous or dried. Then, all isolates were screened to find out the strain producing fibrinolytic enzyme by submerged fermentation.

Screening of isolates producing fibrinolytic enzymes

Bacterial strains were grown on GYP and the fibrinolytic activity of the cell-free culture broth was measured (*Fig. 1)*. In general, 40 out of 73 isolates showed the ability to produce fibrinolytic enzyme at significant levels, which ranged from 6.4 to 98.5 FU/mL. Among them, five isolates, namely THY2.04, THY2.05, TVY.04, THD.01, TTS.05, indicated the highest fibrinolytic activities of 56 ± 4.0, 50 ± 0.5, 98.5 ± 3.5, 49 ± 3.0, and 47 ± 3.0 FU/mL, respectively. Interestingly, these five best isolates were all obtained from the Vietnamese traditional fermented soybean sauce (*Tuong-ban*). These results were consistent with Mine *et al.* (2005), who reported that Asian foods, especially fermented products, are the best source to isolate bacteria producing fibrinolytic enzyme. The strain TVY.04 exhibited the highest fibrinolytic enzyme among the five isolates, reaching 98.5 ± 3.5 FU/mL. This fibrinolytic activity showed great potential compared to values from literature. Using fibrin as substrate for determination of enzyme activity, the marine bacterium *Bacillus subtilis* A26 exhibited 63.45 FU/mL (Agrebi *et al.*, 2009), and strain *Bacillus* sp. HY6 isolated from *Tuong-ban* showed 49.2 FU/mL (Bui *et al.*, 2022). Using fibrinogen as substrate, nattokinase activity of the strain *B. amyloliquefaciens* CB1 isolated from soybean fermented food was 77.95 FU/g (Huy *et al.*, 2016).

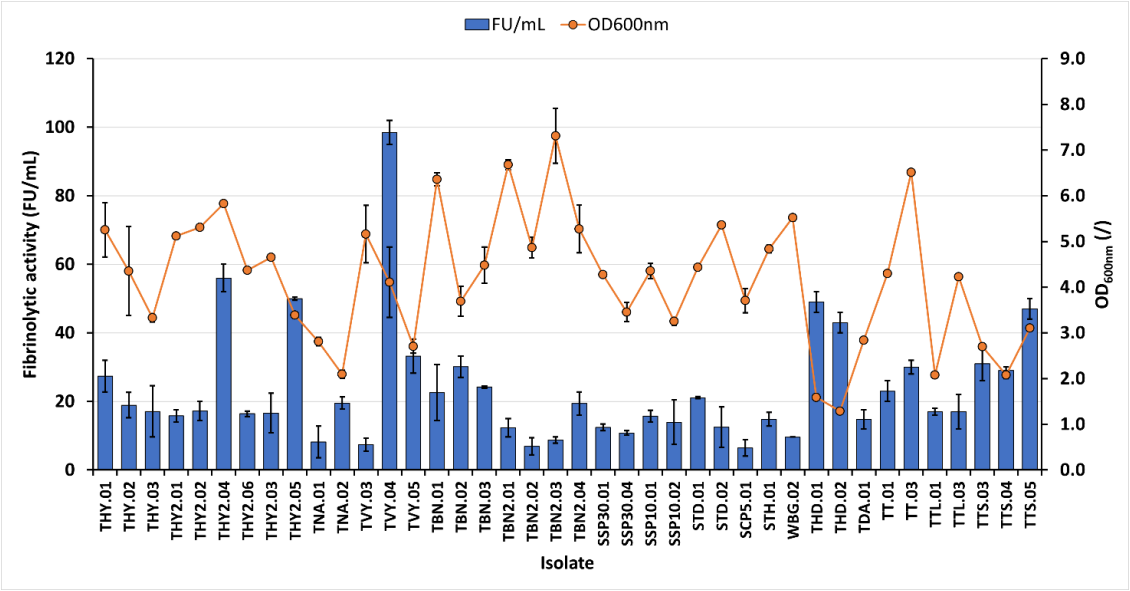

Figure 1. The fibrinolytic activity and OD 600nm measured at t = 24h of 40 isolates

The names of isolates were given according to the sample's code and numbers were added as suffixes to distinguish different isolates from the same sample.

Five isolates (THY2.04, THY2.05, THD.01, TTS.05, TVY.04) were grown on 50 mL GYP medium to evaluate their culture broth's viscosity. Obtained results were represented in *Fig. 2.* In general, the culture viscosity of five isolates showed average differences. Three isolates, THY2.04, THY2.05, and TTS.05, had approximately the same culture viscosity of approximately 5 cP. In contrast, the strain TVY.04 indicated the lowest viscosity and reached 3.87 ± 0.03 cP. Thus, the strain TVY.04 was selected for further experiments.

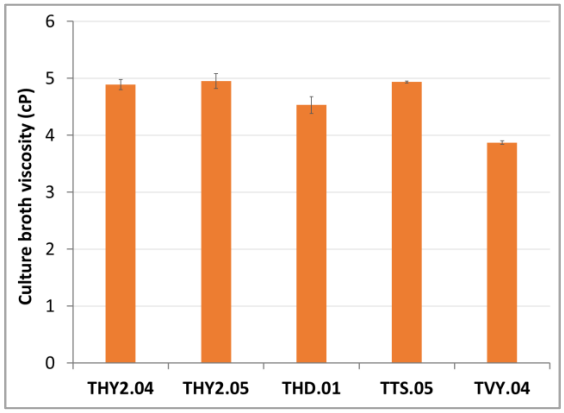

Figure 2. The culture broth viscosity of the best five isolates

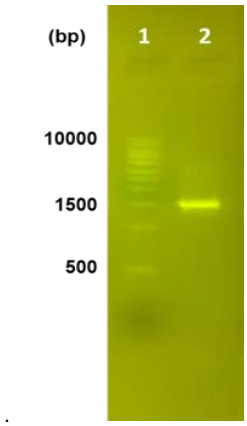

Figure 3. Electrophoresis of 16S rRNA amplified PCR product of the strain TVY.04

Lane 1: Marker; lane 2: The sequence of the strain TVY.04

Morphology and identification of the strain TVY.04

The morphology of strain TVY.04 was shown in *Fig. 3*. The strain TVY.04 had a clear halo on the SMA plate after incubation at 37°C for 15 h. The colony of this strain was milky white, with a smooth and elevated surface, and a rounded edge, and it was a mucinous colony (*Fig. 4A)*. The strain TVY.04 was a gram-positive, rod-shaped, chain-formed, or single-stranded cell (*Fig. 4B).* Based on gram staining and morphology, the strain TVY.04 showed highly homogeneity with the genus *Bacillus*.

The strain TVY.04's DNA was extracted and PCR amplification of 16S rRNA gene sequences was carried out using a pair of primers 27F, 1429R. As expected, the length of the 16S rRNA amplified product was about 1500 bp (*Fig 3*). The amplified PCR products were submitted for sequencing analysis using the Sanger Sequencing technique (1st BASE Sequencing Services) and then the nucleotide sequences were compared with the Genbank database. The sequence of the strain TVY.04 was up to 99.93% homologous with the 16S RNA of *Bacillus amyloliquefaciens* TBMAX73 (Genbank accession number MK834711). Based on these results, the strain TVY.04 was suggested *as Bacillus amyloliquefaciens* TVY.04.

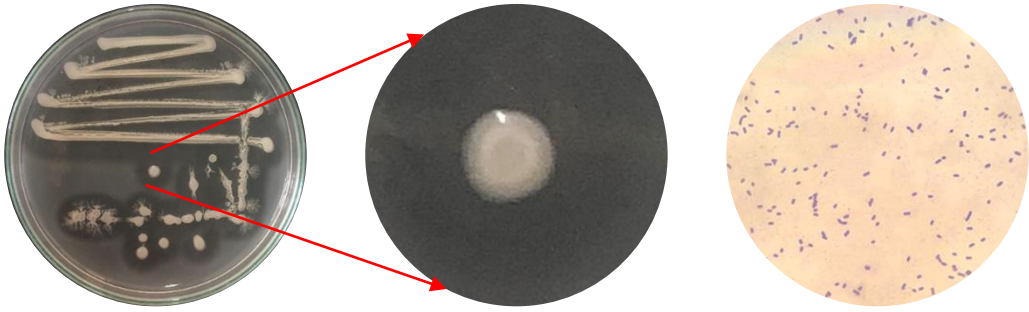

Figure 4. Morphology of strain *B amyloliquefaciens* TYV.04 isolated from *Tuong-ban*

A. Colony on SBM plate, 15 h; B. Gram coloration, magnification 100×

Analysis of exo-polysaccharide (ESP)

In this study, TLC was used to examine the crude EPS produced by *B. amyloliquefaciens* TVY.04. The analysis of hydrolyzed EPS products showed three bands on lane V *(Fig. 5)*. Among these bands, the retention factor of the largest band was similar to L-glutamic acid (lane G). This result suggested that γ-PGA may exist as the main component of EPS from *B. amyloliquefaciens* TVY.04. Previously, Song *et al.* (2019) also pointed out the presence of γ-PGA in *Bacillus* culture broth by TLC analysis.

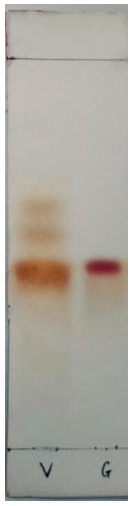

Figure 5. Thin layer chromatography analysis of EPS hydrolysis products

Lane V: EPS hydrolysis products; lane G: standard L-glutamic acid.

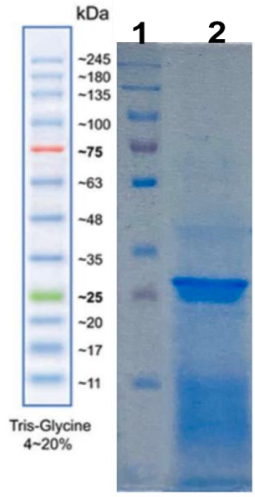

Figure 6. SDS – PAGE analysis of crude fibrinolytic enzyme from *B amyloliquefaciens* TVY.04

Lane 1: Molecular weight of marker protein; lane 2: Crude enzyme. On the left: Gangnam stain protein ladder.

SDS – PAGE analysis

The crude enzyme produced by *B. amyloliquefaciens* TVY.04 was analyzed with SDS-Page. Several bands were observed on stained gel (*Fig. 6*, lane 2). Referring to the protein ladder, the main protein band was identified as 27 kDa, with one extra band at 42 kDa and several at lower than 20 kDa. From the literature, the molecular weight of the bacterial fibrinolytic enzyme, in most cases, Nattokinase, showed some variation from 27 to 30 kDa. (Tran *et al.*, 2015, Yao *et al.*, 2017). It was then strongly recommended that the fibrinolytic enzyme from *B. amyloliquefaciens* TVY.04 is nattokinase.

CONCLUSION

In this study, 73 isolates producing protease on SMA plates were obtained from 25 samples, wherein 40 isolates could produce fibrinolytic enzymes ranging from 6.4 to 98.5 FU/mL. Among them, five isolates had the highest fibrinolytic activity, namely TVY.04, THY2.04, THY2.05, THD.01, and TTS.05. The strain TVY.04 showed the lowest viscosity, reaching 3.87 ± 0.03 cP, and the highest fibrinolytic activity of 98.5 FU/mL. The strain TVY.04 was then identified as *Bacillus amyloliquefaciens* with 99.93% of identity using the 16S rRNA sequence. The analysis of exo-polysaccharide by TLC revealed the presence of γ-PGA as the main component causing viscosity of the culture broth. The result of SDS – PAGE analysis indicated that the molecular weight of fibrinolytic enzyme from *B. amyloliquefaciens* TVY.04 was 27 kDa.

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PHÂN LẬP VÀ TUYỂN CHỌN CHỦNG *Bacillus* sp. SINH TỔNG HỢP ENZYME PHÂN GIẢI FIBRIN VÀ TẠO ĐỘ NHỚT THẤP

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TÓM TẮT

Các bệnh về tim mạch (CVDs) được cho là nguyên nhân hàng đầu dẫn tới các ca tử vong trên thế giới do sự hình thành của các cục máu đông. CVDs có thể được phòng ngừa và điều trị bởi các phương thức khác nhau như áp dụng một lối sống lành mạnh hoặc sử dụng các loại thực phẩm chức năng giúp hỗ trợ giảm thiểu sự hình thành cục máu đông trong mạch máu. Trong bối cảnh này, enzyme tiêu sợi huyết (fibrinolytic enzymes) do các chủng *Bacillus* tự nhiên sản xuất là một tác nhân tiềm năng trong phòng ngừa các bệnh về tim mạch nhờ khả năng làm tan cục máu đông, và có nhiều ứng dụng rộng rãi, đặc biệt được quan tâm trong phòng chống và điều trị CVDs. Tuy nhiên, sự có mặt γ-PGA – một sản phẩm phụ của quá trình lên men – dẫn tới một vài trở ngại trong quá trình thu hồi enzyme. Do đó, nghiên cứu này đã tập trung vào việc phân lập và sàng lọc các chủng *Bacillus* có khả năng sinh tổng hợp enzyme tiêu sợi huyết đồng thời tạo độ nhớt canh trường thấp. Từ 25 mẫu phân lập gồm tương bần – sản phẩm lên men từ đậu tương, đất, nước biển và sản phẩm natto thương mại đã phân lập được 73 chủng vi khuẩn sinh protease. Trong đó, 40 chủng có khả năng sinh tổng hợp enzyme tiêu sợi huyết với hoạt lực enzyme nằm trong khoảng từ 6,4 tới 98,5 FU/mL. Dựa trên độ nhớt của canh trường được tạo ra trong quá trình lên men lỏng, chủng TVY.04 được lựa chọn do tạo độ nhớt thấp (3,8 cP) đồng thời sản xuất enzyme tiêu sợi huyết cao nhất (98,5 FU/mL). Chủng TVY.04 được nhận định là chủng *Bacillus amyloliquefaciens* với độ tương đồng là 99,93 % bằng phương pháp giải trình tự 16S rRNA. Cùng với các kết quả phân tích SDS-PAGE và sắc ký bản mỏng (TLC), bước đầu có thể nhận định chủng *B. amyloliquefaciens* TVY.04 là chủng sinh enzyme nattokinase và tiết ra các polysaccharide ngoại bào có thành phần bao gồm poly-γ-glutamic acid (γ-PGA).

Từ khóa: Bacillus, độ nhớt, enzyme phân giải fibrin, phân lập, sàng lọc, *γ-PGA.*

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