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Clot Lysis Activity of *Bacillus subtilis* G8 Isolated from Japanese Fermented Natto Soybeans

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Abstract

Background and objective: *Bacillus* spp. with potent fibrin digesting enzymes, especially *Bacillus subtilis* (*natto*), has gained favorable insights into the world of cardiovascular health and functional foods. In this study, *Bacillus subtilis* G8 was isolated from Japanese fermented Natto soybean commercialized in Indonesia. The overall activity of *Bacillus subtilis* G8 in resolving blood clot was evaluated under variable conditions quantitatively using clot lysis assay.

Material and methods: Cell-free crude extracts of *Bacillus subtilis* G8 culture were partially purified with acetone precipitation method. Fibrinolytic activity of the epnzymes was verified using fibrin plate assay and zymography. The measurement of clot liquefying activity under physiological temperature and pH was performed using chicken blood. The measurement of clot liquefying activity under physiological temperature and pH was performed using chicken blood. The measurement of clot liquefying activity under physiological temperature and pH was performed using chicken blood.

Results and conclusion: Through zymography, *Bacillus subtilis* G8 expressed four bands of fibrinolytic proteins sized 19 to 30 kDa. Under controlled conditions, a significantly higher clot lysis was seen at 37 and 40°C, compared to lower temperatures ($P \leq 0.05$). In contrast, no significant decrease was seen in blood clot at all tested pH ($P > 0.05$). These findings support the use of functional foods for the prevention of blood clot or as a part of blood health restoration.

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1. Introduction

In general, discovery of potent and affordable thrombolytic agents is important to prevent and treat thrombophilia and hence to decrease cardiovascular risks. The existing treatment of thrombophilia includes invasive interventions or use of antiplatelet and anticoagulant agents, as well as fibrinolytic enzymes. [1]. Unlike antiplatelet and anticoagulant agents that inhibit thrombus formations, fibrinolytic enzymes resolve and remove existing blood clots [2]. The currently available thrombolytic agents act as activators of plasminogen-plasmin system, including streptokinase, urokinase plasminogen activators and tissue plasminogen activators [3,4]. These chemicals are generally safe but costly [5,6].

Potent microorganism genera with fibrinolytic activity have been assessed [1]. Discovery of *Bacillus* spp. with potent fibrin digesting enzymes brought additional favorable insights into the world of cardiovascular health and functional foods, especially *Bacillus* (*B.*) *subtilis natto* [7,8]. This bacterial species is widely known to produce enzymes with fibrinolytic properties. As purified enzymes are expensive, use of *B. subtillis natto* fermented products could be the most cost effective, feasible and safe alternative in prevention of undesired thrombus formation [9]. Generally, *B. subtilis* is recognized as safe used in various fermented soy-based products such as Japanese fermented natto (*B. subtillis* subs. *natto*) [10], Chinese traditional Douchi (*B. subtilis* DC33) [11], buckwheat Sokseongjang (*B. subtilis* HJ18-4) [12] and Chungkokjang/Doenjang (*B. subtilis* C5-1) [13]. Although nattokinases (approximate molecular weight of 27.7 kDa) have

been shown to include the majority of the fibrinolytic activity of *B. subtilis*, other enzymes have been identified as well [14]. Therefore, the current study further evaluated the overall activity of *B. subtilis* G8 from natto to resolve blood clot under a range of physiological conditions. Although quantitative clot lysis has extensively been used in drug assays such as purified streptokinases [15], no study has been carried out on functional foods intended for prevention of thrombophilia, or as a part of blood health restoration.

In this study, in addition to assessing compatibility of thrombolysis activity from *B. subtilis* G8 under physiological conditions, the present study validated the thrombolytic activity by microbial or probiotic isolates. Therefore, *B. subtilis* G8 was isolated from Japanese fermented natto commercialized in Indonesia. Chicken blood samples were used as they are inexpensive and easily obtainable. Results have indicated that thrombolytic studies using clotted chicken bloods provide a rapid, effective and efficient method for the overall clot-lytic effects of the *B. subtilis* G8 extracts isolated from Japanese fermented natto in Indonesia.

2. Materials and methods

Molecular identification of G8 isolate

Bacteria were isolated from Japanese natto, commercialized in Indonesia and streaked on nutrient agar. Total genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, USA). Then, 16S rRNA genes were amplified and sequenced for the molecular identification of *B. subtilis* G8. Polymerase chain reaction (PCR) was carried out in 50-µl reactions using universal primers of 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (IDT, Singapore) and KAPA HiFi™ PCR Kit (Kapa Biosystems, USA). The initial denaturation step was carried out at 95°C for 3 min; followed by 25 cycles of denaturation at 98°C for 20 s, annealing at 55°C for 15 s and extension at 72°C for 60 s. A final extension step was carried out at 72°C for 2 min. The PCR products were electrophoresed on agarose gels and visualized using gel documenting system. Then, 16S rRNA PCR products were sent to 1st BASE Laboratories, Malaysia, for sequencing. The 16S rRNA partial sequences of the selected isolates were processed using Sequence Scanner Software v.2 (Applied Biosystems, USA) and BioEdit Software (Ibis Therapeutics, USA). These sequences were compared to those from NCBI GenBank database using BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Phylogenetic analysis was carried out using MEGA X (Molecular Evolutionary Genetics Analysis).

Protease and fibrin plate assays

Agar well diffusion method was applied by inoculating cell-free supernatant on both protease and fibrin agar plate. *Escherichia* (*E.*) *coli* ATCC 8739 was used as negative control. Protease plates assay were conducted on skim milk agar plates (2% of skim milk and 1.5% of agar) and incubated at 37°C for 24 h upon introduction of supernatant. Formation of clear zones around the wells indicated extracellular protease Fibrin plates were prepared by adding 0.4% of fibrinogen in 1 M PBS (Sigma, USA) to an equal volume of 2% of agarose and 80 µl of 100 NIH unit/ml thrombin (Sigma, USA). Crude extracts of the G8 isolate and *E. coli* ATCC 8739 were added into separate wells once the agarose solidified. Plates were incubated at 37°C for 36 h [16].

Partial purification of the enzyme

The enzyme purification optimization was carried out by saturation adjustment of supernatants containing crude extracts using acetone (chilled at -20°C for 1 h.(

The fractions were then centrifuged and dissolved in minimum volumes of pre-cold phosphate buffered saline (PBS, pH 7.4). Enzyme activities were measured through well diffusions on skim milk agar plates. Pure colony of B. subtilis was inoculated on 1 ml of nutrient broth and incubated at 37°C for 16 h using shaking incubator.

SDS-PAGE and zymography

Briefly, SDS-PAGE was carried out for the partially purified crude extracts of *B. subtilis* G8. Isolates were grown in NB overnight and centrifuged at 3,000 ×g for 5 min. The supernatants containing crude extracts were adjusted to saturation using acetone (chilled at -20°C for 1 h) at fractions of 1:3. These fractions were centrifuged and dissolved in minimum volumes of pre-cold PBS (pH 7.4) and then were analyzed using SDS-PAGE (including 12% of acrylamide). Furthermore, sample fibrin zymogram was carried out for the fibrin acrylamide gels by adding 0.006% (w v^{-1}) of fibrinogen (Sigma, USA) and 50 µl of 100 NIH units ml^{-1} thrombin (MP Biomedicals, USA) to fibrin-polyacrylamide solutions. Then, acrylamide gels were loaded with 10 µl of each sample and electrophoresis was carried out at a constant voltage of 50 mV for 1 h; the voltage was increased to 100 mV for the following hour. These gels were stained in Coomassie blue (0.5% of Coomassie blue, 40% of methanol and 10% of acetic acid glacial in dH_2O) for 20 min, rinsed briefly in dH_2O and destained twice for 1 h followed by overnight incubation in 40% of methanol and 10% of acetic acid glacial solutions. Zones of clearance in gels corresponded to the presence of enzymes with fibrinolytic activities [17].

In vitro blood clot lysis assay

In general, a colony of the identified isolate was inoculated on 1 ml of NB and incubated at 37°C for 16 h using shaking incubator. Then, 1% of the isolate cultures, with OD_{600} values of 0.6-0.8, was added into 1 ml of fresh NB or soy broth (SB) (62 g of soybean powder l-1 in H_2O) incubated at 37°C overnight using shaking incubator. Coagulated chicken blood was purchased from local markets and shifted into clots of 0.09-0.10 g each. Blood clot was then added into 1 ml of the media (with cultures or cell-free extracts). Mixtures were incubated at 37°C and visually studied at 0, 2, 4, 6 and 24 h [15].

Conditions affecting blood clot lysis

A piece of 0.2 g retracted blood clot was added into solutions of 250 µl of 1 M sodium phosphate buffer (pH 7.0) and 750 µl of cell-free crude extracts. Mixtures were then incubated under variable temperatures of 30, 35, 37 and 40°C. To evaluate the effect of pH, clots were incubated at 37°C in cell-free crude extracts with variable pH values adjusted with 1 M phosphate buffer solutions to pH values of 6.4, 6.8, 7.0, 7.4, 7.8 and 8.0. The remaining clots were weighed after 4 h of incubation and dried at 100°C for 20 min using oven. The relative clot lysis activity was calculated using Eq. 1:

Relative blood clot lysis = 100% × (dry weight of blood clots before incubation) – dry weight of blood clots after incubation) / (dry weight of blood clots before incubation) Eq. 1

Statistical analysis

Statistical analysis was carried out for the clot reduction. Mean and standard deviation were calculated. Comparisons between the groups were carried out using analysis of variance test (ANOVA). Generally, $P \leq 0.05$ was considered as statistically significant. All statistical analyses were carried out using Minitab statistical software version 18.

3. Results and discussion

Bacterial verification

In general, 16S rRNA gene partial sequencing of the isolates from the fermented natto produced 1,397 nucleotides. The isolates were assigned as *B. subtilis* G8 and the 16S rRNA gene sequences were annotated in GenBank database with the accession number of MH559564. BLAST results for 16S rRNA gene sequences from cultured isolates showed 100% identities with those for *B. subtilis* subsp. *natto* (KX279936). Sequence alignments of the 16S rRNA genes from *B. subtilis* G8 with *B. subtilis* subsp. *subtilis* strain 168 (NR_102783) and *B. subtilis* subsp. *natto* BEST195 (NC_017196) showed a 99% similarity. However, evolution analysis using phylogenetic tree suggested that *B. subtilis* G8 was closer to *B. subtilis* BEST195. Although, *B. subtilis* BEST195 was reported to be closely linked to *B. subtilis* 168; however, they differently expressed favorable phenotypes for fermentation [18]. Furthermore, *B. subtilis* BEST195 was claimed as the starter strain of natto commercialized and consumed in Japan [10,19]. The subtilisin NAT was demonstrated as an important enzyme contributing to the taste and flavor of natto as well as exhibiting fibrinolytic activities [20]. To better understand of fermenting profile and fibrinolytic activity of *B. subtilis* G8 subtilisin, selected loci must be analyzed using sequence typing, particularly *apr* genes which include regulatory regions [18,21] (Fig. 1).

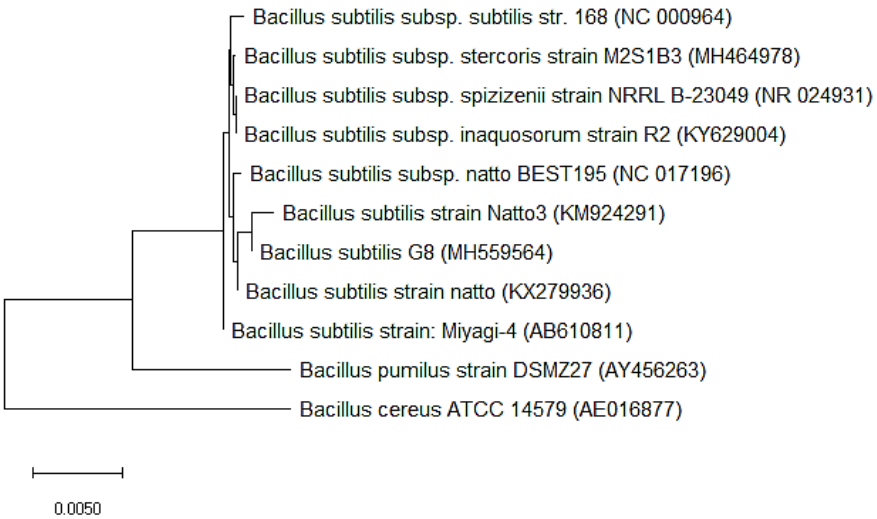


Figure 1. Phylogenetic tree of *Bacillus subtilis* G8 isolated from natto based on 16s rRNA sequences. The phylogenetic tree was constructed using Neighbor-joining method and MEGA X. The phylogram shows phylogenetic relationship between various Bacillus isolates based on the ribosomal 16S rRNA sequences. The scale bar represents 0.005 substitutions per site.

Production of proteolytic and fibrinolytic enzymes by *B. subtilis* G8 was verified by the formation of clear zone around well on skim milk agar plates (Fig. 2A) and fibrin plates (Fig. B), respectively.

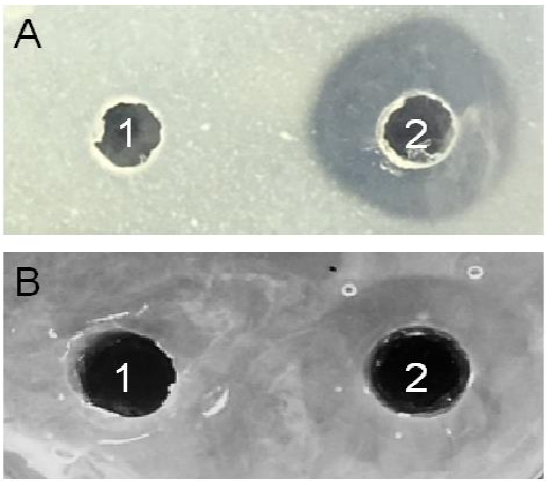


Figure 2. Protease plate assay, cell-free supernatant were added onto wells made from skim milk agar plates (2% of skim milk and 1.5% of agar) and incubated at 37°C for 24 h (A); and fibrin plate assay, cell free supernatant were added onto wells made by adding 0.4% of fibrinogen in 1M phosphate-buffered saline (PBS) to equal volume of 2% of agarose and 80 µl of 100 National Institute of Health (NIH) standard unit/ml of thrombin and incubated at 37°C for 36 h. (B).
1) *Escherichia coli* ATCC 8739, 2) *Bacillus subtilis* G8.

Identification of fibrinolytic enzymes

To specifically identify production of fibrinolytic enzymes by *B. subtilis* G8, crude extracts of *B. subtilis* G8 overnight cultures were partially purified using acetone precipitation 1:3 (v:v) and then were analyzed using SDS-PAGE and zymography. Fibrin zymography of the partially purified samples showed a single band with molecular mass of approximately 19.1 kDa and three other weak bands of approximately 22.5, 27.5 and 29.3 kDa (Fig. 3). Normally, *B. subtilis* G8 produces fibrinolytic enzymes with various molecular weights as compared to other *Bacillus* spp. isolated from traditional foods such as oncom and gembus and *B. subtilis* isolated from jeotgal (small shrimp) [22,23].

Size of nattokinase produced by *B. subtilis* subs. natto was previously reported as 27.7 kDa, which might be produced by *B. subtilis* G8 as demonstrated by one of the enzyme bands on zymogram [8].

Nattokinase, also known as subtilisin NAT, is encoded by *apr*N gene, which belongs to peptidase S8 family [20]. Another homologous gene which demonstrates similar properties to subtilisin N (*apr*N) and commonly found in *B. subtilis* is subtilisin E (*apr*E) [24]. The subtilisin (*apr*) genes have been reported in other Bacillus species, including subtilisin amylosacchariticus in *B. subtilis* subsp. *amylosacchariticus* [25,26] and subtilisin BPN and subtilisin Carlsberg in *B. amyloliquefaciens* and *B. licheniformis*, respectively [27-29].

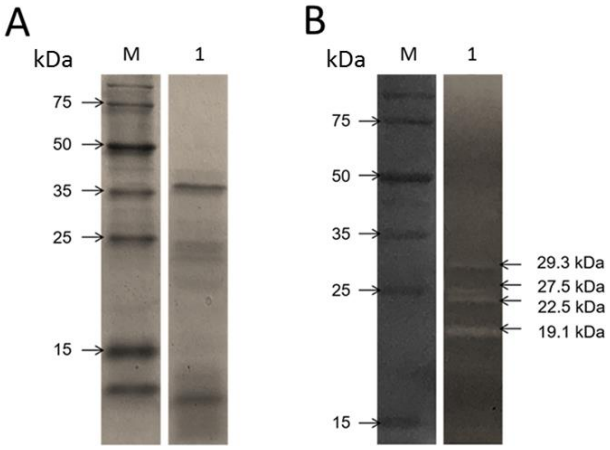


Figure 3. Coomassie blue stained SDS-PAGE gels (A) and fibrin zymograms (B) of the culture supernatants from *Bacillus subtilis* G8. Lane M) V8491 broad-range protein molecular weight markers; and Lane 1) Acetone partially purified crude extracts of *Bacillus subtilis* G8 grown overnight in nutrient broth at 37°C.

Although *B. subtilis* G8 is predicted to express subtilisin (*apr*) protease with a similar fibrinolytic activity to that of nattokinase (*apr*N), more than one zymogram band indicates the presence of other enzymes that contribute to overall clot lysis activity. In addition to *apr* gene, fibrinolytic activity was demonstrated by *vpr* gene, which produces a mature protein of approximately 68 kDa. The *vpr* gene was shown to contain a nucleotide sequence similar to that from the active site of subtilisin [14]. There are more than one peptidase S8 (subtilisin-like serine proteases/subtilases) encoded by the *B. subtilis* genome (380-1500 amino acid residues). Individual isolation of these enzymes is necessary for a precise fibrinolytic enzyme profiling. The exact mechanisms of subtilases in the isolate need to be extensively characterized as these enzymes might play substantial roles in digestion of fibrins.

Blood clot lysis assessment of *B. subtilis*

Potential thrombolytic enzymes were assessed using in vitro models to study their clot lysis activity. The existing blood clot lysis assessment was commonly carried out for qualitative analysis [30]. Previous quantitative analysis was carried out based on the measurement of retracted clot weight before and after particular treatments [15]. However, retracted clots often swell when exposed to liquid enzyme treatments, contributing to errors that result in conflicted weight readings. In the current study, the existing method was further extended to measurement of blood clot dry weight, resulting in further precise and reliable measurements of the reduced clots before and after treatments. Although fibrinolysis activity varies among various species, use of chicken bloods in this study produced substantial reduction in clots [31,32].

Two types of media were used for In vitro blood clot lysis. The growth medium was reported to affect not only

the growth rate but the fibrinolytic enzyme yields of a particular microorganism [33,34]. Previously, the main process in production of fibrinolytic enzymes involved soybean fermentation, which created a remarkable problem for both production and application of the enzymes [7]. The study first hypothesized that soy-based media could promote fibrinolytic activity of *B. subtilis* G8 from natto. It was reported that use of soy-based media could promote conversion of soy into functional ingredients with fibrinolytic enzymes [35,36]. However, relatively no differences were seen in both media (NB and SB). This suggests that other molecules with thrombolysis activity might be produced simultaneously, enhancing clot lysis regardless the involvement of soy fermentation. This suggests that other molecules with thrombolysis activity might be produced simultaneously, enhancing clot lysis regardless the involvement of soy fermentation. The blood clot lysis demonstrated by reduced blood clots was observed as early as 2 h in *B. subtilis* G8 broth cultures and 6 h in centrifuged cell-free supernatants (Table 1A). Moreover, *E. coli* ATCC 8739 showed no reduced blood clot and supernatants remained clear (Table 1B). In addition to macroscopic clot lysis activity, microscopic analysis showed that red blood cells (RBC) preserved their integrity with no hemolysis after the assay (data not shown).

Conditions affecting fibrinolysis activity

In this study, clot lysis activity was assessed under various temperatures (30, 35, 37 and 40°C). Therefore, reduced blood clot progression remained stable through the temperatures. A significantly higher clot reduction was seen at 37 to 40°C, compared to that at 30°C ($P \leq 0.05$) (Fig. 4).

In contrast to temperature values, reduction of clots was constant for all pH values (pH 6.4-8.0). Normal physiological pH of the human arterial blood includes 7.35-7.45, which is achieved by blood pH buffer potency. The highly strict blood pH values result in sustainable regulation of cellular activities ensuring human survival [37]. However, conditions such as metabolic abnormalities could result in pH changes above or below normal ranges [11]. The pH extremities for survival are reported around 6.8-7.8; other values are otherwise considered incompatible with normal health conditions. Extreme alkalosis in humans occurs mostly at pH 7.69 [38]; whereas extreme acidosis mainly occurs at pH 6.52 [39]. Patients with each condition experience extreme body distresses. In this study, ranges of pH tested on blood clot lysis activity stretched beyond pH extremities for survival (pH 6.4-8.0).

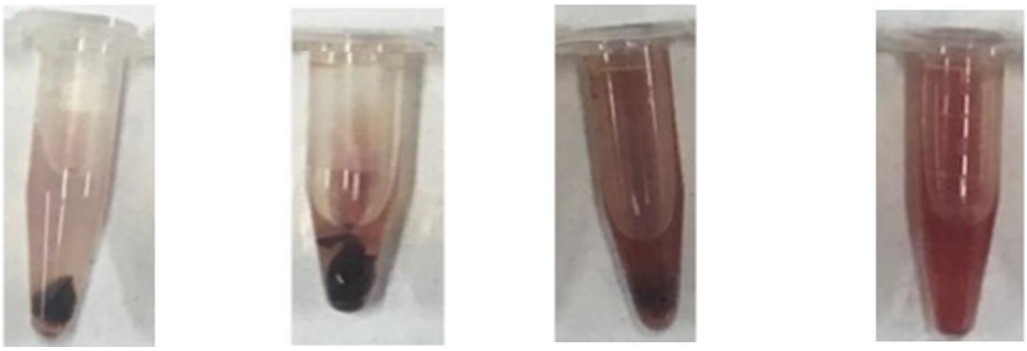
Table 1. A) Blood lysis activity of overnight incubated *Bacillus subtilis* G8 soy broth and nutrient broth media with culture or centrifuged cell-free supernatants; B) Visual reference grading for blood lysis levels.

A. Blood lysis activity

Incubation time (h)	Soy based media		Nutrient broth media	
	Broth culture media	Cell-free supernatant	Broth culture media	Cell-free supernatant
2	+	-	+	-
4	++	-	++	-
6	++	+	+++	+
24	++	++	+++	++

B. Visual reference grading

No clot clearance	Weak blood clot clearance	Moderate blood clot clearance	Absolute blood clearance
-	+	++	+++



Coagulated chicken bloods with concentrations of 0.1 g were added into 1 ml of the culture media (with culture or cell-free extracts). The mixtures were incubated at 37°C for the indicated time (2-24 h).
No clot clearance (-), no color diffuse into supernatants and no changes in blood clot size; weak blood clot clearance (+), supernatants with light blood color and minimal changes in blood clot size; moderate blood clot clearance (++), supernatants with obvious blood color and decreases in blood clots; absolute blood clearance (+++), no blood clots left as blood clots were completely dissolved in the mixture.

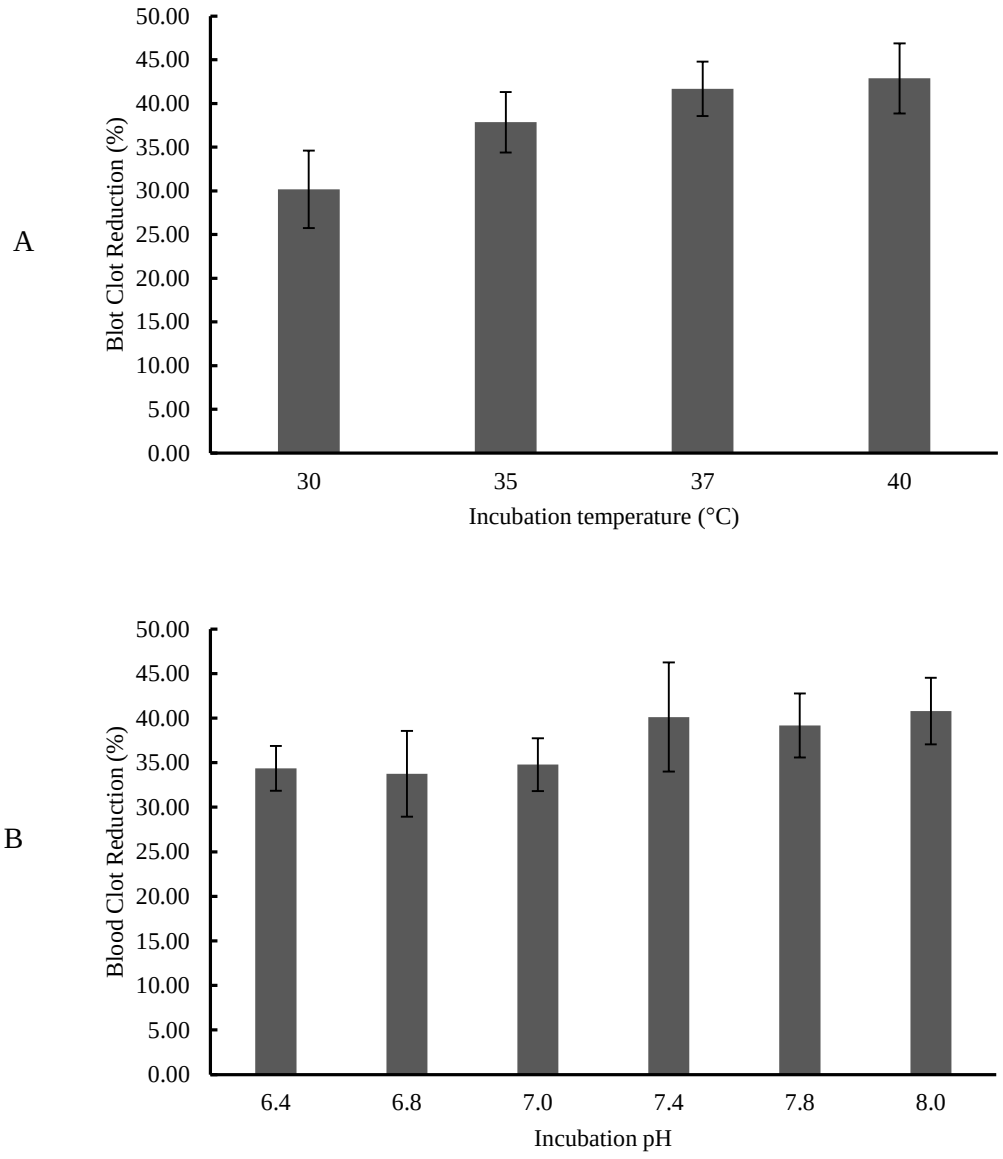


Figure 4. Effects of all various temperatures (30-40°C) (A); and pH (6.4-8.0) incubated at 37°C for 4 h (B), on blood lysis activity of *Bacillus subtilis* G8 cell-free extracts. Results are expressed as mean±SD. Statistical analysis is calculated using ANOVA, different significant levels are denoted by different letters (a, b) ($P \leq 0.05$).

Similar to these findings, working and optimal pH values of nattokinase extracted from *B. subtilis* var. natto B-12 were identified as 6.0-9.0 and pH 8.0, respectively. Thermostability of the nattokinase was observed at temperatures ranging from 30 to 50°C and its optimal activity at 40°C [29].

This study utilized an in vitro model for the reduction clot lysis activity for potential thrombolytic drugs [15]. Although nattokinase has been reported as an enzyme that dominantly play a role in fibrinolytic activity by *B. subtilis*, other molecules of various molecular weights with thrombolysis activities are produced simultaneously, enhancing clot lysis process regardless the involvement of soy fermentation [22,29,40].

4. Conclusion

In conclusion, the current study shows the presence of fibrinolytic activity of *B. subtilis* G8 from commercialized natto using fibrin plates and zymography. Clot lysis activity was seen throughout physiological blood conditions. These findings could support use of functional foods in prevention of thrombophilia or as a part of blood health restoration.

5. Acknowledgements

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6. Conflict of interest

The authors declare no conflict of interest.

References

1. Kotb E. Activity assessment of microbial fibrinolytic enzymes. Appl Microbiol Biotechnol. 2013; 97 (15): 6647-6665.
doi: 10.1007/s00253-013-5052-1

2. Chen C, Yang FQ, Zhang Q, Wang FQ, Hu YJ, Xia ZN. Natural products for antithrombosis. Evid Based Complement Alternat Med. 2015; 2015: 1-17
doi: 10.1155/2015/876426

3. Butcher K, Shuaib A, Saver J, Donnan G, Davis SM, Norrving B, Wong KSL, Abd-Allah F, Bhatia R, Khan A. Thrombolysis in the developing world: Is there a role for streptokinase?. Int J Stroke. 2013; 8 (7): 560-565.
doi: 10.1111/j.1747-4949.2012.00923.x

4. Gomaraschi M, Ossoli A, Vitali C, Pozzi S, Vitali Serdoz L, Pitzorno C, Sinagra G, Franceschini G, Calabresi L. Off-target effects of thrombolytic drugs: Apolipoprotein A-I proteolysis by alteplase and tenecteplase. Biochem Pharmacol. 2013; 85 (4):525-530.
doi: 10.1016/j.bcp.2012.11.023

5. Johnston SC. The economic case for new stroke thrombolytics. Stroke 2010; 41 (10): S59-62.
doi: 10.1161/strokeaha.110.597351

6. Sikri N, Bardia A. A history of streptokinase use in acute myocardial infarction. Tex Heart I J. 2007; 34 (3): 318-327.

7. Sumi H, Yanagisawa Y, Yatagai C, Saito J. Natto *Bacillus* as an oral fibrinolytic agent: nattokinase activity and the ingestion effect of *Bacillus subtilis natto*. Food Sci Technol Res. 2004; 10 (1): 17-20.
doi: 10.3136/fstr.10.17

8. Weng Y, Yao J, Sparks S, Wang KY. Nattokinase: An oral antithrombotic agent for the prevention of cardiovascular disease. Int J Mol Sci. 2017; 18 (3): 523.
doi: 10.3390/ijms18030523

9. Gurewich V. Why so little progress in therapeutic thrombolysis? The current state of the art and prospects for improvement. J Thromb Thrombolys. 2015; 40 (4):480-487.
doi: 10.1007/s11239-015-1217-3

10. Sumi H, Hamada H, Tsushima H, Mihara H, Muraki H. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; A typical and popular soybean food in the Japanese diet. Experientia. 1987; 43 (10): 1110-1111.
doi: 10.1007/BF01956052

11. Wang CT, Ji BP, Li B, Nout R, Li PL, Ji H, Chen LF. Purification and characterization of a fibrinolytic enzyme of *Bacillus subtilis* DC33, isolated from Chinese traditional douchi. J Ind Microbiol Biotechnol. 2006; 33 (9): 750-758.
doi: 10.1007/s10295-006-0111-6

12. Eom JS, Lee SY, Choi HS. *Bacillus subtilis* HJ18-4 from traditional fermented soybean food inhibits *Bacillus cereus* growth and toxin-related genes. J Food Sci. 2014; 79 (11): M2279-M2287.
doi: 10.1111/1750-3841.12569

13. Park S, Kim MJ, Hong J, Kim HJ, Yi SH, Lee MK. Selection and characterization of Cheonggukjang (fast fermented soybean paste)-originated bacterial strains with a high level of S-adenosyl-L-methionine production and probiotics efficacy. J Med Food. 2014; 17 (11): 1170-1176.
doi: 10.1089/jmf.2013.3052

14. Kho CW, Park SG, Cho S, Lee DH, Myung PK, Park BC. Confirmation of Vpr as a fibrinolytic enzyme present in extracellular proteins of *Bacillus subtilis*. Protein Expres Purif. 2005; 39 (1):1-7.
doi: 10.1016/j.pep.2004.08.008

15. Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM, Daginawala HF. Development of an In vitro model to study clot lysis activity of thrombolytic drugs. Thromb J. 2006; 4: 14.
doi: 10.1186/1477-9560-4-14

16. Motaal AA, Fahmy I, El-Halawany A, Ibrahim N. Comparative fibrinolytic activities of nattokinases from *Bacillus subtilis* var. natto. J Pharmaceut Sci Res. 2015; 7 (2):63-66.

17. Jeong SJ, Kwon GH, Chun J, Kim JS, Park CS, Kwon DY. Cloning of fibrinolytic enzyme gene from *Bacillus subtilis* isolated from Cheonggukjung and its expression in protease-deficient *Bacillus subtilis*. J Microbiol Biotechnol. 2007; 17 (6): 1018-1023.

18. Kamada M, Hase S, Fujii K, Miyake M, Sato K, Kimura K, Sakakibara Y. Whole-genome sequencing and comparative genome analysis of *Bacillus subtilis* strains isolated from non-salted fermented soybean foods. Plos One. 2015; 10 (10): e0141369.
doi: 10.1371/journal.pone.0141369

19. Kamada M, Hase S, Sato K, Toyoda A, Fujiyama A, Sakakibara Y. Whole genome complete resequencing of *Bacillus subtilis natto* by combining long reads with high-quality short reads. Plos One. 2014; 9 (10): e109999.
doi: 10.1371/journal.pone.0109999

20. Nakamura T, Yamagata Y, Ichishima E. Nucleotide sequence of the subtilisin NAT gene, aprN, of *Bacillus subtilis* (*natto*). Biosci Biotechnol Biochem. 1992; 56 (11): 1869-1871.
doi: 10.1271/bbb.56.1869

21. Inatsu Y, Nakamura N, Yuriko Y, Fushimi T, Watanasiritum L, Kawamoto S. Characterization of *Bacillus subtilis* strains in Thua nao, a traditional fermented soybean food in northern Thailand. Lett Appl Microbiol. 2006; 43 (3): 237-242.
doi: 10.1111/j.1472-765X.2006.01966.x

22. Afifah DN, Sulchan M, Syah D, Suhartono MT. Isolation and identification of fibrinolytic protease-producing micro-organ-isms from Red Oncom and Gembus, Indonesian fermented soybean cakes. Malays J Microbiol. 2014; 273-279.

23. Yao Z, Kim JA, Kim JH. Properties of a fibrinolytic enzyme secreted by *Bacillus subtilis* JS2 isolated from saeu (small shrimp) jeotgal. Food Sci Biotechnol. 2018; 27 (3): 765-772.
doi: 10.1007/s10068-017-0299-4

24. Jeong SJ, Heo K, Park JY, Lee KW, Park JY, Joo SH, Kim J H. Characterization of AprE176, a fibrinolytic enzyme from *Bacillus subtilis* HK176. J Microbiol Biotechnol. 2015; 25 (1): 89-97.

doi: 10.4014/jmb.1409.09087

25. Yoshimoto T, Oyama H, Honda T, Tone H, Takeshita T, Kamiyama T, Tsuru D. Cloning and expression of subtilisin amylosacchariticus gene. J Biochem. 1988; 103 (6): 1060-1065.
doi: 10.1093/oxfordjournals.jbchem.a122380

26. Yoshimoto T, Oyama H, Takeshita T, Higashi H, Xu Sl, Tsuru D. Nucleotide sequence of the neutral protease gene from *Bacillus subtilis* var. *amylosacchariticus*. J Ferment Bioeng. 1990; 70 (6):370-375.
doi: 10.1016/0922-338x(90)90080-g

27. Jacobs MF. Expression of the subtilisin Carlsberg-encoding gene in *Bacillus licheniformis* and *Bacillus subtilis*. Gene. 1995; 152 (1): 69-74.
doi: 10.1016/0378-1119(94)00655-C

28. Peng Y, Huang Q, Zhang RH, Zhang YZ. Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from douchi, a traditional Chinese soybean food. Comp Biochem Physiol B Biochem Mol Biol. 2003; 134 (1): 45-52.
doi: 10.1016/S1096-4959(02)00183-5

29. Yao Z, Liu X, Shim JM, Lee KW, Kim HJ, Kim JH. Properties of a Fibrinolytic enzyme secreted by *Bacillus amyloliquefaciens* RSB34, isolated from doenjang. J Microbiol Biotechnol. 2017; 27 (1): 9-18.
doi: 10.4014/jmb.1608.08034

30. Vijayaraghavan P, Vincent SG. Statistical optimization of fibrinolytic enzyme production by *Pseudoalteromonas* sp. IND11 using cow dung substrate by response surface methodology. Springerplus 2014; 3: 60.
doi: 10.1186/2193-1801-3-60

31. Haggroth L, Mattsson C, Friberg J. Inhibition of the human tissue plasminogen activator in plasma from different species. Thromb Res. 1984; 33 (6): 583-594.
doi: 10.1016/0049-3848(84)90113-0

32. Siller-Matula JM, Plasenzotti R, Spiel A, Quehenberger P, Jilma B. Interspecies differences in coagulation profile. Thromb Haemost. 2008; 100 (3): 397-404.
doi: 10.1160/TH08-02-0103

33. Afifah DN, Sulchan M, Syah D, Yanti, Suhartono MT, Kim JH. Purification and characterization of a fibrinolytic enzyme from *Bacillus pumilus* 2.g isolated from Gembus, an Indonesian fermented food. Prev Nutr Food Sci. 2014; 19 (3): 213-219.
doi: 10.3746/pnf.2014.19.3.213

34. Chen PT, Chiang CJ, Chao YP. Medium optimization for the production of recombinant nattokinase by *Bacillus subtilis* using response surface methodology. Biotechnol Progr. 2007; 23 (6): 1327-1332.
doi: 10.1021/bp070109b

35. Seo JH, Lee SP. Production of fibrinolytic enzyme from soybean grits fermented by *Bacillus firmus* NA-1. J Med Food. 2004; 7 (4): 442-449.
doi: 10.1089/jmf.2004.7.442

36. Afifah DN, Sulchan M, Syah D, Suhartono MT. The use of red Oncom powder as potential production media for fibrinogenolytic protease derived from *Bacillus licheniformis* RO3. Procedia Food Sci. 2015; 1 (3): 453-464.
doi: 10.1016/j.profoo.2015.01.050

37. Vijayaraghavan P, Vincent SG. Statistical optimization of fibrinolytic enzyme production by *Pseudoalteromonas* sp. IND11 using cow dung substrate by response surface methodology. Springerplus 2014; 3: 60.
doi: 10.1186/2193-1801-3-60

38. Tripathy S. Extreme metabolic alkalosis in intensive care. Indian J Crit Care Med. 2009; 13 (4): 217-220.
doi: 10.4103/0972-5229.60175

39. Di Rollo N, Caesar D, Ferenbach DA, Dunn MJ. Survival from profound metabolic acidosis due to hypovolaemic shock. A world record?. BMJ Case Rep. 2013; 2013: 1-3.
doi: 10.1136/bcr-2012-008315

40. Wang C, Du M, Zheng D, Kong F, Zu G, Feng Y. Purification and characterization of nattokinase from *Bacillus subtilis natto* B-12. J Agric Food Chem. 2009; 57 (20): 9722-9729.
doi: 10.1021/jf901861v

http://dx.doi.org/10.22037/afb.v6i2.22479

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بررسی فعالیت کافت لخته توسط *باسیلوس سوبتیلیس* G8 جداشده از دانه های سویای ژاپنی تخمیر شده ناتو

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چکیده

سابقه و هدف: گونه‌های باسیلوس به ویژه *باسیلوس سوبتیلیس* (ناتو)، به علت محتوای آنزیم‌های هضم کننده فیبرین از جنبه سلامت قلبی-عروقی و مواد غذایی فراسودمند[1] بسیار شناخته شده‌اند. در این مطالعه، *باسیلوس سوبتیلیس* G8 از غذای تخمیری ژاپنی تجاری سویای ناتو جدا شد. فعالیت کلی *باسیلوس سوبتیلیس* G8 در رفع مشکل لخته خونی در شرایط مختلف با روش کافت لخته از نظر کمی مورد بررسی قرار گرفت.

مواد و روش ها: عصاره خام بدون سلول *باسیلوس سوبتیلیس G8* به روش رسوب‌دهی با استون به طور نسبی تخلیص شد. فعالیت فیبرینولیتیکی آنزیم‌ها به روش پلیت فیبرین و زیموگرافی مورد تایید قرار گرفت. این روش بر لخته خون جوجه در درجه حرارت‌ها و pHهای گوناگون انجام گرفت.

یافته‌ها و نتیجه‌گیری: زیموگرافی *باسیلوس سوبتیلیس G8* چهار پهنای پروتئین‌های فیبرینولیتیک در اندازه‌های ۱۹ تا ۳۰ کیلودالتون را نشان داد. تحت شرایط کنترل شده، کافت لخته‌ای در درجه حرارت‌های ۳۷ و ۴۰ درجه سلسیوس در مقایسه با درجه حرارت‌های پایین‌تر به طور معنی‌داری بیشتر بود ($p\leq ۰/۰۵$). در مقابل، کاهش معنی‌داری در لخته شدن خون در تمام pH های مورد مطالعه مشاهده نشد ($p>۰/۰۵$). این یافته‌ها استفاده از مواد غذایی فراسودمند را برای پیشگیری از لخته شدن خون یا به عنوان بخشی از بازیابی سلامت خون تایید می‌کنند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی

- *باسیلوس سوبتیلیس*
- روش کافت لخته
- فیبرینولیتیک
- ناتو
- زیموگرافی

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[1] Functional foods