




Fibrinolytic bacteria of Indonesian fermented soybean: preliminary study on enzyme activity and protein profile

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Abstract

A number of fibrinolytic enzyme producing microorganisms have been identified in various fermented food in Asia, such as from Red *Oncom*, *Tempe*, *Terasi* (Indonesia), *Natto* (Japan), *Douchi*, Soy paste (China), *Meju*, *Kimchi*, *Jeot-gal*, *Tofuyo*, *Chungkook-jang*, Soybean paste and *Doen-jang* (Korea). The high protein content in soybean is potential to promote the growth of proteolytic and fibrinolytic producing microbes. This study investigated several fibrinolytic bacteria, isolated from the moromi stage of the fermented soysauce production. The fibrinolytic activities of the isolates were confirmed in a fibrin plate assay, and all 3 potential isolates (K1, K2, and K3) were identified as Gram positive, rod shaped, and spore forming bacteria. Moreover, analysis of the sequences encoding 16S rRNA gene, revealed K1 as *Bacillus cereus*, K2 as *Bacillus subtilis*, and K3 as *Bacillus cereus*. SDS PAGE analysis demonstrated different protein profile of the cell free supernatant of isolates grown in LB media during fermentation, which ranged from 18.40-45 kDa.

Keywords: fermented soybean; fibrinolytic protease; moromi.

Practical Application: Finding of fibrinolytic activity of bacteria isolated from moromi stage in the fermented soysauce production accentuate the importance development of the enzyme into a powerful health product to treat thrombus related disease.

1 Introduction

Annually, cardiovascular diseases (CVDs), including stroke and heart diseases have been identified as the leading cause of death worldwide. Meanwhile, WHO data estimated about 17.9 million related deaths in 2016, which represents 31% of all global cases, and about 85% occurred due to heart attack and stroke. In addition, out of the 17 million premature demise, i.e., under the age of 70, the proportion attributed to noncommunicable diseases as reported in 2015 were 82% of low- and middle income countries, and 37% were caused by CVDs (World Health Organization, 2017). Moreover, the basic pathophysiology of stroke involves formation and attachment of fibrin clot or thrombus to the injured blood vessel walls.

Fibrin is the major protein component of blood clots, formed from fibrinogen by thrombin (Voet & Voet, 1997), and the fibrinolytic enzymes are thrombolytic agents that are capable of dissolving fibrin clots. In addition, the agents that act as fibrinolytics, including urokinase plasminogen activator (u-PA), tissue plasminogen activator (t-PA), and streptokinase, have been adopted in the treatment of CVDs, while thrombolytic agents, encompassing t-PA and u-PA are classified as generally safe, although very expensive, with the propensity to cause undesirable side effects, including gastrointestinal bleeding (Peng et al., 2003; Killer et al., 2010; Jeong et al., 2015; Yogesh

& Halami, 2017). In recent decades, many related studies on enzymes obtained from food-grade microorganisms have been proven as safe thrombolytic agents (Joo et al., 2002; Mine et al., 2005; Simkhada et al., 2010; Afifah et al., 2014; Stephani et al., 2017; Kurnia et al., 2017).

Therefore, the bacterial strains of *Bacillus subtilis* A26 (Agrebi et al., 2009), *Bacillus amyloliquefaciens* (Wei et al., 2011), *Paenibacillus* sp. IND8 (Vijayaraghavan & Prakash Vincent 2014), *Xenorhabdus indica* KB-3 (Pranaw et al., 2014), and *Bacillus* sp. (Anh et al., 2015) were recently adopted in the production of fibrinolytic enzymes. In addition, a research on such enzymes from food microbes has been conducted in Japan, using *Tofuyo* (Fujita et al., 1993), *Skipjack* (Sumi et al., 1995), and *Natto* (Fujita et al., 1993; Chang et al., 2000; Seo & Lee, 2004), Korea (*Chungkook-jang* (Kim et al., 1996 a, b), *Jeot-gal* (Hyun-Kuk Kim et al., 1997), *Kimchi* (Noh et al., 1999), *Doen-jang* (Kim & Choi, 2000; Choi et al., 2005), *Soybean paste* (Lee et al., 2001), and *Meju* (Jo et al., 2011), China (*Douchi* (Peng & Zhang, 2002) and *Chinese soy paste* (Wei et al., 2011), and Indonesia (*Terasi* and *Jambal roti*, fermented fish (Prihanto et al., 2013), Red *Oncom* and *Gembus Tempeh* (Afifah et al., 2014). Considering the rich fermented local products present in Indonesia, similar research is still highly limited.

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Fermented soy sauce is one of the food products from soybean, obtained through two steps in the manufacturing process, encompassing (1) solid-state (koji fermentation) where the output is dried and (2) salt fermentation, where the dry mass is mixed with brine solution to form a mash, termed “moromi”, which is further fermented within various periods of aging (range from six months to one year) (Lee et al., 2013; Zhu & Tramper, 2013). The high protein contents in the soybean is potential of being utilized as a media for growing proteolytic and fibrinolytic microorganism, and this preliminary study confirmed a strong activity from bacteria isolated at the moromi stage.

2 Materials and methods

In this study, the moromi of fermented soy sauce was obtained from PT. Logan Food Lombok Gandaria (Karanganyar, Central Java, Indonesia), while the bacteriological agar was purchased from Sigma, US and Luria Bertani media was procured from Scharlau, Spain. Fibrinogen sourced from bovine plasma was purchased from Sigma, US, and the API 50CHB kit for bacterial identification was obtained from BioMérieux S.A, France.

2.1 Isolation of proteolytic bacterial strain from Moromi (Afifah et al., 2014)

The process of isolating proteolytic microbe was conducted using Skim Milk Agar media (SMA), with the composition of bacteriological agar and skim milk (1.5%). In addition, a total of 1 mL of moromi was dissolved in 10 mL sterile aquadest, which was then stirred to a point of homogeneity (moromi solution). Therefore, 0.1 mL was collected and spread onto the surface of SMA media, using a sterile hockey stick, which was subsequently incubated at 37 °C for 48 hours, and then the clear zone observed indicated the existence of the protease producing microbes. Furthermore, the protease colonies were isolated and purified until a pure culture in the form of a mono colony was obtained, which was then grown in slant agar, and saved as a stock in glycerol 50% for further analysis.

2.2 Enzyme production (Afifah et al., 2014)

One of the selected microbe was grown in 12.5 mL sterile Luria-Bertani broth media (LB), in order to obtain the optical density (OD) of 0.8 at λ_{620nm} . Therefore, a total of 10% (v/v) fermentation broth recorded to have reached the required value was mixed with 25 mL LB, and further incubated for 1, 2, 3, 4, and 7 days at 37 °C in the shaking incubator (120 rpm) (PolyScience, US). Furthermore, the production media with fibrinolytic protease content was taken at the appropriate time and centrifuged at 6000 g for 30 minutes at 4 °C, and then the cell free supernatant was collected as the enzyme source for subsequent analysis.

2.3 Fibrin plate assay (Astrup & Mullertz, 1952)

The Fibrinolytic bacteria were screened using a modified fibrin plate method (Astrup & Mullertz, 1952), where a total of 0.5% fibrinogen (Sigma F3879, US) solution in 50 mM sodium phosphate buffer (pH 8) was mixed with 2% agarose solution along with 0.02 mL of thrombin (Sigma T4648, US) solution

(100 NIH units). These were then applied to a petri dish, and left for 1 hour at room temperature to form a fibrin clot layer. In addition, a sterile glass tube (diameter 6 mm) was used to make hole in the fibrin plate, to which twenty μ L of the enzyme was dropped and incubated at 37 °C for 20 hours. Therefore, the positive control utilized the commercial fibrinolytic enzyme termed Lumbrokinase from *Lumbricus rubellus* (Disolf DLBS1033, Dexa Medica, Indonesia) (40 mg/mL), prepared in a 20 mM phosphate buffer. Moreover, the activity of fibrinolytic enzyme was estimated by measuring the dimensions of the clear zone on the fibrin plate.

2.4 SDS PAGE (Laemmli, 1970)

SDS-PAGE was conducted with 10% of separating and 4% stacking gel. Also, the cell free supernatant was diluted in a sample buffer 5x (containing 60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, and 0.1% bromophenol blue (BPB)), and the gel was run at 100 V, 100 mA for 1 hour, up to the point where BPB reached the bottom. Therefore, the protein bands were visualized by staining the gel with Coomassie Blue (CBBGR) for 20 minutes, which was sequentially soaked in a destaining solution, in order to attain discoloration, and the apparent molecular mass of the protein was calculated using Broad Molecular Weight standard protein markers (ThermoFisher Scientific, US).

2.5 Protein determination (Bradford, 1976)

The protein concentration was analysed using the reagents consisting of 100 mg Coomassie Brilliant Blue (CBB) G-250 in 50 mL ethanol 95% and 100 mL phosphate acid 85% in 1L. Therefore, Bovine Serum Albumin (BSA) was used as the protein standard, and the experiments were conducted in triplicates for each measurement.

2.6 Identification of microorganisms

The identification of microorganisms followed 3 steps, which include (1) microbiology analysis i.e. Gram staining, spore staining, and morphological examination, using a microscope with 1000X magnification. (2) Biochemical tests with kit API 50CHB (BioMérieux S.A, France) for *Bacillus* spp., followed by identification using Apiweb™ software. (3) Molecular identification, where the selected microorganisms were grown in LB media, and identified at the species level. In addition, the DNA was extracted using a DNA mini kit Qiagen (Qiagen, US), with a reaction mixture (10 μ L) that contained 1 μ L DNA template (50 ng/ μ L), 0.25 μ L forward primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3'), 0.25 μ L reverse primer 1387R (5'-GGG CGG AGT GTA CAA GGC-3'), 5 μ L PCR Master Mix (Qiagen, US), and 3.5 μ L Nuclease Free Water (Qiagen, US). Therefore, the amplification of 16S rRNA gene using a 2720 thermal cycler (Applied Biosystems, US) was performed as follows: pre-denaturation (94 °C, 5 min, 30 cycles), denaturation (94 °C, 30 min, 30 cycles), annealing (50 °C, 1 min, 30 cycles), extension (72 °C, 2 min, 30 cycles), final extension (72 °C, 5 min), and cooling (25 °C, 10 min). Furthermore, the PCR products were purified using Accuprep™ purification kit (Bioneer, Korea), and sequenced using Sanger dideoxy sequencing (genetic analyzer 3730 XI, Applied Biosystems, US) (Sanger & Coulson,

1975), and a sequence homogeneity search was performed using BLAST in NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Moreover, pairwise and multiple sequence alignment were analyzed using Clustal X2 programs, while the Phylogenetic tree analysis required the use of Neighbor-Joining method (Saitou & Nei, 1987).

3 Results and discussion

Microbes usually obtained in fermented food are Gram-positive bacteria, encompassing *Bacillus* sp., *Lactobacillus* sp., *Staphylococcus* sp., and *Streptococcus hemolyticus*, which tend to be easily screened from food and the environment. There are non-toxic and beneficial microorganisms for human health, including *Bacillus pumilus* JB-1 from Korean *Cheonggukjang*, which has long been known to produce immuno-stimulant substances. Research conducted by Wei et al., (2011) reported antioxidant activity in enzymes produced by *B. amyloliquefaciens* LSSE-62, obtained from fermented chickpea. Juan & Chou (2010) and Lee et al. (2008) also reported similar activities in *Bacillus subtilis* and some *fungi* obtained from fermented legumes. Duc et al. (2004) stated the potential of *Bacillus cereus*, *Bacillus clausii*, and *Bacillus pumilus* to confer probiotic properties.

The 3 isolates (K1, K2, and K3) screened from moromi stage of soy sauce production were proven capable of producing fibrinolytic protease activity, as seen on the fibrin plate assay (Figure 1a). Specifically, K2 fermented at 1 day (K21) possessed the highest fibrinolytic activity of all, with the widest clear zone area of 1.50 cm in the fibrin plate. In addition, the measurement for K1, K2, and K3 grown for 1 day (K11, K21, and K31) were between 1.30 cm and 1.50 cm, which was higher than those fermented at 2 days (K12, K22, and K32), within the range of 0.81 cm and 1.08 cm, and 3 days (K13, K23, and K33) of 0.65 cm to 1.23 cm. Furthermore, the extracellular enzyme obtained from K13 possessed the lowest activity of about 0.65 cm. In this

case the protein concentrations of all samples evaluated were within the range 0.92-0.99 mg/mL. The fibrinolytic activity of extracellular enzyme obtained from K1, K2, and K3 isolates were compared with the commercial Lumbrokinase (LK), a fibrin degrading enzyme from earthworm *Lumbricus rubellus*, and Figure 1b indicated a close similarity with the K21 enzyme.

Afifah et al. (2014) reported the production of fibrinolytic enzymes from bacteria isolated from Indonesian fermented *Gembus tempe* and *Red oncom*. Clear zone produced on the fibrin plate using similar method were 1.35 cm and 1.45 cm, respectively. These values were less than that from the best isolate of the current study. Thus, the bacteria obtained from moromi stage of fermented soysauce, were proven capable of producing good fibrinolytic enzymes. *Bacillus licheniformis*, *Bacillus cereus*, *Brevibacillus laterosporus*, and *Bacillus pumilus* were the fibrinolytic bacteria identified from *Red oncom* and *Gembus tempe* (Afifah et al., 2014).

Based on Gram staining, spore staining, and morphological test, all of the isolates (K1, K2, and K3) were identified as rod-shaped Gram positive and spore producing bacteria (Figure 2a). The results of biochemical tests using API 50 CHB kit specifically for *Bacillus* spp, identified isolate K1 as *Bacillus cereus* 1 (99.8%), K2 as *Bacillus subtilis* (97%), and K3 as *Bacillus cereus* (99%). Molecular identification based on 16S rRNA's gene confirmed K1 as *B. cereus* 1 (100%), K2 as *B. subtilis* (99.86%), and K3 as *B. cereus* (97.56%). The nucleotide sequences were deposited at GenBank, with accession number MK045762 for K1, MK652831 for K2, and MK530096 for K3, and the phylogenetic tree was constructed on the basis of the sequences as presented in Figure 2b.

The specific genus from fermented food that were reported earlier capable of producing strong fibrinolytic enzyme include *Bacillus natto* from *Natto* (Fujita et al., 1993), *Bacillus*

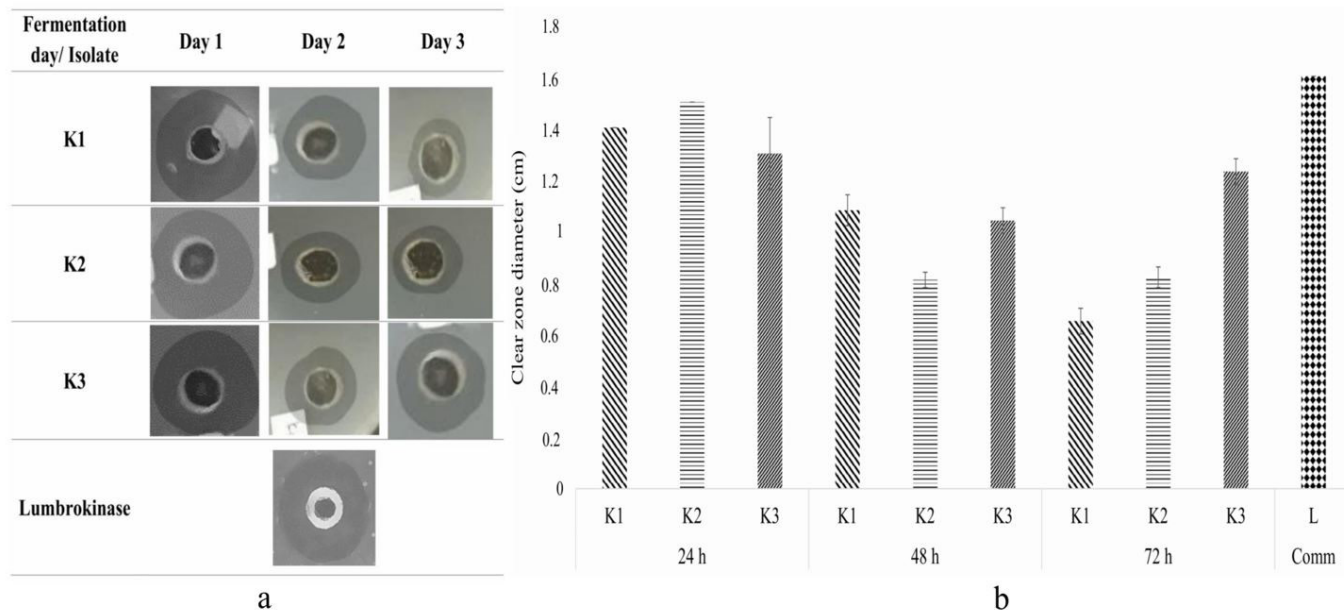


Figure 1. (a) Fibrinolytic activity shows in fibrin plate, (b) Fibrin degrading activity (cm) from fibrinolytic enzymes K1, K2, K3 grown at 24h, 48h, and 72h. L: Lumbrokinase.

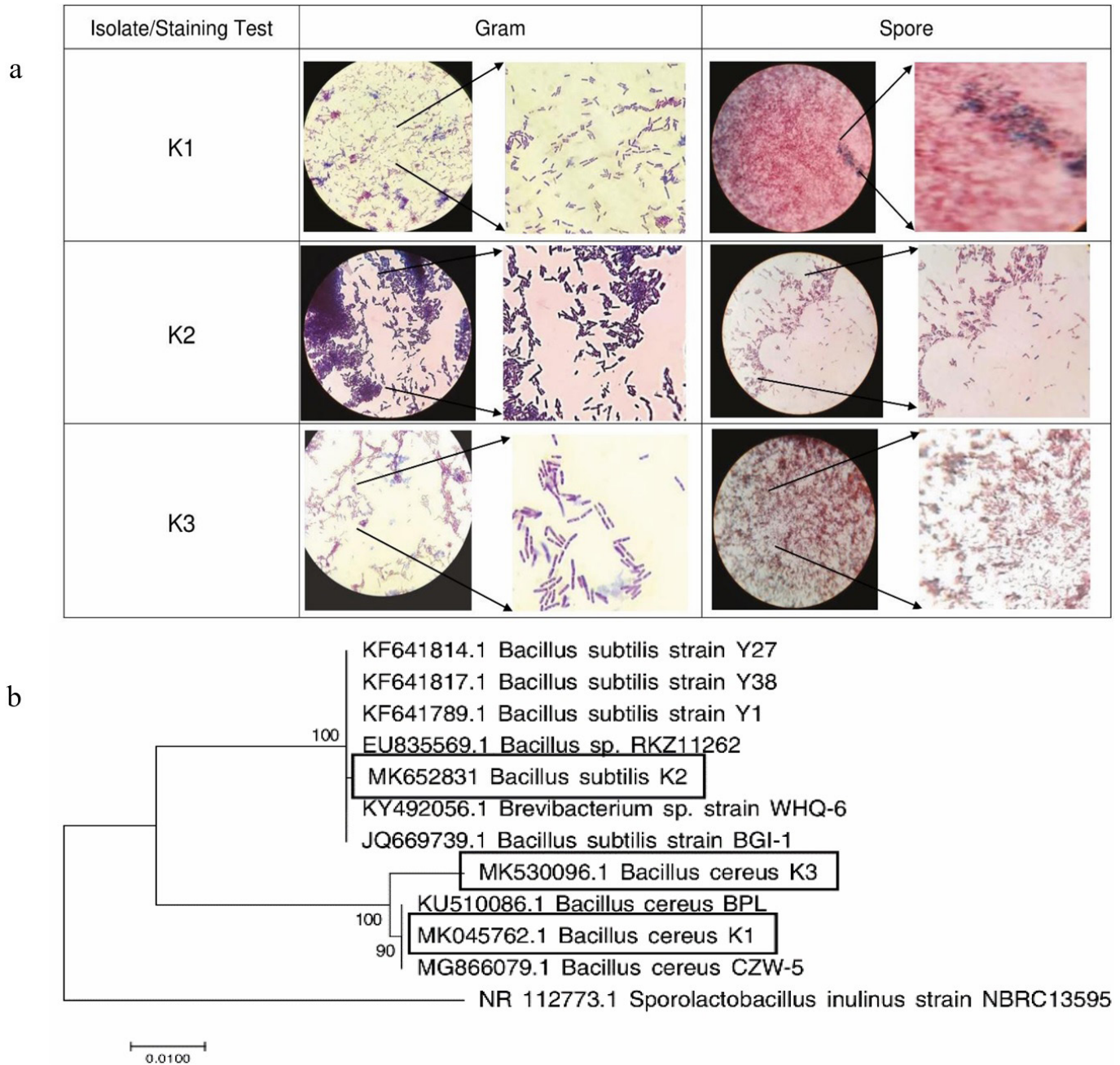


Figure 2. (a) Gram and spore staining of K1, K2, and K3 (magnification 1000 x); (b) Phylogenetic tree of the strains constructed based on sequence analysis of their 16S rRNA gene.

amyloliquefaciens DC-4 from *Douchi* (Peng & Zhang, 2002), *Bacillus amyloliquefaciens* MJ5-41 from *Meju* (Jo et al., 2011), *Bacillus amyloliquefaciens* LSSE-62 from Chinese soy paste (Wei et al., 2011), *Bacillus* sp. DJ-2 from *Doen-jang* (Choi et al., 2005), *Bacillus licheniformis* KJ-31 from *Jeot-gal* (Hwang et al., 2007), and *Bacillus coagulans* from *Terasi*, an Indonesian fermented fish (Prihanto et al., 2013).

Fermented foods are regarded as sources with potential fibrinolytic efficacy, as their active compounds are suspected to affect blood circulation. Data on studies reporting fibrinolytic activity in various fermented foods are shown in Table 1.

The fibrinolytic activity of extracellular enzyme obtained from *Bacillus cereus* K1, *Bacillus subtilis* K2, and *Bacillus cereus* K3 isolate were comparable to the commercial product Lumbrokinase (LK) from earthworm (*Lumbricus rubellus*). This implies the useful potentials of the three new isolates (*Bacillus subtilis* and *Bacillus cereus*) in the production of fibrinolytic enzymes for health application, which in this case act as an anti thrombolytic agent.

Homeostasis is very important in the blood coagulation and fibrinolysis system because of the highly elaborated and complicated system, where both factors ought to be maintained at a balanced and well controlled state. In addition, the fibrin

Table 1. Fermented food and fibrinolytic producing microorganism.

Microorganism	Food	Enzyme	Reference
<i>Bacillus subtilis</i> var natto	Vegetable cheese, <i>Natto</i> , Japan	Nattokinase	Sumi et al. (1987)
<i>Bacillus natto</i> , NK	<i>Natto</i> , Japan	Nattokinase	Fujita et al. (1993)
<i>Bacillus</i> sp.	<i>Tofuyo</i> , Japan	SMCE	Fujita et al. (1993)
<i>Katsuwonos pelamis</i>	<i>Skipjack</i> , Japan	Katsuwokinase	Sumi et al. (1995)
<i>Bacillus</i> sp. CK	<i>Chungkook-jang</i> , Korea	CK	Kim et al. (1996a,b)
<i>Bacillus</i> sp. KA38	<i>Jeot-gal</i> , Korea	Jeot-gal enzyme	Hyun-Kuk Kim et al. (1997)
<i>Bacillus</i> sp.	<i>Kimchi</i> , Korea	Bacillus protease	Noh et al. (1999)
<i>Armillaria mella</i>	<i>Armillaria mella</i>	Neutral metalloprotease	Kim & Kim (1999)
<i>Bacillus</i> sp. DJ-4	<i>Doen-jang</i> , Korea	Subtilisin DJ-4	Kim & Choi (2000)
<i>Bacillus subtilis</i> IMR-NK1	<i>Natto</i> , Japan	Nattokinase	Chang et al. (2000)
<i>Bacillus</i> sp. KDO-13	<i>Soybeanpaste</i> , Korea	-	Lee et al. (2001)
<i>Enterococcus faecalis</i>	<i>Tempe</i> , Indonesia	<i>E. faecalis</i> BRCA-5	Joo et al. (2002)
<i>Bacillus subtilis</i>	<i>Natto</i> , Japan	Nattokinase	Yamashita et al. (2003)
<i>B. amyloliquefaciens</i> DC-4	<i>Douchi</i> , China	Subtilisin DFE	Peng et al. (2003)
<i>Bacillus subtilis</i> QK02	Fermented soybean	QK-1 and QK-2	Ko et al. (2004)
<i>Bacillus firmus</i> NA-1	<i>Natto</i> , Japan	-	Seo & Lee (2004)
<i>Bacillus</i> sp. DJ-2	<i>Doen-jang</i> , Korea	bpDJ-2	Choi et al., (2005)
<i>Rhizopus chinensis</i> 12	Chinese wine, China	-	Xiao-lan et al. (2005)
<i>Fusarium</i> sp	<i>Tempe</i>	-	Sugimoto et al. (2007)
<i>B. amyloliquefaciens</i>	Fermented chickpeas, China	-	Wei et al. (2011)
<i>Bacillus coagulans</i>	Fermented fish, <i>Jambal roti</i> and <i>Terasi</i> , Indonesia	-	Prihanto et al. (2013)
<i>Bacillus licheniformis</i> RO3	<i>Red oncom</i> , Indonesia	-	Afifah et al. (2014)
<i>Bacillus pumillus</i>	<i>Gembus</i> , Indonesia	-	Afifah et al. (2014)
<i>Bacillus licheniformis</i>	<i>Soy flour</i>	-	Gad et al. (2014)
<i>Bacillus amyloliquefaciens</i>	<i>Spoilt milk</i>	-	Gad et al. (2014)
<i>Bacillus subtilis</i> ZA400	<i>Kimchi</i> , Korea	BsfA	Ahn et al. (2015)
<i>Stenotrophomonas</i> sp	<i>Oncom</i> , Indonesia	Stenoprotease	Nailufar et al. (2016), Kurnia et al. (2017)

converted from fibrinogen during thrombosis was identified as an insoluble protein, which is stabilized by thrombin-activated factor XIII through cross-linking (Furlan, 1988). Therefore, it is critical to decrease its redundant deposits at the sites of tissue injury, in order to fully restore potency to the damaged vessel. Furthermore, the fibrinolytic system, consisting of enzymes like plasmin, tissue plasminogen activator (t-PA), plasminogen activator inhibitors (PAIs), and urokinase are identified as the body's main defense mechanism against occlusive vascular diseases (Lijnen, 2001).

Thrombolytic agents have been identified in numerous organism, ranging from earthworm, snake, centipede, mushroom, kelp, some algae and microorganisms (Kotb, 2012; Yogesh & Halami, 2017). Hence, streptokinase from *Streptococcus hemolyticus* and staphylokinase from *Staphylococcus aureus* which act as plasminogen activator, have been adopted as thrombolytic agent's medicine to overcome thrombosis. Meanwhile, two other agents known to be effective (Kotb, 2012) include tissue-type plasminogen activator (t-PA) (Collen & Lijnen, 2004) and urokinase (Duffy, 2002), which both activate plasminogen into active plasmin, in order to properly degrade fibrin. However, in addition to being expensive, certain side effects have been reported through injection application, which is possibly due to the presence of impurities or some immune active material in the

preparation. This has, therefore, ignited the quest for a research on safer sources, including traditional fermented food.

The protein profile of extracellular enzymes produced from each isolate with different fermentation time were analysed with SDS PAGE (Figure 3), using about 0.92–0.99 mg/mL concentrations loaded into the gel. Generally, protein of 20 kDa was present in almost all fermentation extract, and the elevation of fermentation time led to an increase in the protein synthesis performed by the bacterias. Therefore, the profiling illustrated the presence of only 1 or 2 protein bands on day 1 and 2, while higher protein varieties from 14 to 45 kDa, indicative of more protein bands, were found on the fermentation time of day 4 and 7.

Table 2 shows the correlation between protein fractions observed in SDS PAGE profiling and proteolytic activity, pointing 20 kDa protein as responsible for the fibrinolytic effects. In addition, the strongest activity was observed on day 1 of the fermentation extract in all isolates, followed by day 2 and 3, which imply that the fibrin degrading enzyme was synthesized at the early phase of growth.

Enzymes with fibrinolytic activities occur in diversified sizes: 18 kDa from *Bacillus polymaxa* NRC-A (Mahmoud et al., 2011), 18.1 kDa as FCF-11 from *Bacillus amyloliquefaciens* FCF-11 (Kotb, 2014), 18.6 kDa as thrombinase from *Bacillus*

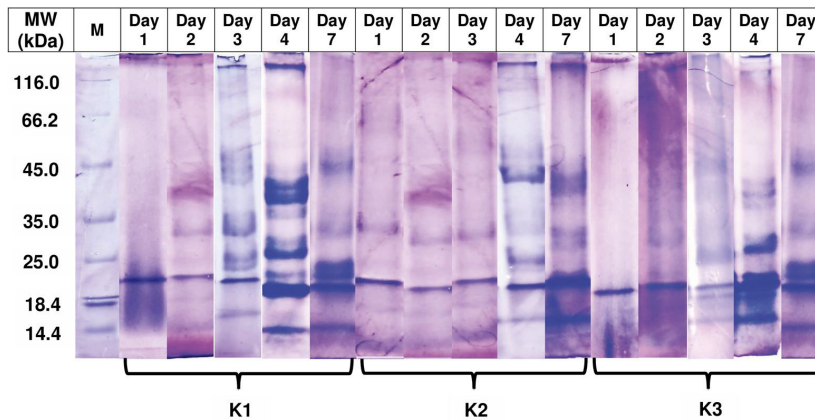


Figure 3. Protein profile in SDS PAGE.

Table 2. Protein fractions with various molecular weight observed during fermentation.

Band (kDa)	Isolate								
	K1			K2			K3		
	1d ^a	2d	3d	1d	2d	3d	1d	2d	3d
<18	-	√	√	-	-	√	-	√	√
18-20	√	√	√	√	√	√	√	√	√
20-25	-	-	√	√	√	√	-	√	√
35-45	-	-	√	-	-	√	-	-	√
45-66	-	-	-	-	-	-	-	-	√
>66	-	-	√	-	-	√	-	-	-
Fibrinolytic activity	+++	++	+	+++	+	+	+++	++	++

note: ^a shows fermentation time in day (d); +++ very strong, ++ strong, + strong enough.

spharicus (Balaraman & Prabakaran, 2007), 14 kDa, 21 kDa, 35 kDa, and 46 kDa as multiple proteases from *Bacillus subtilis* BR-21 (Yogesh & Halami, 2015), and 45 kDa from *Bacillus* sp. KDO-13 (Lee et al., 2001). Afifah et al. (2014) reported the extracellular enzymes with 20 to 41 kDa produced from *Bacillus* isolated from *Gembus tempe* and *Red oncom*. Proteins with 20 to 41 kDa obtained from the *Bacillus cereus* K1, *Bacillus subtilis* K2, and *Bacillus cereus* K3, possibly possess the strongest activity, especially the 21 kDa and their purification is underway.

Fibrinolytic enzyme from bacteria are usually produced as several isozymes, including EfP-0-2 and EfP-II-2, which are the trypsin-like types present in glycosylated forms obtained from earthworm *Eisenia fetida* (Wu et al., 2007). F5 and F6 molecules are another trypsin-like serine protease isozyme isolated from *Lumbricus rubellus* (Cho et al., 2004). Whereas, F-III-2 and F-III-1 were the varieties obtained from *Lumbricus rubellus* (Nakajima et al., 1993). Ahn et al., 2015 reported the purified fibrinolytic BsfA enzyme from *Bacillus subtilis* ZA400 are 18.4-45 kDa proteins, while the enzymes produced from *Stenotrophomonas* sp of *Red oncom* had molecules of 19 to 64 kDa (Stephani et al., 2017).

4 Conclusion

Based on the results, it was established that Moromi from soy sauce contains fibrinolytic bacteria, as confirmed by fibrin plate analysis. Moreover, based on morphological, biochemical and molecular evaluation, K1, K2, and K3 isolates were confirmed

as Gram positive, rod shaped, and spore forming *Bacillus*, where K1 was confirmed as *Bacillus cereus*, K2 *Bacillus subtilis* and K3 *Bacillus cereus*. All three isolates produced several proteins during fermentation process in the LB media.

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