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Screening and identification of *Bacillus* sp. isolated from traditional Vietnamese soybean-fermented products for high fibrinolytic enzyme production

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Abstract

Fibrinolytic enzymes produced by microorganisms have been attractive in prevention and treatment of cardiovascular diseases (CVDs) by their low-cost and safety. This study focused on screening for the existence of fibrinolytic enzymes in Vietnamese traditional fermented soybean paste products and isolation and identification of related bacteria. Sixteen fermented soybean paste samples were collected over three regions of Vietnam in which seven samples gave the positive results on fibrinolytic enzyme activity. Miso (MS) and Green Chili (GC) samples had the highest fibrinolytic enzyme activities (1.81 and 0.77 FU/g, respectively). According to morphological features, four strains of bacteria were isolated and all of them were found to produce fibrinolytic enzymes. The enzyme activities produced by four isolated strains were in a range of 29.7 - 77.9 FU/g after culturing on solid state media for 24 h. The isolated strains were identified as *Bacillus amyloliquefaciens* using 16 rRNA sequence and phylogenetic analysis with 99% similarity.

Introduction

Unhealthy diet, physical inactivity, tobacco use and harmful use of alcohol are considered as among the most common behaviour risk factors of cardiovascular diseases (CVDs) besides other risk factors, taking responsibility about 80% of coronany heart and cerebrovascular diseases. CVDs caused an estimated 17.3 million people died in 2008, occupying 30% of total global deaths and it will develop into 23.3 millions by 2030. Moreover, over 80% of CVD deaths happened in low- and middle-income countries and occurred evenly in men and women (WHO, 2011).

Although the thrombolytic agents have been widely used such as tissue-type plasminogen activator (t-PA) (Collen and Lijnen, 2004), streptokinase (Aylward, 1993) and urokinase (Duffy, 2002), their costly prices and undesirable side effects such as gastrointestinal bleeding, allergic reactions, and resistance to repercussion are still big issues (Blann *et al*., 2002). Fibrinolytic enzyme was firstly discovered in Natto product which is a popular traditional soybean-fermented food in Japan (Sumi *et al*., 1987). Then numerous traditional Asian fermented foods were found to contain fibrinolytic enzymes such as Japanese natto (Sumi *et al*., 1987; Fujita *et al*., 1993), Korean Chungkook-Jang soy sauce (Kim *et al*., 1996), Chinese Douchi (Peng *et al*., 2003), Indonesian terasi and Jambal roti (Asep *et al*., 2013).

Traditional Vietnamese soybean-fermented products have been consumed in daily meal for long time ago, especially in the South and North of Vietnam. This kind of fermented food is made of mainly fried blended soybean, mixing with salt, waxy rice and water, and then fermented under sunlight for about three months. Since the Vietnamese soybean-fermented foods are produced by various ingredients and fermentation conditions, these products possess different tastes and physical properties such as color, moisture contents and flavors. The traditional Vietnamese fermented foods have been investigated to contain fibrinolytic enzymes producing by bacteria existed in these products such as Chao Vinh Phong (Linh *et al*., 2013), fermented shrimp paste products (Anh *et al*., 2013; Anh *et al*., 2015) and fermented fish paste products (Uyen *et al*., 2013). However, the fibrinolytic enzyme activity and sources of microorganism of different products varied depending on the processing conditions and locations. Therefore, the traditional soybean-fermented products produced at different locations in Vietnam were collected and screened for the presence of fibrinolytic enzymes in this study, and then the bacteria producing high fibrinolytic enzyme in these products were isolated and identified to find out the new source of bacteria.

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Materials and Methods

Materials

Sixteen soybean-fermented food samples produced by different companies in Vietnam were collected. ToanThu (TT) in Hai Duong province, HuongDat (HD) and HuongViet (HV) in Hung Yen province, TamDuc (TD), HangHai (HH) and ThuySanKV1 (TSKV1) in Ha Noi Capital were collected from the North of Vietnam. Miso (MS) in Tien Giang province, MinhTam (MT), GreenChili (GC), HoChiMinh (HCM), ChaVa (CV), HuuAi (HA), HauSanh (HS) and TrungNguyen (TN) were collected from Binh Duong province, Ho Chi Minh City and Bac Lieu province in the Southern of Vietnam. NamDan (ND) and MinhChau (MC) were collected from Nghe An province and Hue City in the middle of Vietnam. All of samples had various colors and dry matter contents (13.2 – 65.0%).

The soybean oil cake powder (SOCP) used as substrate was purchased from Quang Minh Company. It was ground into powder form and then measured moisture content. All chemicals used in this study were purchased from Merck Chemical Company (Germany).

Screening of crude fibrinolytic enzyme in the soybean-fermented products

The collected soybean-fermented products were screened for fibrinolytic enzymes, which existed naturally in these samples. Firstly, the samples were diluted with 245 mM phosphate buffer (pH 7) to ensure that all samples had the same dry matter content after checking the moisture contents of the products. Samples were then shaken for 10 min and filtered through Whatman paper. The filtrated solutions were then used to evaluate fibrinolytic enzyme activity using the fibrin degradation assay (Wang *et al*., 2009).

Fibrinolytic activity evaluation

Fibrinolytic enzyme activity was evaluated according to the fibrin degradation assay provided by Japan Bio Science Laboratory Co., Ltd. (JBSL) with slight modifications (Wang *et al*., 2009). First, 0.4 mL of fibrinogen and 0.1 mL of 245 mM phosphate buffer (pH 7) were loaded to a test tube and incubated at 37°C. After 5 min, 0.1 mL of thrombin solution was added and incubated at 37°C for 10 min to form fibrin clot. After adding 0.1 mL enzyme solution, the mixture was incubated at 37°C for 60 min with stirring for 20 min interval, 2.0 mL of 0.2 M trichloroacetic acid (TCA) was added and mixed well to stop enzyme reaction. The reaction mixture was then incubated at 37°C for 20 min, centrifuged at 6,500×g for 5 min and finally removed pellet. Then, absorbance of the supernatant containing fibrinolytic enzyme was measured at 275 nm using a spectrophotometer (UVD-2960, Labomed, USA). The control sample was also measured with the same above process except for the enzyme addition step, in which the enzyme solution was added after terminating the reaction by TCA. In this assay, 1 unit (Fibrin degradation unit, FU) of enzyme activity is defined as a 0.01-per-minute increase in absorbance at 275 nm of the reaction solution.

Enzyme activity was calculated using the following formulas:

FE activity (FU/mL) = $[(OD_s – OD_c)/(0.01 \times 60 \times 0.1)] \times (V/W)$

Where: OD_s, optical density value of sample; OD_c, optical density value of control; V, total volume of solution; W, dry weight of sample.

Isolation of bacteria

The soybean-fermented product which had the highest fibrinolytic enzyme activity was used to isolate the presented bacteria. The sample was diluted at 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} concentration and then 100 µl diluted solutions were spread onto LB agar plates and incubated at 37°C for 24 h. After that, the LB agar plates were observed and sub-cultured until each agar plate had only one kind of colony.

Fibrinolytic enzyme production by isolated bacteria

Each isolated bacterium was cultured into Erlenmeyer flasks containing 30 ml of seed culture (0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_2O$, w/w) and they were incubated at 37°C for 24 h. After incubation, the seed culture (0.1 ml) was transferred to Erlenmeyer flasks containing culture medium in solid state form (Ramakrishna *et al*., 2011) and then incubated at 37°C for 24 h. The culture medium was extracted by adding phosphate buffer and the solution was filtered to obtain crude enzyme. The filtrated solution was then measured for fibrinolytic enzyme activity.

Identification of isolated bacteria

16S rRNA gene sequences of bacteria producing the highest fibrinolytic enzyme activity were identified using a Big Dye Terminator Cycle Sequencing Kit and 3130x1 Genetic Analyzer at Nam Khoa Biotek company in Vietnam. After sequencing, the sequences were compared in the BLAST search tool in the NCBI nucleotide sequence database. Then the sequences were aligned with Clustal X 1.83 software (Chenna *et al*., 2003), an evolutionary distance tree

was built based on the neighbor-joining method using MEGA 4.1 software (Kumar *et al.*, 2001), and the reference sequences used in tree construction were obtained from GenBank database.

Statistical analysis

Analysis of variance (one-way ANOVA) was performed using Duncan's multiple-range test to compare treatment means at $P < 0.05$ using SPSS software version 16 (SPSS Inc., USA).

Results and Discussions

Screening of fibrinolytic enzyme activity in soybean-fermented products

The fibrinolytic enzyme activities (FU/g) in the traditional soybean-fermented products are shown in Figure 1. Among sixteen collected soybean-fermented products, seven samples had positive results of fibrinolytic enzymes with the activities ranging from 0.32 to 1.81 FU/g. Among the enzyme-existed samples, the Miso (MS) and GreenChilli (GC) products, collected from the South of Vietnam, had the highest fibrinolytic enzyme activities (1.81and 0.77FU/g sample, respectively), followed by HCM, MC, MT, HD and HV samples with the enzyme activities ranging from 0.32 to 0.57 FU/g. In contrast, the remaining samples, HA, TT, HS, TN, CV, ND, HH, TD and TSKV1, did not contain the fibrinolytic enzymes. The difference in fibrinolytic enzyme activities in these samples might be due to the different ingredients, fermentation conditions and processing locations. Mine *et al.* (2005) reported that the fermentation process of the natural fermented food products is due to bacteria from air, utensils or ingredients which are present in nature. Therefore, the bacterial strains which produced a strong fibrinolytic enzyme varied in the different products such as *Bacillus* sp. in Chunkook-Jang, a traditional Korean fermented soybean sauce (Kim *et al*., 1996), *Bacillus subtilis* in a domestic "natto" in Taiwan (Chang *et al*., 2000) or *Bacillus natto* in a traditional fermented food in Japan, natto (Sumi *et al*., 1987). As a result, the bacterial strains producing high fibrinolytic enzymes in the MS and GC in this study were isolated and identified to get more information of these bacteria.

Isolation and identification of isolated bacteria

The Miso and GreenChilli soybean-fermented products which produced the highest fibrinolytic enzyme activities were selected to isolate the related bacteria. Based on the morphology of colonies, four strains of bacteria were isolated from these products. Two strains coded as CB1 and CB2 were isolated

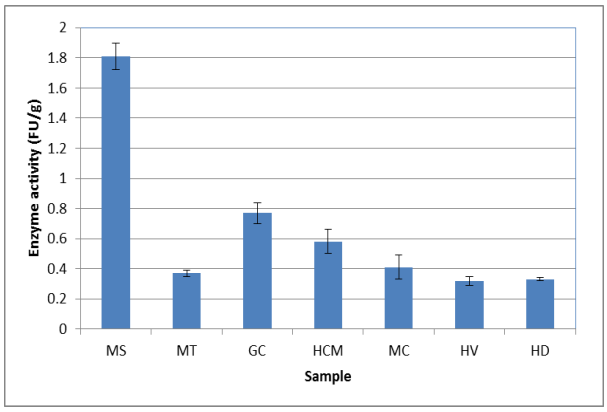

Figure 1. Fibrinolytic enzyme activities of selected soybean-fermented products. MS, Miso; MT, MinhTam; GC, GreenChilli; HCM, HoChiMinh; MC, MinhChau; HV, HuongViet; HD, HuongDat

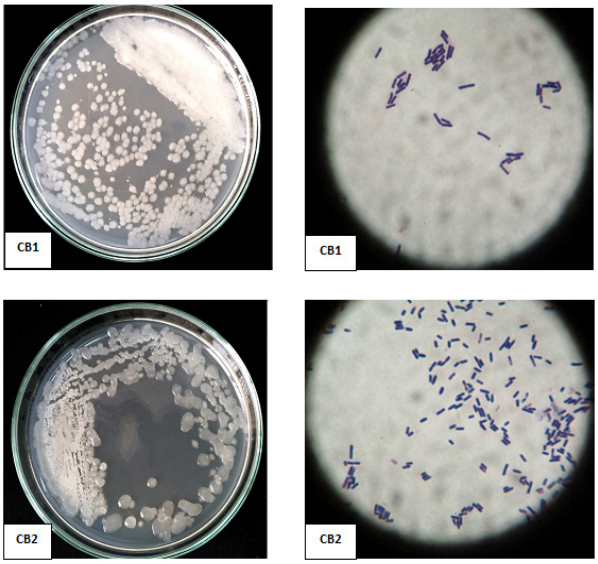

Figure 2. Morphologies of bacteria isolated from GreenChili sample under 100× microscope. CB1 and CB2, bacterium 1 and 2 isolated from Green Chili sample

from the GreenChili sample as shown in Figure 2. The morphologies of both CB1 and CB2 strains were circular, white and glistening. In addition, CB1 colonies had undulate margin, raised elevation, smooth texture and opaque, while CB2 colonies were entire, pulvinate, rough and translucent. Both of isolated bacteria are Gram-positive, motile, rod-shaped, and endospore-forming in overnight incubation on LB plate at 37°C. Figure 3 shows the morphologies of two strains isolated from the Miso sample, which was coded as MB1 and MB2. MB1 colonies were irregular shape, lobate margin, rough texture, raised elevation, cream color and dull appearance, whereas MB2 colonies were circular shape, undulate margin, raised elevation, rough texture, white color and glistening appearance.

The isolated strains were further identified based on the 16S rRNA sequence using a Big Dye

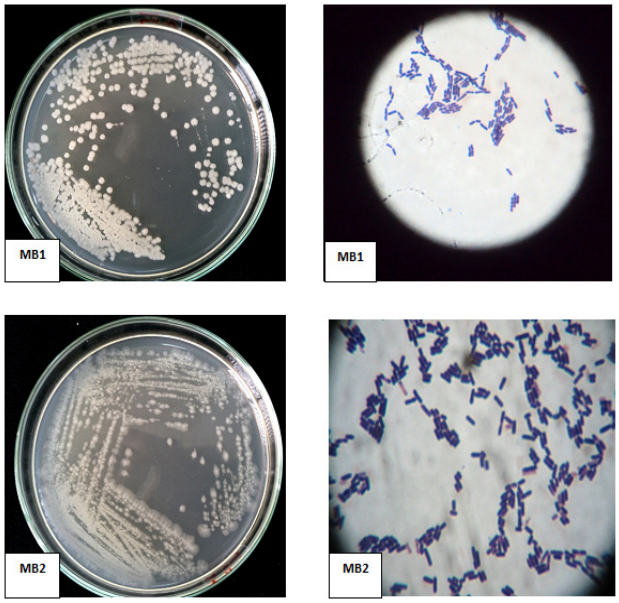

Figure 3. Morphologies of bacteria isolated from Miso sample under 100× microscope. MB1 and MB2, bacterium 1 and 2 isolated from Miso sample

Terminator Cycle Sequencing Kit and 3130x1 Genetic Analyzer. A phylogenetic tree was built by using the neighbor-joining method shown in Figure 4 indicated that four isolated strains belong to the *Bacillus* spp. The aligned results in GenBank database with all available 16S rRNA sequences showed that all four strains were identified as *Bacillus amyloliquefaciens* FZB42 strain with 99% of identities (Genbank accession number NR 075005.1). The total scores of CB1, CB2, MB1 and MB2 strains were 920, 957, 946 and 935, respectively. The results of this study is consistent with the previous studies who reported that *Bacillus amyloliquefaciens* produced high fibrinolytic enzymes in soybean fermented foods in Korea and China (Kim and Choi, 2000; Peng *et al*., 2003). Thus, the results in this study indicated that the Vietnamese soybean-fermented products contained fibrinolytic enzymes produced by *Bacillus amyloliquefaciens* strains. The production of fibrinolytic enzymes was affected not only processing conditions and locations but also depended on the specific strain existed in the product. Suzuki *et al.* (2003) reported that daily consumption of natto shortened euglubulin clot lysis time (ECLT) and did not prolong bleeding time. Like other food-sourced fibrinolytic enzymes, Vietnamese soybean-fermented food products are considered to be kinds of functional foods for thrombolytic therapy.

Production of fibrinolytic enzymes by isolated bacteria

The fibrionolytic enzyme activities produced by four strains, CB1, CB2, MB1 and MB2, are shown in Table 1. All strains produced the fibrinolytic

Table 1. Fibrinolytic enzyme activities produced by isolated bacteria[a,b,c]

Strains	FU/g
CB1	77.95 ± 2.15c
CB2	40.19 ± 2.49ab
MB1	56.33 ± 4.16b
MB2	29.74 ± 3.59a

[a]Values represent the mean of triplicate ±SD.
[b]Mean values of samples followed by the same letter are not significantly different ($P<0.05$).
[c]CB1 and CB2, bacterium 1 and 2 isolated from GreenChili sample. MB1 and MB2, bacterium 1 and 2 isolated from Miso sample.

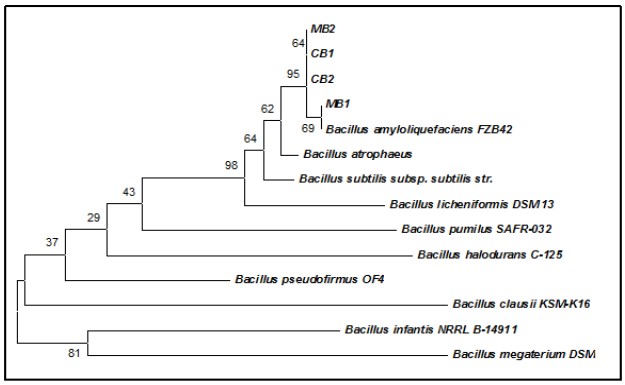

Figure 4. Phylogenetic tree based on 16S rRNA gene sequences between isolated strains and representatives of related species. Bootstrap values (expressed as a percentage of 500 replications) were shown at branch points. Bar 0.01 nucleotide substitutions per position. CB1 and CB2, bacterium 1 and 2 isolated from Green Chili sample. MB1 and MB2, bacterium 1 and 2 isolated from Miso sample

enzymes with different activities under solid state fermentation in which soybean oil cake powder was used as main ingredient. The strain CB1 produced the highest fibrinolytic enzyme activity (77.95 FU/g), whereas the strain MB2 gave the lowest enzyme activity (29.74 FU/g). The enzyme activities produced by CB2 and MB1 strains were 40.19 and 56.33 FU/g, respectively. Thus, the fibrinolytic enzymes produced by the isolated strains were significantly higher than those naturally occurred in the traditional fermented soybean products. Wei *et al.* (2011) reported that a *Bacillus amyloliquefaciens* LSSE-62 strain isolated from Chinese soybean paste produced fibrinolytic enzyme with activities of 39.28 FU/g under the optimal condition using chickpeas-based media. In general, commercial nattokinase functional food showed fibrinolytic activity of 20-40 FU/g (Wei *et al.*, 2011). As a result, primary solid-state fermentation of soybean oil cake powder for fibrinolytic enzyme production using the isolated

Bacillus amyloliquefaciens strains from Vietnamese fermented soybean paste products in this study had high enzyme activity, which might be applied for production of functional food products with high fibrinolytic enzyme activities.

Conclusion

Seven soybean-fermented products gave the positive results on enzyme activity over sixteen samples collected in three regions of Vietnam. Four strains of bacteria were isolated from Miso and GreenChili samples which contained the highest fibrinolytic enzyme activities. All of them are capable of producing fibrinolytic enzymes ranging from 29.74 to 77.95 FU/g under solid state fermentation. All four bacteria were identified as *Bacillus amyloliquefaciens*, closely related to *Bacillus amyloliquefaciens* FZB42 strain with 99% of identities (Genbank accession number NR 075005.1) using the 16S rRNA sequence and a phylogenetic tree. As a result, the isolated strains could be applied for production of functional food products with high fibrinolytic enzyme activities.

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The authors have declared no conflict of interest.

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