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Screening and Identification of Fibrinolytic Bacteria from Malaysian Fermented Seafood Products

Zarita Zakaria[1], Madihah Mohd. Salleh[1], Noor Aini Abdul Rashid[2*]

[1]Dept. of Biotechnlogy and Medical Engineering, Faculty of Biosciences and Medical Engineering, UTM 81310 UTM Skudai, Johor, Malaysia
[2]Dept. of Biosciences and Health Sciences, Faculty of Biosciences and Medical Engineering, UTM 81310 UTM Skudai, Johor, Malaysia.

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ABSTRACT

Natural fibrinolytic enzymes have been focused recently when compared to chemically synthesize thrombolytic drugs as they are more economical and believe to have less adverse reaction. Belacan (shrimp paste), Budu (fish sauce) and Cencaluk (shrimp sauce) were traditional fermented Malay seafood products used as sources for this study. The isolation and screening resulted in the discovery of 43 potential isolates with the ability as an extracellular fibrin degrader. Each isolate were unique due to differences in morphology characteristics and the amounts of fibrinolytic enzyme secretes under standard growth condition. Furthermore, it has been demonstrated that some strains posses the ability to act as tissue plasminogen activator (t-PA) which in deed has beneficial to many medicinal and therapeutic purposes. Of these 43 strains investigated for the fibrinolytic enzyme production, three species, mainly the best producer from each food sample were chosen for further molecular identification. This lead to the discovery of *B. cereus* 13BN, *B. subtilis* 2CN and *B. subtilis* 9BD strains, which posses both high fibrinolytic and t-PA activities, making it extremely valuable and promising producer.

INTRODUCTION

The first fundamental problem occurs when fibrin which plays a vital role in health and healing, has the tendency for an overzealous propensity to form inappropriate clots in the body. Normally, when correctly balanced, deposition and removal of fibrin maintains avoidance of blood loss and adverse viscosity in the vascular system. However, a balance tipped in favor of fibrin overproductions lead to dangerous clotting popularly known as thrombosis, and leading to a major risk factor for chronic diseases such as myocardial infarction and stroke, a types of cardiovascular diseases (CVDs). Although these CVDs can be prevented and treated, an increasing number of the new arising cases and deaths worldwide each year is of very big concern. One of the heartbreaking statistics reported by WHO in 2011 claims that CVDs had taken more lives than anything else, and accounting for nearly one-third of deaths worldwide and those deaths are projected to increase until at least until 2030 (Mendis *et al*., 2011; Wong, 2014). In focus, heart diseases are still on the rise despite the improvements and developments in health services and facilities around the country. Apart from that, these cardiac diseases were the second leading cause of death in Malaysia, accounting for 15.5 per cent of those who died in government hospitals in 2006 and by 2020, they are projected to be the leading cause of death in Malaysia and other developing countries (National Cardiovascular Disease Database, 2006). As an alternative to commonly used chemical drugs, enzyme therapies have becoming more prevalent in medicine today since many therapeutic enzymes have been found to be beneficial in the treatment of various life-threatening diseases. Microbial enzymes, for example subtilases serine proteases possess significant potential in reducing this alarming CVDs and the usage of microorganisms as thrombolytic agent has been extensively reported in many literatures (Peng *et al*., 2005). They are scientifically proven to have the effective ability to degrade excessive blood clots in arteries andthis observation paved the way for the use of microbial serine proteases in medicine.

* *Corresponding Author*
Noor Aini Abdul Rashid, Dept. of Biosciences and Health Sciences, Faculty of Biosciences and Medical Engineering, UTM 81310 UTM Skudai, Johor, Malaysia. Email: nooraini09@gmail.com

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Presently, these enzymes are used as anticoagulant, oncolytics, thrombolytics, anti-inflammatories, mucolytics, antimicrobials and digestive aids (Kotb, 2012). Interestingly, these valuable microorganisms predominantly of bacterial origin as source of serine protease enzymes, gained extra attention in biotechnology, biomedical and pharmacology due to their short generation time, broad biochemical diversity, susceptibility to genetic manipulation and easy large scale production in various sectors (Patel *et al*., 2005). Besides, the inability of plant and animal serine proteases to meet current world demands has led to an increased interest in microbial proteases.

On the other hand, fermented food products are a good sources for the isolation of bacteria with serine protease enzymes since they are preserved by microbes and often manufactured under non-sterile conditions which allow the access of proteolytic bacteria, including the strains of *Bacillus, Pseudomonas, Streptococcus*, *Lactococcus, Lactobacilli, Bifidobacteria, Enterococcus,* and *Pediococci* (Montville and Matthews, 2005). The enzymes released for biochemical proteolysis, lipolysis and glycolysis pathways by these natural microflora or starter cultures during manufacture are main contributors to a specific acidification and flavor profiles of the fermented foods final product (Smit *et al*., 2005).

Finally, while there are many research available involving screening and isolation of microorganisms for enzymes with high fibrinolytic activity, most of the commercially available enzymes still have drawbacks and complications like bleeding, allergenic in nature, secondary immune response and expensive (Chitte, 2013). Therefore, there is an imperative need to search for better and safe fibrinolytic proteases which can be used in preventive medicine. On top of that, it has been a priority for more research and studies to be done on this enzyme considering the lack of satisfactory information although these microbial proteases are executed in a large variety of pharmaceutical and industrial functions. Besides, some of the vast diversity of proteases has low specificity in their mode of action, and therefore attracted worldwide attention in an attempt to exploit more on their physiological and biochemical characteristics (Kotb, 2012). A study of the proteolytic enzymes is therefore valuable, because of their importance as reagents in laboratory and clinical industries (Chitte, 2013).As the demand for novel and better fibrinolytic enzyme is increasing day by day, it is a great opportunity in exploring a potential fibrinolytic enzyme producer from Malaysian fermented foods.

MATERIALS AND METHODS

Isolation of Fibrinolytic Enzyme-Producing Microorganisms

Three different samples of local fermented foods: *Belacan*, *Budu* and *Cencaluk* from grocery stores in the vicinity of Skudai, Johor were used in this study. 1g of sample was suspended in 9 mL sterile saline solution (NaCl 0.85%, w/v), serially diluted ten-fold in saline and then spread on Nutrient Agar (NA) plates containing 1.5% (w/v) skimmed milk beforeincubation for 16 h at 37°C. Colonies, with clearing zone indicating protease activity, were selected and transferred to fresh NA plates. Pure colonies obtained were then tested for fibrinolytic activity.

FIBRINOLYTIC ENZYME ASSAYS

Qualitative Test

Fibrinolytic activity was determined qualitatively with a slight modification of fibrin plate assay (Astrup and Mullertz, 1952). The fibrin plate was made up of the fibrinogen solution (5ml 0.6% (w/v) bovine fibrinogen in 50 mM NaCl (pH 7.4), 0.1 mL of thrombin solution (10 NIH unit/mL) and 5 ml of 2% (wt/vol) agarose solution. To observe the fibrinolytic activity of the enzymes, 10 µL of the enzyme solution was dropped into holes previously made on fibrin plate using a capillary glass tube (5 mm diameter) and incubated at 37°C for 14 h. The activity of the fibrinolytic enzyme was determined by measuring the dimension of the clear zone on the fibrin plate qualitatively by measuring against calibration curve plotted using plasmin as standard at a range of 0.2-2.0 unit/mL.

Quantitative Test

For the quantitative enzyme assay, a fibrin clot suspension was prepared by mixing 500 µL of 0.6% (w/v) fibrinogen solution in borate buffer (pH 7.0) and 20 µL thrombin solution (0.2 mL of 0.85% (w/v) saline containing 200 U thrombin with 3.8 mL borate buffer (pH 7.0). The clot was allowed to form at room temperature. Then 5 µg/mL of partially purified enzyme was added to the clots and the reactions mixture were then pre-incubated at 37°C for 30 minutes in a shaking incubator. After centrifugation at 4°C and 10,000 rpm for 20 minutes, the amount of tyrosine released in the supernatant was determined at 280 nm using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Varying plasmin concentrations were used to construct a standard curve used to calculate the fibrinolytic activity. One unit (U) of fibrinolytic protease activity is defined as 1µg of tyrosine liberated per min per mL of enzyme.

Fibrinolysis Mode of Action

Fibrinolytic mode of action for each enzyme sample was determined by the plasminogen-free fibrin plate method and the plasminogen-rich fibrin plate method. Plasminogen-free fibrin plate was made up of fibrinogen solution [2.5 mL of 1.2% human fibrinogen (Sigma, USA) in 0.1 M sodium phosphate buffer, pH 7.4], 10 U of thrombin solution (Sigma, USA) and 1% agarose. Meanwhile, plasminogen-rich fibrin plate consisted of 2 mL of 1.5% fibrinogen and 5U plasminogen. 10 µl of sample solution was dropped into the wells previously made on a fibrin plate using a capillary glass tube and incubated at 37 °C for 14 h. An equal volume of plasmin solution (1 NIH unit/mL) was used as positive control. The activity of the fibrinolytic enzyme was determined by measuring the dimension of the clear zone on the fibrin plate. The fibrinolytic activity was calculated from the standard curve generated using plasmin.

COLONY MORPHOLOGICAL IDENTIFICATION, GRAM STAINING AND MICROSCOPIC ANALYSIS

Identification of the bacterial isolates was performed, based on a variety of characteristics to include their morphological and physiological characteristics based on Bergey's Manual of Determinative Bacteriology system (Sneath *et al*, 1986). The morphological and physiological characteristics of the isolates were observed using a phase contrast microscope (Leica DME).

Gram staining is a method to discriminate two major groups of bacteria: Gram-positive and Gram-negative (Tortora *et al*., 2004). The isolated bacteria were stained following a standard protocol after the glass slides were washed with ethanol to clean dirt and remove any microbes. A single colony of bacteria was picked with an inoculating loop and smear on the slides with a drop of water. 2-3 drops of crystal violet reagent was flooded on the smear and left for 1 minute. Excess dye was removed gently by rinsing with tap water. The iodine was flooded on the smear and left for 1 minute to form crystal violet-iodine complex. Subsequent to that, the smear was decolorized by using 95% alcohol. The slide then was rinsed with water again follow by a drop of 0.25% safranin onto it. After 30 seconds, the slide was rinsed again with tap water and left to dry. The stained bacteria then was visualized under the microscope under oil immersion at 100x magnification.

DETERMINATION OF PROTEIN CONCENTRATION AND ENZYME ACTIVITY OF ISOLATES

The protein content for every extracellular enzyme was determined by applying the Lowry Assay using varying concentrations of BSA (0 - 0.50 mg mL^{-1}) as standard. The original Lowry method for total protein analysis was first described in one of the most cited papers in biochemistry (Lowry *et al*., 1951). The assay is a colourimetric assay based on cupric ions and Folin-Ciocalteu reagent for phenolic groups.

16S rRNA SEQUENCING AND ANALYSIS

One bacterial strain exhibiting the highest fibrinolytic activity for each fermented food sample was ultimately identified by 16 S rRNA sequencing (Sneath *et al*. 1986). Strains 13BN, 2CN and 9BD were selected for further molecular identification. To confirm the identity of the isolates up to species level, PCR amplification and sequencing of the 16S rRNA gene were performed. The 16S rRNA gene was amplified by PCR using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')according to Lane, 1991; Weisburg *et al*., 1991. PCR mixture was composed of 50 µL of PCR Master Mix, 2X (Promega, USA), 100 ng of each primer, and 100 ng of DNA template. The PCR reaction was performed in 100 µL for 25 cycles of 1 min at 94 °C, 1 min at 55°C, and 2 min at 72°C. Additional extension was carried out for 10 min at 72 °C. DNA amplification was performed using Perkin Elmer Gene AmpR PCR System 9700 thermal cycle (Perkin-Elmer Corp., Emeryville, Calif.). The amplified PCR products were analyzed by 1% (w/v) agarose gel electrophoresis and visualized using Gene Flash gel doc (Syngene). The fragment than was purified using the Promega PCR purification kit to eliminate excess primers and impurities, according to the manufacturer instruction. Finally, the sequencing of the amplified DNA fragments was performed using the services of 1st BASE Pte., Ltd. Similarity searches were performed against the sequences with the database using the BLAST (Basic Local Alignment Search Tool) program from the National Centre for Biotechnology Information (NCBI) website. This program can be accessed at http://blast.ncbi.nlm.nih.gov/Blast.cgi. BLAST uses a heuristic algorithm technique of two or more sequences alignment measurements and is therefore able to detect relationships among sequences which share only isolated regions of similarity (Altschul *et al*., 1990).

CONSTRUCTION OF PHYLOGENETIC TREE

Multiple Sequence Alignments

A set of sequences to be subjected to the homology and phylogenetic analyses is collected and aligned with the 16S rRNA sequences available in the public databases from BLAST (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD, USA), and were identified to the generic level (Altschul *et al*., 1997; Benson *et al*., 1999).Multiple sequence alignments were performed using the Clustal W program (Larkin *et al*., 2007).

MEGA5 Software

A phylogenetic tree was constructed using the neighbour-joining method with a bootstrap value of 1000 replicates using MEGA5 software (Tamura *et al.*, 2011). The sequence alignment was performed using the ClustalW program embedded within the MEGA5 software. It was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset by using complete deletion option in MEGA5 based on Stam *et al.* (2006).

RESULTS AND DISCUSSION

Isolation and Screening of Bacteria Strains Producing Extracellular Fibrinolytic Enzyme

Based on the colonial and cellular morphologies, 43 bacterial strains with extracellular protease and fibrinolytic activities were successfully isolated from the three typical Malaysian fermented foods and listed in Table 1.21 strains were isolated from *Belacan,* 15 isolates were from *Budu* and the 6 were from *Cencaluk*. This suggests that variation in the ingredients added, salt concentration plus the different incubation period, influenced the microbial growth of *Belacan, Budu* and *Cencaluk*; which contributes to the typical and unique flavor of these Malaysian delicacies. As highlighted by Klein *et al*., (2006), the flavour and aroma of foods are generated by the action of

Table 1: The morphology of isolated bacteria.

No.	Name	Gram staining	Bacteria shape	Colony morphology				
				Colour	Form	Margin	Elevation	Size (mm)
				Belacan				
1.	1 BN	-	coccus	yellow	punctiform	entire	convex	0.5
2.	2 BN	-	bacilli	yellowish white	irregular	lobate	umbonate	2.7
3.	3 BN	-	coccobacilli	yellowish white	irregular	entire	umbonate	2.6
4.	4 BN	-	coccobacilli	light yellow	circular	entire	convex	2.5
5.	5 BN	+	streptobacilli	yellow	irregular	entire	convex	3.1
6.	6 BN	+	streptobacilli	yellow	irregular	undulate	convex	3.4
7.	7 BN	-	streptobacilli	yellow	circular	entire	umbonate	2.9
8.	8 BN	-	capsulated bacilli	yellowish white	irregular	curled	raised	3.5
9.	9 BN	-	bacilli	yellowish white	irregular	curled	raised	3.6
10.	10 BN	-	coccobacilli	yellowish white	irregular	entire	convex	2.8
11.	11 BN	-	coccobacilli	yellowish white	circular	entire	convex	2.8
12.	12 BN	-	coccobacilli	yellowish white	irregular	entire	umbonate	2.6
13.	13 BN	+	bacilli	light yellow	circular	entire	flat	3.2
14.	14 BN	-	bacilli	yellowish white	irregular	entire	raised	3.1
15.	15 BN	-	coccobacilli	yellowish white	irregular	curled	raised	3.2
16.	16 BN	-	bacilli	yellow	irregular	entire	convex	1.7
17.	17 BN	+	streptobacilli	yellow	circular	entire	flat	1.8
18.	18 BN	-	coccobacilli	yellow	circular	entire	flat	3.8
19.	19 BN	-	coccobacilli	yellow	irregular	undulate	convex	2.7
20.	20 BN	-	streptobacilli	cream	circular	entire	flat	1.7
21.	21 BN	+	streptobacilli	yellow	circular	entire	flat	1.4
				Cencaluk				
22.	1 CN	+	bacilli	light yellow	circular	undulate	convex	2.4
23.	2 CN	+	bacilli	yellow	circular	entire	raised	2.6
24.	3 CN	-	bacilli	yellow	circular	entire	flat	1.3
25.	4 CN	+	capsulated bacilli	cream	circular	entire	raised	2.3
26.	5 CN	-	bacilli	cream	irregular	entire	umbunate	2.8
27.	6 CN	-	bacilli	light yellow	circular	entire	convex	2.8
28.	7 CN	-	diplococci	yellow	circular	entire	umbunate	1.7
				Budu				
29.	1 BD	+	coccobacilli	yellowish white	circular	entire	raised	2.9
30.	2 BD	-	coccobacilli	yellow	irregular	curled	raised	2.8
31.	3 BD	+	bacilli	cream	irregular	lobate	umbunate	2.7
32.	4 BD	-	coccobacilli	yellow	circular	entire	flat	2.3
33.	5 BD	+	bacilli	yellow	circular	entire	convex	1.2
34.	6 BD	+	coccobacilli	yellow	circular	entire	convex	1.4
35.	7 BD	+	streptobacilli	light yellow	circular	entire	umbunate	2.8
36.	8 BD	-	bacilli	cream	circular	entire	convex	2.6
37.	9 BD	+	streptobacilli	light yellow	circular	entire	convex	2.5
38.	10 BD	+	streptobacilli	yellow	punctiform	entire	umbunate	0.5
39.	11 BD	-	bacilli	yellow	irregular	entire	umbunate	1.6
40.	12 BD	-	coccobacilli	cream	irregular	curled	convex	2.9
41.	13 BD	-	bacilli	cream	circular	entire	umbunate	2.8
42.	14 BD	+	streptobacilli	yellow	circular	lobate	raised	3.5
43.	15 BD	-	streptobacilli	yellow	irregular	curled	convex	1.6

proteolytic microorganisms that survived during the fermentation process. The primary screening of protease producers using skimmed-milk agar plate was regarded as a simpler and faster method of detection where numerous samples could be identified in a day. Besides, the production and secretion of the protease enzymes can be simply identified by the formation of clear zones (of dissolved substrate) around the growing colonies can be easily distinguished from the non-proteolytic ones. Figure 1 shows several random colonies grown on skimmed-milk agar plates and surrounded by a clear zone which corresponds to protease activity. The smaller serially diluted plate shows high density of bacteria growth and bacteria density reduced as dilution times increases, hence enabling the formation of single colony bacteria, which was isolated and examined. Majority of isolated bacteria were bacilli or long rods in shape, suggesting that the bacteria probably fall under the genus *Bacillus*. This result in fact is in good agreement with Peng *et al*., (2005), who concur that the genus *Bacillus* from traditional fermented foods is most notable among the microorganisms to produce the fibrinolytic enzymes. For example, *B. subtilis* strain *natto* from Japanese fermented food *natto* (Sumi *et al*., 1987), *B. amyloliquefaciens* DC-4 from Chinese fermented food *douchi* (Peng *et al*., 2003), *Bacillus sp*. CK from Korean fermented food *chungkook-jang* (Kim *et al*., 1996) and *B. subtilis* TP6 from Indonesian fermented food tempeh (Kim *et al*., 2006).

Table 2: Fibrinolytic enzyme activity of the isolates.

Bil	Name	Fibrin plate (Qualitative)				Fibrinolysis assay (quantitative)	Fibrinolysis action mode		OD_{550}=0.8
		Zone of hydrolysis (cm)	Activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Specific activity (U/mg)	Plasminogen-rich; Zone of hydrolysis (cm)	Plasminogen-free; Zone of hydrolysis (cm)	
Belacan									
1	1BN	0.8	0.611	2.105	0.290	0.301	*	*	F (3-4 hours)
2	2BN	1.8	3.468	2.116	1.639	1.565	-	1.4	S (>5 hours)
3	3BN	1.2	1.753	2.094	0.837	0.856	*	*	M (>4-5 hours)
4	4BN	1.5	2.611	2.100	1.243	1.223	*	*	F
5	5BN	1.8	3.468	2.038	1.702	1.743	2.9	2.4	M
6	6BN	1.4	2.325	2.048	1.135	1.012	*	*	F
7	7BN	1.2	1.753	2.083	0.842	0.890	*	*	F
8	8BN	1.4	2.325	2.123	1.095	1.109	*	*	F
9	9BN	1.2	1.753	2.122	0.826	0.798	*	*	M
10	10BN	1.8	3.468	2.143	1.718	1.699	-	3.0	F
11	11BN	1.8	3.468	2.012	1.723	1.732	-	2.4	M
12	12BN	1.7	3.182	2.158	1.475	1.357	-	2.6	F
13	**13BN**	**1.8**	**3.468**	**1.978**	**1.753**	**1.762**	**3.0**	**3.0**	**F**
14	14BN	1.7	3.182	2.131	1.493	1.512	-	2.4	M
15	15BN	1.5	2.611	2.101	1.243	1.112	*	*	M
16	16BN	0.7	0.325	1.933	0.168	0.172	*	*	F
17	17BN	1.3	2.039	2.077	0.982	0.976	*	*	S
18	18BN	1.3	2.039	2.089	0.976	0.954	*	*	F
19	19BN	1.5	2.611	2.026	1.289	1.221	*	*	F
20	20BN	1.6	2.896	2.085	1.389	1.442	*	*	F
21	21BN	1.7	3.182	2.122	1.500	1.489	-	0.7	F
Cencaluk									
22	1CN	1.7	3.182	2.085	1.526	1.433	-	1.4	F
23	**2CN**	**1.8**	**3.468**	**1.892**	**1.832**	**1.798**	**2.3**	**2.6**	**F**
24	3CN	1.6	2.896	2.020	1.434	1.411	-	2.2	M
25	4CN	1.4	2.325	2.084	1.116	1.012	*	*	M
26	5CN	1.7	3.182	2.036	1.563	1.423	-	1.3	M
27	6CN	1.6	2.896	2.085	1.389	1.372	*	*	S
28	7CN	1.7	3.182	2.074	1.534	1.452	-	2.5	M
Budu									
29	1BD	1.8	3.468	2.118	1.643	1.523	-	2.3	M
30	2BD	1.8	3.468	2.131	1.627	1.592	*	*	S
31	3BD	1.8	2.896	2.063	1.684	1.677	2.9	2.3	F
32	4BD	1.2	1.753	2.118	0.828	0.813	*	*	M
33	5BD	1.0	1.182	2.067	0.572	0.577	*	*	F
34	6BD	1.7	3.182	2.036	1.563	1.571	2.3	2.0	M
35	7BD	1.6	2.896	2.053	1.411	1.423	-	2.5	F
36	8BD	1.6	2.896	2.185	1.325	1.345	*	*	F
37	**9BD**	**1.7**	**3.182**	**1.993**	**1.597**	**1.611**	**2.9**	**2.5**	**F**
38	10BD	1.6	2.896	2.135	1.356	1.366	2.7	1.4	F
39	11BD	1.6	2.896	2.158	1.357	1.292	2.7	2.5	M
40	12BD	1.7	3.182	2.007	1.585	1.576	-	2.3	F
41	13BD	1.7	3.182	2.146	1.483	1.467	-	2.3	M
42	14BD	1.7	3.182	2.146	1.483	1.399	-	3.0	F
43	15BD	1.7	3.182	2.098	1.517	1.521	*	*	F

* Not determined, - No activity

However, microbial fibrinolytic enzymes have also been reported from various other species such as *Pseudomonas* (Imshenetskii *et al*., 1991), *Staphylococcus* (Berdzulishvili *et al*., 1973), *Alteromonas* (Demina *et al*., 1990), *Coryneform* bacteria (Egorov *et al*., 1982), *Penicilium* (Anreeva *et al*., 1972), *Aspergillus* (Ushakova *et al*., 1974; Klocking *et al*., 1975), *Fusarium* (Abdel-Fatah *et al*., 1993), *Trichotecium* (Stepnova *et al*., 1976), *Actinomyces* (Engorov *et al*., 1976), *Streptomyces* (Engorov *et al*., 1984) and *Escherichia coli* (Malke *et al*., 1984). Apart from that, Table 2 summarizes the bacterial growth profile for all 43 isolates. The table reveals that the growth was categorized into three groups based on the time taken for each isolated to reach the OD_{600} of 0.8 after incubation, under sufficient aeration at an agitation speed of 200 rpm, optimum temperature of 37°C and standard nutrients provided for their growth. The fastest (F) group only took about 3-4 hours to reach OD_{600} of 0.8 after the

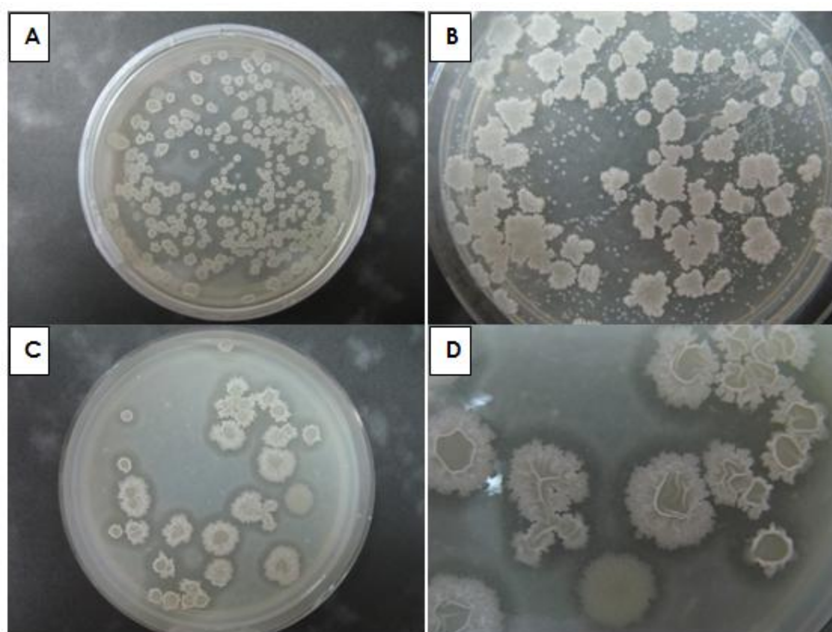

Fig. 1: Mix colonies on skimmed-milk agar with *Belacan* samples at several dilution factors; A: (10^{-1}), B: (10^{-2}) and C: (10^{-3}). Colonies identified with zone of hydrolysis corresponding to protease activity (D).

inoculation. Some bacteria were classified into moderate (M) group which took more than 4 hours until 5 hours to reach the same OD reading. The rest were classified as slow (S) growers which took about 5-7 hours. Even though the provision of sufficient energy is important for the microorganisms to grow and replicate, but the duration for all the strains also varies considerably in length with thecondition of the particular microorganism and the nature of the medium (Shuler and Kargi, 2002).

PROTEIN CONCENTRATION AND ENZYME ACTIVITY OF THE ISOLATES

Total protein assays have been used to analyze hundreds of industrial, agricultural, and biotechnology products. They are also basic for research purposes, especially for determining the specific activity of enzymes. Hence, the protein content for every extracellular enzyme was determined using the Lowry Assay using varying concentrations of BSA (0 - 0.50 mg mL^{-1}) as standard. The values derived were calculated according to BSA protein standard curve plotted, where the equation was $y = 2.0922x + 0.0576$ and $R^2 = 0.9879$. According to the calculated protein content, the concentration of the enzymes measured were between 1.892 to 2.185 mg mL^{-1}as presented in Table 2. This shows that there is no significant variation in protein concentration among the samples obtained.

FIBRINOLYTIC ENZYME ASSAYS

Qualitative fibrinolytic activity in fibrin plate assay

Fibrin plate assay was used to qualitatively investigate the ability of isolates to produce extracellular serine protease as fibrinolytic enzyme. The assay was revealed by incubating the crude enzyme on fibrin plate at 37 °C. After 14 hours incubation, clear zone of hydrolysis around the well (as shown on the fibrin plate in Figure 2), corresponds to the fibrinolysis activity of the extracellular enzyme.

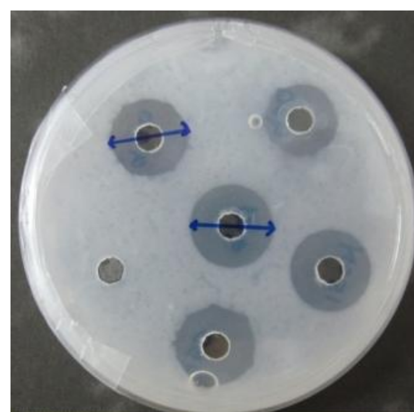

Fig. 2: Zone of hydrolysis on fibrin agar plate assay for extracellular protease from several isolates indicating the activity of fibrinolytic enzyme. Blue arrow represents the diameter measured for the formed fibrinolytic zone of hydrolysis and the well without a zone of hydrolysis represents negative control (LB medium) in this test.

The activity of the enzymes was calculated using the plasmin standard curve as comparison. From the standard curve, the equation was $y = 0.35x\ 0.0389$ and $R^2= 0.9903$. Table 2 summarizes the results for qualitative fibrin plate assay. Qualitatively, strain 2CN strain exhibited the strongest activity with a specific activity of 1.832 U mg^{-1}, followed by 13BN and 11BN with 1.753 U mg specific activity and 1.723 U mg^{-1}, respectively. This suggesting that these fibrinolytic enzyme shows high substrate specificity toward fibrin. Meanwhile, the lowest

activity was obtained from strain 16BN, giving specific activity of only 0.168 U mg^{-1}. This value is almost 10 times lower than the activity of 2CN, 13BN and 11BN.

Quantitative fibrinolytic enzyme assay

In the quantitative fibrinolytic enzyme assay, the activity was measured based on amount of tyrosine released after the hydrolysis of fibrin clots by the extracellular enzyme of the isolates. The results obtained in Table 2 has suggesting that relatively, the specific activity for qualitative and quantitative assays are in agreement with each other. Yet, 2CN demonstrated the highest activity with 1.789U mL^{-1}, followed by 13BN with 1.762 U $mL^{-1}$and 5BN with1.743 U mL^{-1}. On the other hand, the lowest activity was determined from 16BN at only 0.172 U mL^{-1}.

Fibrinolytic Mode of Action

Of 43 isolates, only 23 isolates with relatively exhibited large hydrolysiszone on fibrin plates and higher activity in quantitative enzyme assay were then selected for further investigation on plasminogen-free and plasminogen-rich plates, in order to study the fibrinolytic action mode of each species. This is because there are two types of fibrinolytic enzymes; one is plasmin-like protein such as nattokinase and lumbrokinase, which can directly degrade the fibrins of the blood clots (Peng *et al*., 2005) and another type is tissue-plasminogen activator (t-PA), which responsible to activate zymogen plasminogen into active plasmin which in turn degrades fibrin (Mine *et al*., 2005). Commercial fibrinogen usually contains plasminogen which can be removed by heating at 80 °C (Peng *et al*., 2003) to form plasminogen-free plate. Meanwhile, plasminogen-rich plate consisted of 5 U plasminogen and without heat treatment.

The results summarized in Table 2 has clearly shows that these 23strains possess a distinctive mode of fibrinolytic action. In general, all these 23 strains were able to degrade the fibrins directly. However, only 8 strains (5BN, 13BN, 2CN, 3BD, 6BD, 9BD, 10BD and 11BD) gave positive reaction on both plasminogen-rich plate and plasminogen-free plate indicating two prong action of fibrin degradation. On average, the hydrolysis zone on plasminogen-rich plates were found larger than plasminogen-free plates (Figure 3). It could be hypothesised that fibrinolysis takes place better in the presence of plasminogen. Among all the colonies, strains 13BN shows the strongest fibrinolytic activity in both plasminogen-free and plasminogen-rich plates, securing zones of hydrolysis of 3.0 cm for both plates. Similar mode of action could be observed in strains 5BN and 9BNwhereby the size of the zone of hydrolysis on plasminogen-rich plates for both strains were 2.9 cm and on plasminogen-free plate were 2.4 cm and 2.5 cm. Conversely, 10BN and 14BD only have one fibrinolytic mode of action, although they possess very strong activity with the largest zone of hydrolysis at a diameter of3.0 cm.

Even though the strains in general demonstrated dissimilar mechanism of action, whether they possess only one or both action of mechanisms, the fibrinolytic enzymes are still valuable in reducing unwanted blood clot. But some scientific report construed that t-PA has greater benefit and attracted more considerable attention medically because of its participation in a wide variety of connective tissue matrix alterations (Kruithof *et al*., 1995). Recently, reports have shown that t-PA is upregulated by proinflammatory cytokines and black-pigmented *Bacteriodes* in human pulp cells *in vitro* (Huang *et al*., 2006). These findings imply that t-PA is one of the important proteolysis factors in the pathogenesis of pulpal inflammation (Huang *et al*., 2007). Besides, Tsai (2006), has discovered that t-PA was useful as a therapeutic mechanism as antidepressants. Evidence from animal and human clinical studies suggested that a disturbance in serotonergic activity and/or brain-derived neurotropic factor (BDNF) signaling may be implicated in the pathogenesis of major depressive disorder (MDD). According to Tsai, (2006), MDD is a common disabling psychiatric illness including schizophrenia, bipolar disorder, attention-deficit hyperactivity disorder and Alzheimer's disease. Briefly, the t-PA cascade is implicated in the cleavage of proBDNF to BDNF may act through the t-PA/plasminogen/BDNF pathway to achieve antidepressant effect. This explains the importance of strain 13BN as a dual-function enzyme which not only possesses the fibrinolytic activity but also has t-PA which has many medicinal and therapeutic functions.

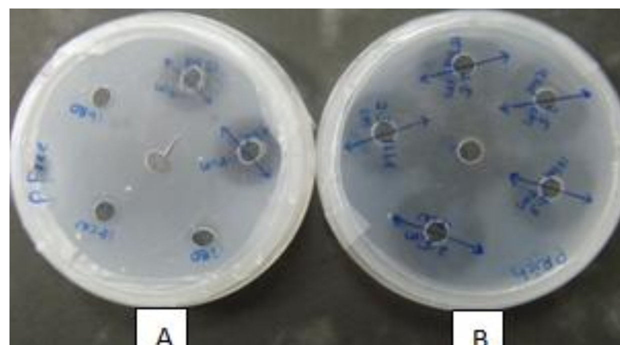

Fig. 3: Zone of hydrolysis on plasminogen-free plate (**A**) and plasminogen-rich plate (**B**).

16S rRNA IDENTIFICATIONOF 13BN, 2CN AND 9BD

Threeenzyme producer, 13BN, 2CN and 9BD strains (representing the best producer from each food sample) were finally chosen as the most promising and potential candidates as an alternative for current chemical anticoagulants. They were categorized as fast grower, gram-positive, rod shaped bacteria (Figure 4) and contained plasminogen activating, t-PA potential. Therefore, further molecular identification of 16S rRNA was accomplished by PCR amplification using a pair of bacterial-specific universal primers in order to determine the species of these bacteria. Then, the presence of 1.6 kb amplicon in Figure 5 verified the presence of amplified 16S rRNA PCR products for the 13BN, 2CN and 9BD strains. The BLAST analysis and alignment with various bacterial sequences in NCBI databases indicates that all three bacterial isolates belonged to the genus *Bacillus*. The top five hits for each 16S rRNA sequence of 13BN, 2CN and 9BD strains are shown in Table 3. The results showed that strain 13BN

Table 3: Top five similarity searches hits using BLAST for r RNA sequences for 13BN, 2CN and 9BD.

Sample	Match	Score	E Value	Identity	Accession
Strain 13BN	*Bacillus cereus* 03BB108, complete genome	2768	0.0	99.9%	CP009641.1
	Bacillus anthracis strain Sterne 16S ribosomal RNA gene, partial sequence	2768	0.0	99.9%	KP942847.1
	Bacillus anthracis strain A1144, complete genome	2768	0.0	99.9%	CP010852.1
	Bacillus cereus strain S2-8, complete genome	2768	0.0	99.9%	CP009605.1
	Bacillus thuringiensis strain 97-27, complete genome	2768	0.0	99.9%	CP010088.1
Strain 2CN	*Bacillus subtilis* strain TO-A JPC, complete genome	2658	0.0	99.9%	CP011882.1
	Bacillus subtilis strain SANN3 16S ribosomal RNA gene, partial sequence	2658	0.0	99.9%	KP975920.1
	Bacillus subtilis strain UD1022, complete genome	2658	0.0	99.9%	CP011534.1
	Bacillus sp, LM 4-2, complete genome	2658	0.0	99.9%	CP01101.1
	Bacillus subtilis HJ5, complete genome	2658	0.0	99.9%	CP007173.1
Strain 9BD	*Bacillus subtilis* strain EBS05 16S ribosomal RNA gene, partial sequence	2680	0.0	99.9%	FJ876834.1
	*Bacillus*sp, E1R-j 16S ribosomal RNA gene, partial sequence	2680	0.0	99.9%	EF502021.1
	Bacillus subtilis strain ZWQ-2 16S ribosomal RNA gene, partial sequence	2678	0.0	99.9%	HQ650841.1
	Bacillus subtilis strain HJ5 16S ribosomal RNA gene, partial sequence	2678	0.0	99.9%	GQ249662.1
	Bacillus subtilis strain UD1022, complete genome	2659	0.0	99.9%	CP011534.1

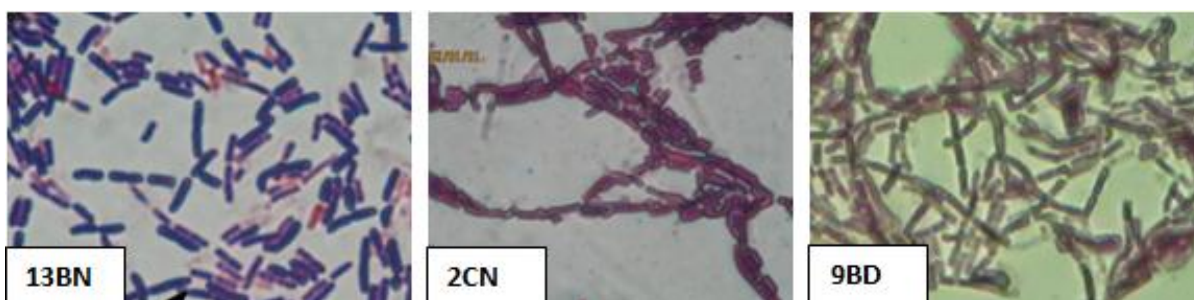

Fig. 4: Gram stained of 13BN, 2CN and 9BD bacteria.

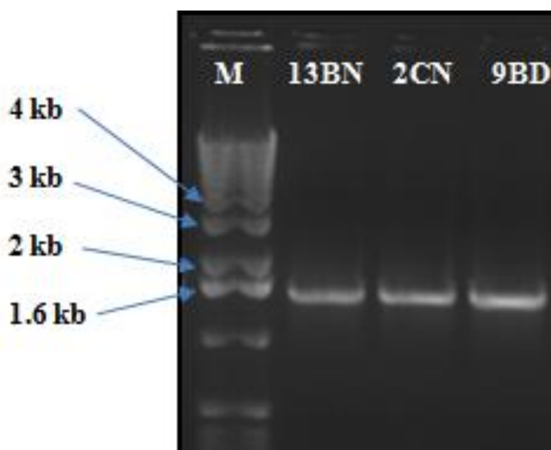

Fig. 5: PCR amplified 16S rRNA gene fragment of ~ 1.6 kb size of DNA samples from 13BN, 2CN and 9BD strains. Lane M is 1 kb DNA Ladder (Promega).

was highly similar to over 50 strains of *B. cereus, B. anthracis* and *B. thurengiensis,* with 99% similarity. Basically, the species of *B. cereus, B. thuringiensis* and *B. anthracis* are genetically very close, as highlighted by Helgason *et al*., (2000), and shown by multilocus enzyme electrophoresis and gene sequence analysis. These members of the *B. cereus* group, demonstrating widely different phenotypes and pathological effects. For example, *B. anthracis* causes the acute fatal disease anthrax and is a potential biological weapon due to its high toxicity. Meanwhile, *B. thuringiensis* produces intracellular protein crystals toxic to a wide number of insect larvae and is the most commonly used biological pesticide worldwide. However, *B. cereus* is a probably ubiquitous soil bacterium and an opportunistic pathogen that is a common cause of food poisoning. Therefore, strain 13BN was designated as *B. cereus* 13BN. On the other hand, both 2CN and 9BD gene sequences have 99% similarity with *B. subtilis* species. Interestingly, they do have 99% resemblance with each (data not shown) other even though they came from different food sources. Therefore, these two strains were designated as *B. subtilis* 2CN and *B. subtilis* 9BD.

PHYLOGENETIC ANALYSIS

For a more precise classification, a phylogenetic tree was constructed for13BN, 2CN and 9BD strains using their 16S rRNA sequences, together with several other well characterized and published *Bacillus* sp., obtained from the GenBank database. The sequences were first aligned using ClustalW programme before a

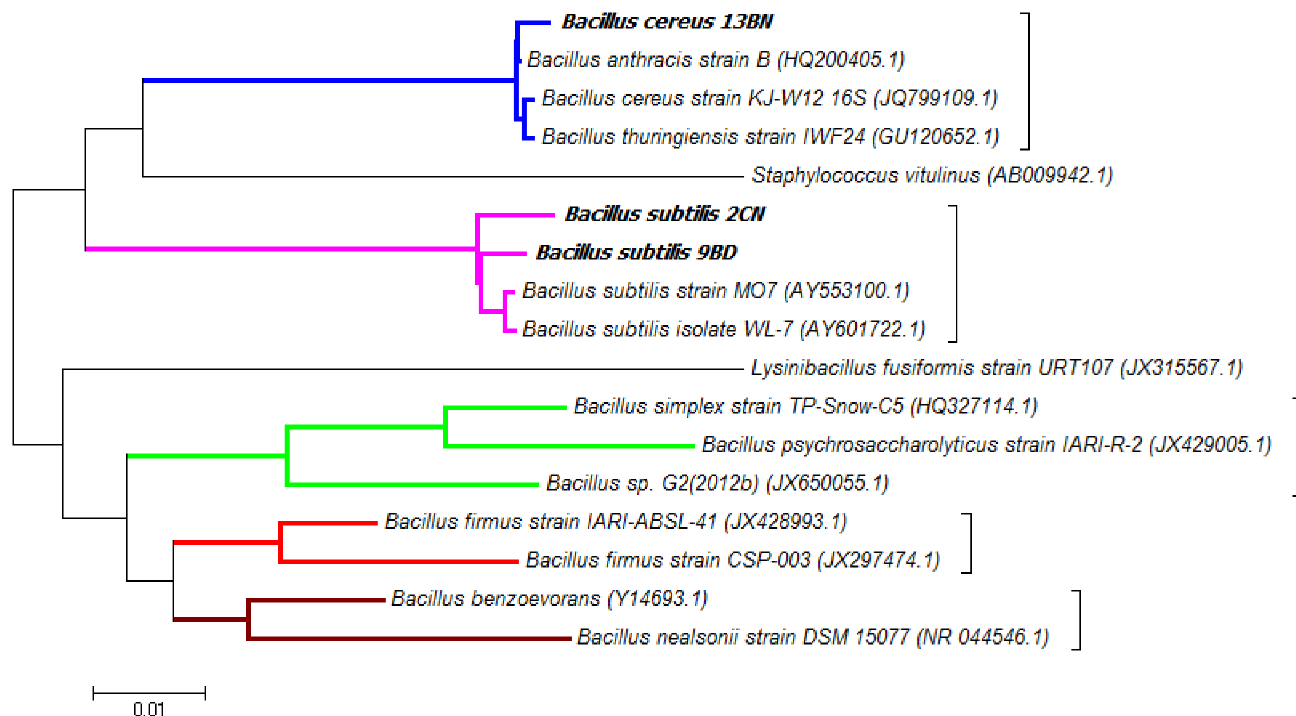

Fig. 6: Phylogenetic tree of the three isolates 13BN, 9BD and 2CN.

neighbor-joining phylogenetic tree was constructed using MEGA5 software. The resulting phylogenetic tree is shown in Figure 6.The tree was generated by neighbor-joining method with the bootstrap values (%) of 1000 replicates. The scale bar represents 0.01 nucleotide substitutions per position. 15 out of 17 strains were divided into 5major subgroups, meanwhile, the rest is remain ungrouped indicating poor DNA-DNA relatedness with each other. As predicted earlier, the constructed phylogenetic tree confirmed that strains 13BN belongs to *B. cereus* species and strains 2CN and 9BD belong to the *B. subtilis* species.

CONCLUSIONS

It can be concluded that Malaysian traditional fermented seafood products are indeed a rich source of microbial biodiversity holding within them immense novelty and are potential sources of new isolates in the production of life saving natural thrombolytic agents. Three best bacteria strains initially designated 13BN, 2CN and 9BDwere successfully isolated from *Belacan, Cencaluk* and *Budu*. They have relatively high activity of fibrinolytic enzyme, as well as the unique possession of a two prong mode of fibrinolysis. They were categorized as fast grower, gram-positive and rod shaped bacteria. From the homology search and phylogenetic analysis, 13BN was designated *B. cereus* 13BN. Meanwhile, strains 2CN and 9BD were designated as *B. subtilis* 2CN and *B. subtilis* 9BD, respectively. Therefore, these novel *B. cereus* 13BN, *B. subtilis* 2CN and *B. subtilis* 9BD can be regarded as having a great potential as a fibrinolytic enzyme producer since the enzyme produced were also able to act as tPA, which is more practical and effective for clinical use. This dual performance provides better fibrinolytic action *in vivo* with the future objective of developing cheaper alternative natural agent directed towards thrombolytic therapy besides the commercially available nattokinase and streptokinase.

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