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ISOLATION OF THERMOTOLERANT BACTERIA PRODUCING FIBRINOLYTIC ENZYME

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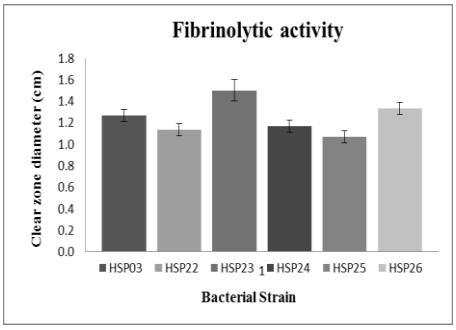
Graphical abstract



Abstract

Fibrinolytic enzymes were widely used in the treatment of cardiovascular diseases. However, the efficiency of the commercial enzymes are still lack of perfection because there are many side effects as well as not tolerant to downstream processing such as heat sensitive during spray drying process. Therefore, this study presents newly isolated thermophiles bacteria producing fibrinolytic enzyme. Sample was collected from Hot Spring Selayang at Selayang Selangor. Spread plate agar containing skim milk powder growth at pH 7, 53℃ for 24 hours was utilized to isolate thermotolerant bacteria producing protease. Further isolation on bacteria producing fibrinolytic enzyme was carried out using fibrin plate. 16S rDNA gene sequence analysis was used to identify the genotype of the isolates. 27 colonies of thermotolerant bacteria were isolated, however, only 19 of them showing proteolytic activity. All of the 19 isolates are motile and cocci in shapes, with 4 types of arrangement, which are single, diplo (pair), strepto (chain) and staphylo (cluster). HSP04 and HSP11 are gram positive bacteria and others are gram negative. From 19 isolates only 6 were chosen for further analysis. HSP23 showed the highest fibrinolytic activity compared with others. HSP23 was identified as *Bacillus licheniformis* with 98 % similarity to *Bacillus licheniformis* DCM 13 and *Bacillus licheniformis* strain ATCC 14580.

Keywords: Fibrinolytic Enzyme, Thermotolerant Bacteria, *Bacillus licheniformis*

1.0 INTRODUCTION

Cardiovascular diseases are globally the main cause of death. According to a report published by the World Health Organization (WHO) in 2011, an estimated 17.3 million people died from the diseases in 2008, representing 30% of all global deaths [1]. The number of people who died from cardiovascular diseases are anticipated to increase to 23.3 million people by 2030. Intravascular thrombosis is one of the major causes of cardiovascular diseases. Clots formed from insoluble fibrin restrict the smooth flow of blood in blood vessels, leading to thrombosis and heart attacks. Insoluble fibrin is the major protein component of blood clots, which are formed from fibrinogen by thrombin [2].

Fibrinolytic enzymes are mainly proteases from two families for instance serine or metalloproteases [3]. Fibrinolytic protease has an ability to degrade fibrin.

The enzymes are exist in plants, animal and microorganisms. Microorganisms are vital resources for thrombolytic agents. Streptokinase from *Streptococcus hemolyticus* and Staphylokinase from *Staphylococcus aureus* were earlier proved to be effective in thrombolytic therapy [4]. Over the years, more fibrinolytic enzymes from various microbes have been discovered in succession such as from soil sample (*Bacillus licheniformis, Bacillus cerus, Staphylococcus aureus, Bacillus sp. Strain AS-S201-I*) [5,6], marine bacterium (*Bacillus subtilis* HQS-3, *Bacillus subtilis* ICTF-1) [7,8], fermented food (*Bacillus subtilis* HK176, *Bacillus pumilus* 2, *Bacillus Sp.* STRAIN B1)[9,10,11] and fish products (*Pseudoalteromonas sp.* IND11, *Bacillus coagulants* TB1, *Virgibacillus halodenitrificans* SK1-3-7[12,13,14].

When producing fibinolytic protease from microorganism, most important factor is their high biomass yield that more enzyme will be harvested in industrial applications. Therefore, it is essential to provide optimal growth conditions in increasing enzyme production. The culture conditions that promote fibrinolytic protease production were found to be significantly different from the culture conditions promoting cell growth [15]. Since different microbes possess diverse physiological characteristics, it is necessary to optimize nutrient components and environmental conditions for cell growth and fibrinolytic enzyme production [16,17]. Besides, the cost for enzyme production and downstream processes are the major obstacle in the successful application of protease in the industry [18]. Moreover, downstream processing of the enzyme production may involve high temperature treatment especially during spray drying which require thermophilic bacteria that produce thermotolerant fibrinolytic enzyme.

Thermophilic bacteria from hot springs contains proteins chaperonins. The chaperonins are thermostable and resist denaturation and proteolysis [19]. This special protein helps other protein to fold correctly after their denaturation and restore their functions[20]. Therefore, this study is presenting newly isolated thermophiles bacteria producing fibrinolytic enzyme as suitable candidate in producing therapeutic enzyme for cardiovascular diseases.

2.0 EXPERIMENTAL

2.1 Isolation of Bacteria

Samples of soil were collected from a Hot Spring Selayang at Selayang Selangor using sterile spatula and transferred into sterile 50 ml tube. Temperature and pH values immediately were recorded. All samples were transported without temperature control and analysed within 24 hours. An amount of 1 g of the soil samples were dissolved in 10 ml of steriled water to make soil suspensions. The media used in this research was nutrient agar medium (Oxoid, England). An amount of 100 µl of the suspension were inoculated onto triplicate nutrient agar by spreading and were incubated at 53℃ for 24 hours. The colonies were stored as stock culture in Nutrient broth containing 30% glycerol at -80℃.

For futher studies, 100 µl were taken from glycerol stock and cultured in 5ml Nutrient broth medium (Oxoid, England). Next, the culture were incubated at 53℃ for 24 hours and centrifuged at 10,000 xg for 45 min at 4℃. The supernatant was used for caseinolytic and fibrinolytic assay.

2.2 Identification of Bacterial Isolates

All isolated strains was grown on nutrient agar plates at 53℃ for 24 hours. Cell morphology and motility was examined under light microscope. Gram staining was performed using Bergey's Manual of Systematic Bacteriology [21].

2.3 Screening for Caseinolytic Activities

Skimmed milk agar medium containing 2% (w/v) skim milk (Oxoid, England) and 28g nutrient agar (Oxoid, England) in 1000ml distilled water were used for enzymatic screening. The respective isolates were inoculated on the plates as a single line and incubated at 53℃ for 24hrs. Comparison of proteolytic activity among the isolates was based on the zone of casein hydrolysis and individual colony diameter. The zone of hydrolysis was measured to the nearest cm. Further enzymatic assay using casein as a substrate used as confirmation for caseinolytic activities. Caseinolytic activities carried out according to universal protease activity assay from Sigma-Aldrich protocol. Caseinolytic activity was calculated by comparing the reading with the tyrosine standard.

2.4 Screening for Fibrinolytic Activities

Fibrin plate assay was carried out according to the Astrup and Mullertz [22] method with modifications as described by Kim *et al*. [23]. The diameter of the halo of clearing zone was measured for fibrinolytic activity. Spectrophotometric assay of the samples were carried out according to Singh *et al*. [24]. Fibrinolytic activity was calculated by comparing with plasmin standard. Fibrinolytic activity equivalent to one plasmin unit (PU) was defined as the A_{280} equivalent of perchloric acid soluble products released from fibrinogen in a reaction volume of 100µl by NIH unit of plasmin in 30 min at pH 7.4 at 37℃. One A_{280} was equivalent to 167 plasmin units (PU) in the above defined conditions.

2.5 16S rDNA Gene Analysis and Sequencing

For 16S rDNA sequencing, total genomic DNA were isolated from culture broth of selected isolates. using Genomic DNA kit (Qiagen, Germany). The partial sequence of the 16S rDNA gene were amplified using a polymerase chain reaction (PCR) and bacterial

universal primers specific to 16S rDNA gene, using forward primer 27f (5'- AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR conditions used were; denaturation at 94℃ for 30 sec, followed by 30 cycles of denaturation at 94℃ for 20 sec, annealing at 53℃ for 30 sec, extension at 68℃ for 1 min and final extension at 68℃ for 5 min. Amplified PCR products were analyzed on an agarose gel and PCR product were sequenced.

The sequence were analyzed at National Centre for Biotechnology Information (NCBI) server (http://ww.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST) and download corresponding sequences and analyse sequence homology.

2.6 Growth Profile

Bacterial strains were cultured in studier medium containing (g/L): Tryptone, 10; Yeast Extract, 5; Na_2HPO_4, 4.45; KH_2PO_4, 3.4; NH_4Cl, 2.67; Na_2SO_4 ,0.71; Glycerol, 5; Glucose, 0.5; a-lactose, 2; $MgSO_4$, 0.49; and Trace element solution (1000X metals) 0.2ml) [25] at 53℃ for 48 Hours. OD_{600} were measured every 4h of cultivation and graph was plotted. Besides, specific growth rate, μ and doubling time were calculated depending on growth profile.

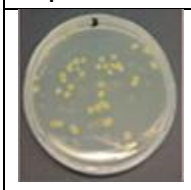
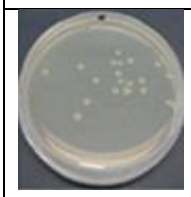
3.0 RESULT AND DISCUSSION

The primary stage in the development of an industrial fermentation process is to isolate strain capable of producing the target product in commercial yields. Screening of a large number of microorganisms is an important step in selecting a highly potent microbial culture for multipurpose utilization. Sample was collected from Hot Spring Selayang, Selangor. The total of 27 colonies of thermotolerant bacteria were isolated on pH7 nutrient agar plate that incubated at 53℃ for 24 hours.

Results showed that, 19 thermotolerant bacteria showing proteolytic activity when cultured on skim milk agar plate. Then, the thermotolerant bacteria secreted protease were classified into 4 groups based on colonial and bacterial morphology. All of them are motile and cocci in shapes, with 4 types of arrangement which are single, diplo (pair), strepto (chain) and staphylo (cluster) (Table 1). HSP04 and HSP11 are gram positive bacteria and others are gram negative.

Normally, fibrinolytic protease organisms are isolated by surface plating on a fibrin agar medium and subsequent screening for the desired characteristics. Proteolytic bacteria were not automatically degrading fibrin. However, only a few of proteolytic bacteria have capability to degrade fibrin [12]. HSP03, HSP22, HSP23 HSP24, HSP25 and HSP26 were showing fibrinolytic activity which ranged as 1.1-1.5 cm when tested by fibrin plate assay . HSP23 showed the highest capability to degrade fibrin with 1.5 cm clear zone diameter (Figure 1).

Table 1 Morphology of isolated thermotolerant bacteria on skimmed milk agar

Spread Plate	Morphology	Strain
	Yellow Circular Raised Entire	HSP03, HSP04, HSP06, HSP12
	Pale Yellow Circular Raised Entire	HSP02, HSP09, HSP10, HSP11, HSP15, HSP18, HSP19
	Pale Yellow Irregular Convex Serrate	HSP14, HSP22, HSP23, HSP24, HSP25, HSP26, HSP27
	Pale Yellow Irregular Convex Filamentous	HSP20

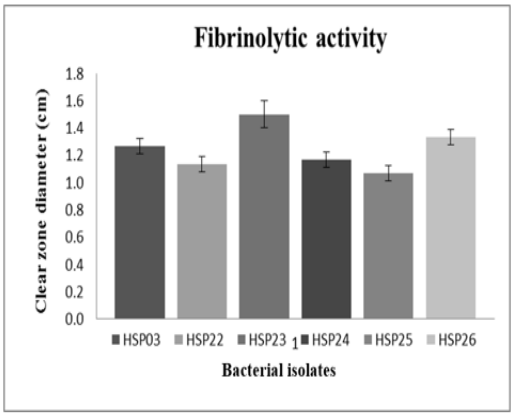


Figure 1 Fibrinolytic activity based on a fibrin plate of various bacteria from soil hot spring

In addition, Chitte and Dey were reported that *Streptomyces megaspores* SD5, isolated from the hot spring water, can produce a strong thermo stable fibrinolytic enzyme [26]. It is interesting to note, other that high in proteolytic activity, the HSP23 strain also shown the highest fibrinolytic activity at the rate of 33.543 U/mg protein (Table 2).

Table 2 Specific fibrinolytic activity from different strains producing fibrinolytic enzyme

Strain	Specific Fibrinolytic Activity (Unit/mgprotein)	
	Casein Assay	Fibrinolytic Assay
HSP03	1.253	27.940
HSP22	1.083	23.364
HSP23	1.503	33.543
HSP24	1.139	30.330
HSP25	1.071	9.152
HSP26	1.307	30.238

Molecular identification were carried out for HSP03, HSP22, HSP23 HSP24, HSP25 and HSP26 to identify the specific strain . Six isolates were taken into 16S rDNA gene sequence analyses. The comparative sequence analyses revealed that similarity values ranged as 93-98%. HSP23 about 98% homology of sequences shows with *Bacillus licheniformis* DCM 13 and *Bacillus licheniformis* strain ATCC 14580. NCBI BLAST results (tree list) are showed (Figure 2) on neighbor joining method. Therefore, the HSP23 strain identified as *B. licheniformis* HSP23.

B. licheniformis is a saprophytic bacterium common in soil and other natural environments. The cells are capable of degrading several substrates and growing on a large diversity of nutrient sources due to its capacity of producing and secreting plenty of hydrolytic enzymes including protease, a-amylase, pectinase and cellulase [27]. Among others, this ability makes *B. licheniformis* an interesting organism for industrial purposes [28,29].

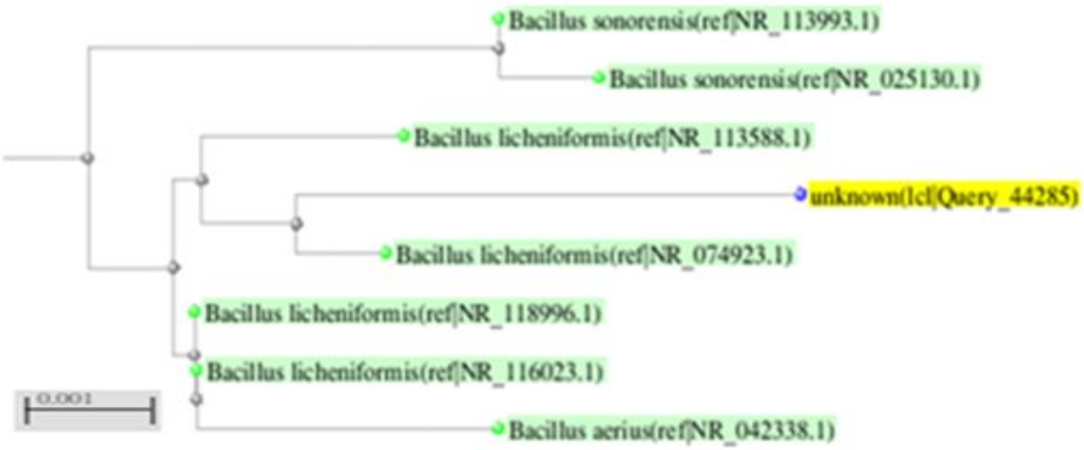


Figure 2 Phylogenetic tree constructed based on 16S rDNA gene sequence analysis

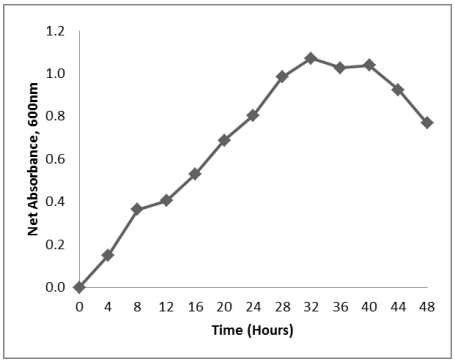


Figure 3 Growth Profile of *B. licheniformis* HSP23 in Studier Autoinduction Medium fermentation

The growth curve was obtained and showed perfect sigmoid with early stationary. From the results it can be seen that 0-4 h was lag phase, 4-32 h was log phase, 32-40 h was stationary phase. Growth was starting turn to death stage after 40h. Therefore, the optimal time to collect bacterial cell for further study is 32-40 h (Figure 3).

4.0 CONCLUSION

The search for promising strains of fibrinolytic proteases producers is a continuous process. The isolate which shows highest fibrinolytic proteases activity were selected and identified as *Bacillus licheniformis* HSP23. Thus, with the availability of thermostable bacteria producing fibrinolytic enzymes gave an advantage to enzyme due to high temperature during downstream processing and suitable candidate in producing therapeutic enzyme for cardiovascular diseases.

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