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ORIGINAL ARTICLE

Isolation of Bacteria from Indigenous Fermented food, studies on Fibrinolytic enzyme production and its Molecular characterization

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ABSTRACT
An Indian traditional fermented food, Hawaijar, was subjected to microbiological studies for the isolation of bacteria endowed with the potential of producing a fibrinolytic enzyme. A total of 12 bacterial isolates belonging to three different species viz., Bacillus subtilis, Bacillus cereus, and Bacillus licheniformis were identified by biochemical studies. Screening of isolates for proteolytic properties on Skim milk agar designated Bacillus subtilis HT-4 as it exhibited a comparatively better activity. Studies on submerged fermentation of potato-soybean medium using this potential isolate indicated the carbon source of fructose, pH of 8, temperature of 50^{o}C and incubation time of 72h as optimal parameters for enhanced production of biomass and enzyme. The crude fibrinolytic enzyme was subjected to sequential purification procedures such as ammonium sulphate precipitation, dialysis, gel filtration chromatography and ion exchange chromatography. Separation on SDS-PAGE deciphered the molecular weight of the enzyme in the range between 29 and 66 KDa. Sequencing of 16s rRNA of the potential isolate Bacillus subtilis HT-4 and phylogenetic studies using BLAST analysis revealed its close similarity with Bacillus subtilis.
Keywords*: Thrombosis, Fibrinolytic enzyme, Bacillus subtilis, Submerged fermentation, Molecular characterization*

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INTRODUCTION

Coagulation of blood resulting in clot (also call a thrombus or thrombosis) is one of the natural defenses against bleeding. Every time the coagulation is initiated, the anticoagulants are also activated to prevent excess clot formation which catalyze the clot breakdown process. In some cases, the blot clot formed in intravascular locations may remain in circulation. As a natural thrombo-resistance mechanism, a complex formed of Protein C, protein S and thrombomodulin causes slowing down or inhibition of the clot formation upon activation by thrombin. Thus, a delicate balancing occurs between bleeding and clotting [1].

Some of the common risk factors which cause intravascular blood clot include, immobility due to prolonged hospitalization, major surgery, catheterization, increased estrogens and other medical conditions such as heart failure, varicose veins, inflammatory disorders and nephrotic syndrome. It may also occur due to inherited disorder, obesity, cigarette smoking and so on [2]. If the intravascular blot clots are not lysed by natural mechanisms, it can lead to complications and even life threatening conditions. In order to circumvent such conditions, infusion of thrombolytic or fibrinolytic drugs is advised.

Thrombolytic agents are used to treat a wide variety of thromboembolic disorders and, in particular, the acute myocardial infarction. These drugs act by triggering the conversion of inactive precursor, plasminogen, which in turn forms a cleaved product called plasmin. Plasmin is a proteolytic enzyme capable of breaking the cross-links between the fibrin molecules, which provide the structural integrity of

blood clots. Conventionally, fibrin specific synthetic drugs, called tissue plasminogen activators (e.g., Alteplase, Reteplase, Tenecteplace), natural drugs of human origin (Urokinase), earchworms (lumbrokinase) and snake venom (e.g., serine proteases, metalloproteinases, disintegrins) are used for fibrinolytic treatment. Their activity resembles that of plasmin, which can degrade fibrin and inhibit fibrin clot formation [3-5]. Owing to their short plasma half-life, high production cost and undesirable contraindications (such as internal bleeding, blood vessel and kidney damage) they are less preferred by the medical practitioners.

Besides the above natural sources, certain bacteria such as group C, ß-hemolytic *Streptococci* and *Staphylococcus aureus* have been employed in the production of fibrinolytic enzymes viz., streptokinase (SK) and staphylokinase (SAK) respectively [6]. Similar to the other natural drugs, these products have been encountered with poor fibrin specificity, antibody-mediated inhibition and bleeding complications [7].

In order to overcome the drawbacks associated with the conventional drugs, there is a growing demand in recent years to explore the enzymes possessing specific fibrinolytic and wide therapeutic properties from alternative sources. Research studies have been attempted to isolate fibrinolytic enzymes from Actinomycetes [8], Algae such as *Coidium intricatum* [9], fungi such as *Aspergillus ochraceus* [10], *Tricholoma saponaceum* [11], *Rhizopus sp.* [12], *Bionectria sp.* [13]. Certain bacterial strains such as *Pseudomanas sp.* TKU015 [14], *Paenibacillus polymyxa* EJS-3 [15] and so on were investigated for the isolation of fibrinolytic enzymes. However, the range of efficacy and the characterization of enzymes isolated in these studies have not been completely documented. Besides, these microbes were isolated from natural sources such as soil and endophytic environments.

The fermented foods are considered as rich source of nutrients and have the tradition of being consumed by human since ancient days. These foods constitute an essential part of the regular diet worldwide as they offer variety of health benefits. The probiotic microbes present in these foods cause biochemical changes with the resultant nutrient chemicals and enzymes which promote natural defense mechanisms and alleviate many health disorders [9]. For the purpose of isolating fibrinolytic enzyme producing microbes research studies have explored many fermented foods such as Japanese 'Shiokara' [4], Korean 'Chungkook-Jang' [16], Saudi Arabian 'Khamir [17], Indonesian 'Tempeh' [18] and so on.

Owing to the changes in human life style, unbalanced diet, irregular sleep pattern and poor physical activities, there is an alarming raise of life threatening cardiovascular diseases worldwide. Thrombosis is a frequently reported symptom of these diseases including hypertension, coronary heart disease, atherosclerosis and acute myocardial infarction. Since the indigenous fermented foods offer health promoting essential nutrients owing their microflora, exploring them for the isolation of fibrinolytic enzymes would help developing an efficient cost-effective measure to meet the high demanding therapy for intravascular thrombosis. Therefore, the present study was conducted to isolate a potential bacterium from an Indian fermented food Hawaijar which could synthesize a promising thrombolytic enzyme, for effective therapeutic applications.

MATERIAL AND METHODS

Collection of fermented food sample

Samples of the fermented food 'Hawaijar' were obtained from the departmental stores located in the market place of the Manipur city, India (Fig. 1). A total of 10 samples were collected using sterile polythene bags and transferred to laboratory under aseptic conditions. The samples were systematically processed for the isolation of thrombolytic enzyme producing bacteria.

Isolation and identification of bacteria

One gram of dried food sample was dissolved in hot water and mixed well to make a uniform suspension. One ml of this sample was mixed with 99mL of sterile distilled water to obtain 10^{-2} dilution. Further to serial dilution up to 10^{-6} dilution using sterile distilled water, 0.1 ml of aliquot from 10^{-3} to 10^{-6} dilutions were spread plated on sterile Nutrient Agar (NA) Medium. The plates were incubated at 37°C for 24-48 hours and observed for appearance of colonies.

Colonies grown on the NA plates were initially subjected to standard bacterial procedures including macroscopic, microscopic and biochemical tests for the identification of bacterial isolates adopting the Bergey's manual of systematic bacteriology (IX Edition).

Screening of isolates for proteolytic activity

The bacterial isolates were screened to detect the proteolytic activity on Skim Milk Agar (SMA) medium. Each bacterial isolate was spotted on the medium and incubated at 37°C for 24 hours to observe the proteolysis in terms of zone of clearance around the colonial growth. Potential isolate was selected based on its ability of causing maximum proteolytic activity.

Production of Fibrinolytic enzyme and optimization of fermentation conditions

Selected potential isolate was subjected to submerged fermentation of Potato-soybean medium prepared with the ingredients of potato, soya bean powder, NH_4NO_3, $Cacl_2$, $MgSO_4$, K_2HPO_4 and KH_2PO_4 for the production of fibrinolytic enzyme. The shake flask fermentation method typically involved the inoculation of one loopful of culture into 50mL of basal medium in 250mL conical flask and maintaining it on rotary shaker at 150 rpm for 24-48 hours at 37°C. After 48 hours of incubation, the medium was centrifuged at 6000 rpm for 30 min and the supernatant was taken for further processing.

In order to optimize the fermentation parameters, the fermentation was carried out in multiple flasks each maintained with different carbon sources (glucose, fructose, sucrose and mannitol), pH (5-8), temperatures (37°C, 40°C and 50°C) and incubation times (24-48h) while maintaining other standard ingredients of the medium. Best parameters of the fermentation were determined based on the maximum yield of the biomass and enzyme.

Purification of and characterization of Enzyme

For the purpose of characterizing the enzyme produced in the present study, the crude enzyme contained in the supernatant of the fermentation spent media was separated by initial centrifugation. The supernatant was subjected to a two-step processing for the partial purification of fibrinolytic enzyme. The first step, ammonium sulphate precipitation, was carried out by the method prescribed by Jeong *et al*. [19]. The second step involved the dialysis of the pellet obtained by the precipitation technique. Briefly, the dialysis bag was filled with the sample, tied and then suspended in a beaker containing 500mL phosphate buffered saline (pH 7.4) overnight at 4°C. Then the content of the dialysis bag (dialysate) was transferred into a leak proof container and stored at 4°C. Protein concentration of the dialysate was determined by the method suggested by Wu *et al*. [20] using bovine serum albumin as standard.

The partially purified fibrinolytic enzyme thus obtained from dialysis was then subjected to ion-exchange and gel filtration chromatography for further purification. For the purpose of determining the molecular weight of the purified protein (enzyme), it was subjected to Poly acrylamide gel electrophoresis (SDS-PAGE) along with standard marker proteins.

Assay of Enzyme activity

The fibrinolytic activity of the enzyme was determined by the method of *in vitro* degradation of artificial blood clot [21]. An artificial blood clot prepared by spontaneous coagulation of fresh blood (2mL) in a glass test tube was rinsed thoroughly with phosphate buffer solution (PBS). Later, it was flooded with 2mL of purified enzyme solution (0.5mg/mL of PBS) and left at room temperature for 1 hour. In the control tube, normal saline was used without the fibrinolytic enzyme. The positive result was confirmed by the complete disappearance of blood clot.

Molecular characterization of the potential bacterial isolate

The fibrinolytic enzyme producing potential bacterial isolate was investigated by genomic studies for determining its molecular characteristics and phylogenetic position among the bacteria of similar group. Extraction of DNA of the bacterial isolate was done by the standard procedure using Phenol: Chloroform mix. The DNA thus obtained was subjected to a 35 cycles PCR for the amplification of the target 16s rRNA. The 16s rRNA obtained was digested with EcoRI and the fragments were identified by native agarose gel electrophoresis.

Fluorescence tagged chain Terminator sequencing [8], a modified method of Sanger Dideoxy sequencing technique, was carried out for 16s rRNA in an automated DNA sequencer. This method utilized fluorescent dye labelled nucleotides which emitted lights as they pass the detector. A tracing electrogram of specific peaks corresponding to light emissions was generated by the computer and the data was decoded into nucleotide sequences. Molecular identification of the potential bacterial isolate was performed by submitting the 16s rRNA nucleotide sequences in GenBank database of NCBI and carrying out the analysis using Basic Local Alignment Search Tool (BLAST). Further to the comparative sequence match analysis and phylogenetic tree construction, the proximity of the isolate with the existing database of bacterial strains was determined.

RESULTS AND DISCUSSION

Selection of fermented food

According to World Health Organization (WHO) the death due to heart diseases accounts to 72 lakhs per year [5]. The Centers for Disease Control (CDC) documented that the cardiac arrest is a major cause for mortality in both developing and developed countries. The intra vascular coagulation or thrombus formation is a risk factor for the development of cardiac arrest. Certain microorganisms have the ability of synthesizing thrombolytic enzymes which have been explored for advanced therapeutic applications. But

the *in vivo* efficacy of these microbial enzymes is altered when injected into animal body. Therefore, there is always a search for an efficacious enzyme of microbial origin to treat this dreadful complication.
For the purpose of isolating bacteria capable of synthesizing thrombolytic enzyme, a traditional fermented food of the people living in the northeastern part of India was used as a source. This food, namely Hawaijar, as has been reported to be rich in protein [22], with the anticipation of occurrence of proteolytic bacteria, it was processed for isolation of thrombolytic enzyme producing bacteria. Similar studies on the isolation of proteolytic bacteria conducted elsewhere had employed fermented foods of different parts of the world. Some of these foods include Natto of Japan [23], Douchi of China [24], Temph of Indonesia [25], Chungkook-Jang of Korea [16], Kishk of Egypt [26].

Table 1. Characterization and identification of bacteria of fermented food

Identification method	**Tests performed**	**Characteristics recorded**		
Macroscopic tests	Growth on Nutrient Agar	Small, white or creamy, irregular, flat, dry colonies	Large irregular and flat colonies with undulate margin	Small, white to brown, irregular, opaque colonies
	Growth on Trypticase Soy Agar	Dry, flat & irregular with lobate margin	--	--
	Growth on Blood agar	Gamma hemolysis	Beta hemolysis	No hemolysis
Macroscopic tests	Bacillus medium	No growth	Growth	Growth
	Gram staining	Gram Positive	Gram Positive	Gram Positive
	Spore Staining	Endospore	Endospore	Endospore
	Motility	Motile	Motile	Motile
Biochemical tests	Catalase	+	+	+
	Oxidase	+	+	+
	Starch Hydrolysis	+	+	+
	Gelatin Hydrolysis	+	-	+
	Glucose	+	+	-
	Mannitol	+	-	+
Bacteria identified		***Bacillus subtilis***	***Bacillus cereus***	***Bacillus licheniformis***
No. of isolates		7	4	1

Table 2. Results of testing of parameters for optimization of fermentation conditions

Parameter of Fermentation	**Variables tested**	**Biomass production (Absorbance @ 560nm)**
Carbon source	Glucose	3.044
	Sucrose	2.254
	Fructose	3.217
	Mannitol	3.000
pH of the medium	5	2.025
	6	2.249
	7	2.406
	8	2.249
Temperature	RT	2.473
	37°C	2.269
	40°C	2.733
	50°C	3.000
Incubation time	24h	2.136
	48h	2.139
	72h	3.000
	96h	2.897

Isolation and identification of bacteria

The serial dilution of the fermented food and plating on isolation medium yielded the growth of various colonies upon incubation. These colonies were subjected to standard bacteriological identification procedures such as macroscopic observation on basic, specific and differential media, microscopic studies on motility and staining characteristics and biochemical tests for physiological characteristics. Kim *et al*.

[16] have adopted similar procedures for the isolation of bacteria from a Korean fermented food Chungkook-Jang.

A total of 12 bacterial isolates showing the characteristic morphology of *Bacillus* were obtained. The results of identification tests determining the characteristics of these isolates are shown in table 1 and fig. 1. Results of these tests indicated the occurrence of three different species of B*acillus* namely, *Bacillus subtilis, Bacillus cereus,* and *Bacillus licheniformis.* The orders of prevalence of these three different species of bacteria are 50%, 30%, and 20% respectively. This finding is in concordance with the study of Wang *et al.* [14] on the isolation of *Bacillus subtilis, Bacillus cereus,* and *Bacillus licheniformis* from the of Chinese fermented food Douchi. Other foods reported to contain *Bacillus sp*. were Temph [25], Chungkook-Jang [16], Jeot-gal [21] and Soibum [27].

Selection of bacteria with thrombolytic potential

Since the main objective of this research was to isolate the bacteria capable of producing fibrinolytic enzyme, the studies on selection of potential bacterial isolate was attempted. The fundamental characteristic essential for enzyme production in terms of proteolytic activity was tested on SMA medium. Upon screening of all the 12 isolates of *Bacillus spp.*, one isolate, namely *Bacillus subtilis* HT-4, exhibited maximum proteolytic activity. Since it produced a larger zone of clearance of medium than that of its counterparts, it was selected as a potential isolate for further studies. Many earlier researchers had investigated the application of SMA for the screening of bacterial isolates for detection of proteolytic activity [28-30].

Enzyme production by fermentation and optimization of culture parameters

The present study further concentrated on the production of fibrinolytic enzyme using the method of submerged fermentation augmented by the potential isolate *Bacillus subtilis* HT-4. The submerged fermentation is recommended as the most suitable method for the production of secondary metabolites and enzymes by the bacteria when involving inorganic substrates. In consonance with our study, Wang *et al.* [14], Kim *et al.* [16], Wang *et al.* [24] and Agrebi *et al.* [31] have followed the submerged fermentation in their studies on fibrinolytic enzyme production.

The earlier studies on the production of fibrinolytic enzyme from *Bacillus subtilis* conducted by utilized Dubus salt medium [32], NYSM medium [33] and so on for the fermentation process. In contrast, the present study employed Potato-Soybean medium for the enzyme production by *Bacillus subtilis* HT-4. Providing a rich source of carbohydrate in the basal medium is critical for facilitating the growth of bacterial inoculum and consequent biochemical changes [24]. The purpose of using potato based medium in our study was to use a cost effective medium that could enhance higher biomass and enzyme production. Care was taken to ensure the nutrient composition and functioning of the present fermentation medium is on par with other media employed by earlier researchers for efficacious production of fibrinolytic enzyme.

The characterization and optimization of fermentation conditions for achieving the maximum production of fibrinolytic enzyme was also done in the present study. In order to standardize the culture conditions that favor the enzyme production by *Bacillus subtilis* HT-4, parameters such as carbon sources, pH of the medium, temperature of the fermentation atmosphere and length of incubation period. The efficacious fermentation parameters are determined based on their impact on enhancing the biomass as well the production of enzyme [2, 11]. The enzyme production at varying conditions of the fermentation was assayed by periodical testing of culture filtrate for the fibrinolytic activity on artificial blood clot. Table 2 presents the results of analysis of different culture parameters and their impact on the fermentation in terms of biomass production (Absorbance values). The fermentation parameters thus standardized, that favoring the maximal enzyme production, were the carbon source of fructose, pH of 8, temperature of 50°C and an incubation time of 72h.

In order to check the production of thrombolytic enzyme and to test its degrading potential on blood clot, the artificial blood clot lysis method as suggested by Hwang *et al*. [21] was adopted in the present study (Fig. 3). The supernatant containing crude enzyme was tested on the artificial blood clot made in test tubes and the lysis of blood clot within the time of one hour was considered positive for the test. The finding of the present study is in agreement with the results of Wang *et al.* [34] and Varol *et al.* [1].

Purification and characterization of Fibrinolytic enzyme

The partial purification of fibrinolytic enzyme from the spent medium by ammonium sulphate precipitation and dialysis techniques indicated that the dialysate held an average concentration of 120µg/mL of protein. Further ultra-purification using gel filtration chromatography and ion exchange chromatography confirmed the presence of enzyme at the retention time of 3 hours. In every step of the purification process, the concentration of the enzyme was determined by Lowry's method of protein estimation. Contemporary studies carried out by Varol *et al.* [1], Hu *et al*. [32] and Agrebi *et al*. [35] have

followed similar pattern of purification and estimation of concentration of enzyme obtained from the spent media of the fermentation.

Further characterization with reference to the determination of molecular weight of the enzyme produced by *Bacillus subtilis* HT-4 was performed using SDS-PAGE technique. Subsequent to the electrophoresis of the samples of the purified enzyme, a total of three bands of protein were obtained on the acrylamide gel (Fig. 4). The molecular weight of these bands were in the range between 29 kDa and 66 kDa with reference to that of the standard marker. The fibrinolytic enzyme produced in our study was assumed to be one among these separated protein bands. This finding resonates with the report of Wang *et al*. [36] which determined the molecular weight of the fibrinolytic enzyme produced from *Bacillus subtilis* LD-8547 as 29 kDa. Concomitant research studies conducted elsewhere on fibrinolytic enzymes have recorded the molecular weights of 22 kDa for Subtilisin DFE [37], 27 kDa for Nattokinase [23] and 44 kDa for KCK 7 [35].

Fig. 1 – Sample of the fermented food Hawaijar

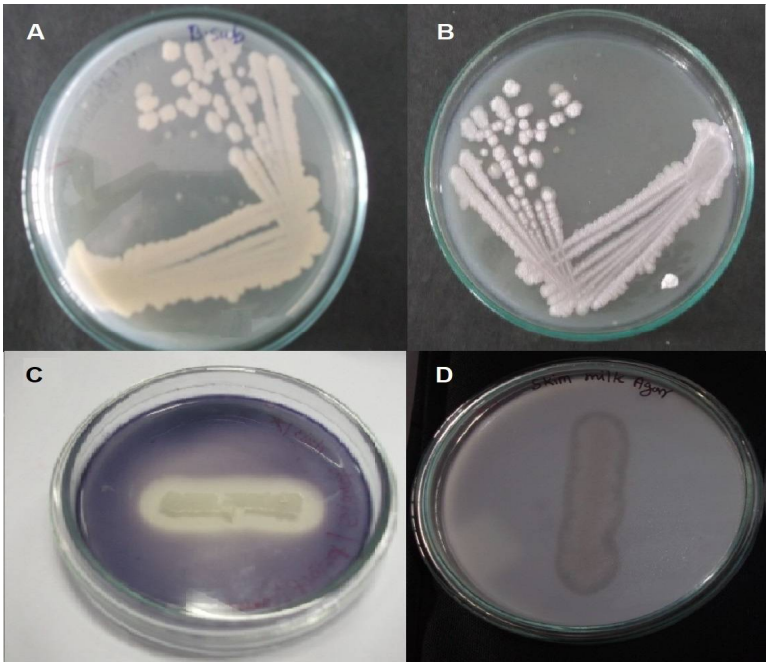

Fig. 2 – Characteristics of Bacillus sp. on A) Nutrient Agar B) Trypticase Soy Agar C) Starch hydrolysis medium and D) Skim Milk Agar

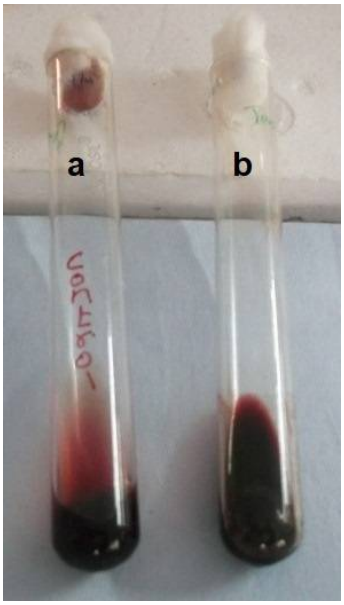

Fig. 3 – Artificial blood clot lysis test for fibrinolytic activity of enzyme
a) Complete degradation b) Control tube

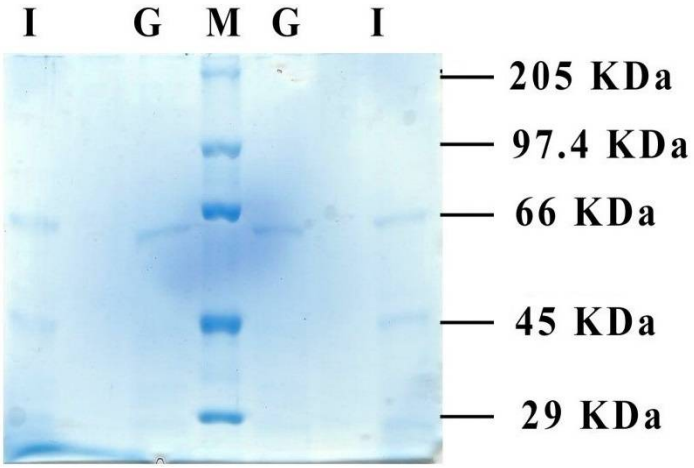

Fig. 4 Protein profiling of fibrinolytic enzyme by SDS-PAGE
I, Ion exchange purification; G, Gel filtration purification; M, Protein marker

Molecular characterization of bacterial isolate

The bacterial isolated *Bacillus subtilis* HT-4 isolated from the fermented food Hawaijar, since proved to encompass the potential of producing fibrinolytic enzyme, further investigations were carried out to determine its molecular and phylogenetic characterization. The sequencing technique deciphered that the 16S rRNA of this potential isolate comprise a total of 812 base pairs (Fig. 5). The BLAST based analysis of this sequence with the available databases of the GenBank was performed for the identification of the potential isolate. The phylogenetic tree developed out of the genomic study indicated its similarity to the extent of 97% with *Bacillus subtilis* strain 36 (Fig. 6). Thus, the isolate *Bacillus sp* HT-4 16s_812 could be confirmed for its identity as *Bacillus subtilis.*

The present study earnest and maiden attempt of investigating an Indian indigenous fermented food Hawaijar for the isolation of fibrinolytic enzyme producing bacteria. Although this food is consumed by a large population for its health benefits, our study has demonstrated the existence of the bacteria *Bacillus subtilis* could contribute to the probiotic characteristics of this fermented food. Exploring the potential isolate of the present study *Bacillus subtilis* HT-4 for the mass industrial production of the thrombolytic enzyme and further insight *in vivo* pharmacological studies would help developing a cost effective and efficacious drug to treat the intravascular thrombosis which is attributed for dreadful coronary diseases.

CGGCGGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTC
TCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGC
ATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGA
CTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTT
CGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGG
GCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCA
CCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGT
TGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACC
ACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGG
ATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCT
CCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCG
TACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAA
ACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCT
AATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAG
AGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACA
CGTGAATTCCACTCTCCTCTCTGCACT

Fig 5 - The Genomic Sequence of 16s rRNA of *Bacillus subtilis* strain HT-4
(16S-F --- 812 Base pairs)

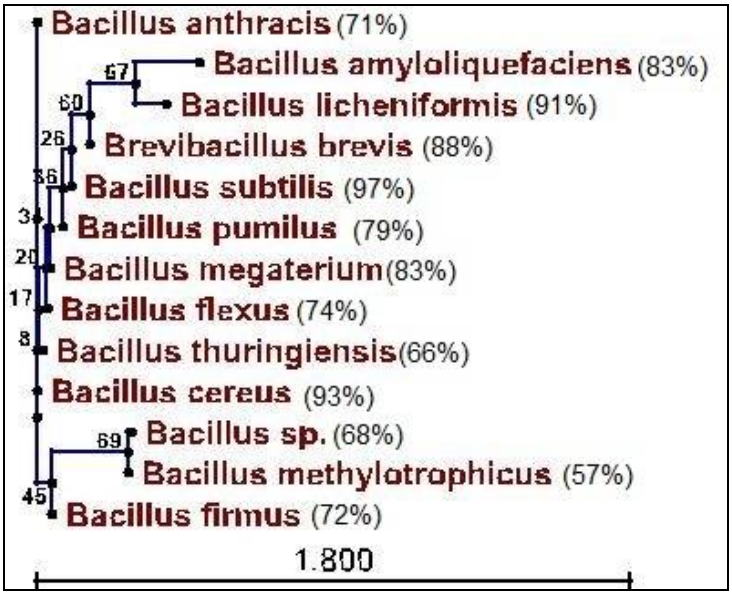

Fig. 6 - Results of phylogenetic study of *Bacillus subtilis* strain HT-4

CONCLUSION

Conventional fibrinolytic drugs of human, animal and microbial origin, owing to the drawbacks, have prompted newer research studies focusing on alternative microbial sources for development of efficacious, cost-effective and safe fibrinolytic drugs. Fermented foods harbor abundant bacteria provided with probiotic characteristics. Bacteriological studies on the fermented food Hawaijar revealed the existence of three species of the bacteria Bacillus viz., *B. subtilis, B. cereus* and *B. licheniformis* in the descending order of their prevalence. Preliminary screening of bacterial isolates on skim milk agar for proteolytic activity facilitates the selection of isolate the isolate with the potential of producing fibrinolytic enzyme. For the fermentative production of enzyme by the potential isolate *Bacillus subtilis* HT-4 potato-soybean medium could be considered owing to its economical and biomass promoting properties. Optimization of culture parameters such as carbon source of fructose, pH of 8, temperature of 50°C and incubation time of 72h help achieving enhanced production of fibrinolytic enzyme by submerged fermentation. Artificial blot clot lysis method could be a useful tool for validation of fermentation process and enzyme activity. The proteins produced by the potential isolate, possessing molecular weights ranging 29 and 66 kDa, could act as the fibrinolytic enzyme. The 16S rRNA based

genomic studies and similarity search studies on GenBank databases confirms the proximal phylogenetic relationship of the potential isolate with the bacterial species *Bacillus subtilis*. Further insight purification and pharmacological studies on the enzyme produced by *B. subtilis* HT-4 would help developing a promising drug for treatment of intravascular thrombosis.

Declaration:
The authors declare that there are no financial or non-financial conflicts of interest.

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