Fibrinolytic Potential Of Novel Bacillus Sp. Strains Isolated From Pulicat Lake, India

K. Gowthami, R. Jaya Madhuri

Abstract: The aim of the present study was to identify the potent microbial strains with anti-fibrinolytic activity from marine sediment. Marine sediments were collected from Pulicat Lake in southern India and were processed to isolate various kinds of microorganisms. The isolated microbes were screening for their fibrinolytic activity. Further, the effect of the crude enzyme extract of potent bacterial strain on RBC, animal tissue and a blood clot was also scrutinized. Out of 20 isolated microorganisms, only one bacterial strain (GPJ3) has shown potential fibrinolytic activity. The cultural, morphological, and biochemical characters and 16S rRNA sequencing suggest that the isolated strain is Bacillus sp. From the results, it can be concluded that the marine bacterial strain, GPJ3 could act as an anti-fibrinolytic agent..

Index Terms: Marine sediment: Bacterial diversity; Protease activity; Fibrinolytic activity.

1. INTRODUCTION

Marine sediment is a prominent source for various potential microorganisms, which are capable of producing fibrinolytic enzymes [1]. Currently, most of the researchers have been working on the potentiality of fibrinolytic enzymes from marine microorganisms due to their growth in extreme environmental conditions, including high salinity, diverse temperature, pronounced biochemical diversity and feasibility of mass culturing and genetic manipulations [2,3]. Furthermore, nowadays the usage of marine bacterial strains is being increased in various pharmaceutical and biotech companies due to their capacity to produce fibrinolytic enzymes with diverse biological activities such as vital role in catalyzing the chemical reactions in biochemical systems and possess high catalytic activity than the synthetic catalysts [4,5]. Fibrinolytic enzymes were identified from a diverse group of microorganism including Streptococcus pyogens, Bacillus natto, Bacillus amyloliquefacens, actinomycetes and Fusarium orysporum, Mucour sp, Armillaria mellea [6]. Further, these fibrinolytic enzymes have also been purified and characterized from many types of foods. Due to their significant role in the biological system, they can be used effectively as therapeutics for the treatment of cardiovascular diseases [7]. In addition, a fibrinolytic enzyme is considered to be the most potent thrombolytic agent in the treatment and prevention of cardiovascular disease [8]. Consequently, the microbial fibrinolytic proteases are majorly employing in the treatment of thrombosis to liquefy the blood clots. Moreover, recombinant fibrinolytic products are expensive and sometimes induce allergic reactions in human beings. Hence, to overcome this difficulty, the present study strives to screen the new fibrinolytic agent from the marine environment

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2. MATERIALS AND METHODS

2.1 Sample Collection and Isolation of marine microorganisms

Marine sediment samples were collected in autoclaved polyethylene covers through sterile containers from the depth of five meters of Pulicat Lake, Tamilnadu, India. Then the samples were immediately transferred to the laboratory and aseptically dried in the laminar air flow for 24 hrs and stored for further use. One gram of the dried sediment samples were serially diluted and inoculated onto the nutrient agar, potato dextrose agar, starch casein agar plates and then plates were incubated at different temperatures (37°-40°) for 1day, 3days and 5daysto isolate marine bacteria, actinomycetes and fungi respectively.

2.2 Preparation of crude fibrin

The goat blood was collected in a sterile screw-cap tube containing anticoagulant (citrate buffer) to prevent the blood coagulation. Then the blood sample was subjected to centrifugation at 7000rpm for 10 minutes to separate plasma. The obtained 100ml of clear supernatant (plasma)was collected and poured in sterile Petri plates that already contained 200µl of $CaCl_2$ and allowed for coagulation. Subsequently, the formed clot was washed twice with distilled water to remove attached globulins and albumins. Followed by clot was dried, powdered and stored as “Crude fibrin compound” [9].

2.3 Screening

Marine microbes were screened for fibrinolytic activity in 3 steps, which includes Primary, Secondary and Tertiary level screening.

2.4 Primary screening:

In the primary level screening, the proteolysis activity of the various isolates was detected by using skim milk agar plate method [10]. In this method, the clear zone of skim milk hydrolysis has been observed around the colonies after the incubation period.

2.5 Preparation of crude enzyme;

After primary screening for fibrinolytic activity, the isolated pure microbial cultures including bacteria, actinomycetes, and fungal strains were inoculated into a nutrient broth, potato dextrose broth and starch casein broth and incubated at

different temperatures (37◦-40◦) for 1, 3 and 7days respectively. After specific incubation period for each microbial type, the broth culture was centrifuged at 10000rpm for 30 minutes at 4^0C, the pellet was discarded and the supernatant was used as crude enzyme source [11].

2.6 Secondary level screening:
In secondary level screening, the fibrinolytic activity of isolated marine microbes was determined by fibrin agarose plate method. The wells were prepared on solidified fibrin agar plates with sterile cork borer, and then each well filled with 200µl of crude enzyme extract of various marine isolates and the plates were incubated at 37^0C for 24 hrs. After incubation, the plates were observed for a clear zone of hydrolysis around the wells, indicating that fibrinolytic activity of the marine isolates [12].

2.7 Tertiary level screening:
In tertiary level screening, blood clot lysis method was used asa confirmatory test for the fibrinolytic activity of the positive isolates in the primary and secondary screening. In this method, three clean grease-free slides were taken and placed a human clotted blood on each slide, then treated with crude enzyme and observed for the lysis of blood clot [13].

2.8 Identification of potent isolates:
The potent isolates having fibrinolytic activity were identified by morphological and biochemical activities as per the standard procedures described in Bergey's Manual of Systemic Bacteriology. Finally, the strain was identified by 16s DNA sequencing.

2.9 Enzyme activity on RBC Cells
The fibrinolytic activity should be specific to fibrin not on the other components of blood. To find the effect of crude enzyme extracts of marine organisms onRBC has also been tested as follows, 1ml of RBC cells were taken, followed by centrifuged and mixed with the 1ml of crude enzyme extract.

2.10. Crude enzyme activity on tissue
To determine the effect of crude enzyme activity on tissue was performed in heart vein of a goat. The combination of crude enzyme extract and methylene blue dye in a ratio of1:2was filled with heart vein and then it was tied and placed in the saline. Furthermore, 125µl of dye was used asa control without crude extract and allowed for 3 hours incubation to interpret the results.

2.11. In vitro clot lysis on different concentration:
The collected blood was centrifuged in pre-weighed Eppendorf tubes and incubated at 37^0C for 45 minutes. After clotting, the serum was completely removed without disturbing the clot and then again weighed the Eppendorf tubes to determine the weight of clot (clot weight = weight of clot containing tube – weight of empty tube). After, the clots were treated with various quantities of crude extract of marine organisms (20µl, 40µl, 60µl, 80µl, 100µl and 120µl). Further, the same quantity of distilled water was also used as a control for each sample. Then all the tubes were incubated at 37^0C for 30 minutes and observed for lysis of the clot. After incubation, the released fluid was removed and again the tubes were weighed to determine the fibrinolytic activity by calculating the percentage of change between before and after clot lysis.

3. RESULT AND DISCUSSION

3.1 Isolation of microbial strains from marine sediment:
In the present work, a total of twenty isolates were isolated from the marine sediment which are dissimilar in morphological appearance and colony characteristics. Among the isolated microorganisms, ten are bacterial strains, four are fungal strains, and six are actinomycetes strains. In marine sediments, the ample portion of taxonomic abundance and biomass are contributed by members of the Bacteria and Archaea, which correspond approximately 90% of the total benthic biomass. Therefore, in the present study, we have isolated a total of 20 isolates of various microorganisms to screen their fibrinolytic activity.

3.2 Screening of microbial strains for fibrinolytic activity:

3.2.1 Primary level screening:
Initially, the identified bacterial strains were subjected to various levels of screening methods to determine the fibrinolytic activity. In the primary level screening, out of 20 isolated various microorganisms, only eight strains (4 bacterial strains (GPJ_1 to GPJ_4), 3 actinomycetes (GPJ_5 to GPJ_7) and 1 fungal strain (GPJ_8) have shown the proteolytic activity by forming the clear zone around the colonies (Fig. 1). Further, these positive strains were used for further levels of screening.

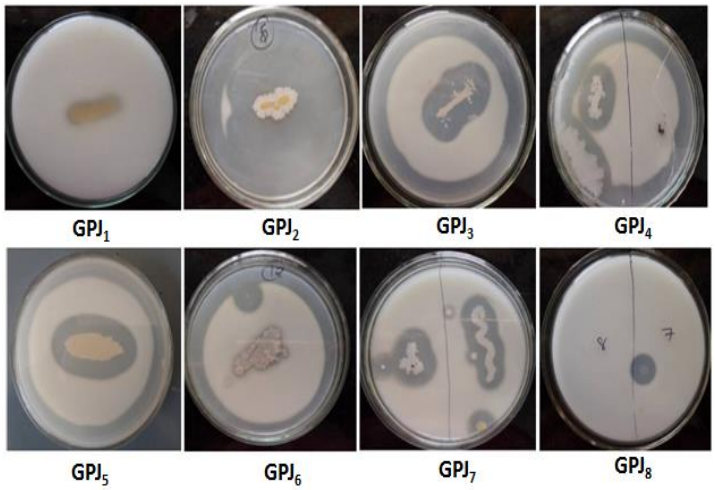

Fig. 1*. Potential isolates of various microbial strains and their proteolytic activity.*

3.2.2 Secondary level screening:
In the secondary level screening, the proteolytic activity-positive isolates were subjected to a screening of fibrinolytic activity. The crude enzyme extracts were prepared from selected microbial strains, loaded in the wells of fibrin agar plates and labeled as GPJ_1 to GPJ_8.After the incubation period, only four bacterial strains viz. GPJ_2, GPJ_3, GPJ_5 and GPJ_6 have shown clear zone of fibrinolysis around the wells (Fig.2). These positive fibrinolytic bacterial strains again used for the determination of lysis of blood clot in the tertiary screening.

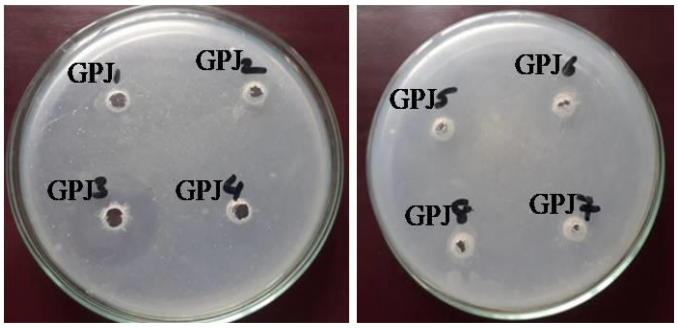

***Fig.2**. Fibrinolytic activity of bacterial strains viz. GPJ_2, GPJ_3, GPJ_5 and GPJ_6 (Secondary screening)*

3.2.3 Tertiary level screening:

Tertiary level screening is the confirmatory test for potential fibrinolytic activity, in which the selected marine bacterial isolates including GPJ_2, GPJ_3, GPJ_5 and GPJ_6 were further tested on blood clots for fibrinolysis. Among these four isolates, GPJ3 has potentially liquefied the blood clot within 20 minutes (Fig. 3). Thus this organism was selected for molecular characterization of strain for its identification.

***Fig. 3**. Blood clot assay by isolated strains of GPJ_2, GPJ_3, GPJ5 (Tertiary screening)*

3.3 Biochemical and Molecular characterization of GPJ3:

The biochemical properties of GPJ_3 are summarized in Table 1. The colonies of GPJ_3 on nutrient media are lucid in nature, smooth, raised colonies having rough margin and light brown in colorafter3 days of the incubation period. The cells of GPJ_3 are Gram-positive, short rods and arranged singly (Fig.4). The taxonomic identification of the GN2 was based on 16s rDNA analysis. The 16s rDNA sequence of the strain was compared with the sequences in GenBank using BLAST and aligned with the sequences retrieved from NCBI GenBank database using the Clustal W method. The database was deposited in NCBI GenBank with an accession number MN102109. Based on the cultural, morphological, physiological, and molecular analysis, the GN2 was identified as Bacillus.sqn Bacillus.

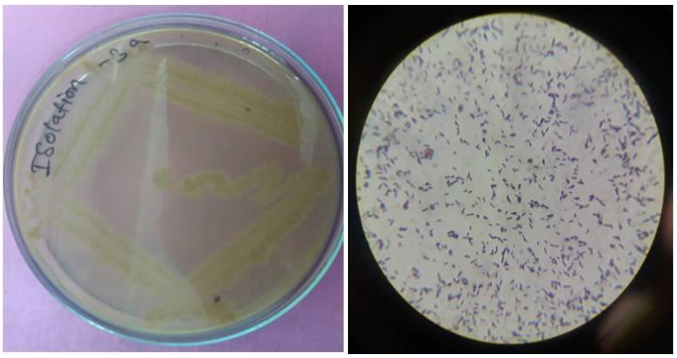

***Fig. 4**. Cultural characteristics of marine bacterial strain, GPJ3*

***Table 1.** Morphological and Biochemical tests of GPJ3 strain*

Characters	Microbial Strain
Colony appearance	Flat , irregular, rough, dull white, undulate (wavy) margin
Motility	Motile - flagellated
Colony Colour	Dull white
Grams stain	Gram (+ ve) rods
Starch hydrolysis	++
Gelatin hydrolysis	++
Casein hydrolysis	++
Indole	+
Methyl red	-
VP	+
Citrate	+
Urease	-
Carbon utilization	
Glucose	+
Sucrose	+
Fructose	++
Maltose	+
Nitrogen utilization	
Glutamic acid	-
Histidine	-
Methionine	+
Leucine	+

3.4 Effect of crude enzyme activity on RBC cells: The crude extract of GPj3 has shown no effect on RBC cells since there is no significant optical density has been observed between the control and GPj3. The results clearly demonstrate that the fibrinolytic activity of GPj3 should act on only fibrin (Table.2).

***Table No.** 2. Effect of crude enzyme activity on RBC cells*

Wavelength	520nm	540nm
GPj3	0.13	0.13
Control	0.11	0.10

3.5 Crude enzyme activity on animal tissue:

To assess the crude enzyme activity on animal tissue, we have performed an experiment with animal tissue, heart vein. In this experiment, no colour change has been observed in the beaker containing the isotonic solution, which clearly indicates that there is no harmful effect on animal tissue by the crude enzyme of GPJ3 (Fig.5.).

Fig. 5*. Crude enzyme activity of GPJ3 on animal tissue (Heart vein)*

3.6 In vitro clot lysis on different concentration:

The assay results clearly indicate that among the various concentrations of GPJ3 crude extract, 120µl was effectively lysed the blood clots within 30 minutes of time intervals (Fig. 6.).

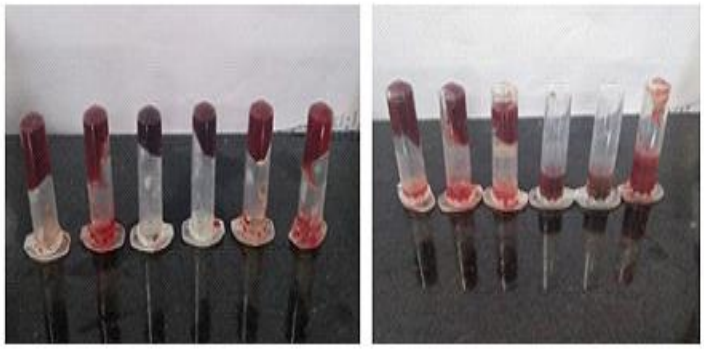

Fig. 6*. In vitro clot lysis assay by GPJ3.*

Nowadays, isolation of fibrinolytic enzymes from marine sources has been gaining a great prominence due to their significant role in clinical practice. In the present study, the marine sediment samples were taken from Pulicat Lake and were used for the isolation and identification of potential anti-fibrinolytic microbial strains. There are 20 different marine microbial strains were isolated and these cultures were used for screening of fibrinolytic enzyme production. Out of twenty isolates, the bacterial strain i.e. GPJ_3 has been identified as a potent fibrinolytic agent. As per the literature, there are several microorganisms that serve as the essential sources of thrombolytic agents for therapeutic purposes. For instance, Streptokinase produced by streptococcus hemolytic and streptococcus aureus is effectively used for thrombolytic therapy. Moreover, various fibrinolytic enzymes produced by different microorganisms viz. Bacillus subtilis, Bacillus mesenericus [14], Altramonaspiscicida [15], Bacillus licheniformis have also had the capability to serve as thrombolytic agents. Currently, the available fibrinolytic agents include tPA and uPA, bacterial plasmogen activators and streptokinase, which are commonly using in clinical use. However, all these agents have been associated with undesirable side effects and low specificity. Recently, an investigation was conducted that the isolation of fibrinolytic enzyme from marine organisms which was used as thrombolytic therapy and exhibits high specificity for fibrin and are relatively inexpensive. From the results that we obtained, the present study will explore the future research directions to screen out the various potent microbial strains with anti-fibrinolytic activity from different marine sources.

5. CONCLUSION

From the obtained results, it can be concluded that the marine bacterial strain GPJ3 has shown a potential fibrinolytic activity and it was identified as Bacillus species. Further, the work is in progress to optimize the cultural and nutritional conditions of the organism for enchanted production of fibrinolytic enzymes along with purification and characterization.

Conflicts of interests

The authors declare no relevant competing financial interests to disclose.

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