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Research Article

IN VITRO THROMBOLYTIC ACTIVITY OF NATTOKINASE FROM *BACILLUS SUBTILIS* ISOLATED FROM MANGROVE SEDIMENTS

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A R T I C L E I N F O

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A B S T R A C T

In the present study, total seven bacterial isolates were obtained from the mangrove sediment. Out of seven isolates, MS2 showed larger clear zone on fibrin place, which indicates the presence of nattokinase enzyme. The thrombolytic effect of nattokinase enzyme obtained from *Bacillus* sp. of the the mangrove sediment was studied in vitro. The enzyme (10µl) obtained from this strain showed fibrinolytic activity on casein agar plate. At 300µl concentration, this enzyme showed thrombolytiv activity (> 50%) blood clot lysis. The enzyme showed optimum stability at 50^0C. for 30 minutes. The results showed that Nattokinase from *Bacillus* sp. played a significant role in thrombolysis and anticoagulation in vitro. The phylogenetic analysis identified the enzyme producing strain as *Bacillus* sp. The results indicate that the pure enzyme has a potential in dissolving blood clot, and the possibility for application in the treatment of thrombosis.

INTRODUCTION

Thrombolytic therapy has become a conventional treatment for acute myocardial infarction (AMI), yet currently, clinically prescribed thrombolytic drugs have problems such as delayed action and other side effects. Fibrinolytic enzymes have attracted interest as thrombolytic agents because of their efficiency in the fibrinolytic process, including plasmin activation. Nattokinase (NK) is a potent fibrinolytic agent for thrombosis therapy. Therapeutic enzymes have been in use since last 4 decades. One of the therapeutic enzyme was reported as a part of replacement therapies for genetic deficiencies (Vellard, 2013) and further research is continuing on this group of enzymes for their possible applications in therapeutic treatment (Mane & Tale 2015). It has been reported that regular consumption of enzymes and enzyme-rich foods is very much useful for prevention of disease as well as aging process. Enzymes are in use as digestion aids but mostly ignored as drugs.

The enzyme nattokinase comes under fibrinolytic enzymes. This group of enzymes can be found in a variety of foods, such as Japanese Natto, Tofuyo, Korean Chungkook-Jang soy sauce and edible honey mushroom (Dubey *et al*. 2011). This enzyme, which is serine proteinase is obtained from *Bacillus subtilis*. This enzyme is very much useful for therapeutic treatment as it can reduce some factors of blood clotting and lipids that are associated with an increased risk for cardiovascular disease (CVD) (Mane and Tale 2015).

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In this context, oral administration of nattokinase could be considered as a CVD neutraceutical. The plasma levels of fibrinogen, factor VII, and factor VIII can be reduced by using this enzyme. (Hsia *et al.,* 2009). This enzyme has haemolytic activity and it shows prolonged action of preventing coagulation of blood and dissolving existing thrombus (Milner, 2008).

MATERIALS AND METHODS

Collection of sediment samples: The sampling area was mangrove creek of Ratnagiri (17 04 03.994 N, 73 17 17.557 E). A small amount of sediment sample was collected in sterile bag and brought to the laboratory.

Isolation of bacteria: Serially diluted sediment samples were spread plated on Nutrient agar plates of 10% concentration and the plates were incubated at 30^0C. After 48 hours of incubation, the plates were observed and the bacterial colonies were purified. Colonies were isolated from the petridishes and streaked onto nutrient agar slants.

Screening of bacteria for fibrinolytic enzyme: For this purpose, fibrin plate assay, described by Liang *et al*., 2007 was carried out. The fibrin plate was made up of 0.4% fibrinogen, 0.6% agarose and 0.5 U mL^1 thrombin, which were dissolved in 50 mM barbitol buffer (pH 7.8) beforehand and mixed in a petridish. The fibrin plate was spot inoculated by seven different bacterial isolates and this plate was incubated overnight at 37ºC. The diameter of the fibrinolytic zone was measured and the bacteria producing fibrinolytic enzyme showed clear transparent zone due to hydrolysis of fibrin due to enzyme. The diameter of clear zone was measured and recorded.

Shake flask fermentation: The potential bacterial strain MS2 (Bacillus sp.) was grown in 500ml nutrient broth (HiMEDIA) containing 50% sea water. The incubation temperature was 30^0C and the culture was kept in shaking condition at 100RPM.

Cell harvestation by centrifugation method: Cells were harvested from the 48 h grown culture by using centrifugation method. The culture broth was centrifuged at 10000 rpm for 10 min at 4°C. The culture supernatant was collected separately and the residual cell pellet was discarded.

Microfiltration of culture supernatant: The cell free supernatant was then filtered through 0.3µm glass fiber filter using vacuum pressure to remove the suspended particles. The micro filtered sample was collected in a separate container and used further for analysis.

Concentration of enzyme for further analysis

The filtered laccase enzyme supernatant was concentrated through Tangential Flow Filtration (TFF) apparatus of PALL with a 10KDa filter. The crude enzyme was concentrated to 10 fold. This concentrated cellulase was used for further studies.

Screening of enzyme using casein agar medium

The enzyme obtained from MS2 culture was checked for nattokinase activity by using casein agar medium (Borah *et al*. 2012). A plate having About 8 mm diameter well was made in petriplates containing casein agar medium was prepared and a well of 10mm diameter was prepared on the plate. Enzyme (10µl) was added in the well and the plate was incubated at 37°C for 24 hrs. Colorless zone of casein hydrolysis around the well was an indication of nattokinase secretion.

Thrombolytic activity of crude enzyme

This assay was carried out by following the method described by Thirunavukarasu *et al*. 2014. The blood samples were obtained from the blood bank, which were taken from the healthy individuals without a history of oral contraceptive or anticoagulant therapy. Three different enzyme concentrations were used and for each concentration 3 replicates were performed. For clotting purpose, blood sample (1 mL) was distributed in pre weighed sterile micro centrifuge tubes and incubated at 37^0C for 60 mins for clot formation. After this, the serum was completely aspirated without disturbing the clot and the tubes were again weighed to determine the clot weight (clot weight = weight of the tube containing clot - weight of the empty tube). To the each micro-centrifuge tube different concentration (100µl, 200µl, and 300µl) of crude enzyme was added. 50µL of sterile distilled water was used as a negative control. All the assay tubes were incubated at 37^0C. for 24 hrs and observed for clot lysis. The fluid obtained after the incubation was removed carefully and the tubes were weighed again to observe the difference in weight after clot disruption. Difference in the weight taken before and after clot lysis was expressed as percentage of clot lysis.

Effect of temperature and pH on crude enzyme: The method followed by Borah *et al*. 2012 was used to check these effects. The reaction mixture containing 2.0ml of 0.5% casein solution in 0.1M carbonate buffer (pH 9.3) and 0.1ml of enzyme solution in the total volume of 2.1 ml was prepared for the assay. For different temperatures (10^0C, 30^0C, 50^0C and 70^0C) were selected for incubating enzyme reaction mixture. After incubation 30 minutes and 60 minutes, the reaction was stopped by adding 3.0 ml of 10% ice cold TCA and centrifuged at 10,000 rpm for 5 min. The optical density was measured at 660 nm in UV- Spectrophotometer to monitor the enzyme activity.

In order to check the effect of pH on enzyme activity, the enzyme (0.1ml) reaction mixture was prepared at four different pH (4, 6, 8 & 10) using 0.1M carbonate buffer. After incubation of 1 hour, the optical density was measured at 660 nm in UV- Spectrophotometer.

Phylogenetic analysis of potent bacterial culture: Molecular phylogeny of bacteria was determined by amplifying genomic 16srRNA region. Two primers specific to 16srRNA region used in this study were 519F and 1385R in order to amplify approx. 850bp sequence of bacterial 16srRNA gene.

***PCR amplification*:** The DNA isolated from bacteria was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). The PCR reaction mix contained 2.5µl of 10X buffer, 1µl of each primer (Table 7), 2.5µl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µl Template DNA and 8.5µl nuclease free water. The PCR amplification cycle consist of, a cycle of 5 min at 94°C; 35 cycles of 1min at 94°C, 1 min at 50°C, 2 min at 72°C; and additionally 1 cycle of 7 min at 72°C. The reagents used are procured from GeNei (Table 8).

***Gel electrophoresis*:** Gel electrophoresis was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplified PCR product. The size obtained was approx. 850bp for partial 16S rRNA region (Figure 7).

***DNA sequencing*:** The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). 100µl of PCR-A buffer was added to the 25µl of reaction. The sample was mixed and transferred to column placed in 2ml collection tube and centrifuge at 10,000 rpm for 1min. the filtrate was discarded. 700µl of W2 buffer was added to the column and centrifuge at 10,000rpm for 2min. This step was repeated twice. The column was transferred to a new tube. 25µl of Eluent was added into the column and incubated at room temperature for 2min. Then centrifuge at 10,000rpm for 5min. It was further sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram was obtained. For sequencing of PCR product 519F - 5' CAGCAGCCGCGGTAATAC 3' sequencing primer was used.

***Bioinformatics analysis*:** The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The BLAST results were used to find out evolutionary relationship of bacteria. Altogether twenty sequences, including sample were used to generate phylogenetic tree (figure 1-6). The tree was constructed by using MEGA 5 software (Saitou N. and Nei M.,1987; Felsenstein J.1985 and Tamura K. *et al* 2011).

RESULTS AND DISCUSSION

Table 1 Morphological characteristics of bacteria isolated from sediment samples.

Bact. Code	Color	Form	Elevation	Margin
MS1	Orange	Circular	Flat	Entire
MS2	Cream	Irregular	Flat	Entire
MS3	Yellow	Irregular	Flat	Lobate
MS4	cream	Circular	Flat	Entire
MS5	Cream	Irregular	Flat	Entire
MS6	Pale yellow	Circular	Umbonate	Entire
MS7	Cream	Circular	Flat	Entire

Table 2 Results of fibrinolytic activity of bacteria using fibrin plate

Bact. Code	Diameter of fibrinolytic zone
MS1	-
MS2	12mm (This bacterium was selected further for enzyme extraction)
MS3	-
MS4	-
MS5	3mm
MS6	3mm
MS7	-

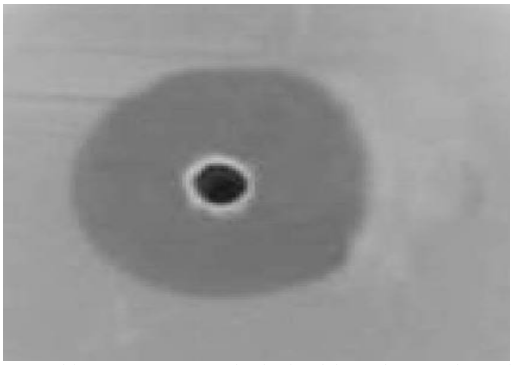


Figure 1 Nattokinase enzyme (10µl) obtained from the MS2 bacterium was added in the well and the plate was incubated at 37°C for 24 hrs. The enzyme showed clear zone on casein agar medium, which is an indication of fibrinolytic activity.

Table 3 Thrombolytic activity of nattokinase enzyme in clot lysis assay

Enzyme concentration	Percentage of clot lysis		
	Replicate 1	Replicate 2	Replicate 3
100µl	-	4%	-
200µl	28%	32%	30%
300µl	56%	57%	51%

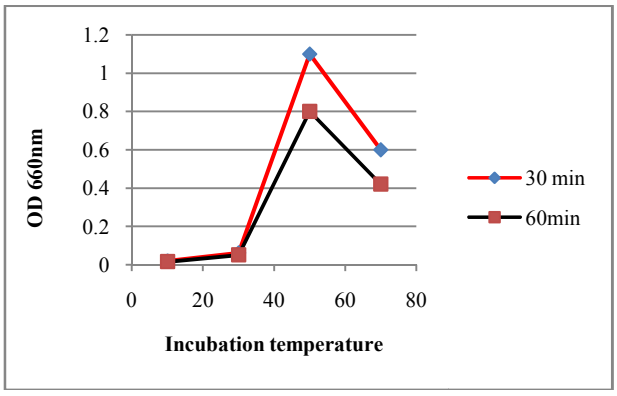


Figure 2 Stability of nattokinase enzyme at different temperature

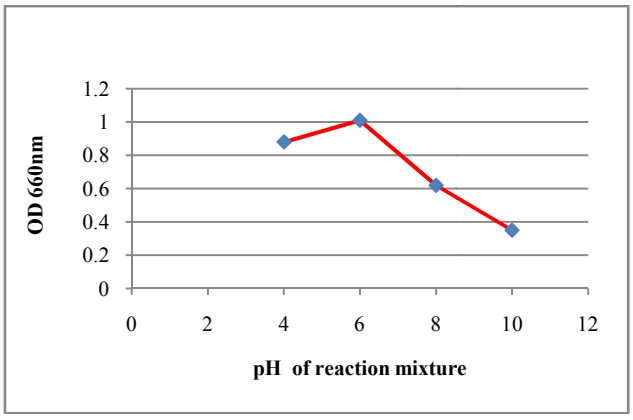
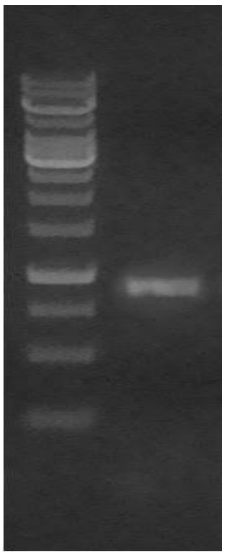


Figure 3 Effect of pH on enzyme activity



Lane 1: 1Kb DNA marker of Fermentas (#SM0311)
Lane 2 Amplified product of bacterium MS2

Fig 4 Phylogenetic analysis results: The potent bacterial culture producing nattokinase activity was identified as Bacillus sp.

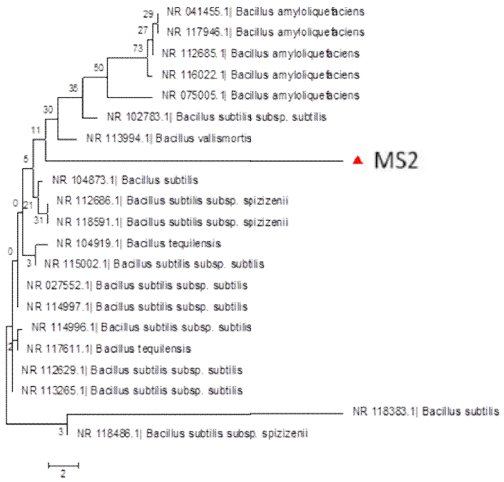


Figure 5 Phylogenetic tree for MS2 using partial 16S rRNA gene sequence

Maximum Parsimony analysis of taxa

The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. The analysis involved 21 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1561 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

CONCLUSION

The results showed that Nattokinase from Bacillus sp. played a significant role in thrombolysis and anticoagulation in vitro. The phylogenetic analysis identified the enzyme producing

strain as Bacillus sp. The results indicate that the pure enzyme has a potential in dissolving blood clot, and the possibility for application in the treatment of thrombosis.

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