Optimization for the production of fibrinolytic enzyme from halophiles and its application

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A b s t r a c t

Halophiles are organism requiring at least 0.2M salt for growth that live in high salt concentrations. They are a type of extremophilic organism. The name comes from the greek word for “salt-loving”. While most halophiles are classified into the archaea domain, there are also bacterial halophiles and some eukaryote, such as algae or fungus. A halophilic Bacillus species was isolated from soil samples collected from Mahabalipuram sea shore (Tamil Nadu). Experiments were set to observe the effects of different pH, temperature, incubation time, carbon and nitrogen sources on the fibrinolytic enzyme production and activity. Optimum fibrinolytic activity was achieved after 24hrs of incubation, at 37°C, pH 8, carbon source (lactose), nitrogen source (tryptone) and substrate concentration [casein] 0.8%. Enzyme was purified by ammonium sulphate precipitation, dialysis, ion exchange chromatography and SDS-PAGE. The purified enzyme was examined for its application by blood clot method, milk clot and detergent activities tests.

INTRODUCTION

The process which prevents blood clots that becoming problematic is called Fibrinolysis [1]. In fibrinolysis, a fibrin clot, the product of coagulation, is broken down. Its main enzyme plasmin cuts the fibrin mesh at various places, leading to the production of circulating fragments that are cleared by other proteases or by the kidney and liver [2]. Fibrin is the main component of the blood clot, and it is normally formed from fibrinogen by the action of thrombin (EC. 3. 4. 21. 5). The accumulation of fibrin in the blood vessels usually results in thrombosis [3]. Microbial fibrinolytic enzymes, especially those from food-grade micro organism, have the potential to be developed as functional food additives and drugs to prevent or cure thrombosis and other related diseases [4]. Halophiles are organisms that live in high salt concentrations and they area type of extremophile organism. While most halophiles are classified into the Archaea domain, there are also bacterial halophiles and some eukaryote, such as the alga Dunaliella salina or fungus. They are also classified into slight, moderate or extreme by the extent of their halotolerance [5]. Thrombus in blood vessels or in a chamber of the heart leads to myocardial infarction and other cardiovascular diseases (CVDs). For thrombolytic therapies, both injection and oral administration of thrombolytic agents have been extensively investigated. Based upon their mechanism of activation of the fibrinolytic system, fibrinolytic agents are classified into two types. One is plasminogen activator such as tissue-type plasminogen activator (t-PA) [6] and urokinase [7]. Halophiles are also important in the food industries where they are used in the preparation of sauces as well as in the production of other products such as cheeses, cured, meats and fish [8]. Halophiles that grow and flourish in saline or hyper saline environments show potential for use in bioremediation [9].

Microbial fibrinolytic protease is considered as a potent fibrinolytic agent to treat CVDs [10]. Many thrombolytic agents have been identified and characterized from different sources [11-14]. Although microbial fibrinolytic enzymes have been extensively studied, only few reports are available concerning statistical medium optimization [3, 15-16]. In recent years, an attempt was made to isolate a potent fibrinolytic enzyme producing organism from marine environment [16]. Till now haloalkaliphiles were studied extensively for the microbiological classification and phylogeny; only limited attempts have been made to explore molecular basis of adaptation, enzymatic potential and their other biotechnological implications. The use of halophiles for bioremediation and biodegradation of various materials from industrial effluents to soil contaminants and accidental spills are being widely explored. The diversity of the halophilic, haloalkaliphilic and alkaliphilic microbes has been studied from the hyper saline and hyper alkaline environment.

MATERIALS AND METHODS

Isolation of halophilic microbes

Soil and water samples were collected from different parts of Tamil Nadu namely the fields of Kovalam (Sample 1), Mahabalipuram (Sample 2 & 3) and Muthukadu (Sample). 1gm of each of the collected soil sample was subjected for serial dilution up to 10-7 and spread plate method was carried out on nutrient media. All the nutrient agar plates were kept for incubation at 37°C in bacteriological incubator for 24hrs. Casein hydrolysis media was used for the growth of micro organisms [17].Nutrient agar medium was used for pure culture preparation. The inoculated cultures were incubated at 37°C for 24 hours and preserved at 4°C.

Identification of bacterial strain
The morphology of the strain was observed in the petri plate i.e., the zone of inhibition and the nature of the colony. Microscopic identification of the culture was done by gram’s iodine method. Various biochemical tests were performed as follows; on verification with “Bergey’s manual of systematic bacteriology”. Hence biochemical tests namely: Methyl red, Voges proskauer, Indole test, Citrate utilization test, Starch hydrolysis, Casein hydrolysis, Catalase, Carbohydrate fermentation (sucrose, lactose, fructose & dextrose), Gelatin and H₂S test were carried out.

Fibrinolytic enzyme assay
500µl of substrate (fibrin) was dissolved in 300µl of Tris Hydrochloric acid (0.02M) at pH 8 and 200µl of enzyme was added and the total volume of the reaction mixture was made up to 1ml. The reaction mixture was incubated in water bath for 10mins at 40°C and added 1ml of 10% TCA (chilled). Kept on ice bath for 20mins and centrifuged the above sample at 8000 rpm for 10 mins and collected the supernatant. 0.5 ml and 2.5 ml of 0.5 M Na₂CO₃ was added to the supernatant and incubated for 10mins at room temperature. Added 0.5 ml of Folin Ciocalteau reagent (1:3 dilutions) and left at room temperature in dark conditions for 30 mins. Then blue color was read at 660nm [18-19].

Optimization of media for the growth of isolate for the production of fibrinolytic enzymes
Optimization of various parameters such as salt tolerance (2.5% - 15% NaCl), pH range (4-9) using different buffers, temperature range (25 – 70°C), substrate concentration (0.2 -1%), carbon source (dextrose, maltose, lactose, starch and cellulose) and nitrogen source (sodium nitrate, potassium nitrate, ammonium nitrate, trypotene and peptone) using 0.1M phosphate buffer of pH 8. Nutrient media was prepared and autoclaved. The culture was inoculated and incubated for 24 hrs and checked for the activity by protease assay method [17].

Protease assay
The alkaline protease activity was assayed by the method of Folin & Ciocalteau with some modifications. One unit of alkaline protease activity was defined as the amount of enzyme that liberated one micromole of tyrosine per ml per minute under experimental condition [20].

Protein estimation by Lowry’s method
Protein estimation of the fibrinolytic enzyme was done by lowry’s method. The best range of enzyme concentration was chosen based on the results [21].

Production of fibrinolytic enzyme
Production broth was prepared by using 0.8% casein and pH was maintained at 8 with temperature-37°C, 5% NaCl, 1.5 g of yeast extract, 1.5g of carbon source (lactose), 1.5g of nitrogen source (trypotene) and the total volume was made up to 150ml and the broth was autoclaved, culture was inoculated and incubated for 24 hrs.

Purification of fibrinolytic enzyme
The enzyme sample was purified by ammonium sulfate precipitation, dialysis, ion exchange chromatography and SDS PAGE.

Preparation of crude enzyme
The 24hours old broth was centrifuged at 6000 rpm for 10 minutes at 4°C. The pellet was collected and dissolved in 10ml of 10mM Tris hydrochloric acid solution and dialysis was carried out [22].

Ion exchange chromatography
The ion exchange column was washed with water then with 95% ethanol and left to dry. It was then packed with DEAE Cellulose. The slurry is added in portions, allowing it to settle at the bottom then adding it slowly by the sides and avoiding disturbing the resin. Set of test tubes was prepared of salt buffer ranging from 25mM, 50mM, 75mM, 100mM, 125mM, 150mM. The enzyme extract after dialysis was added to column and flow was collected. Then low salt buffer is transferred to the column to drain into the resin, and then entire buffer is transferred. The elute was collected and another salt buffer is transferred. Activity of each elute was checked by the Folin-ciocalteau method. The protein content of elutes were determined [23].

SDS-PAGE
One dimensional SDS- PAGE was carried out for determination of molecular mass of the protease in a 4% stacking gel (pH 6.8) and 10% resolving gel (pH 8.8) according to the method of Laemmli [24].

Zymogram
Casein zymography was carried out with slight modifications. Casein (0.2%w/v) was dissolved in 20mM Tris-HCl (pH 8.5) and copolymerized with 10% resolving gel. Samples were prepared by diluting the enzyme in zymogram buffer (0.125M Tris HCl, 2% SDS, 10% glycerol, 0.02% bromophenol blue, pH 6.8). The samples were loaded in to wells and electrophoresed at a constant current 12mA at 4°C. After electrophoresis, the gel was incubated for 30 mins at room temperature in a reaction buffer (10mM Tris-HCl buffer, pH 9) containing 2.5% (v/v) Triton X-100. The gel was then washed with distilled water to remove Triton X-100, incubated in reaction buffer (Tris-HCl pH 9) for 30 mins and destained. The protease activity was destained as a colourless clear zone against dark blue background [25].

Characterization of fibrinolytic enzymes
Buffers of different pH were prepared according to Deutscher [27]. To measurement of optimal pH for enzyme activity, different pH from 5-10 each in a single test tube separately. Phosphate buffers were prepared at pH 7.5 for enzyme activity of different temperature from (20°C-60°C), different substrate concentration (casein) from (0.2%-1.2%), different activator (MgCl₂ ) from (2mm-10mM), different inhibitor (Tri-sodium citrate), from (2mm-10mM) and different time intervals (5min-30min) each in a single test tube separately. Tubes were incubated at 37°C for 30 minutes and the activity of fibrinolytic enzyme was done by Folin-Ciocalteau method.

RESULTS
Isolation of halophilic microbes
6 pure cultures were obtained from 3water and 1 soil sample. Only 1 pure isolate from the soil sample was able to hydrolyse casein media when it was cultured on an enrichment media containing casein.
Figure 1(a). Primary screening
Figure 1(b). Pure cultures of isolates

Table 1: Results of biochemical tests of the isolates

<table>
<thead>
<tr>
<th>Sl no.</th>
<th>Biochemical tests</th>
<th>Results</th>
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<tbody>
<tr>
<td>1</td>
<td>Methyl red</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Voges proskauer(vp)</td>
<td>_</td>
</tr>
<tr>
<td>3</td>
<td>H2S production</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Gelatin hydrolysis</td>
<td>_</td>
</tr>
<tr>
<td>5</td>
<td>Casein hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Starch hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Indole</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Urease</td>
<td>_</td>
</tr>
<tr>
<td>9</td>
<td>Citrate utilization</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Carbohydrate utilization</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Fructose</td>
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<tr>
<td>12</td>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Lactose</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Dextrose</td>
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Estimation of fibrinolytic assay

The activity of the fibrin enzyme was calculated using standard tyrosine graph. The enzyme activity of the fibrinolytic enzyme assay was found to be 181.33 U/ml.

Optimization for the Production of fibrinolytic enzyme

When the salt concentration (NaCl) for production of fibrinolytic enzyme was investigated from 2.5% - 15%, 5% was found to be the optimum salt concentration for fibrinolytic production (70.4 U/ml) as shown in figure 2(a). pH of the growth medium plays an important role by inducing physiological changes in microbes and their enzyme secretion. The obtained results demonstrated that though fibrinolytic production was detected over a broad pH range from 4.0 to 9.0, maximum enzyme production was noted at pH 8 and its enzyme activity is 35.2 U/ml as shown in figure 2(b). When the optimum temperature for the production of fibrinolytic enzyme was investigated from 25°C to 70°C, 37°C was found to be the optimum temperature for fibrinolytic production (70.4 U/ml). The incubation at temperatures more than 37°C was found to decrease the production of fibrinolytic enzyme as shown in figure 2(c). When the substrate concentration (casein) for production of fibrinolytic enzyme was investigated from 0.2% - 2%, 0.8% was found to be the optimum substrate concentration for fibrinolytic production (117.3 U/ml) as shown in figure 2(d). Testing the effect of various carbon sources and nitrogen sources on fibrinolytic production, it was found that Lactose gave the highest enzyme activity of 52.9 U/ml as shown in figure 2(e) and Tryptone gave the highest enzyme activity of 105.5 U/ml as shown in figure 2(f).
Figure 2(e): Effect of carbon source on the growth of isolated Bacillus species for the production of fibrinolytic enzyme.

Figure 2(f): Effect of nitrogen source on the growth of isolated Bacillus species for the production of fibrinolytic enzyme.

Purification of fibrinolytic enzyme
Molecular weight determination
The molecular weight of the partially purified fibrinolytic enzyme, as analyzed by SDS-PAGE, showed a single protein band of approximately 90kDa as shown in figure 3(a).

Characterization of fibrinolytic enzyme
The enzymatic specificity of the partially purified fibrinolytic enzyme of Bacillus sp. The 2-D SDS-PAGE gel was visualized using the SYPRO Ruby staining system. When the three different zymographic maps of enzyme from Bacillus sp. the same enzymatic maps on the fibrin and gelatin gels then show the activity. On the other hand, spots with low molecular weight were not visualized on the casein gel as shown in figure 3(b).

Figure 3(a): SDS-PAGE profile of fibrinolytic enzyme from Bacillus species.

Figure 3(b): Bands representing the activity of fibrinolytic enzyme through Zymography.

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Figure 4(a): Effect of pH on the activity of fibrinolytic enzyme

Figure 4(b): Effect of temperature on the activity of fibrinolytic enzyme

Figure 4(c): Effect of substrate concentration on enzyme activity

Figure 4(d): Effect of activator on activity of fibrinolytic enzyme

Figure 4(e): Effect of inhibitor on activity of fibrinolytic enzyme

Figure 4(f): Effect of time on activity of fibrinolytic enzyme

APPLICATIONS

Blood clot removal
The anticoagulant effect of partially purified fibrinolytic enzyme is shown, no blood clots were observed in the test tubes for 2 minutes. The blood clots were partly formed after 2 minutes. Meanwhile, clotting had occurred in the test tube of blank as shown in figure 5(a). The result indicated that the enzyme exhibited an efficient anticoagulant effect in vitro.

Milk clot removal
The effect of partially purified fibrinolytic enzyme is shown on milk, the removal of milk clots were observed in the petriplate immediately after addition of enzyme. On the other hand, addition water does not show the activity of enzyme in milk clot removal as shown in figure 5(b).
DISCUSSION

Alkaline proteases produced by microorganism have the major industrial applications and the recent trend towards the use of alkaline proteases from these sources in different process like detergents, tanning, food, waste treatment and peptide synthesis has increased because of their high catalytic activity and high degree of substrate specificity [28]. In recent studies, fibrinolytic enzymes for various products and particularly in industrial scale like detergents, milk clot removal and medical field like blood clot. It was found that carbon and nitrogen source of sucrose and peptone induced more enzyme production in submerged fermentation but in present study lactose and tryptone worked as good source of carbon and nitrogen. The protein fraction precipitated with 80% ammonium sulfate had the highest fibrinolytic activity (35.12 x 10^4 units/mg proteins). Ammonium sulfate was found to activate the fibrinolytic activity after dialysis. Fibrinolytic enzyme was partially purified using anion exchange chromatography (DEAE-Sephadex). Purity was increased 86 fold and specific activity of 39.31 x 10^4 units/mg protein was obtained [28]. Bacillus spheacicus produce 64 mg/l of the crude fibrinolytic protease enzyme and after purification it was 6.3 mg/l. The molecular weight of the compound was 18.6 kDa [29]. A isolated from soil and was subjected to fibrinolytic production and its partial characterization. The maximum production of crude fibrinolytic enzyme after incubation of 24 hours was 152 ml. The crude preparation of protease showed higher activity at 40°C with the pH of 7. The enzyme activity of the purified fibrinolytic enzyme through ion exchange chromatography was 83.6 μmole/min/ml. The purity was increased by 23.6 folds and its specific activity was 348.33 U/mg of protein. Industrial productions and medicinal use of the enzyme needs large scale production by some alternative methods and high purity. So, isolation, production, purification, assay and characterization of fibrinolytic enzymes from microbial sources are very effective and useful. In the future, the research will progress into the production of highly purified fibrinolytic enzymes from bacterial sources.

CONCLUSION

The present work deals with optimization for the production of fibrinolytic enzyme from halophiles and its application. Collected soil samples were subjected to identification of organisms. The organism was subcultured using nutrient agar and followed with optimization of media for the production of fibrinolytic enzyme. Purification was done by different methods such as ammonium sulphate precipitation, dialysis, ion exchange chromatography and SDS-PAGE. Fibrinolytic enzyme has wide applications in blood clot removal, as detergent additives, in food industries for the preparation of sauces, other products such as cheeses, salt cured meats and fish. It has environmental applications in bioremediation and biodegradation of various materials from industrial effluence to soil contaminants. It is also used in chicken feather degradation, dehairing of skin, milk clot.

REFERENCES


