Production of a fibrinolytic enzyme by *Streptoverticillium mobaraense*

Moataza MS, El-Shanshory A, Ghada SE

Abstract

Twenty isolates of actinomycetes were isolated from different soil samples collected from different location of El-Gharbia Governorate for their fibrinolytic activity in four different media, for different time intervals and under different culture conditions. The most active isolate for production of fibrinolytic enzyme was identified according to morphological properties and Biochemical tests as *Streptoverticillium mobaraense* GH4. On the other hand using shaken cultures grown on glucose-yeast extract peptone medium for 5 days, optimal conditions for enzyme production were pH 8.0, 2% sorbose (w/v) as carbon sources, yeast extract 2% (w/v) as a nitrogen sources at 28 ºC.

Keywords: *Streptoverticillium mobaraense*, Fibrinolytic enzyme, activity, culture condition

Introduction

Thromboembolic diseases are today a major cause of morbidity and mortality. Anticoagulants are thus being used as aid in preventing the extension of existing thrombi. Microorganisms are important sources for thrombolytic agents. Fibrinolytic proteases are the single class of enzymes which play an important in the metabolism of the almost all organisms (plant, Animal, Fungi, Bacteria and Viruses [1]). For thrombolytic therapy, microbial fibrinolytic enzymes have now attracted much more attention than typical thrombolytic agents because of the expensive prices and the undesirable side effects of the latter. The fibrinolytic enzymes were successively discovered from different microorganisms. Fibrinolytic enzyme has been identified in a number of microorganisms including *Streptomyces* [2, 3, 4], bacteria such as *Bacillus*, [5, 6, 7] *Staphylococcus* sp. [8, 9], Fungi such as *Aspergillus ochraceus* 513 [10], *Rhizomucor miehei* [11]. The aim of the work attempt was made for screening, several actinomycetes isolates for fibrinolytic enzyme production and selected the most active strain was *Streptoverticillium mobaraense* GH4, selected for detailed studies, carbon and nitrogen sources and optimum parameters for the production of the fibrinolytic enzyme.

Materials and method

Screening of fibrinolytic enzyme activity

Twenty isolates of actinomycetes were isolated from different soil samples collected from local Egyptian soil of El-Gharbia Governorate and choosing only 12 isolates could produce fibrinolytic enzyme. This isolates inoculated on fibrin plate agar (Venkata& Divakar2013) containing 1.2%w/v agarose, 0.4% human fibrinogen and 20u/ml human thrombin in a petridisc and incubated at 37 ºC for 3 days. Aclear zone around the growth of Strptomyces was indicated to fibrinolytic activity. The most active organism was identified in our laboratory by chemical methods by [12, 13, 14, 15].

Cultivation conditions

The isolates were grown in media with different chemical compositions. Glucose- yeast extract peptone medium (GYP) was selected for the best fibrinolytic enzyme production and activity. The medium composition was (g/l): (1): Starch-nitrate medium (M1) : Starch 20.0, KNO₃ 2.0, K₂HPO₄ 1.0, MgSO₄ 0.5; NaCl 0.5, CaCO₃ 3.0, FeSO₄0.01, Yeast extract 1.0, Trace salt solution 1.0 ml. Trace salt solution was prepared from the following ingredients (g/100ml) FeSO₄.7H₂O (0.1); MnCl₂.4H₂O (0.1) and ZnSO₄ .7H₂O (0.1).(2): Soybean medium (M2) (16): Starch 20.0;soyabean,17.5;(NH₄)₂SO₄ 0.9; K₂HPO₄ 0.8, CaCO₃,3.0, FeSO₄0.01; MnCl₂.4H₂O 0.01 and ZnSO₄ .7H₂O 0.02. (3): Glucose-yeast extract peptone medium (GYP) (M3) - (2):
glucose 10.0, peptone 5.0, yeast extract 5.0, NaCl 5.0 and CaCl2 0.2(4); Modified glucose yeast extract medium (M4) glucose 10.0, peptone 10.0, yeast extract 2.0, K2HPO4 1.0, MgSO4 0.5; FeSO4.003; MnCl2.4H2O 0.007 and ZnSO4 .7H2O 0.004. The flasks containing medium 3 were inoculated with 1 ml of preinoculated inoculum 72h-old vegetative seed on rotary shaker (150 rev/min) at 30 oC and then the contents were centrifuged at 4000 rpm for 20 min. The mycelium pellets were subjected to dry cell weight measurements and the supernatants were assayed for fibrinolytic enzyme activity. The optimum incubation time was evaluated by varying sampling times (1, 2, 3, 4, 5, 6 and 7 days). The effect of aeration on enzyme production was also determined. The influence of the initial pH by using citrate-phosphate buffer (pH 5.5-7), sodium-phosphate buffer (pH 7.5-8.5) and carbonate-bicarbonate buffer (pH 9-10.5) of the GYP medium was studied. The effect of various carbon and nitrogen sources on fibrinolytic enzyme production was also studied.

Enzyme assay
Citrated human plasma was used for fibrin preparation which used as substrate by adding 1N/40 calcium chloride solution at 37°C with continuous stirring and left to stand for one hour. This was followed by filtration through cloth to collect fibrin, washing several times with distilled water, drying and grinding the fibrin. The fibrinolytic enzyme activity was measured by Lowry method [17]. The reaction was set in 0.5 ml of supernatant containing enzyme, 8 mg of human fibrin, 0.5 ml phosphate buffer pH 6.8. The reaction mixture was incubated for 60 min at 37°C in a water bath, then the reaction was stopped by the addition of 1 ml 5% trichloro acetic acid (TCA). Kept it for another 20 min at the same temperature. This was followed by centrifugation at 4000 rpm for 20 min then the contents were centrifuged at 4000 rpm for 20 min. The mycelium pellets were subjected to dry cell weight measurements and the supernatants were assayed for fibrinolytic enzyme activity. The optimum incubation time was evaluated by varying sampling times (1, 2, 3, 4, 5, 6 and 7 days). The effect of aeration on enzyme production was also determined. The influence of the initial pH by using citrate-phosphate buffer (pH 5.5-7), sodium-phosphate buffer (pH 7.5-8.5) and carbonate-bicarbonate buffer (pH 9-10.5) of the GYP medium was studied. The effect of various carbon and nitrogen sources on fibrinolytic enzyme production was also studied.

Results and discussion
Screening for fibrinolytic enzyme production
From twenty isolates of actinomycetes were isolated from different location of El-Gharbia Governorate (Egyptian soil). The selected actinomycete isolates in the previous experiment were individually used to inoculate flasks containing four different media for the production of fibrinolytic enzyme. The enzyme production after 4 days of incubation with shaking at 28°C was estimated. The results recorded in Table (1) indicated that medium (M3) was more suitable than other media for the production of the enzyme by strain number 4 (623.24 units / ml), in greater than those reported by [21, 22], and therefore it was used for further studies.

Table (1): Fibrinolytic enzyme of different isolated local actinomycetes on different media.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Different media</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>M1</td>
</tr>
<tr>
<td>1</td>
<td>238.3±0.017</td>
</tr>
<tr>
<td>2</td>
<td>54.18±0.024</td>
</tr>
<tr>
<td>4</td>
<td>407.8±0.006</td>
</tr>
<tr>
<td>5</td>
<td>531.72±0.008</td>
</tr>
<tr>
<td>6</td>
<td>291.9±0.12</td>
</tr>
<tr>
<td>8</td>
<td>111.3±0.37</td>
</tr>
<tr>
<td>9</td>
<td>214.2±0.02</td>
</tr>
<tr>
<td>10</td>
<td>599±0.14</td>
</tr>
<tr>
<td>11</td>
<td>4630.05</td>
</tr>
<tr>
<td>12</td>
<td>513.54±0.015</td>
</tr>
</tbody>
</table>

Identification of most strain
Out of 12 actinomycete isolates organism No. 4 was the most active for production of fibrinolytic enzyme. It was subjected to further studies in order to be characterized and identified. The identification study was performed basically according to many different international keys [12, 13, 14, 15] were followed. Bergey's Manual of Systematic Bacteriology [23] and Bergey's Manual of Determinative Bacteriology [24] were being the references of identification. Identification process of the actinomycete isolates was performed to the species level according to their specific characteristics that will be given hereafter. On the basis of the previous characters of isolate No. 4 and comparing with those of the closest reference strains, this isolate showed to be similar to Streptoverticillium mobaraense GH4. Thus, it could be concluded that the actinomycete isolate No. 4 is...
Suggestive of being related to *Streptovercillium mobaraeence* GH4 and thus could be given the name *Streptovercillium mobaraeence* GH4. Fig. 1 (A&B) and Fig. 2 (A&B).

**Effect of cultural condition**

**Inoculum age and Size**

In the present work, we aimed to study optimal conditions for this enzyme production and the results are shown in Table 2. The age of *S. mobaraeence* GH4 used for inoculation of shake flasks seemed to be an important factor for enzymes production. Results obtained indicated that 3 days-old inoculum was the most suitable for the highest enzyme production (902.08 units/ml). In addition 1 ml (v/v) inoculum size supported maximum enzymes production (908.02 units/ml). Several authors studied different effects of inoculum on the production of enzymes by several microorganisms. [25] on *Bacillus subtilis* LD-8547 and [26] on fibrinolytic enzyme from *Bacillus sp. S-05*.

**Incubation period**

Studies on the effect of incubation period on growth and production of fibrinolytic enzyme by *S. mobaraeence* GH4. Data Table (3): showed that there was a gradual increase for both growth and enzyme production by increasing incubation period reaching their maximal activity (908.24 units/ml) after five days of incubation and decreased thereafter. These results were in accordance with [22] who reported that the suitable incubation period for production of fibrinolytic enzyme from *Streptomyces* sp. NRC 411 was seven days. Also, it was found the same result from *Shizophyllum Commune* BL23 by [27]. On the other hand, the use of 3 days and six days incubation period for fibrinolytic enzyme production by *Bacillus amyloliquefaciens* and *Rhizomucor miehei* had been reported by [7] and [11].

**Air: medium ratio**

From the present study the effect of air: medium ratio on enzyme production, data table 4: showed that the flasks containing 50 ml medium were the most effective because it gave the highest fibrinolytic enzyme yield (910 units/ml). However, increasing medium volume decreased the fibrinolytic enzyme yield. This observation may be due to that oxygen was very often the principal limiting factor in the process, since it had a substantial influence on the growth of microorganism and on its metabolism [28]. Our result is in agreement with the results of [25] on *Bacillus subtilis* LD-8547. While, it was disagreement with those obtained by [29] with *Bacillus vallismortis* and [26] with *Bacillus sp. S-05*. They found that the 30 ml medium volume was optimum.
Table 4: Effect of air: medium ratio on fibrinolytic enzyme activity.

<table>
<thead>
<tr>
<th>Air : medium ratio (v/v)</th>
<th>Final pH</th>
<th>Fibrinolytic activity (units/ml)</th>
<th>Growth (dry weight g/50 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>235 : 15</td>
<td>8.26±0.017</td>
<td>504.00±0.33</td>
<td>0.250±0.008</td>
</tr>
<tr>
<td>220 : 30</td>
<td>8.28±0.008</td>
<td>756.00±0.33</td>
<td>0.130±0.003</td>
</tr>
<tr>
<td>200 : 50</td>
<td>8.18±0.0015</td>
<td>910.00±0.15</td>
<td>0.145±0.008</td>
</tr>
<tr>
<td>175 : 75</td>
<td>8.19±0.0012</td>
<td>613.20±0.047</td>
<td>0.239±0.01</td>
</tr>
<tr>
<td>150 : 100</td>
<td>8.27±0.005</td>
<td>571.20±0.037</td>
<td>0.150±0.001</td>
</tr>
</tbody>
</table>

pH medium
The initial pH of growth medium was adjusted to various pH values ranging from 5.5 to 10.0 using different buffers like citrate-phosphate buffer, sodium-phosphate buffer and carbonate-bicarbonate buffer. Results of enzyme production are illustrated in Fig. (3). From the results, it could be noticed that the fibrinolytic enzyme activity was increased with increasing in pH value until reaching their maximum level (914.08 units / ml) at buffered pH 8. Buffered pH 7.5 was the best for maximum growth. In our study, fibrinolytic enzyme produced by *S. mobaraense* GH4 was favoured by slightly alkaline pH 8.0 of sodium phosphate buffered medium thereafter a slight decrease was noticed at higher pH values Fig.(3). In addition, this optimum pH was correlated with highest growth yield. In accordance with our results, [30, 3] reported that for fibrinolytic enzyme production by *B. amylophilum fasicatus & Streptomyces megalosporus* SD5, the best initial pH was 8.0 also [11] for *Rhizomucor miehei*. In addition, the use of acidic pH 6.0 for production of fibrinolytic enzyme by *Schizophyllum Commune* BL23 was also reported by [27] [22] who found that the optimum pH for fibrinolytic enzyme production from *Streptomyces* sp. NRC 411 was 6.0. Whereas, initial pH 7.0 was the most suitable for fibrinolytic enzyme production from *Streptomyces* sp. NRC 411 was 6.0. Whereas, initial pH 7.0 was the most suitable for fibrinolytic.

Carbon sources
In this experiment, six different carbon sources were added separately to basal medium at a final concentration of 1%. The results in Fig. (4) showed that the highest fibrinolytic enzyme activity was produced in the presence of L-sorbose as a sole source of carbon, although lower enzyme production was obtained in the presence of other carbon sources. The results showed that fibrinolytic enzyme produced by *S. mobaraense* GH4 was found to be constitutively synthesized and the highest enzyme production (953.4 units/ml) was obtained with L-sorbose (2%) as a sole source of carbon due to L-sorbose increased the permeability of the cell wall so, increased the enzyme production. Although, a lower enzyme yield was noticed in the presence of sucrose, starch, D-galactose, L-maltose D-glucose and D-fructose (571.62, 569.10, 615.72, 676.20, 842.27, 796.32 u/ml, respectively). Best growth was attained using glucose as C-source. According to these results it was necessary to study the effect of using different concentrations of L-sorbose in the growth and enzyme activity. Fig. (5) showed that the highest fibrinolytic enzyme production was obtained at 2% concentration of L-sorbose but 0.5% L-sorbose was the best for maximum growth. Therefore, L-sorbose at concentration of 2% was used as a sole carbon source for enzyme production in further studies. Results of the used different carbon sources for fibrinolytic enzyme production were in disagreement with the others found in literature. In this respect, [31] used starch as a sole carbon source in medium for production fibrinolytic enzyme by *Actinomyces fradiae*, [32, 4] used glucose (1%) for production of fibrinolytic enzyme from *Penicillium chrysogenum* and *Streptomyces* sp.DPUA1576, also [3, 25] used glucose while [33, 7] used maltose as carbon source for production of fibrinolytic enzyme.

![Fig 3: Effect of buffered pH of the culture medium on the enzyme activity.](image3)

![Fig 4: Effect of different carbon sources on the fibrinolytic enzyme activity.](image4)

![Fig 5: Effect of different L-sorbose concentrations on fibrinolytic enzyme activity.](image5)
**Nitrogen Sources**

Different organic and inorganic nitrogen sources were added separately to basal medium instead of its nitrogen on such amount that the final concentration of nitrogen base in medium remained unchanged. It is worthy to mention that this medium was supplemented with the appropriate carbon source. The obtained results in Fig. (6) showed that yeast extract was the best nitrogen source for enzyme production by strain under study (968.84 units/ml). Lower fibrinolytic enzyme production were observed when NaNO₃, KNO₃, (NH₄)₂H₂PO₄ or (NH₄)₂SO₄ was used as a sole nitrogen source in growth medium. In addition, all organic and inorganic nitrogen sources tested supported good growth for *S. mobaraense* GH4. According to these data, it was necessary to study the effect of different concentrations of yeast extract on growth and enzyme formation. Results, Fig. (7), indicated that yeast extract at final concentration of 2% was found to be the most suitable concentration for growth and enzyme production (977.60 units/ml). In addition, the was found to be the most suitable concentration for growth (7), indicated that yeast extract at final concentration of 2% expressed good growth for *S. mobaraense* GH4. According to these data, it was necessary to study the effect of different concentrations of yeast extract on growth and enzyme formation. Results, Fig. (7), indicated that yeast extract at final concentration of 2% was found to be the most suitable concentration for growth and enzyme production (977.60 units/ml). In addition, the use of higher concentrations of yeast extract was not so useful for both growth and enzyme formation. The use of yeast extract as a sole nitrogen source in medium for the production of fibrinolytic enzyme from culture broth of *S. mobaraense* GH4 was also reported by [3]. Results of the used different nitrogen sources for fibrinolytic enzyme production were in disagreement with others found in literature. In this respect, [31, 7] used soybean as a sole nitrogen source in medium for production fibrinolytic enzyme by *Actinomyces fridei* and *Bacillus amyloliquefaciens*, [22] used casein (1%) as suitable nitrogen sources for the production of fibrinolytic enzyme produced by free cells of *Penicillium chrysogenum*, also [4] used ammonium nitrate as a sole nitrogen for fibrinolytic production by *Strptomyces* sp. DPUA1576.

**Conclusion**

Our results indicate the existence of a novel enzyme excreted by *S. mobaraense* GH4 with an alkaline pH. However, extensive purification and properties of the enzyme studies are required to confirm this conclusion.

**Reference**


