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Moataza MS
Department of Microbial Chemistry, National Research Center, Dokki, Cairo, Egypt.

El-Shanshory A
Department of plant, Tanta University, Cairo, Egypt.

Ghada SE
Department of plant, Tanta University, Cairo, Egypt.

Correspondence
Moataza MS
Department of Microbial Chemistry, National Research Center, Dokki, Cairo, Egypt.

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 pH8.0, 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 attept 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, KNO_3 2.0, K_2HPO_4 1.0, $MgSO_4$ 0.5; NaCl 0.5, $CaCO_3$, 3.0, $FeSO_4$0.01, Yeast extract 1.0, Trace salt solution 1.0 ml. Trace salt solution was prepared from the following ingredients (g/100ml) $FeSO_4.7H_2O$ (0.1); $MnCl_2.4H_2O$ (0.1) and $ZnSO_4$.$7H_2O$ (0.1).(2): Soybean medium (M2) (16): Starch 20.0;soyabean,17.5;$(NH_4)_2SO_4$,0.9; K_2HPO_4 0.8, $CaCO_3$,3.0, $FeSO_4$0.01; $MnCl_2.4H_2O$ 0.01 and $ZnSO_4$.$7H_2O$ 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, K_2HPO_4 1.0, $MgSO_4$ 0.5;, $FeSO_4$0.003**;** $MnCl_2.4H_2O$ 0.007 and $ZnSO_4$.$7H_2O$ 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^{o}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 ^{o}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 applied the method of **[17]**. 5 ml of reagent Lowry C were added to 1 ml of supernatant and kept for 20 min at room temperature, then assaying the solubilized proteins in the supernatant using the phenol color method as described according to [18]. 0.5 ml of Folin Ciocalteu phenol reagent [19] were added to mixture and mixed immediately and left to stand for 10 min at room temperature. The phenol reagent was diluted with two volumes of distilled water before use. After 10 min the blue color was measured in a Spectrophotometer at 750 nm. One unit of enzyme is defined as the amount of enzyme which yields a color-equivalent to µ mole of tyrosine per min in the 1.0 ml of digestion mixture under the standard conditions of pH and temperature.

Statistical analysis

All experiments were carried out in triplicate and repeated twice. One way analysis of variance (ANOVA) and two way variance were used according to [20].

Results and discussion

Screening for fibrinolytic enzyme production

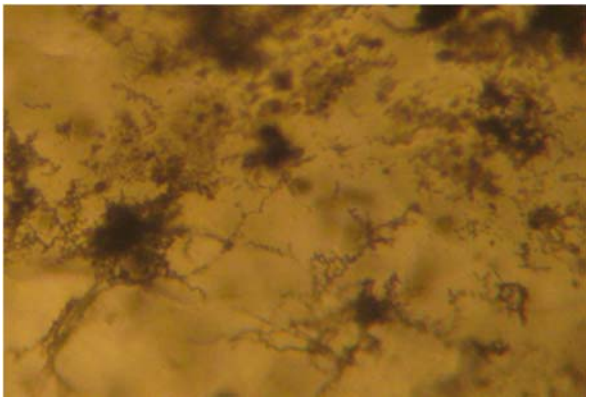
From twenty isolates of actinomycetes were isolated from different location of El-Gharbia Governorate (Egyptian soil). The selected actinomycete isolates in the previous experiment were individully used to inoculate flasks containing four different media for the production of fibrinolytic enzyme. The 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.

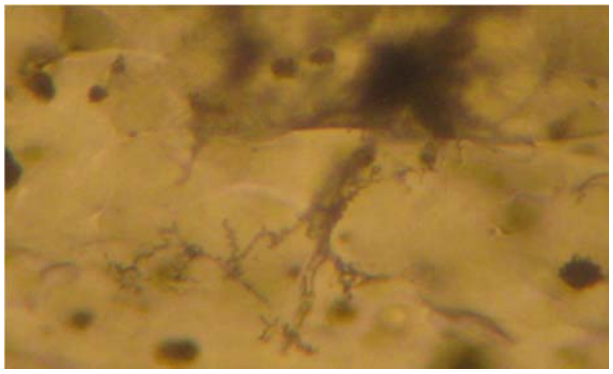
Isolate No.	Different media			
	M1	M2	M3	M4
1	238.56±0.017	567±0.035	602.2±0.021	321.3±0.021
2	54.18±0.024	378±0.028	533.4±0.015	132.72±0.029
4	407.82±0.006	470.82±0.014	623.24±0.012	388.68±0.030
5	531.72±0.008	463.68±0.011	571.16±0.026	253.68±0.023
6	291.9±0.12	606.48±0.030	574.56±0.032	155.4±0.014
8	111.3±0.37	205.8±0.011	463.68±0.031	257.04±0.011
9	214.2±0.02	362.96±0.012	357±0.034	152.88±0.020
10	599.±0.14	540.12±0.014	449.4±0.026	279.3±0.033
11	4630.035	560.56±0.024	546.9±0.031	402.89±0.015
12	513.54±0.015	406.34±0.011	558.98±0.030	295.87±0.012

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



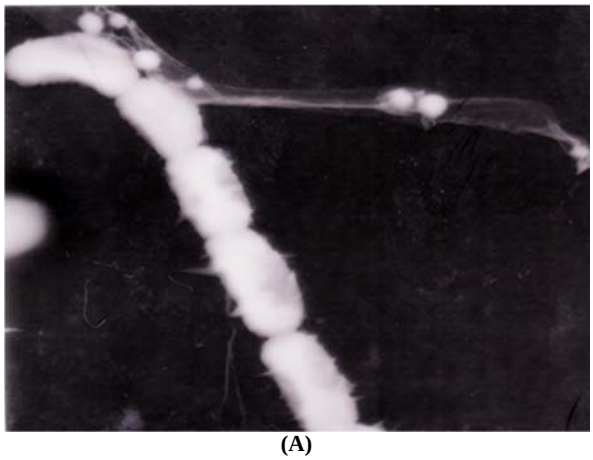
(A)



(B)

Fig 1: (A&B): Light micrograph of the selected actinomycete isolate No. 4 grown on medium (M1) for 7 days at 25 °C (x10)

Suggestive of being related to *Streptoverticillium mobaraense* GH4 and thus could be given the name *Streptoverticillium mobaraense* GH4.Fig.1 (A&B) and Fig.2 (A&B).



(A)

(B)

Fig 2 (A&B): A: Photograph of transmission electron microscope showing the spore surface of the isolate No.4 (x20.000), B: Photograph of transmission electron microscope showing spore chain surface of the isolate No. 4 (x25.000)

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.mobaraense* 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 subtillis* LD-8547 and [26] on fibrinolytic enzyme from *Bacillus sp.S-05*.

Table 2(A): Effect of inoculum age on fibrinolytic enzyme activity.

Inoculum age (days)	Fibrinolytic activity (units/ml)	Growth (dry weight g/50 ml)
2	714.00±0.003	0.233±0.04
3	902.08±0.0011	0.165±0.008
4	806.4±0.0017	0.386±0.054
5	772.8±0.015	0.210±0.025

Table 2(B): Effect of inoculum size on fibrinolytic enzyme activity.

Inoculum size (ml)	Fibrinolytic activity (units/ml)	Growth (dry weight g/50 ml)
1	908.02±0.037	0.45±0.052
2	902.24±0.029	0.26±0.023
3	850.00±0.003	0.21±0.030

Incubation period

Studies on the effect of incubation period on growth and production of fibrinolytic enzyme by *S. mobaraense* 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].

Table 3: Fibrinolytic enzyme activity at different incubation periods.

Incubation period (days)	Fibrinolytic activity (units/ml)	Growth (dry weight g/50ml)
1	210.00±0.35	.101±0.0008
2	354.48±0.008	0.11±0.017
3	616.56±0.027	0.13±0.003
4	880.32±0.03	0.15±0.003
5	908.24±0.011	0.165±0.008
6	523.32±0.002	0.235±0.002
7	504.00±0.04	0.3±0.006

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.

Air : medium ratio (v/v)	Final pH	Fibrinolytic activity (units/ml)	Growth (dry weight g/50 ml)
235 : 15	8.26±0.017	504.00±0.33	0.250±0.008
220 : 30	8.28±0.008	756.00±.33	0.130±0.003
200 : 50	8.18±0.0015	910.00±0.15	0.145±0.008
175 : 75	8.19±0.0012	613.20±0.047	0.239±0.01
150 : 100	8.27±0.005	571.20±0.037	0.150±0.001

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 r 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. amyloliquefaciens & Streptomyces megasporus* 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.

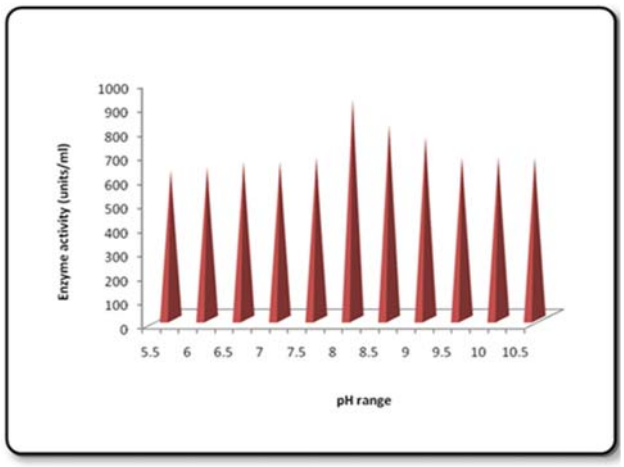


Fig 3: Effect of buffered pH of the culture medium on the enzyme activity.

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.

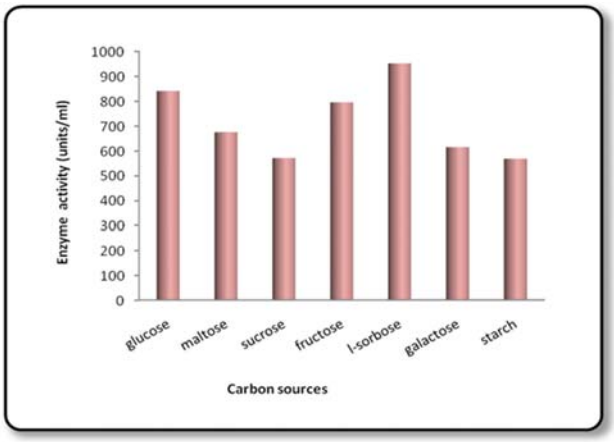


Fig 4: Effect of different carbon sources on the fibrinolytic enzyme activity.

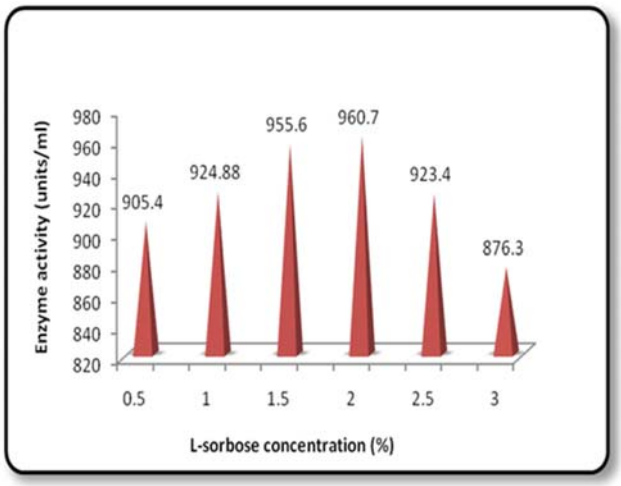


Fig 5: Effect of different L-sorbose concentrations on fibrinolytic enzyme activity.

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(968.84units/ml). Lower fibrinolytic enzyme production were observed when $NaNO_3$, KNO_3, $(NH_4)H_2PO_4$ or $(NH_4)_2SO_4$ 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 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 fradiae* and *Bacillus amyloliquefaciens*. [32] 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.

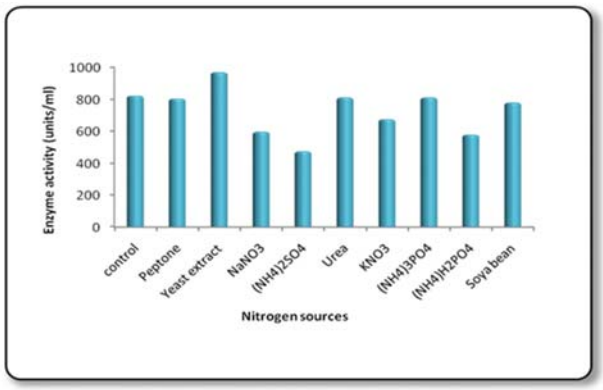


Fig 6: Effect of different nitrogen sources on the enzyme activity

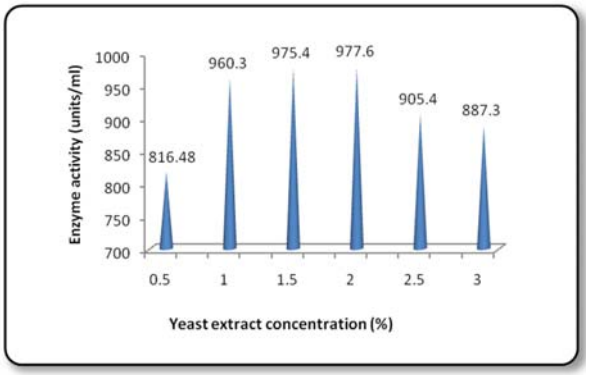


Fig 7: Effect of different concentrations of yeast extract on enzyme activity.

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.

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