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PRODUCTION AND CHARACTERIZATION OF NOVEL FIBRINOLYTIC ENZYME FROM DIFFERENT SOIL FUNGAL SP.

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ABSTRACT

Different fungal species, *Aspergillus niger, Aspergillus flavus* and *Pencillium notatum* selected for the production of extracellular fibrinolytic enzymes, Production rate was enhanced by using different carbon source where maltose increased the production rate of enzymes 2.4mg/ml in *Aspergillus niger,* 2.2mg/ml *Aspergillus flavus* and glucose 2.2mg/ml in *Pencillium notatum*. Enzyme was fractionated using ammonium sulfate fractionation, different characteristic studies like protease activity showed 1.4units/mg of proteins in *Aspergillus niger,* 1.0 units/mg of proteins in *Aspergillus flavus*, 1.3units/mg of proteins in *Penicillium notatum*. Fibrinplate method showed 200 µg/ml, 600 µg/ml, 240 µg/ml in *Aspergillus niger, Aspergillus flavus*, *Penicillium notatum* respectively and anticoagulation clotting time assay showed 25, 20, 15 min delay in clotting activity in *Aspergillus niger*, *Penicillium notatum, Aspergillus flavus* respectively. The Protein bands found on SDS-PAGE for fibrinolytic enzymes from different fungal sp were approximately 14kD from *Penicillium notatum*, 27kD from *Aspergillus niger*, 26kD from *Aspergillus flavus*. The current research showed fibrinolytic enzyme enhancement in maltose and glucose and its characterization specified that be a novel method for yield enhancement.

KEYWORDS: Fibrinolytic enzymes, anticoagulant, fibrin, SDS-PAGE, fungi

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INTRODUCTION

Hemostasis is a complex process obtained through an optimal balance between bleeding and clot formation. Blood clot is composed of fibrin. Fibrin is the main protein component of blood clot and is normally formed from fibrinogen by the action of thrombin. These fibrin clots are dissolved by the hydrolysis of plasmin, which is activated from plasminogen by tissue plasminogen activator [1]. The hydrolysis of fibrin is also known as fibrinolysis. The abnormal clot, called thrombus with in the vascular system obstructs the blood flow. It causes a variety of diseases such as Myocardial Infraction or other cardiovascular diseases and stroke [2]. Fibrinolytic enzymes can be found in a variety of foods such as Tofuyo, Korean Chung-Jang, edible honey and mushroom [3, 4]. The currently used thrombolytic agents include enzymes such as urokinase and tissue pasminogen activators. These enzymes are expensive, thermolabile but these produce undesirable side effects, exhbit low specificity for fibrin and are also relatively expensive [5]. Therefore the searches for other fibrinolytic enzymes from various sources are being continued. Microorganism ate important sources of thrombolytic agents. Streptokinase produced by *Streptococcus hemolyticus* and Staphylokinase produced by *Streptococcus aureus* were proved to be more effective for thrombolytic therapy [6]. In recent years various fibrinolytic enzymes produced by different microorganisms were in succession discovered [7]. The present study investigated the effect of carbon source on production of fibrinolytic enzymes, purification and biochemical characterization of fibrinolytic enzyme from different soil fungus *Aspergillus niger, Aspergillus flavus, Penicillium notatum*.

MATERIALS AND METHODS

a. Soil sampling and isolations [8].

Soil samples were collected from the garden of Shridevi Institute of Engineering & Technology campus, Tumkur, Karnataka, India, during January, 2011. Soil was taken from the 10cm depth and soil was sieved and dried for 3-5d at 18^0C. Serial dilution method was used for the isolation of fungus. A dilution series was made up to 10^{-6}. Aliquots (0.5 ml) and spread onto (9.75 g/liter) PDA plates. The plates were incubated at 30^0C for 5 days. Resulting colonies were screened using standard mycological keys and cultured on PDA plates.

b. Identification of fungi [9, 10, 11]

For characterization of the fungal isolates, slides were prepared from cultures and stained with lactophenol blue stain and examined with a bright-field and phase-contrast microscope. Identification was based on morphological characteristics such as growth pattern, hyphae, colour of colony and medium, surface texture, margin character, aerial mycelium, mechanism of spore production and conidial characteristics, using standard identification manuals.

c. Mass production and culture conditions

The basal medium is modified Czapek dox medium, consisted of the following ingredients (g/l) 5; casein, 5; sucrose, 30; KH_2PO_4, 1.0; $MgSO_4$ .7H O_2, 0.5; KCl, 0.5; Fe_2SO_4 .7H_2O all the ingredients were dissolved in 1000 ml distilled water. Different carbon sources of glucose, lactose and maltose were added to respective conical flask respectively at concentrations 5g/l, 50 ml of the media were transferred to 250ml conical flask and sterilized by autoclaving at 121^0C for 20 min and cooled to room temperature. One ml of uniformly prepared spore suspension (10^5 spores ml^{-1}) from 7 days old cultures was used as inoculums,(*Aspergillus niger ,Aspergillus flavus, Penicillium notatum*) all the cultures were incubated at 35°C for 7 days.

d. Extraction of fibrinolytic enzyme

After 5 days the contents of the flasks were filtered through Whatman No. 41 filter paper discs. Culture filtrates were centrifuged at 5000*g* for 10 min and the supernatant was used as the crude enzyme.

e. Purification of Fibrinolytic Enzyme [12]

The crude enzyme (50ml) was placed in a salt-ice bath $(NH_4)_2SO_4$ was added, at varying concentration of 20-80% saturation with constant stirring under ice for 1h, precipitated protein was removed by centrifugation at 10000 rpm for 20min at 4^0c and the supernatant was discarded. The precipitated protein was dissolved in 15ml of 0.02M phosphate buffer of pH 7.0. Proteins precipitated were determined by measuring absorbance at 280 nm. Ammonium sulfate was removed using dialysis.

f. Screening for Fibrinolytic enzymes by milk clotting activity [13]

Milk clotting activity method was employed for the screening of fibrinolytic enzymes. In this method 500mg of milk powder was dissolved in 0.1 Tris- Hcl Buffer to this 0.2% of agarose was added. The solution was poured in to clean petriplate and allowed to solidify in laminar air flow. Wells was created using the gel puncher, 50µl of the extracellular product obtained from the cultured fungus (*Aspergillus niger, Aspergillus flavus, Penicillium notatum*) were added and incubated at 32^oc for 10h. To observe the lytic circle around the wells.

g. Assay of Protease activity [14,15].

Protease activities of crude and purified enzymes were measured using casein as substrate and measured the release of peptide fragments. Reaction consisting of 0.5 ml of 2 % casein, 0.5ml of 0.2M Sodium phosphate buffer of pH 7.0 and 1ml of crude enzyme, incubated for 20min at 37^0c. Reaction was stopped by the addition of 3ml of 0.15% TCA. Tyrosine was determined in the neat filtrate by measuring the absorbance at 570 nm. One fibrinolytic enzyme Unit is that amount of enzyme which liberates 1µmole of tyrosine in one min. under the assay conditions.

h. Fibrinolytic activity [16]

Fibrinolytic activity was detected by taking 2ml of Human blood (Informed consent) in 0.1MPhosphate Buffer, pH 7.4, to this 0.1% of agarose was added and 0.2% of human fibrinogen was added. It was poured into a 10cm petridish and allowed to clot. The clot was allowed to stand for 1h at room temperature. Using the gel puncher wells was created and twenty micro liters of purified enzyme solution was carefully placed into the wells. The plate was incubated for 18h at 37^0c and the diameter of the lytic circle formed was measured. In this fibrin plate method, a clear transparent region is observed where fibrin is hydrolyzed. The units of the enzyme activities were determined according to the standard curve using streptokinase as standard fibrinolytic enzyme. A series of Streptokinase standards with different dilutions 200µl - 1000µl, were spotted on the artificial fibrin plate and then incubated at 37^0c for 10 hours. The lytic area diameter of each standard was measured. Then a standard curve was established with the logarithm of different activity on the X-axis and the logarithm of the average of each lytic area diameter on the Y –axis. Therefore the acitivity of each sample can be obtained according to the diameter of its lytic area.

i. $CaCl_2$-induced clotting time assay [17]

A CaCl2-induced clotting time assay was developed for the determination of a 50% clotting time and effect on fibrin formation. The assay was performed by adding extracellular fungal extract to human plasma and clotting was induced by the addition of 0.16 M $CaCl_2$, clotting time was observed.

Preparation of plasma

Blood was collected directly from healthy volunteer (Informed consent) into a test tube containing 8% of sodium citrate solution in the proportion of one volume to 19 volumes of blood. The mixtures were immediately agitated, by gentle inversion centrifuge and separated canary yellow plasma was pooled. Three test tubes of 5ml were cleaned by immersion overnight in chromic acid. To each tubes, 0.8ml of Extracellular fibrinolytic enzymes (*Aspergillus niger, Aspergilus falvus, Penicillium notatum*) was added followed by 1ml plasma, 0.2ml of calcium chloride solution (1%) were added. EDTA was used as standard anticoagulant. The time was immediately recorded and each tube was stopped. The contents were mixed by inverting three times in such a way that the

entire inner surface of the tube was wet. The time required for clotting was determined.

j. SDS-PAGE and gel staining [18].

SDS-PAGE was carried out with the SDS tris-glycine system (discontinuous) of Polyacrylamide slab gel of 160x140x1.5 mm (length x width x thickness) dimension was used. Enzyme protein (1.0 mg/ml) dissolved in Tris-HCl buffer (*p*H 6.8) containing 0.5 per cent each of SDS and mercaptoethanol was kept in a boiling water bath for 3 min in a tightly stoppered vial. Fifty microlitres of this sample was loaded on the gel. For molecular weight determination, the standard proteins used were Bovine Serum Albumin (BSA) (66kD), Chicken ovalbumin (45kD), Soybean trypsin (21kD), and Chicken lysozyme (14kD).

RESULTS AND DISCUSSION

a. Fungal identification

After culturing the different sample on PDA media, these cultures were identified based on the morphological, conidial and staining methods using standard manuals. They were identified according to morphologically characters like woolly to cottony, flat, spreading colonies, and color of the colony may be white, black colonies. Fungal cultures was placed on a clean glass slide and dissected using a needle, stained with lactophenol blue stain, observed under microscope. Based on these three fungal species was identified, they are *Aspergillus niger, Aspergillus flavus, Penicillium notatum* (Fig 1-3).

Fungal identification

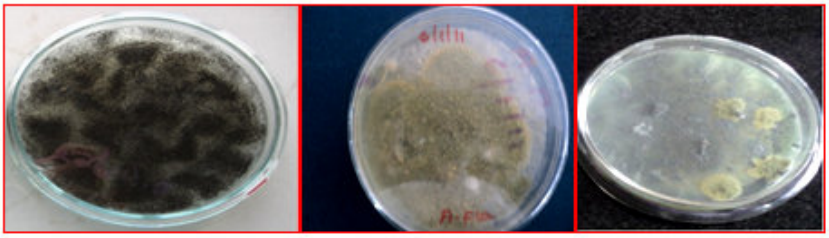


Figure 1 ***Aspergillus niger*** **Figure 2** ***Aspergillus flavus*** **Figure 3** ***Penicillium notatum***

b. Milk clotting assay for screening of fibrinolytic activity

The screening of fibrinolytic activity by milk clotting assay is the primary method to shown the presence of fibrinolytic enzymes. The assay showed that all the extracellular fungal extracts *Aspergillus niger, Aspergillus flavus, Penicillium notatum,* showed clear lytic area around the well confirms the presence of fibrinolytic activity in the fungal extract (Fig 4).

Milk clotting assay

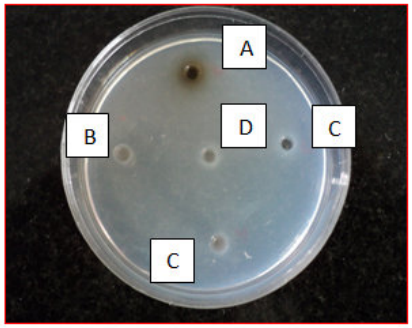
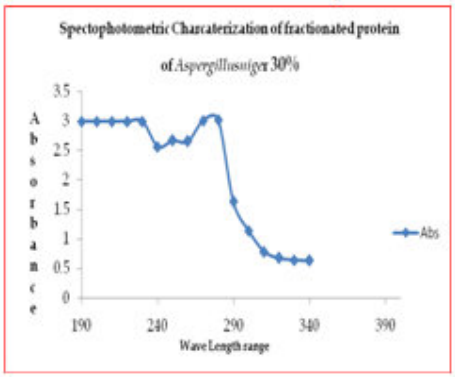


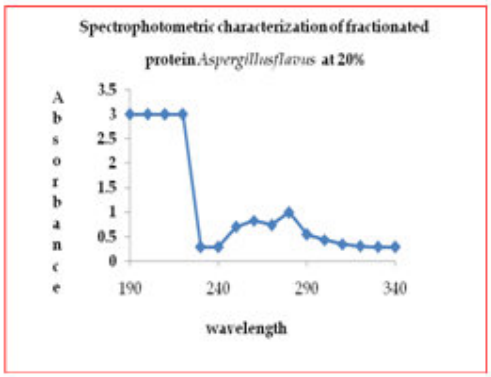
Figure 4: A
Aspergillus niger, B&D: Aspergillus flavus, C & C: Penicillium notatum

c. Purification of Fibrinolytic protease from Aspergillus niger, Aspergillus flavus, Penicillium notatum

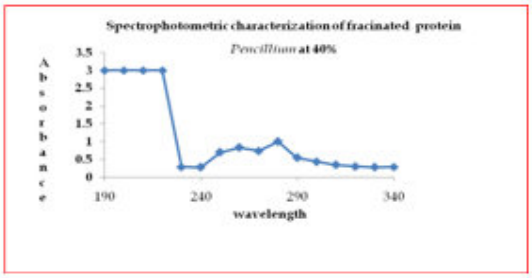
An extracellular enzyme from culture broth was separated by filtration followed by centrifugation at 5000g for 10min at 4^0c. Protein was fractionated using ammonium sulfate fractionation at different concentration from 20 to 80 %, maximum saturation at 30% obtained for *Aspergillus niger*, at 20% saturation for *Aspergillus flavus* and 40 % saturation for *Penicillium notatum.* (Graph1-Graph 3) Protein that was precipitated was confirmed by checking absorbance at 280nm, followed by dialysis to remove the ammonium salts for further studies.

Spectrophotometric characterization of Proteins





Graph 1 ***Protein from Aspergillus niger*** **Graph 2** ***Protein from Aspergillus flavus***



Graph 3 ***Protein from Penicillium notatum***

Spectrophotometric Characterization has been done to characterize the precipitated protein and the substance showed strong absorbance at 280nm confirms the presence of protein. The entire sample showed strong absorbance at 280nm. The protein was estimated using the Lowry's method by all the fungal extracts 1.3µg/ml from *Aspergillus niger*, 1.7µg/ml from *Aspergillus flavus*, 1.4µg/ml *Penicillium notatum*.

d. Assay of proteolytic enzymes

The purified sample from different fungal species was tested to breakdown casein. The results showed that all the purified sample from different fungal source have proteolytic activity. *Aspergillus flavus* showed 1.4 units mg^{-1} protein, *Penicillium* showed 1.3 units mg^{-1} protein and *Aspergillus niger* showed 1 units mg^{-1} protein (Table 1).

Tabular Column of Assay of Proteolytic Enzymes

Table 1
Assay of Proteolytic Enzymes

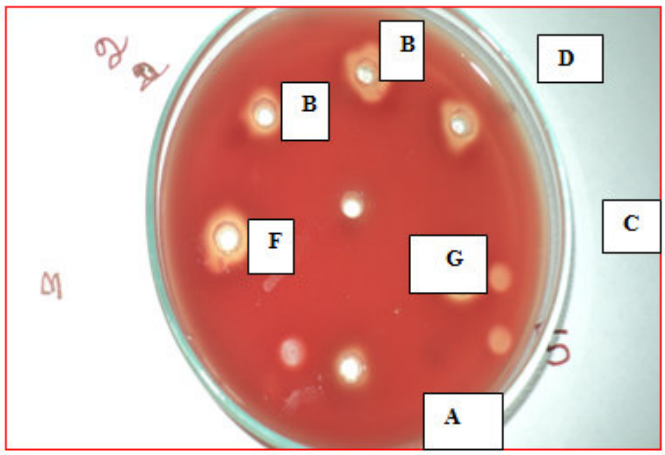
Fungi	Protein mg/ml	Specific activity (Units/mg protein) Casenolytic activity
Aspergillus flavus	1.7	1.4
Aspergillus niger	1.3	1.0
Penicillin notatum	1.4	1.3

e. *Fibrinolytic activity*

Fibrinolytic enzyme activity by fibrin plate method was done, using streptokinase as standard. In Fibrin plate method the lytic area created by the Fibrinolytic enzymes has been measured and compared with the standard Fibrin plate to check the Concentration of Fibrinolytic enzymes (Fig 5) (Table 2). The Final concentration of Fibrinolytic enzymes produced from fungal species *Aspergillus niger* 200µg/ml, *Aspergillus flavus 600* µg/ml and *Pencillium notatum* 240 µg/ml respectively (Fig 6). Graph was plotted using drug concentration vs. lytic area to detect the concentration of fibrinolytic enzyme from the fungal extracts (Graph 4).

Std Drug Fibrin Plate

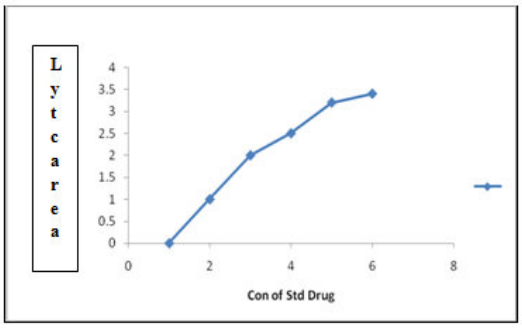
Figure 5
STD. Drug Fibrin Plate



A: 200µg/l , B:400µl, C:600µg/l, D:800µl, A:1000µg/l, G:CONTORL

Table 2
Std. Drug induced lytic area

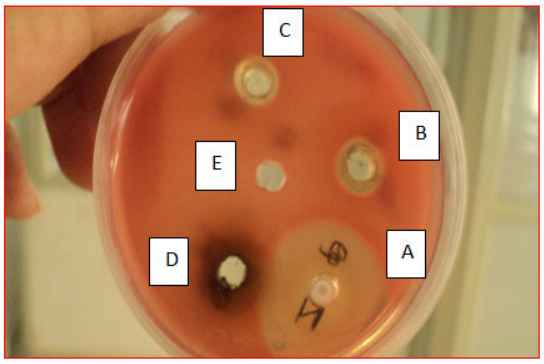
SL.No	Sample Range	Drug Conc	Lytic area
1	0.2	200µg/ml	1Mm
2	0.4	400µg/ml	2Mm
3	0.6	600µg/ml	2.5Mm
4	0.8	800µg/ml	3.2Mm
5	1ml	1000µg/ml	3.4Mm



Graph 4
STD. drug vs Lytic area

Fibrin Plate Method

Figure 6
Fibrin Plate Method



A: Std Drug, B: Aspergillus flavus, C: Penicillium notatum E: Control, D: Aspergillus niger

f. $CaCl_2$-induced clotting time assay

$CaCl_2$-induced clotting time assay important assay to evaluate the anticoagulant activity of Fungal Species. Data concerning the anticoagulant activity of different fibrinolytic enzymes shown in the table 3. Ibuprofen was used as standard anti-Coagulant. Ibuprofen showed higher anticoagulating activity than Fungal Fibrinolytic enzymes, where plasma clotting was occurred after 25Min, *Aspergillus niger* and *Penicillium not*atum fibrinolytic enzyme caused clotting for plasma after 20 and 15 min respectively in *Aspergillus flavus.*

Figure 7
$CaCl_2$ Clotting time assay

1: Plasma with standard drug, 2: Plasma 3: Plasma + Aspergillus flavus extract, 4: Plasma + Penicillium extract, 5: Plasma+ Aspergillus niger extract.

Table 3
$CaCl_2$ Induced clotting time assay.

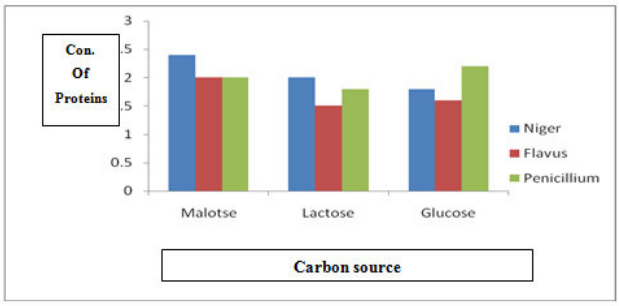
Fibrinolytic Enzymes	Anticoagulant activity
BLANK	_
Std Drug	++++++
Aspergillusniger	++++
Aspergillus flavus	+++
Penicillium	++++

No Clotting, +++++++ : Clotting after 25min
++++: Clotting after 20min +++: Clotting after 15min

Effect of Carbon source

Protein precipitated from all the carbon source, protein was estimated using Lowrys method, maximum amount of fibrinolytic enzymes (protein) that has been produced from the maltose as carbon source in *Aspergillus niger, Aspergillus flavus* and glucose in case *Penicillum notatum.* The amount of protein found is 2.4mg/ml from *Aspergillus nige*r in maltsoe, 2.2mg/ml from *Aspergillus flavus* in maltose and 2.2mg/ml from *Penicillium notatum* in glucose (Graph 5).

Effect of Carbon source

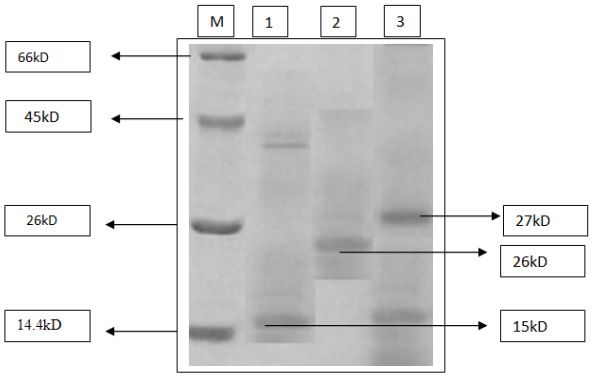


Graph 5
Effect of Carbon source

SDS-PAGE

The protein bands found on SDS PAGE for fungal extract were approximately 15kD from *Penicillium notatum*, 27kD from *Aspergillus niger*, 26kD from *Aspergillus flavus* (Fig 7).

Figure 8
SDS PAGE



M: Marker, 1: Penicillium notatum, 2: Aspergillus flavus 3: Aspergillus niger

CONCLUSION

From the current research work we found that three fungal Spp. selected *Aspergillus niger, Aspergillus flavus* and *Penicillium notatum* were capable of producing fibrinolytic enzymes. Milk clotting assay showed the presence of fibrinolytic activity. The production of fibrinolytic enzymes was enhanced by using different carbon source, Maltose, lactose and glucose. In carbon source, maltose showed the 2.4mg/ml of fibrinolytic enzymes in *Aspergillus niger* and 2.2mg/ml in *Aspergillus flavus*. Glucose showed high production rate in 2.2 mg/ml in *Penicillium notatum* which was relatively higher comparable to 64mg/l in *Bacillus sphaericus* [19]. The extracellular fibrinolytic enzyme was precipitated using ammonium sulfate fractionation where maximum amount of protein precipitated at 20, 30, 40% respectively in *Aspergillus niger, Aspergillus flavus* and *Penicillium notatum*. The presence of Fibrinolytic enzyme was proved in proteolytic assay where that was the index for fibrinolytic activity. $CaCl_2$ induced clotting time assay also provided the good result where coagulation time was delayed with the use of fibrinolytic enzyme in compared to the Standard drug (Ibrofen).where the clotting was delayed for 20 and 15 min than compared with Standard drug having 25Min delay in Coagulation. SDS-PAGE of fibrinolytic enzymes was done to determine the molecular weight of Fibrinolytic enzymes along with other proteins. SDS PAGE showed the presence of 14kD from *Penicillium notatum*, 27kD from *Aspergillus niger* and 25kD from *Aspergillus flavus.* Earlier report showed 52kD size from *Cordyceps militarys* [20]. Finally we concluded that fibrinolytic enzymes produced using different fungal spp. provides good source for fibrinolytic enzymes that can be produced in high quality and quantity in short time by using maltase and glucose and its characterization by above mentioned assay specify that over all activity is too good and may be considered for large scale production.
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