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Optimization of Culture Conditions for the Production of Endoglucanase from *Aspergillus sydowii* using Corn Cobs

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ABSTRACT

Endoglucanase is one of the important industrial enzymes that can be produced from various microorganisms. *Aspergillus sydowii* is a thermophillic fungus that was utilized for the production of endoglucanase. Present study aimed to optimize different culture conditions; temperature, incubation period, pH, moisture level and inoculum size, for maximum enzyme production using agricultural wastes. Three substrates, corn cobs, sugarcane bagasse and rice straw were used in this study. Maximum endoglucanase production was observed from corn cobs, with enzyme activity of 2.01 IU/mL/min after 96 hrs of incubation at 70% moisture level (2.71 IU/mL/min), temperature 55°C (2.98 IU/mL/min) and pH 5.5 (IU/mL/min) and 3mL inoculum size (3.15 IU/mL/min). Sugarcane bagasse and rice straw found to be poor sources with enzyme activity 1.87 IU/mL/min and 1.99 IU/mL/min respectively.

Keywords: Endoglucanases, Rice Straw, *Asperillus sydowii*, Culture conditions

INTRODUCTION

Lignocellulose is the most abundant and renewable biological source of fermentable sugars on biosphere, which has been produced in large amount as agricultural and industrial wastes. It has the potential to be converted it into many useful by-products like human nutrients, biofuel, animal feed with improved quality and other chemicals by enzymatic hydrolysis (Han and Chen, 2010).

Cellulose is a major biopolymer constituent of plant cell walls, that consists of linear chain of D- glucose linked through β (1→4) glycosidic linkage (Jarvis, 2003). Crystalline structure of cellulose makes it difficult to hydrolyze by normal process and to convert into different useful products. Chemical and physical properties of lignocellulosic materials make them good substrate for different biotechnological processes (Malherbe and Cloete. 2003). Most of the hydrolysis of lignocellulosic material is due to synergistic action of a group of cellulolytic enzymes; most important are endo-glucanase, exo-glucanase and β-glucosidase (Bhat, 2000). Endoglucanases, randomly breaks down internal glycosid linkages of the amorphous region of cellulose, releasing polysaccharides with lower degrees of polymerization (DP) than the parent fiber, as well as soluble oligosaccharides, whereas exoglucanase also known as cellobiohydrolases act on reducing and non-reducing ends of cellulosic fibers and releases glucose molecules directly (Teeri *et al*., 2000).

Due to great importance of cellulases in industrial areas, these are mainly produced by fungi and bacteria. Fungal cellulases are mainly produced by *Trichoderma* and *Aspergillus* sp. One of the major producers for endoglucannases is the thermophillic fungus *Aspergillus sydowii,* which is found worldwide and can be easily isolated from air, soil, desert soil, mangrove swapms, sewages and foodstuffs. This is rarely an opportunistic human pathogen causing nail infections (onchomycosis) (Shinn *et al*., 2000). Keeping in view the great importance of endoglucanases in different industrial processes, present study was designed to optimize different physical parameters for enhanced production of endoglucanase from *Aspergillus sydowii*.

MATERIALS AND METHODS

Substrate Preparation

Corn Cobs, sugarcane baggase and rice straw were used as a lignocellulosic substrate for the production of endoglucanase. The substrate was dried at 70°C, ground to

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a powdered form and then meshed with 40mm sieve. It was then stored in air fixed synthetic jar for a later use in the fermentation medium.

Fermentative Organism

Thermophilic *Aspergillus sydowii* isolated from temperate area of Pakistan was used for the production of endoglucanase. The colonies of *Aspergillus sydowii* were maintained on PDA media and fresh cultures were prepared every month. Fungus was stored in form of slants at 4°C a 500 mL inolculum media was made that consisted of: (Potato Starch 10g, Dextrose 10 g, urea 1.50 g, $KH2PO_4$ 0.04g, $ZnSO_4 \cdot 7H_20$ 0.005g, KCl 0.075g, $MgSO_4$. $7H_20$). (Zarofonetis, 1959). The pH of this media was adjusted to 4.5 with the help of 1M HCl / NaOH and autoclaved at 121°C for 15 minutes for sterilization. Inoculum flasks were inoculated with loopful of fungal spores aseptically. After inoculation, flasks were incubated at 50°C in shaking incubator for 72 hrs. The conidial (spores) concentration was adjusted at 10^{7-} 10^{8} spores/mL with the help of heamocytometer.

Solid State Fermentation

Solid state fermentation process was utilized for endoglucanase production by *Aspergillus sydowii.* 5g. of three grounded substrates, (corn cobs, sugarcane bagasse and rice straw) were poured in 500 ml Erlenmeyer flasks. The substrates were then moistened with 3.5 mL of mineral salt solution (gL^{-1} KH_2PO_4 5g; $(NH_4)_2SO_4$; $MgSO_4 \cdot 7H_2O$ 0.2g) and plugged with cotton. The growth medium was autoclaved for 15 minutes. The growth media was inoculated with the inoculum (2mL) and incubated at 55°C under still culture conditions (Krishna *et al*., 1996). All experimental treatments were performed in duplicates. Media was harvested after specified incubation times for the extraction of endoglucanase. 50ml distilled water (pH 5.0) was added to each flask and then placed in shaker incubator at 120 rpm for 1 hr. Enzyme extract was then filter with the aid of filter paper. Crude endoglucanase was centrifuged and the supernatant was assayed for endoglucanase activity (Shafique *et al*., 2004).

Optimization of Conditions

To get the maximum endoglucanase production from *Aspergillus sydowii,* following parameters were optimized during the study.

Optimization of the Substrate

Three substrates, sugarcane bagasse, rice straw and corn cobs were optimized for endoglucanase production by *Aspergillus sydowii* at 55°C for 5 days. Sample was harvested for enzyme activity after 24 hrs interval. Substrate that gave maximum enzyme activity was selected.

Optimization of Fermentation Period

Fermentation time was studied by growing the fungus on 5gsubstrate at 55°C in duplicates, for different time periods (24, 46, 72, 96 and 120 hrs). Sample was harvested every 24 hours. Enzyme assay of crude endoglucanase sample was performed. The sample with maximum enzyme activity was selected for subsequent study.

Optimization of Moisture Level

Less moisture level is required in SSF than LSF. Five varying moisture level were chosen to test the effect on endoglucanase production. The fungus was incubated at 55?C for 96 hrs (optimum) at five different moisture levels (50, 60, 70, 80 and 90 %).

Optimization of pH

Aspergillus sydowii was incubated at 55?C at varying pH (4.0, 4.5, 5.0, 5.5, 6.0 and 6.5) to find out the optimal pH for maximum production of enzyme. Moisture level was set at 70%.

Optimization of Temperature

Aspergillus sydowii has an ability to grow at high temperatures due to its thermophilic nature. The fungus was incubated at six different temperatures (40, 45, 50, 55, 60, 65°C) for 96 hrs, at pH 5.5 and 70% moisture level for the optimization of temperature in which *A. sydowii* give maximum production of enzyme.

Optimization of Inoculum Size

Five different inoculum sizes; 1ml, 2ml, 3ml, 4ml, 5ml were selected for the optimization of inoculum size. Fungus was incubated for 96 hrs at 55°C temperature, 5.5 pH, and 70% moisture level was adjusted to get the maximum production of enzyme.

Standard

Glucose was used as a standard because endoglucanase hydrolyzes carboxymethylcellulose to produce free carboxymethyl glucose units. Different concentrations of standard were prepared. Standard factor (S.F) was determined by taking absorbance at 540 nm for each concentration.

Enzyme Assay

1mL of enzyme solution was incubated with 1mL of CMC (1%) and 1mL ofcitrate buffer to maintain pH for thirty minutes at 50ºC. After that 3ml DNS was added and mixture was boiled for fifteen minutes and cooled immediately on ice. After cooling, absorbance was recorded 540nm using a spectrophotometer (Sherief *et al.*, 2010).One unit of enzyme activity in each case was defined as the amount of enzyme which released one μ-mole of glucose per minute.

$$\text{E.A (IU/ml/min)} = \frac{\text{Absorbance of Enzyme soln. x Standard factor x Dilution factor}}{\text{Time of incubation}}$$

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Where,

S.F = Conc. of Standard (µM/mL)
Abs. of standard at 540 nm

Statistical Analysis

All the data obtained was analyzed by ANOVA, DMRT and LSD using MSTAT"C" software.

RESULTS

Aspergillus sydowii was cultured on PDA media. Three substrates, Sugarcane baggase, corn cobs and rice straw were tested for the production of endoglucanase. Flasks containing 5g of each substrate with 2mL of fungal inoculum were incubated for 120 hrs. Samples were harvested after 24hrs time period. Maximum enzyme activity (2.01 IU/mL/min) was observed from corn cobs sample after 96 hrs of incubation. Lower enzyme activity was observed in case of rice straw while with sugarcane bagasse (1.87 IU/mL/min) it was slight lower than that of corn cobs at 72hrs of incubation period (Figure 1). Based on these results, corn cobs were selected for further experiments. Five different physical parameters (incubation period, inoculum size, temperature, and pH and moisture level) were selected to compare the production of endoglucanase.

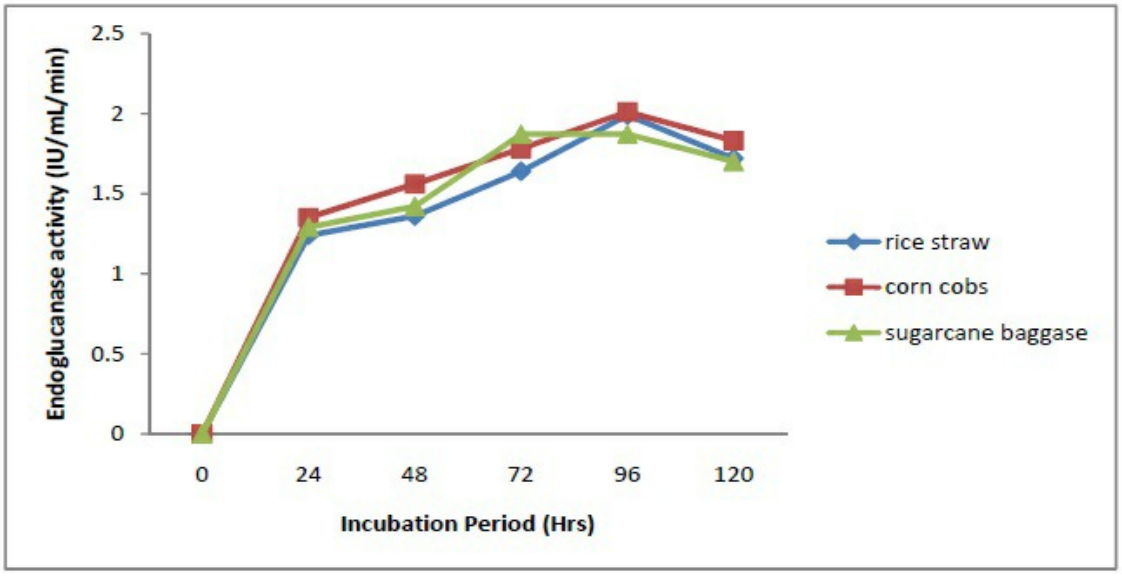

Fig.1: Optimization of substrate
0* incubation period taken as control

Optimization of Incubation Period

Incubation period plays a vital role in enzyme production. Flasks containing 5g of corn cobs and2mL of fungal inoculum were incubated at 55°C. Samples were harvested after 24hrtime intervals. Enzyme production with maximum endoglucanase activity (2.21 IU/mL/min) was observed at 96 hrs of incubation period (Figure 2).

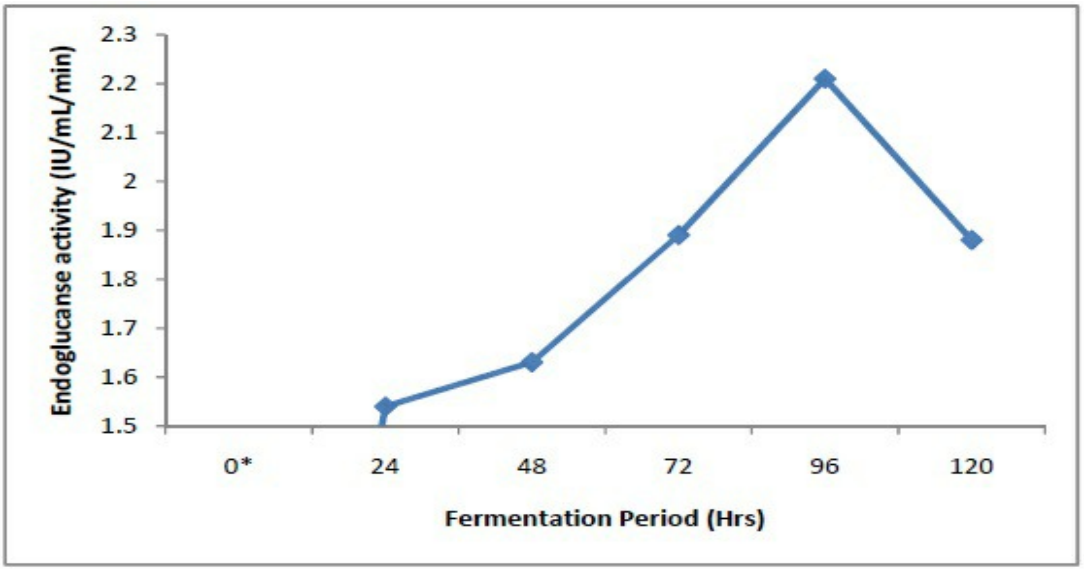

Fig. 2: Optimization of incubation period
0* incubation period taken as control

Optimization of Moisture Level

Solid State fermentation contains less moisture level than liquid state fermentation. Maximum endoglucanase activity (2.71 IU/mL/min) was observed at 70% moisture level (Figure 3). Further increase in moisture level resulted in a decrease in enzyme activity.

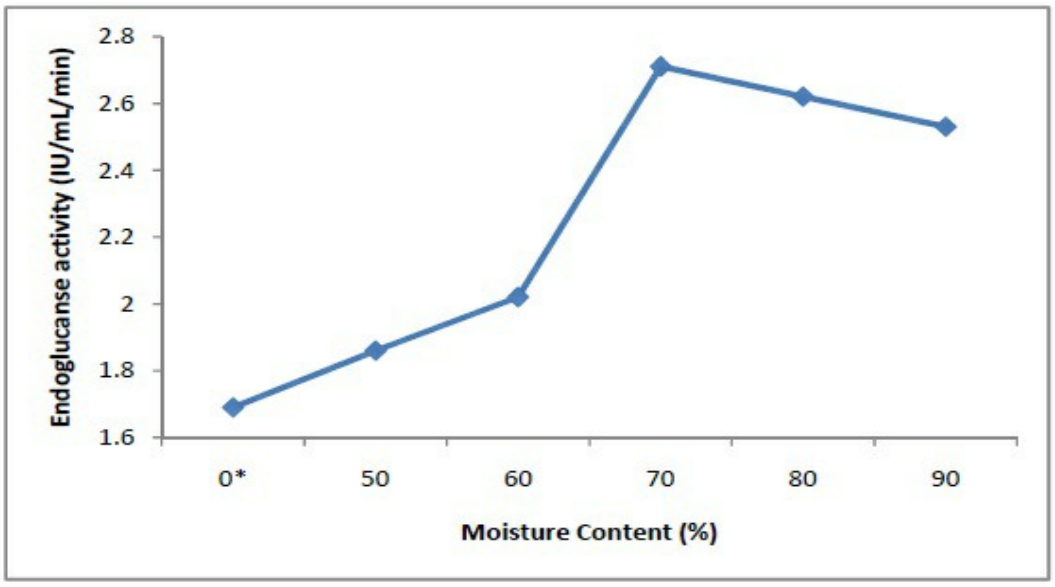

Fig. 3: Optimization of Moisture Level
*Positive control has 0% moisture level

Optimization of incubation temperature

Aspergillus sydowii was incubated with corn cobs at five different temperatures. Optimum temperature for maximum activity (2.98 IU/mL/min) was observed to be 55°C. Further increase in temperature caused as decrease in enzyme activity (Figure 4).

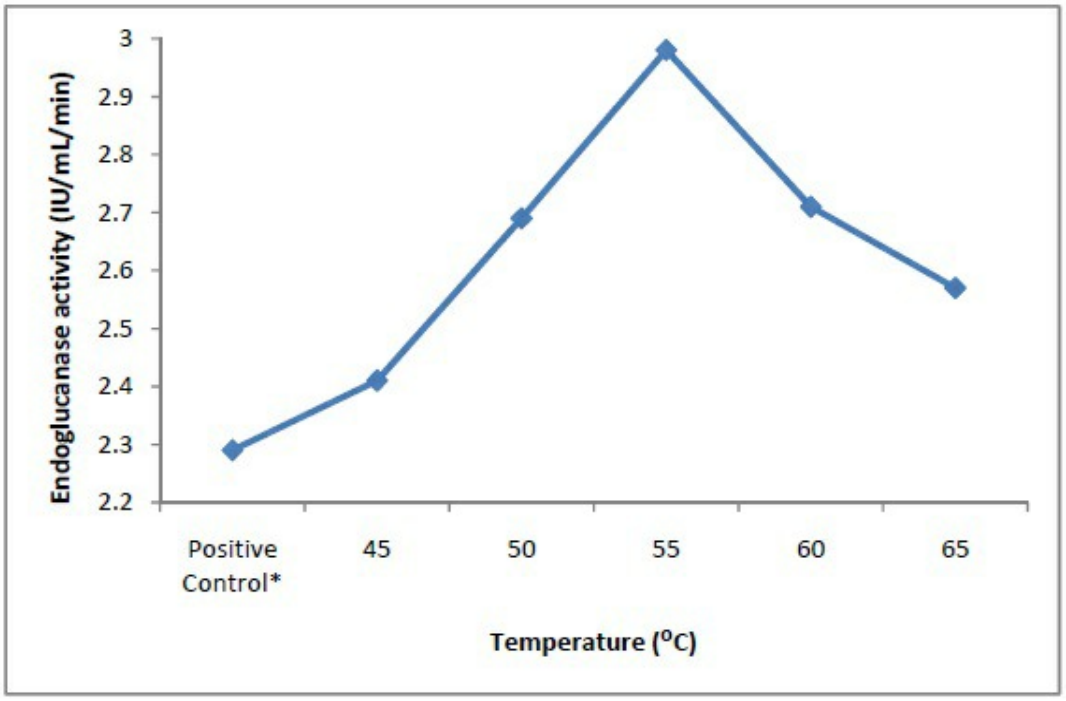

Fig. 4: Optimization of incubation temperature
*Positive control incubated at room temperature

Optimization of pH

Five different pH levels (4-6) were analyzed for enzyme production. Aspergillus sydowii produces maximum endoglucanase at pH of 5.5 with enzyme activity 2.82 IU/mL/min (Figure 5).

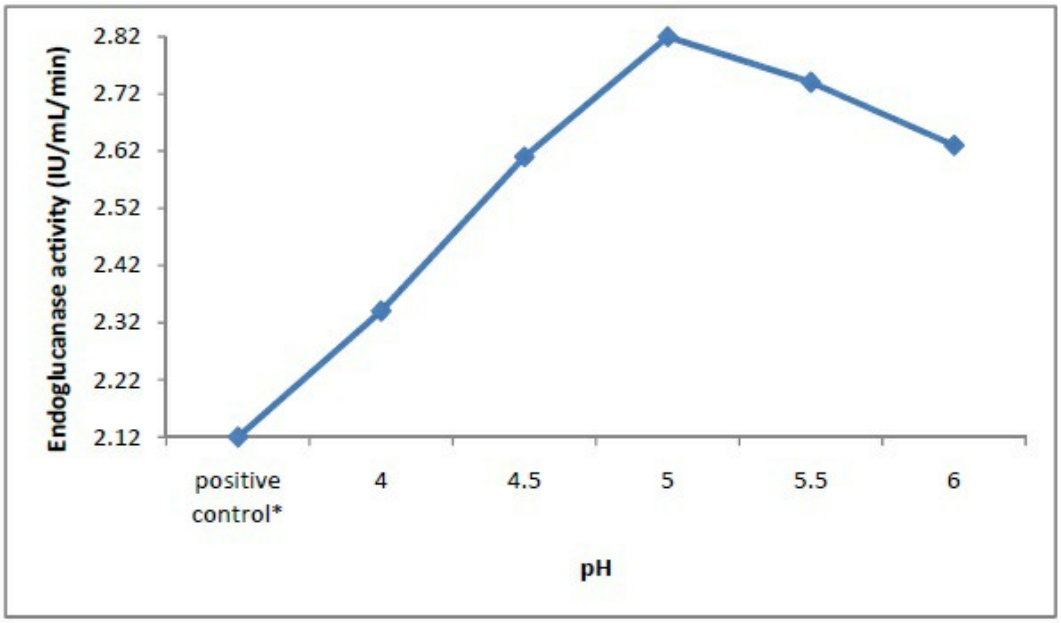

Fig. 5: Optimization of pH

*Positive control has neutral pH

Optimization of Inoculum size

Five grams of corn cobs were incubated with five different concentrations (1-5 mL) of fungal inoculum.Maximum activity (3.15 IU/mL/min) was observed with 3mL of inoculum size (Figure 6).

Fig. 6: Optimization of Inoculum size

*Positive control has 0 mL inoculum.

DISCUSSION

Endoglucanse have been utilized in many industrial processes and are most significant industrial enzymes (Hanif *et al*., 2004; Jamil *et al*., 2005). Due to its diverse applications in industries of starch processing, alcohol fermentation, malting and brewing, separation of fruits and vegetable juices, pulp and paper industry, and textile industry, researchers have deep interest in cellulose (Gao *et al*., 2008; Zhou *et al*., 2008).

The production of ethanol as a fuel from lignocellulosic biomass is a good alternative to gasoline in inner combustion engines and is one of the promising applications of cellulases (Duff and Murray, 1996). The transformation of lignocellulosic biomass to fuel ethanol is based on enzymatic decomposition of cellulose utilizing enzymes and it is one of the most effective technologies (Holker *et al*., 2004; Ahamed and Vermette, 2008). Keeping in mind the above mentioned industrial applications, the present study was designed to optimize physical parameters for the maximum production of endoglucanse from agricultural wastes e.g., corn stover, corn cobs, rice straw etc. *Aspergillus* sp. has been widely used for cellulase production. *Aspergillus sydowii* is a thermophillic fungus that, has an ability to grow at wide

range of temperature range from 50-65°C. Due to its ability to grow at high temperature, it can be utilized for endoglucanase production that will be active at high temperatures. In the present study three agricultural wastes, sugarcane bagasse, corn cobs and rice straw were optimized. Maximum enzyme production was observed with corn cobs (2.01 IU/mL/min) after 96 hrs of incubation and enzyme activity decreased with additional incubation. There was little enzyme activity observed with rice straw (1.99IU/mL/min)at 96 hrs and with sugarcane bagasse it was at 72 hrs (1.87 IU/mL/min). Further increase in incubation period causes decrease in enzyme production. Maximum enzyme production was also reported at 120 hrs by Tao *et al*., 2010 and Kavitha *et al*., 2011. Effect of moisture level was also assessed and maximum production was observed at 70% (w/w). Further increase in moisture caused a decrease in enzyme production probably due to lack of proper aeration. Jecu, 2000 also found that an increase in the early moisture content of the substrate from 55-74% significantly increased the enzyme production. Inoculum size was also as important factor and it was observed that 3mL inoculum yields the maximum amount of enzyme and these levels decreased when 4 or 5 mL inoculum volumes were used. This decrease was due to reduced availability of substrate for degradation. *Aspergillus sydowii* is a thermophillic fungus and produces maximum endoglucanase with activity (2.98 IU/mL/min) at 55°C. The reported optimum temperature of endoglucanase production is differrent from other fungal sources. Samiullah 2009 has reported the optimum temperature to be 60°C, while Elyas e*t al*., in 2010 found optimum temperature for enzyme production from *Aspergillus*-SA 58 to be 35°C. Our results indicate maximum endoglucanase production at pH 5.5 with inoculum size of 3mL.

These results are in accordance with the results reported by Tao et. al, (2010).These findings are helpful for the endoglucanase production on an industrial scale where high temperature, slightly acidic pH, and low moisture is required. Furthermore, agricultural wastes can also be utilized on large scale for endoglucanase production at low cost where availability of substrate is a big problem.

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