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Expression studies of *Bacillus licheniformis* chitin deacetylase in *E. coli* Rosetta cells

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ABSTRACT

Chitin, the biopolymer of the *N*-acetylglucosamine, is the most abundant biopolymer on the planet after cellulose. However owing to its crystalline nature, its deacetylated derivative; chitosan is industrially more potent. This conversion on an enzymatic scale can be made using chitin deacetylase. The metagenomics library constructed from the soil exposed to chitin and chitosan yielded chitin modifying enzymes, one of them being chitin deacetylase (CDA) utilized for the present study. The gene was amplified and expressed using the pET 22b vector in *E. coli* Rosetta cells. The effect of two additives; chitin and glycerol on the CDA activity were studied. The inclusion of glycerol in the medium improved the biomass by 50% from the initial value of 1.25 g/l to 2.5 g/l. The activity of CDA increased from 90 μmol/min/ml to 343 μmol/min/ml. The CDA activity reported in the present paper is the highest observed for any strain. The addition of glycerol to the media not only helped improve the yield of the chitin deacetylase but also imparted value addition to the waste of the biofuel industry.

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1. Introduction

Chitin, the second most abundant biopolymer after cellulose is a linear polymer of β-(1 → 4) linked *N*-acetyl- D-glucosamine (GlcNAc) units [1,2]. In spite of its abundance, the polymer, is not a preferable choice for industrial application due to its crystalline nature owing to which it is not soluble. This shortcoming is addressed by the modifying chitin so as to improve its hydrophilicity and reduce the crystallinity. One such modified derivative of chitin is chitosan; the deacetylated form of the polymer. Chitosan thus is a co-polymer of β-(1 → 4) linked *N*-acetyl- D-glucosamine (GlcNAc) and D-glucosamine units. Due to the reduction in the crystallinity as compared to chitin, chitosan has many biological and industrial applications [2,3]. On an industrial scale, the thermochemical alkaline deacetylation of crustacean chitin yields chitosan. In addition to being an environmentally unsafe process, batch to batch variation of the chitosan products is also observed [1,3]. Parameters such as the chain length and the degree of acetylation which influence the functional value and biological activities of the chitosan are some of the parameters which vary batch wise [3]. The alternative process to the thermochemical could be the enzymatic route employing microbial chitin deacetylase. The process is not only environmentally friendly but also the gives chitosan with consistent properties [1,3–6]. Chitin deacetylase (CDA) is the enzymes that can convert chitin to chitosan [7–10]. The first reports of the enzyme were from the fungal culture of Mucor [11]. Many studies have since reported postulating the role of CDA in different fungi *Absidia coerulea* [12], *Aspergillus nidulans* [13], *Colletotrichum lindemuthianum* [4,14], *Metarhizium anisopliae* [15], *Scopulariopsis brevicaulis* [16] and *Mortierella* sp. [17]. Studies on the presence of the CDA and Peptidoglycan de acetylase (PDA) have and also from some bacterial strains [18–20]. In addition to the roles of the CDA in bacteria and fungi, the importance of the insects has also been extrapolated [21,22]. The initial studies of scaling the CDA production were performed on *Lentinus edode* using both submerged and solid state fermentation techniques to draw a comparison between the two techniques [23]. This study later paved the way to some such studies on different fungi like *C. lindemuthianum* [24], *Rhizopus japonicus* [25], *Penicillium oxalicum* [26] where the yields improved by changing the composition of the media. Raw material costs have the largest share of the overall cost of any bioprocess. Many studies on the inclusion of industrial waste utilization as a supplement of the media to reduce this cost have yielded the inclusion of glycerol [27–29]. It is a major waste out of the bio-diesel. Yang et al. well reviews glycerol Its usage in the production of various products

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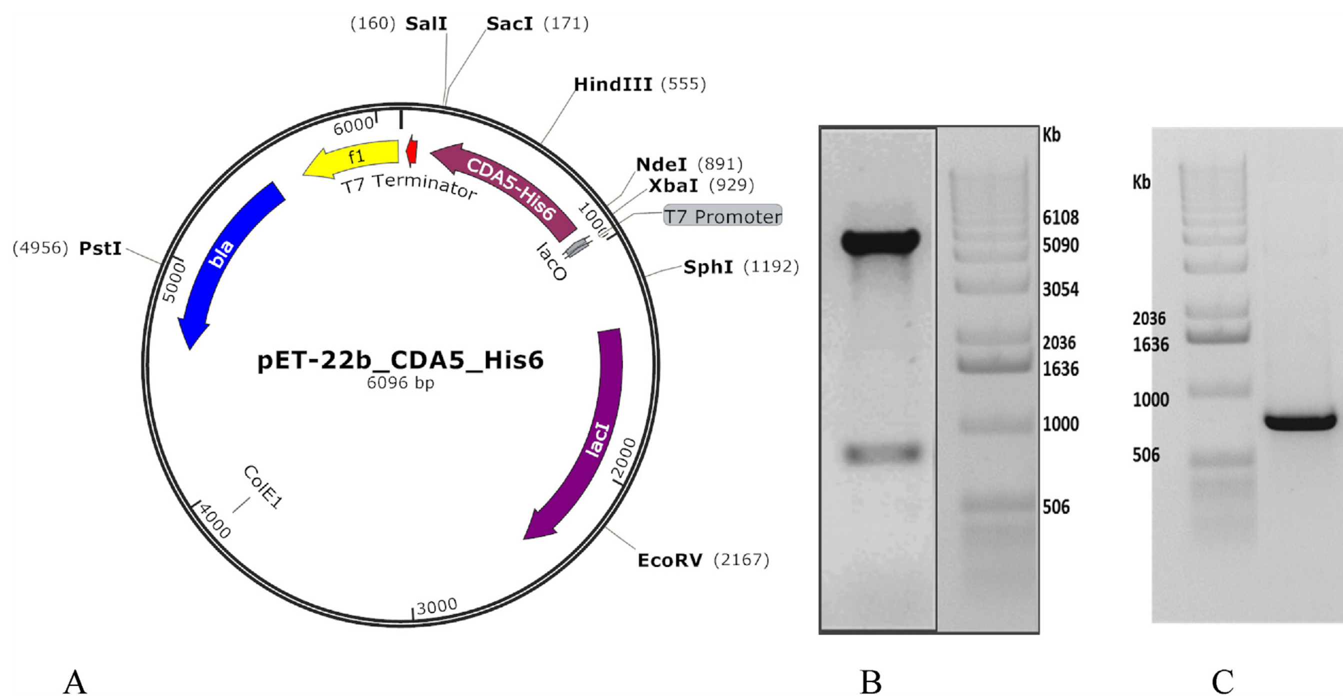

Fig. 1. The CDA gene corresponding to a size of ~750 bp was amplified from the metagenomic library and cloned into pET 22 b (+) vector between the Nde I and Sal I sites. A: The vector map of the pET 22b(+) with the chitin deacetylase gene cloned between NdeI and SalI sites downstream of the T7 promoter. B: The vector was double digested with NdeI and SalI to confirm the size of the insert. C: The amplified product of CDA gene.

such as energy chemicals, propanediol, citric acid, lipids and oils, biopolymers, recombinant protein production. [30].

In the present study, we have attempted to work on chitin deacetylase from *Bacillus licheniformis* and its scale-up using glycerol as an additive.

2. Materials and methods

2.1. Cloning of Blcda in pET22b and its expression in E. coli Rosetta pLysS

The gene for *Blcda* was a kind gift by Prof. Moerschbacher. The gene was an outcome of the metagenomics library constructed from the soil exposed to chitin and chitosan for ten years [31]. The raw sequence of the library has been submitted to the GenBank under accession No. PRJEB6317. The gene; *Blcda* is one of the sequences coding for potential chitin and chitosan modifying enzymes were identified within the metagenomic study. Briefly, the gene was amplified in a 25 μL mixture, including one μl of DNA, one μl of 10 μM of each primer (5′ GG**ATGGGC**CCAAAGCAGGAACCG 3′ and 5′ **GTCGAC**GGAGCTCGAAATTCCATCTG 3′; Sigma Aldrich India)- forward and reverse primers specific for chitin. The PCR (Eppendorf) program was set with an initial denaturation of 95 °C for two mins, followed by 30 cycles at 95 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min. The PCR product with the sites for Nde I (NEB, Germany) and SalI (NEB, Germany) incorporated was ligated with the pET 22b vector after digesting it with the same enzymes. The ligation was performed using T4 DNA Ligase (NEB, Germany) at 16 °C for 16 h. *E. coli* Rosetta pLysS (Novagen) competent cells were transformed with the ligated vector and the transformants screened on LB agar (Himedia, India) supplemented with ampicillin (100 μg/ml)(Himedia, India) and chloramphenicol (35 μg/ml)(Himedia, India). The strain of *E. coli* was chosen for higher expression of the heterologous proteins which might use rare codons. The positive colony screened was inoculated to the liquid broth supplemented with the same antibiotics. The flasks were incubated on the orbital shaker at a temperature of 37 °C and 200 rpm. The expression was induced with one mM Isopropyl-β-D-Thiogalactopyranoside (IPTG, Himedia, India) once the OD (optical density) of the host reached between 0.5–0.6. The intracellular fraction was utilized for the expression studies. For the further experiments, glycerol at a concentration of 2% was incorporated into the culture medium.

2.2. Growth and maintenance of E. coli Rosetta pLysS

The *E. coli* cells were maintained at 37 °C in LB Agar medium supplemented with 1% (v/v) commercial glycerol, ampicillin (100 μg/ml), chloramphenicol (35 μg/ml). Commercial grade glycerol was obtained from biodiesel plant of NITK premises containing 92–94% glycerol, 2–3% salts, 0.01–0.02% methanol and water.

A starter culture of 3 ml prepared in LB broth supplemented with 1% commercial grade glycerol (v/v) was inoculated with a loop of the culture from the agar plate. This plate was incubated at 37 °C until the OD of the culture reached 0.5–0.6. This culture was then used to inoculate the media for further studies. For all shake flask experiments, 15 ml of LB broth was taken in 250 ml conical flasks along with 1% glycerol (v/v) and antibiotics as mentioned above. Two more set-up one without any additives and another with 1% chitin were also made. A 2% (v/v) inoculum culture having OD of 0.5–0.6 was used for inoculation of the liquid medium. The flasks were incubated at 37 °C with shaking at 200 rpm. The samples were taken at regular time intervals for analysis of cell biomass and CDA activity. A calibration curve for biomass versus OD was prepared.

2.3. Scale-up of chitin deacetylase

The fermenter (Scigenics, India) was filled with 1.9 L LB broth containing 1% glycerol and sterilized. The concentration of the antibiotics and the preculture used were the same as in the shake flask. The temperature was set to 37 °C, and stirring was set to 200 rpm. As soon as OD reached 0.5, 2 ml of IPTG was added, and OD

of the samples were measured every hour at 600 nm. The dissolved oxygen (DO) was kept at an average of 66%, and the pH maintained around 6.6. The culture was harvested upon attaining the saturation phase. The pellet thus obtained was resuspended in 100 ml lysis buffer containing 10 mM imidazole.

2.4. Estimation of enzyme activity

The chitin deacetylase activity was measured using glycol chitin as substrate as per the modified method of Kauss and Bauch [32]. Broth samples were centrifuged at 8000 rpm for 15 min at 4 °C. The pellet was suspended in 1 ml of Tris-HCl buffer of pH 6.5 and protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM. The cell lysis was done using the probe-type sonicator (130 W, Inkarp Instruments) for 5 min with 5 s ON and 1 s OFF-pulse at 20% amplitude. The samples were kept on ice during sonication to reduce the adverse effect of rising temperature due to sonication. The sonicated samples were again centrifuged at 10,000 rpm for 15 min at 4 °C, and the supernatant was filtered through a 0.2 μm syringe filter. The filtrate was diluted appropriately and was used to study intracellular CDA activity.

250 μL of glycol chitin (1 mg/ml), 250 μL of Tris-HCl buffer (pH 6.5) and 100 μL of the diluted supernatant were mixed. This mixture was incubated at 37 °C with shaking for 1 h. The reaction of stopped by addition of 500 μL of 5% $KHSO_4$ and incubation for five minutes. Then 0.5 μL of 5% $NaNO_2$ was added to the samples. The samples were incubated overnight in a fume hood for the release of NO_2 gas. The reaction was followed by addition of 500 μL of ammonium sulphamate (12% w/v) and incubated for 10 min. After the addition of 500 μL of 0.5% MBTH (w/v), the samples were kept for 1 h incubation at room temperature. Finally 500 μL of 0.5% eCl_3 (w/v) prepared in 0.1N HCl was added to the samples. The samples were further incubated for 1 h and the absorbance of the resulting color was measured at 656 nm. The color was developed due to the coupling of MBTH with glucosamine formed due to the activity of CDA on the substrate. One Unit of the enzyme is defined as the activity which released 1 μmol of glucosamine from the Glycol chitin per minute. The enzyme activity of the sample was calculated accordingly. Absorbance readings of enzyme blank and substrate blank were deducted from absorbance readings of the sample. Standard Calibration curve was made using known concentrations of glucosamine hydrochloride.

2.5. Purification of CDA

The supernatant obtained after cell lysis as mentioned in Section 2.3, constituting the intracellular fraction was loaded onto the nickel-NTA column (Invitrogen). The column was later washed with 10 column volumes of Washing Buffer (WB) (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole). The washing was followed by the elution of the Nickel-bound CDA with a two column volume of the Elution buffer (EB1) (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole) followed by one column volume with EB2 (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM Imidazole). A fraction size of 0.5 ml of the sample was collected and later the purity of the protein was verified by SDS-PAGE.

3. Results

The metagenomic DNA was extracted from a chitin enriched soil sample and sequenced on a Genome Sequencer FLX platform (Roche) using the Titanium chemistry. The data revealed 851,147,093 sequenced bases corresponding to 2,281,090 reads with an average GC-content of 62.51%. Assembly of the metagenome sequence data resulted in 257,179,783 assembled bases (699,710 reads representing 30.68% of all reads). Within the assembly 6625 contigs comprising 12,812,598 bases were larger than 500 bp. The average contig size was 1933 bp with an N50 contig size of 1952 bp. The largest contig was 39,406 bp [31]. In total, 16,289 genes were predicted by Reganor [33]. The genes encoding target enzymes were identified by applying downstream bioinformatics analyses on the assembled contigs. The gene coding for chitin deacetylase was an outcome of the metagenomics library constructed. The gene with the 5′ NdeI site and 3′ SalI site was amplified and cloned into the multiple cloning sites of pET22 b (+) vectors using the same set of enzymes (Fig. 1). The ligated vector was then used to transform competent E. coli pLysS Rosetta cells with the gene under the control of T7 promoter and pelB signal sequence for the periplasmic expression. An attempt to improve the yield of the expressed CDA was made on the shake flask cultures. One of the factors that were studied was the inclusion of additives; glycerol- the waste from the biodiesel industry and chitin. The Fig. 2 depicts the study conducted on the recombinant *E. coli* for improving the activity of CDA in the intracellular fraction. The results are an average of the triplicate experiments conducted. It was observed that the growth of *E. coli* underwent a sigmoidal curve in all the three media conditions with an initial lag phase followed by the log and stationary phase. As we had maintained a 10% filling volume for better oxygen transfer, the entire growth cycle lasted about 8 h. The culture was induced with one mM of IPTG upon entering the early log phase for the induction of the CDA gene. The cells upon reaching the stationary phase were harvested for their biomass. The cells reached 2.4 g/l total biomass in the presence of glycerol as the additive which was 50% higher than that obtained in LB medium with a value of 1.25 g/l. The media supplemented with chitin had a maximum biomass of 1.1 g/l. In addition to the impact of glycerol on the biomass, its effect on the CDA activity was also studied. The media with glycerol had a value of 343 μmol/min/ml as against 90 μmol/min/ml in the case of unsupplemented media and 32 μmol/min/ml of LB media supplemented with chitin. Thus the conditions of the inclusion of 1% glycerol were taken for future experiments. The media conditions

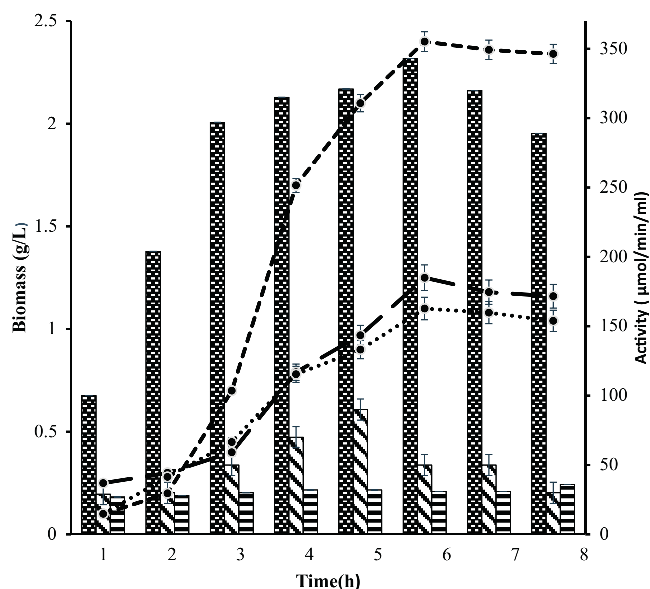

Fig. 2. Growth and CDA activity profile of E. coli cells in (1) LB broth, (2) LB broth + Glycerol, (3) LB broth + chitin. The bars indicate the CDA activity whereas the dotted lines indicate the biomass. The CDA activity in LB, LB+chitin and LB + glycerol are being depicted. The cultures were grown in 250 ml shake flasks, 12 ml filling volume, 200 rpm and 25 mm shaking diameter. The biomass in the presence of LB−−, LB+ glycerol−−− and LB+ chitin ···· has been indicated in the figure. The X- axis depicts the time interval (h) after induction with 1 mM IPTG.

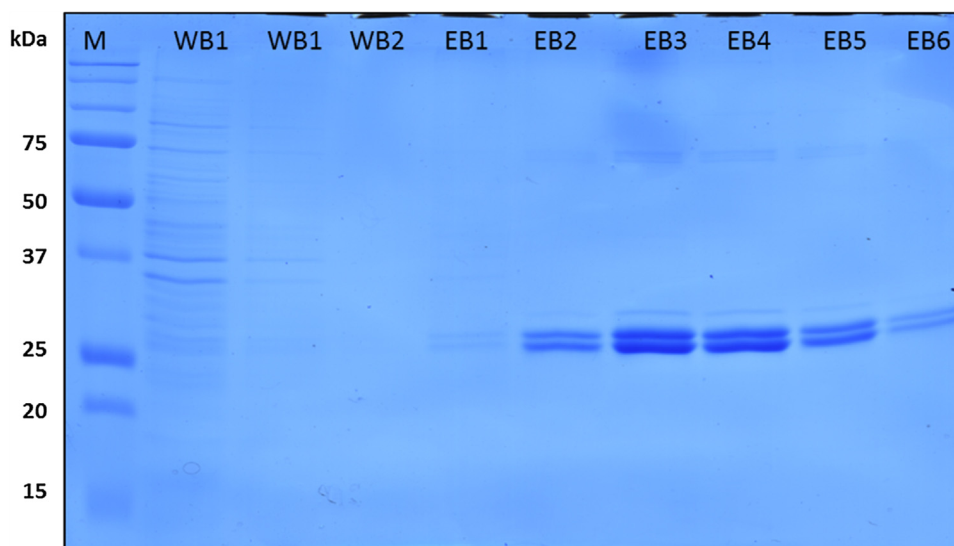

Fig. 3. Purification of chitin deacetylase using Ni-NTA column chromatography. The cells grown in LB+ glycerol, induced with 1 mM IPTG were harvested upon attaining saturation phase. The pelleted cells were sonicated, and the lysate subjected to Ni-NTA purification. First lane, Marker; Second–Third Lane, Wash buffer; Fourth–Eight Lane Eluted fraction with 250 mM Imidazole and Ninth–Tenth Lane Eluted fraction with 500 mM Imidazole.

with glycerol as an additive was later scaled for the 1.9 L fermenter. The cells obtained were harvested for the purification of the CDA from the intracellular fraction upon sonication. The culture lysate was subsequently subjected to Ni-NTA chromatography and the fractions eluted with increasing concentration of Imidazole. This profile has been represented in Fig. 3. The Lanes WB1 and WB2 represent the fractions collected after the initial wash of the column with the wash buffer which include Imidazole at a concentration of 20 mM. The Lanes E1–E4 represents the protein profile with elution buffer 1 (250 mM Imidazole) whereas lane E5 to E6 represents elution buffer 2 comprising 500 mM Imidazole. The gene for CDA is under the pel B signal peptide directing its periplasmic expression. As the protein was a pooled intracellular fraction, two bands were visible, one with the signal peptide and one without it. The two proteins were observed corresponding to 27.5 kDa and 25 kDa band seen in Fig. 3. The enzyme activity of the pooled samples was calculated, and it was observed 282 μmol/min/ml. Thus the activity had reduced from an initial value of 320 μmol/min/ml. But the specific activity had increased from 5.16 μmol $min^{-1} mg^{-1}$ in crude preparation to 1142 ± 43 μmol $min^{-1} mg^{-1}$ yielding a purification fold of 221 folds.

4. Discussion

The metagenomic readout of the library constructed out of the soil exposed to chitin and chitosan waste for ten years had resulted in yielding many enzymes, one of them being the CDA used in the present study. The source organism was also found to be *B. licheniformis*, a soil bacteria. The gene of an approximate size of 750 bp was amplified using the standardized PCR conditions, and the gene was cloned in the pET 22b expression vector for further studies. In the shake flask studies, an attempt was made to include glycerol, one of the by-products of the bio-diesel industry whose disposal is becoming a menace. The main idea behind this was of value addition of the waste. Glycerol has been employed in for solvent production like dihydroxyacetone and 1,3-propanediol [34]. In additon, its usage, can also made as a carbon source to obtain recombinant proteins. Many groups have been working on the value addition of this waste by including it as a media component [35]. The transporters for the glycerol are said to be present in the genome of the *E. coli* but are not utilized in the presence of glucose in the media [36]. Glycerol, when included in the LB medium, has shown to have an impact on delaying the death phase of the *E. coli* [37]. Also, it enhances the induction of recombinant proteins [38,39]. This effect of glycerol might be due to the initiates the glycolytic and gluconeogenic pathways in *E. coli* for efficient utilization of carbon sources [37,40]. Glycerol might be acting as a carbon source once the glucose in the medium depletes. This explains an improved biomass and in turn CDA activity in the presence of glycerol (Biomass 2.7 g/l and CDA activity 343.14 μmol/min/ml; Fig. 2) while no appreciable impact was seen with the same percentage of chitin included in the medium. This enhance effect of glycerol has resulted in the observation of highest reported CDA activity so far. The activity observed can be compared to the earlier studies performed by Tokuyasu wherein 0.01954 Umg^{-1} were obtained with *Colletotrichum lindemuthianum* as the organism. The activity upon purification had improved to 18.4 Umg^{-1} [14]. Another group working on the CDA activity in *Metarhizium anisopliae*, an insect pathogen, reported 12 Uml^{-1} [15]. Another study involving *Penicillium oxalicum* SAE_M-51 yielded an activity of 0.210 Uml^{-1} in submerged fermentation conditions [41] which was improved to 0.414 Uml^{-1} upon using response surface methodology [26]. The study of inclusion of additives has been tried earlier also with a study Shirai et al. working on *Colletotrichum gloeosporioides*, a plant pathogen. They found that the inclusion of phytohormone; abscisic acid (ABA) led to enhancement of the activity by 9.5 folds from an initial of 0.11 Umg^{-1} to 1.05 Umg^{-1} [42]. Thus additives have been shown to improve the yield, and the same result was observed in our study with glycerol as an additive. Thus from 343.14 μmol/min/ml, upon purification we could enhance the yield by 221 folds to obtain the final enzymatic activity against glycol chitin as the substrate as 1142.5 ± 43 μmol min^{-1} mg^{-1}. The future work would involve working on the mode of action of the enzyme on the polymeric chitosans.

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