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Protease Production by *Aspergillus oryzae* in Solid-State Fermentation Utilizing Coffee By-Products

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Abstract: *Aspergillus oryzae* CFR305 was isolated from coffee residues and studied for protease production using coffee substrates under solid-state fermentation. Coffee cherry husk was found to be suitable substrate for protease production. The influence of process parameters such as temperature, pH, moisture, particle size, inoculum size, additives and pretreatments of substrates were evaluated. Maximum protease production of 12236 U/gds was obtained on pretreated coffee cherry husk by steam. The protease was partially purified and characterized. The enzyme was purified 33.76 fold by ammonium sulphate precipitation and ion exchange chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) indicates that the purified protease is a monomeric enzyme with a molecular mass of ~35 kDa. The enzyme is a serine protease as indicated by its inhibition studies. The protease enzyme showed maximum activity at pH 10 at 60°C. Thus, the alkaline serine protease indicate their use as additives in industry and also states the importance of SSF for the production of protease using coffee cherry husk as substrate, which offer significance benefit due to abundant availability and economy. This is the first report on production of protease using coffee by-products.

Key words: *Aspergillus oryzae* CFR305 · Solid-state fermentation · Coffee by-products · Protease · Pre-treatment

INTRODUCTION

The processing of coffee generates expressive amounts of agricultural waste such has coffee pulp, coffee cherry husk, silver skin and spent wastes in more than two million tons quantity yearly. Traditionally these coffee residues have limited application as fertilizer, livestock feed, compost etc., [1] and utilized only a fraction of available quantity and were not technically very efficient. Since these coffee residues contain a good amount of fermentable sugars, they form appropriate substrates for the cultivation of mould and yeasts and further production of enzymes [2]. Solid-state fermentation has gained renewed interest owing to its importance in recent developments in biomass conservation, in solid wastes treatment and its application to produce secondary metabolites. Production of these enzymes using agro-biotech substrates under SSF provides several advantages in productivity, cost-effectiveness in labor, time and medium components in addition to environmental advantages like less effluent production, waste minimization etc., [3, 4].

Protease is one of the three largest groups of industrial enzymes accounting for about 60 % of world wide sale of enzymes and is widely used in detergents, leather processing, meat processing, dairy, digestive aid and silk industry [5]. Among microbes, fungi as enzyme producers have many advantages, since they are normally GRAS (generally regarded as safe) strains and the produced enzymes are extra cellular which makes its easy recuperation from fermentation broth. Reports are available on protease biosynthesis by fungi belonging to the genera *Aspergillus* and *Penicillium* [6, 7]. Proteases are classified into acid, neutral and alkaline proteases on the basis of pH range in which their activity is optimum. The proteases of fungal origin offer an advantage over bacterial proteases because the mycelium can be easily removed from the final product by simple filtration,

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the ability of the fungus to grow on cheaper substrate, easy immobilization of mycelium for repeated use, broad range of pH (4,11), spore substrate specificity and lastly low cost of production. These biocatalysts find applications in food processing industry and pharmaceuticals. Proteases production has been carried out in submerged and solid state fermentation using substrates such as wheat bran, rice bran, agro wastes as mango peel, banana peel etc. In the present communication, we report the potential of coffee residues to be used as a substrate for the production of protease by *Aspergillus orzyae CFR 305* and its properties.

MATERIALS AND METHODS

Microorganisms and Solid–State Fermentation: A fungal strain, *Aspergillus Orzyae CFR* 305 used in the present study was isolated from coffee wastes collected from coffee growing regions (Coorg district), Mysore City. It was grown on potato dextrose agar (PDA) slants for 3-5 days at 27°C and maintained at 4°C. The *Aspergillus Orzyae CFR* 305 (10^6 spores ml) was used as inoculum. Coffee wastes such as pressed coffee pulp (CP), coffee cherry husk (CH), coffee parchment husk (PH), silver skin (SS) and coffee spent wastes (SW) obtained from coffee growing areas, coffee curing works, roasting units and instant factories from Mysore city was used for investigation. Substrates (10 g) were grinded to particle size of 1mm and were taken in 250 ml Erlenmeyer flasks, sterilized, inoculated and incubated for 5 days at room temperature.

Optimization of Process Parameters for Protease Production: Various process parameters affecting enzyme production during SSF were optimized and subsequently optimal conditions were employed in pretreatment studies. Fermentation conditions such as pH (3.5, 4.5, 5.5, 6.5 and 7.5), moisture (50, 60 and 70 %), fermentation time (3, 5 and 7 days), temperature (24, 27 and 32°C), inoculum size (1,2 and 3 %) and particle size with 0.5, 1.0 and 2.0 mm size of the substrates were optimized to obtain best activity with CH.

Effect of Supplementation of Carbon and Nitrogen Sources: Studies were performed to evaluate influence of supplementation of substrate with different carbon sources such as glucose, maltose, xylose, sucrose and lactose (1% w/w) and nitrogen sources such as peptone, Yeast extract, beef extract, casein and ammonium sulphate (1% w/w). Individual substrates as sole carbon source were considered as control.

Pre-treatments: Different pretreatments were investigated on the CH for further improvement of the enzyme activity. The substrates were subjected to steaming at 100°C for one hour and latter provided with 60 % moisture and pH 4.5 was adjusted using acetate buffer and sterilized [2], hydrogen peroxide (1 %) [8], alkali treatment (1 %) [9]. All the experiments were done in triplicates.

Enzyme Extraction and Assay: Activity for alkaline protease was determined spectrophotometrically according to Keay and wildi [10], with a slight modification. Crude enzyme extract of 200 μl, 500 μl of casein (1%) and 300 μl of 0.2 mol/l phosphate buffer (pH 7.0) were added. The reaction mixture was incubated at 60°C for 10 min and arrested by addition of 1ml of 10 % trichloroacetic acid. The mixture was centrifuged at 10,000xg for 15 min and to the supernatant, 5ml of 0.4 mol Na_2CO_3 and 1ml diluted Folin and Ciocalteaus phenol reagent were added. The resulting solution was incubated at room temperature for 30min and the absorbance of the blue color developed was read at 660nm using tyrosine standard. One unit of enzyme activity was defined as the amount of enzyme that liberated 1μg of tyrosine from substrate (casein) per minute under assay conditions. Protease activity was expressed as units per gram dry substrate (U/gds) in the case of SSF and as U/ml for purification and characterization. The total protein in the extract was determined [11].

Purification and Characterization of the Enzyme: The protease produced under SSF was extracted and purified employing ammonium sulphate (20, 40, 60, 80 and 90 %) was used to standardize the precipitation of protease enzyme from the fermented broth. The precipitated protein was re-suspended in 0.1 M phosphate buffer (pH 7), dialyzed against the buffer for 24 h at 4°C with six changes of buffer and assayed for protease activity and protein content. The concentrated sample was applied to a Sephadex G-100 gel filtration column (90 × 12 cm) and equilibrated with the same buffer.

Electrophoretic Methods: The purity of alkaline protease was analyzed by 10 % native and 12 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass (using a standard molecular weight marker) was determined by SDS-PAGE according to the method of Laemmli [12]. The protein was then stained with 0.1% solution of Coomassie Brilliant Blue (CBB) R-250 (Sigma, USA).

Effect of pH, Temperature and Protease Inhibitors on Protease Activity: The effect of assay substrate concentration on the activity of protease was studied using different concentration of casein (5, 10, 15, 20, 25 and 30 mg/ml). Optimum pH of the protease enzyme was determined by using buffer at varying pH of 1-14 the influence of temperature on the activity of protease was studied by incubating the assay reaction mixture at different temperature (30-100 °C). For the determination of protease type, p-Chloromercuribenzoic acid, (p-CMB), ethylene diamine tetra acetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), 8-hydroxyquinoline, urea, trypsin inhibitor and 2-mercaptoethanol (each containing 1mM in a separate tube) inhibitors were tested. The purified protease was pre incubated with each inhibitor in 100 mM Tris-HCl buffer (pH 8.0) for 1h. The control was pre-incubated without any inhibitor and the residual protease activity was measured. Metals including NaCl, $COCl_2$, $CuSO_4$, $CuCl_2$, $MgSO_4$, $MnSO_4$, $BaCl_2$ and $NaNO_3$ at 1mM level were evaluated for their effect on protease activity.

RESULTS AND DISCUSSION

SSF was carried out with coffee by products as substrates for the production of protease with initial moisture of 50 % at 27 °C using *Aspergillus oryzae CFR* 305 for 5 days. Among the different coffee substrates CH showed activity of 7539 U /gds (unit per gram dry substrate) with 320.2 mg protein concentration (Fig. 1). The CP, SS SW and PH showed 6777, 5645, 6079 and 1766 U/ g enzyme activity respectively. The protein content was found to be high in CH and was directly proportional to the protease activity. Each organism or strain has its own special conditions for maximum enzyme production. Hence, optimization of medium composition has to be carried out to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. Results were significant with coffee Cherry husk and further studies were carried with these substrates.

Optimization of Solid–state Fermentation: SSF with good yield might be due to natural growth conditions available to the cells in solid cultures as well as due to nutrient availability in the SSF medium. Initial moisture content is a critical factor in SSF since the moisture of the medium determines microbial growth and product yield. The optimal moisture level for production of protease was

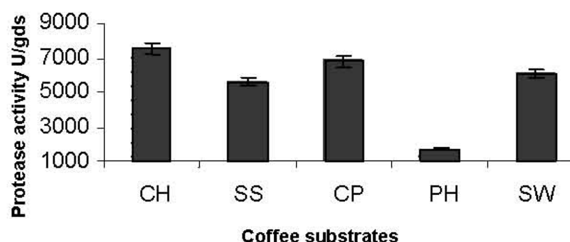

Fig. 1: Production of protease from coffee wastes in SSF

found to be 50 % with 8154 U/gds for CH (Fig 2). The optimum moisture content for growth and substrate utilization depends on the organisms and the substrate used for cultivation. The initial moisture content is a critical factor for solid-state fermentation processes because this variable has influence on growth, biosynthesis and secretion of different metabolites [13]. The higher moisture level may cause reduction in enzyme yield due to steric hindrance of the growth of strain by reduction in porosity of the solid substrate thus interfering with oxygen transfer. The moisture level in SSF varies between 30-80 %.

The effect of temperature on enzyme production varied during fermentation (Fig 2).The optimum temperature for maximum yield of protease was 30 °C with 6584 U/gds for CH. The significance of the temperature in the development of biological process is reported to determine the protein denaturation, enzyme inhibition and cell growth. Fig 2 shows optimum pH for protease productivity as 7.0 for coffee cherry husk with 8569 U/gds. Our reports are supported by Sandhya *et al.* [14] where *Aspergillus oryzae* gave maximum yield for protease production at pH 7.5. The pH of the medium strongly affects many enzyme processes and transport of various components across the cell membrane [15]. The enzyme activity decreased with increase in pH of the substrate. Activity of enzyme on different species of the microorganisms is reported to vary depending on metabolism. As shown in the Fig 3, the enzyme activity was high on 4th day of fermentation with *Aspergillus Oryzae CFR* 305 with 7142 U/gds for CH. Size of inoculum is an important biological factor, which determines biomass production in fermentation. Improved activity was found with 3 % inoculum level compared to 1, 2 and 4 % I noculum size with 8962 U/g of enzyme activity. The particle size of the substrate especially hard surface is important for SSF. Fig 3 showed enzyme production to be high with 1mm particle size for CH with 7998 U/gds. 1mm particle size is found to be more suitable

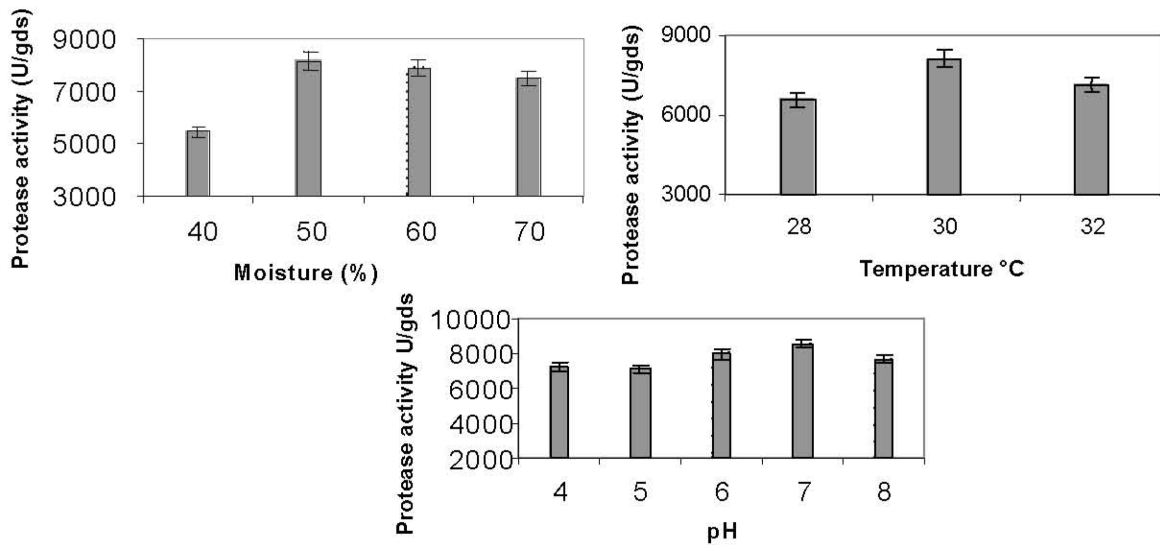

Fig. 2: Effect of Moisture, Temperature, pH on protease production in SSF

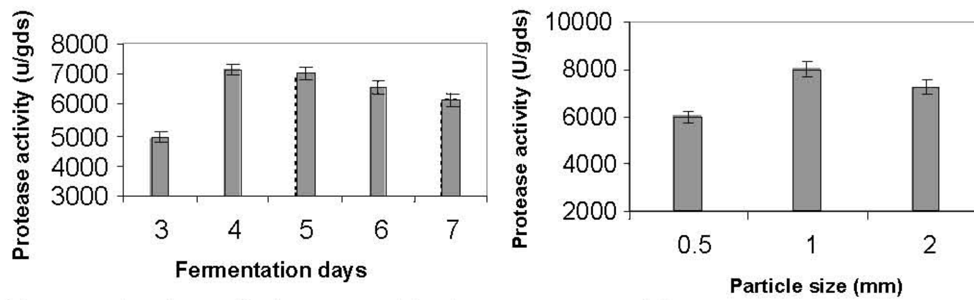

Fig. 3: Effect of fermentation time and substrate particle size on protease activity

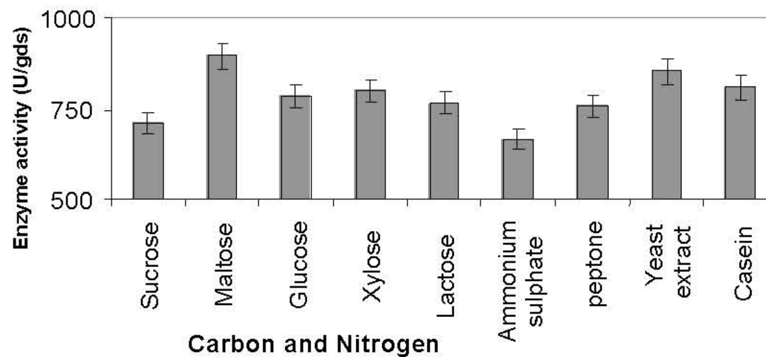

Fig. 4: Effect of Carbon and nitrogen source on protease in SSF

for protease production in case of various agro industry wastes and the small particle (0.6-0.8) have resulted in poor growth and enzyme production by interfering in microbial aeration [4].

Fig. 4 shows protease activity of CH on supplementation with carbon and nitrogen sources. *A.oryzae* with maltose as a carbon source produced maximum enzyme activity with 8954 U/gds with CH. Other reports describe similar findings that sugars induce the production of protease in different strains of *A. oryzae* and *Penicillum* [15]. Nitrogen source with yeast extract produced 8541 U/gds with CH (Fig. 4). The optimized conditions with moisture (50 %), pH (7.0), temperature (30°C), inoculum size (3 %), particle size (1mm) and fermentation time (4 days), carbon source (maltose), nitrogen source (yeast extract), increased protease activity to 9867 U/gds.

Table 1: Influence of pre-treatment of Coffee husk substrates on the production of Protease under SSF using *Aspergillus Oryzae sp*

Pre-treatment of substrate	Protease activity U/gds
Untreated	7539±85
Alkali treatment	9373±100
Acid treatment	7859±75
H_2O_2	6124±90
Methanol	5810±55
Steaming (121°C/ 15min)	12236±29

Table 2: Summary of Purification of crude Protease produced under SSF by *Aspergillus Orzyae* using coffee husk

Purification step	Total activity (U/ml)	Total Protein (mg)	Specific activity (U/mg protein)	Recovery Yield (%)	Purification fold
Crude enzyme	39500	990	39.8	100	1
Ammonium sulphate precipitation (40-90 %)	26450	90	293.8	66.92	7.38
Ion-exchange chromatography (Sephadex G-100)	8065	6	1344	20.4	33.76

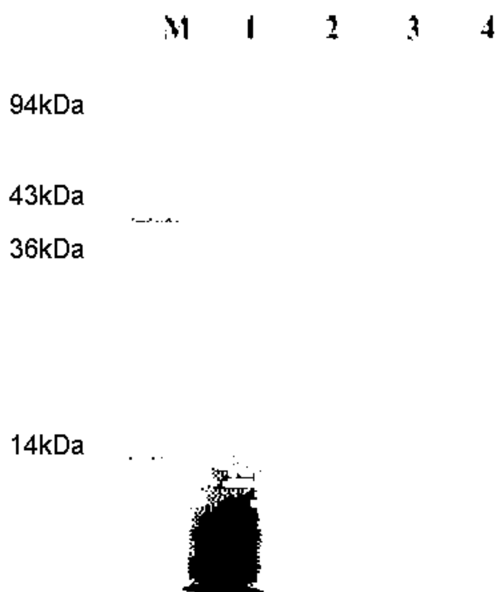

Fig. 5: Electrophoretic profile of protease
Lane M-Phosphorylase b (94 kDa), Ovalbumin-43 kDa, Glyceraldehydes 3 phosphate dehydrogenase -36 kDa, 14kDa- α-lactoabumin. Protein standards, lane 1-Crude extract, lane 2-After ammonium sulfate precipitation, lane 3-Dailysed protease, lane 4-purified protease with sephadex (35kDa).

Pre-Treatments: The pretreatment of wastes has often been found useful to improve its digestibility and easy access for microbial attack by removing core and non-core lignin fractions. Steam treatment was found to be effective on coffee substrates. Maximum protease activity with CH was 12,236 U/gds with steam followed by alkali treatment 9373 (Table 1). Steam increases the porosity of the substrate so that microorganisms can easily take up the nutrients for their growth. The pretreatment of substrates influences the physicochemical properties [2].

Purification and Characterization: Result obtained for purification of crude enzyme is summarized in Table 2. The precipitate formed at 40-90 % saturation of ammonium sulphate, showed 7.38 fold increase in specific activity compared to the crude sample. The sample was further purified employing ion-exchange chromatography. Elution profile from the sephadex column furnished a single peak with protease activity, which could be eluted with buffer containing 0.2 M NaCl. This resulted in 20 % protease recovery with a specific activity of 1344 U/mg protein. The molecular mass of protease estimated by comparing the electrophoretic mobility of marker protein showed molecular mass of 35 kDa (Fig. 5).

The optimum pH of the purified protease was recorded at pH 10 with 100 % relative activity (Fig. 6). More than 80 % of the maximal activity was retained in pH 9-12. Our results are supported by Rashberi *et al*, [16] which reports maximum production of protease with optimum pH 10.5 in case *A .parasiticus*. The enzyme was active over a wide range of temperature and maximal activity was recorded at 60° C with 100 % relative activity. More than 80 % activity was conserved between 50-70°C (Fig. 6). Only few reports on the fungal protease with high temperature are reported [17]. This is an important feature for use of protease as detergents additives. The K_m value was determined for the hydrolysis of casein by purified protease. The Line weaver-Burk plot was drawn between the inverse of different concentrations of casein and the reaction velocity to determine the Michaelis constant. The K_m was 3.0 mg casein/ml and the V_{max} value was 715±2.4μg tyrosine / min/ml.

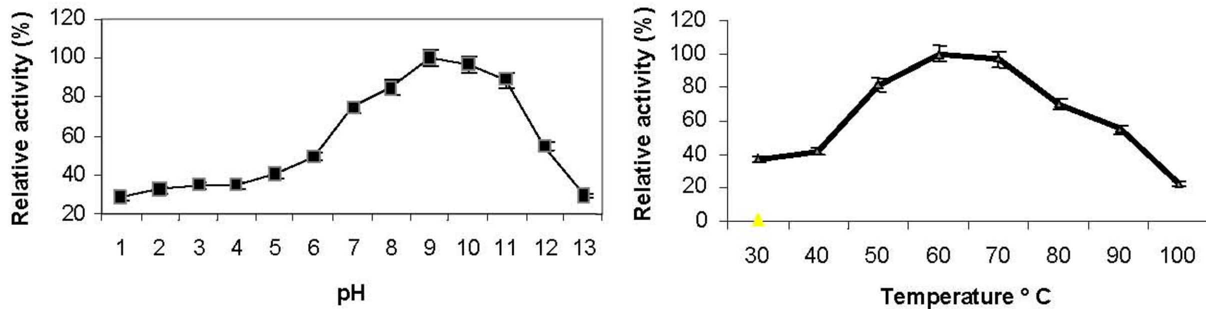

Fig. 6: Influence of pH and temperature on activity of purified Protease from *A.oryzae*
*Reaction mixture contained 1.8 ml of 1% casein and 0.2 ml of enzyme solution.
Relative activity is expressed as a percentage of the maximum.

Table 3: Effect of inhibitors on protease activity

Specific inhibitors (Concentration 1 mM)	Inhibition (%)
Control	0
EDTA	25 ±0.2
2-mercaptoethanol	47 ±0.5
p-CMB	52 ±0.2
PMSF	100 ±0.3
Urea	11

Table: 4 Effect of metallic ions on protease activity

Metal ion (1mM)	Residual activity (%)
Control	100
Mg^{2+}	130 ±0.5
Na^{2+}	112.0 ±0.5
Ca^{2+}	120±0.4
Mn^{2+}	82 ± 0.6
Co^{2+}	100 ±0.5
Ba^{2+}	100±0.5

Effect of Inhibitors/ Activators: The inhibitors/activators used included metal ions, metal chelators and some metabolic inhibitors. Inhibitors such as EDTA, 2-mercaptoethanol and p-CMB inhibited the enzyme activity at 75, 63 and 58 %, respectively. Protease activity produced by *A.oryzae* was 100 % inhibited with PMSF. These results and particularly strong inhibition by PMSF indicated that the active-centre of protease contains serine residues. (Table 3). Similar results of PMSF inhibition has also been reported by *A.parasiticus* and *A.fumigatus*. [18,19]. Metals such as $Co2^{+}$ and Mn^{2+} inhibited enzyme activity (Table 4) whereas the Mg^{2+}, Ca^{2+} and Na^{2+} protected the enzyme from undergoing denaturation and enhanced the proteolytic activity marginally. However, the enzyme was activated by Ca^{2+} and Mg^{2+} by 20 % and 30 % respectively at 1 mM concentration and also indicate that the enzyme require metal ions for its activity (Table 2). These observations indicate that metal-binding region on protease molecule is important for the regulation of the enzyme activity.

CONCLUSIONS

SSF has revealed the possibilities of effective utilization of coffee by-products for value addition through biotechnological means. In the present study, coffee cherry husk with *Aspergillus oryzae* is found to be potential producer of protease. Coffee husk after steam pretreatment have proven to produce maximum of 10236 U/gds. This is the first report on production of protease using coffee cherry husk. The partially purified proteases have molecular weight of ~35 kDa and have potential commercial value due to its properties. Proteases used in industry are mainly derived from bacteria, which have some major limitations whereas fungal origin proteases offer advantages such as the ability of the fungus to grow on cheaper substrate such as cherry husk which pave way in effective solid waste management and value addition .

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