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ORIGINAL PAPER

Chitin-Glucan Complex Production by *Schizophyllum commune* Submerged Cultivation

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Abstract

Chitin-glucan complex is a fungal origin copolymer that finds application in medicine and cosmetics. Traditionally, the mycelium of *Micromycetes* is considered as an industrial chitin-glucan complex source. *Basidiomycete Schizophyllum commune* submerged cultivation for chitin-glucan complex production was studied. In different *S. commune* strains chitin-glucan complex composed 15.2 ± 0.4 to 30.2 ± 0.2% of mycelium dry weight. Optimized conditions for chitin-glucan complex production (nutrient medium composition in g/l: sucrose – 35, yeast extract – 4, Na_2HPO_4*12H_2O – 2.5, $MgSO_4$*7 H_2O – 0.5; medium initial pH 6.5; aeration intensity 2 l of air per 1 l of medium; 144 hours of cultivation) resulted in 3.5 ± 0.3 g/l complex yield. Redirection of fungal metabolism from exopolysaccharide synthesis to chitin-glucan complex accumulation was achieved most efficiently by aeration intensity increase. Chitin-glucan complex from *S. commune* had the structure of microfibers with diameter 1–2 μm, had water-swelling capacity of 18 g/g, and was composed of 16.63% chitin and 83.37% glucan with a degree of chitin deacetylation of 26.9 %. *S. commune* submerged cultivation is a potent alternative to *Micromycetes* for industrial-scale chitin-glucan complex production.

Key words: *Schizophyllum commune*, chitin-glucan, optimized cultivation

List of abbreviations: CGC – chitin-glucan complex, IPA – isopropanol, PDA – potato dextrose agar, rpm – rotations per minute, vvm – air volume per broth volume per minute, YE – yeast extract

Introduction

Chitin-glucan complex (CGC) is general name for a wide variety of biological copolymers composed of chitin macromolecules with covalently linked β-D-glucan chains. The complex naturally occurs in the cellular walls of filamentous fungi, where it forms rigid microfibers that contribute cell wall mechanical strength. CGC can be extracted from fungal mycelium by various physiochemical and enzymatic methods, with the use of inorganic reagents, organic solvents, detergents, *etc.* (Ivshina, 2007). Traditionally CGC is recovered as an insoluble residue after mycelium successive treatments with alkali and acid. Fungal CGC is considered as an alternative source of chitin/chitosan (Wu *et al.*, 2005; Teslenko and Woewodina, 1996) as well as a potent agent for application in medicine for wound-healing management (Teslenko and Woewodina 1996; Valentova *et al.*, 2009), for improvement of desquamation process and xerosis reduction in diabetic patients (Quatresooz *et al.*, 2009), for reduction of aortic fatty streak accumulation (Berecochea-Lopez *et al.*, 2009), *etc.*

Traditionally waste mycelium of *Aspergillus niger* from citric acid production is considered as an industrial chitin(chitosan)-glucan complex source. There are several reports on other *Micromycetes* belonging to genus *Ascomycota* and *Zygomycota* utilization for CGC production (Wu *et al.*, 2005; Teslenko and Woewodina 1996). *Basidiomycetes* have been rarely considered as CGC producers, though they are capable of intensive growth in submerged culture as well. *Schizophyllum commune* is *Basidiomycete* used for β-(1,3;1,6)-D-glucan schizophyllan production. *S. commune* can be a promising culture for industrial scale CGC production if mycelium growth and CGC content in mycelium is increased and exopolysaccharide synthesis suppressed. The aim of the work was to characterize CGC from *S. commune* submerged mycelium and to study the possibility of fungal metabolism redirection from exopolysaccharide synthesis to CGC formation.

Experimental

Materials and Methods

S. commune strains from different microorganism collections were used: F-795 (Czech Collection of Microorganisms), 11223, 1024, 1025 and 1026 (German

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Collection of Microorganisms and Cell Cultures), 127 (Collection of Microorganisms CPN Ltd.). The strains were stored on agar slants with PDA at + 4°C and subcultured regularly. Prior to experiment the strains were inoculated to Petri dishes that were cultivated for 7 days at 29°C. 250 ml Erlenmeyer flasks with 100 ml medium (Inoculum I) were inoculated with 10 pieces 0.5 × 0.5 cm of mycelium from the Petri dishes and incubated in rotary shakers at 29°C, 200 rpm for 5 days. Inoculum I was then homogenized by T 25 digital Ultra-TURRAX (IKA, Germany) and 50 ml of homogenate were used for inoculation of 1000 ml Erlenmeyer flasks with 500 ml medium. 1000 ml Erlenmeyer flasks were cultivated in rotary shakers at 29°C, 200 rpm for 7 days. In case of cultivations in 50 l fermenter 1000 ml Erlenmeyer flasks with 500 ml medium were cultivated for 3 days and then used as seed culture for fermenter inoculation (5% amount of inoculum). Fermenters with working volume 0.3 l were inoculated with 20 ml of homogenized Inoculum I and cultivation lasted 4 days.

Medium for the seed cultures and production cultivations contained (in g/l): 35 – sucrose, 3 – YE, 2.5 – Na_2HPO_4*12H_2O, 0.5 – $MgSO_4$*7 H_2O, initial pH 5.5, unless otherwise specified. Effects of nutrient medium composition and pH were studied in 1000 ml Erlenmeyer flasks with 500 ml medium. Medium pH was adjusted by NaOH or HCl prior to sterilization. Influence of aeration was studied in Sixfors fermenters (INFORS AG, Switzerland) with working volume 0.3 l. The cultivation conditions were as follows: temperature 29°C, agitation 150 rpm, aeration 0.5–2.0 vvm. Effect of cultivation time was studied in fermenter (INFORS AG, Switzerland) with working volume 50 l under the following conditions: temperature 29°C; aeration 2 vvm; agitation 150 rpm. Cultivation medium (in g/l): 35 – sucrose, 4 – YE, 2.5 – Na_2HPO_4*12H_2O, 0.5 – $MgSO_4$*7 H_2O, pH 6.5.

Mycelium and schizophyllan yields were measured as follows: 500 ml of cultural broth were centrifuged (10 000 × g, 25°C, 20 min), supernatant was collected and used for schizophyllan precipitation. Sediment (mycelium) was resuspended in 250 ml of demineralised water, centrifuged again and supernatant was discarded. The process of mycelium washing was repeated 2 more times. The mycelium was placed into a Petri dish, freeze-dried in Heto PowerDry PL 3000 freeze dryer (Thermo Scientific, USA) to constant weight, and mycelium yield in grams of dry mycelium per 1 liter of cultural broth was calculated. Schizophyllan was precipitated from supernatant with triple amount of IPA, dried under 60°C for 24 hours, weighted and schizophyllan yield in grams of dry polysaccharide per 1 liter of supernatant was calculated. CGC amount in mycelium was determined as follows: 2 g of freeze-dried mycelium were resuspended in 60 ml of 4.2 M NaOH. The mixture was heated to 90°C and incubated under this temperature for 3 hours under constant mixing. The mixture was then centrifuged (10 000 × g, 25°C, 10 min), the supernatant was discarded and sediment was resuspended in 300 ml of demineralised water by Ultra-Turrax T25 Digital (IKA, Germany) and centrifuged again. The process was repeated until supernatant pH 7. The sediment was then mixed with 60 ml of 0.25 M HCl, resuspended by Ultra-Turrax T25 Digital (IKA, Germany) and incubated for 2 hours at 50°C. The resulting CGC was centrifuged (10 000 × g, 25°C, 10 min), supernatant was discarded, sediment was resuspended in 300 ml of demineralised water by Ultra-Turrax T25 Digital (IKA, Germany) and centrifuged again. The process was repeated until supernatant pH 7. The CGC was dehydrated by IPA, dried under 60°C for 24 hours and weighed. CGC content is presented as mass fraction (%) in dry mycelium. Residual sucrose in the medium was calculated from glucose that resulted from sucrose degradation by baker's yeast invertase (Sigma, USA). Glucose was measured by L-Glucose assay kit GOD-POD (BioVendor, Czech Republic). pO_2 in the cultivation broth was measured by optical probe Hamilton-Visiferm DO 120 (Hamilton, Switzerland).

CGC swelling capacity was measured as follows: 0.5 grams of sample were placed into spherical container 45 mm diameter made of wire screen. The container was deep into water for 40 sec. to let the substance swell. Then the container was removed from the water, left for 30 sec. to drain and weighed. Swelling capacity (g of water/1 g of sample) was calculated as follows: (weight of container with swelled sample – weight of wet container – 0.5)* 2.

Hydrogen bromide titrimetric analysis was conducted by modified method described by Khan *et al.* (2002) with utilization of 500 mg CGC suspended in 100 ml 0.2 M HBr. Elementary analyses were made on FISONS EA-1108 CHN elemental analyzer (Italy). Electron-scanning microscope image was made by Tescan VEGA II LSU electron microscope (Tescan USA Inc.) under the following conditions: high voltage 5 kV, working distance 4.4 mm, magnification 5000, display mode secondary electrons, high vacuum, room temperature. A15 nm layer of gold particles was applied on the sample by SC7620 Mini Sputter Coater (Quorum Technologies, UK). All experiments were repeated at least 3 times. The data is presented as value ± standard deviation.

Results and Discussion

Mycelium growth and CGC production by submerged culture of six *S. commune* strains was studied. Cultivation lasted 7 days, corresponding to late

Table I
Mycelium yield, CGC content in mycelium,
CGC and schizophyllan production by different *S. commune* strains

S. commune strain	Mycelium yield g/l	CGC in mycelium %	CGC production g/l	Schizophyllan production g/l
F-795	6.0 ± 0.2	15.2 ± 0.4	0.9 ± 0.1	2.3 ± 0.5
11223	11.0 ± 0.4	17.4 ± 0.2	1.9 ± 0.2	1.7 ± 0.2
1024	8.5 ± 0.1	16.1 ± 0.1	1.4 ± 0.3	0.7 ± 0.1
1025	8.6 ± 0.5	17.1 ± 0.1	1.5 ± 0.2	0.7 ± 0.1
1026	12.3 ± 0.3	20.3 ± 0.3	2.5 ± 0.2	1.2 ± 0.3
127	11.4 ± 0.7	30.2 ± 0.2	3.4 ± 0.4	0.5 ± 0.1

stationary growth phase. *S. commune* mycelium yield varied between 6.0 ± 0.2 g/l and 12.3 ± 0.3 g/l (Table I). For comparison, mycelium yields of chitin producers *A. niger* and *Mucor rouxii* are reported about 7 g/l and 5 g/l respectively (Wu *et al.*, 2005; Tan *et al.*, 1996). CGC content in mycelium of different *S. commune* strains ranged within 15.2 ± 0.4% and 30.2 ± 0.2% and was similar to that reported for *Aspergillus* and *Mucor* (Wu *et al.*, 2005, Arcidiacono and Kaplan 1992, Muzzarelli *et al.*, 1980). CGC production by *S. commune* reached 3.4 ± 0.4 g/l that is superior to many reported production values of *Micromycetes*.

The microstructure and chemical composition of CGC from *S. commune* mycelium (Strain 127) were analyzed. The copolymer was a cotton-like substance white to creamy in color without odor. Electron-scanning microscopy showed that even after harsh extraction that removes alkali soluble cell wall polysaccharides, CGC from *S. commune* was composed of microfibers with diameter 1–2 μm, similar to fungal hypha (Fig. 1). Highly developed microstructure determined remarkable swelling capacity of the complex that was 18 grams of water per 1 gram of CGC and was comparable with that measured for cotton wool (35 g/l). This characteristic makes CGC from *S. commune* a valuable product for application in bandages.

Elementary analyses of the complex showed nitrogen content 1.22 ± 0.10%, carbon 42.20 ± 0.24% and hydrogen 6.61 ± 0.15%. Glucosamine in the complex comprised 4.5 ± 0.4%. When these two analyses were combined, the composition of CGC from *S. commune* can be assumed as follows: 16.6% chitin and 83.4% of glucan with chitin deacetylation degree 27%. Although, chitin portion in *S. commune* CGC is lower than in *A. niger*, where chitin content is reported to be about 30% (Wu *et al.*, 2005; Machova *et al.*, 1999), utilization of *S. commune* for chitin production is promising due to high CGC yield.

Fungal mycelium separation from cultivation medium is an essential technological step in CGC production. From this point, filtration of *S. commune* cultural broth is a rather complicated process due to exopolysaccharide content. The possibilities of CGC production increase together with exopolysaccharide synthesis suppression by variation of cultivation technique were studied. The study was conducted on *S. commune* F-795.

CGC accumulation by fungi can be effected by nitrogen source in nutrient medium. Sousa *et al.* (2003) reported that when *Mucor circinelloides* was cultivated in synthetic medium with L-asparagine as single nitrogen source, chitin content in mycelium depended upon amino acid concentration. There was studied effect of yeast extract (YE) in the medium on CGC production by *S. commune.* The amount of YE was varied from 2 g/l up to 5 g/l and CGC content in mycelium, mycelium yield and schizophyllan synthesis were measured. It was found, that CGC content in *S. commune* mycelium is little affected by YE, and it varied in the range of 15.4 ± 0.3% under all studied YE concentrations. Increase of YE content from 2 to 4 g/l increased mycelia biomass production more than 1.5 times (Fig. 2). Further supplementation of the medium with YE increased mycelium growth only slightly. As distinct from mycelium, schizophyllan synthesis was suppressed by YE concentrations over 4 g/l (Fig. 2).

Amorim *et al.* (2001) reported medium pH as a regulating agent for chitosan production by *Mucor racemosus* and *Cunninghamella elegans*. The effect can be due to chitin deacetylase activity modification. It can be expected that medium pH may effect activity of enzymes, involved in *S. commune* CGC formation as well. CGC production by *S. commune* in medium with different initial pH was studied. Again, CGC

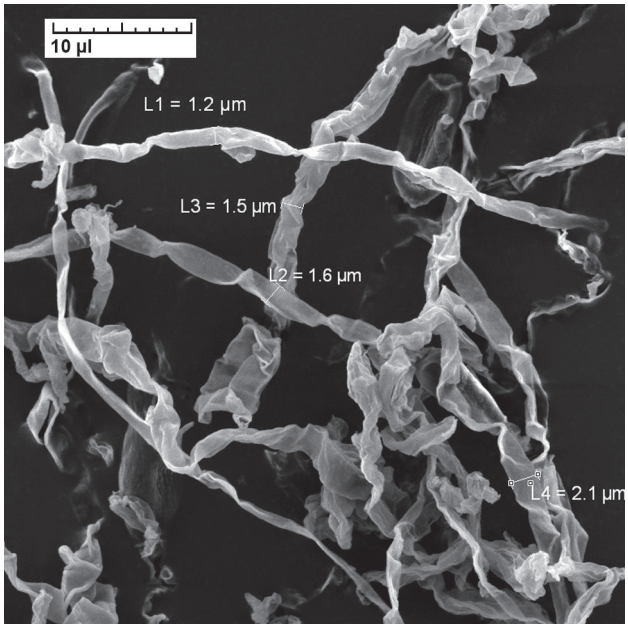

Fig. 1. Electron-scanning microscope image of CGC from *S. commune* submerged mycelium (High voltage 5 kV, working distance 4.4 mm, magnification × 5000).

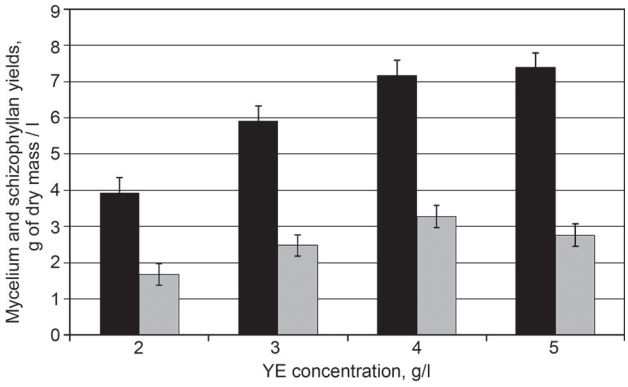

Fig. 2. Effect of YE in nutrient medium on mycelium yield (■) g/l and schizophyllan production (■) g/l by *S. commune.*

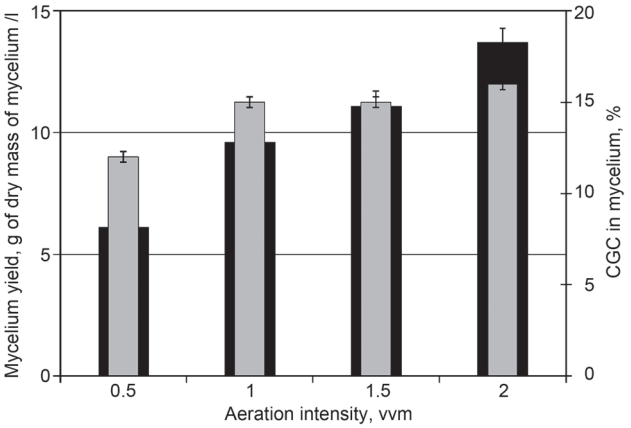

Fig. 4. Effect of aeration intensity on *S. commune* mycelium yield (■) g/l, and CGC content in mycelium (■) %.

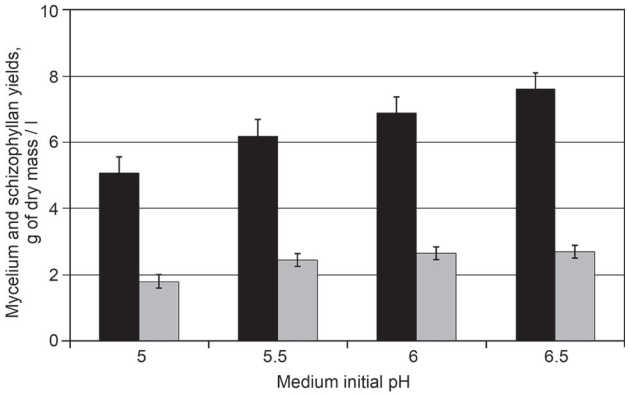

Fig. 3. Effect of initial medium pH on mycelium yield (■) g/l and schizophyllan production (■) g/l by *S. commune.*

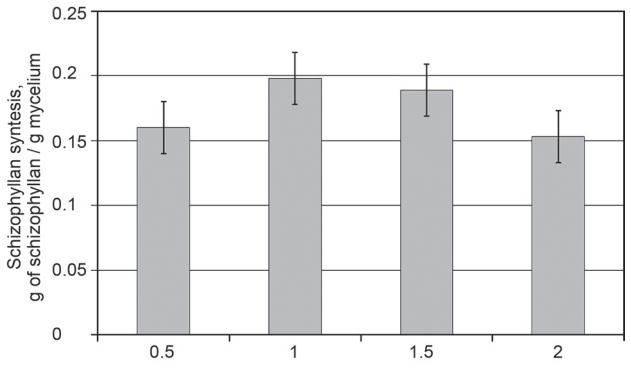

Fig. 5. Effect of aeration intensity on *S. commune* schizophyllan synthesis, grams of schizophyllan per gram of mycelium in the cultural broth.

content in mycelium was not significantly affected by pH and varied in the range of 15.1 ± 0.4%. Mycelium yield reached highest values at medium initial pH 6.5 (Fig. 3). Medium neutralization from pH 5 to pH 6 increased schizophyllan production 1.5 times. Further increase of medium pH left schizophyllan synthesis practically unchanged.

It is reported (Aguilar-Uscanga *et al.*, 2003) that aeration intensity effects cell walls formation in fungi. In agreement with this, our study showed very significant changes in CGC production by *S. commune* during cultivation under different aeration rates. CGC content in mycelium rose from 12.4 ± 0.3% to 15.5 ± 0.3% when aeration increased to 1 vvm (Fig. 4). Further

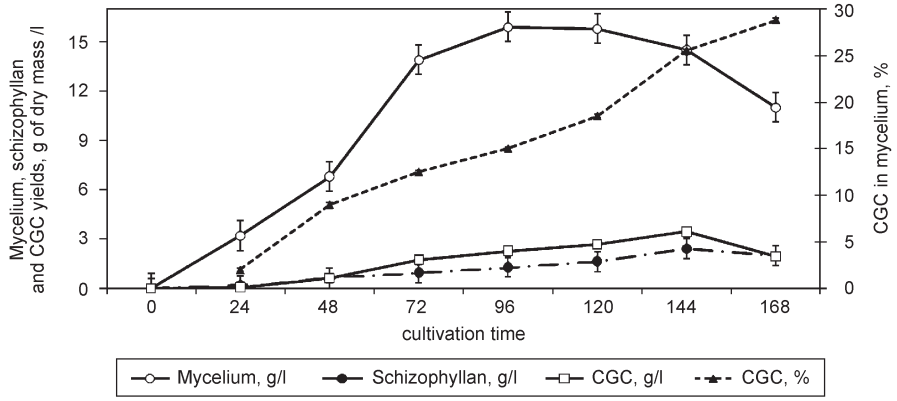

Fig. 6. Change of mycelium yield (g/l), CGC content in mycelium (%), CGC and schizophyllan production (g/l) during *S. commune* cultivation.

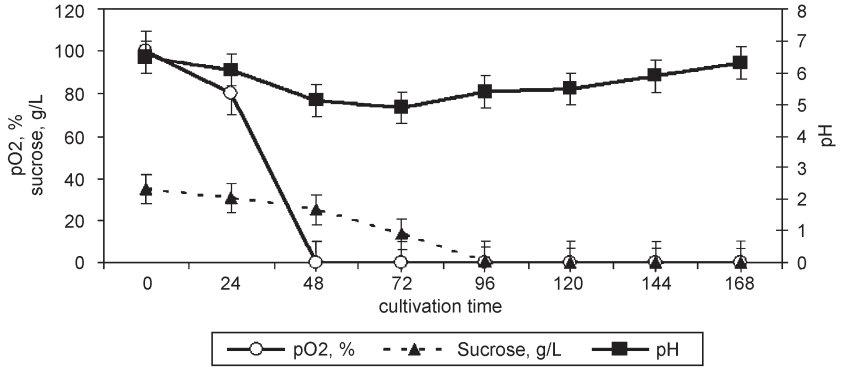

Fig. 7. Change of medium pH, pO_2 (%) and sucrose content in medium (g/l) during *S. commune* cultivation.

aeration increase up to 2 vvm increased CGC content to 16.3 ± 0.3% only. Mycelium yield increased more than 2 times with increase of aeration from 0.5 vvm to 2 vvm and reached the maximum value of 13.7 ± 0.6 g/l (Fig. 4). From the data above, there was calculated maximal CGC production of 2.2 ± 0.2 g/l under 2 vvm aeration.

When the amount of schizophyllan was related to mycelium yield, it was found that the most intensive exopolysaccharide synthesis takes place at aeration of 1 vvm (Fig. 5). These indicate that aeration intensity can be used as the key regulator for redirection of *S. commune* metabolism from schizophyllan synthesis to CGC accumulation.

Cultivation conditions that favored high CGC production (YE 4 g/l, pH 6.5, aeration 2 vvm) were combined and effect of cultivation time on CGC and schizophyllan accumulation was studied in fermenter with 50 l working volume. The culture reached stationary growth phase in 96 hours, the highest mycelium yield of 15.9 ± 0.9 g/l was recorded at that cultivation time as well (Fig. 6). Amount of CGC in mycelium increased during all cultivation and reached maximum value of 28.1 ± 0.2% in 168 hours. The highest CGC production of 3.5 ± 0.3 g/l was recorded in 144 hours of cultivation. The amount of schizophyllan in medium increased until 144 h of cultivation when it reached 2.4 ± 0.6 g/l, whereupon it started to decrease (Fig. 6).

S. commune acidified medium to pH 4.9 in first 72 hours, however then medium pH started to increase and reached 6.3 at the end of cultivation (Fig. 7). All sucrose was consumed within the first 96 hours. pO_2 probe indicated 0% medium oxygen saturation beginning from 48 hours of cultivation (Fig. 7).

By means of cultivation conditions optimisation CGC production by *S. commune* was increased more than 3.8 times. CGC content in mycelium was little sensitive to medium composition, but was increased by aeration intensification and cultivation time prolongation. Mycelium yield was increased by adjustment of YE content in the medium, medium initial pH and aeration intensity. Redirection of fungal metabolism from schizophyllan synthesis to CGC accumulation was achieved most efficiently by aeration intensity increase. The study showed the potential of *S. commune* submerged cultivation for industrial-scale CGC production. CGC from *S. commune* can find application in medicine and chitin/chitosan production as an alternative to CGC from *Micromycetes*.

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