



Characterization of *Monascus purpureus* isolated from Red Yeast Rice and its Evaluation for the Production of Cholesterol Lowering Lovastatin

Preeti Dogra and Dinesh Kumar

School of Bioengineering & Food Technology,
Faculty of Applied Sciences and Biotechnology,
Shoolini University, Solan-173212 (Himachal Pradesh), INDIA

(Corresponding author: Preeti Dogra, preetidogra24@rocketmail.com)

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ABSTRACT: Red yeast is a bio-pigment producing fungi which contain natural form of statins. It is a red mold species which grows on starch containing substrates. Red yeast rice is used as a coloring and flavoring agent beside production of various types of statins. In the present study red yeast was isolated from red rice and screened for the production of lovastatin using solid state fermentation in Rose Bengal Agar after 7 days growth at 35°C. Based on colony morphology, microscopic observations and genetic characterization it was identified as *Monascus purpureus*. Morphological characteristics of the red yeast showed the strain with cleistothecia having oval ascospores and aleuroconidia.

Keywords: Rose Bengal Agar, Red yeast rice, *Monascus purpureus*, SEM, Lovastatin production

INTRODUCTION

Monascus spp. belongs to the family *Monascaceae* of the phylum *Ascomycota*. Based on cultural characteristics 9 *Monascus* species viz., *M. pilosus*, *M. ruber*, *M. purpureus*, *M. floridanus*, *M. eremophilus*, *M. pallens*, *M. sanguineus*, *M. lunisporas*, and *M. argentinensis* are acknowledged internationally (Dikshit and Tallapragada, 2013). Over 20 species have been cited in the literature since the genus *Monascus* was proposed in year 1884 (Hesseltine, 1983). *Monascus* spp. are well known producers of a family of structurally related hexaketide pigments which are yellow and red in colour and are used in Asia for many centuries to colour and flavour food and beverages (Hesseltine, 1983; Yoshimura *et al.*, 1975). These natural colorants are of practical interest because of the difficulties in obtaining red pigments safe for use in food industry (IFT, 1981). *Monascus* pigments typically comprise six major azaphilone pigments: yellow pigments, monascin (C₂₁H₂₂O₅) and ankaflavin (C₂₃H₃₀O₅); orange pigments, monascorubrin (C₂₃H₂₆O₅) and rubropunctatin (C₂₁H₂₂O₅); and red pigments, monascorubramine (C₂₃H₂₇O₄) and rubropuntamine (C₂₁H₂₀O₄). *Monascus* sp. is a non-pathogenic fungus that was reported to produce statins that can lower blood cholesterol in human body and also coproduces mycotoxin (citrinin) as well as other potentially toxic metabolites, such as monascus pyridines (Blanc *et al.*, 1995). The present study was undertaken to isolate the red yeast from red yeast rice and evaluate it for its growth, morphological and

genetic characteristics and for lovastatin production under solid state fermentation process.

MATERIALS AND METHODS

A. Isolation of red yeast

Red yeast culture was isolated from red yeast rice obtained from the local household in Taiwan using Rose Bengal Agar (g/L) (mycological peptone 5g, dextrose 10g, monopotassium phosphate 1g, magnesium sulphate 0.50g, Rose Bengal 0.05g, chloramphenicol 0.10g, agar 15g and pH 6.0). The isolate was maintained on Potato Dextrose Agar (g/L) (Infusion from potatoes 200g, dextrose 20g, agar 15g and pH 6.0) and Rose Bengal Agar medium after incubation at 30°C for 7-8 days. The isolate was sub-cultured once every 4 weeks and stored at 4°C for further use.

B. Taxonomic investigation

The taxonomic investigation of the isolated red yeast culture was performed following the procedure of Hawksworth and Pitt (1983). The isolate was cultivated on the following three culture media before taxonomic investigation viz. a) Malt Extract Agar (MEA) (g/L): malt extract 20g, peptone 1g, glucose 20g, agar 15g and pH 6.0., b) Rose Bengal Agar (RBA) (g/L): mycological peptone 5g, dextrose 10g, monopotassium phosphate 1g, magnesium sulphate 0.50g, Rose Bengal 0.05g, chloramphenicol 0.10g, agar 15g and pH 6.0 and, c) Potato Dextrose Agar (PDA) (g/L): Infusion from potatoes 200g, dextrose 20g, agar 15g and pH 6.0.

All the culture media were sterilized at 121°C and inoculated with 24 h old culture of red yeast and grown for 7-8 days at 28-30°C in a bacteriological incubator. During the growth the samples were taken at intervals using inoculation loop and studied for its morphological and cultural characteristics.

C. Macroscopic and Scanning Electron Microscopic (SEM) investigation of red yeast culture

The isolated red yeast culture was inoculated on Potato Dextrose Agar (PDA) and incubated at room temperature to observe its morphological characteristics viz., colonies, ascospores and conidial stages using light microscopy.

The grown culture was also studied using Scanning Electron Microscopy (SEM). For this study the culture grown on PDA slants was gently scraped and fixed in 0.05 M potassium phosphate buffer (pH 7.3) containing 4% glutaraldehyde. The fixed material was rinsed three times with 0.05M potassium phosphate buffer and distilled water. After dehydration using ethanol (Asensio *et al.*, 2005) the test materials were mounted on stud coated with gold and observed under a LEO 435 VP Scanning Electron Microscope.

D. Molecular identification of red yeast culture

The red yeast culture used in the present study was identified using ITS sequencing. Genomic DNA was isolated from the pellet of red yeast culture using method of Cenis (1992). The isolated DNA was amplified using the ITS primers i.e. GAGGCAATAACAGGTCTGATGC as forward and CCGTGTTTCAAGACGGG as reverse primer. Sequence data was aligned and analyzed for finding the closest homologs for the identification of the red yeast culture. Homology and similarity analysis were done on the ITS sequences of samples using online database, BLASTn. The phylogenetic analysis of sequence was done with the published database using Sea View Version 4 program.

E. Preparation of Red Yeast Rice using *Monascus purpureus* culture

The red yeast rice used in the present study was prepared by inoculating white rice (*Oryza sativa* L.) with 5% (v/w) inoculum of *Monascus purpureus* prepared in Rose Bengal Agar. The rice used in the present study to make red yeast rice was Basmati white rice (from Sirmour, H.P.). The red yeast rice was prepared under controlled condition with appropriate weight, culture age, inoculum volume, temperature, humidity and pH according to Boonsangsom *et al.* (2004). Rice grains were immersed in water for 2 hours following by steaming for 20 minutes. After cooling, 20 grams of steamed rice was put in 100 ml flask and sterilized at 15 psi and 121°C for 15 minutes. One week old pre-cultured *M. purpureus* was used as inoculum and the inoculated mixture was incubated at 30°C for 2-3 weeks. Humidity and pH were measured before and

after inoculation. Samples of red yeast rice were finally taken out after 2-3 weeks of growth and dried in the oven at 65°C for 2 hours to obtain dried red yeast rice and used for the further study.

F. Evaluation of Red Yeast Rice extract for Lovastatin production

For determination of lovastatin production by the fermented red yeast rice a 2g of fermented red yeast rice was suspended in 5 ml ethyl acetate and kept in orbital shaker at 180 rpm and 70°C for 1.5 h. The mixture was centrifuged at 3,000Xg for 10 minutes and the supernatant was collected. To this 1% trifluoroacetic acid was added for the lactonization of the extracted lovastatin. This mixture was further diluted to 10-50ppm.

The presence of lovastatin in the prepared red yeast rice was determined using RP-High Performance Liquid Chromatography (Agilent Technology) with binary pumps using Agilent eclipse XBD column (C₁₈ 4.6 x 150mm bonded with 5 µm particle size) coupled with EZ-Chrome software. The mobile phase used was acetonitrile/water/trifluoroacetic acid (55:45:0.1) with a flow rate of 1ml/min. and the data was recorded with photodiode array detector at a wavelength of 238nm.

Standard were prepared with pure lovastatin (HiMedia). A 5µl injection of each concentration of sample were injected into the RP-HPLC system separately under the standard conditions and analyzed at 238nm. Peak areas were recorded for all the samples and plotted against the standard concentrations to calculate the yield of lovastatin production.

RESULTS AND DISCUSSION

A. Isolation and characterization of red yeast culture

The red yeast used in the present study was isolated from red yeast rice obtained as a gift from local household of Taiwan. The red yeast culture sample isolated from red rice is studied taxonomically according to Hawsorth and Pitt (1983). The morphological and cultural characteristics investigation results of the isolated red yeast culture are shown in Fig. 1. The isolated red yeast showed different qualitative colour response in the Potato dextrose agar (PDA), Malt yeast agar (MYA) and Rose Bengal agar (RBA) media under similar growth conditions. The colonies of red yeast strain isolated on PDA were initially white and later became orange to red. This red yeast isolate released the pigments into the media. The mycelium was white in the early stages but rapidly changed to a rich pink and orange color during the growth. Deep crimson colour was observed as the culture aged at the end of the growth. The septate mycelia are generally coloured and the fungus produces cleistothecium enclosing ascospores similar to the characteristics of red yeast reported earlier by Young (1931) and Carels and Shepherd (1975,1977).

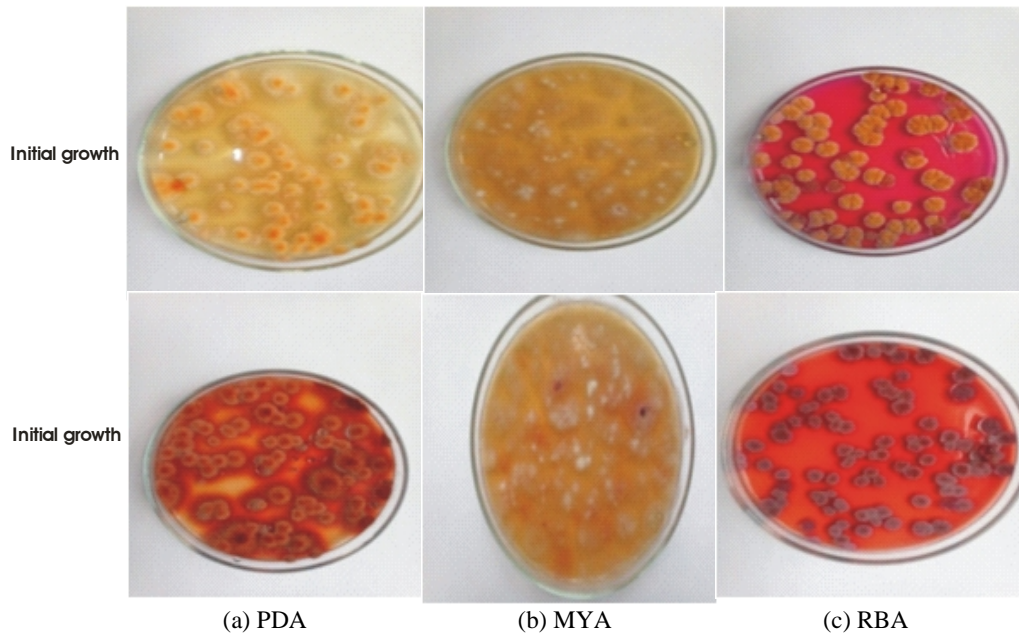


Fig. 1. Growth characteristics of red yeast culture isolated from red yeast rice on different media viz., a) Potato Dextrose Agar (PDA) b) Malt Yeast Agar (MYA) and c) Rose Bengal agar (RBA) after 7 days of growth at 30°C.

The isolated red yeast cultures were further studied by microscopy and the results of this study are shown in Fig. 2. Microscopic observation of the red yeast spores showed presence of perithecia singly on stalk in MEA media. Cleistothecium mycelium was present and ascospores were round, smooth and pigmented. *Monascus* sp. can be easily observed by its ascospores,

which may appear to be spherical in shape or slightly ovoid. The red yeast culture forms active antheridia, ascogonia and conidia from conidiophore. The characteristics of ascomata, ascospores and conidia show similarity to *M. purpureus* strain CMU001 as described by Chairote (2007).

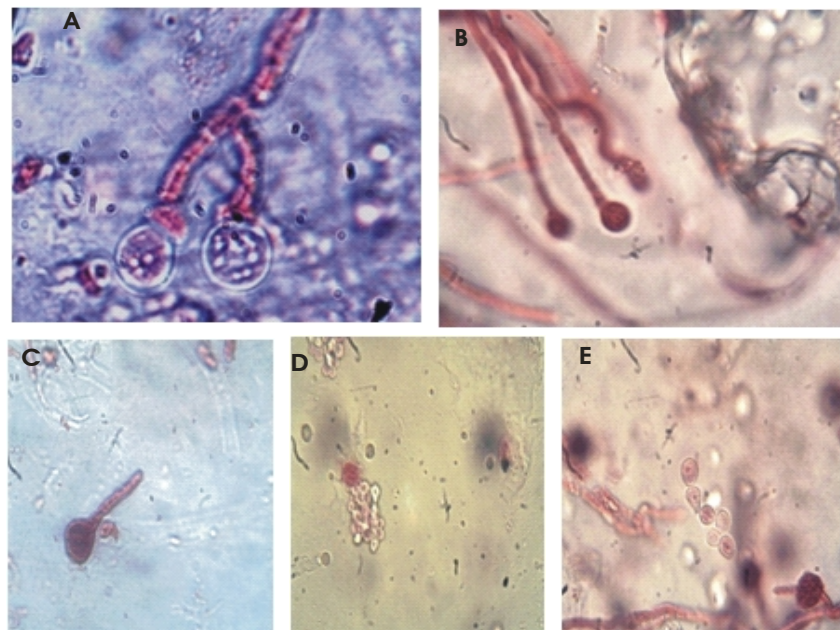


Fig. 2. Microscopic study of red yeast culture isolated from red yeast rice. a) Asci developed in stalked cleistothecium b) Conidiophore bearing conidium and c) Sub-globose or ellipsoidal ascospores d) Conidiophore released from the conidium e) Cluster of Conidiophores.

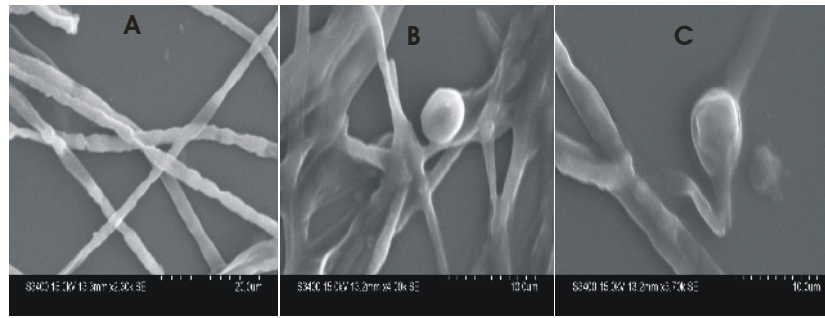


Fig.3. Scanning Electron Micrographic (SEM) study of culture of red yeast isolated from red yeast rice. a) Septate hyphae b) oval shape spore and,c) formation of conidiophores

The colony shape, colour, aerial mycelium and exudates formation between two strains i.e. *M. purpureus* CBS 109.07 and *M. purpureus* 94–25 (Rasheva *et al.*, 1998) are similar to the *M. purpureus* isolated from the red yeast rice reported in the present study. The isolated red yeast culture was also studied using scanning electron microscopy (SEM) and the results are shown in Fig. 3. The SEM study of red yeast showed septate hyphae and oval shaped spore presence in the red yeast due to ascocarp rupture. In general, ascospores of filamentous fungi are more heat resistant than mycelia, conidia and yeast ascospores

(Dijksterhuis and Samson, 2006). Ascospores are widely formed sexual spores within the Ascomycetes with a high survival capability. After the final growth of the culture the red yeast structure mainly composed of mycelium network. The formation of conidiophores in red yeast culture was also recorded in SEM study.

B. Molecular identification of the red yeast culture

The isolated red yeast culture was also characterized by ITS RNA sequencing for its identification. The following data was revealed after PCR amplification and ITS RNA sequencing of the isolated red yeast:

Forward sequence:

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GGAGCCAGGGACTGAAGGTTACTGAACATAAACACTGGGTATATACGCCCTCCGGGGTCCGGTTGTA
CTTTGGGCATTAGGCTTCCCGCTCAACCCAGTCTAACTGCCCTGGAACGAGCCGTCCGACAGGGTGA
GAATCCCGTCCGGGAAGGGGTGTCTGATTCCAAGTGATTCTCCTTCAAAAATTCGAGTTGTTTGGGAAT
GTCCTCTAAATGGGTGGTAAATTTTCATCTCACTCTAAATGTGGCCGGAGGTCTCCGGCCAGTATTTAAC
TGATCATGAAATTTACCACCTTTTAAAGCTGCATTCCCAACAACCTCGACTCGTCAAAGAGATTCA
CATGGACCCAGGACCCCGTCCCAGACGGGATTCTACCCTCCGACTGCCCGTTCCAGGGCACTTA
AACGGGGGTGAGCCCCGAAGTCCCTCTGCAAATTACTACGCGGACCCCGGAGGGCCATCTTTCTATT
TGAGCTCGCGCCGCTTCACTCGCCATTACGGAGGCAATCCCGGTTGGTTTCTTTTCCGCCCTAGAGAT
ATGCTTAAAATACAGGCAGGTAGAGCCTTGGTGAAAGGGGACAAGGAAG
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Reverse sequence:

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TCATGGACATCTGTAAGGCACCTCGCTTAAGTTCTGCAGCATAAACCCCTCGGGATATACGACCCCGG
GAGGGCGCCTTGTTTCTTGGGCCTTAGGCCGCCACCCGAACCGACTCTGGCTGGCCTGCGGGGAAGT
ACTCCGACAGGGTGGCAATCCCGAACCGGGAAGGGGAGTCTGATCCCAAGCGCTTCTCCTTCAACAAT
TTCAAGTTGTTTGTAAATGTCTTTTCAATGGCTGGTAAATTTTCGATCACTCTACTGCTGCGCTATCAC
CTCCGGCCGTATTTAACTGATCATGAAATTTACCACCTTTTAGAACTGCATTCCCAACAACCTCGACT
CGTCAAAGAGCTTACATGGACCCAGGACCCCGTCCCAGTTGGGATTCTACCCTTCAGACTGCG
TGTTCCAGGGCACTTACACGGGGCTGAGTCCGAAGTTCGCTGCTTATTAATGCGCGCCCCCGGAG
CGCCATATTTCAAGGGGTCTTGCCGCTTTTCACTACCTATTACAGCCGCAATCCGGGGTTGTTTCTTTTC
CTCCCTTGAGATGTGTTTTATACGGGGGACAGGCCATCTG
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Both forward and reverse sequence were obtained after sequencing the amplified ITS product fragment of expected size and further assembly sequence was generated. But in the present study only forward sequence was used for the further analysis. The BLASTn result showed the present isolate with highest possibility score of 95% with 99% query coverage to *Monascus* sp. (Accession number JN940514.1) and was therefore finally identified as *Monascus purpureus*. The

comparative analysis of present sequence with the published sequences using the Sea View Version 4 program further confirmed its similarity with the *Monascus purpureus* JN940514.1 and subsequently demonstrated its phylogenetic relation to a generated Neighbor-joining rooted tree as shown in the Fig. 4. The assembly sequence was further deposited at NCBI with accession number KU897090.



Fig. 4. Phylogenetic tree (neighbour joining) of isolated *Monascus purpureus* based on ITS sequencing. The scale bar represents the relative sequence similarities.

C. Production of lovastatin in red yeast rice prepared with Monascus purpureus

The red yeast rice is prepared using local white Basmati rice. The stepwise preparation of red yeast rice is shown in Fig. 5. The fermented red yeast rice was evaluated further for the presence of Lovastatin (Monacolin) an important statin used in lowering of cholesterol level in

human beings. The lactonized red yeast fermented rice extract was filtered through 0.25 μ m membrane filter and analyzed quantitatively using HPLC and the chromatograms of lovastatin (standard) and lovastatin produced by the isolated *Monascus purpureus* are shown in Fig. 6.

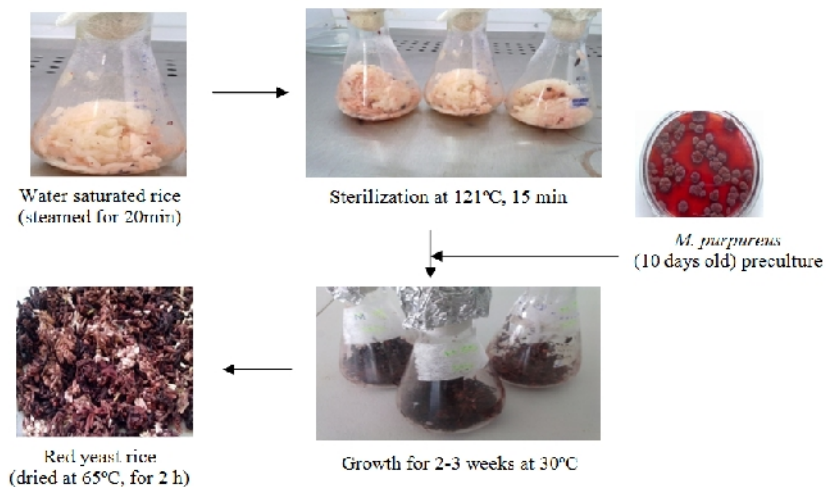


Fig. 5. Stepwise preparation of Red Yeast Rice after fermentation of white rice (*Oryza sativa* L.) with red yeast (*Monascus purpureus*) culture.

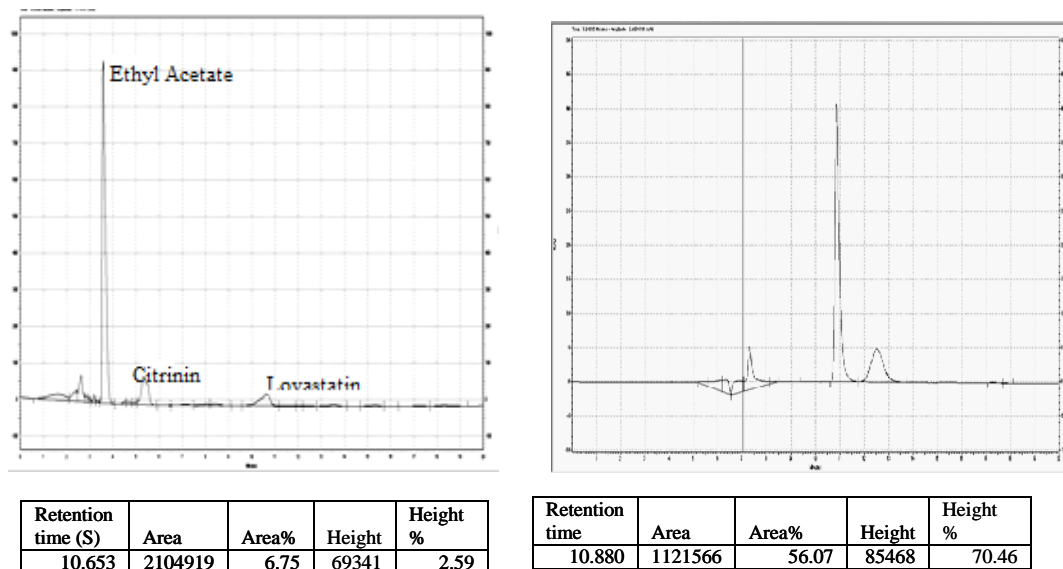


Fig. 6. HPLC chromatogram of lovastatin produced by *Monascus purpureus* (a) produced Lovastatin with a retention time of 10.653 min and b) standard of pure lovastatin with a retention time of 10.880 min.

From the chromatogram it was evident that the retention time of lovastatin produced by *M. purpureus* resembled (Fig. 6a) with retention time of standard lovastatin (Fig. 6b) and 0.00922gm/100gm of lovastatin was recorded from the red yeast rice under solid state fermentation in the present study.

CONCLUSION

All morphological, microscopic and genetic investigations in the present study concluded that the red yeast strain isolated from the fermented red yeast rice is *Monascus purpureus*. The solid state fermented red yeast rice prepared using this culture produced a significant amount of lovastatin in the preliminary stages (0.00922gm/100gm). Further detailed optimization of this culture is under process to improve the production of this important statin (lovastatin) used in lowering the cholesterol level in the human being through its consumption as fermented red yeast rice or as purified preparations.

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