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Characterization of monascorubrin pigment isolated from *Monascus purpureus*

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Abstract
Synthetic colors are major threat and liability to human race. Many naturally ocuuring microorganisms synthesize naturals colors as potential as synthetic colors that can be used as an replacement for synthetic colors in food industry. One such fungal organism is *Monascus purpureus. Monascus purpureus* was isolated from soil samples collected from various regions of Ooty district. Soil samples were analysed and *M. purpureus* was identified and screened for pigment production. The identification of strain was done using Lacto phenol cotton blue staining. Monascorubrin pigment was extracted by standard solvent extraction method, solvent used was ethanol (V/V). Extracted pigment was concentrated using rotary vacuum evaporator. Purified by HPLC (C17 column) and characterized by UV-Vis absorption spectroscopy, Particle size, zeta potential, and LC-MS.

Keywords: *M. purpureus,* monascorubrin, HPLC, Particle size, zeta potential, LC-MS

Introduction
Monascus purpureus is a speices of fungi that are known to produce well-known azaphilone pigments like monascorubrin rubropunctatin. Monascorubrin and rubropunctatin belonging to Monascaceae family. These pigments have a unique structure responsible for their high-affinity for compounds with primary amino groups. Reactions with amino acids yield the water-soluble red pigments, monascorubramine and rubropunctamine (Dufosse, 2009).
Monascus purpureu is a species of mold that is purplish-red in color. It is also known by the names ang-khak rice mold, corn silage mold, maize silage mold, and rice kernel discoloration. During growth, *Monascus* spp. break down starch substrate into several metabolites, including pigments produced as secondary metabolites. The structure of pigments depends on type of substrate and other specific factors during culture, such as pH, temperature, and moisture content (Panda *et al.,* 2010). Monascus can produce red, yellow and orange, pigments. The condensation of one mole of acetate with five moles of malonate in the cytosol leads to the formation of a hexaketide chromophore by the multienzyme complex polyketide synthase (Yang *et al.*, 2006) [12-13].
Medium chain fatty acids, for example, octanoic acid, are synthesized by the fatty acid pathway and bind to the structure of the chromophore through a trans-etherification reaction, generating the orange pigment monascorubrin or rubropunctatin by trans-etherification of the octanoic acid. The reduction of the reddish orange pigment monascorubrin forms the yellow pigment ankaflavin, or monascin for rubropunctatin, whereas the amination of orange pigments gives rise to red pigments monascorubramine and rubropunctamin (Mukherjee and Singh, 2011). Due to the affinity by amine groups, the Monascus pigments are frequently associated to proteins or to the cellwall, forming complex pigments, difficult of being extracted (Meinicke *et al.,* 2012). Several factors such as strain selection, substrate, pH, nitrogen source, light intensity temperature, broth rheology, and oxygen can influence the red pigment production (Vendruscolo *et al.,* 2014) [4]. The objectives of this study were 1) to isolate pigment producing *M. purpureus* from different soil samples; 2) purify and characterize red pigment by standard protocol and techniques; 3) to characterize the red pigment.

Materials and Methods
This chapter contains the details of methodology and materials used during the experimentation. The investigation entitled "Characterization of red pigment isolated from

Monascus purpureus". The details of location, soil and material used and techniques adopted in the present experiment are described in this chapter under the following heads:

Sample collection
The soil samples were collected from different area of Ooty, Tamil Nadu. The soil sample are collected in sterile plastic (zipper) polythene bags by digging 5-10 cm deep from different industrial sites around the rhizospheric soil. A total of 5 soil samples were collected, about 50 g of soil was collected in sterile bags for each sample.

Isolation of microorganisms
The collected soil samples were spread on potato dextrose media (10^{-4} and 10^{-5} dilution) and incubated overnight at 37 °C. After incubation pigmented colonies were selected. The identification of fungal species was done based on morphological, cultural, biochemical and physicochemical characteristics as suggested by Sinha *et al*., (2017) [10].

Identification and Characterization of fungi
The pigment producing fungal cells that appeared on the agar plates were picked for identification. Isolates were identified using its morphological characteristics and lactophenol cotton blue staining (AOAC, 2016) [1].

Lactophenol cotton blue staining
A drop of lactophenol cotton blue stain was placed on a clean slide under aseptic conditions. Nichrome wire was used to tease the fungal culture into a thin smear. Cover slip was placed and slide was viewed. The smear was observed by 100x magnification using a bright field microscope.

Extraction of Monascorubrin
To prepare the inoculum, a 7-day-oldmycelium of *M. purpureus* in a PDA slant was washed by 6 ml distilled water and then transferred to the culture medium by adjusting the concentration of suspension spores at about 2.5 -107 spores/ml. The inoculum was incubated in a 500-ml Erlenmeyer flask containing 50 ml of the culture medium for 7 days in a rotary shaker at 180 rpm under 30 °C. The 7 day culture was taken and filtered using whatman No.1 filter paper the cells were harvested and 60% (w/v) absolute ethanol was added to the cells and placed in a magnetic stirrer. The suspension was heated up to 65 °C to remove the pigments from the cells. After complete extraction the suspension was centrifuged at 8000 rpm for 15 mins. The collected supernatant was fed into rotary vacuum evaporator (SARE-T43 Model, SPAN Automation) and heated upto 70 °C at 80 rpm for 2 hrs to concentrate the pigment and remove ethanol from pigment. The concentrated pigment solution was used as a source of monascorubrin. Monascorubrin was quantitatively assay based on measuring the absorbance of monascorubrin in the acidic form at Max λ according to the following equation. (Vendruscolo *et al.*, 2014) [4].

Concentration of monascorubrin (µg/ml) = O. $D_{(Max\lambda)}$×17.072

Characterization of monascorubrin
High Performance Liquid Chromatography (HPLC) purification
HPLC determination of the lactoferrin separation module Alliance 2695 with diode-array detector PDA 2996 (Waters, Millford, USA) were used. Detection was carried out at the wavelength 205 nm. Separation was performed on a chromatographic column Poroshell 300SB-C-17, 2.1 × 75 mm, 5 µm particle size (Agilent, Santa Clara, USA). Linear gradient and flow rate 1 ml/min were used. Mobile phase A consisted of water/acetonitrile/trifluoracetic acid (95:5:0.1) and mobile phase B water/acetonitrile/trifluoracetic acid (5:95:0.1). The column temperature was set at 45 °C and injection volume was 10 µl. Data were collected and evaluated by software Empower (Waters, Millford, USA).

FTIR analysis
Methods used by Ahamad *et al.* (2013) was followed. The transformation of the interferogram into spectrum was carried out mathematically with a dedicated on-line computer. The Bruker IFS66v FT-IR instrument (VERTEX model, Bruker optics, Germany) consists of globar and mercury vapor lamp as sources, an interferometer chamber comprising of KBr (Potassium Bromide) and Mylar beam splitters followed by a sample chamber and detector. The spectrometer works under vacuum and the entire region of 10 – 10000 Cm^{-1} was covered. Solid samples were dispersed in KBr or polyethylene depending on the region of interest.

Particle size analysis
Particle size analysis is performed by dynamic light scattering (DLS). Depending on the physical properties of the sample, the dynamic range is 0.3nm – 0.8µm. The temperature of the system is set to 20 °C and the injection volume was 2ml.

Zeta potential
The charge on the surface of particles is characterized by measuring the zeta potential. The measurement range is -200 to + 200mV. The temperature of the system is set to 20 °C and the injection volume was 2ml.

Results and Discussion
Soil samples were serially diluted up to 10^8 dilutions. 10^4 and 10^5 dilutions were plated on sabouraud dextrose agar for fungi isolation. Pigmented colonies were observed in different soil samples. White colour cottony fungal colonies (Isolate F1) with reddish orange colour diffused pigments were observed on the 3rd day on sabouraud dextrose agar plates in 10^5 dilution of soil sample. The fungal colonies were picked and purely spread on sabouraud dextrose agar plates. Intense pigmentation was observed on the 7th day of incubation at 30 °C. The colony morphology of isolate F1 which had an apical hyphal growth, with wall extension to the hemispherical apex of the hyphae. The hyphal branching occurred to give an approximately circular color (Fig 1).

Fig 1: Fungal isolate F1

On LPCB staining isolate F1 (Fig 2) was found to be well-developed, branched and septate, saprophytic, forming conidia and sporangium-like asci (ascocarps and

cleistothecium, terminal with and individual hyphal walls), with asci that are enclosed by interwoven hyphae. It can reproduce sexually (involving the formation of cleistothecium with ascospores) and asexually (by the formation of conidia), which strictly aerobic.

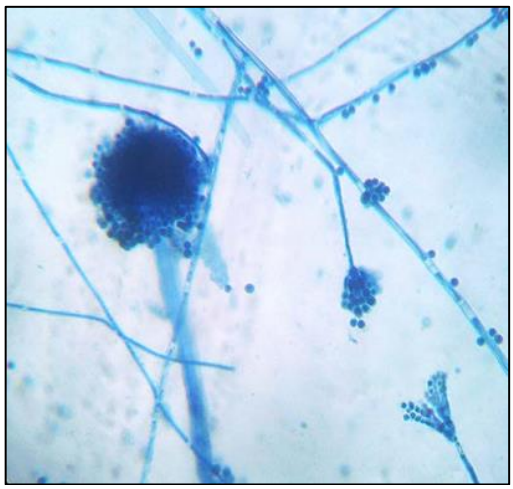

Fig 2: LPCB staining of Isolate F1

Monascorubrin was extracted from 7 day culture using solvent extraction and concentrated by rotary vaccum evaporator. The extract obtain after concentrating was used as a pigment source (Fig 3). The pigment was further purified using HPLC (C17) column. The extract was further eluted using absolute ethanol. The resultant pigment was stored at 4 °C for characterization. The concentration of the monascorubrin pigment was 40.25 µg/ml

Fig 3: Concentrated monascorubrin pigment

The absorbance spectrum of purified monascorubrin was estimated from 200nm to 800nm using UV-Vis spectrophotometer. The concentrated was reconstituted in methanol and absorption was estimated. The absorption was maximum at 470nm (Fig 4).These results were in accordance to the previous research findings of Vendruscolo *et al*., 2014 [4] studied the UV-Vis spectrum of monascorubrin and found its peaks at 450 nm.

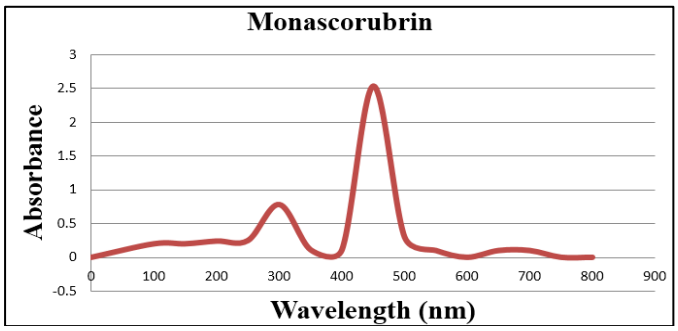

Fig 4: UV-Vis absorption spectrum of purified monascorubrin dissolved in methanol

The particle size and zeta potential of the monascorubrin was determined using nano particle instrument. The purified monascorubrin had a particle size of 3.84 nm (Figure 5) and the zeta potential of the purified Monascorubrin was 2.9 mV (Figure 6). This result was in acceptance to the previous research findings of Lau *et al*., (2004).

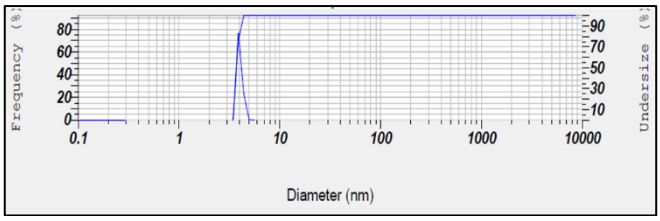

Fig 5: Particle size of monascorubrin

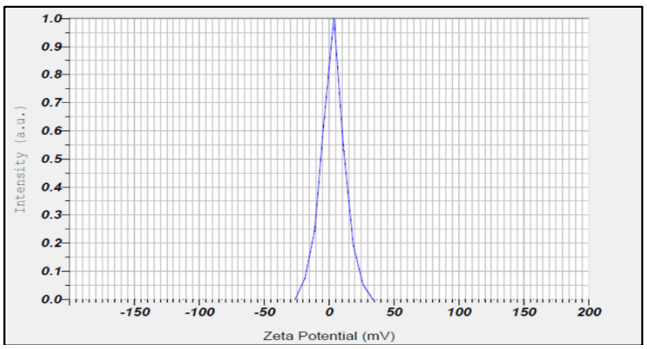

Fig 6: Zeta potential of monascorubrin

FTIR spectroscopy of the methanolic pigments filtrate was done in order to detect chemical classes of compounds based upon their functional groups present in the cultural filtrate (Fig. 7). In monascorubrin, a peak at 877.63cm-1 showed C=O stretch indicating presence of pyrone of coumarin ring and peaks at 1578.29 cm-1, 1639.40 cm-1 corresponded to benzene ring's C=C stretch. There was C-H bending peak at 1454.11 cm-1 and 1383.26cm-1 respectively. C=O stretching band at 1044.89cm-1, 1084.89cm-1 and 635cm-1 in the culture filtrate indicated the presence 1.4-quinones of anthraquinones respectively. The bands at 3355.96 cm-1,2362.71 cm-1 and 2981.75cm-1 corresponded to C-H stretch which has been reported in some alkaloids and hydrogen bonded OH moiety.

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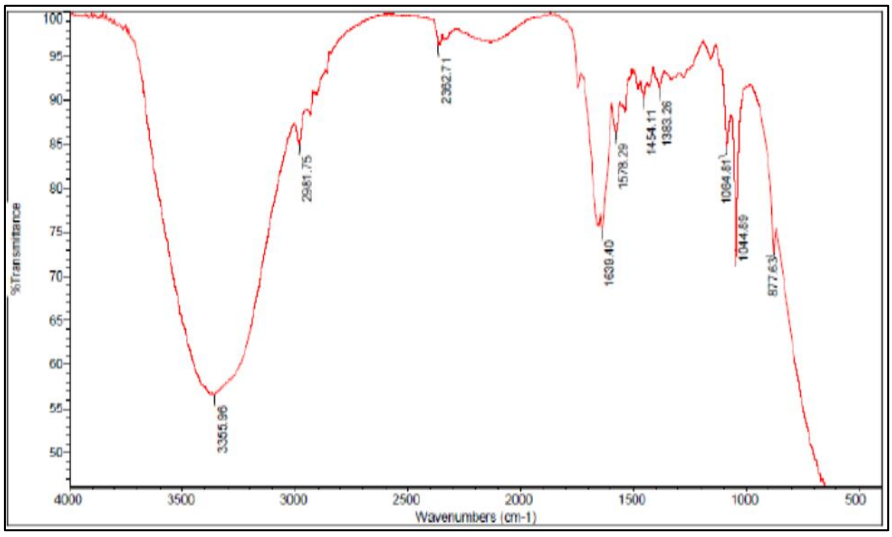

Fig 7: FT-IR spectroscopy of monascorubrin

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