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Bio-Synthesis of silver nanoparticles using a food colorant produced by *Monascus purpureus* MTCC 410 and its inhibitory effect on the food-borne human pathogen, *Aspergillus flavus*

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Abstract
Aflatoxins are secondary metabolites produced by species of *Aspergillus* fungus with toxic, carcinogenic, mutagenic, and teratogenic effects. *Aspergillus* spp. often grows on the food commodities such as peanuts, rice, corn and cottonseed, so contamination of these commodities with aflatoxin makes them unsuitable for human and animal consumption. With the aim to inhibit the growth of mycotoxigenic fungus *Aspergillus flavus* NCIM 538, silver nanoparticles were biologically synthesized using a red pigment produced by *Monascus purpureus* MTCC 410 (the Red Yeast Rice Fungus). The red pigment production was studied in the cooked rice and jack fruit seed powder medium by solid state fermentation. The extracellular pigment was effectively extracted using one step method by treating it with 95% ethanol followed by filtration and air drying. The pigment was characterized using the chromatographic and spectrophotometric method. The silver nanoparticles (AgNPs) synthesized using the red pigment were characterized by UV Spectrophotometry, transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR). TEM images showed that the generated nanoparticles were varied in size (20-50 nm) and shape, and exhibited a surface plasmon resonance at around 437 nm. The inhibitory effect of AgNPs was assessed against *Aspergillus flavus* NCIM 538 at various concentrations (10 µg/ml-100 mg/ml). The fluorescence of the aflatoxin was observed to decrease with increase in the concentration of AgNPs which indicated the inhibitory effect of AgNPs on growth of *Aspergillus flavus* and aflatoxin production. The biological property of AgNPs can be effectively altered to work at a lesser concentration by manipulating its physical and biochemical properties.

Keywords: *Monascus purpureus,* aflatoxin, silver nanoparticles, *Aspergillus flavus*

Introduction
Aflatoxin, produced by *Aspergillus* spp., is the main mycotoxin reported to contaminate crops and food products in India (Reddy and Chinnam, 2007) [22] (Kumar *et al*., 2017) [15]. This not only imposes a threat to the agro-economy but also threatens the human and animal health since it causes various complications such as hepatotoxicity, teratogenicity, and immunotoxicity (Amaike and Keller, 2011) [2]. Silver nanoparticles (AgNPs) have been most effective as a bio-control agent since it has eminent antimicrobial efficacy against bacteria, viruses and other eukaryotic micro-organisms. Also, they are relatively easy to prepare and can be studied widely for its activity by altering their physical, chemical and biological properties (Panja *et al*., 2021) [20]. Whole microbial cells and cellular products have been used for the synthesis of biogenic silver nanoparticles (Klaus *et al*., 1999) [13]. Extracellular components produced by microbial cells can help in economizing AgNPs synthesis because the extensive extraction procedure can be avoided. This focuses on the necessity of search for such extracellular products that are inexpensive, easier to produce and purify. Therefore, pigments derived from microbes have been recently attracting considerable attention due to their metal reduction ability. In this work, silver nanoparticles synthesized using a red yeast pigment were recruited so as to inhibit the growth and toxin production of *Aspergillus flavus*.

Materials and Methods
Standard Strains
Monascus purpureus MTCC 410 was revived *on* Malt Extract agar slants and incubated at 30°C for 6-7 days. *Aspergillus flavus* NCIM 538 was maintained on Sabouraud Dextrose Agar (SDA) and incubated at 37°C for 3-5 days.

Production, extraction and characterisation of the red pigment: White arils of Jackfruit seeds (seed coats), were peeled off and sliced into thin chips, dried at 60°C for 12 h and ground. A mass of 5 g of seed powder was taken into 250 ml Erlenmeyer flask and a salt solution (2 ml) containing (in g/l): KH_2PO_4 2gm, NH_4NO_3 5gm, NaCl 1gm, and $MgSO_4 \cdot 7H_2O$ 1 g was added. Initial moisture was set at 65% by adding the requisite amount of distilled water. The contents of the flasks were mixed thoroughly, autoclaved at 121°C for 20 min and cooled to room temperature. It was inoculated with the spore suspension containing 10^5 spores/ml of *M. purpureus* MTCC 410 and incubated at 30°C with 50% humidity for 6-7 days (Babitha *et al.*, 2006) [3].

Similarly, 50 gm of rice was soaked overnight in 150 ml tap water. Water was drained off from rice and it was spread over paper so as to absorb the extra water, and further rice was crushed with mortar and pestle. Rice was supplemented with 0.5% monosodium glutamate and autoclaved at 121ºC for 20 min and cooled. The substrate base natural medium was inoculated with 10^5 spores/ml of seed culture of *Monascus purpureus* MTCC 410 and kept at 30ºC for 10 days. Moisture content was maintained up to 35-40% (Neera *et al.*, 2017) [18].

Red pigment was extracted from different substrates using 95% ethanol, at the rate of 5 ml ethanol per gm of fermented mass and kept at shaking condition on rotary shaker at 180 rpm under 30ºC for overnight. It was filtered through Whatmann filter paper No. 1, and then kept for drying. After drying, pigment was collected in microfuge tubes for further characterization study (Neera *et al.*, 2017) [18].

Pigment was analyzed by Thin Layer Chromatography (TLC). Concentrated ethanol extracts were applied to Silica Gel 60 plates (Merck, Darmstadt, Germany) and developed using following solvent systems, Benzene: Chloroform: Methanol (85:12:3), (Benzene: Methanol: Chloroform (30:10:9) and Chloroform: Methanol: Water (90:25:4) (Bau and Wong 1979) [4]. Pigment was assessed for absorption maxima using spectrophotometer (Chemito UV 2100).

Synthesis and characterization of Silver Nanoparticles

Synthesis of AgNPs was achieved by reacting silver ions with red pigment solution. Briefly, the red pigment was reacted with 10^{-3} M silver sulphate aqueous solution under dark conditions for 24-48 h (EI-Baz *et al.*, 2015) [7]. Silver nanoparticles were characterized using UV -visible spectroscopy. The DMSO extract was assessed for absorption maxima using spectrophotometer (Chemito UV 2100) and FTIR (Agilent Cary 630, RAP Analytical, India) (EI-Baz *et al.*, 2015) [7]. Further, TEM grids were prepared by sonicating AgNPs sample solution for 10 min and placing a few drops on the carbon coated copper grid and dried until complete evaporation. Transmission electron microscope (PHILIPS CM 200) operated at 200 Kv accelerating voltage (IIT, Mumbai) (Ahmad *et al.*, 2003) [1].

Antifungal activity of silver nanoparticles: *Aspergillus flavus* NCIM 538 was grown in SDA and incubated at 37˚C for 4-5 days. For detection of Aflatoxin TLC was performed using a solvent system of chloroform: Acetone (8.5: 1.5 ml). It was observed under UV light Chamber (Damann and Kenneth, 2014) [6].

Antifungal activity of AgNPs was assessed using broth dilution method by attaining various concentrations of (10 µg/ml to 100 mg/ml) in SDB (4.5 ml). In indigenous tube, fixed quantity i.e., 250 µl of *Aspergillus flavus* NCIM 538 (10^3 spores/ml) was added. The tube without AgNPs served as a control. All the tubes were Incubated for 2-3 days at 37˚C (Sahu Sonam *et al.*, 2015) [23]. Tubes were visually observed for fungal mycelium and confirmed by spot inoculating the culture broth on SDA plate whereas Aflatoxin was detected under UV light and fluorescence was measured using Photoflorimeter (Equiptronics EQ 870 Digital Photoflorimeter).

Results and Discussion

Red pigment was extracellularly produced by *Monascus purpureus* MTCC 410 using Rice and Jackfruit seed powder substrates. The Red pigment was extracted using 95% ethanol and evaporated and collected for further biological studies (Fig 1) The TLC study revealed a red-orange fluorescent band on the TLC plate under ultraviolet light (365 nm). The spectrum depicted absorption maxima of the red pigment to be 480 nm (Fig 2) which corresponds to the absorption maxima of Monascorubrin, a food colorant (pigment) secreted by *Monascus* spp. Shi *et al* (2017) [25] and Mullaiselvan *et al* (2020)[10] reported absorption maxima of intracellularly and extracellularly produced red pigments by *Monascus* sp. in the range 430 to 470 nm.

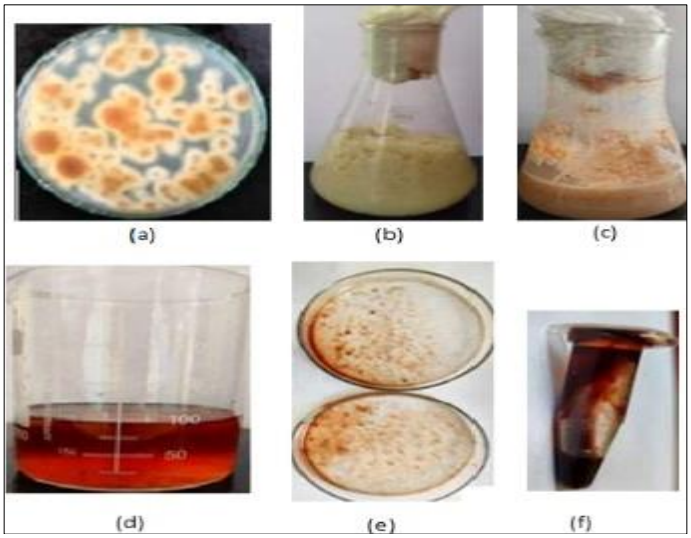

Fig 1: Red pigment production and extraction (a): Growth of *Monascus purpureus* MTCC 410 on SDA (b): Red pigment production in cooked Rice (c): Red pigment production in Jackfruit seed powder (d): Ethanolic extract of the pigment (e): Solvent Evaporation (f): Pigment collection

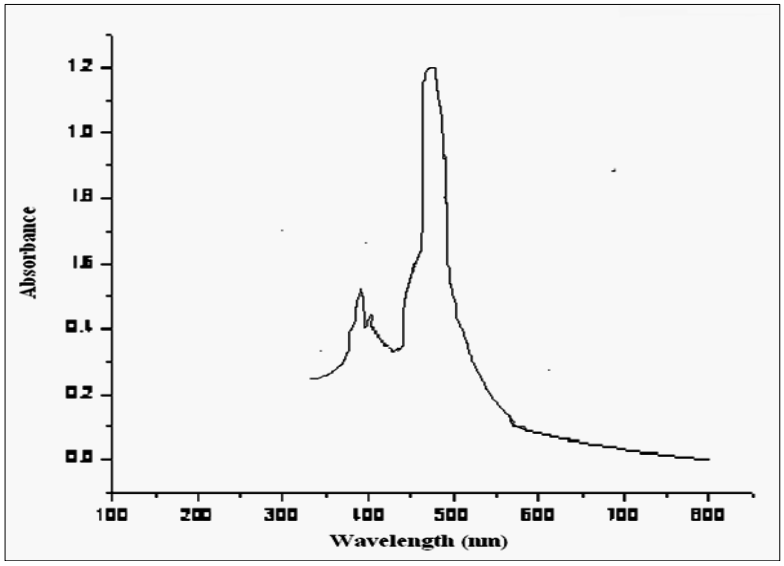

Fig 2: UV-Visible spectrum of the red pigment produced by *Monascus purpureus* MTCC 410

Biological method of AgNPs synthesis is a one-step, simple, inexpensive, environment-friendly method and does not demand for elevated temperatures (300-600°C), pressure, laser ablation, gamma irradiation, electron irradiation and harmful chemicals (Iravani *et al*., 2014) [11] (Gamboa *et al*., 2019) [8] (Koli *et al*., 2018) [14]. Since AgNPs have a high surface area-to-volume ratio, it confers unique properties on them and enhances their catalytic, magnetic, mechanical, and optical properties, thereby expanding scope in the medical field (Sanchez *et al*., 2020) [24]. Therefore, the emphasis for nanoparticle synthesis is drifting away from the conventional methods (physical and chemical) toward "green" chemistry, i.e., biological synthesis. Plants and microbes have become an essential target in this quest because of their abundant availability and diversity. Hence in the present work red pigment-synthesied Silver nanoparticles were characterized using UV spectroscopy, which exhibited strong absorption peak at 437 nm (Fig 3), it is a typical absorption band of spherical AgNPs due to surface Plasmon resonance phenomenon. The red pigment on reacting with silver salt displayed a typical dark brown coloration which is due to reduction of Ag ions and formation of surface plasmon resonance, whereas the metal solution without pigment remained colorless for the same period and under the same conditions (Ahmad *et al*., 2003) [1]. From the TEM results it is evident that most of the AGNPs are irregular in shape (Fig 4.) and the size varied between 20-50 nm (Bhatnagar *et al*., 2019) [5]. The FTIR Spectroscopy confirms the functional group present. In our study, FTIR spectroscopy of the silver nanoparticles was done in order to detect functional group responsible for bioreduction of Ag+ and involved in capping of silver nanoparticles. The observed intense bands were compared with standard values to identify the functional groups (Kumar et. al., 2015) [16]. FTIR spectrum shows absorption band at 1636.729 cm^{-1} was assigned to C=C (alkene) stretching. Band at 2111.885 cm^{-1} in the spectra corresponds to C≡C (alkyne) (Fig 5). The bands 3325.764cm^{-1} was assigned to N-H (amine group) stretching vibration which indicates presence of proteins (Zhang et. al., 2020) [26]. It was documented that protein molecules co-operate with silver nanoparticles via free amide groups. FTIR data demonstrated that amide linkage of the protein possessed the higher potential to join silver and consequently forming protein covering around silver nanoparticles to prevent agglomeration and thereby stabilizing the medium. Presence of C=C (alkene) and C≡C (alkyne) stretching involve in stabilizing nanoparticles by proteins which possibly attach silver and perform both capping and stabilizing process of nanoparticles (Hamouda *et al.*, 2019) [9].

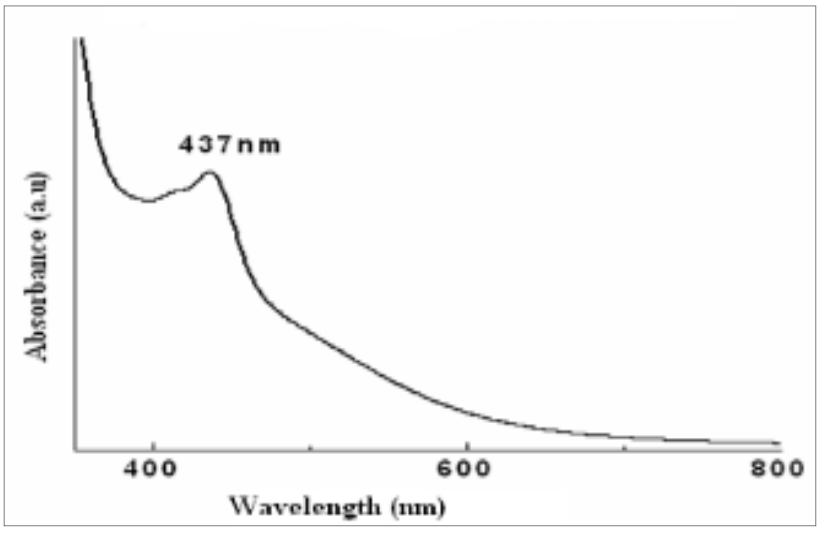

Fig 3: UV-Visible spectrum of the silver nanoparticles synthesized using the red pigment

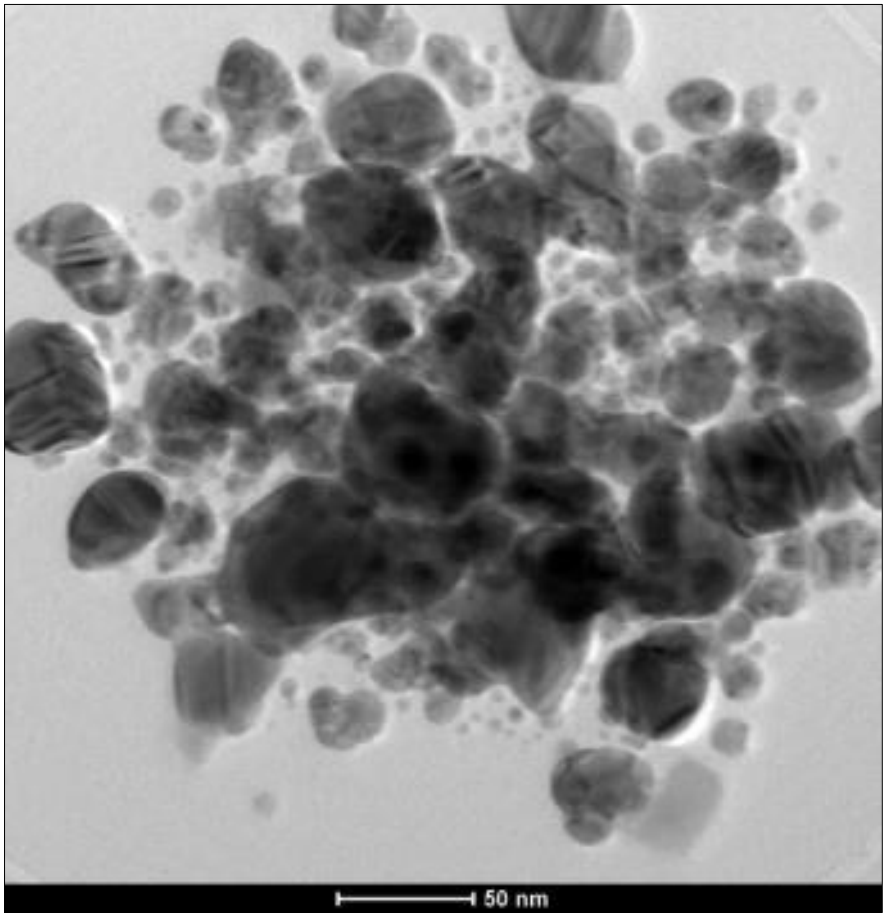

Fig 4: TEM analysis of the silver nanoparticles synthesized using the red pigment

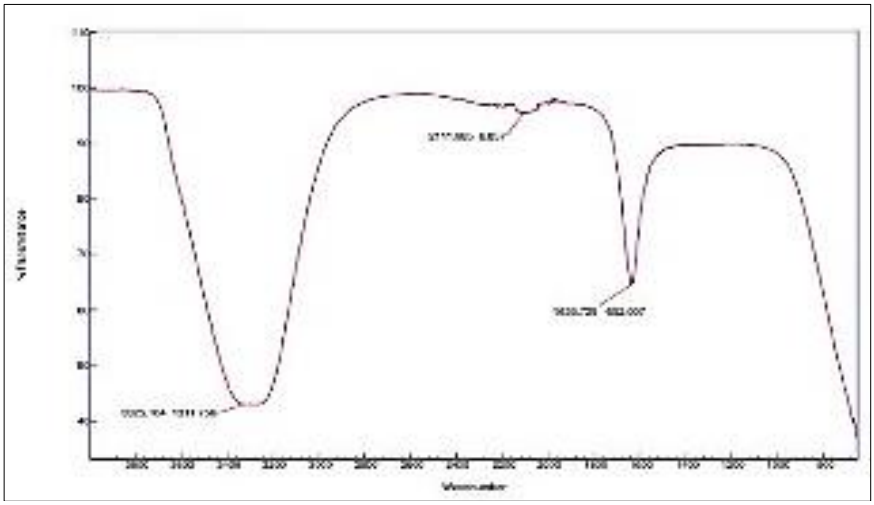

Fig 5: FTIR Analysis of silver nanoparticles synthesized using Red pigment

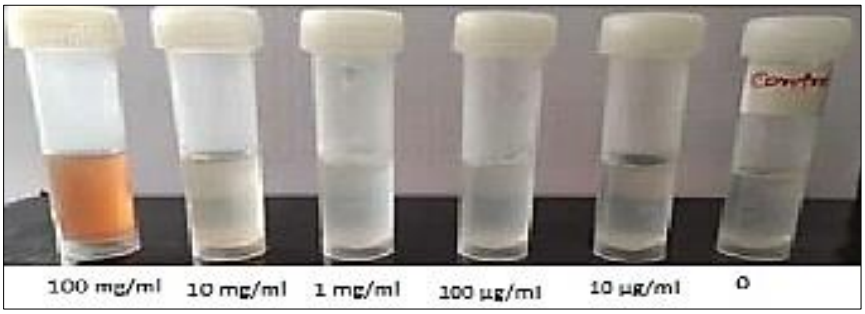

Fig 6: Inhibitory effect of AgNPs on *Aspergillus flavus* NCIM 538 by broth dilution

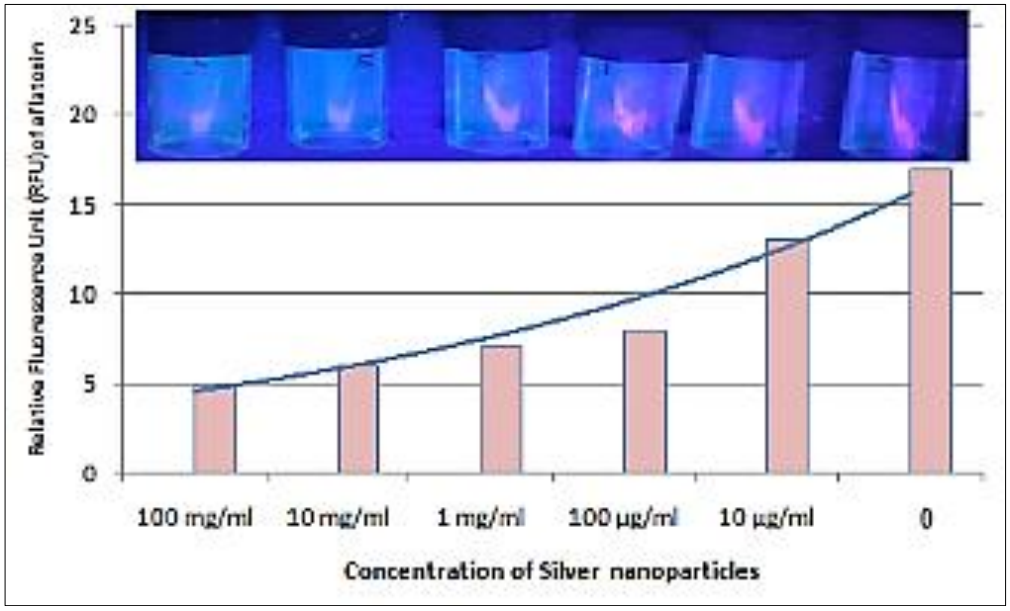

Fig 7: Inhibitory effect of silver nanoparticles on aflatoxin production

Antifungal activity of silver nanoparticles using Broth Dilution method showed that a minimum inhibition concentration (MIC) equal to 0.1gm/ml of Silver nanoparticles was required against *Aspergillus flavus* (Fig 6) which was confirmed by spot inoculating the culture broth on SDA plate. Silver nanoparticles effectively inhibited the growth of the fungus and the Aflatoxin production (Fig 7). Panacek et. al. has claimed that nanosized silver has significant antifungal activity against *Candida albicans* (Panacek *et al*., 2009) [19]. Also in study done by Kim *et al*, the MIC values indicated that silver nanoparticles inhibited growth of *Candida albicans* at relatively lower concentrations (Kim *et al*., 2008) [12]. This difference in observation could be due to the different species of the test fungi and also the biological module used as a reducing agent in the process of nanoparticle synthesis. Results of past studies showed that low concentration of silver nanoparticles could inhibit fungi and those levels had no toxic effects on human health (Rathnayake *et al*., 2012) [21]. Silver is the most effective and least toxic bioactive substances known used to treat human aliments for over 100 years due to its natural anti-microbial properties (koli *et al*., 2018) [14]. Since nanosized silver material has stronger antimicrobial activity than bulk silver material, it has recently attracted great attention (Mousavi *et al*., 2015) [17].

Conclusions

Silver nanoparticles synthesized using the Red pigment (monascorubrin) produced from *Monascus purpureus* MTCC 410 effectively inhibited the growth and the aflatoxin production of the food-borne human pathogen, *Aspergillus flavus*.

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Ethical Matters: No animal model has been used for the present work.

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