

Antibacterial and Antioxidant Activity of the pigment produced by *Monascus purpureus*

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Abstract: Polyketide pigments were isolated and purified from *Monascus purpureus* (MTCC 1090). The quantity of pigment was higher in intracellular than extra cellular. The pigment was produced by submerged and solid state fermentation. Pigment accumulation was observed on 3rd day during solid state fermentation and the SEM spore morphology reveals that the size of spore was 10µm long. The 3 different pigments Red, orange, yellow were separated from the methanol extract of intracellular pigment. The antimicrobial activity of isolated pigment were done towards 4 bacteria namely *S.aureus*, *E. coli*, *Klesiella* sp., *Providencia* sp. Out of four test pathogen .three were sensitive for red and all are sensitive for crude pigment but not against purified yellow and orange. The antioxidant activity of orange pigment isolated from *M. purpureus* was confirmed by FRAP assay and compared with ascorbic acid. The reducing power of the extract might be due to their hydrogen-donating ability. Microbial pigments can be an alternative to the use of synthetic compounds in food and pharmaceutical technology. Further it was also observed that these pigments were non toxic confirmed by in vivo larvicidal assay. The present study concludes pigments from *Monascus purpureus* holding various pigments having various potent biological activities like antibacterial, and antioxidant effect without any side effect.

Key Words: *Monascus purpureus*, Antimicrobial activity, Antioxidant activity, in vivo larvicidal assay.

INTRODUCTION

Monascus purpureus is a red mold species which may be cultivated on starch -containing substrates. The solid -state fermentation of rice by *Monascus* has a long tradition in East Asian countries which dates back at least to the first century A.D. (Meyer, 1990). The microorganisms used for fermenting red yeast rice are various species of a filamentous fungus known as *Monascus*. The *Monascus* group includes *M. anka*, *M. ruber*, and a strain of *M.ruber* known as *M. Purpureus*, among others. These fungi can produce an intense red pigment as well as other metabolic by -products when cultivated on cooked nonglutinous rice (Ma et al. 2000).

Red yeast rice also contains unsaturated fatty acids that may also help reduce serum lipids (Wang, 1997). Red yeast rice extract may help to reduce total cholesterol levels, lower levels of LDH (bad) cholesterol, increase levels of HDL (good) cholesterol, and lower the level of unhealthy fats called triglycerides. *Monascus* pigments are a group of fungal metabolites called azaphillones which are synthesized from polyketide chromophores and β keto acids by esterification (Ming-Jen Ch et al., 2016).

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The red pigment is also reported to have the potential for therapeutic use particularly when produced in red rice (Zhou et al., 2009). Red pigments are formed by the chemicals modification of orange pigments.

MATERIALS & METHODS

Collection of Samples

Monascus purpureus was obtained from IMTECH Culture Collection Centre, Chandigarh, India and maintained on potato dextrose agar slants. Morphology of *Monascus purpureus* was observed under Photomicroscope.

Growth on Different Medium

PDA, YPSS and Corn meal agar were separately prepared and supplemented with 10µg streptomycin and allowed to solidify. *Monascus purpureus* from stock slant was picked and inoculated on the plates and incubated under 28°C for 7 days.

Submerged fermentation for pigment production (Spepherd, 1977)

Fermentation was carried out in a One ml of spore suspension was inoculated into Erlenmeyer flask containing 100 ml of sterile synthetic medium. This was incubated at $28 \pm 2^\circ\text{C}$ for 7 days.

Solid state fermentation for pigment production

20 g of rice were soaked in water for 30 min and then sterilized for 5 min at 80°C. Sterile fungal spores were inoculated and incubated under 28°C for ten days. The pigment production were monitored by the addition of 1% peptone, Yeast extract and compared with control. After incubation the pigments were extracted (1:50) with 80% Ethyl acetate, 80% of Methanol, acetone and water. The OD was taken at 670 nm.

Scanning Electron Microscope (SEM) Analysis

A few granules and fragments of biomass was washed twice with phosphate buffer saline by adding 5 ml PBS to the sample and kept at room temperature for 10 minutes. Then the sample was fixed with by treating with 5 ml of 2.5% glutaraldehyde and kept at 4°C overnight. Then the sample was gradually dehydrated by placing it sequentially in 5 ml of 10% distilled ethanol for 10 minutes till 100% distilled ethanol. After dehydration, the sample was placed on a slide and kept in desiccators for drying followed by observation under Scanning Electron Microscope.

Extraction of cellular pigments

The washed mycelia was taken in Erlenmeyer flask with 25 ml of 75% methanol, incubated on a rotary shaker (150 rpm) at $28 \pm 2^\circ\text{C}$ for 30 minutes and filtered through pre-weighed Whatman No. 1 filter paper.

Estimation of pigment

Pigment estimation was done as described by Tseng in which the optical density at its absorbance maxima were expressed as the concentration of pigment produced. Optical density (absorbance) was measured for both extra-cellular and intra-cellular pigments at λ_{650} , λ_{590} and λ_{570} corresponding to red, orange and yellow pigments respectively in UV-visible spectrophotometer.

Thin Layer Chromatography (TLC)

12 g of silica gel (TLC grade) was mixed well with 24 ml of distilled water in a 250 ml Erlenmeyer flask with cork. The slurry was spread on glass TLC plate measuring 20×20 inches. TLC was run using the mobile phase - methanol; chloroform; distilled water in the ratio 25:65:4.

Purification of Pigment by Colum chromatography

The silica slurry was prepared in petroleum ether and acetone mixture and poured on column. After, the excesses solvent was drained in the column. The pigment sample was loaded on the column and eluted with methanol; chloroform; distilled water in the ratio 25:65:4.

Antibacterial activity of crude and purified pigments

The Muller Hinton agar was prepared and test pathogens were swabbed on the surface of agar medium. Wells were made by using gel puncture and about 100µl of Red, Yellow and orange pigments were loaded on the respective well. The center well was replaced with crude pigment disc incubated under 37°C for overnight.

Estimation of total antioxidant activity of the compound

Ferrous chloride in the tubes will be 1,000, 500, 250, 125, 62.5, and 31.25 μM used as standard. Crude extract of crude pigment (150 μL from 1mg / ml) is mixed with 2850 μL of the working FRAP reagent for 30 min in the dark condition and absorbance was recorded at 593 nm. Sample was placed at 37 ° C in water bath and absorption is again measured after 4 minutes at 593nm. Ascorbic acid standards (250, 500, 750 and 1000 μg).

Analysis of purified pigment fractions by High Performance Liquid Chromatography (HPLC)

The partially purified active pigment fractions were analysed by HPLC under following system condition.

Larvicidal assay

For the larvicide evaluation, World Health Organization protocols were used with modifications. Eggs of *A. aegypti* submerging them in chlorine free water at $27 \pm 2^\circ\text{C}$, with $80 \pm 5\%$ of relative humidity. The larvae were fed with fish food until L3 larval instar. Stock solutions of crude pigment were prepared diluting at 1:10 1:50 and 1:100 dilution and the stock solutions were placed in containers with 25 L3 larvae. Each concentration had 60 larvae in triplicate.

RESULTS AND DISCUSSION

Morphology of *Monascus purpureus*

The growth of *M. purpureus* (MTCC 1090) was periodically monitored at intervals of 2, 4 and 7th days of incubation. The entire tested medium showed production of pigment except corn meal agar (plate-1). Colonies of *M. purpureus* on all tested media showed small white colonies on 3rd day of incubation and become orange on PDA (plate -2). The pigments that diffused into the surrounding agar medium made it red in color at the end of 5th day incubation. The orange colored pigmentation was observed only on PDA medium. Accumulation of red pigment was observed on 3rd day while growing on YPSS medium and reached maximum on 5th day.



Figure 1: Morphology of *Monascus purpureus*

Submerged fermentation

Growth of *M. purpureus* on three different broths were investigated and growth rate was recorded at 600 nm after 48 h. Maximum growth was recorded at YPSS broth followed by PDA and chemically defined medium. 5th day Microscopic and SEM analysis of *M. purpureus* grown on YPSS medium (Plate-2). SEM analysis showed 5 μm long spore production without pigment accumulation spore production of pigment by submerged fermentation in YPSS and PDA medium was observed only on 7th day of incubation. Initially the pigmentation was pink in color. The influence of incubation period on production of polyketides red pigments was observed. The initial pH of the medium was 5.5. The pH was found to increase from 5.5 to 6.5. It was observed that the total dry cell mass were 1.1 to 1.5 g/100 ml on 7th day.

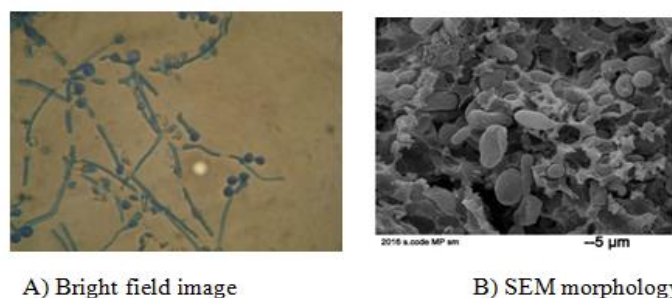


Figure 2: Plate 2 Microscopic study of *M. purpureus* during submerged fermentation

Solid state fermentation

During the solid state fermentation the red rice was produced at the end of 5th day. Direct estimation of biomass or mold mycelium to elucidate its growth in solid-state fermentation is usually impossible, because it is homogeneously adsorbed onto the substrate matrix. Pigment production by *Monascus* has been investigated in both solid-state and submerged fermentation. The luxuriant growth of *Monascus* was observed on the flask supplemented with 1% Yeast extract than peptone, soy protein and control flask. The effect of nitrogen source on cell growth and pigment production was studied in flask cultures with various nitrogen sources, since nitrogen sources have been found to have a great effect on the quality and quantity of *Monascus* pigments produced (Spepherd, 1977). High concentration of biomass was obtained with soybean and yeast extract, whereas the specific productivity of red pigments was reduced to about a third of that obtained with yeast extract.

Estimation of pigment

Data's given in (Table 1) represents the amount of pigment extracted from *M. purpureus* followed by submerged fermentation. It was estimated that the maximum of pigmentation yield was found to be 68% from YPSS medium followed by PDA (60%) and CD medium (58%). Among the submerged and solid state (Table 2) the higher yield of pigment (Fig 2) was observed on solid state fermentation which gave 1.8 OD units/ gm of rice and the yield of pigmentation was 90% by methanolic extract followed by 82% from acetone extract and 60% from ethyl acetate extraction. The synthesis of orange, yellow and red pigment was observed on 3rd, 5th and 7th day respectively extracted during solid state fermentation. Compare to extra cellular pigment the methanolic extract of intracellular red pigment was found to be higher and the productivity was 90 OD units/ gm of mycelium.

Solid state culture gave a higher pigment yield than cultivation in a shaken flask due to enhanced secretion of pigments into the grains of the solid-state substrate. In submerged fermentation, pigment is accumulated in the mycelium. At the same time, *Monascus* spp. has been reported to co-produce the mycotoxin citrinin, a hepato-nephrotoxic compound in humans. In addition to citrinin, other potential toxic metabolites, such as monascopyridines have also been reported in *Monascus* fermented red rice (Sameer et al. 2010). Solid state cultivation resulted in a higher pigment yield than cultivation in shaken flasks and concluded that this phenomenon could be due to a minor inhibition by the product.

Antibacterial activity of TLC separated Pigments

The 3 different Pigments such as Red, Orange and Yellow were eluted on Column Chromatography and confirmed by TLC. The total pigment extract in ethanol was purified into 3 bands and the R_f values were found to be identical 0.66, 0.70 and 0.64 respectively for red, orange and yellow. Among the three purified pigments the red pigment showed significant antibacterial activity against all test pathogens. Out of four test pathogen three were sensitive for red (75%) and the maximum zone of inhibition was found to be 16 mm against *S.aureus*. No antibacterial activity was recorded by yellow and orange pigment against test pathogens. The crude pigment also showed potent antibacterial inhibitory against all test pathogens (Table -3). The maximum zone of inhibition was 18 mm against *S.aureus* more superior to standard antibiotic. The antibacterial activity of crude pigment methanol extract against non pathogenic *E. coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* were previously reported by (Broder and Kohler, 2012). Antimicrobial activity of pigment produced by *Monascus anka* was studied. The crude pigment of Y7 showed antimicrobial activity against some bacteria and yeasts. The diameter of inhibition zone against gram-positive bacteria was a little smaller than that of gram-negative bacteria to the crude yellow pigment (Ho jaa lee and mi yeon park, 2002). Thus, the crude pigment of could be used as a useful alternative colorant for food industry, having the advantage of antimicrobial activity. Application of natural pigments in the control of plant pathogens also reported widely for crop protection (Misato, 1983).

Antioxidant activity

The anti oxidant activity of crude pigment isolated from *Monascus* sp. were determined and the results shows that the concentration of ascorbic acid increases with increase in the optical density. The total Ferric reducing activity of isolated red orange pigment from *Monascus* sample were determined as 280 μ M (Table 7). The ferric reducing power assay of pigment found to be equivalent to 1000 μ g of standard ascorbic acid. The reduction assay was given in (plate 7). The reducing power of the extract, which may serve as a significant reflection of antioxidant activity, was determined using a modified Fe^{3+} to Fe^{2+} reduction assay, where by the yellow colour of the test solution changed to various shades of green and blue, depending on the reducing power of the samples. The presence of antioxidants in the samples causes the reduction of the Fe^{3+} /ferricyanide complex to the Fe^{2+} form, and Fe^{2+} can be monitored by

measurement of the formation of Perl's Prussian blue at 700 nm. Rajasekaran and Kalaivani (2011) studied antioxidant activity of aqueous extract of *Monascus* fermented Indian variety of rice in high cholesterol diet fed-Streptozotocin (STZ) induced diabetic rats. The oxidative parameters like lipid per oxidation (LPO), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase activities were assessed. High cholesterol diet and STZ increased the level of LPO and reduced the level of SOD, GSH and catalase, whereas on administration of aqueous extract of *Monascus* fermented Indian variety of rice reduced the level of LPO to almost normal and increased the levels of GSH, SOD and catalase dose dependently. The animals treated with *Monascus* fermented rice extracts showed reduced LPO than diabetes induced animals fed only with high cholesterol food. This is better than the animals receiving the standard drug having hypoglycemic and antioxidant properties (glibenclamide) (Elmali et al., 2011)

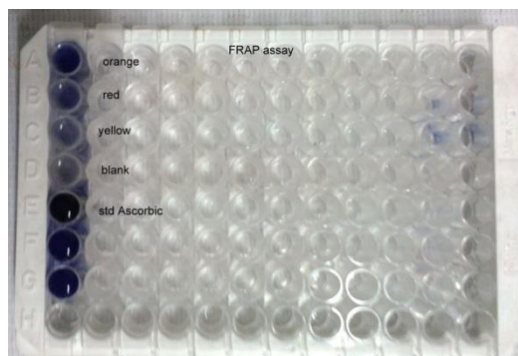


Plate 7

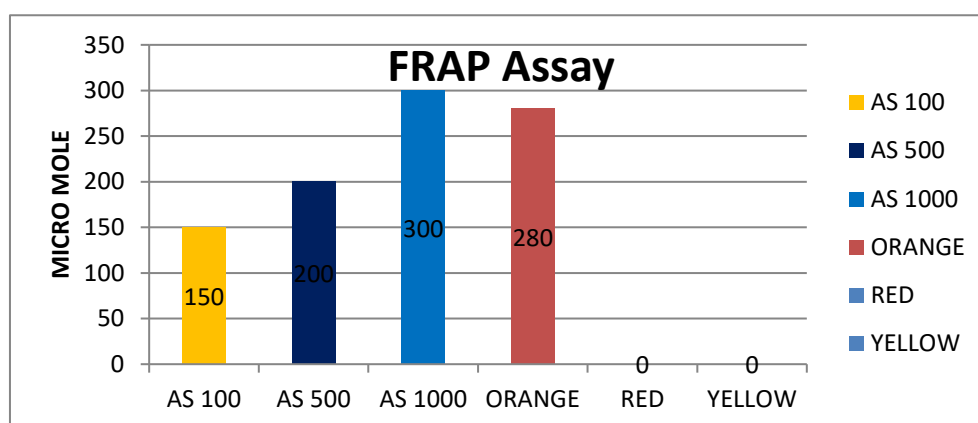


Figure 3: Ferric reduction power assay

High performance liquid Chromatography

HPLC analysis of fraction 2 (orange) showed peaks at retention time of 28.467, 30.008 and 30.642 minutes at 390 nm as shown in figure 4.6

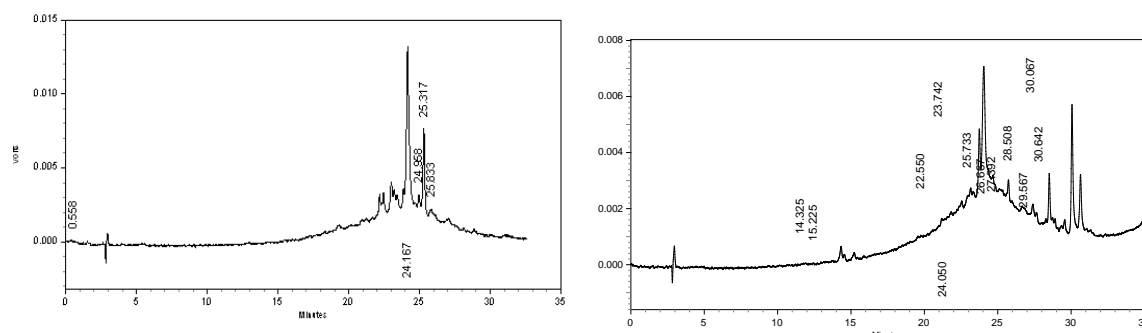


Figure 4: HPLC profile of pigment fraction 1

HPLC profile of pigment fraction 1 (red) showed peaks at retention time of 23.675, 24.208, 30.058 and 30.633 minutes at 425 nm as shown in figure 4.

Larvicidal effect of crude red pigment

The percentage of larvicidal was not recorded at 1:100 dilution and 12% at 1:10 dilution of pigment. The pupation rate was also not affected at 1:100 (Table 5). There was no adult emergence was observed at least dilution 1:10. There were no previous reports about the larvicidal effect of *Monascus* pigment. This is the first report of larvicidal activities of *Monascus purpureus* Pigment against *A. aegypti*. This result correlates the findings of Patil et al (2011). Many researchers reported the potential of larvicidal activity of natural pigments from fungi specially from ascomycetes (Hafeez et al 2011; Vijayan and Balaraman, 1991).

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Table 1: Estimation of pigment extracted from *Monascus* by submerged fermentation

Medium	Dry wt g/ltr	DF	OD	Tot volume	% of pigment
CD	2.4	10	0.48	25 ml	50
PDA	2.6	10	0.64	25 ml	61
YPSS	3.2	10	0.88	25 ml	68

Table 2: Estimation of pigment extracted from *Monascus* by submerged fermentation

Medium	Dry wt g/ltr	DF	OD	Tot volume	% of pigment
Ethylacetate	10	10	1.20	50 ml	60
Acetone	10	10	1.64	50 ml	82
Methanol	10	10	1.80	50 ml	90

Figure 5: Estimation of crude Pigment produced by submerged and solid state

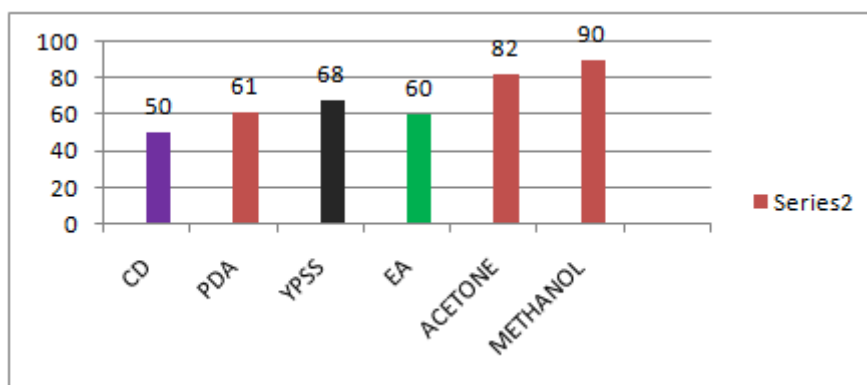


Table 3: Antibacterial activity of pigments against test pathogens

Culture	Red	Yellow	Orange	Crude pigment	Vancomycin
<i>S.aureus</i>	16 mm	Nil	Nil	16 mm	18mm
<i>E. coli</i>	Nil	Nil	Nil	18mm	15mm
<i>Klesiella sp</i>	14 mm	Nil	Nil	17mm	16 mm
<i>Providencia</i>	14 mm	Nil	Nil	16 mm	17 mm

Table 4: Larvicidal activities of pigments produced by fungi against *A. aegypti*

Sample	Number of larva tested	live	Dead	Number of pupa	Adult	Percentage (%) larvicidal
1:100 %	25	25	0	8	4	0
1:50	25	23	2	6	2	8
1:10	25	22	3	6	0	12
Control	25	25	0	10	4	0