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Original Research

Efficacy of polyketide pigment produced by *Monascus purpureus* and its biological activity

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Abstract

Monascus purpureus (MTCC 1090) was obtained from the IMTECH Culture Collection Centre, Chandigarh, India.

Extracellular and intracellular polyketide pigment was produced by solid-state fermentation using red rice production and pigments were extracted with methanol solvent.

Maximum pigment production was found with intracellular extraction and the total yield of pigment was 41 U/g followed by 33 U/g for extracellular production by substrate fermentation.

Crude pigments were separated by column chromatography and an antibacterial study revealed that the yellow pigment was most effective against all test pathogens and the red pigment was found to be a potent antioxidant.

The greatest antagonistic activity was almost 91% against *Trichophyton rubrum* followed by 88% against *Microsporum canis*.

Keywords: Polyketide, homothallism, *Monascus purpureus*, antibacterial, red rice

Introduction

Polyketide pigments are produced by *Monascus* sp. Polyketides are a large family of structurally diverse natural products usually biosynthesized through the decarboxylative condensation of malonyl-CoA-derived extender units in a similar process to fatty acid synthesis. Microbial secondary metabolites have a variety of biological properties that make them useful as antibiotics, anticancer drugs and antiviral drugs, and in other applications [1].

The red polyketide pigments are reported to be of potential therapeutic use, particularly when produced in red rice [2].

Red pigments are formed by the chemical modification of orange pigments. Ascomycetous fungi are very important natural pigment-producing microbes, providing an alternative to synthetic dye. This fungus is so important because it produces pharmacologically active lovastatin analogues such as the monacolins K, L and J, and is used to form red yeast rice and other popular fermented foods in China.

The antimicrobial effect of pigment extracted from *Monascus* sp. was reported against pathogenic and food-spoilage bacteria such as *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus* sp., *Yersinia enterocolitica* and *Listeria monocytogenes*.

In addition, the antifungal activity of the pigment extract was studied against *Aspergillus flavus* and *Fusarium* sp.

Comparison of the effects of polyketide pigments with current antibiotics reveals that pigments can be employed as replacements or as adjuncts to chemotherapeutic agents.

Streptomyces griseoruber produces hedamycin, an aromatic polyketide with anticancer activity [3].

Mosquitoes can transmit more diseases than any other group of arthropods and affect millions of people throughout the world, with the WHO having declared the mosquito 'public enemy number one' [4]. Today, mosquitoes play a predominant role in the transmission of dengue, malaria, yellow fever, filariasis and several other diseases which are currently among the greatest health problems in the world [5].

An antioxidant is a molecule that can inhibit the oxidation of other molecules by preventing formation of free radicals that can cause damage or death to the cell. Antioxidants terminate chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols [6].

Canthaxanthin is produced as the major carotenoid pigment by orange- and dark-pink-pigmented bacteriochlorophyll.

Canthaxanthins are potent antioxidants and inhibit the oxidation of lipids in liposomes.

Carotenoids are yellow to orange-red pigments that are ubiquitous in nature, which are widely used as food and feed supplements along with being used as antioxidants in the pharmaceutical industry.

A number of microorganisms produce these pigments such as *Serratia* and *Streptomyces*. Carotenoids are effective antioxidants and are widely used as food colourants [7].

Astaxanthin, a lipid-soluble carotenoid pigment produced by the red basidiomycetous yeast *Xanthophyllomyces dendrorhous*, is reported to have antioxidant properties [8].

Materials and methods

Morphological study of *Monascus purpureus*

Monascus purpureus was obtained from the IMTECH Culture Collection Centre, Chandigarh and growth on PDA, YPSS, YM and cornmeal agar was evaluated. The morphology of *Monascus purpureus* was observed using a photomicroscope.

Pigment production

Pigmentation was produced by submerged and solid-state fermentation. For submerged fermentation PDA, YPSS and a chemically defined medium was used. Solid-state fermentation was carried out using rice as a substrate along with 0.1% yeast extract. Optical density (OD; absorbance) was measured for both extracellular and intracellular pigments at λ650, λ590 and λ570, corresponding to red, orange and yellow pigments, and the pigment yield was calculated using the following formula:

$$\frac{\text{OD (DILUTION FACTOR) (TOTAL VOLUME OF PIGMENT)}}{\textit{DRY WEIGHT OF BIOMASS}}$$

Antimicrobial activity of crude and purified pigments

Mueller–Hinton agar was prepared and test pathogens were swabbed on the surface of agar medium. Wells were made using gel puncture and approximately 100 µl of red, yellow and orange pigments were loaded on the respective wells. Crude pigment activity was evaluated using a new method, by replacing the centre well with a crude pigment-loaded plain agar which had not been done in earlier studies. All of the plates were incubated at 37°C overnight and the zone of inhibition was recorded. The antifungal activity of *Monascus purpureus* was assessed using the dual culture method described by Evans and Wang [9] to evaluate the antagonistic activity of *Monascus* against *Trichophyton* sp. and *Microsporum* sp.
Data were obtained for the percentage inhibition of radial growth (100 × (R1-R2)/R1) where R1 = radial growth of the pathogen in control, and R2 = radial growth of the pathogen in dual culture with the antagonist.

Larvicidal activity of pigment produced by *Monascus purpureus*

Twenty-five *Culex* larvae were inoculated onto 50 ml of both 0.5 and 1% crude red pigment and incubated for 24–48 hours.
The control tubes were maintained as tap water (50 ml). The experiment was checked daily and the biological effects were recorded.
The larval mortality percentage was estimated using the following equation:

$$\text{Larval mortality \%} = \text{A-B/A} \times 100$$

where A = number of tested larvae and B = number of tested pupae.

Protease inhibitor activity of pigments produced by *Monascus purpureus*

Skimmed milk agar was prepared and wells were made by well puncture. 10 µl of crude protease culture filtrate along with 10, 20 and 30 µl of pigment were loaded onto the respective wells.
10 µl of culture filtrate alone was used as a positive control and the plates were incubated at 37°C overnight and casein lysis was recorded.
Detection of carbonyl functional groups such as in aldehydes and ketones was carried out using the DNPH test and Tollens' test.

Estimation of total antioxidant activity of the compound

Total antioxidant activity is measured by the Ferric Reducing Antioxidant Power (FRAP) assay [10]. The concentration of ferrous chloride in the tubes was 1000, 500, 250, 125, 62.5 and µM, used as standards. A crude extract of the pigment (150 µl from 1 mg/ml) was mixed with 2850 µl of the working FRAP reagent for 30 min in dark conditions and absorbance was recorded at 593 nm immediately after vortexing.
Thereafter, the sample was placed at 37°C in a water bath and absorption was again measured after 4 min at 593 nm.
Ascorbic acid standards (250, 500, 750 and 1000 µg) were processed in the same way.

Results and discussion

Colonies of *M. purpureus* (MTCC 1090) displayed as small white colonies on the 3rd day of incubation and became orange on the PDA plate (**Fig. 1a**). Microscopy examination revealed the brownish-red pigmentation of the mycelium along with ascospores, which were observed on the 3rd day of incubation.

After 72 hours of growth, conidiophores and conidia were seen. Accumulation of pigments was clearly seen on the 5th day with a greater number of fruiting bodies (**Fig. 1b**).

Between the submerged and solid-state fermentation methods, a higher yield of pigment was observed in solid-state fermentation using rice with extraction by methanol (**Fig. 1c, d**) which gave an OD reading of 2.4 at 570 nm.

The total pigment yields with respect to submerged and solid-state fermentation are given in **Table 1**.

Table 1 Yield of pigment with submerged and substrate fermentation

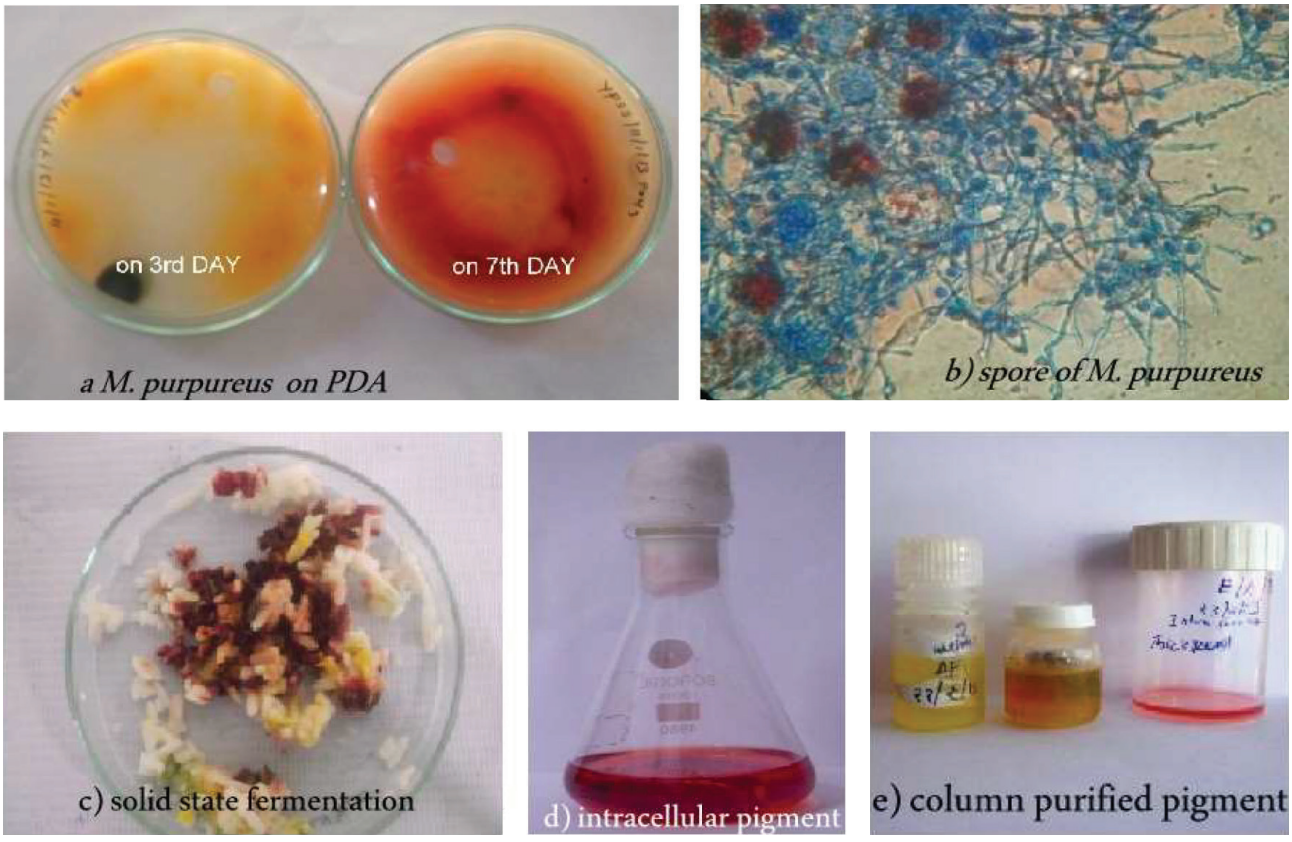
Fermentation	OD	Dilution factor	Dry weight of biomass (g)	Yield (U/g)
Submerged-PDA, extracellular	0.03	2	38	0.15
Submerged-YPSS, extracellular	4.48	2	5.4	16.5
Submerged-chemically defined medium	--	--	--	--
Substrate, intracellular	2.4	4	2.34	41
Substrate, extracellular	1.8	4	2.18	33

Compared to the submerged method, solid-state fermentation was found to be effective.

A higher productivity of 41 U/g was observed for the intracellular crude pigment and this value was 33 U/g for the extracellular pigment using the substrate method (**Fig. 1c, d**).

The maximum pigmentation gave OD readings of 0.03 in PDA broth and 4.48 in YPSS broth,

Figure 1 Colony morphology and pigment production of *M. purpureus*



while the yield was calculated as 0.15 and 16.5 U/g, respectively. There was no pigmentation using a chemically defined medium.

The intensities of red, orange and yellow were recorded at different wavelengths following column separation (**Fig. 1e**) and the maximum productivity was 19.46 units/g for yellow pigment, followed by orange and red. The Growth and Production of Pigment was evaluated and it was found that the solid-state and intracellular pigment was greater than with submerged fermentation. Lee *et al.* [11] state that solid-state cultivation results in a higher pigment yield than cultivation in shake culture and this phenomenon is due to the fact that pigments are released into grains under solid-state culture and the pigments accumulate in the mycelium under submerged cultivation.

Studies of red pigment synthesis by various strains of *M. purpureus* in submerged cultures revealed that the yield is affected by medium composition, pH and agitation [12]. The effect of the nitrogen source on cell growth and pigment production was studied in flask cultures with various nitrogen sources, since these have been found to have a great effect on the quality and quantity of *Monascus* pigments produced [13].

Ammonium nitrate, sodium nitrate and monosodium glutamate (MSG) had good results on pigment production. Concentration of biomass was obtained with soybean and yeast extract, whereas the specific production of red pigments was reduced to about a third of that obtained with MSG. Under yeast extract-stimulated conditions, the sexual cycle was repressed and there was increased biomass production [14]. In submerged fermentation, pigment accumulates in the mycelium. At the same time, *Monascus* spp. have been reported to co-produce the mycotoxin citrinin, a hepato-nephrotoxic compound in humans. In addition to citrinin, other potentially toxic metabolites, such as monascopyridines, have been reported in *Monascus*-fermented red rice [15].

In the present study, solid-state cultivation resulted in a higher pigment yield than cultivation in shaken flasks and productivity was dependent on the composition of the medium. It has been reported that *Monascus ruber* can produce pigment by utilizing corn steep liquor as a nitrogen source instead of yeast extract and that *Monascus purpureus* can produce pigment using grape waste [16].

During submerged cultivation, pigment production was higher in a complex medium than a chemically defined medium [17].

The total pigment extract in ethanol was purified into three bands and the Rf values were 0.66, 0.70 and 0.64, respectively, for red, orange and yellow. Three different pigments such as red, orange and yellow were eluted using column chromatography and confirmed by TLC. Among the three purified pigments, the yellow pigment showed significant antibacterial (72%) activity against test pathogens.

Out of seven test pathogens, five were sensitive to the yellow pigment and the maximum zone of inhibition was found to be 18 mm against *S. aureus*. Orange pigment was less active against *Proteus* sp., with a 12 mm zone of inhibition.

Red pigment failed to demonstrate antibacterial activity against the test organisms. The crude pigment also showed good antibacterial inhibitory activity against five test pathogens (**Table 2**). The maximum zone of inhibition was 24 mm against *Pseudomonas* sp., which is superior to the standard antibiotic.

Table 2 Antibacterial activity of pigments against test pathogens

Culture	Red	Yellow	Orange	Crude	Ampicillin
S. aureus	Nil	18 mm	Nil	Nil	Nil
E. coli	Nil	12 mm	Nil	18 mm	12 mm
Pseudomonas sp.	Nil	12 mm	Nil	24 mm	21 mm
Bacillus sp.	Nil	14 mm	Nil	16 mm	13 mm
Klebsiella sp.	Nil	14 mm	Nil	17 mm	15 mm
Proteus sp.	Nil	13 mm	12 mm	19 mm	12 mm
Providencia	Nil	14 mm	Nil	Nil	Nil

Antifungal results showed that the *Monascus purpureus* tested in this study exhibited antagonistic activity against the dermatophytic fungi *Microsporum* sp. and *Trichophyton* sp. Radial growth of the pathogen was considerably hindered by *Monascus purpureus* antagonists under the conditions of this study.

The most significant antagonistic effect was observed against *Trichophyton* rubrum (90.9%), while *Microsporum* canis was inhibited at a level of approximately 88% (**Table 3**).

Table 3 Antagonistic activity of *Monascus purpureus*

S.NO	R1 (control)	R2 (test)	Percentage Zone of Inhibition
Microsporum sp.	4.1	0.48	88
Trichophyton sp.	5.5	0.5	90.9

This is the first report of the antagonistic activity of *Monascus* sp. against dermatophytic fungi.

The antibacterial activity of crude pigment ethanol extract against non-pathogenic *E. coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* was previously reported by Kumar *et al.* [18]. The antimicrobial activity of yellow pigment produced by *Monascus* anka Y7 (Y7) was studied. The crude yellow pigment of Y7 showed antimicrobial activity against some bacteria and yeasts. The diameter of the inhibition zone against gram-positive bacteria was a little smaller than that for gram- negative bacteria for the crude yellow pigment. Thus, the yellow pigment could be used as a useful alternative colourant within the food industry, having the advantage of antimicrobial activity. *Monascus* pigment, when added to sausages, showed 90% stability at 4°C for three months with antibacterial and antifungal activity against some foodborne pathogens [19, 20].

The percentage larvicidal activity was found to be 100% with 1% crude pigment and 92% with 0.5% pigment. The pupation rate was not observed with 1% and was 8% with 0.5% (**Table 4**).

Table 4 Larvicidal activities of pigments produced by *Monascus purpureus*

Sample	Number of larvae tested	Live	Dead	Number of pupae	Adult emergence	Larvicidal (%)
0.5%	25	17	8	2	Nil	92
1%	25	0	25	Nil	Ni	100
Control	25	25	0		Nil	80

No adult emergence was observed.

This is the first report of the larvicidal activity of *Monascus purpureus* pigment against *Culex* sp. At a higher concentration the microbial pigment prodigiosin produced by Serratia marcescens NMCC46 is effective against Aedes aegypti and Anopheles stephensi and mortality is seen within the first six hours of exposure [21].

The protease inhibitory activity of the pigments produced by *Monascus purpureus* was found at 30 µl. Crude pigment mixed with proteolytic culture filtrate brought about reversal of casein hydrolysis, which indicates the protease inhibitory properties of the pigment.

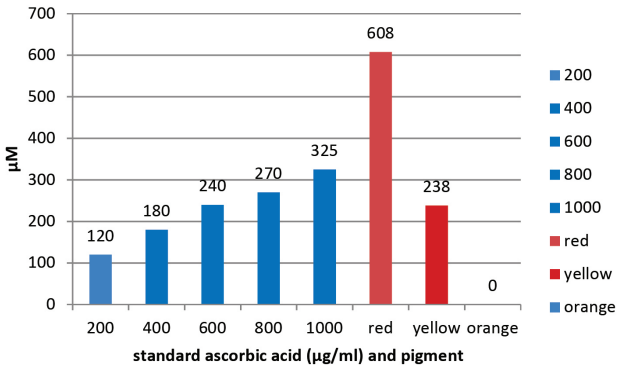
Assessment of the antioxidant activity of the red pigment showed that the metabolite of *M. purpura* has a higher antioxidant capacity than ascorbic acid at 1000 µg/ml.

The methanol extract of mycelia filtrate red pigment demonstrated the highest ferric reducing activity, followed by the purified yellow pigment. No antioxidant activity was found with respect to the orange pigment.

The reducing power of the extract, which may serve as a significant reflection of antioxidant activity, was determined using a modified Fe3+ to Fe2+ reduction assay, whereby the yellow colour of the test solution changed to various shades of green and blue, depending on the reducing power of the samples.

Among the three different separated pigments, red showed the maximum antioxidant effect, giving a value of 608 µM in the FRAP assay, and was more effective compared to yellow (**Fig. 2**).

Figure 2 FRAP antioxidant assay for extracted pigments



The presence of antioxidants in the samples causes the reduction of the Fe3+/ferricyanide complex to the Fe2+ form, and Fe2+ can be monitored by measurement of the formation of Perls' Prussian blue at 700 nm. Rajasekaran and Kalaivani [22] studied the antioxidant activity of an aqueous extract of *Monascus* sp. from a fermented Indian variety of rice in diabetic rats fed a high-cholesterol diet.

2,4-Dinitrophenylhydrazine (DNPH) can be used to qualitatively detect the carbonyl functional group of ketones or aldehydes. A positive test is signalled by a yellow, orange or red precipitate (known as a dinitrophenylhydrazone).

If the carbonyl compound is aromatic, then the precipitate will be red; if aliphatic, then the precipitate will have a more yellow colour.

The carbonyl test shows the presence of aromatic carbonyl groups. Microbial pigments such as carotenoids, naphthoquinones, anthraquinones and melanin from the endophytic fungus, *Stemphylium lycopersici*, and the bacterial species, *Streptomyces spinoverrucosus* and *Streptomyces glaucescens*, are reported to have antioxidant properties [23–25].

Conclusion

Eco-friendly microbial pigments from *Monascus purpureus* used as food colourants from red rice fermentation were extracted and found to be potent antibacterial, antifungal and antioxidant agents. Extensive research is needed to develop microbial pigments in order to fulfill market demand by replacing eco-toxic synthetic dyes.

Conflict of Interest

The authors declare that they have no conflict of interest.

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