

# Production and evaluation of physicochemical properties of red pigment from *Monascus purpureus* MTCC 410

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## Abstract

Microbial pigments are secondary metabolites, which are produced during stationary or late log phase by a variety of microorganisms. *M. purpureus* MTCC 410 produces an extracellular red pigment in solid state fermentation on cooked autoclaved rice and MEA supplemented with ammonium nitrate. Pigment was extracted with ethanol and further quantified by taking OD at 500 nm. Anaerobic condition and low oxygen pressure inhibited *Monascus* growth as well as pigment production. *Monascus* pigment extracted from solid-state fermentation was less sensitive to light than from submerged fermentation. Purified red pigment is thermolabile over 70 °C heating and show a colour change from red to blackish when exposed to 100 °C for 15 min. Stability of the pigment at high temperature and low pH can be enhanced by modifying nitrogen source and substrate for culturing *M. purpureus* MTCC410.

## INTRODUCTION

*Monascus purpureus* belongs to the family Monascaceae and to the class Ascomyceta whose characteristic feature is the ability to produce secondary metabolites with strong yellow, orange or red pigmentation (Juszlova et al., 1996; Pitt and Hocking, 1997). *Monascus* a native organism of China and Thailand can easily grow in several ecosystems and finds several uses, from conferring color to food products to medicinal uses and as meat preservative (Wong and Koehler, 1981; Watanade et al., 1997). *Monascus* helps to lower blood cholesterol, prevent cancer, osteoporosis, stroke, alzheimer's disease and other dementias and muscular degeneration (Cesar et al., 2005).

Among the important metabolites of *Monascus* are the pigments, citrinin, and also a series of anti-hyperlipidemics as monacolins K and L (Ma et al., 2000). At least six different pigments are synthesized by *Monascus* sp. via the polyketide pathway being the red ones; rubropunctamine and monascorubramine are of particular interest for food use as a colorant or as a spice (Campoy et al., 2006). Toxicology studies on *Monascus* pigments show that the biopigment is apparently safe in the quantities tested and have low or non-existent toxicity (Lin and Demain, 1992; Ma et al., 2000).

Pigment production varies greatly with the species and cultivation conditions (Cesar et al., 2005). There are several adequate culture media used for the cultivation of *Monascus*,

but the most common are PDA and malt-extract-agar (MEA). Relatively few articles deal with stability of *Monascus* preparations and their red pigments. According to certain reports these pigments are reasonably stable to autoclaving, in a wide range of pH and show a residual color of 92 to 98% after three months at 4°C, with good sensorial acceptance (Lin and Demain, 1992; Fabre et al., 1993). However, these pigments are unstable towards light. Despite of its poor stability, *Monascus* pigments are still enjoying a promising status as color additive. The present study was carried out with aim to extract red pigment from *M. purpureus* MTCC 410 and evaluate its physicochemical properties.

## MATERIALS AND METHODS

### MICROBIAL STRAINS AND CULTURE CONDITIONS USED

*Monascus purpureus* MTCC 410 was employed in the study. The culture was maintained in Malt extract agar (MEA containing malt extract-20g/l, glucose-20g/l, peptone-1g/l, agar-15g/l) at 30-32°C for 10 days. *Monascus* culture was streak inoculated on plates of MEA, PDA and MEA + NH<sub>4</sub>NO<sub>3</sub> media and incubated at 300 C for 10 days for comparing the amount of pigment produced. For solid-state fermentation autoclaved rice and boiled autoclaved rice were used and for submerged fermentation MEB and PDB were used to propagate *M. purpureus* MTCC 410 culture

(Pastrana et al., 1994; Cesar et al., 2005).

## **PREPARATION OF SPORE SUSPENSION**

After incubation, a spore suspension was prepared by pouring 5 ml of 0.1% v/v Tween 80 directly over the Petri dishes. The spore suspensions were standardized to  $1 \times 10^6$  spores/ml by addition of sterile water (Cesar et al., 2005).

## **PIGMENT PRODUCTION**

### **A) SOLID STATE FERMENTATION**

10g autoclaved and boiled-autoclaved rice with 56% humidity, pH adjusted to values 5 and 6 under two conditions i.e. low (use tight cotton plugging) and high (use loose cotton plugging) oxygen pressure were prepared and inoculated with 0.5 ml of spore suspension. Cultures were then incubated at 300 C for 10 days. Fermented rice was then air-dried at room temperature; pigment was extracted with 95% v/v ethanol with the proportion of 5 ml ethanol/g dry fermented mass, with occasional agitation, for 24h, then centrifuging for 15 min at 10,000 rpm. The extracts were diluted and the absorbance was measured against pure solvent at 500 nm, near the absorbance peak of red pigments as described in literature (Johns and Stuart, 1991; Lin & Demain, 1992; Cesar et al., 2005).

### **B) SUBMERGED FERMENTATION**

3 ml of spore suspension was cultured on sterile PDB and MEB Broths for 10 days at 300C. After cultivation, the fermented broth was filtered through muslin cloth followed by centrifugation to separate biomass and other impurities. Biomass was washed twice with deionized water, drained and stored at - 40C. Red pigment from culture broth was extracted with four volumes of 95% v/v ethanol (Cesar et al., 2005). After 1h extraction, with periodic agitation, the mixture was filtered through Whatmann filter paper and alcoholic extract of red pigment was separated from media by separating funnel and used as a raw pigment extract. The amount of pigment produced under submerged and solid state conditions was compared for 5 days consecutively.

## **PHYSICOCHEMICAL ANALYSIS OF PIGMENT SOLUTION**

### **PH STABILITY**

5 ml of the raw alcoholic extracts were diluted in enough water to complete 500 ml. From this solution, other solutions were prepared, with pH adjusted to several values, from 2 to 10 with 0.1N NaOH or dil. HCl (Cesar et al., 2005). These solutions were incubated at 300C for 1 to 48 h. The color

intensity was read as absorbance at 500 nm, directly for each tube, against water as blank.

### **LIGHT SENSITIVITY**

Pigment solution was exposed to sunlight for several hours and light sensitivity was determined by taking OD of the pigment solution.

### **HEAT STABILITY**

For determining heat stability of the red pigment solution, different aliquots were incubated at temperatures viz. 70, 80, 90 and 100°C for 15 min and absorbance was read at 500 nm.

## **RESULT AND DISCUSSION**

### **GROWTH CHARACTERISTICS OF MTCC 410**

*Monascus purpureus* MTCC 410 formed a typical fungal colony with its orange or red centre and was flat slightly rose in the centre when cultivated on MEA, PDA or MEA supplemented with ammonium nitrate plates (Fig. 1). Morphological characteristics of *Monascus purpureus* could be correlated with the descriptions given by Rasheva et al. in 1998. Maximum pigment production was obtained on MEA plates supplemented with ammonium nitrate as compared to MEA and PDA. The role of inorganic nitrogen in enhancing yield of *Monascus* red pigments is already indicated in literature. The *Monascus* red pigment could be best excreted in the medium after reacting with NH<sub>2</sub> group of nitrogen source (Pastrana et al., 1994). That's why supplementations of MEA with ammonical nitrogen have influenced pigment production (Fig. 2).

## **PIGMENT PRODUCTION**

### **A) SOLID-STATE FERMENTATION**

High amount of *Monascus* growth was also observed on cooked autoclaved rice (4.4OD/g) than autoclaved rice (3.11OD/g), because of higher degree of starch hydrolysis. It was also indicated in the experiment that high oxygen pressure supported three folds higher growth (3.11 OD/g autoclaved rice) than low oxygen pressure (1.14 OD/g autoclaved rice) condition. Levels of oxygen and carbon dioxide environment affects pigment production significantly and also growth to a lesser extent on solid substrates like rice (Han and Mudgett, 1992). An initial pH of the substrate also has profound influence on the growth as well as pigment yields from *Monascus*. At pH 5, very less pigment production was observed than pH 6 on autoclaved rice (Fig. 3). Chen and Johns (1993) also observed that a

higher ratio of extra cellular to intra cellular red pigment absorbance was obtained in batch culture of *Monascus purpureus* sp. at pH 6.5 when compared to pH 4.0.

## **B) SUBMERGED FERMENTATION**

In MEB broths (0.176 OD/ml), yields of red pigment were comparatively higher than PDB broth (0.123 OD/ml), probably due to the reason that MEB broth contains excess of free amino acids than in PDB broth (Chen and Johns, 1993). Solid-state fermentation enhanced pigment production in *Monascus purpureus* MTCC 410 compared to submerged fermentation (Fig. 4). Results indicated that an amount of pigment produced was more on boiled autoclaved rice (4.4 OD/g) and autoclaved rice (3.11 OD/g) than MEB broth (0.176 OD/ml) and it was also observed that from solid-state fermentation, more biomass was obtained than submerged fermentation, as *Monascus* requires more oxygen and surface for attachment of fungal hyphae. 1.09g wet biomass was obtained from solid-state fermented autoclaved rice while 0.44g wet biomass could be recovered from submerged fermented MEB. Similar results were obtained, as in a previous study on *Monascus* where more pigment was extracted through solid-state fermentation using a polymeric resin (Patrick and Henry, 1984).

## **PHYSICOCHEMICAL ANALYSIS OF PIGMENT SOLUTION:**

### **I) LIGHT SENSITIVITY**

Red pigment, which was produced under solid-state conditions (4.4 OD/g) was more than submerged fermentation (0.176 OD/ml) (Fig. 4) and was less sensitive towards light (4.4 OD units decreased to 1.55 OD units after 120 h of direct exposure to sunlight) than pigment produced through submerged fermentation from (0.176 to 0.11 OD units/ml after 120 h of exposure) (Fig. 5 and Fig. 6). Its sensitivity towards light is relatively high, that's why OD<sub>500</sub> red pigment solution decreased to 35% of the original color intensity within 120 h of exposure to direct sunlight..

Reports available in the literature also highlighted similar results that direct exposure to sunlight could inhibit the growth of *Monascus* and pigment production during fermentation and incubation in total darkness could raise red pigment production from 14.5OD/g dry substrate to 22OD/g dry substrate (Babitha et al., 2008). *Monascus* pigments are unstable towards light (only 20% residual color after 50 days) probably due to rapid degradation of these secondary metabolites (Fabre et al., 1993, Lee and Chen, 2000). Degradation patterns and derivatization from red color

gradually to brown pigment were also confirmed by HPLC analysis by Jung et al. (2005). Supplementation of the culture media with amino acids could help to increase the half-life of the pigment during exposure to sunlight or UV light, probably due to their derivatization or complexation.

### **(II) PH STABILITY:**

Ethanol extract of *Monascus* pigment was relatively stable at pH from 6.0 to 8.0 (Fig. 7). Persistent exposure to acidic or basic environments for > 48 h resulted in complete loss of red color in the *Monascus* culture extracts. Degradation of red pigment was comparatively faster above pH 8.0 or below pH 4.0. A good pH stability of *Monascus* pigment could be achieved by maintaining pH in the range from 6.0 to 8.0 by addition of the appropriate buffers and or solvents (Fabre et al., 1993; Lee and Chen, 2000).

### **(III) HEAT STABILITY**

From the thermal profile analysis, it was observed that pigment is comparatively stable at 70°C for 15 min, but color intensity decreased above 70°C. At 100°C, its color changes to blackish red, due to breakdown of pigment molecules in solution. Instability of *Monascus* pigments at high temperature thus could be correlated with its rapid breakdown (45% residual color after 2h at 100°C) (Fabre et al., 1993, Lee and Chen, 2000). When heat stability of pigment produced was compared for solid-state and submerged fermentation methods, a reduction in color intensity with increase in temperature was more under submerged conditions (Fig. 8). After 15 min of heat treatment, 3.31 OD units could be recovered from 4.4 OD/g of dry rice than 0.153 OD/ml units recovered from 0.176 OD/ml from submerged fermentation at 70°C. With increase in temperature from 70 to 100°C, color intensity rapidly decreased from 3.31 to 1.13 OD/g units for solid state condition and from 0.153 to 0.12 OD/ml units for submerged condition. Thus, it indicated the thermolabile nature of *Monascus purpureus* MTCC 410 red pigment.

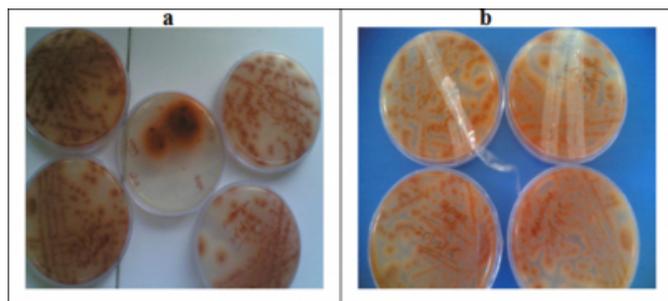
## **CONCLUSION**

The biophysical specification of pigments depends greatly on the structure of the pigment molecule and amino acid or protein with which the pigment was associated and determines color of this polyketide pigment. When the pigment is added to food items, presence of acid, alkali, salt, and exposure to light and heat, change of oxidation state, alone or in combination could lead to a change in pigment color and stability. However, further studies are required to

indicate amount and stability of *Monascus* pigments by addition of amino acids (glutamic acid, methionine, phenylalanine etc.) and role of other environmental factors. As a variety of amino acid derivatives of *Monascus* pigments with better color stability can be produced and further evaluated for their antioxidant and biopreservative potential in raw and cooked packaged meat system.

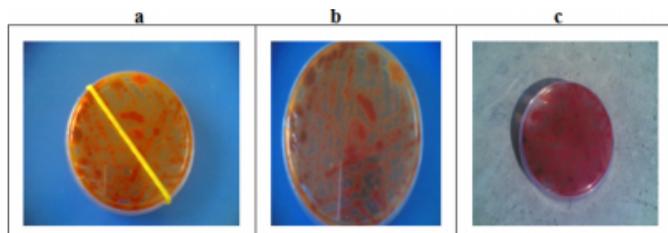
**Figure 1**

Figure 1: Growth of MTCC 410 on MEA plates



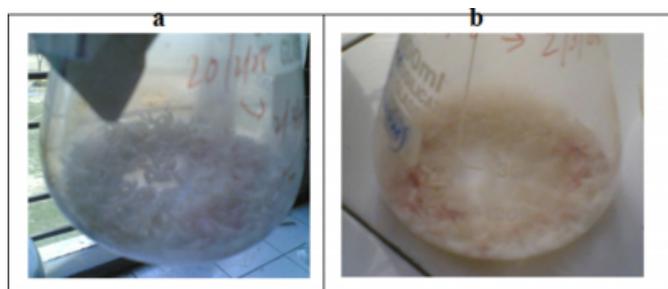
**Figure 2**

Figure 2: Growth of on agarified media; a) PDA, b) MEA and c) MEA supplemented with ammonium nitrate



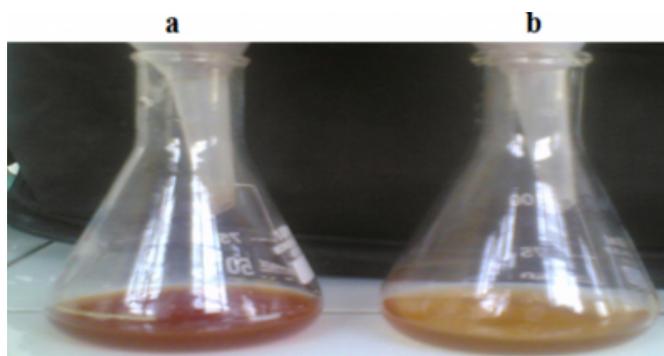
**Figure 3**

Figure 3: Growth of on autoclaved rice; a) at pH 5 and b) at pH 6



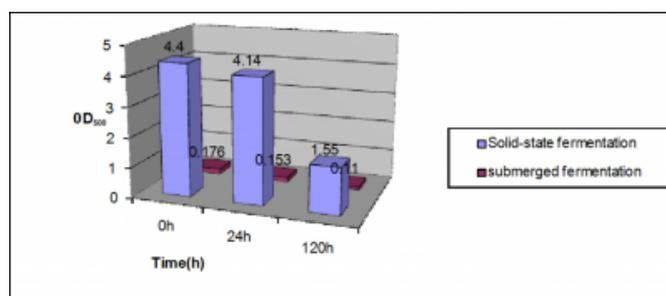
**Figure 4**

Figure 4: Pigment extracted from; a) solid-state fermentation and b) submerged fermentation



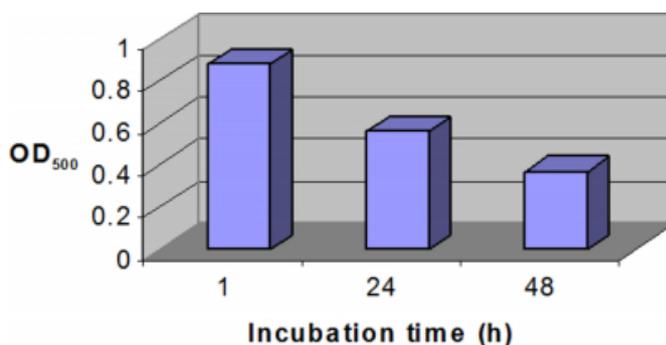
**Figure 5**

Figure 5: Comparison of light sensitivity of red pigment



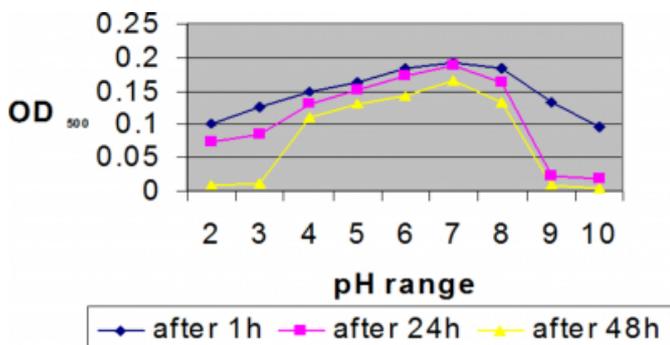
**Figure 6**

Figure 6: Light sensitivity of pigment obtained from solid-state fermentation



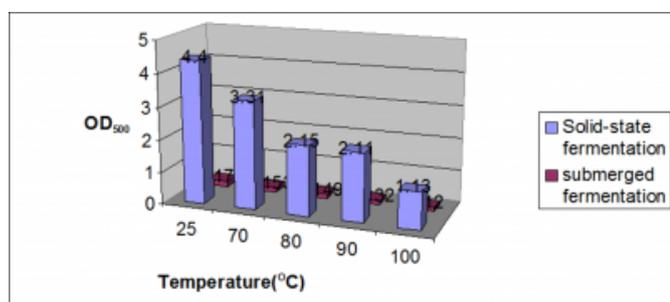
**Figure 7**

Figure 7: pH stability of pigment



**Figure 8**

Figure 8: Heat stability of pigment



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