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# Thermal stability of natural pigments produced by *Monascus ruber* in submerged fermentation

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ABSTRACT

The aim of this work was to present new data on the thermal stability of orange and red pigments produced by *Monascus ruber* CCT 3802 in submerged fermentation. The pigments were evaluated under different temperature and pH conditions in glass bottles with the temperature controlled using a thermostat bath. Samples of orange and red pigments were collected and submitted to measurement of the absorbance at 470 and 510 nm, respectively. The thermal degradation of the orange and red pigments followed a first-order kinetic reaction. The response surface models and empirical results described the behavior of the responses of color degradation and half-life of the pigments, and the temperature dependence of the degradation constants followed the Arrhenius model. The activation energies of the orange and red pigments were 12.65 and 11.49 kcal $mol^{-1}$, respectively.



## 1. Introduction

Color plays an important role in our enjoyment of foodstuffs and is appreciated both for its aesthetic and as a basis for the assessment of quality. In the latter respect, color gives visual clues to flavor identification and taste thresholds (Bridle and Timberlake, 1997). Color is added to food for one or more of the following reasons: to replace color lost during processing, to enhance color already present, to minimize batch to batch variations and to color otherwise uncolored food (Aberoumand, 2005). It is the first sensory attribute used in the selection and purchase of a food product and is widely used by food industries to attract the attention of consumers, especially children.

The production of pigments by microorganisms capable of producing natural dyes, including seaweed, algae, yeasts and filamentous fungi, is a way of increasing the production of natural compounds as an alternative to the use of synthetic ones. The use of fungi for the production of commercially important products has increased rapidly over the past half century, and pigment-producing microorganisms and microalgae are quite common in nature. The molecules produced include carotenoids, melanins, flavins, quinones and more specifically monascins, violacein, phycocyanin and indigo (Dufossé et al., 2005).

Species of the genus *Monascus* have been used as coloring agents for many years in the manufacture of traditional foods (red wines, tofu, sausages, hams, meats and other products) in East Asian countries (Jung et al., 2003). *Monascus* can produce yellow, orange and red pigments (Meinicke et al., 2012). 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. 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—$C_{23}H_{26}O_5$ or rubropunctatin—$C_{21}H_{22}O_5$. Oxidation of the orange pigment monascorubrin gives rise to the yellow pigment ankaflavin—$C_{23}H_{30}O_5$, or monascin—$C_{21}H_{26}O_5$ for rubropunctamine. The red pigments monascorubramine—$C_{23}H_{27}NO_4$ and rubropunctamine—$C_{21}H_{23}NO_4$ are produced by the reaction of the orange pigment with compounds containing $NH_3$ and $NH_2$ in the molecule (Dufossé et al., 2005; Jung et al., 2003; Hajjaj et al., 2000; Jung et al., 2005). The color specification of monascus red pigments depended greatly on the amino acid or protein with which the pigment was associated (Carvalho et al., 2005).

Color degradation is common for natural pigments, and is therefore a major concern in coloring foods, frequently compensated by the proper dosage of the pigment (Lian et al., 2007). Optimizing thermal processing to improve product color requires data on color degradation kinetics and on the temperature dependence that can be described by its activation energy (Chutintrasri and Noomhorm, 2005). However, replacing synthetic

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dyes with natural colorants (carotenoids, anthocyanins, chlorophylls, monascins and other) offers a great challenge, due to the higher stability of synthetic dyes with respect to temperature, pH and emerging technologies such as high pressure, amongst other factors (Dufossé et al., 2005; Cevallos-Casals and Cisneros-Zevallos, 2004; Valadez-Blanco et al., 2007).

Thermal treatment is one of the most important methods of food preservation. However, excessive heating produces considerable loss in the quality and particularly in the sensory properties of foods (Lund, 1975; Hayakawa and Timbers, 1997; Ou et al., 2009). During processing, deterioration reactions contribute to the formation of brown pigments, which is undesirable with respect to the color, flavor and market value (Chutintrasri and Noomhorm, 2005; Buckow et al., 2010). Kinetic models for thermal denaturation are essential to design new processes to guarantee a safe food product and maximum retention of the quality factors (Lund, 1975; Ávila and Silva, 1999). However, the availability of information on reaction kinetics is still quite limited and modeling of the thermal degradation kinetics for color in the temperature range of pasteurization and sterilization is necessary. Based on the above mentioned facts, the aim of this work was to investigate and to present new data on the effects of temperature and pH on the thermal stability of orange and red pigments produced by *Monascus ruber* CCT 3802 in submerged fermentation, using response surface methodology.

## 2. Material and methods

### 2.1. Microorganism and culture media

*Monascus ruber* CCT 3802 was obtained from the Tropical Culture Collection André Tosello (Campinas—SP, Brazil). The strain was frozen at −20 °C after adding 100 μL glycerol $mL^{-1}$ spore suspension as a cryoprotector. The culture was maintained on potato dextrose agar sterilized at 121 °C for 15 min, incubated at 30 °C for 7 days and subsequently stored at 4 °C. The inoculum and culture medium were prepared according to Vendruscolo et al. (2009) containing, per liter: 20 g glucose, 5 g glycine, 2.5 g $KH_2PO_4$, 2.5 g $K_2HPO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 0.03 g MnSO4 and 0.01 g $ZnSO_4$. After sterilization the initial pH was adjusted with 1 M NaOH or HCl to 3.0 or 5.0 for the production of orange and red pigments, respectively (Meinicke et al., 2012).

### 2.2. Inoculum preparation

*Monascus ruber* CCT 3802 was initially grown on PDA medium in a Roux bottle incubated at 30 °C for 7 days and subsequently stored at 4 °C (Vendruscolo et al., 2009). A spore suspension was obtained by washing the Roux bottle cultures with a sterile aqueous solution of 0.1% Tween 80. Fungal mycelia were obtained by germination of the spores suspended in a 1000 mL baffled flask containing 400 mL of inoculum culture medium at 30 °C, and shaken on a rotary shaker at 120 rpm for 60 h. This suspension was used for further inoculation in a bioreactor.

### 2.3. Stirred tank fermentation

Fermentation was carried out in a 6 L (working volume of 4 L) batch bioreactor (Bioflo III from New Brunswick Scientific Co., New Jersey, USA) for 96 h (Vendruscolo et al., 2010). The bioreactor has a vessel with an internal diameter of 0.170 m, a rounded bottom and a height/diameter ratio of 1.4. Agitation was provided by two six-blade Rushton turbines with a $d/t$ ratio of 0.38 and a $w/d$ ratio of 0.18. The culture medium (3.6 L) was inoculated with 0.4 L of the inoculum culture (10% v/v;≈0.5 g $L^{-1}$ dry equivalent of cells), under the following conditions: temperature 30 °C; stirring speed of 300 rpm and specific aeration rate of 0.6 vvm (volume of air per volume of batch per minute). The initial pH was adjusted to 3.0 and 5.0 for orange and red pigment production, respectively.

### 2.4. Production and pigment preparation

#### 2.4.1. Orange pigment

The orange pigment was produced by growing *Monascus ruber* CCT 3802 in the Bioflo III with the pH maintained at 3.0. Under these conditions the pigment has low solubility in the culture medium (crystals). After 96 h of growth, the fermentation was stopped and the biomass and pigments were separated from the liquid media by filtration. The pigment retained on Whatman no 1 filter paper (Madiston, England) was washed with distilled water and the orange pigment was dissolved in 95% ethanol (v/v) and the biomass was discarded. The alcoholic solution of orange pigment was submitted to crystallization by adding water in the proportion of 1:3 and refrigerating at −20 °C for 2 h, followed by filtration through Whatman no 1 filter paper (Madiston, England). The filtrate was discarded and the solid orange pigment stored at −20 °C. The solid pigment was used to prepare pigment solutions with absorbance of around 1.0 unit at 470 nm (≈1.0 $UA_{470\ nm}$).

#### 2.4.2. Red pigment

The red pigment was produced by growing *Monascus ruber* CCT 3802 in the Bioflo III at a pH maintained at around 5.0. Under these conditions the pigment has high solubility in the culture medium. After 96 h of growth, the fermentation was stopped and the pigments separated from the biomass by filtration through cotton gauze and Whatman no 1 filter paper (Madiston, England). The filtrate (50 mL) was mixed with 25 mL of n-hexane in a separating funnel then the n-hexane layer containing orange pigment was removed. This procedure was repeated twice. Red pigment was concentrated under a reduced pressure and stored at −20 °C. The pigment was used to prepare pigment solutions with absorbance of around 1.0 unit at 510 nm (≈1.0 $UA_{510\ nm}$).

### 2.5. Heat treatment

The orange or red pigments were diluted in citrate phosphate buffers ($C_6H_8O_7$—0.1 M citric acid and $Na_2HPO_4$—0.2 M sodium phosphate dibasic anhydrous) to the desired pH values. The pH values of the pigment and buffer solutions were measured using an Analion AN 2000 pH meter calibrated with pH 4.0 and 7.0 buffer solutions.

The effects of the temperature and pH on the heat degradation of the orange and red pigments produced by *Monascus ruber* CCT 3802 were determined in a jacketed glass bottle. The orange or red pigments were added to 300 mL of buffer solution and the initial absorbance adjusted to approximately 1 $UA_{470\ nm}$ and 1 $UA_{510\ nm}$, respectively, in a Spectronic Unicam Genesys *10vis* spectrophotometer. The pigment solution was transferred to a 400 mL jacketed glass flask with water circulation at the temperature of the assay. The temperature was controlled by circulating water from a Microquímica Model MQBMP-01 water bath through the jacket of the glass flask. The flask was maintained at the selected temperatures and periodically agitated with a magnetic stirrer to ensure a uniform temperature throughout the bulk of the sample. The temperature of the sample at its geometric center was monitored using a thermometer. The pigment solutions were heated for approximately 120 min, and samples collected after pre-determined time intervals. The first sample was collected after attaining constant temperature and immediately transferred to an ice water bath. The absorbance was measured in a spectrophotometer.