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# Isoelectric point of amino acid: Importance for monascus pigment production

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ABSTRACT

This study aimed to determine the effect of pH on radial growth rate and pigments production from *Monascus ruber* CCT 3802. Fermentations were carried out in solid medium and submerged fermentations under different pH conditions and the pigments produced were submitted to scanning in spectrophotometer. Higher radial growth rate was obtained when *M. ruber* was growing at pH 4.0 and the production of yellow pigment was obtained at low pH (2.0–3.0), while the production of orange and red pigments were in the pH range from 3.0 to 4.0 and above of 5.0, respectively. The results showed that pH has a significant influence on the radial growth rate, pigment production and particularly on the profile of orange and red pigments. pH proved to be an extremely important factor for obtaining orange (precursor) or red pigment, since the isoelectric point of $NH_3^+$ sources is critical to converting orange pigment into red pigment.

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## 1. Introduction

Color plays an important role in visual recognition and assessment of the surface and subsurface properties of the object. It has a great influence on appearance, processing and acceptance of food products. The first quality impact by which consumers make the decision to purchase a product is its visual appearance.

The great advantage of using living cells as chemical factory is the ability to procedure complex chemical structures from simple and inexpensive precursors with exquisite specificity in effectively a one-stage process. Microbial pigments such as carotenoids (Pennacchi et al., 2015), melanins, flavins, quinones (Dufossé et al., 2005) and more specifically monascins (Vendruscolo et al., 2013; Bühler et al., 2013; Prajapati et al., 2014; Hsu et al., 2002) are among the molecules produced. An alternative route for the production of natural food colorants is through the application of biotechnological tools to microorganisms (Vendruscolo et al., 2014). *Monascus* is an ascomycetes fungus traditionally used for the production of food colorants, fermented foods, alcoholic beverages, red soybean curd, meat, and vegetables in southern China, Taiwan, Japan, Thailand, Indonesia and the Philippines (Tseng et al., 2000; Suh and Shin, 2000).

Monascus can produce yellow, orange and red pigments. Medium-chain fatty acids, for example octanoic acid, are synthesized by the fatty acid pathway and bind to the chromophore structure through trans-etherification reaction, generating the precursor orange pigment monascorubrin ($C_{23}H_{26}O_5$) and/or rubropunctatin ($C_{21}H_{22}O_5$) by trans-etherification of the octanoic acid. The reduction of the orange pigment monascorubrin forms the yellow pigment ankaflavin – $C_{23}H_{30}O_5$, or monascin – $C_{21}H_{26}O_5$ for rubropunctatin, whereas the amination of orange pigments with $NH_3^+$ units gives rise to red pigments monascorubramine – $C_{23}H_{27}NO_4$ and rubropunctamine $C_{21}H_{23}NO_4$. Due to the affinity by amine groups, the Monascus pigments are frequently associated to proteins or to the cell wall, forming complex pigments, difficult of being extracted (Hajjaj et al., 2000; Jung et al., 2003, 2005).

The quantitative and qualitative production of monascus pigments is related to several factors such as the type of fermentation (submerged or state solid fermentation); the species of the microorganism; pH; carbon, nitrogen and nutrients sources; dissolved oxygen; stirring frequency and flow aeration (Lian et al., 2007; Orozco and Kilikian, 2008; Bühler et al., 2013; Kang et al., 2013, Vendruscolo et al., 2016). Among these factors, the pH has proved essential in the production of pigments by *Monascus* species, for this reason the present work aimed to study the effect of pH on the development and production of pigments by *Monascus ruber* CCT 3802.

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## 2. Material and methods

### 2.1. Microorganism and culture media

*M. 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 (Vendruscolo et al., 2010). The culture was maintained on potato dextrose agar (PDA) (Biolife Italiana, Milan, Italy) tubes sterilized at 121 °C for 15 min, incubated at 30 °C for 7 days and subsequently stored at 4 °C.

### 2.2. Radial growth

#### 2.2.1. Measurement of radial growth rates

PDA was prepared, sterilized for 15 min at 121 °C, cooled and pH was adjusted (2.0a 10.0) with HCl or NaOH (10% w/v). To measure radial growth rates, we adapted the methods of Gabiatti et al. (2006) and inoculated the center of each plate with 0.1 mL of spore suspension containing 0.1% (w/v) bacteriological agar (Biolife Italiana, Milan, Italy). Bases of plates were divided into three segments that allowed the calculation of an average diameter in cases where growth was irregular. Plates were incubated at 30 °C and the colony diameter was measured with a scalimeter every 24 h and the radial growth rate was determined from the slope of the linear regression using Eq. (1).

$$r(t) = V_{cr}t + a \quad (1)$$

where $r$ is the radius of the colony (mm), $V_{cr}$ is the radial growth rate (mm $h^{-1}$), $t$ is the cultivation time (h) and $a$ is the linear regression constant. All experiments were performed in quintuplicate and were repeated at least twice.

#### 2.2.2. Pigment extraction and analysis

The growth of *M. ruber* CCT 3802 colonies on PDA were scraped and added of 30 mL 95% ethanol (v/v), incubated at 30 °C for 2 h and filtered in Whatman #1 filter paper. The filtered was submitted to scanning in spectrophotometer Spectronic Unicam Genesys 10vis in the range from 325 to 600 nm.

### 2.3. Submerged fermentation

#### 2.3.1. Inoculum preparation

*M. 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. Fungal mycelia were obtained by germination of the spores suspended in a 1000 mL baffled flask containing 400 mL of inoculum culture medium containing the following 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 $MnSO_4$ and 0.01 g $ZnSO_4$. After sterilization, the initial pH was adjusted with 1 M HCl to 3.0 for the production of orange pigments (Vendruscolo et al., 2013). This suspension was maintained at 30 °C on a rotary shaker at 120 rpm for 60 h (exponential phase). This inoculum was used for further inoculation of Erlenmeyer flasks and Bioflo III bioreactor.

#### 2.3.2. Stirred fermentation

Submerged fermentation under different pH values (2–5) were carried out in 1000 mL triple-baffled Erlenmeyer flasks containing 360 mL of medium, incubated on rotary shaker (Tecnal, model TE 421) at 120 rpm and 30 °C. The culture medium was inoculated with 40 mL of the inoculum culture (10% v/v; ≈ 0.5 g $L^{-1}$ dry equivalent of cells). After 96 h of growth, fermentation was stopped and the biomass and pigments were separated from the liquid media by filtration on Whatman #1 filter paper. Under these conditions, the orange pigment has low solubility in the culture medium.

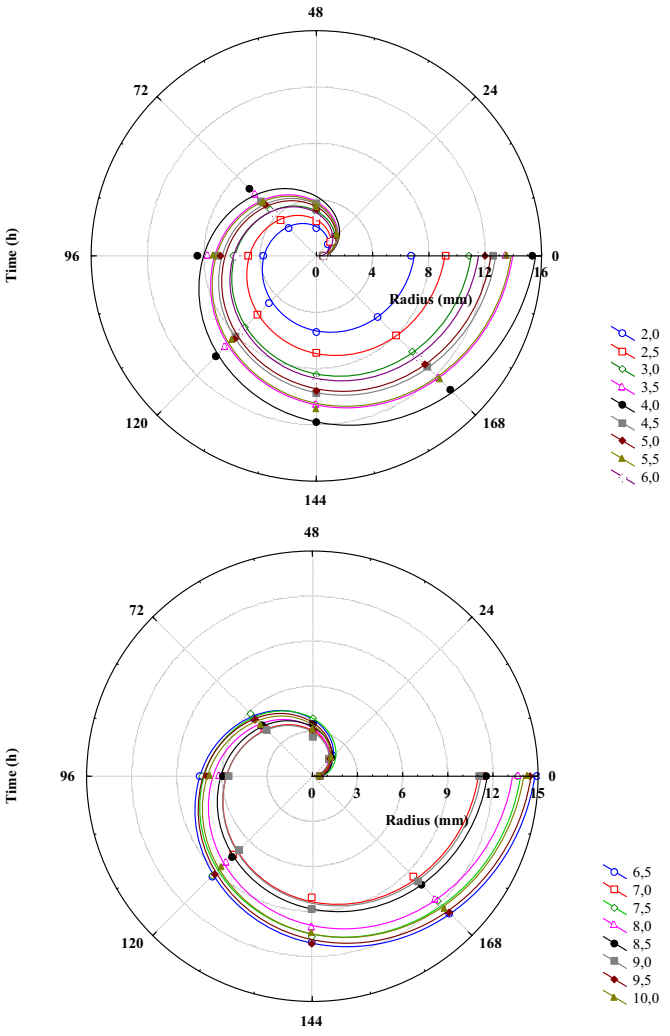


**Fig. 1.** Radial growth curves of *Monascus ruber* CCT 3802 cultivated on different pH values at 30 °C on potato dextrose agar.

**Table 1**
Radial growth rate, equation of radius as a growth time function, correlation index and visual aspects of *Monascus ruber* CCT 3802 colonies cultivated on potato dextrose agar at 30 °C.

| pH | $V_{cr}$ (mm $h^{-1}$) | Equation $r=f(t)$ | $R^2$ | Visual aspect |
|---|---|---|---|---|
| 2.0 | 0.0339 | $r=0.0339t+0.5$ | 0.9969 | Yellow |
| 2.5 | 0.0456 | $r=0.0456t+0.5$ | 0.9992 | Yellow |
| 3.0 | 0.0542 | $r=0.0542t+0.5$ | 0.9998 | Yellow |
| 3.5 | 0.0702 | $r=0.0702t+0.5$ | 0.9924 | Orange |
| 4.0 | 0.0789 | $r=0.0789t+0.5$ | 0.9952 | Orange |
| 4.5 | 0.0640 | $r=0.0640t+0.5$ | 0.9967 | Orange |
| 5.0 | 0.0619 | $r=0.0619t+0.5$ | 0.9956 | Orange |
| 5.5 | 0.0701 | $r=0.0701t+0.5$ | 0.9967 | Orange |
| 6.0 | 0.0587 | $r=0.0587t+0.5$ | 0.9985 | Orange |
| 6.5 | 0.0762 | $r=0.0762t+0.5$ | 0.9986 | Red |
| 7.0 | 0.0554 | $r=0.0554t+0.5$ | 0.9958 | Red |
| 7.5 | 0.0691 | $r=0.0691t+0.5$ | 0.9955 | Red |
| 8.0 | 0.0699 | $r=0.0699t+0.5$ | 0.9949 | Red |
| 8.5 | 0.0581 | $r=0.0581t+0.5$ | 0.9989 | Red |
| 9.0 | 0.0573 | $r=0.0573t+0.5$ | 0.9962 | Red |
| 9.5 | 0.0760 | $r=0.0760t+0.5$ | 0.9961 | Red |
| 10.0 | 0.0737 | $r=0.0737t+0.5$ | 0.9956 | Red |

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