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# Monascus pigment production in bioreactor using a co-product of biodiesel as substrate

## Produção de pigmentos monascus em biorreator utilizando um co-produto do biodiesel como substrato

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### Abstract

The study and use of natural pigments in food industries have increased in recent years due to the toxicity presented by artificial pigments. *Monascus ruber* is a filamentous fungus that produces red, orange, and yellow pigments under different growing conditions. The growth of health food market has increased in parallel with the growth in biofuels production, such as biodiesel, which generates a concomitant increase in the production of glycerin that can be used in bioprocesses. The objective of this study was to use glycerin and glucose as substrates in the production of natural pigments in a bioreactor. The culture of *Monascus ruber* was carried out in a Bioflo III reactor with 4 L of working volume and pH, temperature, aeration, and agitation control. The highest pigment production was observed after 60 hours of fungal culture with 8.28 UA<sub>510</sub> of red pigment. The pH range remained from 5.45 to 6.23 favoring the release of red pigment in the medium. This study shows the feasibility of the production of natural pigments by *Monascus ruber* in a bioreactor using a co-product of biodiesel without previous treatment as a substrate.

**Keywords:** *Monascus ruber*; glycerin; bioreactor.

### Resumo

O estudo e o uso de pigmentos naturais nas indústrias de alimentos têm aumentado nos últimos anos devido à toxicidade apresentada pelos pigmentos de origem artificial. *Monascus ruber* é um fungo filamentosos conhecido por produzir pigmento vermelho, laranja e amarelo sob diferentes condições de cultivo. Paralelo ao crescimento do mercado de alimentos naturais cresce o de biocombustíveis, como é o caso do biodiesel, que gera concomitantemente um aumento na produção de glicerina, podendo esta ser utilizada em bioprocessos. O objetivo deste estudo foi utilizar glicerina e glicose como substratos para produção de pigmentos naturais em biorreator. O cultivo foi realizado em biorreator Bioflo III, com volume útil de 4 L, equipado com controle de temperatura, pH, vazão de aeração e frequência de agitação. A maior produção de pigmentos foi observada em 60 horas de cultivo com 8,28 UDO<sub>510</sub> de pigmento vermelho. O pH permaneceu na faixa de 5,45 a 6,23, favorecendo a liberação de pigmentos vermelhos. O estudo realizado mostra a viabilidade da produção de pigmentos naturais por *Monascus ruber*, em biorreator, utilizando resíduos da produção de biodiesel, sem tratamento prévio.

**Palavras-chave:** *Monascus ruber*; glicerina; biorreator.

## 1 Introduction

The use of natural dyes in food has increased recently due to the marketing advantages with the development of natural ingredients and the consumer concern about the harmful effects of synthetic pigments on health (DUFOSSÉ, 2006). Pigments are derived from natural sources such as plants, insects, and microorganism. There has been much interest in the development of new natural colorants for use in the food industry owing to strong consumer demand for more natural products. It is technologically feasible to prepare new colorants from locally known plants or microorganisms that have not yet been investigated scientifically (WISSGOTT; BORTLIK, 1996).

The genus *Monascus* involves three main species (*M. pilosus*, *M. purpureus*, and *M. ruber*) pertaining to the family *Monascaceae* and class *Ascomyceta*, whose most important trait is the ability to produce secondary metabolites of polyketides structures, some of them with yellow, orange, and red

pigmentation. Easily found in many ecosystems, this fungus was originally used in China and Thailand in the preparation of *angkak*, dark red colored rice with various uses. It gives color to other products such as wine, cheese, and meat; it also serves medicinal purposes and can be used as a preservative in meat (DUFOSSÉ et al., 2005; MAPARI, 2005).

The traditional process of pigment production involves solid state fermentation. Submerged techniques for *Monascus* pigment production have been used to minimize problems of space, scale, and process control (HAMDI; BLANC; GOMA, 1996; VENDRUSCOLO et al., 2010).

Some pigments produced by *Monascus* sp. are intracellular and insoluble in water, but growth conditions such as nitrogen source, pH, and aeration can result in the formation of extracellular and water soluble pigments (HAJJAJ et al., 1998).

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Changes in the *Monascus sp.* culture medium pH alter the proportion between the formation of different pigments and extracellular production (MUKHERJEE; SINGH, 2011).

Extracellular pigments are preferred because they are soluble in the medium in which they are produced, and downstream processes are simpler and cheaper (VELMURUGAN et al., 2010). However, the development of less expensive processes for the production of natural pigments is one of the challenges to enable production in large scale. The cost of the substrate has an important contribution to the overall production cost, and it can be minimized by using low-cost organic waste.

The production of *Monascus* pigments in submerged cultures has been investigated using glucose as main substrate. However, there are studies reporting the use of alternative substrates such as ethanol (HAMDI et al., 1997) and agricultural waste. Red pigments have been obtained from growth on solid medium of cassava (BABITHA; SOCCOL; PANDEY, 2006), corn syrup (HAMANO; KILIKIAN, 2006), wheat flour (DOMÍNGUEZ-ESPINOSA; WEBB, 2003), shrimp flour and crab shell (WANG et al., 2002), pear juice (HAMDI; BLANC; GOMA, 1996), and grape waste (SILVEIRA; DAROIT; BRANDELLI, 2008).

Biodiesel is produced by transesterification of a triglyceride with a mono-alcohol (methanol or ethanol) in the presence of a catalyst forming mono-alkyl esters and glycerol. Brazil will become a major producer and consumer of biodiesel for two reasons: first, the use of alcohol to fuel cars has long tradition in the Brazilian culture and, secondly, the conditions for cultivating oleaginous plants are extremely favorable in many areas. Furthermore, the agricultural know-how to grow these plants is available, and also, large areas of cultivatable land have not yet been explored (SILVA; MACK; CONTIERO, 2009).

Glycerin is formed as a by-product of this reaction. There is a wide range of biotechnology products with high added value such as 1,3 propanediol and biosurfactants (AMARAL et al., 2009), citric acid (IMANDI et al., 2007) and ethanol (JARVIS; MOORE; THIELE, 1997), which can be produced using glycerin. The high amount of glycerin generated from biodiesel production, in Brazil and the world, means an abundant and cheap source of renewable raw materials in the coming years (MOTA; SILVA; GONÇALVES, 2009).

Glycerin is successfully used as carbon source to produce a variety of microbial metabolites. Enteric bacteria of the genera *Klebsiella*, *Enterobacter*, *Clostridium*, and *Citrobacter* degrade glycerin to obtain 1,3 propanediol, which is a monomer used to produce polyesters such as propylene. The production of biosurfactants by microorganism, such as *Pseudozyma antarctica*, *Rhodococcus erythropolis*, *Bacillus circulans*, *Candida bombicola*, and *Pseudomonas aeruginosa*, using glycerin obtained as by product of biodiesel as a substrate is viable (AMARAL et al., 2009).

For each 90 m<sup>3</sup> of biodiesel obtained by transesterification in Brazil, approximately 10 m<sup>3</sup> of glycerin are generated. The projections show a production of about 250,000 t each year with the introduction of B5 (fuel: 5% biodiesel and 95% petrodiesel) in 2013. These figures are much higher than the current

consumption and production levels, and they are estimated to reach 30,000 t per year. This indicates that the commercial viability of biodiesel goes beyond the consumption of this extra volume of glycerin seeking large-scale applications and adding value to the production chain (MOTA; SILVA; GONÇALVES, 2009).

With the growth of biodiesel production, it is important to develop new applications for glycerin and expand the existing ones.

Based on the aforementioned, the main objective is to study the production of pigments by the fungus *Monascus ruber* in a bioreactor using glycerin obtained as a by-product from a biodiesel industry as a substrate.

## 2 Materials and methods

### 2.1 Microorganism

*Monascus ruber* CCT 3802 was obtained from the Tropical Culture Collection André Tosello (Campinas-SP, Brazil). The stock culture was maintained on potato dextrose agar (PDA) tubes. Tubes and Roux bottles were inoculated, incubated at 30 °C, for 7 days, and subsequently stored at 4 °C.

### 2.2 Culture medium and inoculum preparation

The growth medium contained per liter: 10 g glucose, 10 g glycerin, 5 g glycine, 5 g K<sub>2</sub>HPO<sub>4</sub>, 5 g KH<sub>2</sub>PO<sub>4</sub>, 0.1 g CaCl<sub>2</sub>, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g ZnSO<sub>4</sub>·7H<sub>2</sub>O, and 0.03 g MnSO<sub>4</sub>·H<sub>2</sub>O (PASTRANA et al., 1995).

The glycerin used in this study was obtained from a biodiesel production plant located in Brazil. Crude glycerin is a co-product of biodiesel production and was used without purification or pretreatment.

The inoculum was obtained by the germination of *Monascus ruber* spores suspended in 1 L baffled flasks, containing 0.4 L of culture medium incubated at 30 °C, in an orbital shaker at 120 min<sup>-1</sup> for 60 hours (VENDRUSCOLO et al., 2010).

The batch runs were performed in a Bioflo III reactor (New Brunswick Scientific, New Jersey, USA) with 4 L of working volume. After sterilization, 0.4 L of the inoculum culture was filled into the bioreactor containing 3.6 L of medium (VENDRUSCOLO et al., 2012). The following operational conditions were kept constant: temperature at 30 °C, stirring speed of 350 rpm, and aeration rate of 1 vvm. The initial pH of the medium was adjusted to 6.5 with HCl or NaOH.

The end of the fungal culture was defined when the production of pigments decreased and the concentration of biomass remained constant.

### 2.3 Biomass concentration

The biomass was quantified gravimetrically. Each sample (10 mL of culture medium) was weighed on an analytical balance after being vacuum filtered through previously weighed quantitative filter paper (J. Prolab, Brazil), and the retained material was submitted to microwave (Consul, Brazil) drying

at 180 W for 15 minutes (PEREIRA; KILIKIAN, 2001). After cooling in a desiccator for 15 minutes, it was weighed in order to determine the dry weight of the biomass retained on the filter paper.

## 2.4 Quantification of pigments

The pigments were quantified using a spectrophotometer (SP-1100 Series Model SP-105, Tecnal, Brazil). The concentration of extracellular pigment was estimated by measuring the absorbance of filtrates at 510 nm (red pigments), 470 nm (orange pigments), and 400 nm (yellow pigments) (JUZLOVÁ; MARTÍNKOVÁ; KREN, 1996; KIM et al., 2002). The pigments produced by *Monascus ruber* CCT 3802 were expressed in absorbance units (UA).

## 2.5 Kinetic parameter

The maximum specific growth velocity was calculated from the slope of the linearized curve of logarithm residual biomass over time, according to Equation 1.

$$\ln(X) = \ln(X_0) + \mu_{max} t \quad (1)$$

where  $X$  is the biomass in the exponential phase ( $\text{g.L}^{-1}$ );  $X_0$  is the biomass in the initial exponential phase ( $\text{g.L}^{-1}$ );  $\mu_{max}$  is the specific growth velocity ( $\text{h}^{-1}$ ) and  $t$  is time (hours).

The maximum productivity of cells was calculated by the difference between the greatest amount of biomass at a time  $t$ , and the initial amount of biomass divided by the corresponding period of time, as seen in Equation 2,

$$P_{cells} = \frac{(X_{MAXt}) - (X_{t_0})}{t - t_0} \quad (2)$$

where  $P_{cells}$  is the maximum productivity of biomass at the period of time  $(t - t_0)$  ( $\text{g.h}^{-1}$ );  $X_{MAXt}$  is the maximum amount of biomass at time  $t$ ;  $X_{t_0}$  is the amount of biomass at time  $t_0$ ;  $t$  is the time to achieve the maximum value of biomass; and  $t_0$  is the initial time of cultivation.

The maximum productivity of pigments was calculated by the difference between the greatest amount of pigment ( $\text{UA}_{510}$ ) at a time  $t$ , and the initial amount of pigments divided by the corresponding period of time, as seen in Equation 3,

$$P_M = \frac{(UA_{MAXt}) - (UA_{t_0})}{t - t_0} \quad (3)$$

where  $P_M$  is the maximum productivity of pigments at the period of time  $(t - t_0)$  ( $\text{UA.h}^{-1}$ );  $U_{At}$  is the maximum amount of pigment at time  $t$ ;  $U_{At_0}$  is the amount of pigment at time  $t_0$ ;  $t$  is the time to achieve the maximum value of UA; and  $t_0$  is the initial time of cultivation.

The specific production rate of red pigments was calculated using Equation 4, according to the methodology proposed by Le Duy and Zadic (1973).

$$\mu_p = \frac{1}{X} \frac{dP}{dt} \quad (4)$$

where  $\mu_p$  is the specific production rate of red pigments ( $\text{UA}_{510} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ ),  $X$  is biomass ( $\text{g.L}^{-1}$ ) at time  $t$ ,  $P$  is production of red pigment ( $\text{UA}_{510}$ ), and  $t$  is time (hours).

The specific growth rate was calculated using Equation 5, according to the methodology proposed by Le Duy and Zadic (1973).

$$\mu_x = \frac{1}{X} \frac{dX}{dt} \quad (5)$$

where  $\mu_x$  is the specific growth rate ( $\text{h}^{-1}$ ) and  $X$  is biomass ( $\text{g.L}^{-1}$ ) at time  $t$ .

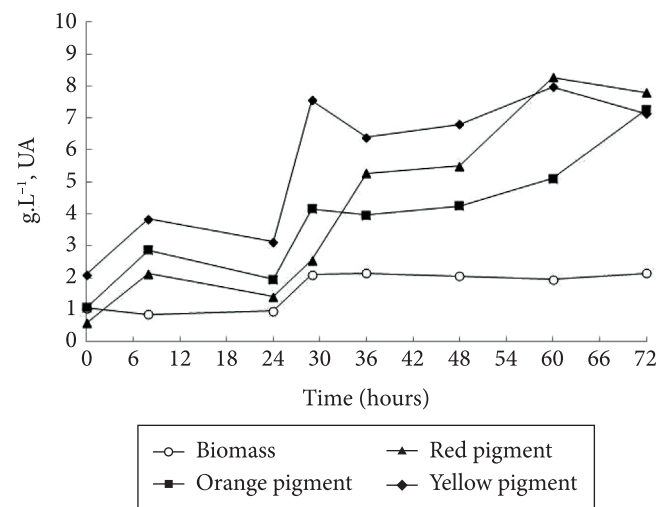
## 3 Results and discussion

The kinetics of biomass and pigment production are presented in Figure 1. The maximum yield of pigments was observed after 60 hours of cultivation with the formation of 8.28  $\text{UA}_{510}$  of red pigment and 7.13  $\text{UA}_{400}$  of yellow pigment and after 72 hours of cultivation with production of 7.26  $\text{UA}_{470}$  of orange pigment.

The cultivation time was 72 hours, and the pigment production was growth associated (HAJJAJ et al., 1998; LEE et al., 2001). Figure 2 shows the specific growth rate ( $\mu_x$ ) and the specific production rate of red pigments ( $\mu_p$ ) obtained in the cultivation, indicating that pigment production is growth associated.

In previous studies (MEINICKE et al., 2012), maximum production of 7.38 UA of red pigments in flasks using glycerol as the only synthetic substrate was observed. Pastrana et al. (1995) studied the production of pigments in a 20 L bioreactor using glucose as a substrate, and they obtained 9.7 UA of red pigment.

The optimum conditions of cultivation in a 3 L bioreactor obtained by Lee et al. (2001) were: 5.9  $\text{g.L}^{-1}$  of biomass and 13.37  $\text{UA}_{510}$  of red pigments, in 80 hours of cultivation.



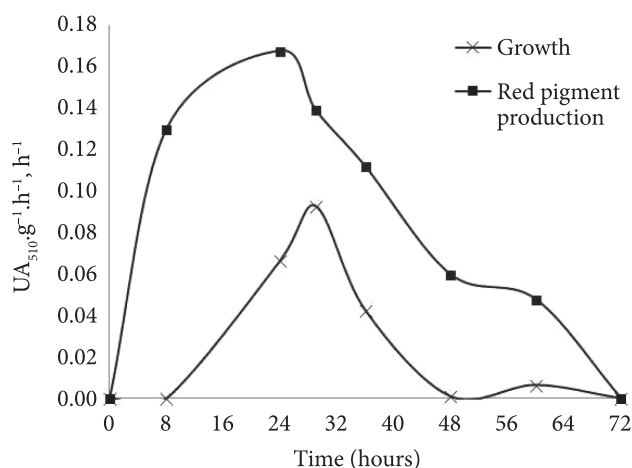
**Figure 1.** Biomass and pigments production kinetics using glucose and crude glycerin as substrate.



Orozco and Kilikian (2008) also studied the production of biomass and pigments and obtained the kinetic parameters in a 4 L bioreactor. They obtained 5.2 to 10.4 g.L<sup>-1</sup> of biomass and 11.3 UA of red pigments using *Monascus purpureus* and glucose as a substrate. Maximum production of pigment was obtained at pH ranging from 6.5 to 8.5.

In order to increase the scale of pigment production, it is important to obtain the kinetic parameters. Studying cell behavior and maintaining the parameters affect the final yield of pigment production and growth (KIM et al., 2002).

The pH remained in the range of 5.45 to 6.23 favoring the release and production of red pigments (CHEN; JOHNS, 1993; BLANC et al., 1995). There was a decrease in pH in the initial stage of the cultivation, followed by a slight increase in the next stage remaining constant. This behavior was also observed by Teng and Feldheim (2001) in cultures with *Monascus purpureus*. Domínguez-Espinosa and Webb (2003) also observed a decrease in pH at the beginning of the cultivation. When pigments are excreted in the medium, their pH value remains equal to or slightly greater than its original level (6-7). Table 1 shows the kinetic parameters, and the specific red pigment rates are shown in Figure 2.



**Figure 2.** Specific growth rates and red pigment production using glucose and crude glycerin as substrate.

**Table 1.** Growth kinetic parameters and pigment production using glucose and crude glycerin as substrate.

Parameters	Value
$\mu_{max}$ (h <sup>-1</sup> )	0.087
$P_{cells}$ (g.h <sup>-1</sup> )	0.003
Maximal specific pigment production rate (UA.g <sup>-1</sup> .h <sup>-1</sup> )	0.167
Red pigment production (UA <sub>510</sub> )	7.69
Orange pigment production (UA <sub>470</sub> )	6.19
Yellow pigment production (UA <sub>410</sub> )	5.86
$P_M$ Red pigment (UA <sub>510</sub> .h <sup>-1</sup> )	0.13
$P_M$ Orange pigment (UA <sub>470</sub> .h <sup>-1</sup> )	0.09
$P_M$ Yellow pigment (UA <sub>410</sub> .h <sup>-1</sup> )	0.10

The specific growth rate ( $\mu_x$ ) obtained by Hamdi, Blanc and Goma (1996) was 0.1 and 0.05 h<sup>-1</sup>, and the specific production rate of red pigments was 0.08 and 0.2 UA.g<sup>-1</sup>.h<sup>-1</sup>. The specific production rate of red pigments increased continuously with red pigment(s) formation and reached 7.85 at the end of the culture.

In a 20 L bioreactor, Pastrana et al. (1995) found a maximum specific growth of 0.04 h<sup>-1</sup> and 0.08 UA.g.h<sup>-1</sup> of maximum specific production rate of red pigments in a culture that lasted 180 hours using *Monascus ruber* and glucose as a substrate.

## 4 Conclusions

The results obtained in this study show that crude glycerin derived from biodiesel production has a high potential for pigment production. The greatest production of red pigments obtained using glycerin and glucose as substrates was 8.28 UA<sub>510</sub> with a productivity of 0.13 UA<sub>510</sub>.h<sup>-1</sup> and 2.15 g.L<sup>-1</sup> of biomass. The production of pigments is growth associated. The nitrogen source and the pH had great effects favouring the production of red pigments and *Monascus* sp. growth.

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