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ASSESSMENT OF PRE-INOCULUM PREPARATION METHODS IN THE PRODUCTION OF *Monascus ruber* BIOPIGMENTS

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ABSTRACT

The fungus *Monascus ruber* is notable for producing a range of microbial biopigments, such as red, yellow, and orange ones, by biotechnological processes. The production of fungal biopigments is influenced by various factors such as culture medium composition, inoculum, aeration, and temperature. The method of pre-inoculum preparation significantly impacts the fermentation process. There are several methods described in the literature for inoculating filamentous fungi, and spore suspension and mycelial discs are commonly used. This study aimed to compare different pre-inoculum preparation methods and surfactant supplementation to determine their effectiveness in enhancing biopigments production by *Monascus ruber* from xylose-based medium. The results indicated that the pre-inoculum preparation with fractionated mycelium disc supplemented with surfactant showed the best performance among the evaluated methods, resulting, in the preparation step itself, in biopigments production of 11.78 AU (yellow), 10.95 AU (orange), 13.12 AU (red), biomass production of 16.69 g/L, and xylose consumption of 24.69%. In the subsequent fermentation process, the selected pre-inoculum preparation method resulted in biopigments production of of 9.75 AU (yellow), 10.19 AU (orange), 11.79 AU (red), besides 11.57 g/L biomass, and 80.19% of xylose consumption.

Keywords: Biopigment. *Monascus ruber*. Surfactant. Pentose. Fermentation.

1 INTRODUCTION

The production of biopigments from *Monascus ruber* has garnered increasing interest in biotechnology due to the wide-ranging applications of these compounds in the food, pharmaceutical, and cosmetic industries. The natural biopigments produced by this fungus possess antibacterial and antioxidant properties, making them valuable as natural food colorants. Utilizing *M. ruber* for biopigment production offers a sustainable and safe alternative to synthetic dyes, which pose environmental and health concerns[1].

The benefits of *Monascus* biopigments can be better utilized by society if production costs are reduced, which can be achieved by selecting lower-cost raw materials and optimizing the process. Regarding raw materials, the evaluation of alternative substrates, such as pentoses (xylose, for example), can help in utilizing the hemicellulosic fraction of lignocellulosics[2]. The production of fungal biopigments is directly influenced by factors such as the composition of the culture medium, inoculum, aeration, and temperature[3]. Besides, adequate inoculum preparation impact the lag phase, specific growth rate, biomass yield, and the quality of the final product. The method of pre-inoculum preparation significantly influences the fermentation process by resulting in different initial cell density, physiological state, and nutrient utilization efficiency. Although there are many methods in literature to inoculate filamentous fungi, spore suspension and mycelial discs are the most used ones[4].

Another promising approach for increasing biomolecules production is the modification of cell membrane structure, an interesting strategy to increase the diffusion of microbial metabolites across the cell membrane. Supplementing the fermentation medium with surfactants has proven to be an effective strategy for optimizing *Monascus* biopigment production[5]. Surfactants are substances that alter the surface tension between liquids or between a liquid and a solid, facilitating nutrient dispersion and oxygen transfer in the culture medium. Adding surfactants can improve fungal cell permeability, leading to increased biopigment release and production. Studies indicate that selecting the right type and concentration of surfactants can maximize biopigment production, making the process more efficient and economically viable[6,7,8]. Therefore, this study aimed to evaluate different pre-inoculum preparation methods to determine their effectiveness in enhancing biopigment production by *Monascus ruber*, in addition to evaluating the effect of surfactant supplementation on pre-inoculum preparation step.

2 MATERIAL & METHODS

Microorganism: The *Monascus ruber* Tieghem IOC 2225 fungal strain was kindly donated by the Filamentous Fungi Culture Collection (CCFF) of the Oswaldo Cruz Foundation (IOC/FIOCRUZ, Rio de Janeiro, Brazil). The microbial culture was kept viable with periodic subculturing on sterile potato dextrose agar (PDA) plates, with the strains maintained for a period of 10 days of incubation at 30 °C to achieve the desired growth.

Composition of the Culture Medium: Xylose (53.38 g/L); yeast extract (4.37 g/L); K_2HPO_4 (5 g/L); $CaCl_2{\cdot}2\ H_2O$ (0.1 g/L); $MgSO_4{\cdot}7\ H_2O$ (0.5 g/L); $FeSO_4{\cdot}7\ H_2O$ (0.01 g/L); $ZnSO_4{\cdot}7\ H_2O$ (0.01 g/L); $MnSO_4{\cdot}7\ H_2O$ (0.03 g/L). The pH of the culture medium was adjusted to 6.0 with concentrated NaOH and HCl solutions.

Pre-Inoculum Method and effect of Surfactant supplementation: Three different methods of inoculating *Monascus ruber* were carried out: cell suspension, mycelial disc, and fractionated mycelial disc. For each inoculation method, assays containing or not 10 g/L of the surfactant Tween-80 were conducted. For the cell suspension method, cells from a Petri dish were scraped with either 10 mL of distilled water or 10 mL of a Tween-80 solution. A volume of 1 mL of this suspension was used as inoculum. For the mycelial disc inoculation, a mycelial disc approximately 8 mm in diameter was cut out and inoculated into the culture medium. For the fractionated mycelial disc method, two additional transverse cuts were made in the 8 mm mycelial disc, fractionating it, and these fragments were used for inoculation. 50 ml Erlenmeyer flasks, containing 20 mL of culture medium, in triplicate, were

placed in an incubator (rotary shaker) in the absence of light at 30 °C and shaken at 150 rpm for 5 days of cultivation. Samples were evaluated for biopigment production, biomass concentration, and xylose concentration at the end of the process.

Evaluation of Inoculation Method in the Production of Biopigments by *M. ruber*: To evaluate the performance of each inoculation method in this study, fermentation assays were performed in triplicate. The pre-inoculation process was carried out as previously described and, after 5 days of cultivation, the biomass produced was inoculated into a new culture medium with the same composition used to prepare the pre-inoculum. Cultivations were carried out for 15 days in 250 mL Erlenmeyer flasks, with a culture medium volume of 100 mL. The samples were evaluated for biopigment production, biomass concentration and xylose concentration at the end of the process.

Biomass concentration: The fungal biomass concentration was determined by measuring the dry mass. For this purpose, the content of each collected cultivation flask centrifuged (Beckman, model J6-HC, California, USA) at 2,465×g for 10 minutes. The supernatant was collected for subsequent evaluation, while the biomass obtained was washed with ethanol. After washing, the biomass was dried in an oven at 60 ºC until a constant mass was obtained[9].

Biopigment Production: The production of biopigments by the fungus *M. ruber* was determined, using absorbance measurements at specific wavelengths: 400 nm (yellow), 450 nm (orange) and 490 nm (red). Measurements were conducted using an Eppendorf spectrophotometer (Eppendorf AG, Germany), and the absorbance results obtained were multiplied by the respective dilution factor. Biopigment production was expressed in absorbance units (AU)[1].

Concentration of Xylose: The concentration of xylose was determined using High-Performance Liquid Chromatography (HPLC)[10].

3 RESULTS & DISCUSSION

Different methods were evaluated as pre-inoculum methods for the fungus *M. ruber*, including cell suspension (TW-80 Free - Suspension), mycelium disc (TW-80 Free - Mycelium Disc), and fractionated mycelium disc (Fract Mycelium Disc). Additionally, the supplementation of Tween-80 (non-ionic surfactant) was also assessed for each pre-inoculum protocol (TW-80 - Suspension, TW-80 - Mycelium Disc, TW-80 Fract Mycelium Disc). The results obtained in this pre-inoculum preparation stage are described in Figure 1.

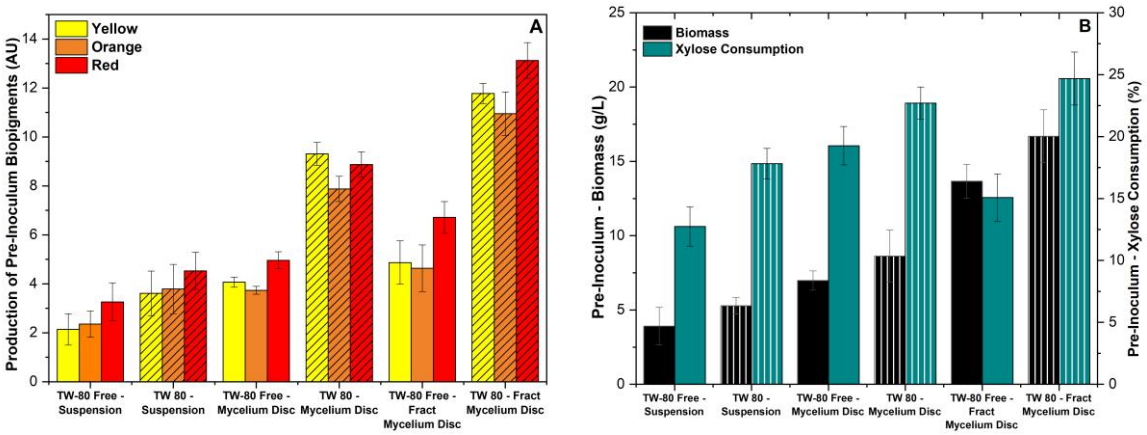

Figure 1 Evaluation of the pre-inoculum protocol, with 5 days of cultivation: Biopigment Production (AU) (A), Biomass (g/L) and Xylose Consumption (%) (B). TW-80 free and TW 80 correspond to experiments without and with addition Tween- 80, respectively. Fract: fractionated.

As can be observed, among the evaluated methods, the cell suspension resulted in the lowest biopigment production (Figure 1A) and cellular biomass (Figure 1B). Cell suspension assays, without surfactant, initially yielded of 2.14 AU (yellow biopigment), 2.35 AU (orange biopigment) and 3.26 AU (red biopigment), with a biomass of 3.91 g/L and xylose consumption of 12.75%. The addition of Tween-80 increased biopigment production to 3.61, 3.79 and 4.53 for yellow, orange and red biopigments respectively and xylose consumption to 17.81%, with biomass rising to 5.28 g/L. This increase suggests that Tween-80 enhanced xylose consumption efficiency, resulting in higher biopigment and biomass production. It was also noted that only fractionation of the mycelium disc led to an increase in biopigment production. Among the biopigments, the red biopigment exhibited the most substantial increase, rising from 4.96 AU to 6.72 AU with the fractionated inoculum, in experiments without Tween- 80 addition. Additionally, in this case, biomass production was significantly impacted by the fractionation of the inoculum disc, nearly doubling the cell biomass from 6.98 g/L to 13.66 g/L.

Regarding the effect of surfactant supplementation, it is also noticeable that it promoted a positive effect on the studied variables. Biopigment production was particularly more influenced than biomass production or xylose consumption. Using surfactant, the production of all biopigments was almost double, both for assays with whole mycelium disc (4.07 to 9.31 AU, yellow; 3.73 to 7.87 AU, orange; and 4.96 to 8.87 AU, red) and for the cut mycelium disc (4.87 to 11.78 AU, yellow; 4.63 to 10.95 AU, orange; and 6.71 to 13.12 AU, red). The same effect was also observed in the study by Wang (2013), wherein among the surfactants of the polysorbate class, Tween-80 showed the best performance in increasing the production of biopigments from *M. purpureus* H1102.This result can be explained by the effect that the surfactant has on the fatty acids of the cell membrane, increasing their fluidity and permeability, which facilitates the secretion of intracellular biopigments and thus increases the productivity of extracellular biopigments. Even so, the application of surfactants as a carbon source or the understanding of their mechanism of action in the production of extracellular biopigments is still being developed[11,12].

The results indicate that the pre-inoculum protocol conducted with fractionated mycelium disc and medium supplemented with surfactant (10 g/L) was the most efficient among the evaluated methods. The obtained values were: 11.77 ± 0.41 AU (yellow biopigment), 10.95 ± 0.89 AU (orange biopigment), 13.12 ± 0.73 AU (red biopigment), 16.69 ± 1.78 g/L (biomass production), and 24.69 ± 2.14% (xylose consumption). In summary, the fractionation of the mycelium disc and the surfactant showed more pronounced effects on the different response factors. After evaluating the pre-inoculum methods, the biomass obtained from each method was inoculated into a new medium to analyze its performance in the subsequent fermentation process. The production of *M. ruber* biomass, biopigment production, and xylose consumption were evaluated at the end of the 15-day fermentation process. The results obtained are shown in Figure 2.

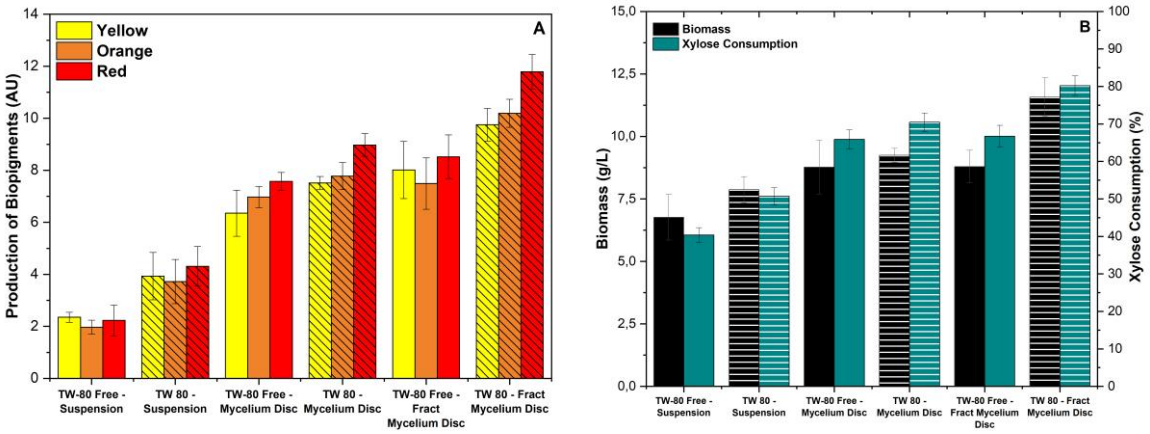

Figure 2 Evaluation of pre-inoculum methods, in the 15-day fermentation of *M. ruber*, regarding the production of Biopigments (AU) (A), cell biomass (g/L) and xylose consumption (%) (B). TW-80 free and TW 80 correspond to experiments without and with addition Tween- 80, respectively. Fract: fractionated.

As shown in Figure 2, the cell suspension protocol again resulted in lower biopigment production, biomass, and xylose consumption. The suspension test without surfactant resulted in the lowest biopigment production, with values of 2.35 AU (yellow), 1.97 AU (orange), and 2.23 AU (red). The addition of Tween-80 (TW 80 Suspension) promoted an increase in biopigment production, reaching 3.93 AU (yellow), 3.72 AU (orange), and 4.31 AU (red), along with an increase in biomass production (from 6.76 to 7.87 g/L) and xylose consumption (from 40.36 to 50.71%). The difference in growth and biopigmentation between the cell suspension and the mycelium inoculation method, may be due to the cell suspension may have a long lag phase due to the time required to adapt to the new culture medium. This physiological stress results in less growth, as cells need to recover from erosion of the mycelial surface during suspension preparation. Furthermore, inoculation with mycelium disc can have more initial biomass, reducing the lag phase due to the greater concentration of cells, enzymes and important metabolites already produced during the preparatory phase of the inoculum[11].

The mycelium disc inoculation methods proved to be more efficient. In the mycelium disc assays, evaluating only the effect of fractionating the mycelium disc, an increase in biopigment production was obtained with values ranging from 6.35 to 8.01 AU (yellow biopigment), 6.97 to 7.77 AU (orange biopigment) a and 7.57 to 8.52 AU (red biopigment), in experiments without addition of Tween-80. However, in this case, regarding cell biomass production and xylose consumption, similar values were obtained in tests with and without fractionation of the mycelium disc in the pre-inoculum. With the addition of surfactant (Tween-80), both the production of all biopigments and biomass production and xylose consumption were favored with its supplementation both for the fractionated mycelium disc tests or not. With the addition of Tween-80 and fractionated mycelium disc, biopigment production reached 9.75 ± 0.63 AU (yellow), 10.19 ± 0.54 AU (orange) and 11.79 ± 0.66 AU (red), biomass production of 11.57 g/L and xylose consumption of 80.19%.

This study focuses on the initial steps of using Tween-80 in biopigment production. New studies will be developed to better understand the mechanism of action and optimal application methods of the surfactant. The ultimate objective is to increase biopigment production and reduce fermentation time by leveraging the biosurfactant's effect on the excretion of extracellular biopigments from *M. ruber*.

4 CONCLUSION

The results of this study demonstrate that the choice of pre-inoculation method has an impact on *Monascus ruber* biopigment and biomass production, as well as xylose consumption. The use of mycelium disc, especially when fragmented and supplemented with Tween-80 surfactant, proved to be highly efficient, resulting in substantial increases in biopigment and biomass production. The combination of mycelium disc fractionation with Tween-80 supplementation proved to be the most promising strategy, maximizing the productivity of extracellular biopigments and the efficiency of substrate consumption.

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