BIOPROCESS OPTIMIZATION FOR PRODUCTION OF *Monascus* PIGMENTS: INFLUENCE OF XYLOSE AND YEAST EXTRACT

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ABSTRACT

Natural pigments are widely used in the food, pharmaceutical, and textile industries for their coloring properties and biological roles, including antimicrobial, anti-inflammatory, antioxidant, and anticancer effects. The fungus *Monascus ruber* is notable for producing a range of microbial pigments, such as red, yellow, and orange, through biotechnological processes. In this study, the initial concentrations of xylose and yeast extract were evaluated for the production of *M. ruber* pigments, aiming to optimize the process in experiments performed according to a central composite experimental design. The results highlighted the influence of xylose and yeast extract concentrations, indicating optimized conditions of 53.38 g/L and 4.37 g/L of these medium components, respectively. Under the optimized conditions, values of 21.29 g/L of biomass and 40.59% of xylose consumption were obtained, reaching 11.98, 10.45, and 14.02 AU of production of yellow, orange, and red pigments, respectively. The potential application of xylose-rich substrates was shown for the production of pigments by the fungus *M. ruber*.

Keywords: Biopigment. *Monascus ruber*. Optimization. Pentose. Microbial pigments.

1 INTRODUCTION

The increase in public awareness regarding environmental issues and the pursuit of healthy eating have driven the growth of the natural products industry. As a result, many organizations are developing green marketing strategies to meet customer needs. Considering that a product's image is one of the first criteria for consumer choice, the color selected for the product is crucial in the decision-making process[1]. Colors have been explored by humanity for millennia, from ancient body paintings and cave art, using parts of plants, insects, and clays. With scientific and technological advancements, the first synthetic organic dye was produced in the 19th century, revolutionizing the use of synthetic colorants. However, due to the use of fossil-derived chemicals and the environmental and toxicological impact of these dyes, the searching for methods to obtain natural pigments has gained prominence[2].

Natural pigments can be obtained from plants, animals, or microorganisms such as bacteria, yeasts, or fungi. Although numerous, plant-derived pigments have some disadvantages, such as the loss of valuable species due to improper exploitation, growth time, the quantity of raw material needed, and dependence on climatic conditions and seasonality[3]. Microbial biopigments have advantages such as simplified production processes, rapid growth, higher yields, stability, and not competing with agricultural land for food production[4]. Among biopigment-producing microorganisms, the fungus *Monascus* stands out because it not only provides color but also exhibits various biological functions such as antioxidant, antibacterial, cholesterol-regulating, anti-inflammatory, anticancer, anti-obesity, and antitumor activities, increasing its interest for industrial-scale production[5]. Biopigments synthesized by the filamentous fungi of the *Monascus* genus present yellow, orange, and red colors[6]. Their production is commonly conducted in solid media; however, recently, submerged culture has shown promise. This approach offers advantages such as ensuring nutrient homogeneity, stable yields, operational ease, and monitoring[7].

The cultivation medium for biopigment production can represent a large portion of the costs involved in the process, making it vital to optimize the used nutrients concentration, especially the carbon and nitrogen sources[8]. Carbon supplementation in the cultivation medium for cell and biopigment production by *Monascus* is of great importance, as it is crucial for the biosynthesis metabolism of various compounds such as carbohydrates, proteins, and lipids, besides its bioconversion into energy for cell maintenance[9]. Nitrogen supplementation is also significant in microbial metabolism and can be of inorganic (nitrite and nitrate) or organic (peptone and yeast extract) origin[10]. Among the monosaccharides applied as carbon sources for *Monascus* biopigment production, xylose has been underexplored despite presenting itself as a potential candidate as a substrate, enabling the utilization of agro-industrial by-products containing xylan in their composition, such as sugarcane bagasse[11]. Therefore, in this study, the initial concentrations of xylose and yeast extract were evaluated for the production of *Monascus ruber* biopigments, aiming to optimize production by performing experiments according to a central composite experimental design. By varying xylose and yeast extract concentrations in the medium, the better C/N ratio can also be determined, a fundamental parameter for biopigments production.

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 (25 – 55 g/L); yeast extract (2,5 – 5,5 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.

Evaluation of the C/N Ratio in biopigment Production by the Fungus *M. ruber*: The response surface methodology was used to evaluate the interaction between the variables under study and their influence on pigment production, biomass, and xylose consumption. Two factors were evaluated during the fermentation: initial xylose concentration and yeast extract concentration, allowing the assessment of the influence of the C/N ratio on the process. A total of 13 experimental trials were conducted according to a face-centered central composite design. The obtained data were analyzed using Design-Expert 13 software (Stat-Ease, Inc., USA). The concentrations of the evaluated factors for initial xylose concentration were 25 (-1), 40 (0), and 55 g/L (+1), while the initial yeast extract concentration was 2.5 (-1), 4.0 (0), and 5.5 g/L (+1). The flasks inoculated with a disc of mycelium (8 mm in diameter) and 20 mL of culture medium were placed in an incubator (rotary shaker) in the absence of light at 30°C and shaking at 150 rpm for 20 days of cultivation. Samples were taken and evaluated for pigment 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 was added to a 50 mL Falcon tube and then centrifuged (Beckman, model J6-HC, California, USA) at 2,465×g for 10 minutes. The supernatant was collected for subsequent analysis of xylose concentration and biopigments production, 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[12].

Biopigment Production: The production of biopigments by the fungus *Monascus ruber* was determined using absorbance measurements at specific wavelengths: 400 nm (yellow), 450 nm (orange) and 490 nm (red). An Eppendorf spectrophotometer (Eppendorf AG, Germany) was used, and the absorbance results obtained were multiplied by the respective dilution factor. Absorbances were expressed in absorbance units (AU)[8].

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

3 RESULTS & DISCUSSION

The results of the experimental design are presented in Table 1.

Table 1 Experimental Design Matrix of Central Composite Face-Centered Design used to evaluate the influence of xylose and yeast extract concentrations in the performance of biopigments production process (coded values in parenthesis)

Factors			Response Variables				
N° Exp.	Xylose (g/L)	Yeast Extract (g/L)	Biomass (g/L)	Xylose Consumption (%)	Yellow biopigment (AU)	Orange biopigment (AU)	Red biopigment (AU)
1	25 (-1)	2.5 (-1)	9.10	47.63	5.02	4.52	6.15
2	25 (-1)	4.0 (0)	11.31	37.21	6.08	6.81	8.23
3	25 (-1)	5.5 (+1)	11.57	59.51	5.60	5.96	7.92
4	40 (0)	2.5 (-1)	12.60	83.63	4.37	4.63	5.47
5	40 (0)	4.0 (0)	13.55	86.21	10.89	10.32	12.55
6	40 (0)	4.0 (0)	13.56	76.94	10.12	10.45	14.25
7	40 (0)	4.0 (0)	13.99	88.79	13.05	10.98	14.02
8	40 (0)	4.0 (0)	13.42	69.80	11.83	9.56	12.84
9	40 (0)	4.0 (0)	12.34	76.47	10.05	10.52	12.58
10	40 (0)	5.5 (+1)	15.28	82.23	6.94	7.57	8.58
11	55 (+1)	2.5 (-1)	18.16	47.10	4.53	4.80	7.30
12	55 (+1)	4.0 (0)	22.10	31.71	10.40	8.25	11.50
13	55 (+1)	5.5 (+1)	25.53	35.30	11.16	8.48	11.25

The results obtained demonstrated that the conditions promoting the highest dry biomass production were achieved in experiments 11 to 13, with a xylose concentration of 55 g/L and yeast extract concentrations of 2.5, 4.0, and 5.5 g/L, yielding biomass values of 18.16, 22.1, and 25.53 g/L, respectively. Regarding the influence of xylose and yeast extract concentrations on biomass production, increasing the concentrations of both xylose and yeast extract increased microbial cell growth. Furthermore, increasing the yeast extract concentration for each xylose level also resulted in increased biomass production. In the experiments in which the xylose concentration was 25 and 55 g/L, regardless of the yeast extract concentration, the xylose consumption and biopigment production were lower than those obtained at the central points. The conditions at the central point resulted in the highest xylose consumption and formation of the three biopigments, with respective values ranging from 69.8% to 88.79% for substrate consumption, 10.05 to13.05 AU for yellow biopigment, 9.56 to 10.98 AU for orange biopigment, and 12.55 to 14.55 AU for red biopigment. It is worth noting that, for all conditions evaluated, the red biopigment was produced in the highest amounts among the biopigments.

The results presented in Table 1 were analyzed using the Design-Expert software. This analysis enabled the development of significant models ($p<0.05$) representing the production of cellular biomass. The model equations enabled the creation of the corresponding response surfaces (Figure 1) for the variables biomass production, yellow, orange, and red biopigments, and xylose consumption, providing a more efficient visualization of the effects of the studied variables. In Figure 1A, it can be seen that the region for the highest production of yellow biopigment occurred at xylose concentrations of 50 to 55 g/L and yeast extract concentrations of 4.0 to 5.5 g/L. Low levels of yeast extract (< 3.0 g/L) indicate low biopigment production for any xylose

concentration. For the orange biopigment (Figure 1B), an optimal region near the central point was found. For the production of the red biopigment (Figure 1C), in addition to the behavior similar to all biopigments, low levels of yeast extract (< 3.0 g/L) indicated low biopigment production for any xylose concentration. The obtained response surface was similar to that of the yellow biopigment, but it presented a broader region of higher production. The optimal region for biopigment production was close to the range of 45 to 55 g/L of xylose and 3.5 to 5.0 g/L of yeast extract. Figure 1D shows for higher production of *M. ruber* biomass, higher concentrations of xylose and yeast extract should be applied. For biomass production above 24 g/L, the culture medium should consist of xylose and yeast extract at concentrations of 55 and 5.5 g/L, respectively. A broad region was characterized as resulting in low biomass production, comprising the range of 25 to 45 g/L of xylose combined with a concentration of 2.5 to 5.5 g/L of yeast extract. Regarding substrate consumption (Figure 1E), a different behavior was evidenced. At the extremes of xylose levels, between 25 and 30 g/L and 50 and 55 g/L, for all yeast extract concentrations, there was lower xylose consumption by the microorganism. The optimal regions to provide high xylose consumption by *Monascus ruber* are in the central region of xylose.

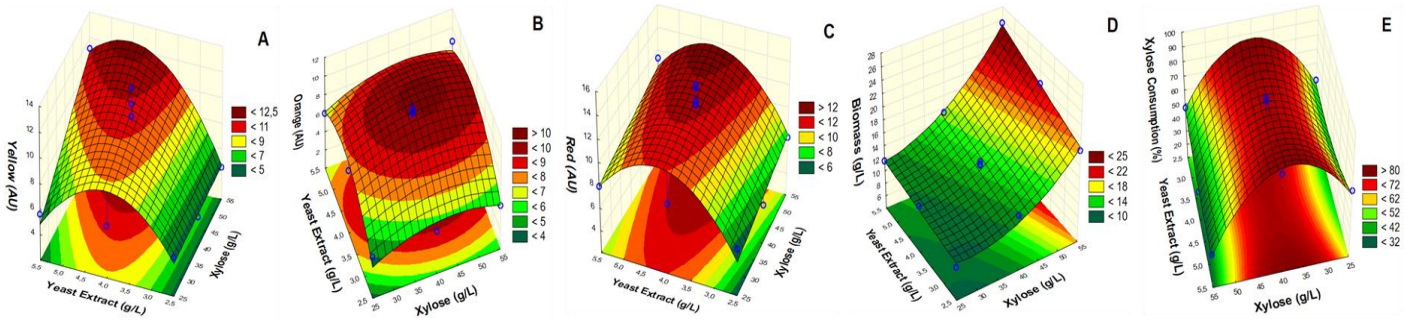

Figure 1. Response surface for pigment production (A, B, C), biomass (D), and xylose consumption (E).

Using an specific optimization tool of the software Design-Expert, the optimized conditions for biopigments production (considering as goal to optimize the production of the three biopigments simultaneously) corresponded to 53.38 g/L of xylose and 4.37 g/L of yeast extract (corresponding to a C/N ratio of 48.8), with predicted values between (95% confidence interval) 20.66 to 22.41 g/L of biomass, 10.22 to 14,22, 8.23 to 10.86, 11.67 to 15.36 AU for yellow, orange, and red pigments, and xylose consumption between 31 to 50 %. An experiment under the optimized conditions was conducted, obtaining values of 21.29 g/L of biomass, 11.98, 10.45, and 14.02 AU of yellow, orange, and red pigments, and xylose consumption of 40.59%. These results indicate that the obtained models were efficient in predicting the results in the range of the studied variables.

The results obtained in this study corroborate findings reported in the literature, indicating that although xylose has a lower assimilation rate in *Monascus* species compared to monosaccharides like glucose[11,14], it still yields satisfactory results for biomass and pigment production[11]. Yeast extract stimulates conidiation, inhibits the sexual cycle, and enhances biomass[15] and pigment production, particularly in the formation of red pigment[10,16].

4 CONCLUSION

The experimental design highlighted the influence of the studied variables (xylose concentration and yeast extract) on pigment production and biomass yield. The optimized conditions were to 53.38 g/L of xylose and 4.37 g/L of yeast extract, corresponding to a C/N ratio of 48,8, and resulting in 11.98, 10.45, and 14.02 AU of yellow, orange, and red biopigments production. The findings here reported show the potential of using xylose as carbon source for *Monascus* biopigments production, a very interesting alternative for using cheap agricultural residues and by-products as raw material for this bioprocess.

REFERENCES

1 CAM, L. N. T. 2023. Heliyon. 9(9). 2023.
2 MERUVU, H., DOS SANTOS, J. C. Critical reviews in biotechnology. 41(8). 1153-1177.
3 LAGASHETTI, A. C. et al. 2019. Microorganisms. 7(12). 604, 2019.
4 KALRA, R., CONLAN, X. A., GOEL, M. 2020. Frontiers in Chemistry. 8. 369.
5 QIN, X., XIE, B., ZONG, X., YU, X., FENG, Y. 2023. Food Bioscience, 103233.
6 AGBOYIBOR, C., KONG, W. B., CHEN, D., ZHANG, A. M., NIU, S. Q. 2018. Biocatalysis and Agricultural Biotechnology. 16. 433-447.
7 WANG, Y., ZHANG, B., LU, L., HUANG, Y., XU, G. 2013. Journal of the Science of Food and Agriculture, 93(13), 3339-3344.
8 VENDRUSCOLO, F., BÜHLER, R. M. M., DE CARVALHO, J. C., DE OLIVEIRA, D., MORITZ, D. E., SCHMIDELL, W., NINOW, J. L. 2016. Applied Biochemistry and Biotechnology. 178. 211-223.
9 CHEN, M., JOHNS, M. R. 1994. Enzyme and microbial technology. 16(7). 584-590.
10 FENG, Y., SHAO, Y., ZHOU, Y., CHEN, W., CHEN, F. 2016. Industrial Biotechnology of Vitamins, Biopigments, and Antioxidants. 497-535.
11 HUAWEI, Z., CHENGTAO, W., JIE, Q., BINGJING, Z., BING, Z., CHUANGYUN, D. 2019. Pigment & Resin Technology. 48(6). 547-554.
12 WANG, M., HUANG, T., CHEN, G., WU, Z. 2017. Applied microbiology and biotechnology. 101(8). 3121-3130.
13 AHMED, M. A., REHMAN, M. S.U., TERÁN-HILARES, R., KHALID, S., HAN, J.I. 2017. Energy conversion and management. 141. 120-125.
14 Moritz, D. E. 2005. Produção de pigmentos Monascus por *Monascus ruber* CCT3802 em cultivo submerso.
15 MANAN, M. A.; MOHAMAD, R.; ARIFF, A. 2017. J Microbiol Exp. 5(3). 00148.
16 MOSTAFA, M. E., ABBADY, M. S. 2014. Global Journal of Biotechnology and Biochemistry. 9(1). 1-13.

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