PRODUCTION OF CAROTENOIDS IN STIRRED TANK BIOREACTOR FOR INDUSTRIAL USE AS FOOD COLORANTS

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ABSTRACT

The employment of artificial colors, such as synthetic carotenoids, within the food industry raises concerns regarding their safety and impact on human health. Some artificial compounds have been linked to adverse reactions, including allergies, hyperactivity in children, sleep disorders, and even cancer. As a substitute, there is a growing interest in natural bioactive compounds, such as microbial carotenoids, which are found in a variety of organisms. The production of carotenoids in red yeast, such as *Rhodotorula glutinis*, can be enhanced through physical and mechanical optimization processes in bioreactor. This study explored the production of natural carotenoids by *R. glutinis* in Stirred Tank Bioreactor, followed by application of carotenoids produced on gelatin, assessing their stability against temperature and time. The results indicated that the combination of agitation at 300 rpm and aeration at 1 vvm (volume of air to volume of liquid per minute) maximized the carotenoid production, particularly torularhodin (2.76 UA_{480nm}). Furthermore, application experiments revealed that the carotenoid extract has potential as a food coloring agent for edible gelatins, demonstrating stability even after exposure to high temperatures (100 °C) and during refrigerated storage for 10 days. These findings underscore the feasibility of carotenoids as an alternative to synthetic colorants, fostering the production of safer and healthier food options.

Keywords: Bioreactor. Natural bioactive compounds. Health concerns. Gelatin. Temperature stability.

1 INTRODUCTION

Artificial carotenoids, synthesized through chemical processes, are extensively employed in the food industry to enhance product appearance and promote consumption. Nevertheless, the application of these additives in food products has triggered debates regarding their potential impact on human health.[1] Simultaneously, there has been a growing interest in healthier additives including with biological activity devoid of artificial counterparts.[2]

Certain artificial colororants have been linked to various adverse effects, including allergic reactions, increased hyperactivity in children,[3] urticaria, adverse immune responses, asthma, hyperthyroidism, anemia, cancer, and even sleep disorders.[4] Responding to these concerns, there has been a surge in interest in natural bioactive compounds for human consumption, such as carotenoids.[5]

Carotenoids are natural compounds present in a diverse array of organisms, including plants, algae, cyanobacteria, certain archaea, bacteria, fungi, and even non-photosynthetic organisms.[6] These fat-soluble pigments are responsible for the vibrant colors observed in many plants, offering a spectrum of hues ranging from yellow to red.[7]

The production of carotenoids in red yeast, particularly *Rhodotorula glutinis*, can be influenced by several factors, such as the carbon/nitrogen ratio (C/N), the components of the culture medium (*e.g.*, carbon and nitrogen sources and salts), as well as physical factors such as lighting and temperature.[8] Additionally, mechanical factors like agitation and aeration can impact the biosynthesis process.[9] This aspect is of paramount importance for yeast bioproducts, considering the cultivation time and, consequently, the electrical energy consumed in the process.

To improve the production of carotenoids through physical and mechanical stress, this study investigated the production of natural colorants by *R. glutinis* in stirred tank bioreactor, followed by studies on their application in gelatin, while also assessing their stability against temperature and time. The resulting product may offer an alternative to synthetic colorants, which have been associated with adverse effects on human health.

2 MATERIAL & METHODS

2.1. Production and quantification of carotenoids

The yeast used was *R. glutinis* CCT-2186, acquired from the Tropical Culture Collection André Tosello (Campinas, SP, Brazil). Cultivation was carried out in a 6 L stirred tank bioreactor (Minifors II) equipped with two Rushton impellers submerged in the bulk liquid, placed 14 cm apart, with a working volume of 4 L in three stages: pre-inoculum, inoculum, starter culture, and cultivation.

For the pre-inoculum, *R. glutinis* was grown on a solid YPD-A (Yeast extract, bacteriological peptone, dextrose, and agar) medium. A loop of the microorganism was inoculated onto a YPD-A plate and incubated at 30 °C for 48 h. The starter culture was prepared

in an orbital shaker (Tecnal, model TE-421, Piracicaba, SP, Brazil) for 48 h at 170 rpm and 30 °C, using an initial optical density (OD) of 0.1 at UA_{600nm} of fungi taken from the inoculum. This process was performed by transferring 50 mL of starter culture medium to 500 mL Erlenmeyer-type flasks. Then, 400 mL of the starter culture was transferred to the bioreactor, completing a volume of 4 L with culture media.

The starter culture medium (inoculation) consisted of (g/L): dextrose (10), bacteriological peptone (5), yeast extract (3), and malt extract (3). The culture media in the bioreactor was composed of (g/L): dextrose (10), KH_2PO_4 (0.52), $MgSO_4$.$7H_2O$ (0.52), NH_4NO_3 (4), malt extract (7.93), and tween 80 (1.71 % v/v), with the pH adjusted to 5.0. The bioreactor vessel containing the cultivation medium was sterilized at 121 °C for 30 min.

The influence of agitation (300 and 500 rpm) and aeration (1.0 and 1.5 vvm) on bioreactor cultivation was studied. Bioreactor cultivation was continuously monitored using pH, dissolved oxygen, and temperature probes. Samples were taken every 24 hto evaluate carotenoid production and glucose concentration over 144 h.

Afterward, the fermented medium was centrifuged at 10,600 x*g* for 5 min at 4 °C. The clarified broth was used to determine the final glucose concentration, while the pellets containing carotenoid-rich biomass were washed three times with a monobasic phosphate buffer (0.2 M) solution at pH 7, and then frozen for 24 h. Subsequently, they were dried at 35 °C for 24 h in a circulating oven. The plates were scraped, and the cells were macerated for 2 min with a mortar and pestle, then frozen for 12 h.

The carotenoids were extracted as follows: 0.25 g of dried cells and 0.50 g of glass beads were resuspended in 10 mL of acetone, and extraction cycles (30 s of vortexing followed by 5 min of centrifugation at 10,600 x*g*, at 4°C) were performed. Then the acetone was rotaevaporated and the carotenoids were solubilized in 2 mL of ethanol. Carotenoid quantification was carried out based on the absorbance spectrum using a UV-vis spectrophotometer (model Genesis 10S, China) at a wavelength of 480 nm corresponding to torularhodin and expressed as Units of Absorbance (UA).

2.2. Application of the carotenoid-rich extract colorants for gelatin

The carotenoids extract obtained in the section "*Extraction and analysis of carotenoids*" was suspended in water (6 mg/mL) and mixed 1:1 with water (control) or colorless and flavorless gelatin Dr. Oetker purchased at the local market (48 mg/mL). The gelatin was dissolved in water and kept at 40 °C for 20 min to form a homogenous solution before incorporation of the carotenoid's suspension. The control sample (carotenoids in water) was kept a 25 °C for 15 min. The samples (carotenoids in gelatin) were maintained at 40 °C for 5 min and 50, 60, 80, and 100 °C for 5 and 15 min. The absorbance spectra of the samples were evaluated in the EnSpire multimode plate reader from Perkin Elmer after the thermal treatments, and after 1, 4, 10, and 24 days at 4 °C. Before each reading, the samples were maintained for 30 min at 25 °C for stabilization of the temperatures. The results are the average of a triplicate after blank (water or gelatin) removal.

3 RESULTS & DISCUSSION

3.1. Production of carotenoids

Improving agitation and aeration conditions during cultivation yields significant benefits for biocompounds synthesis by microorganisms, primarily by enhancing the mass transfer of substrate, product, and oxygen, thereby promoting specific metabolic pathways.[10] In this context, this study aimed to assess carotenoid production by *R. glutinis* in stirred tank bioreactor with varying levels of agitation and aeration, aiming to establish optimal ratios to enhance microorganism productivity. Analysis of cultivation kinetics for torularhodin production revealed distinct responses among cultures. It was observed that the maximum torularhodin production occurred after 72 h of cultivation under conditions of 300 rpm and 1vvm (**Figure 1**). Conversely, conditions with 500 rpm did not induce sufficient stress to increase carotenoid production. Additionally, in all conditions, a decline in production was noted after reaching the maximum point, possibly attributed to cell death.

The impact of aeration on yeast carotenogenesis was previously demonstrated by Davoli *et al.,* [11] who observed that *R. glutinis* yeast produced higher carotenoid concentrations with increased aeration rates, showing a 45% increase in carotenoid content. Malisorn and Suntornsuk[13] also emphasized the positive influence of aeration on carotenoid production by *R. glutinis* (DM28) in a 3 L stirred tank bioreactor, achieving a maximum of β-carotene (0.21 mg/L after 24 h of fermentation) by increasing dissolved oxygen in the medium from 60 % to 80 %.

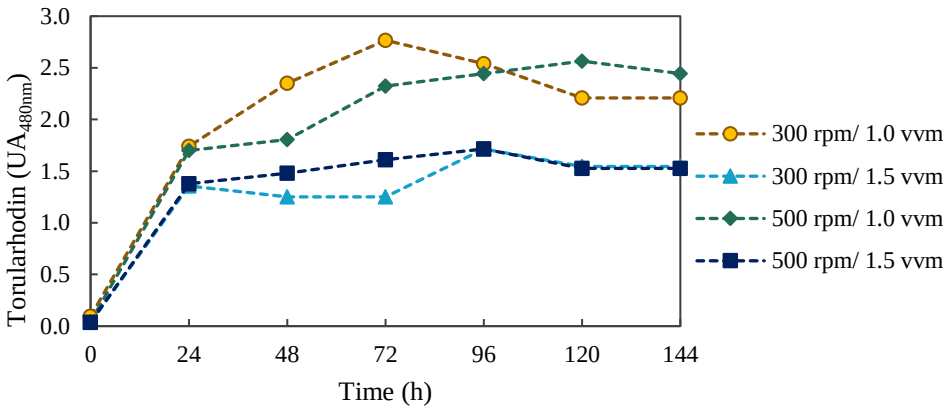

Figure 1 Cultivation of *Rhodotorula glutinis* CCT-2186 over 144 h in stirred tank bioreactor at different agitations and aerations. The dotted lines highlight the trend of the curve.

3.2. Application of the carotenoid-rich extract

To assess the viability of applying the carotenoid extract as food colorants, we examined their incorporation into edible gelatins. Given the typical gelatin preparation process, the retention of carotenoid absorbance in gelatin after exposure to high temperatures (ranging from 50 to 100 °C) for 5 and 15 min was investigated, as well as after refrigerated preservation at 4 °C for up to 24 days.

Despite the generally limited solubility of carotenoids in water, the concentration of 6 mg/mL of the extract produced a visually uniform blend exhibiting a vibrant orange hue. Regarding the thermal treatment of carotenoids in gelatin, it was observed that such treatments did not suppress or alter the absorbance profile of the biomolecules compared to the control in water at 25°C. Conversely, exposure to higher temperatures, such as 15 min at 60 °C and 5 to 15 min at 80 °C and 100°C, even marginally enhanced their absorbance. This enhancement is likely attributed to the increased solubility of the carotenoid suspension in water and gelatin at elevated temperatures, consequently intensifying their absorbance. Moreover, the absorbance of carotenoids remained largely unchanged for 10 days, with peaks registering absorbance levels between 1.7 and 1.9 units at day 0, compared to 1.7 and 2.0 units at day 10. However, a slight decrease was observed after 24 days, with peaks registering absorbance levels between 1.3 and 1.6 units, marking approximately a 20 % decrease from day 0. Furthermore, after 10 days at 4 °C, the gelatin exhibited a consistent and intense orange color across all treatments. Considering the typical recommendation of refrigerating gelatin for 7 to 10 days, this outcome underscores the feasibility of utilizing carotenoid extracts as a food coloring agent for edible gelatins.

4 CONCLUSION

The agitation results at 300 rpm and aeration at 1 vvm demonstrated a significant impact on carotenoid production, particularly torularhodin. These stress parameters proved crucial for optimizing the synthesis of these desired compounds. Furthermore, application tests showed the potential of the carotenoid extract as a food colorant for edible gelatins.

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