CULTURE MEDIA SCREENING FOR CAROTENOID PRODUCTION BY *RHODOTORULA RUBRA* ICCF 220 PRIOR TO BIOREACTOR SCALE-UP

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Optimizing culture media is essential for improving the biosynthesis of carotenoid pigments produced by Rhodotorula rubra ICCF 220. In this study, the yeast was grown in two media with different carbon–nitrogen balances to observe how nutrient composition influences growth and pigment formation. Cultures were monitored under controlled conditions using optical density, viable cell counts, pH, and soluble solids content. The novelty of this study lies in demonstrating that a glucose–nitrate medium (MS3) with a specific carbon–nitrogen balance markedly enhances both biomass growth and carotenoid biosynthesis in Rhodotorula rubra ICCF 220, providing a clear basis for selecting optimal culture conditions prior to bioreactor scale-up.

Keywords: *Rhodotorula rubra* ICCF 220; carotenoid pigments; culture media optimization; microbial growth; bioprocessing.

1. Introduction

Carotenoid pigments produced by microorganisms have received increasing attention over the last decade because of their antioxidant, coloring, and health-promoting potential. Compounds such as β-carotene, torulene, and torularhodin are now frequently used in food, cosmetics, and pharmaceutical formulations, where they contribute both to product color and to oxidative stability. Beyond their industrial applications, carotenoids also play a biological role, helping cells to neutralize reactive oxygen species and resist oxidative stress [1–3]. Among

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microbial producers, yeasts from the genus *Rhodotorula* have become particularly attractive due to their non-pathogenic character, simple nutritional requirements, and ability to accumulate considerable amounts of intracellular pigments even under moderate stress [4]. *Rhodotorula rubra* stands out for its metabolic versatility, being capable of using a wide range of carbon and nitrogen sources. This adaptability makes it a suitable candidate for pigment biosynthesis and other biotechnological applications [5]. The composition of the culture medium represents one of the most critical factors influencing carotenoid production. Both the type and the ratio of carbon and nitrogen sources determine the metabolic balance between cell proliferation and pigment accumulation. Generally, media with higher carbon-to-nitrogen (C/N) ratios favor carotenoid formation by inducing a mild nitrogen limitation, while lower ratios tend to enhance biomass growth [6,7]. Thus, adjusting the C/N ratio remains an efficient and low-cost way to improve yield prior to process scale-up. Environmental factors—such as temperature, pH, aeration, or light—also affect pigment formation, but the nature of the carbon and nitrogen substrates remains the easiest parameter to control in laboratory studies. Preliminary or pre-bioreactor experiments carried out in shake flasks are therefore essential for understanding growth kinetics and pigment synthesis trends, helping to identify the most promising formulations for later bioreactor trials [8-10].
In this study, we investigated how different combinations of carbon and nitrogen sources affect the growth and pigment production of *Rhodotorula rubra* ICCF 220. Four media with distinct C/N ratios were tested, with the goal of determining which formulation best promotes biomass accumulation and carotenoid synthesis. The findings of this work provide a practical foundation for optimizing culture conditions before transferring the process to controlled bioreactor environments.

2. Experimental

2.1 Microorganism and maintenance

The yeast strain *Rhodotorula rubra* ICCF 220 was selected for this study. The strain was maintained on Sabouraud agar slants and periodically subcultured, approximately every four weeks, to preserve its stability and viability. Prior to each experimental run, a small amount of fresh biomass was transferred onto new agar plates and incubated for 48 hours at 28 ± 1 °C. Microscopic observations showed oval to nearly spherical cells, measuring between 3 and 6 μm, with smooth surfaces and the characteristic morphology of yeast. The colonies presented orange to deep red coloration, reflecting the intracellular accumulation of carotenoid pigments typical of *Rhodotorula* species.

2.2 Culture media composition

To assess the effect of different nutrient sources on yeast development and pigment formation, four liquid media were initially formulated, varying in the nature and concentration of their carbon and nitrogen components. These variations generated distinct C/N ratios and nutrient complexities. All media also included mineral salts and small amounts of growth-promoting factors to sustain both biomass accumulation and carotenoid biosynthesis.

For comparative analysis, two representative formulations were chosen for detailed study: MS3, based on glucose and nitrate, and M1, containing glycerol and ammonium sulfate. The two media were intentionally selected because they represent opposite metabolic conditions — one built around a readily fermentable sugar and an oxidized nitrogen source, the other relying on a slower carbon substrate and a reduced nitrogen form.

The MS3 medium (glucose/nitrate) consisted of 4% (w/v) glucose as the main carbon source, 0.15% yeast extract supplying trace vitamins and amino acids, and 0.5% ammonium nitrate as the primary nitrogen source. The inorganic balance was completed with 0.1% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, and 0.04% NaCl. This synthetic formulation provided glucose in an easily assimilable form and nitrate as a more oxidized nitrogen source, conditions that typically promote balanced cellular metabolism and enhanced pigment accumulation.

The M1 medium (glycerol/sulfate) contained 6.7% glycerol serving as the sole carbon source, 0.05% peptone as a trace organic supplement, and 2% ammonium sulfate as the main nitrogen donor. The mineral component included 0.15% $MgSO_4 \cdot 7H_2O$, 0.2% Na_2HPO_4, and 0.4% KH_2PO_4. Compared with MS3, this formulation provided a slower-metabolized carbon source and a more reduced nitrogen form, ensuring sufficient sulfur availability for amino acid biosynthesis but leading to a generally milder growth profile.

All media were adjusted to an initial pH of 6.0 ± 0.1 prior to sterilization (121 °C for 15 min). The C/N ratio for each formulation was calculated from the theoretical carbon and nitrogen content of the substrates. These compositions were designed to enable a direct comparison between two contrasting metabolic routes — fermentative versus oxidative — in *Rhodotorula rubra* ICCF 220.

2.3 Cultivation conditions

Batch cultivations were performed in 500 mL Erlenmeyer flasks, each containing 100 mL of sterile culture medium. The inoculum represented 5% (v/v) of a 24-hour preculture grown in Sabouraud broth under standard conditions. After inoculation, the flasks were incubated at 30 °C on a rotary shaker operating at 150 rpm to ensure proper aeration and homogeneous mixing.

The total cultivation time was 144 hours, during which samples were taken every 24 hours to monitor both growth dynamics and changes in key biochemical

parameters. This time frame was sufficient to capture the full evolution of the culture — from the initial adaptation phase to exponential development and finally to the stationary stage, where pigment accumulation typically becomes more pronounced.

2.4 Analytical determinations

Cell growth was monitored spectrophotometrically by measuring the optical density at 600 nm (OD_{600}) using a Jenway UV–VIS spectrophotometer.

The pH of the culture broth was measured with a digital pH meter (WTW Inolab 7110, Germany), while soluble solids (SU %) were determined refractometrically using a Digital Refractometer DR201-95 (A. Krüss Optronic). Together, these measurements provided complementary information about nutrient consumption and metabolic activity throughout cultivation.

Microscopic cell counts were carried out with a Thoma counting chamber, and biomass concentration was expressed as cells per milliliter. At each sampling point, the cells were collected by centrifugation at 5000 rpm for 10 minutes, washed twice with distilled water, and stored at −20 °C prior to carotenoid extraction.

2.5 Statistical analysis

All measurements were carried out in triplicate, and the results are presented as mean values accompanied by the corresponding standard deviations. The experimental data were processed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to evaluate significant differences between samples. A significance threshold of $p < 0.05$ was applied throughout the analysis to ensure statistical reliability. All calculations and graphical representations were performed using *Microsoft Excel 365* and *OriginPro 8.5* software.

3. Results and discussions

All experimental data were statistically processed using one-way ANOVA followed by Tukey's post hoc test, with $p < 0.05$ considered as the threshold for significance. The temporal evolution of optical density (OD_{600}), cell number, soluble solids (SU%), and pH was monitored over a 144-hour cultivation period for *Rhodotorula rubra* ICCF 220 grown in two representative media: MS3 (glucose/nitrate) and M1 (glycerol/sulfate).

The comparative analysis revealed clear and statistically significant differences between the two media in terms of growth kinetics, nutrient utilization, and overall metabolic behavior. The following sections present a detailed discussion of these trends, highlighting the physiological responses of the yeast to each combination of carbon and nitrogen sources.

3.1. Growth kinetics and optical density (OD_{600}) profile

The evolution of optical density during cultivation displayed a typical sigmoidal pattern in both media (Figure 1). After a short adaptation phase of about 24 h, the cells entered a rapid exponential growth stage that lasted up to roughly 72 h. In the glucose/nitrate medium (MS3), OD_{600} values increased steadily and reached their peak between 120 h and 144 h. In contrast, cultures grown in M1 (glycerol/sulfate) exhibited a slower rate of increase and tended to reach the stationary phase earlier.

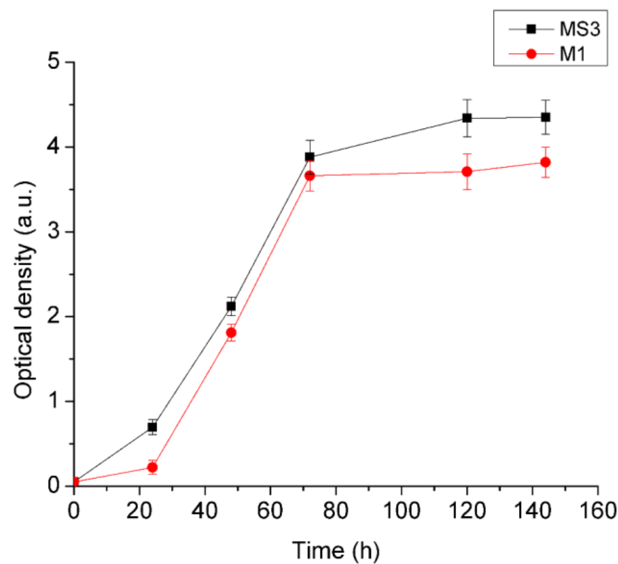

Fig 1. Evolution of optical density (OD_{600}) during the cultivation of *Rhodotorula rubra* ICCF 220 in MS3 and M1 at 30 °C and 150 rpm.

At the end of the experiment, the maximum OD_{600} measured in MS3 was around 14 % higher than in M1, a difference confirmed as statistically significant by Tukey's test ($p < 0.05$). This result indicates that glucose was metabolized more efficiently than glycerol, providing faster energy release and supporting higher rates of cell division. Glycerol, on the other hand, is typically assimilated through oxidative routes that require additional enzymatic steps, which may explain the slower biomass accumulation.

The superior growth observed in the nitrate-containing medium also points to a more balanced nitrogen metabolism. Nitrate, being an oxidized nitrogen form, tends to support a smoother metabolic flow and may indirectly promote the biosynthetic pathways responsible for carotenoid formation.

3.2. Cell number variation and correlation with optical density

The evolution of cell numbers closely mirrored the optical density profile (Figure 2). Cultures cultivated in the glucose/nitrate medium (MS3) showed a steady and more pronounced increase in cell density, reaching about 2×10^7 cells/mL after 144 hours of incubation. In contrast, those grown in the glycerol/sulfate medium (M1) displayed slower multiplication rates, stabilizing earlier at around $1.6–1.8 \times 10^7$ cells/mL.

A slight drop in viable cell count was noted in M1 at 120 hours, most likely indicating the onset of the stationary phase and the beginning of partial cell lysis due to nutrient depletion. Such variations are commonly observed in batch cultures and fall within normal biological fluctuation ranges.

According to the statistical analysis, significant differences between the two media appeared from 48 hours onward ($p < 0.05$). These findings reinforce the idea that nitrate ions were metabolized more efficiently than ammonium sulfate, allowing continuous biomass growth throughout the cultivation period. The strong correlation between optical density and cell number ($R^2 > 0.95$) confirms that OD_{600} can be used as a dependable and quick indicator of yeast growth in such experimental systems.

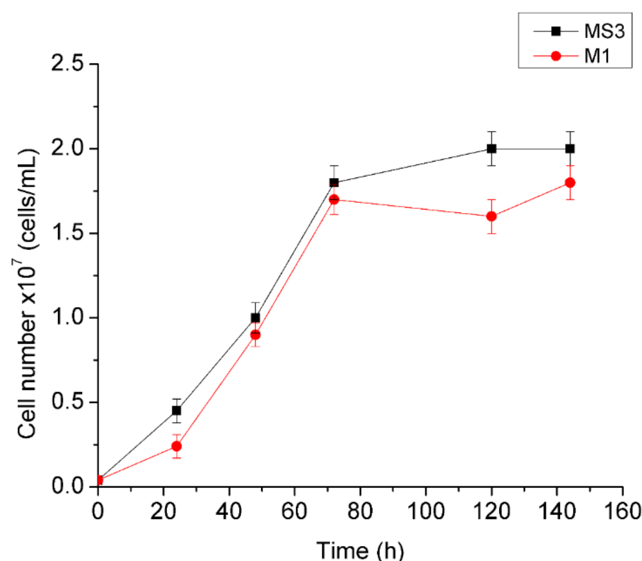

Fig. 2. Variation of cell number (cells/mL) during the cultivation of *Rhodotorula rubra* ICCF 220 in MS3 and M1 media.

3.3. Soluble solids (SS%) variation as an indicator of nutrient consumption

The evolution of soluble solids (SS%) reflected the dynamics of nutrient utilization during cultivation. As illustrated in Figure 3, refractometric values decreased steadily in both media over the 144-hour experimental period. In the glucose/nitrate medium (MS3), the initial SU% of about 5.0 gradually declined to roughly 4.0 by the end of cultivation. In contrast, the reduction observed in M1 (glycerol/sulfate) was slower, with values dropping from 9.2 to 7.8 %.

This continuous decrease indicates the metabolic conversion of soluble carbohydrates and other organic compounds into cellular material and secondary metabolites. The sharper decline recorded in MS3 aligns well with the higher OD_{600} and cell density values, suggesting a more efficient substrate assimilation. Statistical analysis confirmed that differences between the two media became significant after 72 hours ($p < 0.05$), according to Tukey's test.

The earlier depletion of carbon sources in MS3 may have induced a mild nutrient limitation, a condition known to trigger secondary metabolism in *Rhodotorula* species. Such metabolic shifts often coincide with increased carotenoid biosynthesis, particularly during the stationary growth phase.

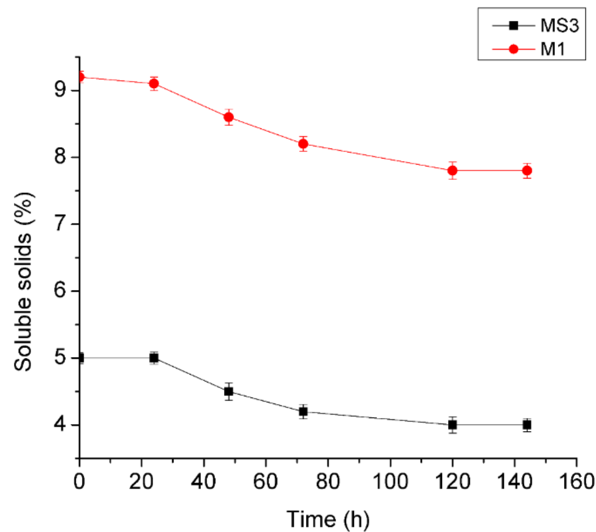

Fig. 3. Changes in soluble solids content (SS%) during the cultivation of *Rhodotorula rubra* ICCF 220 in MS3 and M1 media.

3.4. pH evolution during cultivation and its link to metabolic activity

The variation of pH during cultivation reflected the metabolic activity of the yeast in both tested media (Figure 4). Gradual acidification was observed, mainly

due to the accumulation of organic acids formed during carbohydrate metabolism. The decrease was more pronounced in the glucose/nitrate medium (MS3), where pH dropped from an initial value of 6.0 to nearly 5.0 after 144 hours of incubation. In contrast, cultures grown in the glycerol/sulfate medium (M1) maintained a relatively stable pH around 6.0 throughout most of the experimental period.
The stronger acidification observed in MS3 corresponds well with its higher growth rate and more intense sugar utilization. Such pH reduction is typically associated with active primary metabolism and often signals a shift toward secondary metabolic pathways. For the M1 medium, pH values showed minimal variation across replicates, resulting in negligible standard deviation; therefore, error bars are not visible in the corresponding plot. These conditions are known to favor the biosynthesis of carotenoid pigments during the stationary phase, when cell growth slows and metabolic energy is redirected toward secondary metabolite production. According to Tukey's post hoc test ($p < 0.05$), the pH values recorded in MS3 were significantly lower than those in M1 after 96 hours, confirming a clear medium-dependent difference in metabolic intensity.

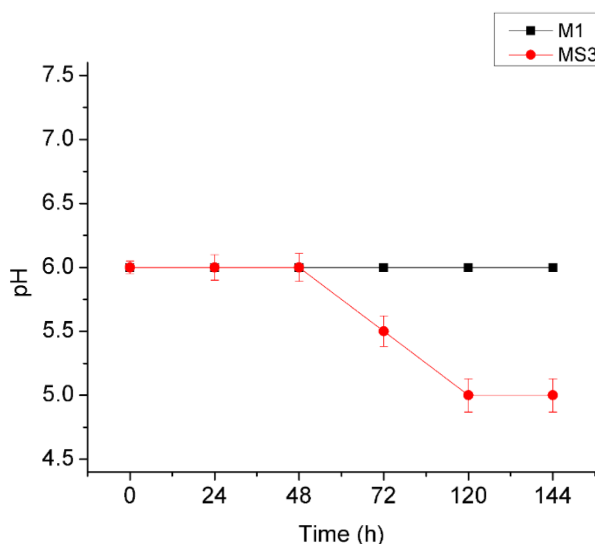

Fig. 4. Variation of pH during the cultivation of *Rhodotorula rubra* ICCF 220 in MS3 and M1 media at 30 °C and 150 rpm.

When all experimental parameters are considered together—growth kinetics, cell number, soluble solids, and pH variation—it becomes clear that the glucose/nitrate medium (MS3) provided the most favorable environment for *Rhodotorula rubra* ICCF 220. The rapid rise in OD_{600} and cell density, accompanied by faster nutrient depletion and a stronger pH decrease, reflects a dynamic and well-balanced metabolism in this medium.

In contrast, the glycerol/sulfate medium (M1) supported slower growth and a more moderate metabolic response, which can be attributed to the slower assimilation of glycerol through oxidative pathways and to the possible inhibitory influence of ammonium ions under high sulfate concentrations.
Overall, MS3 proved to be the most effective formulation for biomass formation and carotenoid accumulation. Its performance highlights the synergistic effect of glucose and nitrate in maintaining both energy production and biosynthetic activity. These findings establish a reliable basis for transferring the process to controlled bioreactor conditions, where further optimization of aeration, pH, and nutrient feeding could enhance pigment yields.

4. Conclusions

The experimental findings showed that *Rhodotorula rubra* ICCF 220 displayed clearly distinct metabolic responses depending on the composition of the culture medium. Among the tested variants, the glucose/nitrate medium (MS3) proved to be the most favorable for both cell proliferation and overall metabolic performance, resulting in the highest biomass accumulation and the most efficient substrate utilization. The differences between MS3 and M1 (glycerol/sulfate) were statistically significant according to Tukey's test ($p < 0.05$), confirming the beneficial influence of glucose and nitrate on yeast metabolism.

The continuous reduction in soluble solids and the stronger acidification recorded in MS3 reflected an active nutrient conversion, indicative of an intensified primary metabolism that later favored carotenoid biosynthesis during the stationary phase. By contrast, the glycerol/sulfate medium supported slower and less intense growth, most likely due to the more complex assimilation route of glycerol and the limited bioavailability of nitrogen in ammonium form.

Overall, these results emphasize the decisive role of the carbon and nitrogen sources, as well as their ratio, in optimizing culture media for *R. rubra*. The MS3 formulation emerges as a promising candidate for subsequent experiments in a bioreactor, where controlled aeration and agitation could further enhance carotenoid yield and process stability. Taken together, the findings provide a consistent experimental foundation for scaling up microbial pigment production under reproducible and well-defined bioprocessing conditions.

These findings also open concrete directions for future research, particularly the evaluation of MS3 under controlled bioreactor conditions, where aeration, agitation, C/N feeding strategies, and dissolved oxygen regulation can be further optimized to maximize carotenoid yield. The insights obtained from this study therefore provide a solid foundation for scaling up microbial pigment production using reproducible and well-defined bioprocessing parameters.

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