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Two-Stage Fermentation for Torularhodin Production from *Rhodotorula mucilaginosa* Using Molasses

Nang Minh Nguyen[1,2], Lan Anh Dang[1], Thao Vy Tran[1], Tuan Anh Pham[1,*]
[1]Hanoi University of Science and Technology, Ha Noi, Vietnam
[2]Military Academy of Logistics, Ha Noi, Vietnam
[*]Corresponding author email: anh.phamtuan2@hust.edu.vn

Abstract

Torularhodin is a carotenoid of considerable interest due to its potent antioxidant activity and potential applications in the food, pharmaceutical, and cosmetic industries. In the study, the influence of culture conditions, including pH, temperature, and medium composition (molasses concentration and urea supplementation) on biomass formation and torularhodin biosynthesis in Rhodotorula mucilaginosa RL 231-1.51 was systematically investigated. Optimal biomass production was achieved at pH of 6.5, 30 °C, and 10 °Bx molasses, whereas maximum torularhodin accumulation was observed at pH of 7.5, 25 °C, 22 °Bx molasses, and 18 g/L urea. Implementation of a two-stage fermentation strategy, designed to decouple biomass growth from carotenoid production, resulted in a maximum torularhodin content of 2628.1 µg/g dry cell weight, corresponding to a yield of 91.98 mg/L. These findings demonstrate that the two-stage fermentation approach is an effective strategy to enhance torularhodin production and holds promise for industrial-scale applications.

Keywords: Molasses, *Rhodotorula mucilaginosa*, torularhodin.

1. Introduction

Torularhodin (3',4'-didehydro-β,ψ-caroten-16'-oic acid; IUPAC name) is a carotenoid pigment with the molecular formula $C_{40}H_{52}O_2$ and a molecular weight of 564.8 g/mol. It is classified as a xanthophyll due to the presence of a terminal carboxyl group. Structurally, torularhodin contains thirteen conjugated double bonds, which confer strong antioxidant activity [1]. Previous studies have demonstrated that torularhodin exhibits higher peroxyl radical scavenging capacity than β-carotene, along with protective effects against alcoholic liver damage and prostate cancer, as well as notable antibacterial activity [2].

Torularhodin is biosynthesised by red yeasts, including species of *Rhodotorula* and *Sporobolomyces* [3], through a metabolic pathway originating from acetyl-CoA, which is further converted into carotenoids such as γ-carotene, β-carotene, torulene, and torularhodin [1]. The yield and distribution of these carotenoids are not constant but vary depending on medium composition and cultivation parameters. For example, *Sporobolomyces ruberrimus* H110 produced 31.54 mg/L torularhodin [1], while 41.1 mg/L was achieved by *Rhodotorula rubra* Persian Type Culture Collection (PTCC) 5255 in a bioreactor [3].

Cultivation conditions significantly affect torularhodin biosynthesis. Temperature not only influences total carotenoid production but also alters the relative distribution of β-carotene, torulene, and torularhodin. *Rhodotorula glutinis* produced the highest torularhodin yield at 15 °C, whereas temperatures between 25–30 °C were more favourable for biomass accumulation and β-carotene production [4]. Likewise, pH strongly modulates carotenoid composition: maximum carotenoid production in *R. mucilaginosa* and *R. glutinis* occurred at pH of 5, while neutral initial pH favoured torularhodin synthesis, and acidic conditions promoted β-carotene production [5, 6].

In addition to physical parameters, chemical supplements may enhance carotenoid synthesis and alter pigment profiles. For instance, supplementation with 1 g/L urea during fermentation of *R. glutinis* DSM 70398 increased the proportion of torularhodin in total carotenoids from 6.6% to 14.88% and β-carotene from 12.18% to 39.45%, while decreasing torulene from 78.24% to 43.79% [6].

Molasses, a by-product of the sugarcane industry, contains high concentrations of sucrose, glucose, fructose, and essential minerals that support microbial growth.

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Owing to its abundance and low cost, molasses is widely used as a substrate for microbial cultivation [7]. In this study, a UV-mutagenized strain of *R. mucilaginosa* was employed to biosynthesise torularhodin using molasses as a carbon source. The effects of molasses concentration, urea supplementation, pH, and temperature on torularhodin production were systematically evaluated. Furthermore, a two-stage fermentation strategy was developed to enhance torularhodin yield.

2. Materials and Methods

2.1. Materials and Culture Conditions

The yeast strain used in this study was *Rhodotorula mucilaginosa* RL 231-1.51, provided by the Fermentation Laboratory – Centre for Research and Development of Biotechnology, Hanoi University of Science and Technology. This strain was UV-mutagenized from the wild-type strain *Rhodotorula mucilaginosa* RL. The wild-type strain RL was isolated from sweet potato leaves, exhibiting a torularhodin accumulation capacity of 109.54 µg/g analyzed using High-Pressure Liquid Chromatography (HPLC). A mutant strain was generated by exposing a suspension of the wild-type strain (10^4 cells/mL) to UV irradiation at 254 nm for 1.5 min, with a lamp-to-sample distance of 10 cm. After screening, a mutant strain designated RL 231-1.51 was obtained, which demonstrated a torularhodin accumulation capacity of 1008.7 µg/g (HPLC).

The molasses, with a soluble solids content of 85 °Bx, supplied by Lasuco, a sugarcane production company in Vietnam, underwent a double dilution process and was adjusted to pH of 2 using H_2SO_4 before filtration. It was then heated at 110 °C for 30 min. Subsequently, the pH was adjusted to 6 using NaOH, followed by centrifugation. Before fermentation, the molasses was diluted to the desired concentration, and the concentration was monitored using a refractometer.

The microorganism was activated in an agar plate composed of 20 g/L glucose, 10 g/L yeast extract, 10 g/L peptone, and 20 g/L agar at 25 °C, for 3–5 days.

The pre-cultures were grown in 250 mL baffled flasks containing 50 mL pre-culture medium (20 g/L glucose, 10 g/L yeast extract, and 10 g/L peptone), incubated at 25 °C, 125 rpm for 24 hours.

All experiments were performed in three replicates and in the Bio-Shaker RB-3000 LF at 25 °C, 125 rpm. The experiments were carried out in 250 mL baffled flasks containing 50 mL of LS10 medium (Glucose 150 g/L; $MgSO_4 \cdot 7H_2O$ 1.5 g/L; KH_2PO_4 0.4 g/L; $CaCl_2$ 0.167 g/L; Urea 0.47 g/L; yeast extract 8.05 g/L; $ZnSO_4$ 1.91×10^{-4} mM; $MnCl_2$ 1.22×10^{-4} mM; $CuSO_4$ 10^{-4} mM). The inoculum was adjusted to achieve an initial OD_{660nm} of 2.

The effect of molasses concentration was examined by replacing the glucose with molasses at different concentrations ranging from 6 °Bx to 26 °Bx.

For the pH control experiments, the pH of culture media was adjusted to the desired value (varying from 4 to 8.5) by supplementing with 3 M H_3PO_4 and 3 M NaOH. The adjustment was performed every 24 h.

A two-stage fermentation process was performed in a 2-L Sartorius Biostat B plus bioreactor containing 1 L of LS10 medium, replacing glucose with molasses to achieve 10 °Bx in the bioreactor. The bioreactor was sterilized at 110 °C for 30 min before use. In stage I, fermentation was conducted at 30 °C with agitation at 1000 rpm, aeration at 2 vvm, and pH maintained at 6.5 by automatic addition of 3 M NaOH or 3 M HCl. After 30 h of fermentation, 688 mL of molasses (52 °Bx) and 61.2 mL of urea (500 g/L) were supplemented to achieve final concentrations of 22 °Bx and 18 g/L, respectively. In stage II, when the yeast culture entered the stationary phase (60 h), the pH was increased to 7.5 and the temperature was reduced to 25 °C to promote torularhodin accumulation. Samples were collected daily to quantify biomass, carotenoids, and torularhodin production.

2.2. Methods

Dry biomass determination

Cells were harvested by centrifugation at 8000 × *g*, 10 min, then washed twice with distilled water and dried at 50 ºC to constant weight.

Torularhodin and carotenoids measurements

Spectrophotometric measurement: carotenoids were extracted from the dried yeast biomass according to Michelon *et al.* [8]. Briefly, 0.1 *g* of dry biomass was added to 1 mL of 4 M HCl and maintained at 65 °C for 1 hour, then centrifuged at 8000 × *g* for 10 min. The supernatant was discarded, followed by adding 1.5 mL of acetone for carotenoids extraction, followed by 800 µL of petroleum ether and vortexing for another 5 min. The mixture was centrifuged at 8000 × *g* for 10 min. The supernatant was measured at wavelengths of 470 nm and 500 nm. Subsequently, 1 mL of the supernatant was mixed with 0.05 *g* of $Ca(OH)_2$, and the mixture was centrifuged at 8000 × *g* for 10 min. The supernatant was measured at wavelengths of 500 nm. The total carotenoid content was calculated using the following equation:

$$\text{Carotenoids} = (A\times V\times 10^4)/(\varepsilon\times m)\ (\mu g/g) \quad (1)$$

where A is absorbance at 470 nm; V is total volume (mL); m is weight of the sample (g); ε is extinction coefficient for carotenoids in acetone 2140 [4].

The concentration of torularhodin was determined by measuring the carotenoid content at a wavelength of 500 nm before complexation with $Ca(OH)_2$, followed by measurement at the same wavelength post-complexation. The difference in absorbance values was then used to calculate torularhodin content, employing an extinction coefficient of 2040 [4].

For HPLC measurement: The carotenoids, including β-carotene, γ-carotene, torularhodin, and torulene were analyzed using HPLC, Agilent 1200 system, equipped with a C18 column (250 × 4.6 mm, 5 µm, Phenomenex, USA), and a Diode Array Detector (DAD) at 450 nm. The mobile phase was acetone as the solvent A and water as the solvent B. The solvents were used as 70% solvent A at 0–15 min, 100% solvent A at 15–25 min, 70% solvent A at 25–30 min with the flow rate of 1 mL/min [9].

Reducing sugar analysis

Reducing sugars were determined following the Miller method [10].

Specific growth rate calculation

The specific growth rate in log phase was determined by equation:

$$\mu = \frac{\ln X - lnX_0}{t - t_0} \quad (2)$$

where μ is specific growth rate (1/h); $X\ is$ biomass at time t (g/L); X_0 is biomass at time t_0 (g/L); $t,\ t_0$ are time (h).

Statistical analysis

The mean values and standard deviations (SD) were calculated from three replicates and expressed in average and SD. The significant difference of mean values was assessed using the analysis of variance (ANOVA) at a significance level of 95% ($p \leq 0.05$).

3. Results and Discussion

3.1. Influence of Molasses Concentration on the Growth and Torularhodin Accumulation

The strain *Rhodotorula mucilaginosa* RL 231-1.51 was cultivated in molasses-based medium with varying concentrations of 6 °Bx, 10 °Bx, 14 °Bx, 18 °Bx, 22 °Bx, and 26 °Bx. The strain exhibited optimal growth performance within the range of 6 °Bx to 22 °Bx (Fig. 1a). In contrast, no detectable growth was observed at 26 °Bx, suggesting inhibition, potentially due to elevated osmotic pressure or inhibitory effects associated with high sugar concentrations.

At molasses concentrations of 6 °Bx and 10 °Bx, the average specific growth rates were comparable, measuring 0.085 h^{-1} and 0.087 h^{-1}, respectively. Further increases in molasses concentration resulted in a progressive reduction in growth rate, declining to 0.081 h^{-1} at 14 °Bx and 0.071 h^{-1} at 22 °Bx. The highest cell density, with an OD_{660nm} of 156, was obtained at 10 °Bx.

Carotenoid and torularhodin accumulation were presented in Fig. 1b and Fig. 1c. At both 96 h and 120 h of cultivation, the highest accumulation levels were observed at a molasses concentration of 22 °Bx, exceeding those obtained at other tested concentrations. Maximum carotenoid and torularhodin contents reached 560.6 µg/g and 338 µg/g (dry cell weight), respectively. For comparison, *Rhodotorula glutinis* cultivated on beet molasses has been reported to produce torularhodin at 268.6 µg/g [7], whereas *R. mucilaginosa* F-1 grown in molasses yielded only 23.37 µg/g [5].

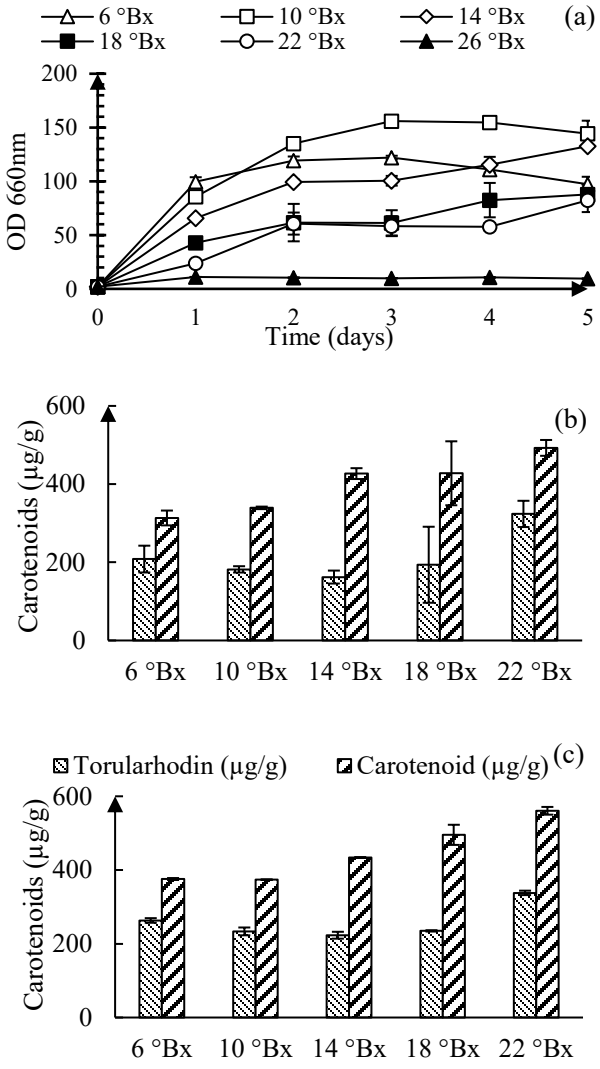

Fig. 1. Effect of molasses concentration on *R. mucilaginosa* RL 231-1.51 growth (a), carotenoid accumulation after 96 h (b), and after 120 h (c)

The OD_{660} and specific growth rate data indicated that a molasses concentration of 10 °Bx supported the highest growth rate and biomass accumulation of *R. mucilaginosa* RL 231-1.51 among the tested conditions. In contrast, torularhodin production was maximized at 22 °Bx. Zoz *et al*. [11] reported that torularhodin biosynthesis occurs predominantly after the exponential growth phase and during the stationary phase. At 22 °Bx, the culture entered the stationary phase as early as day 2, which may account for the elevated torularhodin yield observed under this condition. Future investigations will focus on defining optimal cultivation parameters to maximize yeast biomass at 10 °Bx, followed by targeted optimization of torularhodin production at 22 °Bx to achieve the highest possible yield.

3.2. Influence of pH and Temperature on the Growth of Rhodotorula mucilaginosa RL 231-1.51 at 10 °Bx Molasses Concentration

To examine the impact of pH and temperature on yeast growth, *Rhodotorula mucilaginosa* RL 231-1.51 was cultivated in molasses medium at a concentration of 10 °Bx under various pH and temperature conditions.

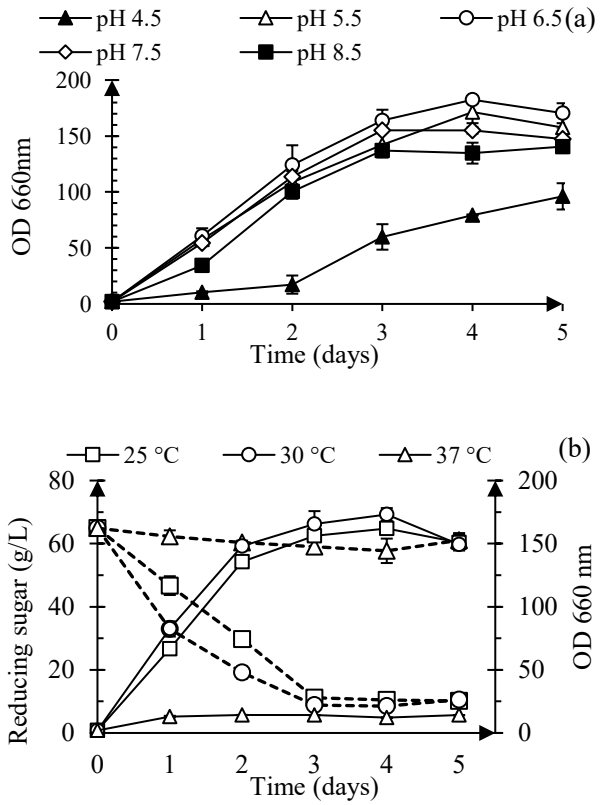

Fig. 2. Growth of *R. mucilaginosa* RL 231-1.51 on different pH (a), and temperature (b) continuous line: OD 660 nm, dot-line: reducing sugar

R. mucilaginosa RL 231-1.51 was cultivated in 250 mL Erlenmeyer flasks containing 50 mL culture. The pH was adjusted to 4.5, 5.5, 6.5, 7.5, or 8.5 every 24 h using 3 M H_3PO_4 (Fig. 2(a)). The strain exhibited growth across the entire pH range tested (4.5–8.5), with specific growth rates of 0.045, 0.083, 0.086, 0.084, and 0.082 h^{-1} at pH of 4.5, 5.5, 6.5, 7.5, and 8.5, respectively.

Under pH-controlled conditions at 5.5 and 6.5, the exponential growth phase was extended to 4 days (Fig. 2a), one day longer than in the uncontrolled-pH experiment (Fig. 1a). At pH of 7.5 and 8.5, the exponential phase lasted only 3 days, after which the strain entered the stationary phase. The maximum OD_{660nm} of 182.4 was achieved at pH of 6.5, which was 1.06, 1.17 and 1.35-fold higher than the maximum OD observed at pH of 5.5, 7.5 and 8.5, respectively. These results indicate that pH of 5.5–6.5 is more favorable for biomass production of this yeast strain. Growth at pH of 4.5 was the lowest and required a two-day adaptation period before the onset of the exponential phase. These results are consistent with those reported by Naoto Urano *et al.* [12], who observed optimal growth of *R. mucilaginosa* K12 in YPD medium at pH of 4–8, with reduced growth at pH of 3 and 9.

The effect of temperature on *R. mucilaginosa* RL 231-1.51 was examined at three temperature levels: 25, 30, and 37 °C. The experiments were performed in baffled flasks, and the pH was adjusted daily to 6.5.

Temperature optimization assays demonstrated that *R. mucilaginosa* RL 231-1.51 is heat-sensitive, with complete growth inhibition observed at 37 °C (Fig. 2b). The strain exhibited favorable growth at 25 °C and 30 °C, with specific growth rates of 0.088 h^{-1} and 0.090 h^{-1}, respectively, indicating minimal difference between these two temperatures. These findings align with those of Luciana Daniela Lario *et al*. [13], who reported maximum growth of *R. mucilaginosa* CBMAI 1528 at 30 °C, followed by 25 °C and 20 °C, with markedly reduced growth at 15 °C and complete inhibition at 10 °C and 35 °C.

3.3. Influence of Cultivation Conditions on Torularhodin Accumulation at 22 °Bx Molasses Concentration

Effect of urea concentration on yeast biomass and torularhodin accumulation

In the LS10 medium [14], nitrogen sources consisted of yeast extract and urea. The role of yeast extract in promoting yeast biomass production has been well documented [15]. Furthermore, yeast extract has been reported to enhance torularhodin biosynthesis when supplied at an appropriate ratio, although its mechanism of action remains unclear [16]. In LS10 medium, the concentration of urea is relatively low (0.047%). However, studies have shown that urea has a strong impact on

torularhodin biosynthesis [4]. Therefore, in the present study, the effect of urea concentration on torularhodin biosynthesis was investigated.

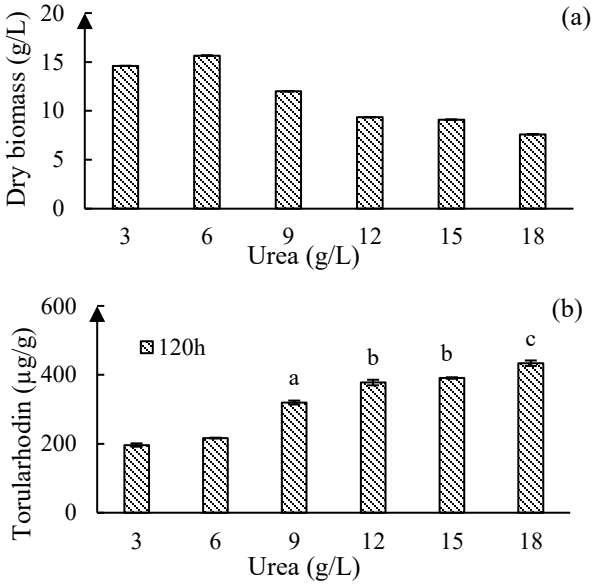

Fig. 3. Dry biomass (g/l) (a), and torularhodin (b) of *R. mucilaginosa* RL 231-1.51 on urea medium (Different letters indicated statistically significant differences ($p \leq 0.05$))

The nitrogen content in molasses is relatively low; thus, urea is frequently utilised as a cost-effective nitrogen source in industrial applications. The effect of urea concentration on the growth of *R. mucilaginosa* RL 231-1.51 was illustrated in Fig. 3a.

As the urea concentration increased from 3 to 6 g/L, yeast biomass exhibited a slight increase. However, biomass content gradually declined when the urea concentration exceeded 6 g/L (Fig. 3a).

In contrast to its effect on biomass formation, urea had a positive influence on torularhodin biosynthesis. Increasing urea concentrations enhanced torularhodin accumulation, reaching a maximum of 437.8 µg/g at 18 g/L. Consistent observations were reported by El-Banna *et al*. [4], who demonstrated that at a carbon-to-nitrogen ratio of 10:1, urea as the primary nitrogen source resulted in reduced biomass formation in *Rhodotorula glutinis* var. glutinis compared with organic nitrogen sources such as peptone, soy peptone, and casein.

Influence of pH and temperature on torularhodin accumulation

As noted earlier, temperatures of 25 °C and 30 °C were favorable for the growth of *R. mucilaginosa* RL 231-1.51 in molasses medium at 10 °Bx. To further assess the influence of temperature on pigment biosynthesis, experiments were conducted at a molasses concentration of 22 °Bx (Fig. 4a and Fig. 4c).

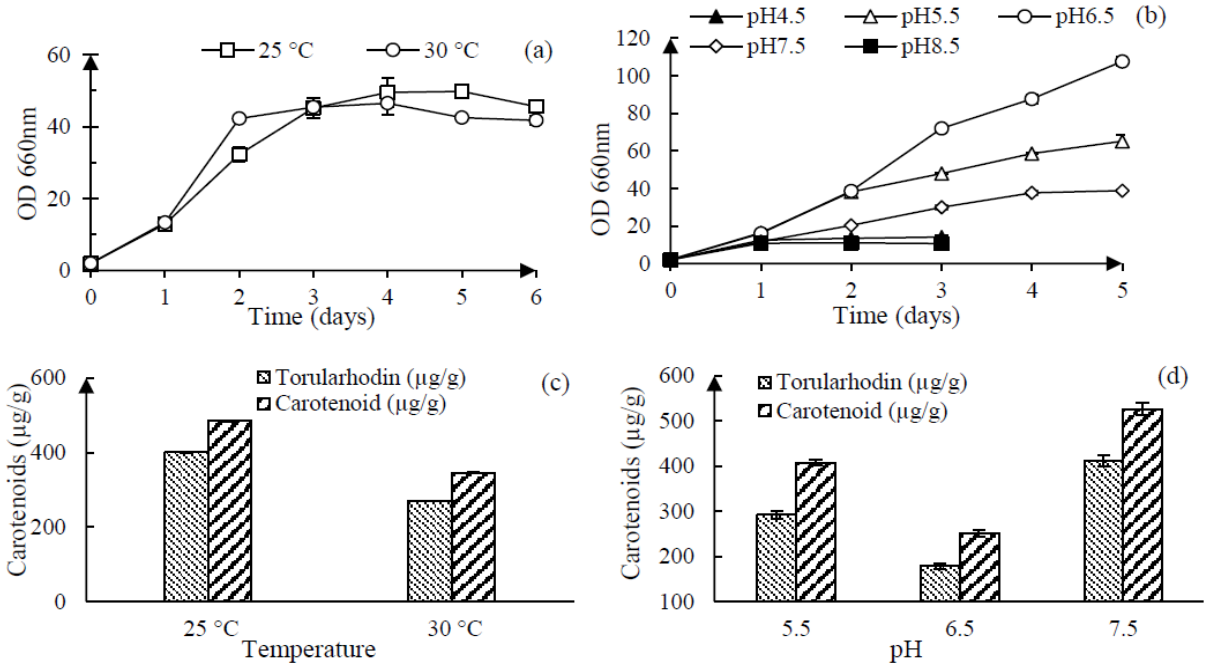

Fig. 4. Effect of temperature and pH on torularhodin accumulation by *R. mucilaginosa* RL 231-1.51 at 22 °Bx after 120 h of fermentation

At 25 °C, torularhodin accumulation reached 400.7 µg/g, representing a 1.37-fold increase compared to 30 °C (Fig. 4c). In contrast, no significant differences in biomass growth were observed between the two temperatures (Fig. 4a). These results agreed with the findings of El-Banna *et al.* [4], who demonstrated enhanced torularhodin synthesis in *R. glutinis* at lower cultivation temperatures. Similarly, Rui Guo *et al.* [17] reported that reduced temperatures promote the generation of reactive oxygen species (ROS) as part of the cellular response to abiotic stress, thereby stimulating carotenoid biosynthesis.

The effect of pH on *R. mucilaginosa* RL 231-1.51 was evaluated at 25 °C in 22 °Bx molasses medium, with pH maintained at 4.5, 5.5, 6.5, 7.5, and 8.5 through twice-daily adjustments (Fig. 4b, Fig. 4c). Growth was inhibited at pH of 4.5 and 8.5, contrasting with results obtained at 10 °Bx. The strain was able to grow at pH of 5.5, 6.5, and 7.5; however, the average growth rates at pH of 5.5 and 7.5 were 0.044 h^{-1} and 0.037 h^{-1}, respectively, both lower than the rate of 0.049 h^{-1} observed at pH of 6.5.

Torularhodin and total carotenoid accumulation were maximised at pH of 7.5, followed by pH of 5.5 and 6.5 (Fig. 4d), exhibiting an inverse relationship with biomass growth. As previously reported by Zoz *et al.*[11], torularhodin biosynthesis occurs primarily during the stationary phase. In this study, cultures at pH of 7.5 entered the stationary phase earlier than at pH of 5.5 and 6.5, which likely explains the enhanced torularhodin yield under these conditions. This observation was consistent with Cheng and Yang [5], who reported greater torularhodin accumulation at pH of 7 compared to pH of 5, despite total carotenoid levels peaking at pH of 5. Similarly, Kot *et al*. concluded that a slightly alkaline environment is favourable for torularhodin biosynthesis in yeast [1].

3.4. Two-Stage Fermentation

In the two-stage fermentation process, the first phase was conducted in batch mode with a 1 L working volume at 30 °C and pH of 6.5 to promote biomass accumulation. After 30 h, the reducing sugar content was nearly depleted, prompting supplementation with molasses and urea to prevent nutrient limitation. The final concentrations in the bioreactor were adjusted to 22 °Bx molasses and 18 g/L urea (Fig. 5) by adding 688 mL of 52 °Bx molasses and 61.2 mL of 500 g/L urea. pH and temperature were maintained under these conditions to support continued growth, biomass production, and adaptation to elevated soluble solid concentrations. By 60 h of fermentation, the specific growth rate declined markedly, indicating the onset of the stationary phase. At this point, cultivation parameters were shifted to pH of 7.5 and 25 °C to initiate the product accumulation stage. The culture entered the stationary phase at 72 h (Fig. 5a), and the maximum biomass concentration achieved was 35 g/L, corresponding to a 1.66-fold increase compared to batch fermentation.

Carotenoid accumulation analysis demonstrated that supplementation of molasses and urea did not markedly enhance carotenoid or torularhodin production between 31 h and 60 h (Fig. 5b). However, following the shift to 25 °C and pH of 7.5 after 60 h, pigment accumulation increased progressively, with carotenoid and torularhodin contents rising from 417.9 µg/g and 316.6 µg/g at 60 h to 578.1 µg/g and 463.1 µg/g at 108 h, respectively. Torularhodin accumulation in the bioreactor was 1.12-fold higher than in flask-scale fermentation.

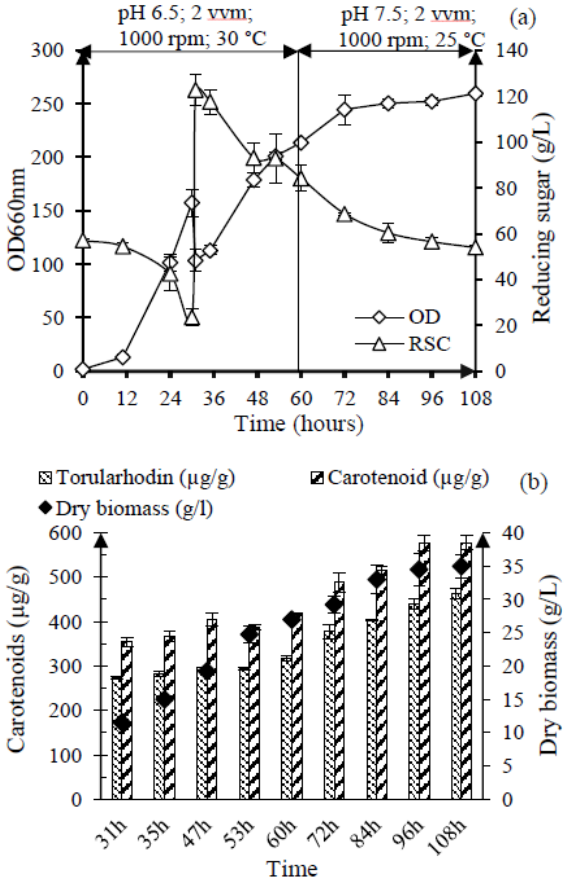

Fig. 5. Kinetics of *R. mucilaginosa* RL 231-1.51 on two-stage fermentation (a), and carotenoid production (b)

Carotenoid composition analysis during the fermentation process revealed that the predominant carotenoids produced by *R. mucilaginosa* RL 231-1.51 were torularhodin, torulene, γ-carotene, and β-carotene (Fig. 6). This profile was consistent with previous reports on carotenoid biosynthesis in red yeasts [9]. The proportion of torularhodin within the total carotenoid

fraction increased from 84% at 60 h to 89% at 108 h. HPLC quantification showed that torularhodin reached an accumulated concentration of 2628.1 µg/g. As a result, the two-stage fermentation process achieved an overall torularhodin yield of 91.98 mg/L (Table 1).

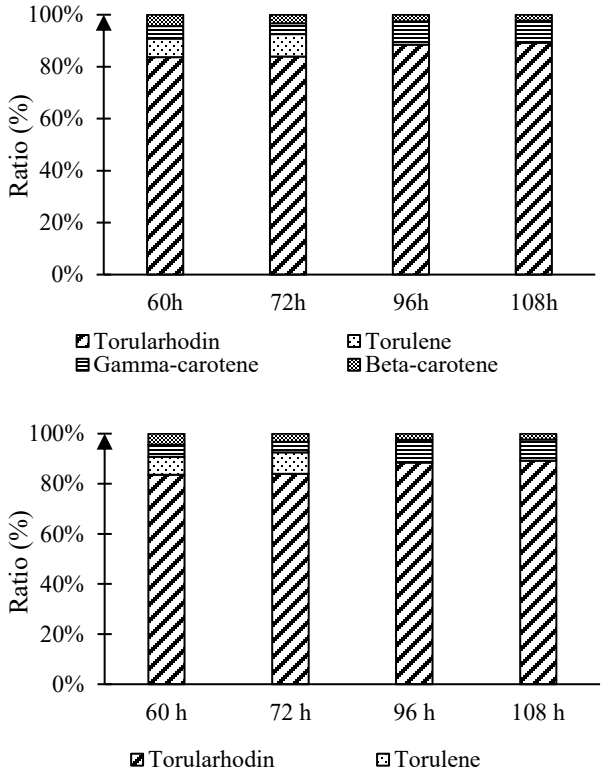

Fig. 6. Carotenoid composition changed during two-stage fermentation, analysed by HPLC

Table 1. Microbial torularhodin yield

Strain	Torularhodin accumulation (µg/g)	Torularhodin production (mg/L)	Ref.
R. toruloides A1-15-BRQ	5100	21.3	[18]
R. rubra PTCC 5255	5188.4	35.6	[3]
R. toruloides RM18	481.9	5.18	[2]
Rhodotorula spp. ELP2022	151.8	10.1	[19]
R. mucilaginosa RL 231-1.51	2628.1	91.98	this study

4. Conclusion

In this study, molasses was employed as a cost-effective carbon source to promote both biomass growth and torularhodin biosynthesis in *Rhodotorula mucilaginosa* RL 231-1.51. The results demonstrated that critical parameters, including molasses concentration, pH, temperature, and urea supplementation, significantly affected cellular proliferation and carotenoid accumulation. Based on these findings, a two-stage fermentation strategy was developed, with optimized conditions applied separately for biomass expansion and torularhodin production. This approach enhanced torularhodin accumulation by 1.66-fold compared with single-stage flask fermentation, resulting in a final torularhodin concentration of 91.98 mg/L.

Acknowledgments

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