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Optimization of *In Vitro* Carotenoid Production by *Rhodotorula Toruloides*

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Carotenoids are widely researched due to their correlation with the mitigation of severe illnesses such as cancer, cardiovascular disease, and macular degeneration, which points out the critical significance of natural pigments. Carotenoid production from microorganisms has many advantages compared to plants or algae because of its rapid growth rate, cost-effectiveness, and independence from geographical factors. The genus *Rhodosporidium*, specifically the species *Rhodosporidium toruloides*, is renowned for generating carotenoid-rich biomass in biotechnology. However, the quantity and composition of carotenoid products obtained highly depend on the specific medium and culture conditions. In this research, carotenoid extraction by combining DMSO and acetone can result in high efficiency. Furthermore, the fermentation medium for *R. toruloides* was successfully optimized with affordable ingredients, including glucose, NaCl, H_2O_2, KH_2PO_4, $MgSO_4.7H_2O$, NH_4Cl_2, Na_2HPO_4, and yeast extract. The reassessment of the optimal model revealed strong compatibility between the predictive and experimental models regarding dry biomass and carotenoid content. The low residual glucose amount ultimately revealed adequate glucose consumption as a substrate for growth and carotenoid production.

1. Introduction

Carotenoids constitute a class of compounds renowned for their immune-enhancing and antioxidant characteristics (Bhatt et al., 2020). Carotenoids, which function as colourants, antioxidants, and vitamin A precursors, are employed in the pharmaceutical and food industries (Park et al., 2018). At this point, using microbes to make carotenoids shows a promising alternative to chemical compounds due to the sustainable characteristics of biological materials as being natural, renewable, and cost-effective (Zhao Y. et al., 2021).
R. toruloides, first identified in 1922, is a nonpathogenic dimorphic red yeast (Wen et al., 2020). *R. toruloides* is a natural source of carotenoids responsible for the red colour of cells, so it is a microbial factory promising for carotenoid production. In recent years, the production performance of *R. toruloides* strains has been improved through genetic modification thanks to the availability of genetic and metabolic engineering (Zhao Y. et al., 2021). Additionally, this organism can exploit glycerol and lignocellulosic hydrolysates as substrates to generate lipid compounds that account for more than 70% of the cell's dry weight. This capability is advantageous for the commercial use of many cheap carbon sources (Viñals et al., 2023). Therefore, the survey of the cultivation parameters and the advancement of industrial fermentation systems contribute to an increase in the production of carotenoids at economic expense (Zhao Y. et al., 2021). Thus, this study aims to optimize *R. toruloides* fermentation medium and product recovery as prerequisites for biotechnological potential.

2. Material and methods

2.1 Material

R. toruloides (ATCC 1056) was derived from a soil sample in Japan. Chemicals, solvents, and Sabouraud dextrose medium were provided by Xilong (China), Sigma (USA), and Merck (USA), respectively.

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2.2 Investigation of carotenoid extraction methods from *R. toruloides*

R. toruloides was cultured for 120 hours at a shaking speed of 200 rpm in Sabouraud dextrose broth. After washing biomass twice with distilled water, carotenoid extraction was performed with various chemicals and mechanical techniques (Table 1).

Table 1: Different methods for carotenoid extraction from R. toruloides

Solvent	Process
DMSO	2 mL of DMSO was added to 1 g of wet biomass, vortexed for 1 min, and incubated at 55 °C for 15 min without agitation. These steps were repeated within 1 h or until the cells exhibited the greatest discolouration (Michelon et al., 2012)
DMSO + acetone	2 mL of DMSO was added to 1 g of wet biomass, vortexed for 1 min, and incubated in a water bath at 55 °C for 1 h. Subsequently, the mixture was centrifuged at 20°C to collect the supernatant. The mentioned steps were repeated 5-7 times with the DMSO. 2 mL of acetone was added to the pellet and centrifuged to collect the extract. The experiments were repeated until the cell colour was completely lost. The final step was when the acetone and DMSO extracts were combined (Moliné et al., 2012)
HCl/ CH_3COOH + acetone	7.5 mL HCl or CH_3COOH at a concentration of 4 mol/L were added to each test tube containing 1 g of wet biomass, vortexed for 1 min and incubated at 55 °C for 15 min in a water bath. Next, the pellet was gathered by centrifugation and washed twice with 7.5 mL distilled water to remove residual acids entirely. The chemically ruptured cells were resuspended in 6 mL of acetone to extract carotenoid (Ni et al., 2008)
Acetone + glass beads	After glass beads at 1.1 g/mL (0.5-0.59 mm) were placed into tubes with 1 g wet biomass and 6 mL of acetone, the mixture was vortexed for 10 min and centrifuged to achieve the acetone extract from mechanically disrupted yeast (Schüler et al., 2020)

2.3 Determination of total carotenoid content

After the final steps of the abovementioned methods, 10 mL NaCl 20 % (w/v) and 10 mL of diethyl ether were added to acetone supernatants to extract the carotenoid. The ether phase was removed from the trace of water with anhydrous sodium sulfate, fulfilled to precisely 2 mL and then quantified by UV-Vis spectroscopy at 485 nm. The total carotenoid content (TC) could be estimated according to the formula (Lopes et al., 2017).

$$TC\ (\mu g/g) = \frac{OD_{485nm} \times V \times 10^6}{A^{1\%}_{1cm} \times 100 \times m_{sample}} \quad (1)$$

V: carotenoid extract volume (mL)
m_{sample}: cell mass (g)
$A^{1\%}_{1cm}$: specific absorbance

2.4 Determination of glucose utilization efficiency

The DNSA method is based on the detection of the free carbonyl group (>C=O) of the reducing sugar. Initially, the ketone and aldehyde group of fructose and glucose are oxidized by 3,5-dinitrosalicylic acid (yellow) to 3-amino-5-nitrosalicylic acid (red-orange) in alkaline condition (Tchakouteu et al., 2017). The colour intensity of the reaction mixture is proportional to the concentration of reducing sugars within a certain linear range.
DNSA reagent was prepared by the 250 mL mixture of solution A (2.5 g of 3,5-dinitrosalicylic acid in 50 mL NaOH 2 M) and solution B (75 g of sodium potassium tartrate in 125 mL of distilled water). Samples were centrifuged to obtain supernatant and diluted (if needed). Subsequently, the mixture of 750 µL of the diluent solution and 250 µL of DNSA reagent was boiled for 5 min and maintained a steady state at ambient temperature. 750 µL Na_2SO_4 was added for colour stabilization before OD_{540} nm values were recorded. The glucose linearity was established over the 10-70 µg/mL concentration range. The reducing sugar concentration (µg/mL) was calculated through the calibration curve $y = 0.0011x + 0.1041$, $R^2 = 0.9629$.

2.5 Optimization of *R. toruloides* fermentation medium

Glucose, malt extract, KH_2PO_4, $MgSO_4.7H_2O$, NH_4Cl, yeast extract (Saran et al., 2017), H_2O_2, NaCl (Marova et al., 2010), and culture conditions (pH 5.6 ± 0.2, 200 rpm, 25-30 °C) were chosen to study factors affecting *R. toruloides* yeast biomass and carotenoid content. The experiment was based on the Plackett-Burman matrix with eight factors in 12 experiments. Table 2 lists low (-1) and high (+1) levels of eight factors.

Table 2: Input variables in the Plackett-Burman matrix

Factors		X1 Glucose (g/L)	X2 Malt extract (g/L)	X3 Yeast extract (g/L)	X4 KH_2PO_4 (g/L)	X5 $MgSO_4.7H_2O$ (g/L)	X6 NH_4Cl (g/L)	X7 H_2O_2 (mL/L)	X8 NaCl (g/L)
Levels	Low	5	0	0	2.5	0	0	2.8	8
	High	10	10	5	7.5	2	2	11.2	18

Twelve experiments with components designed as Table 3 were prepared for each in 50 mL of 250-mL Erlenmeyer flask. The database obtained after 120 h of cultivation was processed using Design Expert® 11.0.0.

Table 3: Experimental design matrix according to Plackett-Burman

Run	1	2	3	4	5	6	7	8	9	10	11	12
X1	5	10	10	5	10	5	5	10	5	10	10	5
X2	10	10	0	10	10	0	0	0	10	0	10	0
X3	0	5	0	5	0	0	5	5	0	0	5	5
X4	7.5	2.5	2.5	2.5	2.5	2.5	2.5	7.5	7.5	7.5	7.5	7.5
X5	0	0	2	2	0	0	2	0	2	2	2	0
X6	2	0	0	2	2	0	2	2	0	2	0	0
X7	11.2	11.2	11.2	2.8	2.8	2.8	11.2	11.2	11.2	2.8	2.8	2.8
X8	8	8	18	18	18	8	8	18	18	8	8	18

Following the statistical results of the Plackett-Burman experimental design, the main factors that significantly affected responses were evaluated at three levels (-1, 0, +1) in the Box-Behnken model. The Design Expert® 7.0.0 software was used to analyze the data and determine the optimal values of component variables for maximum biomass and carotenoid production. The response function was expressed as a quadratic equation.

$$Y = b_o + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{12} x_1 x_2 + b_{23} x_2 x_3 + b_{13} x_1 x_3 \quad (2)$$

b_1, b_2, b_3: coefficients of order 1.

b_{11}, b_{22}, b_{33}: coefficients of order 2.

b_{12}, b_{23}, b_{13}: interaction coefficients of each pair of factors.

x_1, x_2, x_3, x_{11}, x_{22}, x_{33}, x_{12}, x_2 , x_{13}: independent variables.

2.6 Re-evaluation of the optimization model

The model compatibility between predicted and experimental results was compared and evaluated based on *R. toruloides* dry weight (g/10 mL), carotenoid content, and glucose utilization efficiency.

3. Results

3.1 Investigation of carotenoid extraction methods from *R. toruloides* cells

The results showed that DMSO had the best carotenoid extraction efficiency (Table 4).

Table 4: Effects of different methods for total carotenoid extraction

Solvent	DMSO	DMSO + acetone	HCl + acetone	CH_3COOH + acetone	Glass beads + acetone
Carotenoid content (µg/g)	91.4	74.7	44.3	38.7	31.0
Carotenoid extraction efficiency (%)	100	81.7	48.5	42.3	33.9

3.2 Optimization of *R. toruloides* fermentation medium

Three factors (glucose, NaCl, and H_2O_2) have a significant and positive impact on the *R. toruloides* dry biomass and carotenoid content ($p < 0.05$), which are chosen for the optimization in the design of RSM experiments.

Table 5: Experimental data from Plackett-Burman matrix

Experiment test		1	2	3	4	5	6	7	8	9	10	11	12
Dry weight (g/10 mL)	Experiment	0.0489	0.0579	0.0306	0.0977	0.0894	0.0236	0.0816	0.0316	0.0704	0.0769	0.1066	0.0284
	Model	0.05	0.05	0.03	0.09	0.08	0.02	0.08	0.03	0.07	0.07	0.11	0.03
Carotenoid (OD_{485nm})	Experiment	0.798	0.753	0.870	0.775	0.721	0.293	0.694	0.317	0.590	0.621	0.732	0.251
	Model	0.80	0.75	0.29	0.77	0.70	0.30	0.70	0.30	0.60	0.60	0.70	0.25

Table 6: Variables in the Plackett-Burman matrix and their effects

Factors (g/L)		X1	X2	X3	X4	X5	X6	X7	X8
Levels of influences	Biomass	0.033	0.007	0.001	0.004	-0.007	0.006	0.034	0.023
	Carotenoid	0.320	0.005	0.035	-0.04	0.007	-0,160	0.140	0.170
P-value	Biomass	0.0113	0.1847	0.6721	0.3016	0.1808	0.8707	0.0107	0.0227
	Carotenoid	0.0059	0.9188	0.4910	0.4873	0.8760	0.0393	0.0445	0.0328

Table 7: RSM-Box-Behnken model with three selected factors

	Factors	Range	Level		
			-1	0	1
A	Glucose (g/L)	5-10	5	7.5	10
B	NaCl (g/L)	8-18	8	13	18
C	H_2O_2 (mL/L)	2.8-11.2	2.8	7	11.2

Table 8: Experimental results according to RSM design

Experiment		1	2	3	4	5	6	7	8	9	10	11 *	12 *	13	14	15 *
Factors	Glucose (g/L)	5	10	10	5	7.5	5	10	10	5	7.5	7.5	7.5	7.5	7.5	7.5
	NaCl (g/L)	18	13	8	13	8	8	18	13	18	13	13	13	18	8	13
	H_2O_2 (mL/L)	11.2	11.2	7	11.2	7	2.8	7	2.8	7	2.8	7	7	2.8	11.2	7
Dry biomass	Experiment	0.0328	0.0488	0.0666	0.0362	0.0398	0.0386	0.057	0.0578	0.0564	0.0476	0.0476	0.0424	0.0472	0.0334	0.0508
(g/10 mL)	Model	0.033	0.05	0.067	0.04	0.04	0.039	0.06	0.06	0.055	0.048	0.047	0.04	0.05	0.03	0.05
Carotenoid	Experiment	0.818	0.434	0.757	1.17	0.945	0.81	1.1	0.962	0.482	1.009	1.061	0.909	0.86	0.942	0.907
(OD_{485nm})	Model	0.80	0.43	0.75	1.18	0.1	0.80	1.05	0.98	0.60	1.10	0.98	0.91	0.88	0.95	0.90

(*): Center experiments

The analysis of the processing outcomes suggested that the *R. toruloides* dry biomass and carotenoid content aligned with the predictions of the first-order and second-order (Quadratic model) models, with respective correlation coefficients of 0.9529 and 0.9741, respectively. These values meant that the model would precisely calculate the corresponding responses with 95.29 and 97.41 % accuracy. Meanwhile, analysis of variance (ANOVA) was utilized to determine the model significance; the resulting P-values of 0.0075 and 0.0264 indicated that the tested model has statistical significance and was compatible with the experiment. After regression coefficients with P-values greater than 0.05 were excluded, the obtained regression equation can be deployed as a predictive model to estimate the dry biomass and carotenoid.

$$Dry\ biomass\ (g/10\ mL) = 0.026 + 0.0084A + 0.0009B + 0.0035C - 0.0009C^2 \quad (3)$$

$$Carotenoid\ (OD_{485nm}) = 1.38 - 0.072A + 0.027AB - 0.052AC \quad (4)$$

In order for the objective function to be set up based on the maximum responses, Design Expert 7.0.0 software predicted the optimal parameters: glucose 5 g/L, NaCl 8 g/L, H_2O_2 5.2 mL/L. Hence, the *R. toruloides* fermentation medium is thoroughly investigated, which included (g/L) glucose 5.0, NaCl 8.0, KH_2PO_4 2.5, $MgSO_4.7H_2O$ 2.0, NH_4Cl 2.0, Na_2HPO_4 6.0, yeast extract 5.0, H_2O_2 5.2 mL/L, pH 5,6 ± 0.2, 25-30 °C and 200 rpm. The regression equation calculated that the biomass obtained per 10 mL of the fermentation medium was 0.046 g or 4.6 g/L, 1.8 times higher than the biomass from the Sabouraud medium (0.025 g/L). To some extent, a similar pattern is observed in the carotenoid content, whose figure is 2.2 times higher than the Sabouraud medium, with 1.07 and 0.484, respectively.

3.3 Re-evaluating the optimization model by experiments

The optimization model was re-assessed to obtain the compatibility between experimental and predicted models. Simultaneously, OD_{540} results of 15 samples in the RSM-Box-Behnken model were measured, and the remaining glucose content (µg/mL) could be calculated based on the standard curve equation (Table 9, 10).

Table 9: Results of dry biomass and carotenoid content between experimental and predicted models

Run	Dry biomass (g/L)		Carotenoid content (µg/g)	
	Experimental	Predicted	Experimental	Predicted
1	4.45		33.33	
2	4.53		30.70	
3	4.73		30.30	
Average	4.57	4.60	31.44	33.02
Compatibility (%)	99.3		95.2	

After the optimal model was re-evaluated experimentally, the dry weight and carotenoid content compatibilities in predicted and experimental models were 99.3 % and 95.2 %, respectively, demonstrating a high correlation between the two data.

Table 10: Data of glucose content (µg/mL)

Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
OD_{540nm}	0.1380	0.1495	0.1489	0.1465	0.1369	0.1422	0.1571	0.1348	0.1233	0.1318	0.1245	0.1215	0.1489	0.1587	0.1361
Glucose content (µg/mL)	30.82	41.27	40.73	38.55	29.82	34.64	48.18	27.91	17.45	25.18	18.55	15.82	40.73	49.64	29.09

4. Discussion

In terms of the carotenoid extraction method, during a simultaneous investigation of various solvents, it was observed that a yield of 81.7% was achieved with a mixture of DMSO and acetone. In contrast, the mechanical method yielded the lowest result at 34%. Furthermore, it is apparent that the combination of DMSO and acetone yields greater results and reduces the need for additional solvents and time. The use of natural carotenoids as a suitable alternative to synthetic colourants has attracted increasing global attention because of their safety profile. Therefore, the toxicity drawbacks of organic solvents may not be favourable for acceptance on an industrial scale. However, the technique could be employed to quantify carotenoid content for monitoring the cultivation process due to its simplicity, convenience, and cost-effectiveness.

The target was maximizing biomass and carotenoid accumulation using the Plackett-Burman matrix and RSM design. From statistical results, the regression equation in Eq(3) and Eq(4) provided evidence that the concentrations of glucose (A), NaCl (B), and H_2O_2 (C) impacted the yields of biomass and carotenoids. Further clarification is required regarding the weak proportionality of glucose and NaCl to output responses. It is elucidated that the presence of complex sources of carbohydrates and other salts in the fermentation medium still provided a partial guarantee for the *growth of R. toruloides*. The order-1 and regression coefficients of H_2O_2 (0.0035 and -0.0009, respectively) indicated a negative second-order effect, which indicates that the biomass increases proportionally with the gradual increase in concentration of this factor throughout the survey range. However, a further rise in H_2O_2 concentration results in a reverse tendency, which is consistent with the characteristic of an external stress factor. Finally, the experimental results of the carotenoid content were 31.44 µg/g, which was two times higher than the value of 14.8 µg/g obtained from *R. toruloides* strain CBS 14 cultured on a substrate of wheat hydrolysate (Nagaraj et al., 2022). Meanwhile, the combination with batch fermentation enhanced the carotenoid content of 78 µg/g (Freitas et al., 2014) compared with classical cultivation.

Carotenoids were formed in a significant shift when the medium composition was optimized in the presence of glucose. *R. toruloides* is a potential organism for producing lipids and other compounds, such as carotenoids from different carbohydrate sources. Lipids are generated and accumulated when glucose is rapidly metabolized during the log phase. The synthesis of secondary metabolites, such as carotenoids, only starts from the stationary phase and continues until the death phase onset, when glucose gradually decreases (Singh et al., 2016). In our approach, the residual glucose content determined after 120 h of growth was found to be particularly low, ranging from 15.82-49.64 µg/mL, which proved that *R. toruloides* efficiently used a large amount of glucose for growth and secondary compound production.

The effects of chemical stress, such as osmotic (NaCl) and oxidative stress (hydrogen peroxide), and the combined effects of these stress factors on growth and metabolite production were demonstrated (Marova et al., 2010). Accordingly, the addition of peroxide and salt led to the stimulation of carotenoid production in the logarithm phase as well as in the steady-state phase (Marova et al., 2010) with the improvement of the three-time yield of carotenoids compared with the Sabouraud traditional medium. NaCl is thought to induce osmotic stress, leading to a significant impact on bacterial cell metabolism and forcing the cellular machinery towards lipid synthesis and accumulation (Singh et al., 2016) to improve stress tolerance in yeast by supporting membrane integrity (Illarionov et al., 2021). Salt stress is also a causative factor for the biosynthesis of osmoprotectants, such as glycerol, which increases osmotic tolerance (Illarionov et al., 2021). On the other hand, adding hydrogen peroxide to the culture medium increased carotenoid biosynthesis, showing high activity against reactive oxygen species. In particular, thanks to the double bond in molecular structure, torularhodin (a group of carotenoids) can neutralize the damaging effects of H_2O_2 and resist substrate decomposition caused by singlet oxygen more strongly than β-carotene (Kot et al., 2019). In general, carotenoids play a protective role against oxidative stress caused by adverse environmental conditions that yeast cells can be exposed to throughout the life cycle (Zhao D. et al., 2023).

5. Conclusion

The carotenoid extraction with the mixture of DMSO and acetone gave a high efficiency of 81.7 %, which is exceptionally suitable for laboratory conditions. The *parameters of R. toruloides* fermentation media have been thoroughly investigated. The low residual glucose indicated that glucose is the primary source of growth and

carotenoid formation. Furthermore, it is crucial to consider the impact of chemical stress factors on synthesizing secondary compounds. Additional investigation is required to acquire more comprehensive insights into the intricate metabolic functions of carotenoids in red yeast. Based on the results obtained from this investigation, *R. toruloides* exhibits promise as a viable candidate for biotechnological uses involving the production of carotenoid-rich biomass.

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