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Fermentative production, comparative characterization with carrots, and functional analysis of β-carotene produced from *Rhodotorulla toruloids*

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ABSTRACT

INTRODUCTION. Beta-carotene, a widely demanded red-orange pigment, has multiple applications in the food, cosmetic, and textile industries. Synthetic pigments are associated with harmful side effects, which necessitates the exploration of natural alternatives. *Rhodotorula toruloides*, a yeast strain, offers a promising natural source of β-carotene, potentially overcoming the limitations posed by traditional plant-based sources like carrots.

MATERIALS AND METHODS. In this study, β-carotene was extracted from *R. toruloides* using submerged fermentation with YPD medium, optimized for maximum pigment production. Characterization was performed using spectrophotometry, Thin Layer Chromatography (TLC), and High-Performance Liquid Chromatography (HPLC). The antimicrobial and antioxidant activities of the extracted β-carotene were analyzed, and its suitability as a dye was tested.

RESULTS. The yield of β-carotene from *R. toruloides* was 0.36 g/L, with significant antimicrobial activity against *Salmonella sp.* (25.3 ± 0.3 mm inhibition zone), outperforming carrot-derived β-carotene. Additionally, the DPPH assay revealed strong antioxidant activity. The β-carotene extracted from *R. toruloides* also successfully dyed cotton fabric, demonstrating its potential as an organic dye.

CONCLUSIONS. The study concludes that β-carotene extracted from *R. toruloides* exhibits superior antimicrobial and antioxidant properties compared to carrot-derived β-carotene. With its ease of cultivation and cost-effectiveness, *R. toruloides* presents a viable, natural, and efficient alternative source for β-carotene production, suitable for various industrial applications.

KEYWORDS

BETA-CAROTENE

RHODOTORULA TORULOIDES

ANTIMICROBIAL PROPERTIES

ANTIOXIDANT PROPERTIES

INTRODUCTION

Carotenoids are composed of isoprene units forming a 40-carbon polyene skeleton with three to fifteen double bonds, which are responsible for their significant coloration. They are abundantly present in *Daucus carota* (carrots) and some other plants1. Among carotenoids, β-carotene, a strong red-orange pigment, has gained immense importance due to its rising demand in pharmaceutical, medical, cosmetics, food, and textile industries[2].

In 2018, the global production value of β-carotene[3] was approximately $309 million, with an annual growth rate of 3.6%. This demand is expected to increase by 2.9% annually. By 2023, the global market for β-carotene[4,5]

was estimated at 303.8 million USD and is projected to reach 372 million USD by the end of 2030. However, synthetic pigments have several side effects, including causing allergies, toxicity, and even cancer. This has led the scientific community to explore natural sources of pigments[6,7].

Several plants and fruits, such as carrots, tomatoes, oranges, grapefruits, and olives, contain high concentrations of β-carotene. However, being seasonal and requiring long growth periods, they are not suitable for commercial-scale pigment production. Microorganisms such as fungi and algae can serve as alternative sources for β-carotene production8. Several yeasts, including *Phaffia*, *Rhodotorula*, and *Sporobolomyces*, and filamentous fungi like *Blakeslea trispora*, are reported as efficient β-carotene producers in the presence of significant inducers such as dextrose[9-11].

Yeasts produce β-carotene as a secondary metabolite during their stationary phase, and the production can be scaled up via fermentation by providing optimized conditions such as suitable temperature, pH, and sugar source[12,13]. Biotechnological production of natural colors using low-cost substrates like agro-industrial residues is an economical approach for natural pigment production[14]. In this regard, the present study aimed to isolate β-carotene-producing fungi followed by its fermentation to produce β-carotene. The functional analysis of β-carotene was also conducted to determine its antimicrobial and antioxidant potential along with its ability to dye cotton, compared to β-carotene extracted from carrots.

MATERIALS AND METHODS

Isolation and Characterization

Oranges (*Citrus sinensis*) were used for fungal cultivation and placed for 15 days under damp conditions to rot. The rotten peels were scraped, dipped, and shaken in sterile saline water. The suspension of this prepared sample was inoculated in sterilized Sabouraud Dextrose Agar medium (SDA) (pH 6.5) and incubated for 5 days at 25°C. After incubation, pink-colored colonies were selected for further culturing on Yeast Peptone Dextrose Agar (YPD) medium to obtain the pure culture. The isolated pink-pigmented pure culture was characterized at morphological, microscopic, and biochemical levels via colony morphology examination, lactophenol cotton blue staining[15], and carbohydrate utilization test[16], respectively. For molecular characterization, the strain was further sent to the School of Biological Sciences (SBS), Punjab University Lahore, for 18S rRNA sequencing.

Optimization Conditions for Fermentation

Different fermentation parameters, such as cultivation media, carbohydrate source, temperature, and pH, were optimized for maximum pigment production.

Optimization of Cultivation Media

Three different culture media were used for the cultivation of the isolated strain:

- **Basal media:** 2% glucose, 0.4% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4$; pH 8.5.
- **MS3:** 3% glucose, 0.15% yeast extract, 0.1% KH_2PO_4, 0.04% $MgSO_4$, 0.5% NH_4NO_3, 0.04% NaCl, 0.04% L-alanine; pH 5.5.
- **Yeast Malt (YM) extract medium:** 1% glucose, 0.3% yeast extract, 0.5% peptone, 1.5% malt extract; pH 6.2.

The cell density in each incubated medium was estimated by measuring optical density at 600 nm every 24 hours[17].

Optimization of Sugar Source for Pigment Production

Three different propagation media with the composition of 1% yeast extract, 2% peptone, 2% sugar, and 1.5% agar were used to analyze maximum pigment production. The sugar constituent varied in each medium:

- **Yeast Peptone Dextrose (YPD) agar:** 2% dextrose; pH 6.8.
- **Yeast Peptone Glucose (YPG) agar:** 2% glucose; pH 6.5.
- **Yeast Peptone Sucrose (YPS) agar:** 2% sucrose; pH 6.0.

Each medium was inoculated with fresh inoculum and grown in optimized cultivation media for five days. The optical density of each batch[18] was observed after every 24 hours of incubation at 450-500 nm.

Optimization of Various Production Parameters

Optimization of different parameters is essential to assess the best conditions for pigment production. Response surface methodology (RSM) was employed with five levels of central composite rotatable design (CCRD). The combined effect of three independent variables was examined:

- **pH range** of 6-10 (X_1).
- **Sugar concentration** of 2-6% dextrose (X_2).
- **Temperature range** of 20-30°C (X_3).

These were checked on the response variable, highest pigment yield (Y_1). A significant relationship among the variables was predicted via a second-order polynomial quadratic model.

Fermentative Production of Pigment
After optimization of all parameters, submerged fermentation was carried out to produce the pigment. About 3% of inoculum, cultured in basal media with predetermined cell density and cell count (5.6×10^8 CFU/mL), was added to 100 mL of YPD media and incubated at 25°C for 120 h. After fermentation, the content was centrifuged at 4000 rpm for 15 min. The supernatant was discarded, and the pellet was washed with distilled water, dried, and stored for further analysis.

Downstream Processing
The pigment produced during the fermentation process was further extracted, purified, and characterized.

Pigment Extraction and Partial Purification
The pigment present in dried yeast cells was extracted by mixing it with DMSO and then subjected to 10 minutes of discontinuous sonication at 20 kHz. Ten-second pauses were maintained to ensure complete cell disruption and maximize pigment collection in the solvent (DMSO). After sonication, the processed DMSO was mixed with 99.9% pure chloroform for partial purification of the pigment[19]. The chloroform layer was separated and subjected to evaporation. For complete purification, the dried product was mixed with 97% methanol. The pigment-containing layer was separated, dried, and weighed to obtain the pigment yield.
Alongside pigment production from the yeast source, β-carotene was also extracted from carrots, a rich β-carotene source. The idea was to compare the efficiency of yeast-extracted β-carotene and carrot-extracted β-carotene. The crushed carrots were dipped into 1% $CaCO_3$ solution, which was further subjected to ethanol extraction. The filtrate was evaporated and dried to obtain powdered β-carotene[20].

Pigment Characterization
The characterization of the extracted pigment was carried out by spectrophotometry and chromatographic techniques. UV-visible spectroscopic analysis was done in the light range of 450-500 nm to determine the maximum absorption peak[21]. Thin-layer chromatography (TLC) analysis was conducted using Silica Gel 60 F_{254} glass plates (2×5 cm²), and a mobile phase solvent of acetone and distilled water in a 3:1 ratio was used to characterize the pigment based on Rf value calculated using the formula[22]:

$$\text{Rf} = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

Fourier-transform infrared (FTIR) spectroscopy analysis was conducted using an FTIR spectrophotometer (Thermo Nicolet Model-iS50) with a spectral range of 15-35,000 cm^{-1} at Applied Chemistry Research Centre, PCSIR, Lahore[23]. For high-performance liquid chromatography (HPLC), the standard and the extracted pigment were prepared using acetone. A C18 column with a mobile phase of acetonitrile:2-propanol:ethyl acetate (40:40:20) at a flow rate of 1 mL/min was used for the detection of the extracted pigment[24].

Biological Activity Analysis of Pigment
Microbial pigments are in high demand in medicinal, food, and textile industries due to their eco-friendly nature and ease of cultivation. Besides coloration, they possess significant biological activities. Therefore, the antimicrobial and antioxidant activities of the extracted pigment were assessed, along with its dyeing ability.

Antimicrobial Activity
The antimicrobial potential of the extracted pigment against *Staphylococcus sp.*, *Salmonella sp.*, and *E. coli* was tested using the agar well diffusion method[25].

Antioxidant Activity
Antioxidant activity analysis was performed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. DPPH was first mixed with methanol and homogenized under dark conditions to prevent radical oxidation. Yeast-derived β-carotene (50 µL) and carrot-extracted β-carotene (100 µL) were added to separate mixtures of DPPH and methanol, followed by incubation for 2-3 min until color change. Samples were then subjected to spectrophotometry; absorbance was recorded at 520 nm, and data were plotted with concentration on the x-axis and percentage antioxidant activity on the y-axis[25].

Dyeing Ability of Pigment
To evaluate the application of the extracted pigment in dyeing, cotton fabric was dyed in a defined ratio of pigment, organic solvent, and water. The extracted pigment was dissolved in dilute acetone, and the pH of the dye bath was adjusted to 6.5 and the temperature to 40°C for a few hours. The fabric was then washed to remove any excess dye[26].

RESULTS

Isolation and characterization of fungal strain
In the present study, pink-colored microbial colonies, suspected to be the producers of β-carotene, were isolated from rotten orange peels on PDA. The morpho-

logical characteristics, i.e., pink, mucoid, and round colonies (Figure 1a), as well as the microscopic characteristics of the isolated strain, indicated it as yeast. Further screening was conducted based on biochemical tests. As carbohydrate is the main inducer of β-carotene production, the ability of isolated yeast strains to ferment different sugars (dextrose, fructose, sucrose, and glucose) was estimated. The results of the carbohydrate assimilation test showed that the yeast strain fermented glucose and dextrose with gas production, indicated by a color change from red to yellow and bubble formation in Durham tubes. Finally, 18S rRNA sequencing was conducted for phylogenetic analysis of the isolated strain. The results of 18S rRNA sequencing of the isolated strain showed 100% similarity with Rhodotorula toruloids, as indicated in Figure 1b.

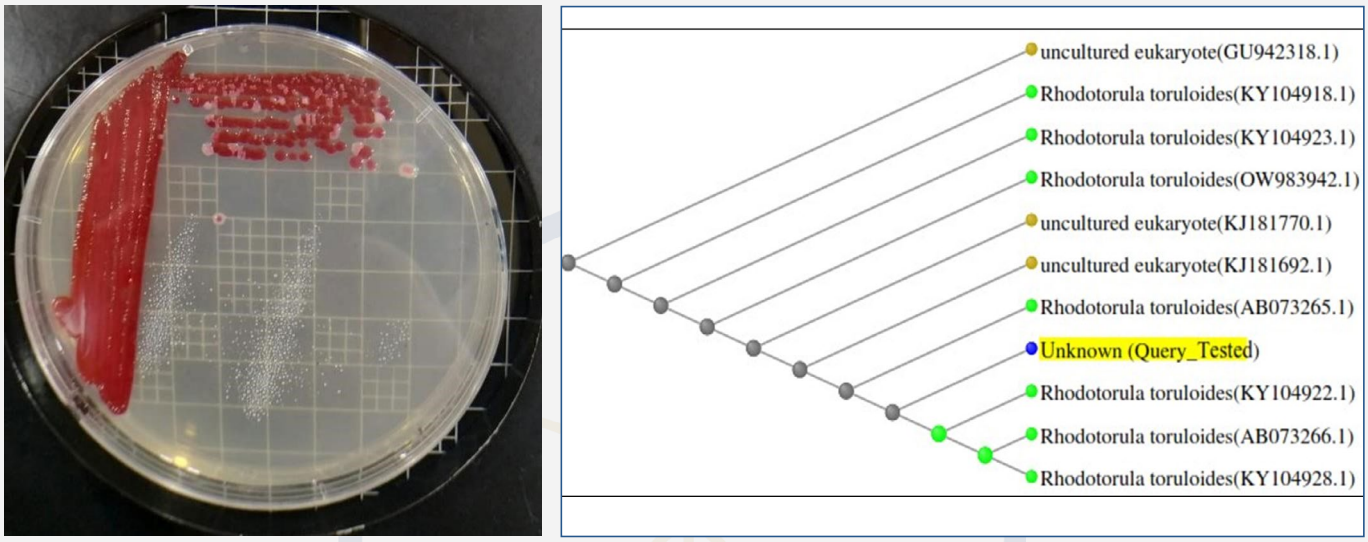


Figure 1. Isolation and characterization of yeast. a) Isolated pink-colored round, mucoid entire and smooth microbial colonies. b) Phylogenetic tree showing 100% similarity of an isolated yeast strain with *Rhodotorula torulo*.

Optimization conditions for fermentation

To maximize the yield of pigment extraction, different parameters such as culture media (Basal, MS3, and YM media), sugar source (Dextrose, glucose, and sucrose), and physical parameters (pH and temperature) were optimized. Among the three-cultivation media, basal media facilitated maximum yeast growth (1.83±0.02 OD at 600 nm) within 120 hours of incubation as indicated in (Figure 2a). Maximum pigment production was induced by dextrose as maximum OD was recorded in YPD media after 120 hours of incubation. Figure 2b shows the effect of different sugar-containing media on pigment production, measured via OD at 460 and 490 nm respectively.

Response surface methodology (RSM), employing a central composite design (CCD), was utilized to optimize the conditions for maximum β-carotene yield. The optimal conditions for maximum yield of β-carotene production are mentioned in Table 1 and indicated in Figure 3. The results revealed that pH had no significant effect on pigment yield, whereas temperature exerted a notable effect, with optimal production occurring at 25°C and declining thereafter. Additionally, sugar concentration significantly impacted pigment yield, with maximum production observed at 4% dextrose.

In the ANOVA quadratic model (Table 2), the F-value of 1.42 suggests that the model lacks significance compared to noise, with a 29.37% probability of this F-value arising due to noise. Factor coding was performed, and model terms were deemed significant if the p-value was below 0.0500. However, no significant model terms were observed in this case, indicating the need for model reduction. The standard plot of the pH effect on pigment extraction indicates no significant effect on pigment yield, while the curve in the standard plot of temperature effect indicates its significant effect on pigment yield. The graph shows minimum β-carotene yield at a lower temperature, maximum at 25oC, and again less at a higher temperature. The effect of sugar concentration on the yield of pigment showed a straight-line standard curve, indicating maximum pigment production at 4%. The final equation provided by Design Expert-V 11 allows the prediction of β-carotene yield based on specified levels of pH, temperature, and sugar concentration.

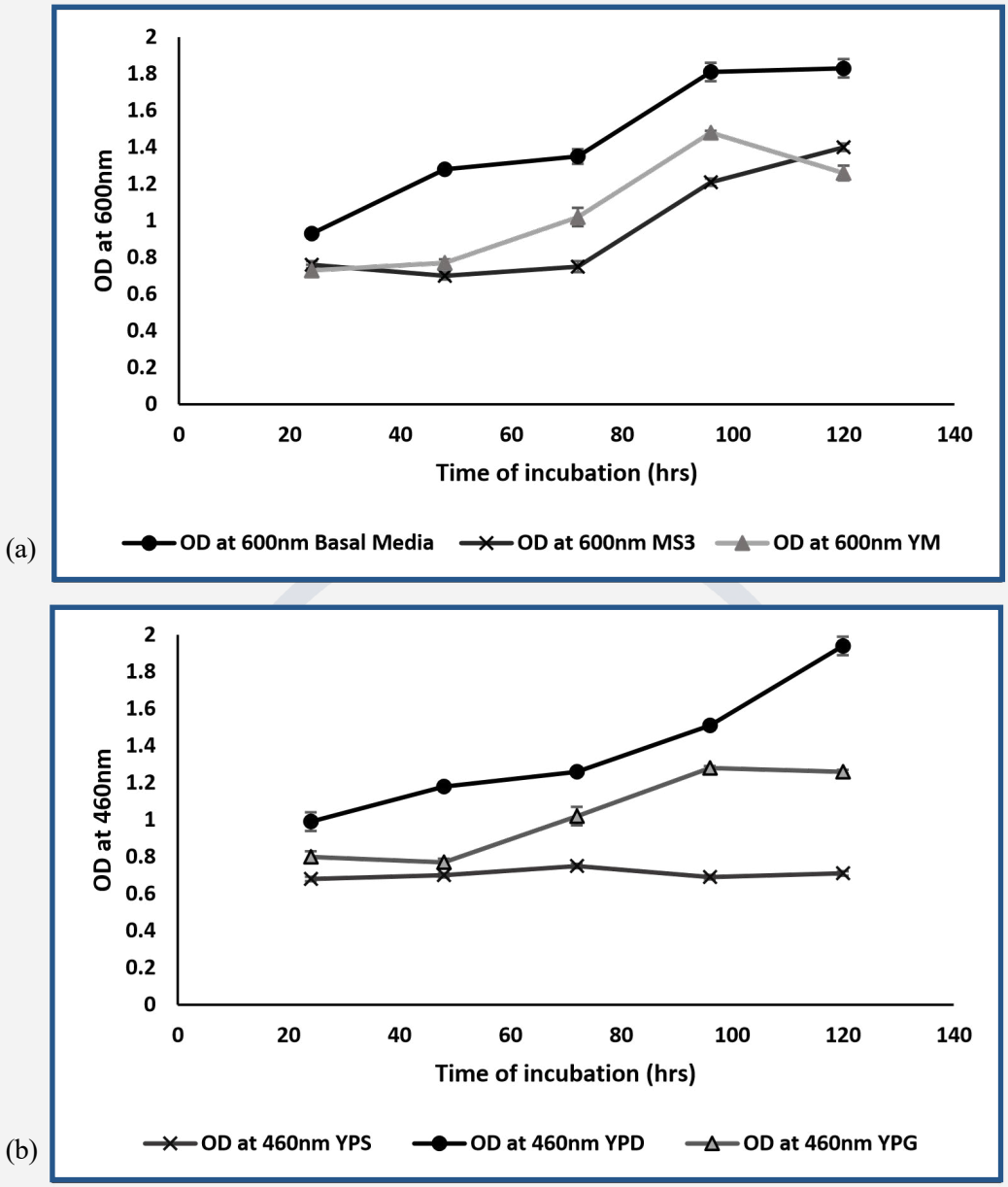


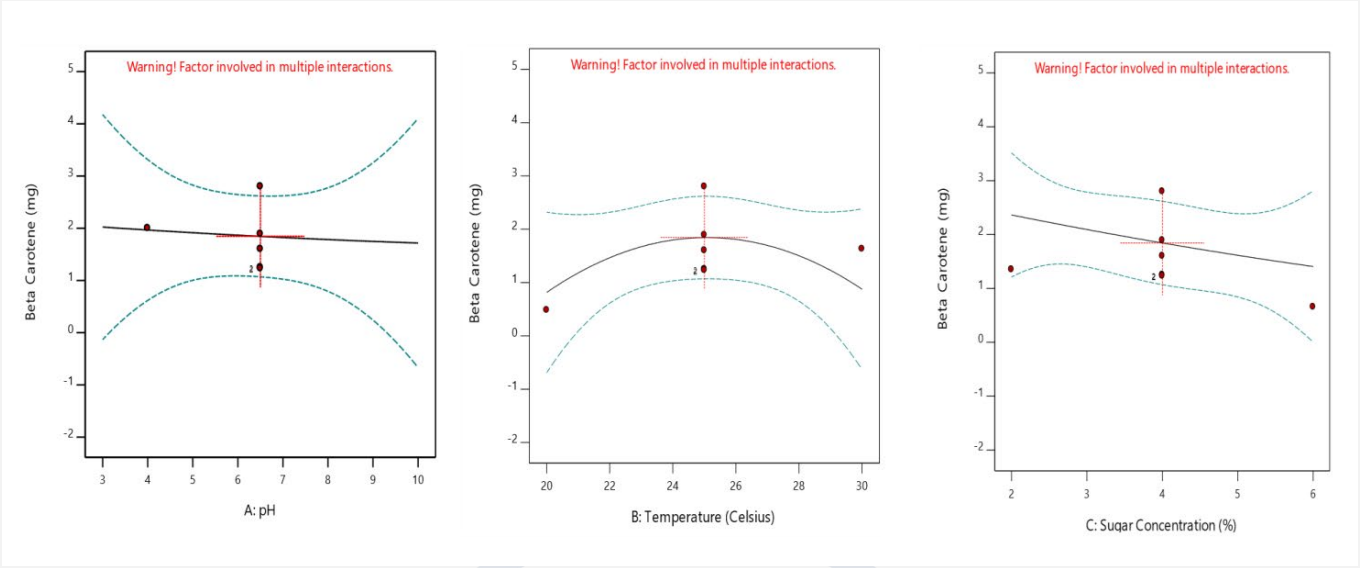
Figure 2. Optimization of culture media. a) Cultivation media optimization on the basis of *R. toruloids* cell density in terms of OD measured at 600 nm. Basal media was shown to facilitate higher cultivation of *R. toruloids*. b) Optimization of media for β-carotene production indicated in a graph showing peaks of maximum pigment production in terms of OD measured at 460 nm.YPD media was shown to facilitate maximum β-carotene production.

Table 1. Optimized experimental design for β-carotene production.

Std	Run	Factor 1: A-pH	Factor 2: B-Temperature (Celsius)	Factor 3: C-Sugar Concentration (%)	Response 1: β-Carotene (mg)
3	1	3	30	2	0.3
6	2	10	20	6	0.21
7	3	3	30	6	1.5
19	4	6.5	25	4	1.23
1	5	3	20	2	2.39
9	6	6	25	4	2
17	7	6.5	25	4	1.25
18	8	8	25	2	3.6
15	9	6.5	25	4	1.6
14	10	10	25	6	0.655
5	11	3	20	6	0.32
4	12	10	25	4	0.76
13	13	6.5	25	2	1.35
11	14	6.5	30	4	1.63
8	15	15	30	6	0.46
10	16	8	25	2	3.31
2	17	10	20	4	0.945
16	18	8	25	4	1.89
12	19	6.5	20	4	0.48
20	20	6.5	25	4	2.8

Table 2. Response of β-carotene based on ANOVA quadratic model.

Source	Sum of Squares	Df	Mean Square	F-value	*p*-value	Significance
Model	10.59	*9*	1.18	1.42	0.2937	Not significant
A – pH	0.1972	1	0.1972	0.2387	0.6357	Not significant
B – Temperature	0.0093	1	0.0093	0.0113	0.9176	Not significant
C – Sugar Concentration	2.54	1	2.54	3.08	0.1098	Not significant
AB	0.1188	1	0.1188	0.1438	0.7124	Not significant
AC	0.1552	1	0.1552	0.1879	0.6739	Not significant
BC	1.72	1	1.72	2.08	0.1801	Not significant
A^2	0.0008	1	0.0008	0.0009	0.9764	Not significant
B^2	1.99	1	1.99	2.41	0.1513	Not significant
C^2	0.0041	1	0.0041	0.0050	0.9452	Not significant
Residual	8.26	10	0.8262			
Lack of Fit	6.55	5	1.31	3.84	0.0830	Not significant
Pure Error	1.71	5	0.3414			
Core Total	18.86	19				



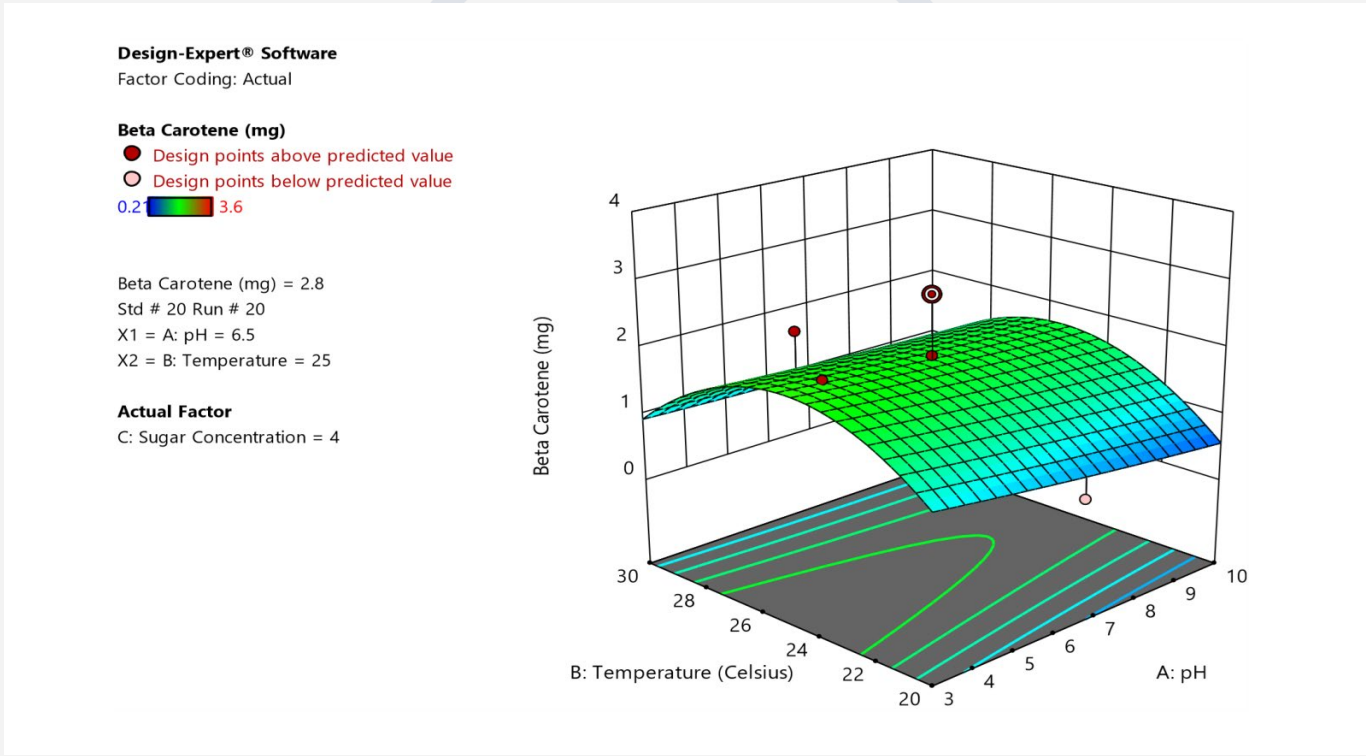


Figure 3. Response surface methodology plots achieved through design experts where RSM plots showing the optimization of three variables (a) pH (b) Temperature °C (c) Sugar Concentration for maximum production of β-carotene. The plot indicated 2.8 mg β-carotene production, taking sugar concentration as 4%, pH as 6.5, and temperature at 25°C.

Beta-Carotene = -16.52492-0.166324 pH+1.77265 Temperature

-1.35210 Sugar Concentration+0.006964 pH * Temperature

-0.019668 pH * Sugar Concentration

+0.046313 Temperature * Sugar Concentration

+0.002095 pH^2-0.039941 $Temperature^2$

+0.010361 $Sugar\ Concentration^2$

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor, and the intercept is not at the center of the design space.

Pigment extraction, purification, and characterization

After the optimization of parameters, pigment production was conducted via submerged fermentation taking the inoculum of cell density of 0.57 OD at 600 nm and cell count of 5.6×108 CFU/ml in YPD media incubating for 120 hours at 25°C in the static incubator. After fermentation, yeast biomass (0.6 g/L) was separated via centrifugation, and the pellet was subjected to solvent-based extraction using chloroform and methanol to get pigment as shown in Figure 4a. Moreover, β-carotene was also extracted from crushed carrots, as indicated in Figure 4b.

The maximum yield of β-carotene was reported as 0.36 g/L and 1.58 g/kg from yeast and carrots respectively. A higher yield of β-carotene from carrots may indicate its worth, but *R. toruloids*, being easily available and cost-effective production can prove a better β-carotene source as compared to carrots.

Characterization of the extracted pigment was conducted using spectrophotometry, TLC, FTIR, and HPLC respectively. The spectrophotometry analysis revealed that standard β-carotene absorbed maximum wavelength at 460 nm and 490 nm. The optical density (OD) of *R. toruloids* extracted β-carotene was measured as 0.56 and 0.60 while that of carrot extracted β-carotene was 0.55 and 0.59 at 460 nm and 490 nm respectively as indicated in Figure 5.

Thin layer chromatography (TLC) was performed for the detection of extracted pigment based on retardation factor (Rf) in a solvent system of diluted acetone (acetone and water in 3:1). The Rf value of pigment extracted from yeast and carrots was calculated as 0.91 and 0.9 respectively (Figure 6) as compared to that of standard β-carotene (Rf =0.92)[27].

For further confirmation and characterization of extracted pigment, its FT IR analysis and HPLC were performed.

The FTIR analysis indicates the presence of specific functional groups in the tested compound. For β-carotene, the FTIR spectra typically reside in the range of 3500 cm^{-1} to 700 cm^{-1}. While comparing the IR spectra of yeast and carrot extracted β-carotene with the standard β-carotene, three types of key peaks were identified. Firstly, a peak between 3000 cm^{-1} - 2500

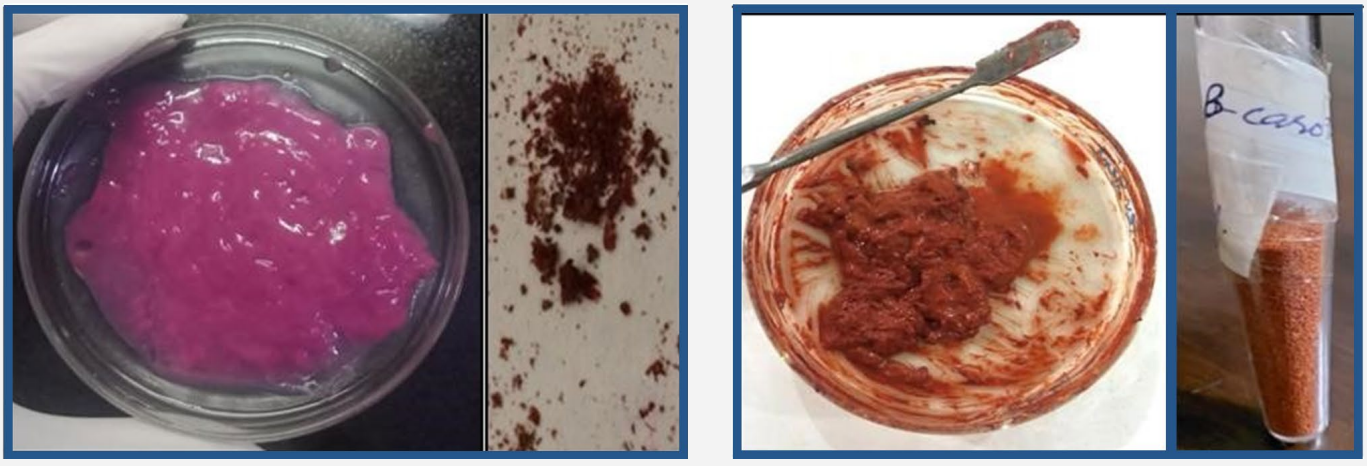


Figure 4. Filtered and dried pigment. (a) β-carotene extracted from *R. toruloids*. (b) β-carotene *extracted from carrots*.

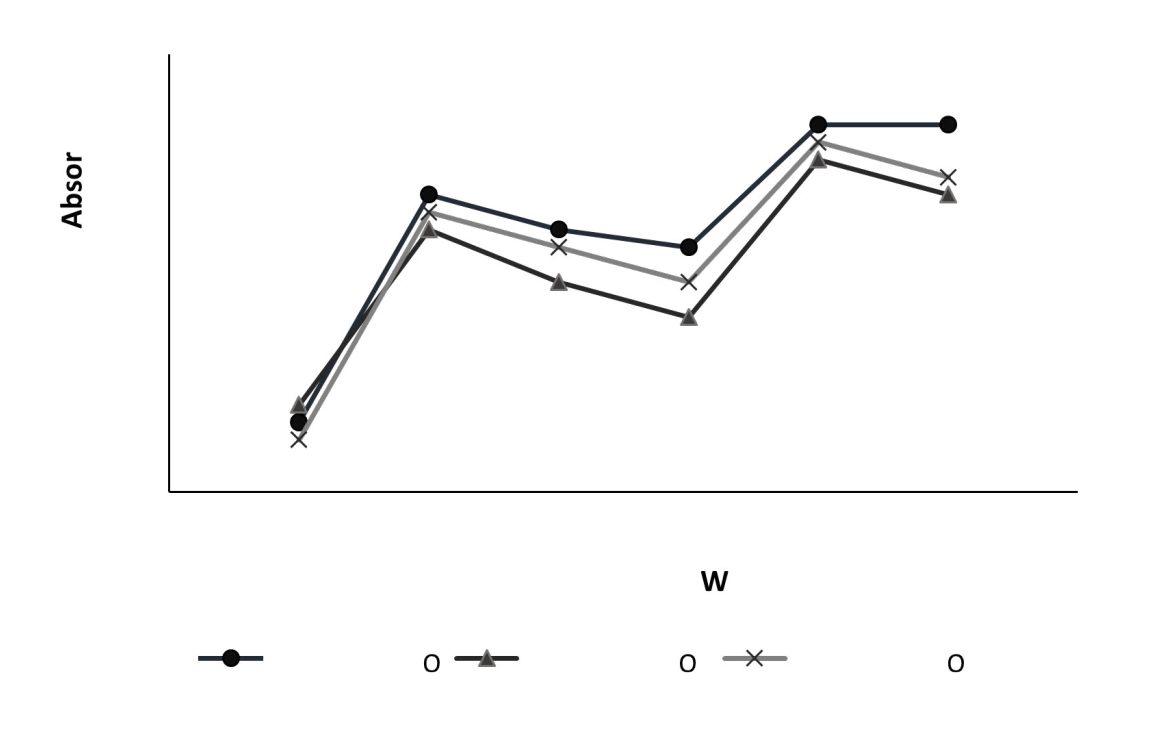


Figure 5. Absorbance of pigments. The graph indicates the spectrophotometric analysis of carrot extracted β-carotene (pigment 1), *R. toruloids* carrot extracted β-carotene (pigment 2) in comparison with standard β-carotene.

(a) (b) (c)

Figure 6. TLC analysis of (a) β-carotene standard, (b) pigment extracted from yeast, and (c) pigment extracted from carrots respectively under UV at 354 nm.

cm^{-1} indicated the presence of C-H stretches in all three spectra as indicated in Figure 7. The second significant peak resided in the range of 1600 cm^{-1} to 1700 cm^{-1}, representing C=C stretching. However, peaks in this region were observed at different wavenumbers for each sample, i.e., 1629 cm^{-1} for the microbial pigment, 1713 cm-1 for carrots, and 1637 cm^{-1} for standard β-carotene. This suggests slight variations in the chemical structure or molecular environment of the C=C bonds in each sample. Furthermore, the di-substitution in the C=C. group was detected at 935 cm^{-1} for microbial pigment, 964 cm-1 for carrots, and 1003 cm^{-1} for standard β-carotene. The overall IR spectra confirmed the successful extraction of β-carotene[28].

Finally, the samples were further characterized via HPLC. The β-carotene from yeast and carrots was run against HPLC grade standard β-carotene. The Peaks on the HPLC spectrum were recorded at the RT of 6.632 mins for standard β-carotene while 6.624 and 6.629 mins for *R. toruloids* extracted β-carotene and carrot extracted β-carotene, respectively as shown in Figure 8. The noise that appeared in the spectra of carrots and yeast is caused by the presence of slight impurities or other compounds in the extracts. The height of the peaks was recorded as 587 for *R. toruloids* extracted β-carotene, 4435 for carrot extracted β-carotene, and 13724 for standard β-carotene[29,30].

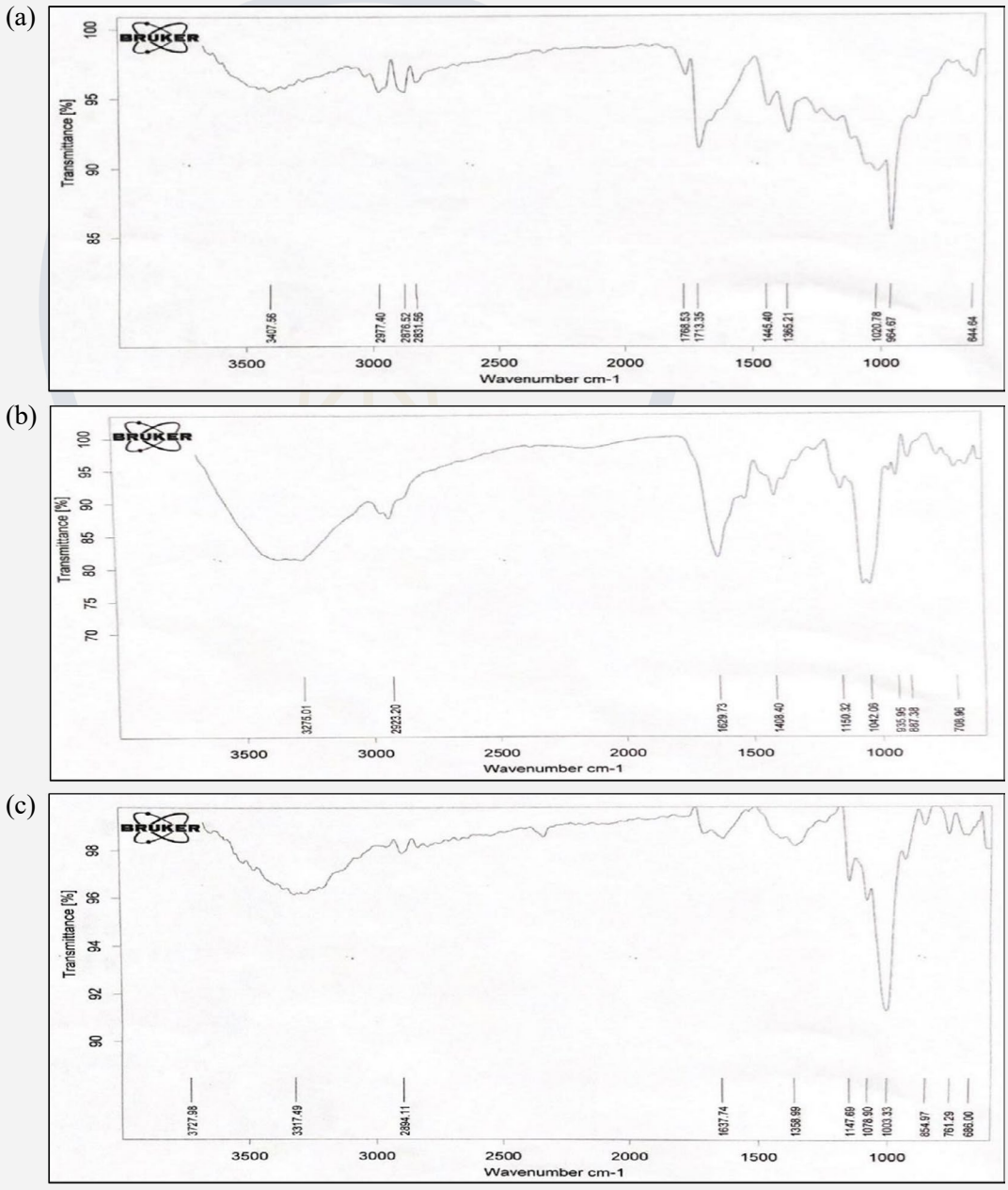


Figure 7. FT-IR spectra of β-carotene. (a) Standard, (b) extracted from Yeast and (c) extract from carrots.

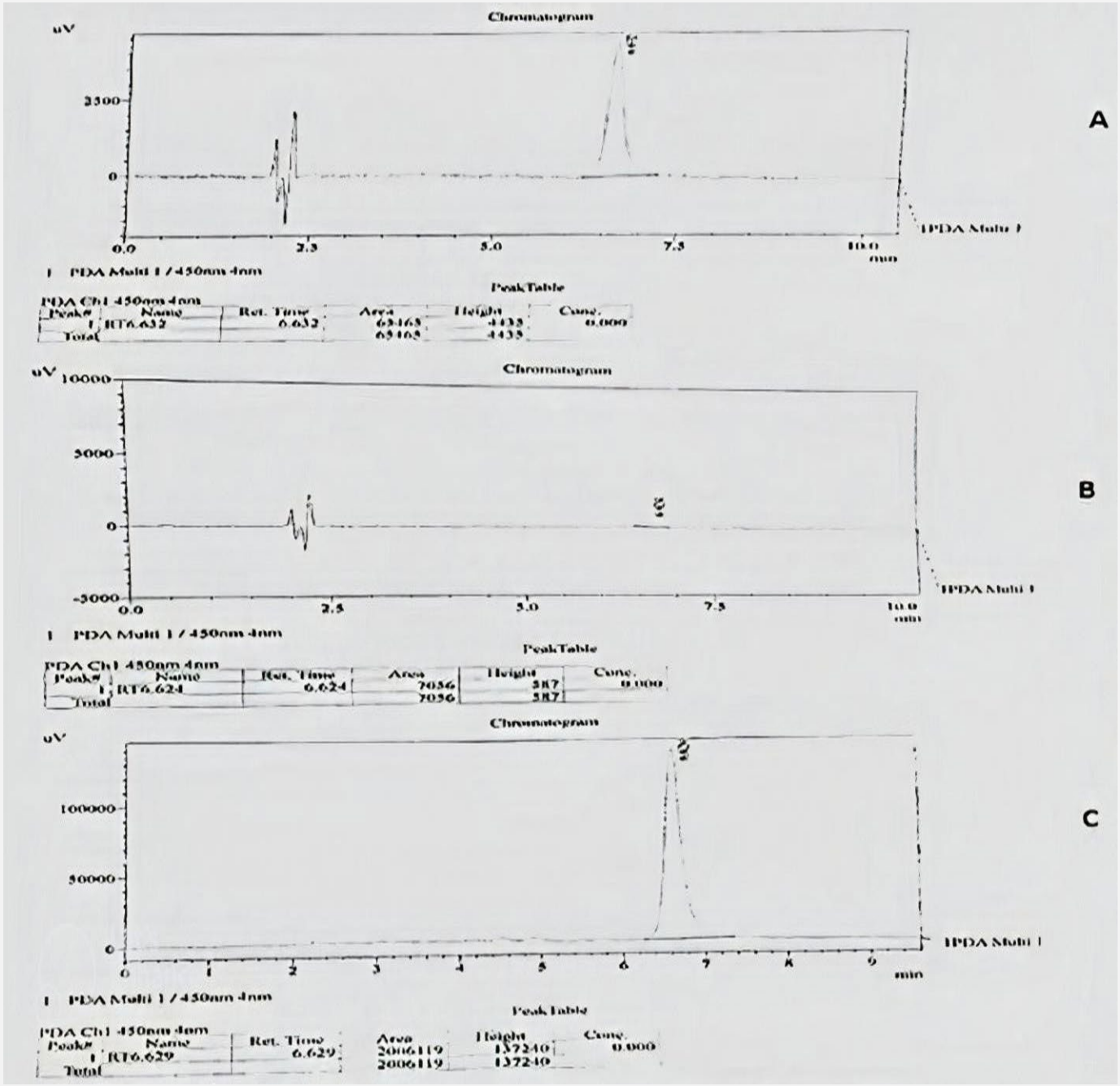


Figure 8. HPLC analysis of (A) standard β-carotene (B) *R. toruloids* extracted β-carotene (C) carrot extracted β-carotene. The peak heights representing the concentration of β-carotene, varied among the samples. The HPLC chromatograms displayed a peak height of 13724 for standard, 587 for *R. toruloids* extracted β-carotene, and 4435 for carrot extracted β-carotene. The difference in peak heights indicates variation in β-carotene concentration among samples.

Functional analysis

The functional properties, i.e., antimicrobial potential and antioxidant potential, were determined by the good diffusion method and DPPH assay, respectively. Moreover, the application of yeast extracted β-carotene as a dye was also checked.

The antimicrobial potential of extracted β-carotene was checked against one gram-positive (*Staphylococcus sp.*) and two gram-negative (*Salmonella sp.*, and *E. coli*) bacteria. For positive and negative control, standard β-carotene and dimethyl sulfoxide (DMSO) were used respectively. After 24 hours of incubation, zones of inhibition were recorded. *R. toruloids* extracted β-carotene remained highly effective against *Salmonella sp.*,

with the formation of (25.3±0.3 mm) zone of inhibition. For *Staphylococcus spp.* and E. coli, *R. toruloids* β-carotene produces inhibition zones of 19.8±0.3 and 19.6±0.5 mm respectively. The β-carotene extracted from carrots also showed the best antibacterial activity against Salmonella sp. as compared to *Staphylococcus spp.* and *E. coli*. However, the antibacterial potential of R. toruloids β-carotene was reported to be greater than carrots β-carotene, but less than the standard β-carotene as indicated in Figures 9 and 10.

The antioxidant activity of β-carotene extracted from *R. toruloids* and carrot was analyzed via DPPH assay. Upon addition of the antioxidant agent, a prominent shift in color from purple to yellow was observed, indicative of the positive antioxidant activity of β-carotene (Figure 11a). The spectroscopic analysis revealed that the pigment had OD lesser than the blank indicating positive antioxidant activity (Figure 11b). Finally, the application of *R. toruloids* extracted β-carotene was determined by dying a piece of cotton in dilute acetone solution as devised by Bahaa Edeen[31]. Figure 12 shows the comparison of dyed cotton pieces by *R. toruloids* extracted β-carotene and carrot extracted β-carotene. The dyeing ability of *R. toruloids* extracted β-carotene was reported similar to that of carrot extracted β-carotene.

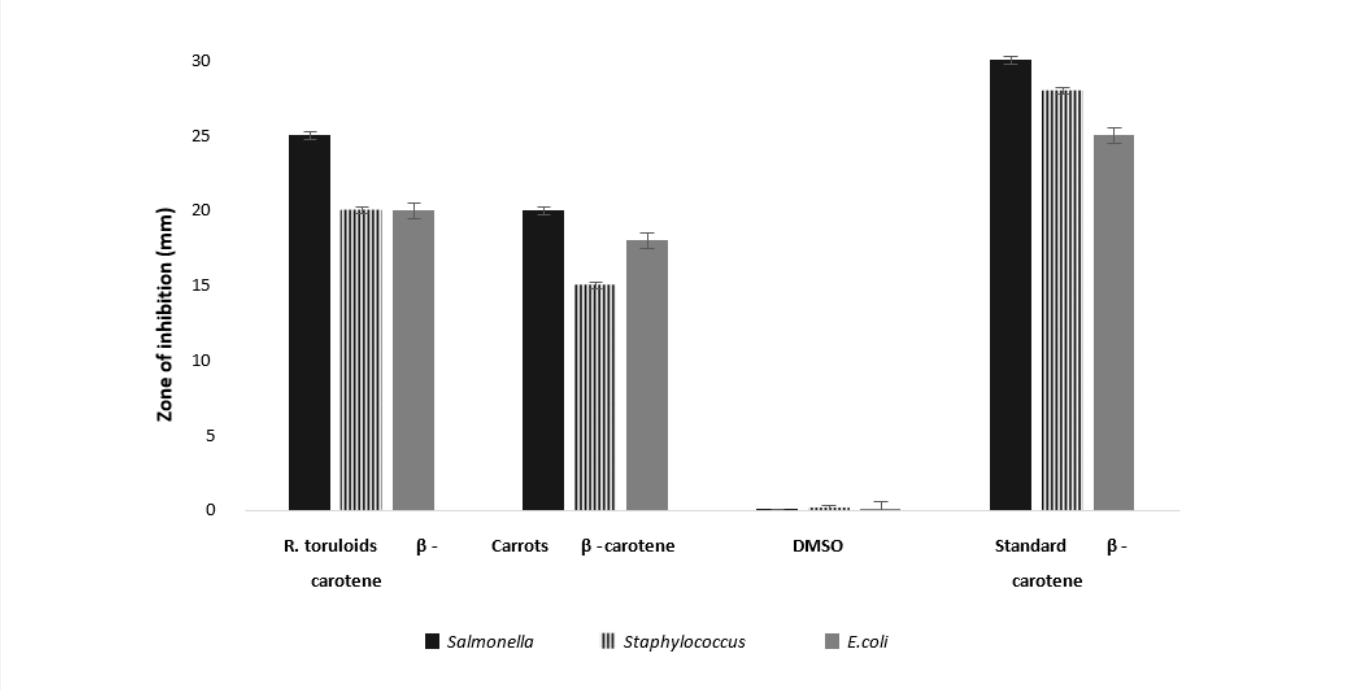


Figure 9. Antibacterial activity of β-carotene extracted from *R. toruloids* and from carrots in comparison with DMSO as negative control and standard β-carotene as a positive control against *Salmonella sp.*, *Staphylococcus sp.*, and *E. coli*.
*The error bars indicate (±SD) of three replicates of each reading.

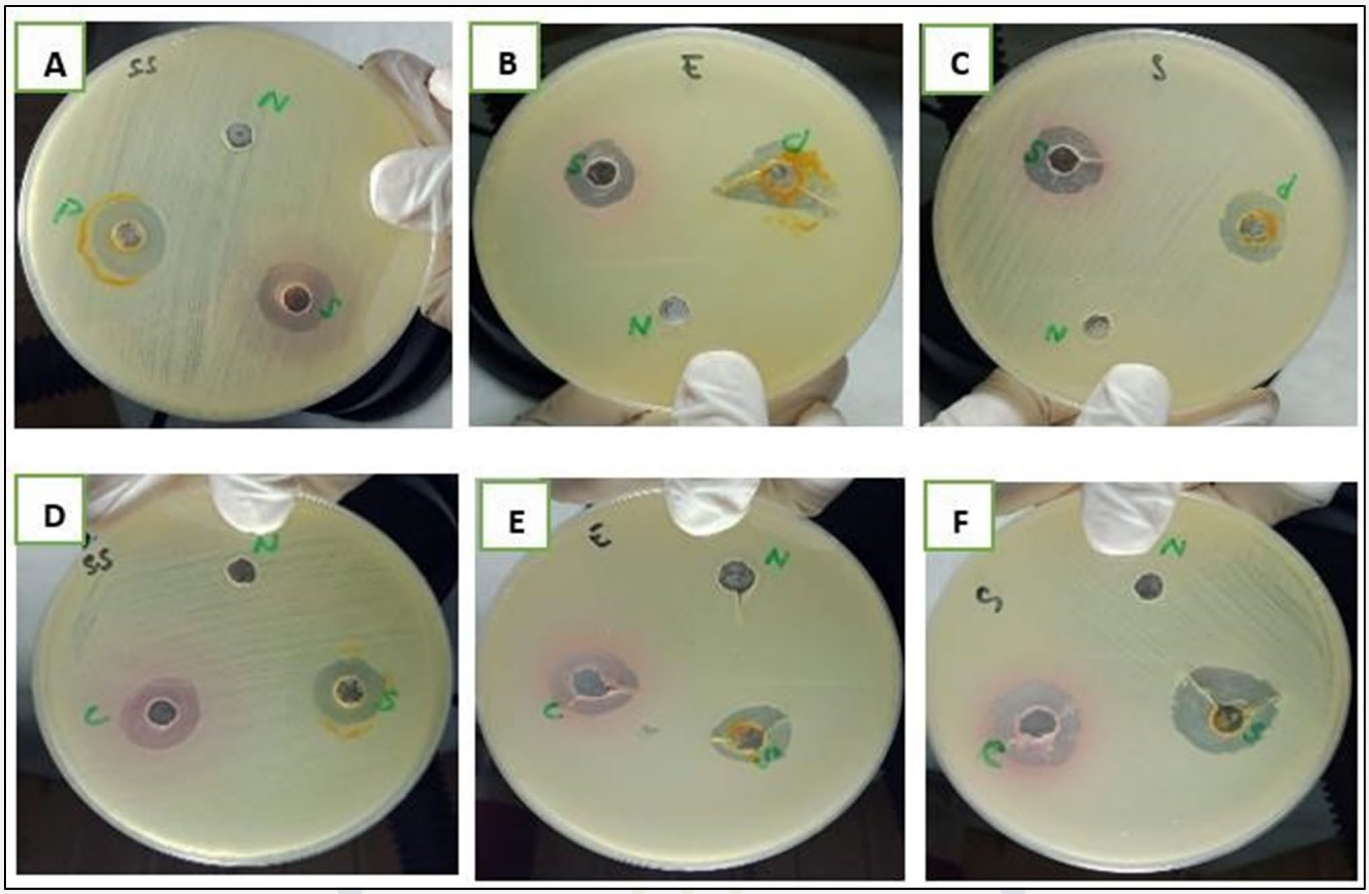
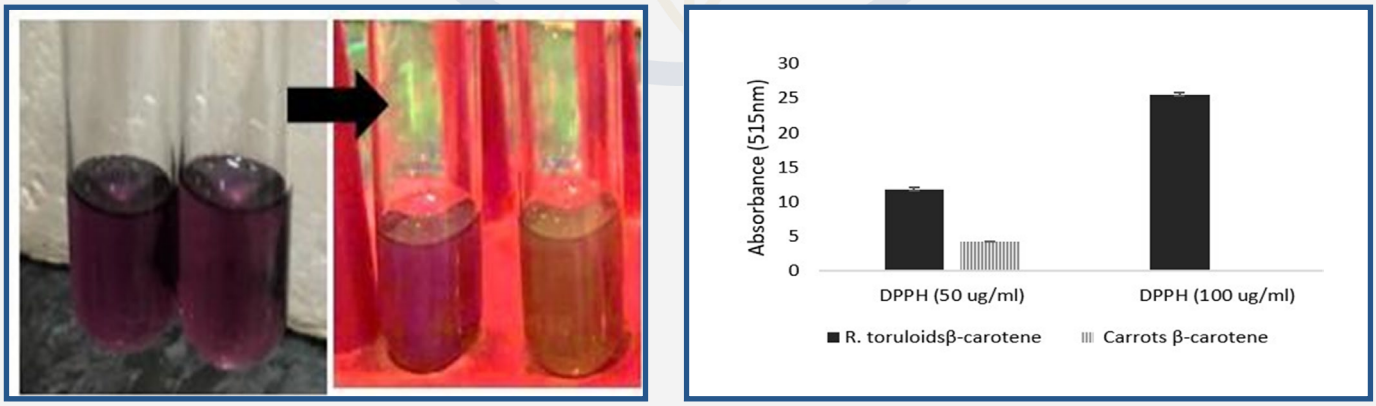


Figure 10. Inhibition zones of *R. toruloids extracted* β-carotene *against* (A) *Salmonella sp.* (B) *E. coli* (C) *Staphylococcus sp.* and carrot extracted β-carotene against (D) *Salmonella sp.* (E) *E. coli* (F) *Staphylococcus sp.*
*Here P or C= Standard β-carotene as positive control, N= DMSO as negative control and S= sample as β-carotene extracted from *R. toruloids* and carrots, respectively.



(a) (b)

Figure 11. Antioxidant activity of *R. toruloids* extracted β-carotene and carrot extracted β-carotene (a) color change before and after the reaction, (b) absorbance at 515 nm of DPPH assay.

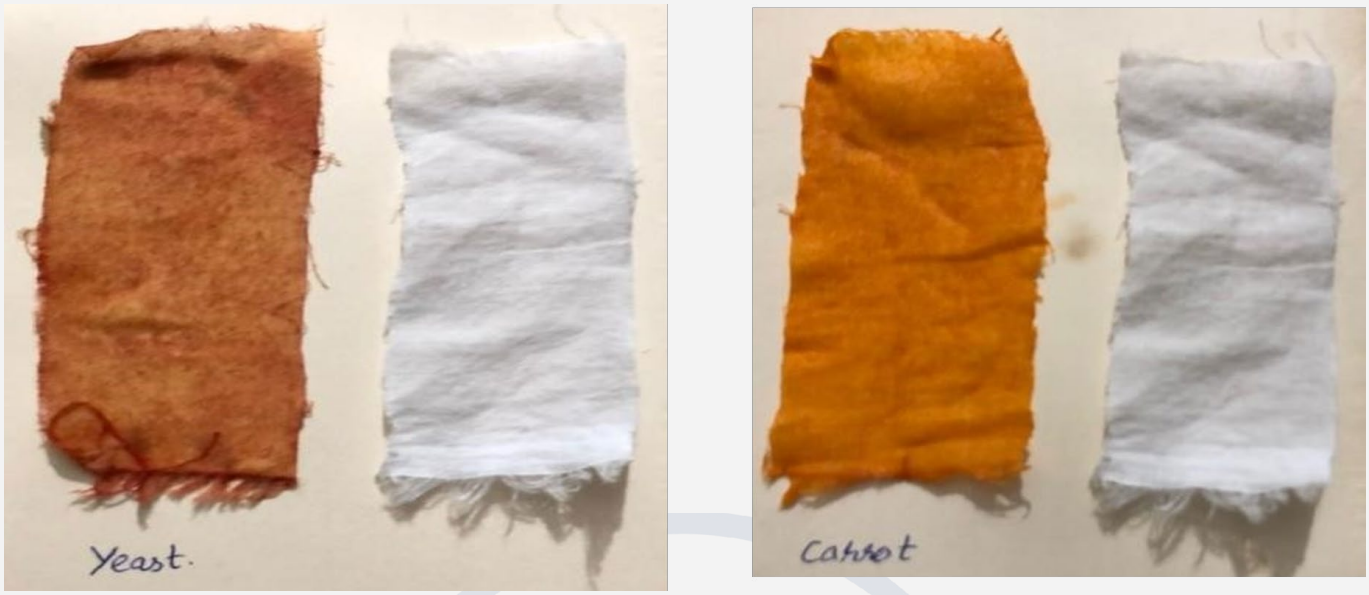


Figure 12. Application of β-carotene in dyeing cotton. (a) Cotton dyed with *R. toruloids extracted* β-carotene; (b) Cotton dyed with carrot extracted β-carotene.

DISCUSSION

Higher plants produce natural carotenoids in very small amounts due to their slow growth rates. Meanwhile, microorganisms such as algae and yeast produce natural carotenoids in higher concentrations. Among microorganisms, yeasts such as *Phaffia sp.*, *Rhodotorula sp.*, *Sporobolomyces sp.*, *Rhodosporidium sp.*, and *Sporidiobolus sp.* efficiently produce profuse concentrations of β-carotene due to higher cell density production at less cost compared to algae[32]. In the present study, pink-colored yeast colonies were isolated from rotten orange peels. The pink color is suspected to be due to the production of β-carotene, an orange-red dye[33]. Carbohydrates are the main inducers of β-carotene production[32-34]; hence, biochemical identification of β-carotene-producing yeast strain was also conducted on the basis of carbohydrate assimilation test. The results indicated the assimilation of glucose and dextrose with gas production, indicating that the yeast strain has the necessary enzymes (zymase) and specific metabolic pathways to break down and utilize these sugars9. Further, the 18S rRNA sequencing confirmed the yeast strain as *Rhodotorula toruloides*. Kim et al[35] also reported the isolation of β-carotene-producing red-colored yeast strain, *Rhodosporidium babjevae*, from rotten citrus fruit. The difference in colony color is due to the difference in strains.

After the isolation and identification of *R. toruloides*, β-carotene production was conducted in different culture media (Basal, MS3, and YM). Figure 2a shows that basal media (2% glucose, 0.4% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4$) gave higher growth of yeast compared to the other culture media used, similar to the results of Hu et al[36]. The presence of glucose provides an efficient energy source, yeast extract provides amino acids and peptides[37], while KH_2PO_4 and $MgSO_4$ maintain optimal ionic balance, which ultimately facilitated higher yeast production in 120 hours of incubation. Moreover, among different carbohydrate sources (glucose, dextrose, and sucrose), YPD culture media with 1% dextrose induced maximum pigment production as indicated in Figure 2b. Gerelmaa et al[17] reported the fermentation of glucose, dextrose, and maltose by the yeast strain *Rhodotorula glutinis*. Saha et al[38] described that yeast produces secondary metabolites, mostly pigments, in its stationary state. They also reported a high yield of β-carotene on YPD media till the end of the stationary phase of the yeast growth period. Kaur et al39 also indicated that *Blakeslea trispora* can grow in media containing fruits and vegetable waste as substrate and gave a good yield of β-carotene, with respect to synthetic media containing all nutrient sources.

Optimization of different parameters is also important to achieve the specific conditions for maximal growth and pigment production. Response surface methodology is considered a more efficient optimizing technique due to its systematic approach and ability to model and predict responses compared to conventional optimizing approaches[40]. The results of RSD indicated the optimal

conditions, i.e., pH 6.5, temperature 25°C with 4% sugar concentration, can give 2.8 mg/L production of β-carotene. Sharma and Ghoshal41 reported optimum conditions for β-carotene production were pH 6.1, and temperature 25.8°C. This minor variation in results is due to change in yeast species, and they used a bioreactor for fermentation, which provides more controlled conditions than a flask. Rodríguez et al[42] also reported 2968 µg/L (2.96 mg/L) β-carotene production by *Rhodotorula mucilaginosa* at pH 5, at 30°C using RSD. The slight change in parameters is due to the change in *Rhodotorula* strain. The ANOVA quadratic model indicated a significant interaction between temperature and pigment production. The standard plot showed that temperature had a significant impact on pigment yield with maximum production at 25°C. Zarandi-Miandoab et al43 also reported maximum pigment production at 25°C.

The characterization of extracted β-carotene was conducted via spectrophotometry, TLC, FTIR, and HPLC. The spectrophotometric analysis showed maximum absorbance at 460 nm and 490 nm in standard, carrot-extracted, and *R. toruloides-extracted* β-carotene. Karnjanawipagul et al44 and Nagaraj et al[45] reported similar results for β-carotene extracted from carrots and *R. toruloides* CBS 14, respectively. The TLC results also gave similar Rf values in the range of 0.9 to 0.92, similar to that of standard β-carotene[46,47]. Moreover, the functional group analysis was conducted on the basis of FTIR spectroscopy. The spectra obtained by FTIR confirmed the presence of C–H (stretch between 3000 cm^{-1} to 2500 cm^{-1}) and C=C–H (stretch between 1629 cm^{-1} to 1713 cm^{-1}), the prominent functional groups in β-carotene also reported by Trivedi et al28. The HPLC results confirmed the extraction of β-carotene because the retention time peaks for carrot-extracted and *R. toruloides-extracted* β-carotene were 6.629 and 6.624 min, similar to that of standard β-carotene as reported by Rashid et al[30] and Grigoryan et al[29]. However, the presence of noise in carrot- and *R. toruloides-extracted* β-carotene respectively indicated the presence of some other metabolites along with the β-carotene.

After the characterization of β-carotene, its biological activities, such as antimicrobial and antioxidant activity, along with its dyeing ability, were analyzed. Both the carrot- and *R. toruloides-extracted* β-carotenes showed the highest antibacterial activity for *Salmonella sp.* Keceli et al[25] also reported that β-carotene has high antimicrobial activity against *Salmonella* and *E. coli*. Moreover, the DPPH assay indicated that both carrot- and *R. toruloides-extracted* β-carotenes have greater antioxidant activity than the carrot-extracted β-carotenes. The DPPH assay is based on the measurement of scavenging activity of antioxidant toward it. The starting point is violet color, which reduces in the presence of antioxidant molecules and loses its color. The valence electron of the nitrogen atom in DPPH is actually reduced by gaining a hydrogen atom from the antioxidant to the corresponding hydrazine[48]. Müller and Böhm[49] described that β-carotene exhibits its antioxidant activity because of its specific chemical structure, which facilitates interactions with biological membranes, helping it to exhibit antioxidant activity. Jaber and Majeed[50] also reported that β-carotene shows high antioxidant activity up to 72%. Basically, the Z-isomers present in β-carotene are responsible for its antioxidant ability.

Finally, the dyeing ability of β-carotene was evaluated by dyeing a piece of cotton. When cotton is immersed in β-carotene solution, the pigment molecules adhere to the cotton fibers via weak bonds. The dyeing ability of β-carotene is because of the conjugated double bonds, which absorb light in the visible spectrum resulting in its characteristic orange color[51]. The similarity of dyeing ability of *R. toruloides-extracted* β-carotenes with the carrot-extracted β-carotene is very significant as it shows its potential application in the textile and food industry.

CONCLUSIONS

In recent years, the demand for natural dyes has increased in the food, cosmetics, and textile industries because of their significance over synthetic dyes. Several plants, fruits, algae, and fungi are natural sources of pigments. Plants are a rich source of pigment, but their slow growth makes them a less preferable source. In this study, β-carotene an orange red pigment was produced from yeast Rhodotorula toruloids and its biological activities were evaluated and compared with carrot extracted β-carotene. The results of the study indicate that β-carotene extracted from R. toruloids showed better antimicrobial and antioxidative potential along with its efficient ability to dye cotton as compared to the carrots β-carotene. Hence, R. toruloids, being easily available and cost-effective production can prove better β-carotene source as compared to carrots.

Ethics Approval

Not applicable.

Consent for publication

This manuscript has not been published and is not being considered for submission to another journal for publication.

Data availability
Will be available on request.

Declaration of competing interest
The author declares no conflict of interest.

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References
1. Berman J, Zorrilla-López U, Farré G, Zhu C, Sandmann G, Twyman RM, Christou P. Nutritionally important carotenoids as consumer products. Phytochem Rev 2015; 14: 727-743. Doi: 10.1007/s11101-014-9373-1.
2. Wang J, Ma W, Ma W, Fang Z, Jiang Y, Jiang W, Jiang M. Strategies for the efficient biosynthesis of β-carotene through microbial fermentation. World J Microbiol Biotechnol 2024; 40: 167. Doi: 10.1007/s11274-024-03955-7.
3. Bogacz-Radomska L, Harasym J. β-Carotene – properties and production methods. Food Qual Saf 2018; 2: 69-74. Doi: 10.1093/fqsafe/fyy004.
4. Kresnowati MTAP, Lestari D. Carotene production from biomass waste. In: Biomass Conversion and Sustainable Biorefinery: Towards Circular Bioeconomy. Singapore: Springer; 2024: pp. 269-278. Doi: 10.1007/978-981-99-7769-7_12.
5. Luthra U, Babu P, RR R, Julius A, Patel Y, Jajula VR, Majeed I. Medium optimization and downstream process design for the augmented yield of β-carotene using fungi Blakeslea trispora. Pigment Resin Technol 2022; 51: 574-580. Doi: 10.1108/prt-05-2021-0055.
6. Li Z, Sun M, Li Q, Li A, Zhang C. Profiling of carotenoids in six microalgae (Eustigmatophyceae) and assessment of their β-carotene productions in bubble column photobioreactor. Biotechnol Lett 2012; 34: 2049-2053. Doi: 10.1007/s10529-012-0996-2.
7. Sereti F, Alexandri M, Papadaki A, Papapostolou H, Kopsahelis N. Natural lycopene and β-carotene synthesis by Rhodosporidium kratochvilovae yeasts: sustainable production, chemical characterization and antioxidative properties. Food Biosci 2024; 57: 103425. Doi: 10.1016/j.fbio.2023.103425.
8. Sankari M, Rao PR, Hemachandran H, Pullela PK, Tayubi IA, Subramanian B, Ramamoorthy S. Prospects and progress in the production of valuable carotenoids: insights from metabolic engineering, synthetic biology, and computational approaches. J Biotechnol 2018; 266: 89-101. Doi: 10.1016/j.jbiotec.2017.12.010.
9. Wang L, Liu Z, Jiang H, Mao X. Biotechnology advances in β-carotene production by microorganisms. Trends Food Sci Technol 2021; 111: 322-332. Doi: 10.1016/j.tifs.2021.02.077.
10. Díaz-Ruiz E, Balbino TR, Dos Santos JC, Kumar V, da Silva SS, Chandel AK. Fermentative production of β-carotene from sugarcane bagasse hydrolysate by Rhodotorula glutinis CCT-2186. Appl Biochem Biotechnol 2024; 196: 4188-4204. Doi: 10.1007/s12010-023-04761-x.
11. Naz T, Ullah S, Nazir Y, Li S, Iqbal B, Liu Q, Song Y. Industrially important fungal carotenoids: advancements in biotechnological production and extraction. J Fungi 2023; 9: 578. Doi: 10.3390/jof9050578.
12. Mapelli-Brahm P, Barba FJ, Remize F, Garcia C, Fessard A, Khaneghah AM, Meléndez-Martínez AJ. The impact of fermentation processes on the production, retention and bioavailability of carotenoids: an overview. Trends Food Sci Technol 2020; 99: 389-401. Doi: 10.1016/j.tifs.2020.03.013.
13. Iqbal H, Rahman A, Khanum S, Arshad M, Badar IH, Asif AR, Hayat Z, Iqbal MA. Effect of essential oil and organic acid on performance, gut health, bacterial count and serological parameters in broiler. Braz J Poult Sci 2021; 23: eRBCA-2021. Doi: 10.1590/1806-9061-2021-1443.
14. Kaur P, Singh S, Ghoshal G, Ramamurthy PC, Parihar P, Singh J, Singh A. Valorization of agri-food industry waste for the production of microbial pigments: an eco-friendly approach. In: Advances in Agricultural and Industrial Microbiology: Volume 1. Singapore: Springer; 2022: pp. 137-167. Doi: 10.1007/978-981-16-8918-5_8.
15. Leck A. Preparation of lactophenol cotton blue slide mounts. Community Eye Health 1999; 12: 24.
16. Young H, Paterson IC, McDonald DR. Rapid carbohydrate utilization test for the identification of Neisseria gonorrhoeae. Sex Transm Infect 1976; 52: 172-175. Doi: 10.1136/sti.52.3.172.
17. Gerelmaa Z, Z Ch, Batjargal B, R Ts. Selection of culture media for the production of carotenoids with antioxidant activity by Rhodotorula glutinis. Proc Mong Acad Sci 2018; 58: 31-38. Doi: 10.5564/pmas.v58i4.1047.
18. Sumerta IN, Yuliani Y, Kanti A. Determining the potential indigenous red-yeasts producing β-carotene and their phylogenetic relationship. J Microb Syst Biotechnol 2019; 1: 27-33. Doi: 10.37604/jmsb.v1i2.31.
19. Kim JI, Lee NK, Yeo IC, Ryu YJ, Park HS, Kim BY, Hahm YT. Isolation of carotenoid-producing yeast, Rhodosporidium babjevae JI-1, and evaluation of cell extract toxicity against rat hepatic cells. J Korean Soc Appl Biol Chem 2012; 55: 137-140. Doi: 10.1007/s13765-012-0024-1.
20. Taswin M, Mangunsong S. How to extract and to examination of β-carotene in carrot (Daucus carota). In: First International Conference on Health, Social

Sciences and Technology (ICOHSST 2020). Atlantis Press; 2021: pp. 252-256.
21. Tao Z, Wang G, Xu X, Yuan Y, Wang X, Li Y. Monitoring and rapid quantification of total carotenoids in Rhodotorula glutinis cells using laser tweezers Raman spectroscopy. FEMS Microbiol Lett 2011; 314: 42-48. Doi: 10.1111/j.1574-6968.2010.02139.x.
22. Latha BV, Jeevaratnam K. Purification and characterization of the pigments from Rhodotorula glutinis DFR-PDY isolated from natural source. Glob J Biotechnol Biochem 2010; 5: 166-174.
23. Tarangini K, Mishra S. Carotenoid production by Rhodotorula sp. on fruit waste extract as a sole carbon source and optimization of key parameters. Iran J Chem Chem Eng 2014; 33: 89-99.
24. Moliné M, Libkind D, van Broock M. Production of torularhodin, torulene, and β-carotene by Rhodotorula yeasts. In: Microbial Carotenoids from Fungi: Methods and Protocols. New York: Humana Press; 2012: 275-283. Doi: 10.1007/978-1-61779-918-1_19.
25. Keceli TM, Erginkaya Z, Turkkan E, Kaya U. Antioxidant and antibacterial effects of carotenoids extracted from Rhodotorula glutinis strains. Asian J Chem 2013; 25: 42-46. Doi: 10.14233/ajchem.2013.12377.
26. Venil CK, Velmurugan P, Dufossé L, Renuka Devi P, Veera Ravi A. Fungal pigments: potential coloring compounds for wide ranging applications in textile dyeing. J Fungi 2020; 6: 68. Doi: 10.3390/jof6020068.
27. Starek M, Guja A, Dąbrowska M, Krzek J. Assay of β-carotene in dietary supplements and fruit juices by TLC-densitometry. Food Anal Methods 2015; 8: 1347-1355. Doi: 10.1007/s12161-014-0019-0.
28. Trivedi N, Tandon S, Dubey A. Fourier transform infrared spectroscopy (FTIR) profiling of red pigment produced by Bacillus subtilis PD5. Afr J Biotechnol 2017; 16: 1507-1512. Doi: 10.5897/AJB2017.15959.
29. Grigoryan AA, Hayrapetyan AA, Azaryan ZA, Harutyunyan SV, Yengoyan AP. HPLC analysis of vitamins C, E, beta-carotene, and some flavonoids in Armenian red wines. Curr Anal Chem 2024; 20: 109-114.
30. Rashid MM, Islam S, Uddin MN, Al Mamun MZU, Abedin MJ, Bhuiyan MHR, Miah MAS. HPLC-DAD analysis of water-soluble vitamins (B1, B2, B3, B5, B6, C and biotin) and fat-soluble vitamins (A, D, E, K1 and β-carotene) in commonly consumed pulses in Bangladesh. Appl Food Res 2024; 4: 100424. Doi: 10.1016/j.afres.2024.100424.
31. Bahaa Edeen A. Dyeing of Egyptian cotton fabrics with orange peel using padding technique. Int Des J 2015; 5: 511-516. Doi: 10.21608/idj.2015.101805.
32. Singh RV, Sambyal K. An overview of β-carotene production: current status and future prospects. Food Biosci 2022; 47: 101717. Doi: 10.1016/j.fbio.2022.101717.
33. Soliman H, Elsayed A, Dyaa A. Antimicrobial activity of silver nanoparticles biosynthesised by Rhodotorula sp. strain ATL72. Egypt J Basic Appl Sci 2018; 5: 228-233. Doi: 10.1016/j.ejbas.2018.05.005.
34. Ansari AR, Arshad M, Masood S, Huang HB, Zhao X, Li N, Sun Z, Cui L, Hu Y, Liu HZ. Salmonella infection may alter the expression of toll like receptor 4 and immune related cells in chicken bursa of Fabricius. Microb Pathog 2018; 121: 59-64. Doi: 10.1016/j.micpath.2018.05.019.
35. Kim JK, Kim JI, Lee NK, Hahm YT, Baik MY, Kim BY. Extraction of β-carotene produced from yeast Rhodosporidium sp. and its heat stability. Food Sci Biotechnol 2010; 19: 263-266. Doi: 10.1007/s10068-010-0038-6.
36. Hu P, Mao J, Zeng Y, Sun Z, Deng H, Chen C, Tang Z. Isolation, identification, and function of Rhodotorula mucilaginosa TZR2014 and its effects on the growth and health of weaned piglets. Front Microbiol 2022; 13: 922136. Doi: 10.3389/fmicb.2022.922136.
37. Tao Z, Yuan H, Liu M, Liu Q, Zhang S, Liu H, Wang T. Yeast extract: characteristics, production, applications and future perspectives. J Microbiol Biotechnol 2023; 33: 151-159. Doi: 10.4014/jmb.2207.07057.
38. Saha N, Samanta AK, Chaudhuri S, Dutta D. Characterization and antioxidant potential of a carotenoid from a newly isolated yeast. Food Sci Biotechnol 2015; 24: 117-124. Doi: 10.1007/s10068-015-0017-z.
39. Kaur P, Ghoshal G, Jain A. Bio-utilization of fruits and vegetables waste to produce β-carotene in solid-state fermentation: characterization and antioxidant activity. Process Biochem 2019; 76: 155-164. Doi: 10.1016/j.procbio.2018.10.007.
40. Nguyen TM, Nguyen TT, Nguyen TT, Vo NN, Vo NT, Nguyen YT. Optimization of in vitro carotenoid production by Rhodotorula toruloides. Chem Eng Trans 2024; 108: 55-60.
41. Sharma R, Ghoshal G. Optimization of carotenoids production by Rhodotorula mucilaginosa (MTCC-1403) using agro-industrial waste in bioreactor: a statistical approach. Biotechnol Rep 2020; 25: e00407. Doi: 10.1016/j.btre.2019.e00407.
42. Rodríguez NAT, Quiñones-Cerna CE, Castillo HMR, Cruz-Monzon JA, Butrón FJH, Soto JCR. Optimization of total carotenoid production by Rhodotorula mucilaginosa from artichoke agroindustrial waste using response surface methodology. Environ Res Eng Manag 2023; 79: 111-121. Doi: 10.5755/j01.erem.79.2.32468.
43. Zarandi-Miandoab L, Hejazi MA, Bagherieh-Najjar MB, Chaparzadeh N. Optimization of the four most effective factors on β-carotene production by Dunaliella salina using response surface methodology.

Iran J Pharm Res 2019; 18: 1566-1577. Doi: 10.22037/ijpr.2019.1100752.
44. Karnjanawipagul P, Nittayanuntawech W, Rojsanga P, Suntornsuk L. Analysis of β-carotene in carrot by spectrophotometry. Mahidol Univ J Pharm Sci 2010; 37: 8-16.
45. Nagaraj YN, Burkina V, Okmane L, Blomqvist J, Rapoport A, Sandgren M, Passoth V. Identification, quantification and kinetic study of carotenoids and lipids in Rhodotorula toruloides CBS 14 cultivated on wheat straw hydrolysate. Fermentation 2022; 8: 300. Doi: 10.3390/fermentation8070300.
46. Payne TD, Dixon LR, Schmidt FC, Blakeslee JJ, Bennett AE, Schultz ZD. Identification and quantification of pigments in plant leaves using thin layer chromatography-Raman spectroscopy (TLC-Raman). Anal Methods 2024; 16: 2449-2455. Doi: 10.1039/D4AY00082J.
47. Arshad M, Hussain T, Chaudhry N, Sadia H, Aslam B, Tahir U, Abbas M, Qureshi Q, Nazir N, Rajoka MI, Iqbal M. Enhancing profitability of ethanol fermentation through gamma ray mutagenesis of Saccharomyces cerevisiae. Pol J Environ Stud 2019; 28: 1-9. Doi: 10.15244/pjoes/78708.
48. Kedare SB, Singh RP. Genesis and development of DPPH method of antioxidant assay. J Food Sci Technol 2011; 48: 412-422. Doi: 10.1007/s13197-011-0251-1.
49. Müller L, Böhm V. Antioxidant activity of β-carotene compounds in different in vitro assays. Molecules 2011; 16: 1055-1069. Doi: 10.3390/molecules16021055.
50. Jaber BA, Majeed KR. Antioxidant and antibacterial activity of β-carotene pigment extracted from Parococcus homiensis strain BKA7 isolated from air of Basra, Iraq. Ann Rom Soc Cell Biol 2021; 25: 14006-14028.
51. Suryana MR, Haziman ML, Islamawan PA, Hariadi H, Yusuf D. Use of beta-carotene pigment to improve food product chemical and sensory qualities: a review. J Funct Food Nutraceutical 2023; 4(2). Doi: 10.33555/jffn.v4i2.92.