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## Taguchi-Based Optimization of Culture Conditions for Enhanced Carotenoids and Astaxanthin Production in *Thraustochytrium* sp. 3F

**Touba Nakhzari Khodakhier**

Ph.D. Candidate in Cellular and Molecular Biology, Department of Biotechnology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran.  
[nakhzari.touba1986@gmail.com](mailto:nakhzari.touba1986@gmail.com)

**Elham Iranmanesh**

Assistant Professor, Department of Chemical Engineering, Faculty of Chemistry and Chemical Engineering, Graduate University of Advanced Technology, Kerman, Iran.  
[e.iranmanesh1@gmail.com](mailto:e.iranmanesh1@gmail.com)

**Farshad Darvishi**

Professor of Applied Microbiology, Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran.  
[f.darvishi@alzahra.ac.ir](mailto:f.darvishi@alzahra.ac.ir)

**Shahryar Shakeri**<sup>\*1</sup> 

Associate Professor, Department of Biotechnology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran.  
[Shahryar.shakeri@yahoo.com](mailto:Shahryar.shakeri@yahoo.com)

### Abstract

Astaxanthin is a xanthophyll carotenoid with potent antioxidant activity that has attracted substantial attention due to its wide pharmaceutical, nutritional, and industrial applications. It is also utilized as a natural and safe pigment in food product and aquaculture feed. In the cosmetic industry, astaxanthin is recognized for its anti-aging and photoprotective properties. Recently, marine thraustochytrids have emerged as promising cell factories for eco-efficient and large-scale biosynthesis of astaxanthin and total carotenoids, providing a sustainable alternative to traditional chemical synthesis. In this study, the cultivation conditions of *Thraustochytrium* sp. 3F, a marine protist isolated from mangrove leaf litter along the southern coast of Iran, were optimized to enhance carotenoid biosynthesis, particularly astaxanthin. A Taguchi L9 orthogonal array design was applied to evaluate the effects of glucose and yeast extract concentration, seawater proportion, and agitation speed on biomass growth and carotenoid accumulation. Cultivation conditions significantly influenced both biomass formation and carotenoid yields. Based on Taguchi analysis, an optimal set of cultivation parameters, comprising 10 g/L glucose, 3 g/L yeast extract, 20 % (v/v) seawater, and 170 rpm was predicted and experimentally validated, resulting in balanced biomass production and substantial carotenoid accumulation. Time-course analysis indicated that carotenoid and astaxanthin biosynthesis primarily occurred during the stationary phase of biomass growth, accompanied by depletion of glucose and nitrogen sources. These findings highlight the importance of fine-tuning carbon, nitrogen, and salinity inputs to optimize biomass growth and secondary metabolite production that demonstrates the potential of *Thraustochytrium* sp. 3F as a sustainable natural source of carotenoids.

**Keywords:** Marine protist, Natural pigment, Antioxidant carotenoids, Culture optimization, Orthogonal array design.

<sup>1</sup> Corresponding Author  
[3060-7647](mailto:3060-7647) / © 2026 The Authors

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## Introduction

Thraustochytrids are marine heterotrophic protists widely distributed in coastal and mangrove ecosystems and are increasingly recognized for their capacity to synthesize high-value biomolecules. These microorganisms are notable producers of carotenoids, including  $\beta$ -carotene, canthaxanthin, and astaxanthin, as well as long-chain polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (1). These metabolites not only play ecological roles in marine environments but also hold substantial industrial and nutraceutical potential. Among these compounds, astaxanthin is a prominent factor as a particularly powerful antioxidant (2, 3). Astaxanthin is a lipid-soluble xanthophyll carotenoid characterized by a conjugated polyene chain and polar functional groups, which enable strong interactions with biological membranes and efficient scavenging of reactive oxygen species (4, 5). As a result, astaxanthin often exhibits higher antioxidant activity than other well-known carotenoids and antioxidants, such as  $\beta$ -carotene and vitamin E (6, 7). Although synthetic astaxanthin has been widely used in industrial applications, increasing concerns regarding safety, sustainability, and consumer preference have driven a growing demand for naturally derived sources (8, 9). In nature, astaxanthin is mainly synthesized as a secondary metabolite by microalgae, yeasts, and certain marine protists. Currently, the production of natural astaxanthin is dominated by microalgae- and yeast-based systems, particularly *Haematococcus pluvialis* and *Xanthophyllomyces dendrorhous* (10). However, despite their well-established biosynthetic capacity, these production platforms face several limitations that hinder large-scale industrial implementation. Algal cultivation systems are often constrained by slow growth rates, strong dependence on light availability, complex two-stage cultivation strategies, and limited volumetric productivity, all of which significantly increase production costs (11, 12).

Similarly, yeast-based systems, although easier to cultivate under heterotrophic conditions, often exhibit lower astaxanthin yields and require stringent control of fermentation parameters to maintain product stability and productivity. Collectively, these challenges reduce process efficiency and restrict the economic feasibility of large-scale natural astaxanthin manufacturing (13). In this context, thraustochytrids have emerged as attractive yet underexplored alternative platforms for carotenoid production. As fast-growing heterotrophic marine protists, thraustochytrids can be cultivated independently of light under controlled

fermentation conditions, enabling higher cell densities, improved process robustness, and easier scalability in industrial bioreactors compared to photoautotrophic microalgae. Moreover, their metabolic versatility allows the simultaneous accumulation of lipids and carotenoids, offering opportunities for integrated bioprocesses and improved overall production economics. Despite these advantages, the potential of thraustochytrids as efficient astaxanthin producers remains insufficiently characterized, particularly with respect to the optimization of cultivation parameters governing pigment biosynthesis and accumulation (14).

Carotenoid biosynthesis in thraustochytrids is strongly influenced by environmental and nutritional factors, including the carbon-to-nitrogen ratio, salinity, temperature, and agitation speed. Modulation of these parameters can redirect cellular metabolism toward enhanced secondary metabolite accumulation; however, such shifts are frequently accompanied by reduced biomass formation (15). Consequently, identifying optimal cultivation strategies requires systematic evaluation of multiple interacting factors, rather than conventional one-variable-at-a-time approaches.

Statistical optimization techniques, particularly Taguchi's design of experiments (DOE), provide an efficient framework for investigating multifactorial effects while minimizing experimental workload. Taguchi DOE employs orthogonal arrays to assess the relative contribution of individual factors and their interactions using a limited number of experiments. Previous studies have demonstrated the effectiveness of Taguchi-based optimization for improving lipid and pigment production in microorganisms. For example, Shene et al. (16) applied a Taguchi orthogonal array to a native *Thraustochytrium* strain, showing that precise optimization of carbon and nitrogen sources significantly enhanced both biomass and DHA accumulation. Similarly, Korumilli et al. (17) used a Taguchi L9 orthogonal array design to optimize astaxanthin production in *X. dendrorhous* cultured on fruit-waste extracts. Their results demonstrated that simultaneous evaluation of key factors, including pH, temperature, and agitation speed, enabled identification of conditions that significantly enhanced pigment yield in the yeast. In addition, Tharek et al. (18) applied Taguchi optimization to *Coelastrum* sp., identifying optimal salinity, nutrient concentrations, and light conditions that nearly doubled astaxanthin output. These findings underscore that Taguchi DOE is not only adaptable but also experimentally economical, and its successful application across thraustochytrids, yeasts, and microalgae highlights its promise as a

tool for enhancing secondary metabolite production. Despite these advances, studies specifically applying Taguchi DOE to optimize astaxanthin biosynthesis in *thraustochytrids* remain limited. Therefore, the present study employs a Taguchi L9 orthogonal array design to systematically evaluate the combined effects of glucose concentration, yeast extract concentration, seawater proportion, and agitation speed on biomass growth and astaxanthin and total carotenoid production in *Thraustochytrium* sp. 3F. This work aims to establish an efficient and sustainable cultivation strategy and to clarify the potential of this marine protist as a natural producer of high-value carotenoid pigments.

## Materials and Methods

### Microorganism and Culture Preparation

The *thraustochytrid* strain used in this study, *Thraustochytrium* sp. 3F, was originally isolated from decaying mangrove leaves collected along the southern coast of Iran (19). Initial isolation was performed on potato dextrose agar (PDA) at 30 °C for 48 h. Emerging colonies were subsequently subcultured on glucose–yeast extract–peptone (GYP) agar to obtain pure cultures. Stock cultures were maintained in 20% glycerol at –80 °C until required for experimental uses.

### Experimental Design

Optimization of culture conditions was performed using a Taguchi orthogonal array design (L9), implemented through Design-Expert software. Four independent factors were selected for evaluation: glucose concentration (10, 15, 20 g/L), yeast extract concentration (1, 3, 5 g/L), seawater content (20, 60, 100% v/v), and agitation speed (140, 170, 200 rpm). Nine experimental runs, each representing a unique combination of factor levels, were generated by the software (Table 1). All cultures were grown in 250-mL Erlenmeyer flasks containing 50 mL of medium.

Table 1. Taguchi L9 design used to evaluate the effects of key cultivation factors on biomass growth, total carotenoid and astaxanthin production.

| Run | Factor 1<br>(Glu) | Factor 2<br>(YE) | Factor 3<br>(Seawater) | Factor 4<br>(rpm) |
|-----|-------------------|------------------|------------------------|-------------------|
| 1   | 10                | 1                | 20                     | 140               |
| 2   | 15                | 1                | 60                     | 200               |
| 3   | 15                | 3                | 100                    | 140               |
| 4   | 15                | 5                | 20                     | 170               |
| 5   | 20                | 3                | 20                     | 200               |
| 6   | 20                | 1                | 100                    | 170               |
| 7   | 10                | 5                | 100                    | 200               |
| 8   | 20                | 5                | 60                     | 140               |
| 9   | 10                | 3                | 60                     | 170               |

Abbreviations: Glu; glucose, YE; yeast extraction. All experiments were conducted in three independent replicates

### Cultivation Conditions

For each experimental run, culture media were prepared according to the factor-level combinations generated by the Taguchi L9 orthogonal array in Design-Expert software. The required concentrations of glucose, yeast extract, and seawater were adjusted by mixing appropriate proportions of distilled water and seawater to achieve the designated salinity levels. The media were then sterilized by autoclaving at 121 °C for 20 min. Following sterilization, *Thraustochytrium* sp. 3F was inoculated into each medium and incubated at 30 °C for 5 days in a shaking incubator to the specified agitation speed (20).

### Biomass Determination

At the end of the cultivation period, cells were harvested by centrifugation at 7000 × g for 10 min. The pellets were washed with distilled water and dried at 60 °C to constant weight. Biomass concentration was expressed as grams of dry weight per liter (g/L).

### Carotenoids and astaxanthin extraction

Total carotenoids were extracted from the biomass using a chloroform: methanol mixture. First, the biomass was sonicated to disrupt the cells and release the pigments. The resulting suspension was centrifuged to remove cell debris. Subsequently, chloroform: methanol (mL) (3:1, v/v) was added to the supernatant, and the mixture was stirred with a magnetic stirrer to ensure complete extraction. After thorough mixing, the sample was centrifuged again to separate the phases, and the lower organic layer was collected as the carotenoid fraction. This fraction was stored at –80 °C until further analysis (20, 21).

### Thin-Layer Chromatography (TLC)

TLC was employed to separate and preliminarily identify carotenoids extracted from the microbial biomass. Silica gel 60 F<sub>254</sub> plates were used as the stationary phase, and 5 µL of each sample was applied onto the plate. The mobile phase consisted of hexane: acetone (7:3 v/v), and the plates were developed to a distance of approximately 7 cm. After development, carotenoids appeared as distinct colored spots under visible light. The retention factor ( $R_f$ ) for each compound was calculated by using Equation 1:

$$R_f = \frac{Z_s}{Z_F - Z_0} \quad (1)$$

Where  $Z_s$  is the distance traveled by the compound,  $Z_F$  is the distance traveled by the solvent front, and  $Z_0$  represents the initial spotting line. The

standard  $R_f$  values for major carotenoids are summarized in Table 2 and were used as reference benchmarks for identifying the separated carotenoid bands. This approach enabled the preliminary identification of astaxanthin and other carotenoids by comparing their  $R_f$  values with those of authentic standards (20, 22).

Table 2. Reference  $R_f$  values of standard carotenoids used for TLC identification.

| Carotenoids            | $R_f$ values |
|------------------------|--------------|
| $\beta$ -Carotene      | 0.99         |
| Echinenone             | 0.87         |
| Astaxanthin Diesters   | 0.75         |
| Astaxanthin Monoesters | 0.5          |
| Canthaxanthin          | 0.4          |
| Astaxanthin Free       | 0.33         |
| Lutein                 | 0.25         |

### UV-Vis Spectral Characterization of Carotenoids

Carotenoid extracts obtained from the culture media were centrifuged at 5000 rpm for 8 min to obtain a clear supernatant suitable for spectral analysis. The supernatants were subsequently diluted in acetone and thoroughly mixed to ensure complete solubilization of the pigments. The absorbance profiles were recorded within the wavelength range of 300–750 nm using a UV-Vis spectrophotometer. Characteristic absorption bands of carotenoids were observed in the same range, showing a distinct maximum around 470 nm. These spectral profiles were used for qualitative evaluation of pigment composition and the assessment of carotenoid purity. The total carotenoid content (C) was determined according to the Equation 2:

$$C = \frac{A_{470nm} V_{extract} DF}{0.2 W_{sample}} \quad (2)$$

Where  $A_{470nm}$  is the absorbance measured at 470 nm,  $V_{extract}$  represents the total volume of the extract (mL), DF is the dilution factor, 0.2 denotes the specific absorption coefficient of astaxanthin, and  $W_{sample}$  corresponds to the dry weight of sample (g) (20, 21).

### HPLC analysis

For astaxanthin quantification, pigment extracts were analyzed using high-performance liquid chromatography (HPLC, Agilent 1100 series) equipped with a C<sub>18</sub> column and photodiode array detector (PDA). Sample preparation involved

dissolving the extracted carotenoids in 800  $\mu$ L of the appropriate solvent, followed by filtration through a 0.2  $\mu$ m syringe filter to remove particulate matter. The prepared extracts were subsequently injected into a C18 column (25  $\times$  4.6 mm) connected to the Agilent HPLC system. The mobile phase consisted of a mixture of methanol (Me), ethyl acetate (EA), and water (W) applied in a gradient elution program. The gradient was initiated with 75% Me, 23% EA, and 2% W for the first 10 min, followed by adjustment to 48% Me, 50% EA, and 2% W between 10 to 35 min. The flow rate was maintained at 1 mL/min, and detection was performed at 460 nm. Retention times were recorded for identification of individual carotenoids. Peak identification and quantification were based on retention times and comparison with authentic standards. Method validation was carried out to ensure accuracy and reproducibility. Finally, astaxanthin concentrations were reported as  $\mu$ g/g DW (21, 23).

### Determination of residual glucose concentration

Residual glucose in the culture medium was quantified by HPLC (Agilent 1100 series) equipped with a refractive index detector (RID). Separation was performed on a Rezex RCM-Monosaccharide column (300  $\times$  7.8 mm, ion-exclusion resin, Phenomenex) under isocratic elution with 5 mM sulfuric acid as the mobile phase, operated at a flow rate of 1.0 mL/min. The column temperature was maintained at 60 °C throughout the chromatographic run. Glucose concentration in the samples were determined by comparison with a calibration curve constructed using known glucose standards (24).

### Measurements of amino acids

Total amino acid content in the samples was determined spectrophotometrically using OPA<sup>2</sup> reagent. For each measurement, 100  $\mu$ L of the culture sample was mixed with 1 mL of OPA reagent, incubated for 2 min, and the absorbance was measured at 340 nm. The OPA reagent was freshly prepared by dissolving 5 mg OPA in 100  $\mu$ L absolute ethanol, followed by the addition of 5  $\mu$ L of 2-mercaptoethanol and 10 mL 50 mM sodium carbonate buffer (pH 10.5) (25, 26). The reagent alone was used as a blank control. Calibration was carried out using L-lysine standards for quantification of total amino acids.

## Results

### Optimization of Culture Conditions

The Taguchi design of experiments revealed that a balanced combination of carbon, nitrogen, salinity, and agitation is critical for achieving optimal biomass growth and secondary metabolite synthesis in *Thraustochytrium* sp. 3F. Employing a Taguchi L9 orthogonal array, the effects of glucose

concentration, yeast extract, seawater concentration, and agitation speed on biomass yield and carotenoid productivity were systematically analyzed. The nine experimental runs revealed distinct trade-offs between cell growth and pigment accumulation (Table 3).

Table 3. Biomass concentration, total carotenoid content, and astaxanthin production obtained under different Taguchi L9 experimental runs.

| Run | Biomass (g/l) | Total Carotenoids (µg/gDW) | Astaxanthin (µg/gDW) |
|-----|---------------|----------------------------|----------------------|
| 1   | 0.788         | 150.09                     | 44.34                |
| 2   | 1.2           | 61.31                      | 12.11                |
| 3   | 2.74          | 41.83                      | 8.26                 |
| 4   | 3.22          | 40.18                      | 26.27                |
| 5   | 1.45          | 74.68                      | 32.07                |
| 6   | 4.36          | 28.1                       | 5.59                 |
| 7   | 3.38          | 40.9                       | 7.99                 |
| 8   | 1.96          | 48.1                       | 15.3                 |
| 9   | 3.18          | 40.6                       | 35.9                 |

Experimental runs with high glucose but low yeast extract (e.g., Run 6: 20 g/L glucose, 1 g/L yeast extract, 100% seawater, 170 rpm) achieved the highest biomass accumulation (4.36 g/L) but showed markedly reduced carotenoid yields (< 30 µg/gDW). This pattern indicates that nitrogen limitation under high carbon availability redirected cellular metabolism toward rapid biomass formation, thereby suppressing secondary metabolite synthesis. In contrast, runs conducted with moderate glucose and higher yeast extract (e.g., Run 2: 15 g/L glucose, 1 g/L yeast extract, 60% seawater, 200 rpm; and Run 8: 20 g/L glucose, 5 g/L yeast extract, 60% seawater, 140 rpm) resulted in reduced biomass formation (< 2 g/L) but substantially elevated carotenoid accumulation (> 50 µg/gDW). This findings suggests that sufficient nitrogen availability combined with moderate carbon supply enhanced pigment biosynthesis. Runs with low salinity (20% seawater) consistently favored carotenoid production compared to high salinity (100% seawater), highlighting the inhibitory effect of osmotic stress on pigment accumulation. Meanwhile, agitation at 170 rpm generally improved astaxanthin yields, whereas agitation at 140 rpm promoted total carotenoid accumulation, suggesting a differential oxygen requirement for specific pigment pathways. Among the nine experimental runs, the optimized condition (10 g/L glucose, 3 g/L yeast extract, 20% seawater, 170 rpm) provided the most favorable trade-off, yielding balanced biomass

production (1.7 g/L) alongside elevated carotenoid content (76 µg/gDW).

### Analysis of carotenoids using TLC

The carotenoid extract obtained from *Thraustochytrium* sp. 3F was analyzed using TLC. Each separated band on the TLC plate corresponded to a specific carotenoid. The  $R_f$ -value for each band was calculated using Equation 1, and individual carotenoids were identified by comparing their  $R_f$  values with the corresponding standards shown in Table 2. This figure depicts the bands developed on the TLC plate. Based on the analysis, Band 1, originating from the baseline, corresponds to free astaxanthin with a  $R_f = 0.35$ , Band 2 ( $R_f = 0.46$ ) as canthaxanthin, Band 3, ( $R_f = 0.51$ ) astaxanthin monoesters, finally band 4 was identified astaxanthin diesters with  $R_f = 0.70$ , while band 5 corresponds to  $\beta$ -carotene ( $R_f = 0.87$ ) (Table 4).

Table 4. Identification of carotenoid fractions produced by *Thraustochytrium* sp. 3F based on TLC analysis.

| N | Carotenoids           | $R_f$ values |
|---|-----------------------|--------------|
| 1 | Astaxanthin Free      | 0.35         |
| 2 | Canthaxanthin         | 0.46         |
| 3 | Astaxanthin Monesters | 0.51         |
| 4 | Astaxanthin Diesters  | 0.7          |
| 5 | $\beta$ -Carotene     | 0.87         |

Carotenoid identification was performed by comparison of  $R_f$  values with authentic standards under identical chromatographic conditions.

The relative positions and intensities of these bands are illustrated in Fig. 1, clearly showing the separation pattern of the major carotenoid components produced by *Thraustochytrium* sp. 3F.

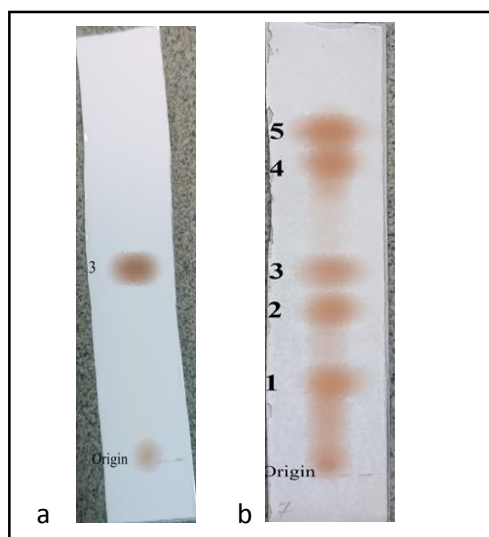


Fig. 1. Comparative TLC analysis of carotenoids from *Thraustochytrium* sp. F and authentic astaxanthin standard: a) astaxanthin reference. b) microbial carotenoid extract.

### Spectrophotometric analysis of Carotenoids

As shown in Fig. 2, the UV–Vis absorption spectrum of the extract was recorded in the wavelength range of 470–477 nm. The observed absorption maximum at 470 nm indicates the presence of the secondary carotenoids. The spectrum recorded in the broader range of 450–480 nm was consistent with standard reference values, confirming the identification of astaxanthin. The relative absorbance at the maximum wavelength was determined, demonstrating that the carotenoid extract contains significant amounts of astaxanthin.

### Analysis of Carotenoids Using HPLC

The carotenoid composition of the *Thraustochytrium* sp. 3F culture was analyzed using HPLC. As shown in Fig. 3, the chromatogram exhibits clear separation of individual carotenoids based on their retention times. The major peak corresponds to astaxanthin, indicating that this compound is produced in considerable amounts under the evaluated culture conditions. Minor peaks were also observed, representing additional carotenoids present in smaller quantities. Quantitative analysis based on peak areas revealed the relative abundance of each carotenoid, confirming the high production of astaxanthin in the optimized culture medium. These results demonstrate the effectiveness of HPLC for the accurate identification and quantification of carotenoids in microbial extracts.

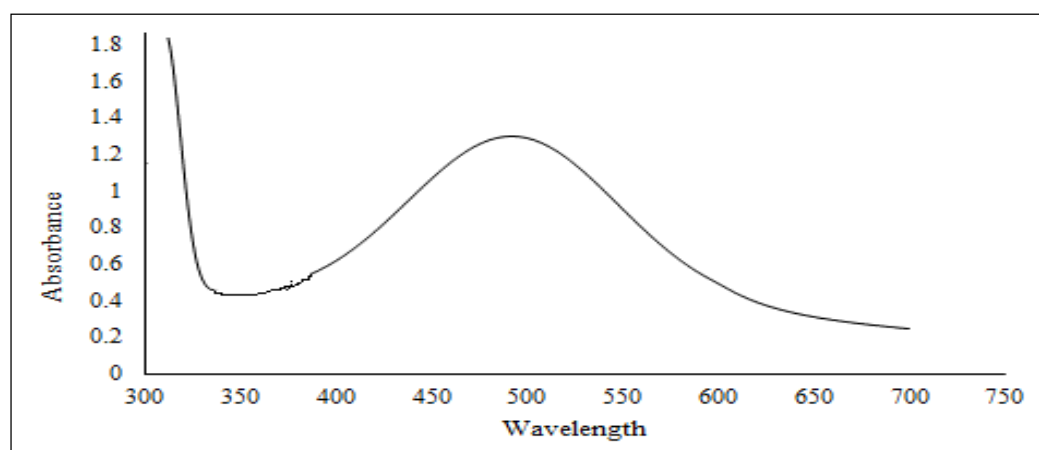


Fig. 2. UV–Vis absorption spectrum of carotenoid extract from *Thraustochytrium* sp. 3F, showing a characteristic peak at 470 nm indicative of astaxanthine.

### Effect of salinity and agitation

Salinity and agitation were identified as critical determinants influencing culture performance. Excessive seawater concentration (100%, Run 6) appeared to impose osmotic stress, which reduced carotenoid accumulation despite maintaining

substantial biomass formation. In contrast, moderate salinity (20%) supported optimal pigment biosynthesis, likely by alleviating oxidative stress while sustaining metabolic activity. Similarly, elevated agitation (170 rpm) enhanced oxygen transfer and stimulated astaxanthin synthesis,

whereas reduced agitation (140 rpm; Run 1) limited cell proliferation but coincided with relatively higher carotenoid accumulation. These findings highlight the intricate interplay between

hydrodynamic and osmotic factors in modulating the organism's metabolic allocation between growth and secondary metabolite production.

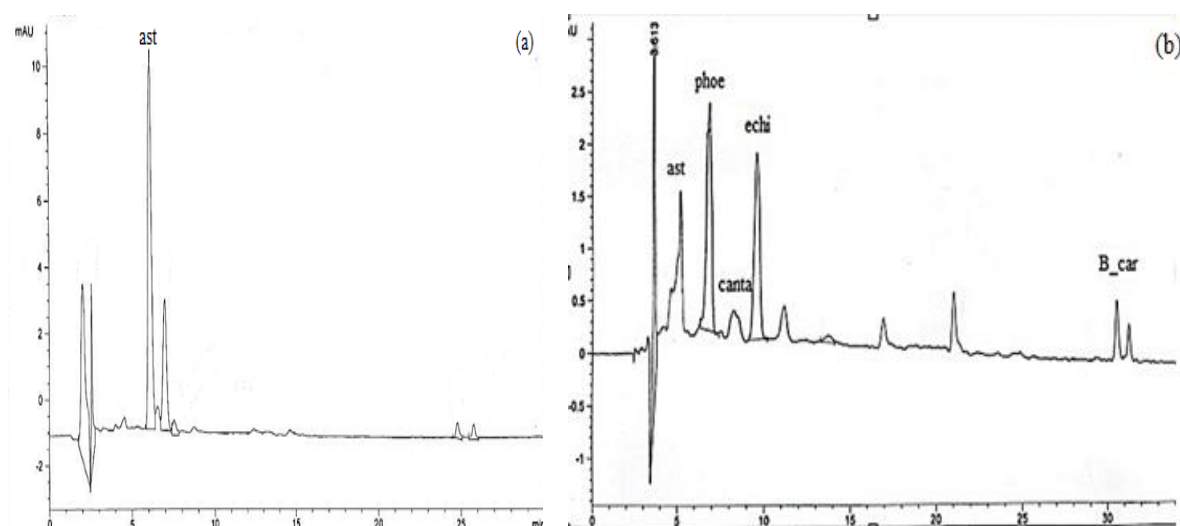


Fig. 3. HPLC chromatographic analysis of carotenoids. (a) HPLC chromatogram of an authentic astaxanthin standard. (b) HPLC chromatogram of the carotenoid extract obtained from *Thraustochytrium* sp. 3F under optimized cultivation conditions. Carotenoid compounds were identified by comparing the retention times of sample peaks with those of the corresponding standards.

To further illustrate the interactive effects of the evaluated cultivation conditions on growth and pigment biosynthesis, a comparative analysis of biomass and pigment production across the Taguchi design runs was performed. A clear trade-off was observed between cellular proliferation and secondary metabolite formation. Cultures achieving higher biomass yields, such as Run 6, exhibited reduced carotenoid accumulation, whereas moderate growth conditions (e.g., Runs 1 and 5) favored enhanced synthesis of total carotenoids and

astaxanthin. All values represent the mean of three independent replicates, with variability indicated by error bars in Fig. 4. This pattern suggests that nutrient limitation and adaptive environmental stress redirect cellular metabolism toward secondary carotenoid pathways. Consequently, these findings highlight that maximizing pigment yield in *Thraustochytrium* sp. 3F requires maintaining a delicate balance between growth-promoting and stress-inducing factors.

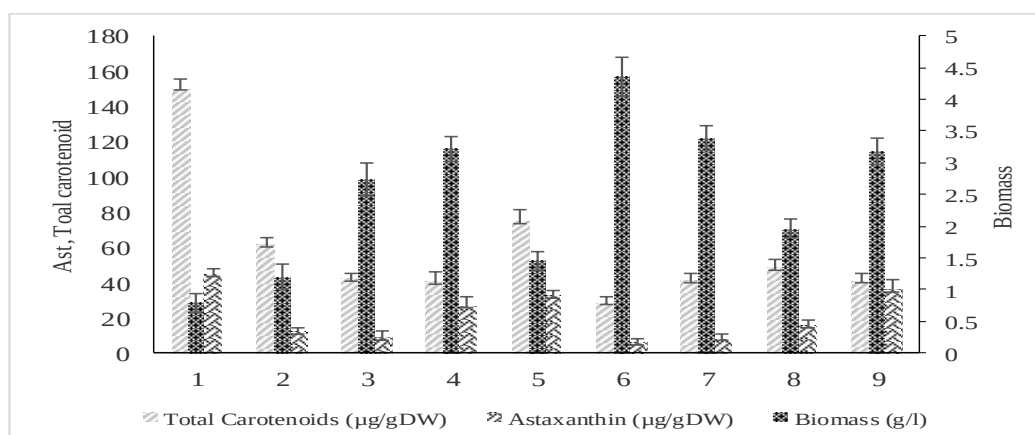


Fig. 4. Comparative analysis of biomass yield, total carotenoids, and astaxanthin content across the nine Taguchi L9 experimental runs. The figure illustrates the trade-off between cellular growth and secondary metabolite accumulation.

### The effect of selected factors on astaxanthin production

As illustrated in Fig. 5, the effects of selected cultivation parameters on the growth performance of *Thraustochytrium* sp.3F for astaxanthin production were investigated. The culture conditions were systematically optimized by varying yeast extract

concentration (3 g/L), glucose concentration (10 g/L), salinity corresponding to 20% seawater, and agitation speed (170 rpm). These parameters were identified as key factors influencing both cell biomass accumulation and carotenoid biosynthesis efficiency under optimized cultivation conditions.

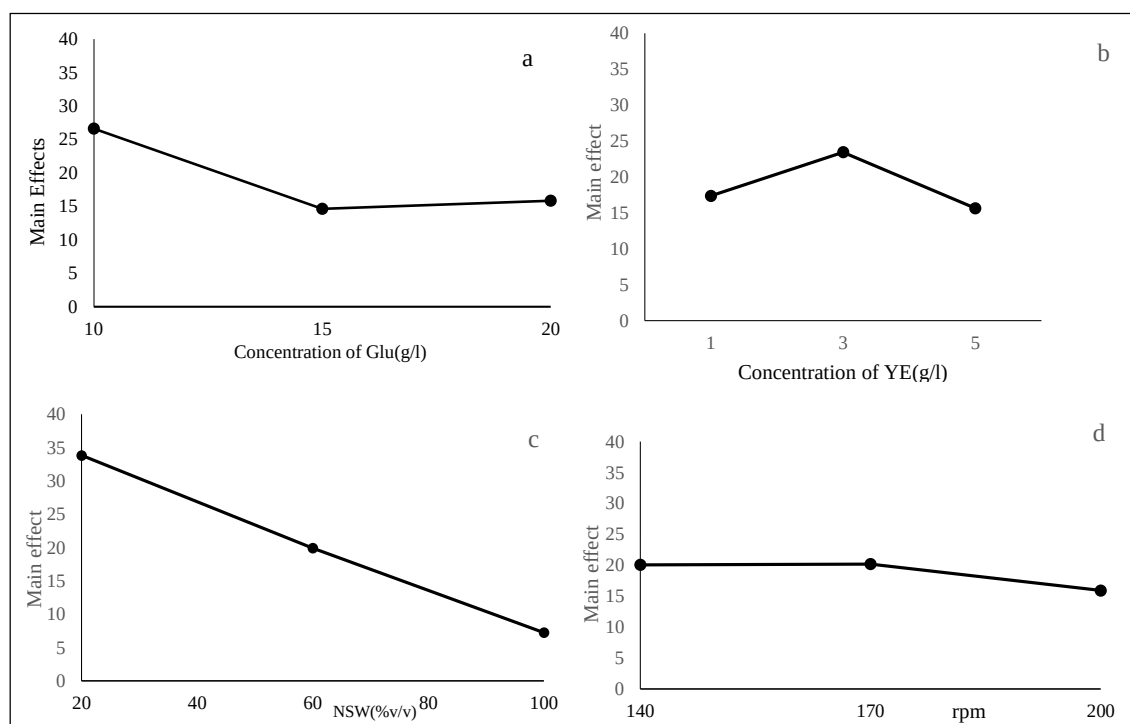


Fig. 5: Main effects of cultivation conditions, including (a) glucose concentration, (b) yeast extract concentration, (c) seawater proportion, and (d) agitation speed, on astaxanthin accumulation in *Thraustochytrium* sp. 3F.

### Dynamic Cultivation under Optimized Conditions

A detailed time-series experiment was conducted under the optimized conditions (10 g/L glucose, 3 g/L yeast extract, 20% seawater, 170 rpm) to monitor substrate consumption, biomass growth, and product accumulation over 120 h. Glucose decreased steadily from ~33.5 mM to near zero (~0.07 mM), reflecting continuous utilization for metabolic needs. Nitrogen, however, declined more sharply—from ~0.0046 mM at inoculation to only ~0.0004 mM by ~95 h—indicating that it became limiting much earlier in the process. Correspondingly, biomass remained minimal during the initial 8 h but increased significantly between 23–95 h, peaking at 2.4 g/L. After 95 h, biomass began to decline, reaching 1.3 g/L by 125 h, likely due to nutrient depletion, oxidative stress, or cell lysis. Meanwhile, carotenoid accumulation showed a delayed onset: the first detectable concentration (~36 µg/gDW) appeared at 23 h, followed by a steady increase to a maximum (~203 µg/gDW) at 95

h, and then a decline to ~84 µg/gDW by 120–130 h. This analysis demonstrates a clear separation between the growth and pigment biosynthesis phases, with nitrogen exhaustion acting as a metabolic switch (Table 5).

The time-course analysis of *Thraustochytrium* sp. 3F cultivation (Fig. 6) revealed distinct metabolic transitions throughout the growth period. Biomass progressively increased, attaining a peak of 2.4 g/L at 95 h, followed by a slight decline, indicating entry into the stationary phase. Carotenoid accumulation paralleled this phase, reaching a maximum level of 203 µg/gDW at approximately 80–95 h, indicating that secondary metabolite biosynthesis predominantly occurs under growth-limiting conditions. The glucose concentration consistently decreased, nearing exhaustion by 128 h, concomitant with the depletion of nitrogen availability. These findings demonstrate that biomass formation and carotenoid accumulation proceed concurrently with nutrient utilization. Altogether, the obtained time-course data provide a comprehensive depiction of

the dynamic interplay among growth kinetics, secondary metabolite production, and nutrient consumption under optimized cultivation conditions.

Table 5: Time-dependent changes in glucose and nitrogen concentrations, biomass production, and carotenoid content during cultivation of *Thraustochytrium* sp. F under optimized conditions (10 g/L glucose, 3 g/L yeast extract, 20 % seawater, 170 rpm).

| Time(h) | Glucose(mmol/l) | Nitrogen(mmol/l) | Biomass(g/l) | Total. C( $\mu$ g/gDW) |
|---------|-----------------|------------------|--------------|------------------------|
| 0       | 33.48           | 0.00457          | 0            | 0                      |
| 8       | 33.092          | 0.00356          | 0            | 0                      |
| 23      | 32.481          | 0.00302          | 0.3          | 36                     |
| 32      | 30.538          | 0.00255          | 0.5          | 54                     |
| 47      | 28.317          | 0.0021           | 0.7          | 64                     |
| 56      | 24.986          | 0.00179          | 1.2          | 89.6                   |
| 71      | 20.433          | 0.00138          | 1.27         | 128.3                  |
| 80      | 16.657          | 0.00098          | 1.8          | 157                    |
| 95      | 8.051           | 0.00047          | 2.4          | 203                    |
| 104     | 3.498           | 0.00029          | 2.2          | 124                    |
| 119     | 1.665           | 0.00019          | 1.7          | 76                     |
| 128     | 0               | 0.000096         | 1.3          | 84                     |

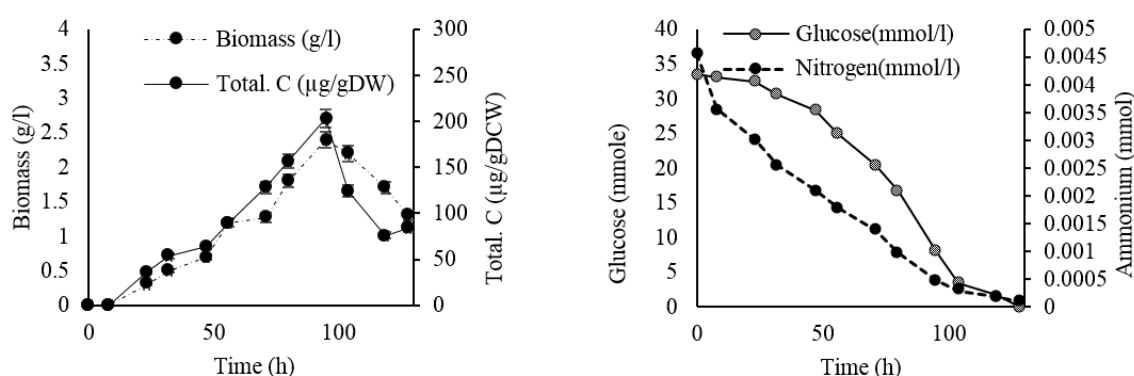


Fig. 6. Time-course profiles of biomass growth, total carotenoid accumulation and substrate consumption during cultivation of *Thraustochytrium* sp. 3F under optimized conditions (10 g/L glucose, 3 g/L yeast extract, 20% seawater, 170 rpm). Error bars represent  $\pm$  standard deviations calculated from three independent experimental replicates.

## Discussion

The present study elucidated the metabolic behavior of *Thraustochytrium* sp. 3F under optimized culture conditions, as evidenced by dynamic profiles of substrate utilization, biomass accumulation, and pigment biosynthesis. Glucose steadily declined throughout cultivation, reaching near depletion by 120 h, whereas total nitrogen was exhausted within the first 80 h. This temporal imbalance redirected cellular metabolism from primary growth toward secondary carotenoid biosynthesis, consistent with previous reports in which nitrogen depletion acts as a regulatory trigger for pigment induction (27, 28). Biomass exhibited a biphasic growth pattern, characterized by a brief lag phase, exponential growth between 23 and 71 h, and a peak concentration of 2.4 g/L at 95 h. The subsequent

decline in biomass was likely attributable to nutrient exhaustion, oxidative stress, or the accumulation of inhibitory byproducts, consistent with observations reported for *Schizochytrium* species under nitrogen-limited conditions (29). Maximum pigment accumulation occurred during the transition from exponential to stationary phase, rather than coinciding with peak biomass, indicating nutrient-stress-driven induction. Following nitrogen depletion, intracellular carotenoid content increased markedly to 203  $\mu$ g/g DW at ~95 h, representing a six-fold increase compared with the early cultivation stage. The later decline in carotenoid content, despite stable biomass, may reflect pigment degradation via non-enzymatic oxidation or a shift toward cellular maintenance, in line with previous

findings on photooxidative stress in marine protists (29, 30).

The kinetic cultivation results strongly support the Taguchi DOE outcomes, which identified culture conditions that balance biomass formation with enhanced pigment accumulation. Maintaining sufficient carbon while allowing controlled nitrogen depletion was critical for maximizing carotenoid productivity, consistent with earlier optimization studies showing that moderate C/N ratios promote carotenoid and astaxanthin biosynthesis without excessive biomass allocation (31, 32). Salinity and agitation also exerted significant regulatory effects: moderate salinity alleviated osmotic stress and favored pigment biosynthesis, whereas excessive seawater reduced carotenoid accumulation despite sustaining biomass. Elevated agitation enhanced oxygen transfer and stimulated astaxanthin synthesis, aligning with reports in *Aurantiochytrium* species that emphasize oxygen availability and hydrodynamic conditions in carotenoid productivity (33, 34).

In summary, these findings reveal a two-phase metabolic strategy in *Thraustochytrium* sp. 3F: an initial nutrient-sufficient growth phase followed by a nitrogen-limited induction phase that triggers carotenoid synthesis. Integration of Taguchi DOE results with kinetic cultivation analysis provides mechanistic insight, paralleling strategies in other carotenoid-producing systems such as *Haematococcus lacustris*, where nitrogen starvation

induces astaxanthin accumulation (35), and similar nutrient-regulated shifts in *Schizochytrium* and *Aurantiochytrium* species (36, 37). Altogether, the present findings emphasize the potential of heterotrophic thraustochytrids as scalable platforms for high-value carotenoid biosynthesis.

## Conclusion

The study demonstrates that the productivity of *Thraustochytrium* sp. 3F is governed by the interplay of carbon and nitrogen availability, salinity, and agitation. Taguchi-based optimization identified culture conditions with moderate glucose, sufficient nitrogen, low salinity, and enhanced agitation as optimal for balancing biomass growth and carotenoid production. Nitrogen limitation induced a metabolic shift toward carotenoid and astaxanthin biosynthesis, reaching a maximum of 203 µg/g DW. The temporal separation between biomass accumulation and metabolite formation underscores the critical importance of harvest timing. These findings validate Taguchi DOE as an effective approach for optimizing cultivation parameters and provide a foundation for scaling up sustainable carotenoid and astaxanthin production. Future studies should investigate fed-batch or continuous cultivation systems, stress induction strategies, and omics-based analyses to further elucidate regulatory mechanisms and enhance industrial applicability.

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