

Maximizing beta-carotene production from *Dunaliella salina* using different concentrations of ferrous sulfate and potassium nitrate under *in situ* and induced cultivation conditions

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ABSTRACT

Natural beta-carotene, a valuable antioxidant and food additive, is conventionally derived from vegetables and microalgae such as *Dunaliella salina* (*D. salina*). Growing concerns over the safety of synthetic carotenoids and increasing demand for natural alternatives in food, nutraceutical, and cosmetic industries have accelerated the development of controlled cultivation systems. However, unstable environmental conditions in outdoor production systems can hinder consistent carotenoid yields. In this context, *D. salina* is recognized as a promising candidate for beta-carotene production through indoor cultivation, which allows for precise control over key growth parameters and reduces dependency on fluctuating environmental factors. This study investigated how nitrogen availability and ferrous ion supplementation affect the growth and beta-carotene accumulation of *D. salina* under both *in situ* and stress-induced conditions. Results show that nitrogen supports robust biomass accumulation, while ferrous ions stimulate beta-carotene synthesis via oxidative stress. Notably, the combined application of these two nutrients produced a synergistic effect, achieving both high cell density ($2.4079 \pm 0.00432 \times 10^7$ cells mL⁻¹) and elevated beta-carotene content (27.12 ± 1.41 pg cell⁻¹) after 20 days of induced two-stage cultivation in a 15 L indoor culture system. These findings contribute to the development of optimized cultivation strategies for sustainable, high-yield beta-carotene production.

Introduction

In recent years, over 750 carotenoids have been identified, with beta-carotene standing out for its diverse applications in cosmeceuticals, skincare, aquaculture feed, nutraceutical, animal feed additive, coloring agents, and preventing diseases (Sharma et al., 2024; Viana et al., 2024). Beta-carotene is widely distributed in higher plants and microorganisms, where it functions as an accessory pigment in photosynthesis—enhancing light energy capture and protecting the photosynthetic apparatus from photo-oxidative damage (Morais, 2006; Morowvat and Ghasemi, 2016). Beta-carotene is a carotenoid and an

organic compound with the molecular formula C₄₀H₅₆. It is a red-orange pigment precursor to the human body's vitamin A (Borowitzka, 1995; Wu et al., 2016).

The global beta-carotene market has grown consistently, driven by increasing consumer interest in natural and organic products (Kaur et al., 2025; Severo et al., 2024; Sportiello et al., 2024). According to market reports, the beta-carotene market was valued at approximately USD 0.6 billion in 2022 and is projected to reach over USD 1.03 billion by 2030, with a compound annual growth rate of 6 % attributed to the increasing global consumption demands of beta-carotene in foods, beverages, dietary supplements, personal care, cosmetics, and animal

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feed industries. Beta carotene is widely utilized due to its antioxidant functions and ability to protect the skin against free radical-induced damage (Research, 2023). In general, chemical synthesis accounted for 84.8 % of global beta-carotene consumption, whereas only 8.4 % was derived from algal synthesis (Ye et al., 2008). Following a series of food safety crises, naturally synthesized pigment products have become highly valued and favored, driving increased demand (Sharma et al., 2024). However, this surge in demand, coupled with limited supply, has exacerbated the supply-demand imbalance, leading to price increases (Zhang et al., 2014).

Natural beta-carotene is mainly from vegetables, fruits, carrots, as well as algae (Schendel et al., 2022). An important consideration is the percent yield of beta-carotene, with 10 % of dry weight regarded as an ideal source (Schendel et al., 2022). Notably, microalgae, particularly the *Dunaliella* genus, are exceptional producers, achieving 10–12 % beta-carotene yield through optimization of cultivation processes, showcasing their high potential as a resource for large-scale beta-carotene production for future foods (Saha et al., 2018). Powder from *Dunaliella salina* has ‘GRAS’ status approved by the US FDA (Food and Drug Administration), meaning that it is ‘Generally Regarded as Safe’ (El-Baz et al., 2019). The high salinity *Dunaliella salina* cultivation also reduces risks from pathogenic bacteria (El-Baz et al., 2019). Furthermore, artificial large-scale cultivation of *Dunaliella* species has been practiced for decades, with several practical scaling factors identified through scientific research and industrial applications. These factors include species selection (within the *Dunaliella* genus), as well as salinity, light intensity, nutrient availability, oxidative stress, chemical treatments, and culture methods—all of which influence beta-carotene accumulation (Keramati et al., 2021; Morowvat and Ghasemi, 2016; Yuan et al., 2019; Zhang et al., 2014). Various factors influence beta-carotene production from *D. salina*, including the type of culture system and the optimization of cultivation conditions to maximize both biomass yield and intracellular beta-carotene content (Pourkarimi et al., 2020). Following the previous reports, batch cultivation strategies, particularly under nutrient-limited conditions, have demonstrated significant potential for enhancing carotenoid production in *Dunaliella*. Stress conditions, including nitrogen depletion, intense light, and high salinity, are known to enhance beta-carotene synthesis in *D. salina*, enabling accumulation of up to 10 % of dry cell weight, although typically accompanied by reduced biomass productivity (Gallego-Cartagena et al., 2019; Mojaat et al., 2008; Saha et al., 2018; Sui et al., 2019). Nitrogen limitation induces oxidative stress responses that enhance carotenoid biosynthesis and redirects metabolic pathways toward increased antioxidant production (Saha et al., 2018; Sui et al., 2019).

However, comprehensive studies on staged cultivation and identifying key nutrients for β-carotene production remain limited. Further investigation is crucial given the increasing demand for large-scale commercial production of β-carotene. Advancing the scientific understanding of *Dunaliella* and refining cultivation strategies will be essential for improving production efficiency and meeting market needs.

The research hypothesized that nutrient availability (nitrogen and iron) and cultivation strategies (*in situ* or induced conditions) affect growth and beta-carotene accumulation in the microalga *D. salina*. The *in situ* means exhausted original medium in all 20 days' cultivation without any medium change; the “induced” means changing the other half volume of medium once at the midpoint of 20 days' cultivation to maximize beta carotene production. Employing different cultivation strategies may possible to induce carotenogenesis by medium composition and or two-stage cultivation.

The goal was to develop an efficient cultivation strategy for industries focused on large-scale pigment production while uncovering the mechanisms driving these processes. Response surface methodology (RSM) was utilized to optimize and analyze microalgal growth and pigment accumulation under specific nitrogen and iron conditions to achieve this.

Materials and methods

Microalgal strain and cultivation conditions

D. salina was isolated from solar evaporation salt ponds in Jing Zai Jiao, Beimen District, Tainan, Taiwan. Single-cell isolation was conducted using a capillary tube under a microscope, and the isolate was cultivated in Ben-Amotz-modified medium (Koberg et al., 2011) (Table 1). Species identification was confirmed via ITS region rDNA gene sequencing. Cultures were maintained at 28 °C, 100 μmol photons m⁻² s⁻¹ light intensity, 8.0 % (NaCl w/v) salinity, and a 12:12 h light-dark photoperiod. The medium was replenished every four days, with subculturing performed biweekly.

Effect of nutritional factors on *D. salina* under *in situ* and induced cultures

Experiments were conducted in 500 mL Erlenmeyer flasks containing 0.4 L of Ben-Amotz-modified medium (excluding ferrous sulfate and potassium nitrate; Table 1). Initial cell densities were $1.69 \pm 0.93 \times 10^6$ cells mL⁻¹ (*in situ*) and $1.54 \pm 0.43 \times 10^6$ cells mL⁻¹ (induced). Ferrous sulfate and potassium nitrate were applied in the following combinations: *in situ* (μM) (assigned as: 0.4L0F0N, 0.4L0.3F0N, 0.4L0.3F3N, 0.4L0.3F6N, 0.4L0.3F9N, 0.4L0.6F0N, 0.4L0.6F3N, 0.4L0.6F6N, 0.4L0.6F9N); Induced (mM) (0.4L0.3F3N, 0.4L0.3F6N, 0.4L0.3F9N, 0.4L0.6F3N, 0.4L0.6F6N, 0.4L0.6F9N).

For induced cultures, potassium nitrate was added on day one, and ferrous sulfate on day ten. Cultures were maintained at 28 °C with 100 μmol photons m⁻² s⁻¹ illumination. Cell density and β-carotene content were measured every two days and at the end of the cultivation period, respectively. Optimization of culture conditions was performed using response surface methodology (RSM) with Design-Expert software v12.0 (Stat-Ease Inc., USA).

Large-Scale cultivation of *D. salina*

Optimal conditions identified via RSM were validated in 15-L plastic bottles. For *in situ* cultivation, the optimal ferrous sulfate and potassium nitrate concentrations were 0.42 mM and 0.0 mM, respectively and assigned as code 15L0.42F0N. For induced cultivation, optimal concentrations ferrous sulfate and potassium nitrate concentrations were 0.6 mM and 3 mM, respectively and assigned as code 15L0.6F3N.

Cell density and β-Carotene content analysis

Cell density was determined by flow cytometry (Chen et al., 2022). β-Carotene was extracted using a methanol/methylene chloride solution (75:25, v/v) and analyzed via HPLC (Tzeng et al., 2004). Detection was performed at 450 nm, with concentrations calculated using β-carotene standards (Sigma-Aldrich, USA).

Table 1
Formula of Ben-Amotz medium.

Ingredients	Concentration, g/L
Magnesium sulfate heptahydrate, MgSO ₄ ·7 H ₂ O	1.23
Calcium chloride dihydrate, CaCl ₂ · 2 H ₂ O	0.147
Potassium dihydrogen phosphate, KH ₂ PO ₄	0.0275
Ethylenediaminetetraacetic acid, Na ₂ EDTA	0.04
Manganese(II) Chloride-4-hydrate, MnCl ₂ · 4H ₂ O	0.0001
Cobalt(II) chloride hexahydrate, CoCl ₂ · 6H ₂ O	0.0001
Ammonium heptamolybdate tetrahydrate, (NH ₄) ₆ Mo ₇ O ₂₄ · 4H ₂ O	0.00124
Copper(II) chloride dihydrate, CuCl ₂ · H ₂ O	0.0001
Zinc chloride, ZnCl ₂ · H ₂ O	0.0001
Sodium hydrogen carbonate, NaHCO ₃	4.20
Ferrous Sulphate (FeSO ₄)	4.555
Potassium Nitrate, KNO ₃	121.32

Statistical analysis

Data were analyzed using SPSS Statistics 22.0 (IBM, USA). One-way ANOVA and Duncan's multiple range test were used, with $p < 0.05$ considered statistically significant.

Results

Effect of nutritional factors on *D. salina* in situ culture

The addition of iron altered algal morphology, transitioning cell color from green to yellow-orange (Fig. 1). After 20 days, the 0F/6 N group achieved the highest cell density ($240.79 \pm 4.32 \times 10^6$ cells mL $^{-1}$), followed by 0.3F/6 N ($1.44 \pm 12.57 \times 10^7$ cells mL $^{-1}$) ($P < 0.05$). The lowest growth was observed in the 0.6F/0 N ($2.71 \pm 5.47 \times 10^6$ cells mL $^{-1}$) and 0.6F/3 N ($3.65 \pm 9.63 \times 10^6$ cells mL $^{-1}$) groups. Cell growth was negatively correlated with iron concentration at fixed nitrogen levels but positively correlated with nitrogen concentration at fixed ferrous levels (Fig. 2).

Regarding β -carotene content, the 0.6F/0 N group exhibited the highest levels (14.42 ± 3.08 pg cell $^{-1}$), followed by 0.3F/0 N (11.48 ± 5.22 pg cell $^{-1}$) and 0.6F/3 N (11.21 ± 2.60 pg cell $^{-1}$) ($P < 0.05$). The 0F/

6 N group showed no detectable β -carotene. β -Carotene content was positively correlated with iron concentration and negatively correlated with nitrogen concentration under fixed conditions (Fig. 2).

Effect of nutritional factors on *D. salina* in induced culture

In induced cultures, cell biomass increased steadily during the first 14 days. Between days 4 and 8, the 9 N group had significantly higher cell numbers than the 3 N and 6 N groups ($P < 0.05$). By day 20, the lowest cell densities were observed in 0.6F/3 N ($9.73 \pm 14.60 \times 10^6$ cells mL $^{-1}$) and 0.3F/3 N ($1.10 \pm 3.12 \times 10^7$ cells mL $^{-1}$) ($P < 0.05$) (Fig. 3).

β -Carotene content was negatively correlated with nitrogen concentration and positively correlated with iron concentration. The highest β -carotene levels were recorded in 0.6F/3 N (18.75 ± 2.06 pg cell $^{-1}$) and 0.3F/3 N (17.64 ± 1.57 pg cell $^{-1}$) ($P < 0.05$), while the lowest was found in 0.6F/9 N (6.74 ± 0.32 pg cell $^{-1}$) ($P < 0.05$) (Fig. 3).

Large scale cultivation of *D. salina*

For *in situ* cultivation, the RSM model predicted a cell density of 88.70×10^6 cells mL $^{-1}$ and β -carotene content of 36.14 pg cell $^{-1}$ under 0.0 mM nitrogen and 0.42 mM iron concentrations. In the 15-L scale-up

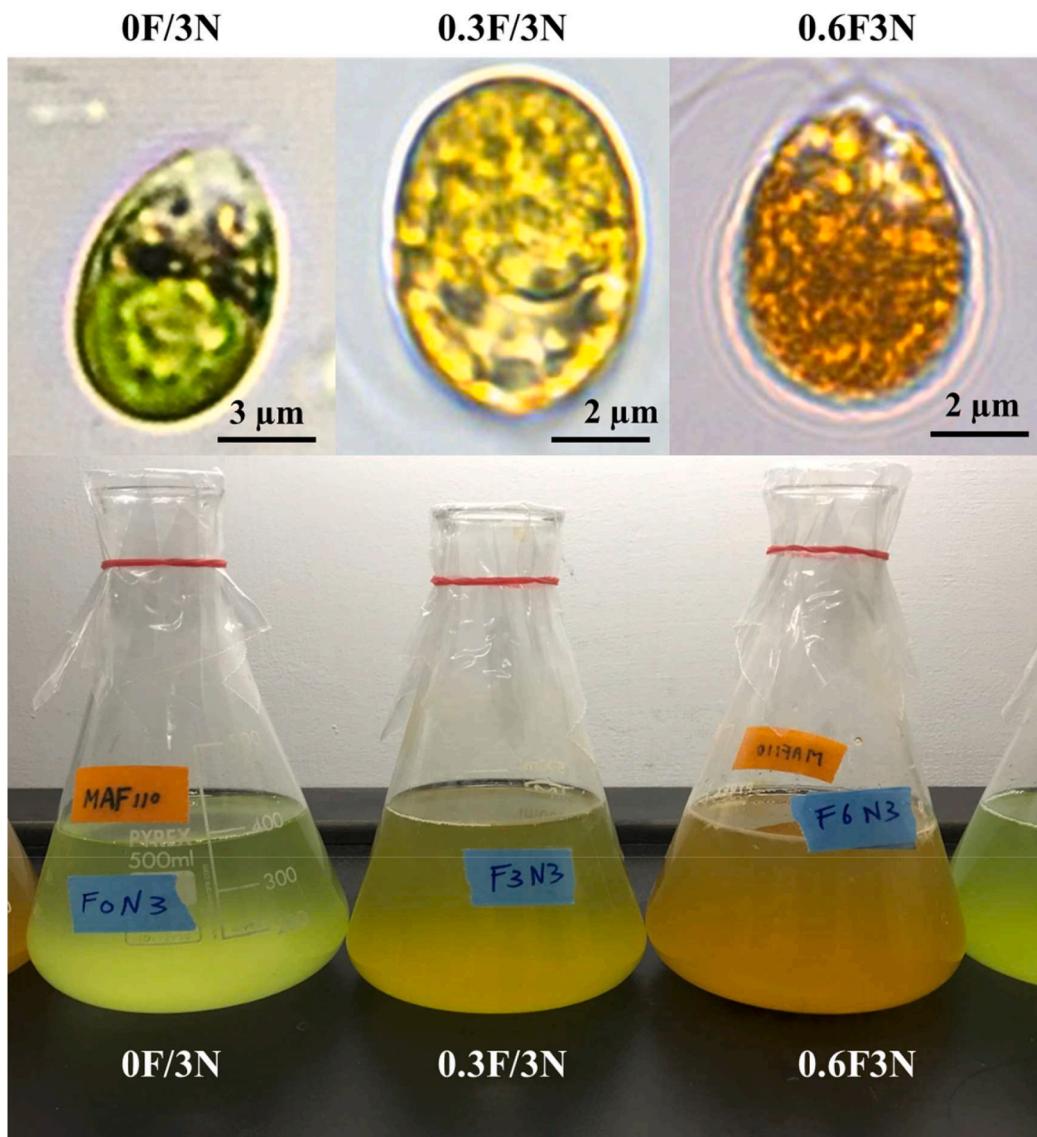


Fig. 1. Effects of iron and nitrogen concentrations on pigmentation in *Dunaliella salina*.

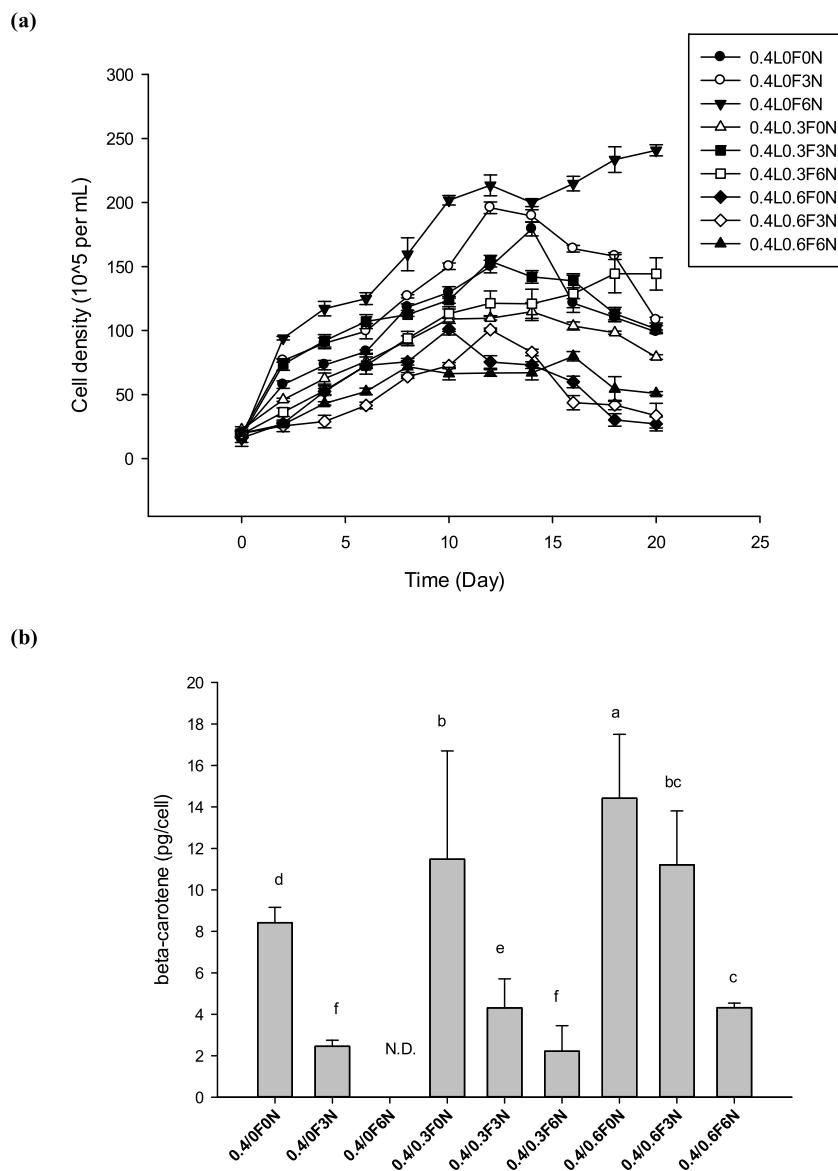


Fig. 2. Effect of nitrogen and iron concentrations on (a) cell density and (b) beta-carotene accumulation in *Dunaliella salina* under *in situ* conditions during a 20-day culture period. Data are shown as mean \pm standard deviation ($n = 3$).

experiment, actual values were $1.15 \pm 1.89 \times 10^7$ cells mL^{-1} and 16.73 ± 2.13 pg cell^{-1} . While cell density exceeded expectations, β -carotene content was lower than predicted (Fig. 4 and Fig. 6).

For induced cultivation, the predicted values at 3.00 mM nitrogen and 0.60 mM iron were 197.71×10^6 cells mL^{-1} and 26.02 pg cell^{-1} . The 15-L scale-up yielded $9.73 \pm 4.89 \times 10^6$ cells mL^{-1} and 27.12 ± 1.41 pg cell^{-1} , with β -carotene content surpassing predictions, though cell density was lower than expected (Fig. 4 and Fig. 6). (Fig. 5)

Discussion

Nitrogen plays a pivotal role in supporting cell division, which explains the positive correlation observed between nitrate concentration and cell density in this study (Liu et al., 2020). Under nitrogen-deficient conditions, nitrate reductase activity, essential for nitrate assimilation, declines significantly (Ip et al., 2004), disrupting cellular metabolism and slowing growth. Consequently, biomass production is compromised

when nitrate levels are insufficient (Mojaat et al., 2008). These findings highlight the necessity of maintaining optimal nitrogen concentrations to sustain *D. salina* growth.

Nitrogen deficiency also induces carotenoid accumulation in *D. salina*. Limited nitrogen reduces chlorophyll and protein synthesis, disrupting photosynthesis and leading to excess intracellular light energy. This imbalance creates oxidative stress, prompting carotenoid production, such as β -carotene, to mitigate photodamage by scavenging reactive oxygen species (Im et al., 2006; Lamers et al., 2008; Wilson et al., 2003). Additionally, nitrogen limitation redirects carbon flow from primary metabolic pathways toward carotenoid and lipid synthesis, enhancing pigment accumulation ((Ben-Amotz, 1995; Im et al., 2006; Lamers et al., 2008; Pourkarimi et al., 2020; Wilson et al., 2003; Xi et al., 2021)). The observed negative correlation between nitrogen concentration and β -carotene content supports these findings.

This study also explored the role of iron in algal growth. Interestingly, iron supplementation decreased cell density across all *in situ*

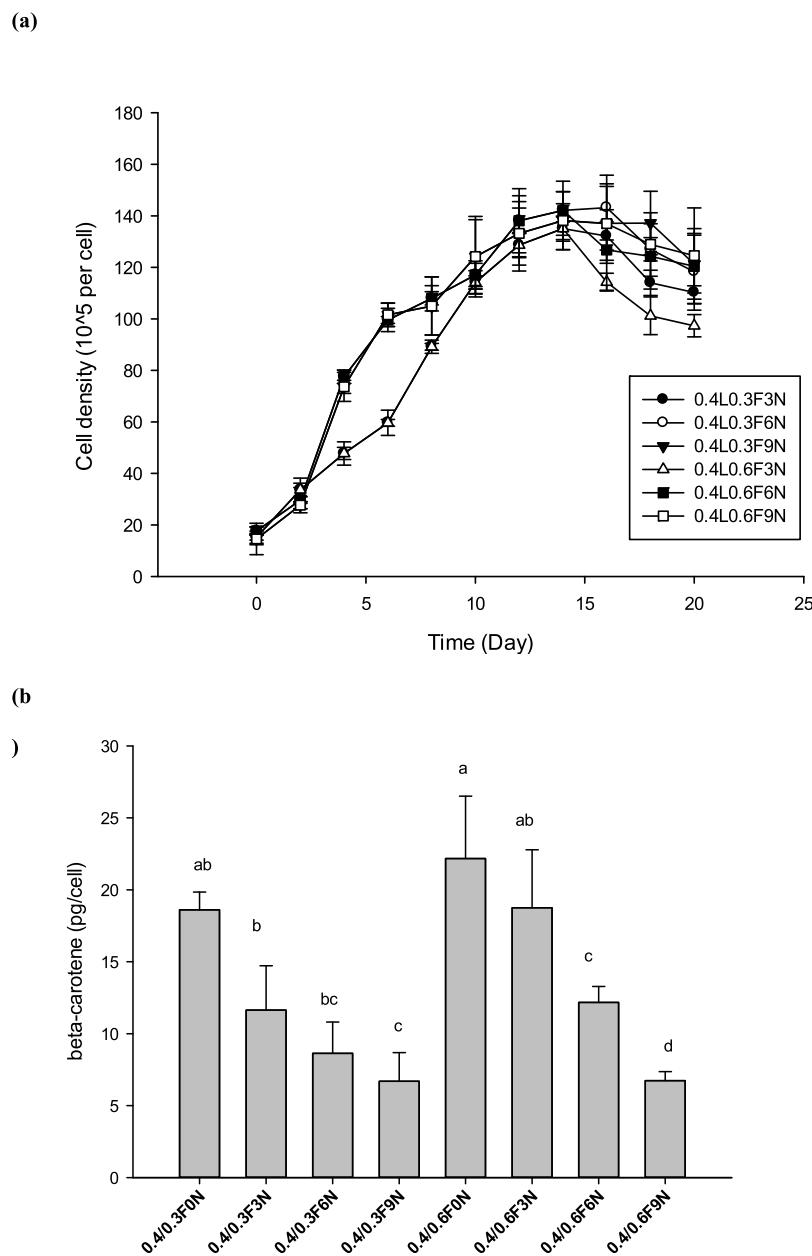


Fig. 3. Effect of nitrogen and iron concentrations on (a) cell density and (b) beta-carotene accumulation in *Dunaliella salina* under induced conditions during a 20-day culture period. Data are shown as mean \pm standard deviation ($n = 3$). N.D are means non detected.

groups. Excessive Fe^{2+} can generate reactive oxygen species through Fenton reactions, causing oxidative damage to DNA, proteins, and membrane lipids (Mojaat et al., 2008). Moreover, intracellular iron overload disrupts metal homeostasis, impairing essential metabolic processes, such as photosynthesis and respiration. Excess ferrous ions may also precipitate, reducing bioavailability and altering medium pH, further stressing cells (Gallego-Cartagena et al., 2019). These results emphasize that while iron is crucial, its optimal concentration must be finely balanced to avoid growth inhibition.

The two-phase cultivation system proved highly effective. In the initial phase (green phase), high nitrogen concentrations facilitated biomass accumulation. Subsequently, nutrient stress, induced by limited nitrogen and controlled ferrous levels, optimized carotenoid synthesis. The importance of temporal nutrient management in enhancing metabolite production while sustaining growth aligns with previous findings (Ben-Amotz, 1995; Pourkarimi et al., 2020; Xi et al.,

2021). Response surface methodology (RSM) analysis further confirmed that optimal biomass under *in situ* cultures occurs at low iron and high nitrogen concentrations, while induced cultures benefited from separate nutrient additions, allowing precise control over β -carotene synthesis.

Additionally, this study revealed a strong positive correlation between iron concentration and β -carotene accumulation. Iron-induced oxidative stress likely triggers β -carotene synthesis, as observed by the absence of carotenoids in iron-free *in situ* cultures. In induced cultures, β -carotenoids were detectable after 10 days of iron exposure, underscoring the significance of stress induction for pigment accumulation. Notably, nitrogen-rich conditions during the first phase led to competitive iron absorption during the second phase, reducing β -carotene content per cell. These observations corroborate earlier reports that nitrate-free conditions yield the highest β -carotene concentrations.

Furthermore, the ratio of chlorophyll to β -carotenoids emerged as a reliable stress indicator (Pourkarimi et al., 2020; Xi et al., 2021). Under

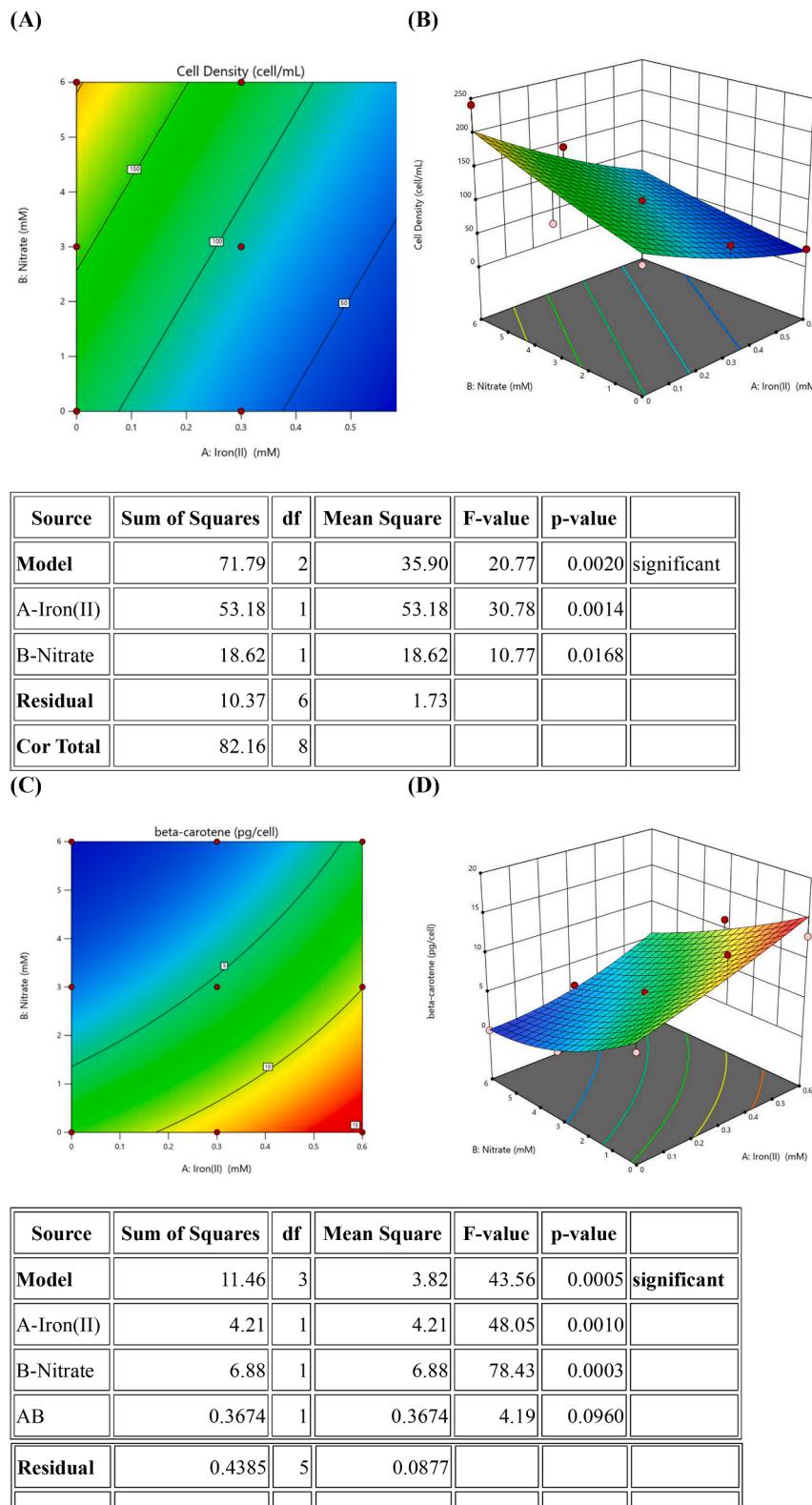
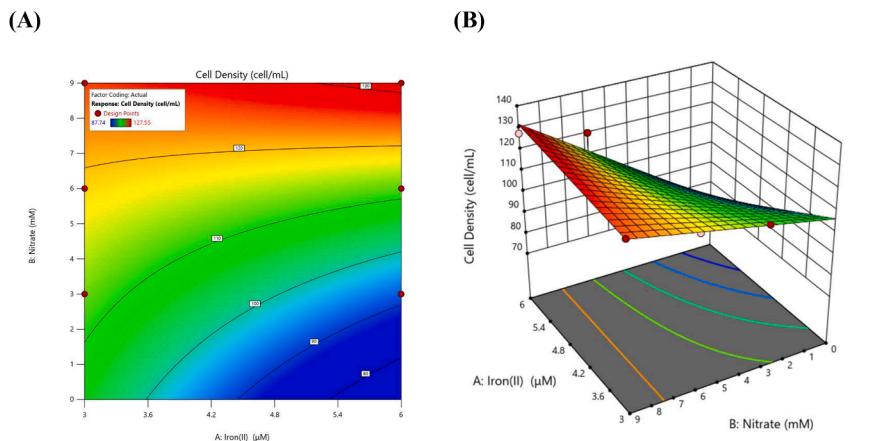


Fig. 4. Response surface and interaction plots for (a) cell density and (b) beta-carotene content, including contour plots depicting binary interaction effects of nitrogen and iron concentrations in *Dunaliella salina* under *in situ* conditions during a 20-day culture period.

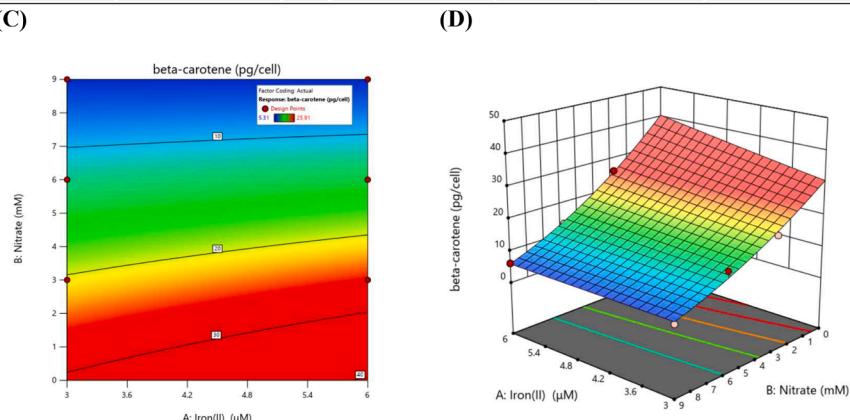
normal conditions, high chlorophyll content supports photosynthesis, resulting in green pigmentation. Stress conditions shift the balance toward β -carotenoids, conferring an orange hue and enhancing anti-oxidative protection (Orosa et al., 2005; Pourkarimi et al., 2020; Xi

et al., 2021).

Large-scale cultivation trials demonstrated the practical applicability of the two-phase system. Although *in situ* cultures exceeded predicted biomass yields, carotenoid levels were lower than expected. Conversely,



Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	936.97	3	312.32	5.69	0.1530	not significant
A-Iron(II)	208.48	1	208.48	3.80	0.1906	
B-Nitrate	673.66	1	673.66	12.28	0.0727	
AB	191.96	1	191.96	3.50	0.2023	
Residual	109.72	2	54.86			
Cor Total	1046.69	5				



Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	5.67	3	1.89	28.30	0.0343	significant
A-Iron(II)	0.1728	1	0.1728	2.59	0.2489	
B-Nitrate	5.49	1	5.49	82.25	0.0119	
AB	0.0747	1	0.0747	1.12	0.4009	
Residual	0.1335	2	0.0667			
Cor Total	5.80	5				

Fig. 5. Response surface and interaction plots for (a) cell density and (b) beta-carotene content, including contour plots depicting binary interaction effects of nitrogen and iron concentrations in *Dunaliella salina* under induced conditions during a 20-day culture period.

induced cultures achieved higher-than-predicted β -carotene concentrations despite lower biomass yields. This trade-off highlights the need for precise nutrient management to optimize both growth and metabolite production.

Conclusion

This study employed *in situ* and induced culture methods to investigate the effects of ferrous and nitrogen on algal cell proliferation and

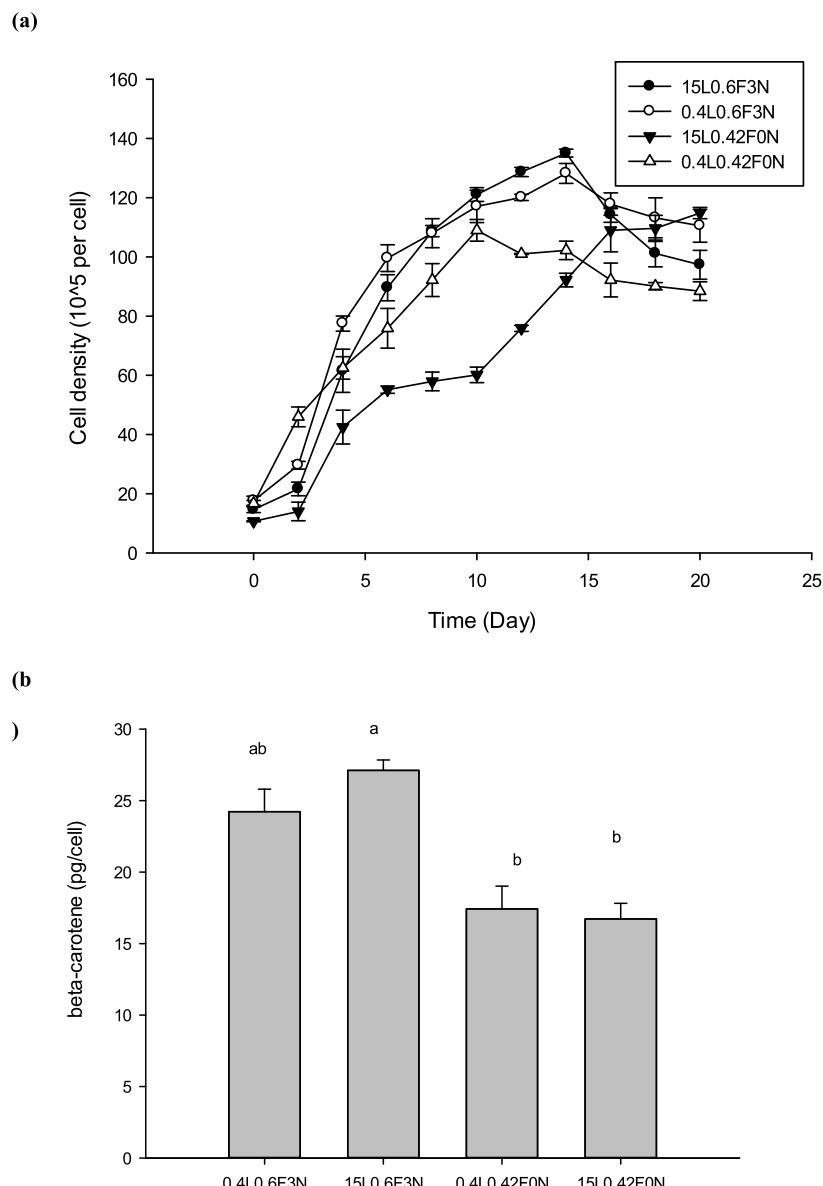


Fig. 6. Effect of nitrogen and iron concentrations on (a) cell density and (b) peak value beta-carotene accumulation in *Dunaliella salina* under 0.4-L and 15 L plastic bottles during a 20-day culture period. Data are shown as mean \pm standard deviation ($n = 3$).

β -carotenoid synthesis. The findings confirmed that nitrogen is essential for promoting algal growth, while ferrous, despite inhibiting growth, plays a critical role in enhancing β -carotene production. The two-phase cultivation system effectively optimized biomass and β -carotene yields, demonstrating its potential as a practical and scalable approach for maximizing secondary metabolite production in industrial applications. Fine-tuning nutrient availability and stress induction timing will be essential for commercial-scale production of high-value beta carotene from *D. salina*.

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Ethical statement

The present study does not relate any data from human or animal experiments.

CRediT authorship contribution statement

Han-Yang Yeh: Investigation. **Po-Yen Yu:** Data curation. **Meng-Chou Lee:** Supervision. **Congo Tak Shing Ching:** Funding acquisition. **Fan-Hua Nan:** Funding acquisition. **Chao-Ling Yao:** Validation. **Yung-Kai Lin:** Conceptualization, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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