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Production of Natural Pigment by *Dunaliella salina:* Key Factors Screening through Plackett-Burman Design

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ABSTRACT: The distinctive biological and technical characteristics of *Dunaliella*, including the need for cheap culture medium, fast growth rate, simple genetic manipulation, and easy scale-up methods, have made this microorganism the prime candidate for molecular agriculture, and a suitable host for the production of antibodies, vaccines and valuable compounds such as carotenoids, glycerol, unsaturated fats, vitamins, proteins, and bioactive substances. Therefore, this alga may be one of the most appropriate models to investigate and utilize to produce useful compounds by optimizing its environment. This study investigated the feasibility of high biomass and pigment (chlorophyll a, chlorophyll b, and carotenoids) accumulation in a species of *Dunaliella salina* native to Iran by creating mixotrophic conditions using the Plackett-Burman screening design. In this design, the effects of 10 variables, including pH, light intensity, carbon source (date waste), Nitrogen source, NaCl, Fe (ferrous sulfate), vitamin B_1, vitamin B_{12}, Incubation time and Inoculum concentration were investigated. The results showed the significant effects of carbon source, sodium chloride, pH, inoculum concentration, and incubation time on biomass accumulation the value of which varied from 1.90 to 8.54 g/100. All variables except vitamins had a significant effect on the accumulation of chlorophyll and increased its amount from 0.60 to 1.35 mg/l. While variables such as pH, incubation time, sodium chloride, light intensity, and iron effected the accumulation of chlorophyll b significantly. pH, carbon source, sodium chloride, nitrogen source, and light intensity affected the accumulation of carotenoids, and the highest amounts of chlorophyll b and carotenoids were obtained as 2.8 mg/l and 8.6 mg/l, respectively.

Keywords: Carotenoid, *Dunaliella salina*, Plackett-Burman.

Introduction

Microalgae are the source of varieties of natural products including high-value nutrients therefore they are considered durable food and nutritional sources making further study and development for their introduction into main production lines a necessity (Spolaore et al., 2006; Priyadarshani and Rath, 2012). Microalgae are generally referred to as single-celled eukaryotic organisms. Being microscopic gives them advantages over their macroscopic counterparts, such as:

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1. Easier genetic manipulation
2. Easier processes for production
3. High biomass yield per unit area
4. Producing biomass with high protein content and bioactive substances
5. Cultivation in non-cultivable land using non-potable water or even salt water (Gantar and Svirčev, 2008; Pal et al., 2014).

Therefore, today, one of the recommended platforms for molecular agriculture is the use of microalgae as green "micro-bio-factories". These micro-organisms are the best candidates for this purpose because they have the advantages of bacteria and yeast (growth speed and low cultivation costs) as well as animals and plants (ability to make modifications) (Kadkhodaei et al., 2015).

Dunaliella is a valuable and high-quality microalga which have a high nutritional value due to its protein, vitamins, minerals, unsaturated fats, carotenoids, and other beneficial compounds. The beneficial compounds in these microalgae lead to their increasing use in the pharmaceutical and medical industries. *Dunaliella* belongs to the green flagella genus and *Volvocales* class. Instead of a cell wall, these microalgae are surrounded by a plasma membrane, which contracts and expands in hypertonic and hypotonic conditions, respectively. Moreover, an amorphous mucilage layer with variable thickness called glycocalyx appears in most old cells. A big cup or bell-shaped Chloroplast takes up most of the space within the cell. *Dunaliella* are known as Eukaryotes and have high tolerance in the face of environmental conditions. Thus, this species can survive high salinity (3% Sodium Chloride) and saturated (31% Sodium Chloride) waters (Borowitzka and Siva 2007; Pal *et al*., 2014). It should be noted that factors such as growth stage and the environment's cultivation conditions, including nutritional availability, light intensity, pH, and temperature fluctuations influence *Dunaliella's* morphological and physiological characteristics. This issue has made it impossible to give an accurate and complete classification of *Dunaliella* microalgae species classification. A halophytic species of *Dunaliella* capable of accumulating β-Carotene is *Dunaliella salina* (Polle et al., 2020). Carotenoids are produced to protect these microalgae from sunlight and they do so by forming oil globules and reducing fatty acid saturation, which in turn causes the accumulation of β-Carotene and certain fatty acids (Araj-Shirvani et al., 2024; Lamers et al., 2012). Glycerol protects them from the osmotic pressure inside the algae body. Therefore, these microalgae can be used in the commercial production of β-Carotene and glycerol (Spolaore et al., 2006).

Many factors affect the growth and accumulation of bioactive compounds in *Dunaliella salina* including the quality and quantity of light, salinity, temperature, pH, and available nutrients, and strains isolated from different areas have had different performances (Koyandea et al., 2019). Some microalgae are able to change their growth habits from photoautotroph to heterotroph (utilization of organic materials as energy sources for growth) or mixotroph (combination of organic nutrition and light). Heterotroph systems which are wholly dependent on organic carbon sources may be more expensive than their photoautotroph counterparts, while mixotroph conditions can decrease cultivation costs by shortening the growth cycle (Koutra et al., 2019; Aziz et al.,2020). Agricultural and food industrial wastes which are rich in mineral and organic carbons, macro and micronutrients can be used in microalgae cultivation.

Using of these wastes induce mixotroph conditions in microalgae not only solving an environmental challenge but also decreasing the cost of providing microalgae growth conditions (Koutra et al., 2019; Kumar et al., 2022). Up-to-date, several materials such as date waste and sugarcane molasses used by *Aurantiochytrium sp.* for the production of biomass and fat with high DHA (Abdel-Wahab et al., 2022), whey used to cultivate *Chlorella protothecoides* in order to produce biomass with high fat accumulation (Espinosa-Gonzalez et al., 2014), sugarcane molasses and date waste to pigment production by *Chlorella* sp (Liu et al., 2013; El-Awady et al., 2020) and date waste and molasses for mixotroph cultivation of *Spirulina platensis* (Andrade and Costa, 2007; Banayan et al.,2022), have been used as substrates for microalgae cultivation (Malakar et al., 2022).

Plackett-Burman Design (PBD) is a two-level fractional factorial design which is a well-known screening method utilized to determine the major effect between various factors in biotechnology research. By applying this method, the effect of a large number of variables on the system response can be checked independently using a limited and effective number of experiments (Vanaja and Shobhai, 2007). PBD has been used to produce bioactive metabolites from microalgae such as *Nannochloropsis oculate* (El-Sheekh et al., 2016), *Spirulina* (Banayan et al.,2022; Abedin and Taha, 2008), and *Chlorella pyrenoidosa* (Priyadharshini and Bakthavatsalam, 2016) .

Date is widely produced in the Middle East. A large quantity of date is wasted during picking, packing, and processing. Iran is the second producer of dates in the world, with 40 % of its production being low-quality dates which do not enter the consumer market and must be used in alternative industries (Najafi et al., 2009). Low-quality dates contain the same amount of sugar as high-quality ones and can be suitable sources of carbon for microorganisms such as microalgae (Besbes et al., 2009; El-Awady et al., 2020). Although the use of various wastes as carbon sources has been studied for the biomass, pigment, and bioactive compounds production in microalgae, no efforts have been carried out to use date wastes as carbon sourced and induction mixotroph growth to produce biomass and pigment using *Dunaliella salina*. In the present study, PBD was applied to screen important significant factors from 10 factors including pH, Light intensity, carbon source (date waste), Nitrogen source, NaCl, Fe, vitamin B_1, vitamin B_{12}, Inoculum concentration, and Incubation time on cell growth and pigment production (chlorophyll a, chlorophyll b, carotenoids) in *Dunaliella salina* (50030) indigenous to Iran from a lake in Semnan province.

Materials and Methods

- ***Microalgae strain and preculture conditions***

Dunaliella salina (IBRC-M 50030) isolated from a lake in Semnan province (34° 30' 0" N, 51° 52' 0" E), was obtained from Iran's National Center of Genetic Resources, Tehran, Iran. Inoculums of *Dunaliella salina* were cultivated in a modified Janson culture medium, pH 7, and 250-ml glass flasks containing 150 ml of the cell suspension under sterile conditions (Besbes et al., 2020; Araj-Shirvani et al., 2024).

- ***Culture condition***

Test cultures were grown in 500-ml glass flasks with 250-ml Janson culture medium. A five-day-old culture of green

cells consisting of inoculum with 10-20 % was used as inoculum according to Table 1. The culture was incubated at 25 °C ± 1 at 100 rpm (Behdad, 40-200 rpm, Iran) with 16/8 h light/dark cycle. Variables are listed in according to (PBD) (Table 1).

- ***Substrate Source Feeding***

All substrates (carbon, nitrogen, iron sources, NaCl, and vitamins B_1 and B_{12}) were fed in a batch system. Date waste syrup as a carbon source (equivalent to 1 g l^{-1} of sugar), urea as a nitrogen source (equivalent to 400 g l^{-1} nitrogen), and iron sulfate (equivalent to 5 mg l^{-1} iron) were added to the culture medium before inoculation, according to Table 1.

A microalgal wet biomass equivalent to $30*10^4$ cells/m^{-1} was used as a source for inoculation.

- ***Assessment of biomass***

Biomass as a dry weight (DW) was determined using 50 ml culturing broth separated from the cultivated solutions and filtered with a pre-weighed Whatman GF/C filter (0.45 µm), then it was dried at 60°C for 24 h (Luo et al., 2020).

- ***Pigment content***

Biomass collection was carried out using centrifugation (Universal 320R made in Iran) followed by washing with deionized water. It was mixed with 3 ml acetone (90 % w/w) for 20 sec, finally, the mixture was centrifuged (Universal 320R made in Iran) at 3500 rpm for 10 minutes. The absorption quality of the higher clear solution was read by spectrophotometer for chlorophyll a, chlorophyll b, and carotenoids at 662 nm, 645 nm, and 470 nm wavelength, respectively. Finally, total carotenoids and chlorophylls were calculated using equations 1, 2, 3, and 4 (Lichtenthaler and Buschmann, 2001; Schoefs, 2002).

$$\text{Chla } (\mu g\ ml^{-1}) = 11.24\times A662-2.04\times A645 \quad \text{(eq 1)}$$

$$\text{Chlb } (\mu g\ ml^{-1}) = 20.13\times A645-4.19\times A662 \quad \text{(eq 2)}$$

$$\text{T Chl } (\mu g\ ml^{-1}) = \text{Chla} + \text{Chlb} \quad \text{(eq 3)}$$

$$\text{Cart } (\mu g\ ml^{-1}) = (1000\times A470-1.9\times \text{Chla}-.14\times \text{Chlb})/21 \quad \text{(eq 4)}$$

Chla = chlorophyll a
Chlb = chlorophyll b
Chl= total chlorophyll
Cart = carotenoid

Table 1. Various levels of experimental variables used in production biomass and pigments (chlorophyll a, chlorophyll b and carotenoids) in *Dunaliella salina* using Plackett-Burman design.

Code	Variables	Unit	Low level (-1)	High level (+1)
A	Date waste	g $100ml^{-1}$	0	1
B	Urea	mg l^{-1}	0	400
C	NaCl	g $100ml^{-1}$	3	8
D	Vitamin B_1	mg l^{-1}	0	2.5
E	Vitamin B_{12}	mg l^{-1}	0	0.5
F	Iron Sulfate	mg l^{-1}	0	25
G	Light intensity	µmol m^{-2} s^{-1}	50	150
H	pH		6.5	7.5
I	Incubation time	day	14	20
J	Inoculum concentration	%	10	20

- ***Statistical analysis***

The current study was carried out to investigate variables with significant effects on biomass and pigments (chlorophyll a, chlorophyll b, and carotenoids) production in *Dunaliella salina* using PBD. Results were analyzed using MINITAB software (version 17) and significance was reported when $p < 0.05$. Variables including pH, light intensity, carbon source (date waste), nitrogen source, NaCl, Fe, vitamin B_1, vitamin B_{12}, inoculation concentration, and incubation time of culture and their two different levels (+1 as high level and –1 as low level) were demonstrated in Table 1. Selected experimental factors and PBD for concluding 12 experimental trials were shown in Table 2.

Results and Discussion

The current study was carried out to investigate important factors that affect *Dunaliella salina* growth conditions using the PBD. Effects of the investigated factors on response variables, including biomass and pigments such as chlorophyll a, chlorophyll b, and carotenoids were assessed as well.

- ***Biomass content***

According to the conducted PDB, the biomass content varied from 1.90 to 8.54 (g/100) in trials 1 and 8 respectively (Table 2). Statistical analysis is summarized in Table 3. The p- values were reported as significant when $p<0.05$. Results showed that only five out of nine factors, including carbon sources (date waste), salt (NaCl), pH, Inoculum concentration and incubation time, were significant ($p<0.05$). The carbon source (date waste) included the highest significant positive effect (2.56) on biomass production (Table 4) because the highest growth rate was achieved in mixotrophic culture (Mojaat et al., 2008; Fawzy and Gomaa, 2020). Utilizing glucose, malonate and hydrolyzed fucoidan and alginate separated from *Cystoseira trinodis* brown alga as a carbon source in the cultivation of *Dunaliella salina* increased the biomass and protein productivity (Mojaat et al., 2008; Kadkhodaei *et al.*, 2015; Fawzy and Gomaa, 2020).

Table 2. Twelve trail PBD to study the effect of ten factors on the biomass content, chlorophyll a, chlorophyll b and carotenoids production by *Dunaliella salina.*

Run	Variables										Biomass g 100 DWC		Chlorophyll a mg l^{-1}		Chlorophyll b mg l^{-1}		Carotenoid mg/l	
	A	B	C	D	E	F	G	H	I	J	Experimented	Predicted	Experimented	Predicted	Experimented	Predicted	Experimented	Predicted
1	+1	-1	-1	+1	+1	-1	-1	+1	+1	-1	1.90	2.44	0.72	0.71	1.54	1.63	3.14	3.64
2	-1	+1	-1	-1	+1	-1	+1	+1	+1	+1	2.62	1.43	0.82	0.81	1.07	1.07	4.22	3.92
3	+1	-1	+1	-1	-1	-1	+1	-1	+1	+1	6.89	7.33	1.14	1.19	2.52	2.55	8.02	7.90
4	+1	+1	-1	-1	-1	+1	-1	+1	-1	+1	3.72	4.23	0.60	0.64	1.28	1.25	4.35	4.57
5	-1	+1	+1	+1	-1	-1	+1	+1	-1	-1	2.91	3.12	0.93	0.94	1.24	1.18	4.59	4.87
6	+1	-1	+1	-1	+1	+1	+1	+1	-1	-1	5.9	5.69	1.05	1.04	1.72	1.75	5.32	5.45
7	+1	+1	-1	+1	-1	+1	+1	-1	+1	-1	4.92	4.62	1.35	1.31	2.8	2.7	8.6	7.86
8	+1	+1	+1	+1	+1	-1	-1	-1	-1	+1	8.54	7.57	0.97	0.92	2.14	2.15	7.92	7.96
9	-1	+1	+1	-1	+1	+1	-1	-1	+1	-1	2.84	3.22	1.09	1.12	1.95	1.98	5.95	6.45
10	-1	-1	+1	+1	-1	+1	-1	+1	+1	+1	2.44	2.58	0.78	0.74	1.59	1.56	3.9	3.1
11	-1	-1	-1	+1	+1	+1	+1	-1	-1	+1	2.89	3.85	0.81	0.80	1.57	1.70	4.66	5.42
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	2.91	2.3	0.59	0.60	1.16	1.10	4.70	5.01

Table 3. Twelve-trial Plackett-Burman design for ten variables

Source	DF	Adj MS				P-value			
		Biomass	Chlorophyll a	Chlorophyll b	Carotenoid	Biomass	Chlorophyll a	Chlorophyll b	Carotenoid
Main Effects	10	8.82	0.11	0.65	6.66	0.00	0.00	0.00	0.00
pH	1	14.76	0.18	2.27	35.58	0.00	0.00	0.00	0.00
Light intensity	1	2.26	0.30	0.25	4.36	0.05	0.00	0.003	0.01
Carbon source	1	39.43	0.10	1.93	13.55	0.00	0.001	0.00	0.00
Nitrogen source	1	1.27	0.07	0.02	5.17	0.13	0.003	0.28	0.006
NaCl	1	18.86	0.18	0.52	5.46	0.00	0.00	0.00	0.005
Fe	1	1.63	0.03	0.25	0.002	0.09	0.02	0.004	0.94
B_1	1	0.31	0.01	0.23	0.002	0.44	0.13	0.005	0.98
B_{12}	1	0.09	0.01	0.06	1.79	0.67	0.61	0.10	0.07
Incubation time	1	4.41	0.14	0.91	0.66	0.01	0.000	0.00	0.25
Inoculum concentration	1	5.19	0.06	0.01	0.04	0.007	0.006	0.50	0.76
Residual Error	13	6.62	0.005	0.26	0.47	0.00			
Lack of Fit	1	6.59	0.01	0.02	3.06		0.10	0.25	0.005
Pure Error	12	0.03	0.004	0.23	0.25				
Total	23	94.89							

Table 4. Effect of factors and statistical analysis of factors using Plackett–Burman Design

Term constant	Effect				Coefficient			
	Biomass	Chlorophyll a	Chlorophyll b	Carotenoid	Biomass	Chlorophyll a	Chlorophyll b	Carotenoid
pH	-1.56	-0.17	-0.61	-2.43	-0.78	-0.08	-0.30	-1.21
Light intensity	0.61	0.22	0.20	0.85	0.30	0.11	0.10	0.42
Carbon source	2.56	0.13	0.56	1.50	1.28	0.06	0.28	0.75
Nitrogen source	0.46	0.11	0.06	0.92	0.23	0.05	0.03	0.46
NaCl	1.77	0.17	0.29	0.95	0.88	0.08	0.14	0.47
Fe	-0.52	0.08	0.20	-0.02	-0.26	0.04	0.10	-0.01
B_1	-0.22	0.04	0.19	-0.006	-0.11	0.02	0.09	-0.003
B_{12}	0.12	0.01	-0.10	-0.54	0.06	0.008	-0.05	-0.27
Incubation time	-0.85	0.15	0.39	0.33	-0.42	0.07	0.19	0.16
Inoculum concentration	0.93	-0.10	-0.03	0.08	0.46	-0.05	-0.01	0.04

* Different letters indicate significant differences (p<0.05).

NaCl has had a positive significant effect (effect = 1.77) on the isolated *Dunaliella salina* biomass content in the two analyzed levels (Table 4). This microalgae can bare a wide range of salinity and geography is influential in the width of this range (García *et al*., 2007). The impact of salinity in the three phases of high, medium, and low on a *Dunaliella salina* species showed the highest number of cells occur in medium salinity with 1.5 molar NaCl (Vo *et al*., 2017). Salinity shocks in a *Dunaliella salina* have shown that cell growth exists in various salinities but the optimum biomass accumulation occurs in a specific range and in high-stress situations, there are fewer cells with higher cell volumes (Vo *et al*., 2017; Qin *et al*., 2021).

pH has negatively affected (-1.56) the biomass content. It shows that biomass production when the pH of culture media before inoculation was 6.5 was more (Table 4). In general, hydrogen ion concentration (pH) of the growth environment affects many processes related to microalgal growth, metabolism, and ion absorption (Borowitzka and Siva, 2007). Maximum growth and biomass production of *Dunaliella salina* was reported at pH 7 (Reshma *et al*., 2021). However, changes in culture conditions such as salinity may alter the optimal pH for biomass production (McLachlan, 1964).

The inoculum concentration in the two investigated levels significant y positive effect on biomass content. Suitable

inoculation can effectively shorten the delay stage of the microalgae cell and make it enter the growth phase faster (Baquerisse, 1999; Zhu and Jiang, 2008). Thus, shortening the cultivation cycle, increasing efficiency, and reducing cost (Zhu and Jiang, 2008).

Incubation time showed a significant negative effect (-0.85) on biomass content (Table 4). Microalgae growth curve in optimal conditions is divided into four stages (lag phase, exponential phase (EX), or log phase, stationary phase, and death phase). Phototroph microalgae go through an extra growth phase, namely linear growth. The transformation from the logarithmic phase (growth phase) to linear growth in such microalgae including *Dunaliella salina* is caused by limited light in the dense algae cultures. *Dunaliella salina* showed the highest biomass accumulation on the 12^{th} and the 14^{th} day and after that biomass cultivation decreased (Ra et al., 2015; Aziz et al., 2020). Therefore, the linear growth phase in this microalga can be delayed or prevented by slightly increasing the light intensity depending on the cell concentration. The limitations of the culture condition can affect the duration of these phases and thus the cultivation time it takes for the microalgae to reach equilibrium or the optimum completion of the phases (Aziz *et al*., 2020). If the algae reach equilibrium after passing the exponential phase, biomass accumulation decreases as seen in this study.

- ***Pigments content (Chlorophyll a, b, and Carotenoids)***

The highest and lowest contents in *Dunaliella salina* belonged to Trials 7 and 1, respectively. Carotenoid, Chlorophyll a and b content of *Dunaliella salina* varied from 3.43-8.65 (trial 1-7), 0.63-1.36 (4-7) and 0.93-2.98 mg/L (2-7). Regression analysis revealed that the pH, carbon source, NaCl, nitrogen source and light intensity had significant effects on carotenoid production, while light intensity, NaCl, pH, Incubation time, carbon source, nitrogen source, inoculum concentration, and Fe affect chlorophyll a and pH, carbon source, incubation time, NaCl content, light, Fe and B1 content affect chlorophyll b production significantly (Table 3) ($p<0.05$).

pH has a significantly negative effect -0.17, -0.61, and -2.43 on chlorophyll a, b, and carotenoid production by *Dunaliella salina* (Table 4). Thus, maximum pigment accumulation occurred in pH 6.5 ($p\leq0.05$). Significant changes in the environment's pH may cause stress in the growing cells thus launching the stress prevention process which has a considerable impact on the production of chlorophylls, carotenoids, and other metabolites. Modifying culture conditions such as salt concentration can change the optimal pH for cell growth. Suitable pH for microalgae can change depending on their species (McLachlan, 1964). In this regard, in this research, maximum chlorophyll and carotenoid production of *Dunaliella salina* occurred at pH = 7 (Reshma et al., 2021).

Light intensity showed positive and significant effect on pigment production (Table 4). Linear correlation between light intensity and the accumulation of pigments was proved (Xu *et al*., 2018). In this regard, it has shown that the greatest effect of light intensity (+0.22) is in the accumulation of chlorophyll a. Chlorophyll a is the main photochemical pigment in photosynthetic organisms and its accumulation increases with the increase of light intensity (Wood, 1979). Carotenoid production in *Dunalliela salina* occurs in order to control the stress caused by high-light-intensity (Xu et al., 2016).

The addition of date waste as a rich source of organic carbon, in a culture medium affect positively on pigment production ($p<0.05$). Evidently, an organic carbon source can provide mixotroph growth conditions and cause cell growth and pigment accumulation (Chavoshi and Shariati, 2019). Research shows that a mixotroph culture can positively impact carotenoids in *Dunaliella salina* by optimization of its components including organic carbon (Morowvat and Ghasemi, 2016).

Investigating NaCl in two levels in the PBD showed that with the increase of NaCl, pigments accumulation increases. This factor is valuable for Chlorophyll accumulation. Chlorophyll a is the most important photosynthetic pigment, one which in the right conditions has a positive impact on cell growth. However, in stress conditions including high salinity, this pigment is damaged, and thus protective pigments such as Chlorophyll b and Carotenoids come into action (Goericke and Montoya, 1998). In this study, the addition of 8 % NaCl to the culture medium was suitable for *Dunaliella salina* growth and pigment accumulation. A significant correlation between the salt stress and growth rate, pigments and lipid accumulation, and lipid compounds in *Dunaliella salina* was asserted (Benavente-Valdés et al., 2016; Qin *et al*., 2021).

Insert of urea as a nitrogen source had a positive effect on chlorophyll a (+0.11) and carotenoids (+0.92) production by *Dunaliella salina* (Table 3 and 4). In the presence of nitrogen source at concentration 400 mg l^{-1}, high accumulation of chlorophyll a and carotenoids is observed. *Dunaliella salina* carried out carotenogenic activity in order to control stress, which in turn increases the accumulation of carotenoid pigments (Pereira and Otero, 2019). Addition of nitrogen may be stressful for *Dunaliella salina* and induce carotenogenesis activity therefore, production and accumulation of carotenoids was increased (Giordano, 2001).

Addition of iron sulfate as a source of Fe^{2+} had a significant and positive effect on Chlorophyll a and b accumulation ($P\leq0.05$) but this factor did not have a significant effect on Carotenoids (Table 4). Research has shown that the stress caused by the lack of this ion in the culture medium of photosynthesizing microalgae dramatically changes photosynthetic activity and cell function so much so that it causes chloroplasts shrinkage, thylakoid membrane reduction, and great reduction in Chlorophyll content which in turn lead to energy yield reduction and mutual photochemical transformations in photosystem 2 (PS-II). Adding iron to the culture reverses all the above-mentioned alteration (Vassiliev *et al*., 1995; Varsano et al., 2003). The added iron in this study increases Chlorophyll content in order to optimize photosynthetic activity. However, this level of iron did not have much effect on Carotenoid content. Higher than optimal level of iron causes stress by producing active oxygen molecules and therefore has a negative impact on cell growth and Chlorophyll accumulation while having a positive impact on Carotenoids (Mojaat *et al*., 2008). This occurrence is due to Carotenoids' role in protecting the photosynthetic mechanism against environmental stress especially oxidative ones caused by active oxygen. In this research, iron ion concentration was not as high thus did not cause a significant effect on carotenoid content.

Incubation time significantly affected chlorophyll a and b accumulation while this effect was not significant for carotenoid content ($p<0.05$). Research has

shown that in addition to light intensity, incubation time, or the time that microalgae are exposed to light, can also affect growth rate (Rehman *et al*., 2022). Under different exposures, in terms of exposure time and frequency, the growth rate of microalgae may differ (Carvalho *et al*., 2010). In this research, incubation time increased chlorophyll accumulation during growth but did not cause stress and did not increase the accumulation of protective pigments such as carotenoids (Xu *et al*., 2016).

This level of B_1 concentration was not significant to chlorophyll a and carotenoid but positively affected Chlorophyll b (Table 4). Changing the levels of B12 did not significantly alter this pigment\s content. Many studies explain that alga species require various combinations of B_{12} (cobalamin), B_1 (thiamin), and B_2 (biotin), and must obtain them from the culture medium. Among the 306 analyzed species, more than half required cobalamin, 22% required thiamin, and only 5% required biotin (Croft *et al*., 2006). Many such vitamins become available to the alga through a symbiotic relationship with bacteria. In a study, *Dunaliella salina* was cultivated in various cultures. The difference between two of these cultures was in their B_1 and B_{12} contents. Biomass, carotenoid pigment, and chlorophyll accumulation significantly increased in the culture with higher vitamin content, especially in the first seven days (Colusse *et al*., 2020). In the recent research, however, the investigated levels only showed a significant effect on chlorophyll. This could be because photochemically active pigments such as chlorophyll b are less sensitive and less likely to decrease compared to chlorophyll a in the conditions of heat and light intensity (Xu *et al*., 2016).

Change in the Inoculum concentration showed significant negative effect on Chlorophyll a while it did not show a significant effect on the other three pigments. Research has shown that Carotenoid content was higher in high inoculation concentration than in low concentration. However, this increase in concentration is limited, and does not occur in every high inoculation concentration (Zhu and Jiang, 2008). In this study, the higher concentration has probably reduced the delay phase and the cells reached equilibrium faster, thus a reduction in Chlorophyll content is seen. This change is not visible in other pigment contents.

Conclusion

The screening phase of research by PBD is the first step of the optimization process. Out of the ten factors in this study (pH, light intensity, carbon source (date waste), Nitrogen source, NaCl, Fe (ferrous sulfate), vitamin B_1, vitamin B_{12}, Incubation time and Inoculum concentration) that affected the pigment production and growth of *Dunaliella salina*, factors affecting each response were screened using PBD. It has been shown that the use of agricultural wastes such as date wastes in *Dunaliella salina* culture medium can increase biomass and pigment (chlorophyll a, chlorophyll b, and carotenoids) productions while decreasing production costs. It is necessary to optimize and modeling growth factors of *Dunaliella salina* using appropriate statistical methods for the scale up of this cultivation. In the recent study, this significant increase in carotenoids due to the increase of organic carbon levels was seen which can be a solution to optimize the conditions of this microalgae in order to increase the carotenoid pigment in future research.

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