Enhancement of β-Carotene Biosynthesis in Microalga *Dunaliella salina*: Mixotrophic Cultivation and Static Magnetic Field Treatment

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Abstract

Carotenoids, such as β-carotene and astaxanthin, are the most important photosynthetic metabolites found in microalgae. They have several properties such as antioxidant and antidiabetic activities. β-Carotene is one of the precursors of vitamin A possessing useful applications in the food industry, cosmetics, and pharmaceuticals. The microalga *Dunaliella salina* is known as one of the biological producers of this valuable pigment, thanks to the lack of a rigid cell wall and shorter cultivation time. The optimization of culture medium for *D. salina* through the application of appropriate operational conditions such as static magnetic fields, can significantly impact biomass and carotenoids production. In this study, mixotrophic cultivation of *D. salina* was examined by adding different organic carbon sources, including glucose, fructose, acetate, malonate, glycerol, and starch, each at two levels of concentration. The inhibitory effect of malonate and glycerol on both growth and β-carotene production was observed. Starch demonstrated higher biomass production (1.22 g L^{-1}) and β-carotene accumulation (10.12 mg L^{-1}) compared to other carbon sources. Based on its superior performance, the optimization process was continued using 7.0 g L^{-1} of starch as the optimum concentration. Subsequently, static magnetic fields with two intensities (10 and 30 mT) were applied to the mixotrophic samples at various exposure times (1 and 24 h day^{-1}). The results revealed that the 24-hour treatment with both intensities improved biomass production (2.18 g L^{-1}) and β-carotene concentration (6 mg L^{-1}) by up to 25% in the culture of *D. salina* enriched with an organic carbon source.

Keywords: β-Carotene, *Dunaliella salina*, Static magnetic fields, Mixotrophic cultivation

Introduction

β-Carotene is a natural red-orange carotenoid that is widely distributed in some

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plants, bacteria, fungi, and microalgae. It serves as an accessory pigment for photoprotection and photosynthesis. Besides, it is commonly used in the food, feed, aquaculture, pharmaceutical, and cosmetics industries owing to its essential properties such as a colorant agent, antioxidant, anticancer, etc.(Comstock et al., 1992; Hejazi and Wijffels, 2003). Chemical synthesis of β-carotene is not extensively demanded due to its numerous side effects and lack of economic profitability. Hence, recent studies have focused on the natural biosynthesis of β-carotene from algal sources. Among these sources, microalgae, with their ten times higher carotenoid content and high biomass production, along with continuous stock supply, are considered better sources than macroalgae. Furthermore, microalgae have a unicellular structure, which allows for more energy to be directed towards photosynthesis, growth, and propagation processes. (Gong and Bassi, 2016; Griffiths et al., 2011; Mohamadnia et al., 2020a). Therefore, recent studies have been conducted to economically produce microalgae by stimulating growth and modifying metabolic behaviors. In other words, optimizing factors such as light intensity, salinity, nutrient deficiency, and physical treatment like exposure to a static magnetic field (SMF) (intensity and exposure time) can lead to the accumulation of more β-carotene in microalgae cells (Small et al., 2011; Zarandi-Miandoab et al., 2019).

It is found near electrical devices that SMF has higher power, despite the earth generating only 0.05 mT by itself. Researchers are concerned about the risk possibility of SMFs disordering functions in biological systems. This concern arises due to the relatively high strength of SMFs measured in various environments, such as rail systems(2 mT), welding (50 mT), power plants (50 mT), aluminum production facilities (100 mT), and even small bar magnets (1–10 mT) (Bauer et al., 2017; Organization 2006). Despite several disadvantages of SMF, magnetic treatments are broadly used due to their non-toxic, secure, low-cost nature, and absence of secondary pollutants (Deamici et al., 2016b). Besides, SMFs have numerous consequences on microorganisms, such as increasing seed germination, growth, pigment content, oxidative stress, and changes in gene expression (Small et al., 2012). SMFs enhance the lifetime, concentration, and activity of free radicals by transforming the spin orientation and energy levels of electrons. On the other hand, the development of metabolic pathways leads to the augmentation of carotenoids (antioxidants), which help cells defend against oxidation under various stresses (Wang et al., 2008).

The magnetic field effects were investigated on the growth profile of different microorganisms. SMFs motivate or inhibit the growth of microorganisms and their metabolisms also will be affected by modifying the synthesis of carbohydrates, proteins, and accumulation of essential amino acids (Veiga et al., 2020). It has been observed cell membrane breakage at 100 mT (Bajpai et al. 2012), higher growth rate of 10–15%, and maximum growth at 76 ± 5 mT in *Escherichia coli* (Letuta and Berdinskiy, 2017). Moreover, it has been

reported the increment of the growth rate in some bacteria and colony formation ability at 35 mT (Letuta and Tikhonova, 2019), cell proliferation, and changes in gene expression at 300 mT (Potenza et al., 2004). In other research, it was shown the larger size of colonies at 0.4–0.8 mT (Haque et al., 2016) improvement in catalase activities and glutathione peroxidase, reduction in growth and viability under 250 mT SMF in *Saccharomyces cerevisiae* (Kthiri et al., 2019).

Lately, magnetic fields have been potentially considered as a physical treatment to enhance both cell growth and bio-products synthesis in microalgae such as fatty acids, pigments, carotenoids, etc., simultaneously (Bauer et al., 2017). For instance, biomass productivity and biochemical contents of *Chlorella fusca* have risen to 60 mT (Deamici et al., 2016a). Small et al. (Small et al., 2012) found more amount of biochemical compounds such as carbohydrates, protein, lipids, and carotenoids were produced alongside doubled biomass production at 10 mT in *Chlorella kessleri*. In another study, Bauer et al. (Bauer et al., 2017) exhibited cell density enhanced up to 50%, bio-compounds hardly changed under 60 mT and 1 h day^{-1} exposure time of SMF. Although there are many reports about the effect of SMF on microalgae e.g. *Spirulina* sp. (Deamici et al., 2016b; Shao et al., 2018; Veiga et al., 2020), *Nannochloropsis oculate* (Chu et al., 2020), *Scenedesmus obliquus* (Tu et al., 2015), etc. there is a necessary need to examine the effect of SMFs on the biomass production, biochemical, and carotenoids contents, particularly β-carotene, in the microalga *Dunaliella salina*.

The present study aims to investigate the effect of SMF on the growth of *D. salina*, biochemical composition changes, and antioxidant activity under mixotrophic conditions.

Material and methods

Medium preparation

Dunaliella Salina CCAP 19/18 was kindly donated by the Agricultural Research, Education, and Extension Organization (AREEO), Iran. The experiments were carried out in 1000 mL sterilized flasks with 500 mL working volume at 25 ± 1 °C. The flasks reached 500 mL with Modified Johnson medium followed by inoculation with 100 mL of *D. salina* (X_0 = 7.5×10^5 cells m L^{-1}) (Johnson et al., 1968). The light was provided by 60 W fluorescent lamps under 60 µmol m^{-2} s^{-1} illumination intensity with (16:8) light: dark cycle in phytotron. Cultures were aerated by air supplemented with 1% (v/v) CO_2 through a 0.45-µm filter at 5 L min^{-1}. All experiments were done in triplicate.

Mixotrophic cultivation

The carbon substrates (fructose, glucose, acetate, malonate, starch, and glycerol) were added to the prepared medium after filtration through 0.22 µm membranes, separately. The concentrations of carbon sources are described in Table 1. The mentioned concentrations were extracted from the literature to achieve maximum cell density and β-carotene production and then applied to the Johonson medium (Eijckelhoff and Dekker, 1997; Folch et al., 1957; Frings and Dunn, 1970; Johnson et al.

1968; Mohamadnia et al., 2020b). Similar to the screening step cell density, β-carotene content, and biochemical compositions were measured.

SMF effect on mixotroph cultivation

SMF effect was performed by ferrite magnets adaptation with a mean intensity of 30 mT (150 × 100 × 20 mm, cubic) at 15 cm departing or 10 mT (160 × 120 × 20 mm, cylindric) at 18 cm departing. Thus, the SMF generated was concentrated in the inner part of the bottles and the SMF intensities were measured by a Gauss meter (Magna, MG-701, Japan). The control cultures (CC) were carried out under the same conditions of the cultivation with only an SMF of 0.005 mT (Earth's SMF). To evaluate the exposure time of MF in the cultures, the magnets were applied in two ways: throughout all cultivation periods (24 h day^{-1}) and for 1 h day^{-1} at light cycle.

Chemicals and reagents

Analytical grade chemicals and solvents like phenol, vanillin, glucose, fructose, sodium acetate, sodium malonate, glycerol, starch, acetone, sulfuric acid, and phosphoric acid were purchased from Merck Chemical Company. Distilled water was used for all experiments, other chemicals consumed in modified Johnson media, sodium dodecyl sulfate (SDS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (USA).

Growth analysis

Algal growth was measured through three approaches; cell counting utilizing a hemocytometer after lugol dying on a microscope (Nikon YS100 Biological Microscope) at 100x magnification, absorbance using a spectrophotometer at 680 nm (Pharmacica LKB. Ultrospec III), and dry cell weight. The maximum specific growth rate (μ_{max}, day^{-1}) was calculated using Eq. (1):

$$\mu_{max} = \ln (N_t/N_0)/(t_t-t_0) \quad \text{Eq. (1)}$$

Where N_0 and N_t are cell density (cell mL^{-1}) at the beginning and end of the logarithmic growth phase, respectively. t_0 and t_t are the duration of the exponential phase (day). The doubling time (t_d) was measured by Eq. (2):

$$t_d = \ln 2(\mu_{max})^{-1} \quad \text{Eq. (2)}$$

The maximum biomass productivity (P_{max}, mg L^{-1} day^{-1}) was acquired by Eq. (3):

$$P_{max} = (X_t-X_0)/t*1000 \quad \text{Eq. (3)}$$

Where X_t and X_0 are the biomass concentrations at the end and beginning of the cultivation, respectively (Deamici et al. 2016a). Besides, the maximum biomass concentration (X_{max}, g L^{-1}) was obtained on the last day of the culture.

β-Carotene and chlorophyll determination

β-Carotene and chlorophyll contents were measured daily by taking the samples from the medium and centrifuged at 3000 rpm,

Table 1. The concentration of carbon sources.

Nutrient Concentration	Glucose (M)	Fructose (M)	Sodium acetate (M)	Sodium malonate (M)	Glycerol (M)	Starch (g L^{-1})
Low	0.50	0.10	0.05	0.05	0.01	0.50
High	0.90	0.40	0.07	0.07	0.20	5.00

5 min. Supernatants were ignored, and biomass was rinsed with distilled water and centrifuged again. Remained biomass vortexed with 80% acetone (1:1 v/v) for a minute. Finally, the samples were centrifuged at 3000 rpm for 5 min. The extraction process was repeated until the biomass was colorless. Equations (4), (5), and (6) were used to determine the chlorophyll a (chl a), chlorophyll b (chl b), and β-carotene (bc) concentration (µg mL^{-1}) (Eijckelhoff and Dekker 1997):

$C_{chl\ a} = -1.709\ A_{412} + 11.970\ A_{431} - 2.998\ A_{460} - 5.708\ A_{480}$ Eq. (4)

$C_{chl\ b} = -0.171\ A_{412} - 0.230\ A_{431} + 11.871\ A_{460} - 13.248\ A_{480}$ Eq. (5)

$C_{bc} = -0.430\ A_{412} + 0.251\ A_{431} - 4.376\ A_{460} + 13.216\ A_{480}$ Eq. (6)

Where the A_{412}, A_{431}, A_{460}, and A_{480} are the absorbance at 412, 431, 460, and 480 nm.

Biochemical composition assay

To determine the amount of total lipid, carbohydrate, and protein, the algal cultures were harvested by centrifugation at 5500 rpm for 5 min and then freeze-dried at -55 °C for 48 h. The samples were kept at −20°C for further analysis. All analyses were done in triplicate.

Total lipid

Lipid content was estimated according to the Sulfo–Phospho–Vanillin (SPV) method (Frings and Dunn 1970) with a few modifications. 5 mL of chloroform-methanol (2:1 v/v) was added to 50 mg freeze-dried algal powder and vortexed according to Folch et al. (Folch et al. 1957) pre-treatment method. Afterwards, 5 mL of NaCl 0.9% (w/v) was combined with the mixture and centrifuged at 3000 rpm for 3 min. The algal residues remained in the aqueous phase and the organic phase (chloroform-methanol) contained lipids. Then, 0.2 mL of the organic phase was evaporated in the oven at 50°C. Lastly, 0.1 mL of 98% sulfuric acid and 2.4 ml of phospho-vanillin reagent (30 mg vanillin in 25 mL of 68% (v/v) phosphoric acid) were put together. The absorbance of the samples was read at 540 nm. Lipid concentration was measured throughout the standard curve of canola oil (0–80 µg in chloroform).

Total carbohydrate

50 mg of freeze-dried algal biomass was pretreatment and hydrolyzed by a mean of 2 N HCl for 1 hr. according to (Mohamadnia et al., 2020b). Next, the analysis was continued by adding 5% phenol and 98% sulfuric acid to the samples. The absorbance was read at 490 nm followed by 20–min staying at 30 °C in a water bath. The calibration curve was attained using a standard glucose (Laurens et al., 2012).

Total protein

50 mg of powdered microalgae were broken with 0.2 mL ultra-pure water and up to 2% sodium dodecyl sulfate (SDS). After sonication for 10-min and centrifugation at 3000 rpm for 5-min, the supernatants were kept at 4 °C for 12 hours. Subsequently, 100% acetone was mixed with proteins (2.5:1, v/v) and sonicated in an ice bath for 30 min. The proteins of the supernatant were washed with 70% acetone (2.5:1, v/v) followed by re-centrifugation. At last, the supernatants were removed by centrifugation at 3000 rpm for 2 min 0.5 mL of ultra-pure water was added to proteins and solubilized proteins were quantified at 595 nm (He 2011). Total

proteins were determined by a standard curve obtained by the known concentrations of bovine serum albumin (BSA) standard solutions (150–350 μg mL^{-1}).

Antioxidant activity assay

The scavenger effect of free radical 2,2–diphenyl–1–picrylhidrazol (DPPH) was estimated by the Rufino et al. (Rufino et al. 2007) method with a few modifications. Samples of 3.9 mL DPPH (0.06 mM) soluble in methyl alcohol kept in the dark, were added to 0.1 mL methanol extract (2.5 mg mL^{-1}). After 60 min at ambient temperature, the optical densities were read at 517 nm, and calculation was done according to Equation 7.

Inhibition (%) = (1–A sample/$A_{control}$) ×100 Eq. (7)

The lowest absorbance represented a greater scavenging capacity of the DPPH radical and antioxidant activity (Bauer et al. 2017).

Results

Carbon source screening

Growth profile

Algal growth in heterotrophic or phototrophic cultures can be potentially enhanced by enriching their medium with an appropriate organic carbon (Mohamadnia et al., 2023; Alkhamis and Qin, 2013). In the present study, the Modified Johnson medium was supplemented by different organic carbons to investigate the growth profile of *D. salina*. As shown in (Figure 1a), the medium containing 0.5 M glucose reached a stationary phase at day 9 (μmax = 0.23 day^{-1}) almost the same as the control (cell density (CD) = 6×106 cells mL^{-1}, μmax = 0.20 day^{-1}) at day 12. Besides, 0.9 M glucose (CD = 6×106 cells mL^{-1}, μmax = 0.17 day^{-1}) showed an inhibitory behavior in comparison with the control.

Furthermore, the low and high concentrations of fructose reached the late exponential phase after 9 and 7 days, respectively, with the same cell density and growth rate (CD = 6×10^6 cells mL^{-1}, μ_{max} = 0.22 day^{-1}) (Fig.1b). On the other hand, as manifested in Fig.1c,

Table 2. Kinetic parameters at different carbon sources.

Nutrient	X_{max} (g L^{-1})	P_{max} (g L^{-1} day^{-1})	μ_{max} (day^{-1})	t_d (day)
Control (Autotrophic)	1.05	0.07	0.20	2.30
Glucose (0.5 M)	1.07	0.09	0.23	2.16
Glucose (0.9 M)	1.33	0.11	0.17	2.47
Fructose (0.1 M)	0.85	0.07	0.22	2.20
Fructose (0.4 M)	1.08	0.09	0.22	2.20
Sodium acetate (45 mM)	1.09	0.09	0.20	2.30
Sodium acetate (67 mM)	1.16	0.09	0.17	2.47
Sodium malonate (45 mM)	0.50	0.04	0.20	2.30
Sodium malonate (67 mM)	0.91	0.07	0.19	2.35
Glycerol (5 mM)	0.51	0.04	0.20	2.30
Glycerol (200 mM)	0.73	0.06	0.16	2.52
Starch (0.5 g L^{-1})	1.00	0.06	0.20	2.30
Starch (5.0 g L^{-1})	1.22	0.08	0.22	2.20

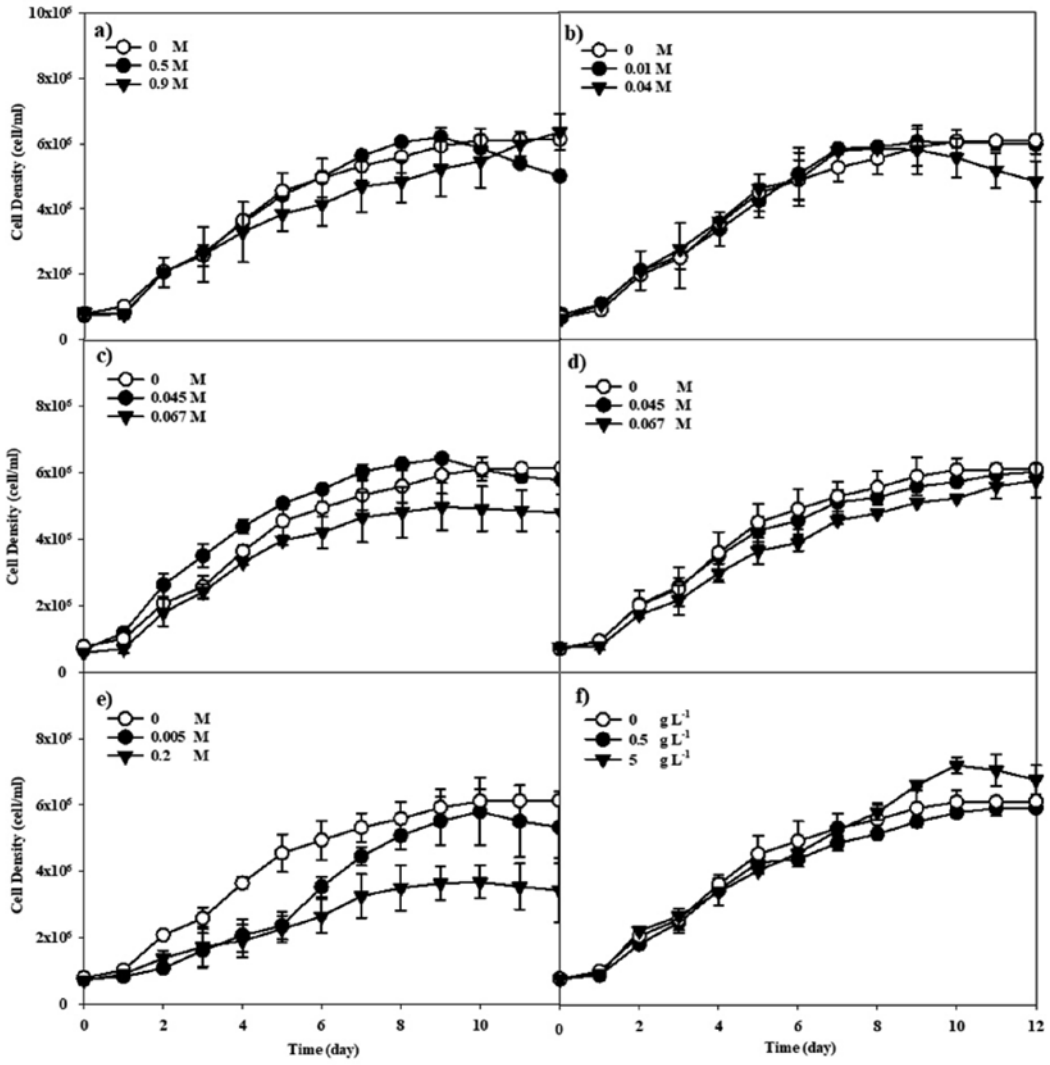

Fig. 1. Effect of different carbon sources on cell density a) glucose, b) fructose, c) sodium acetate, d) sodium malonate, e) glycerol, and f) starch data is shown as mean ± SD (n = 3).

sodium acetate at low concentration (0.045 M) had a better performance during the exponential growth phase (9–days) with approximately 6.5×10^6 cells mL^{-1} and μ_{max} = 0.17 day^{-1} rather than its high concentration (0.067 M), (CD = 5×10^6 cells mL^{-1}, μ_{max}=0.17 day^{-1}).

In addition, sodium malonate in both 0.045 and 0.067 M had no significant effect on growth (CD = 5×10^6 cells mL^{-1}, μ_{max} = 0.17 day^{-1}) and (CD = 5×10^6 cells mL^{-1}, μ_{max} = 0.17 day^{-1}), respectively (Fig. 1d). Glycerol had a negative effect and inhibited the growth of *D. salina* by 0.16 day^{-1} (Table. 2) at 0.2 M in the present work (Fig. 1e). At last, a high concentration of starch (5 g L^{-1}) has the most effectiveness on cell density (7.2×10^6 cell m L^{-1}) 20% higher than the control in 10 days (Fig.1f).

β-carotene production and chlorophyll to β-carotene ratio (CBR)

Maximum β-carotene content and minimum chlorophyll to β-carotene ratio (CBR) were obtained (7.4 mg L^{-1}, 2.5) and (6.3 mg L^{-1}, 2.5) for 0.5 and 0.9 M glucose, respectively, in comparison to photoautotrophic growth

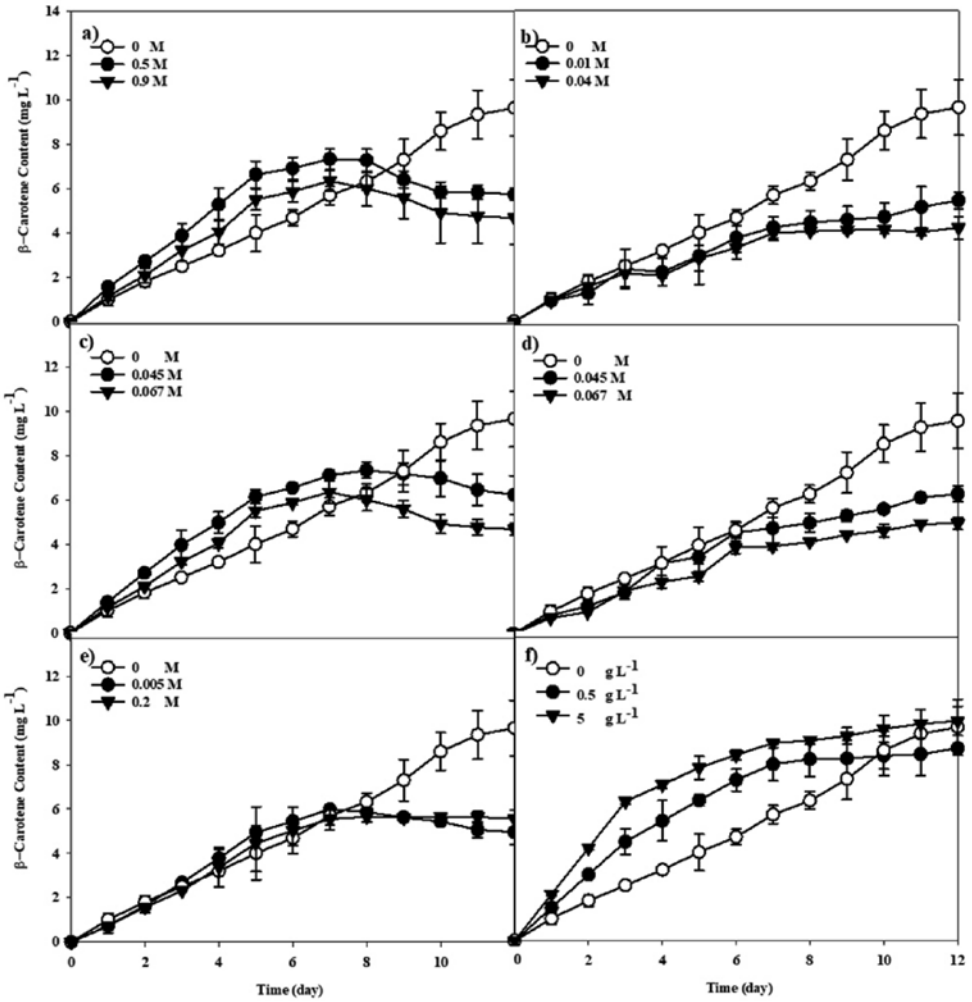

Fig. 2. Effect of different carbon sources on β-carotene content a) glucose, b) fructose, c) sodium acetate, d) sodium malonate, e) glycerol, and f) starch, data is shown as mean ± SD (n =3).

(9.7 mg L^{-1}, 3.2) (Fig. 2, 3 a).

The amount of β-carotene in the medium containing fructose (both concentrations) was less than the control (5.3 mg L^{-1} for 0.01 M and 4.3 mg L^{-1} for 0.04 M), in contrast, CBR was lower in comparison with control (2.0 at 0.01 M and 2.9 at 0.04 M), (Fig. 2, 3 b).

Sodium acetate and sodium malonate addition to the medium had not any positive effect on the production of β-carotene at both their low and high concentrations. Even though the maximum amount of β-carotene in glucose and sodium acetate medium was the same (7.4 mg L^{-1}), the CBR in glucose medium (2.5) was preferable rather than sodium acetate (4.8). On the other hand, β-carotene content and CBR in the medium enriched with fructose (5.3 mg L^{-1}, 2.0), and sodium malonate (7 mg L^{-1}, 4.6) were not affected significantly.

It was observed the glycerol-containing medium had less β-carotene (6.0 mg L^{-1} in both concentrations) and more CBR (4.5 at 0.005 M and 3.3 at 0.2 M). Besides, as the starch concentration in the medium was increased, the production of β-carotene (10.12 mg L^{-1}) and CBR were promoted (Fig. 2, 3 f). Also, the most biomass productivity (P_{max} = 0.09 g L^{-1} day^{-1}) was determined at 5

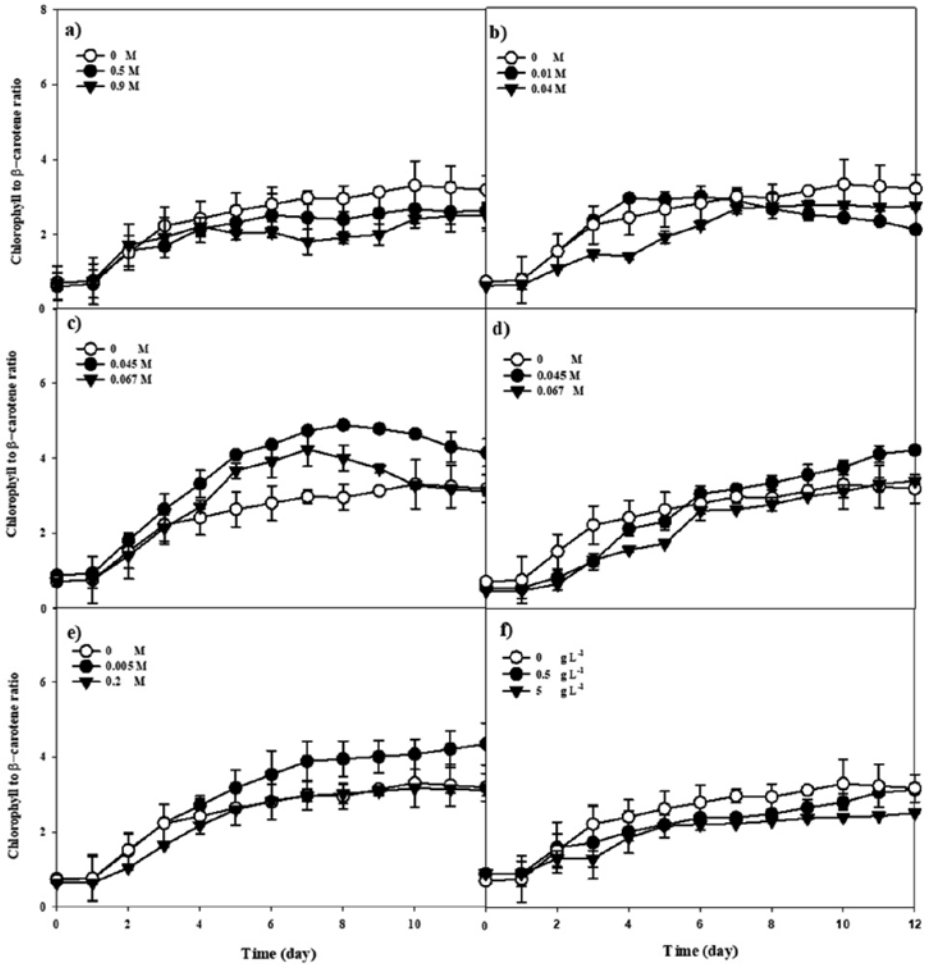

Fig. 3. Effect of different carbon sources on chlorophyll to β-carotene rate a) glucose b) fructose c) sodium acetate d) sodium malonate e) glycerol f) starch, data is shown as mean ± SD (n = 3).

Table 3. Kinetic parameters of growth at various concentrations of starch.

Starch Concentration (g L^{-1})	X_{max} (g L^{-1})	P_{max} (g $L^{-1}day^{-1}$)	μ_{max} (day^{-1})	t_d (day)
0.0	1.03	0.09	0.21	2.25
3.0	0.77	0.06	0.19	2.35
4.0	0.80	0.07	0.20	2.30
5.0	1.15	0.08	0.21	2.25
6.0	1.48	0.13	0.21	2.25
7.0	1.65	0.15	0.26	2.00

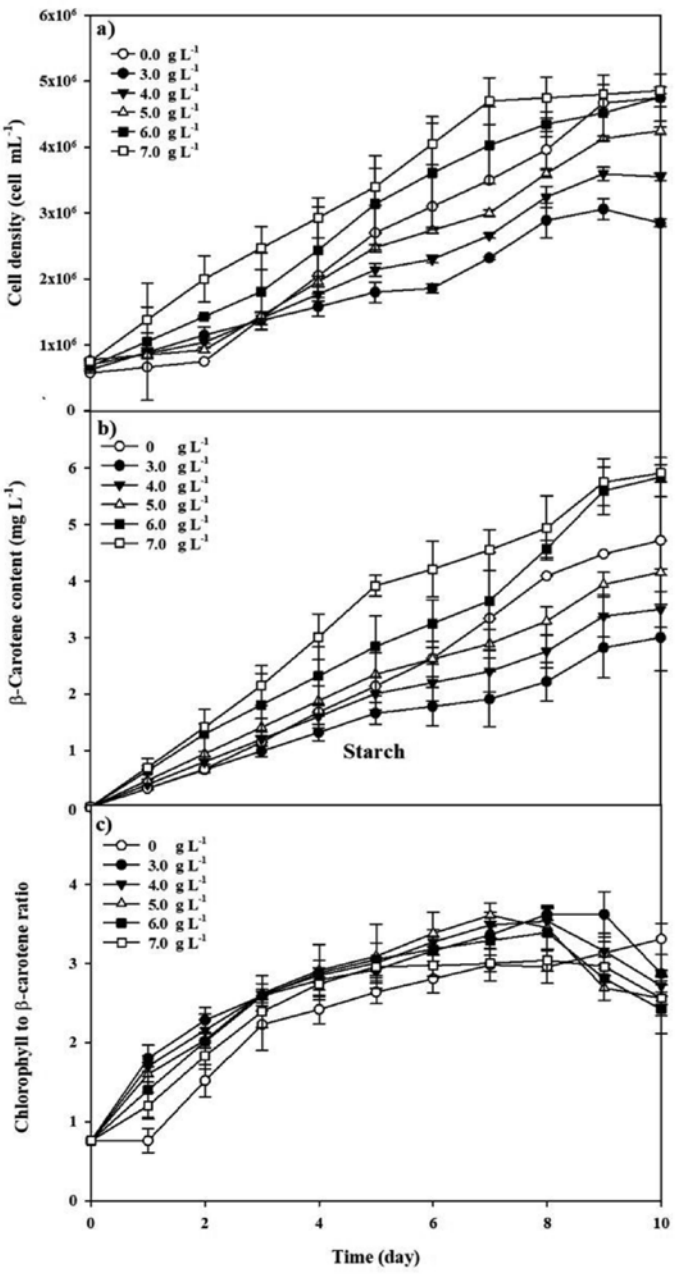

Fig. 4. Effect of different concentrations of starch on a) cell density, b) β-carotene content, and c) chlorophyll to β-carotene ratio, data is shown as mean ± SD (n = 3).

g L^{-1} of starch among tested carbon sources (Table 2).

Starch optimization

Starch containment in the medium was optimized using a one-factor-at-a-time (OFAT) approach to maximize microalgae growth and β-carotene accumulation. The one-factor-at-a-time approach involves studying about the responses when one factor changes while the other factors are constant. Cell density and β-carotene content were improved followed by the starch concentration increased in the medium. According to Fig. 4a, cell number reached 4.8×10^6 cells mL^{-1} on day 7 by the supplementation of the medium with 7.0 g L^{-1} starch (μ_{max} = 0.26 day^{-1}), while the control achieved μ_{max} = 0.21 day^{-1} after 10

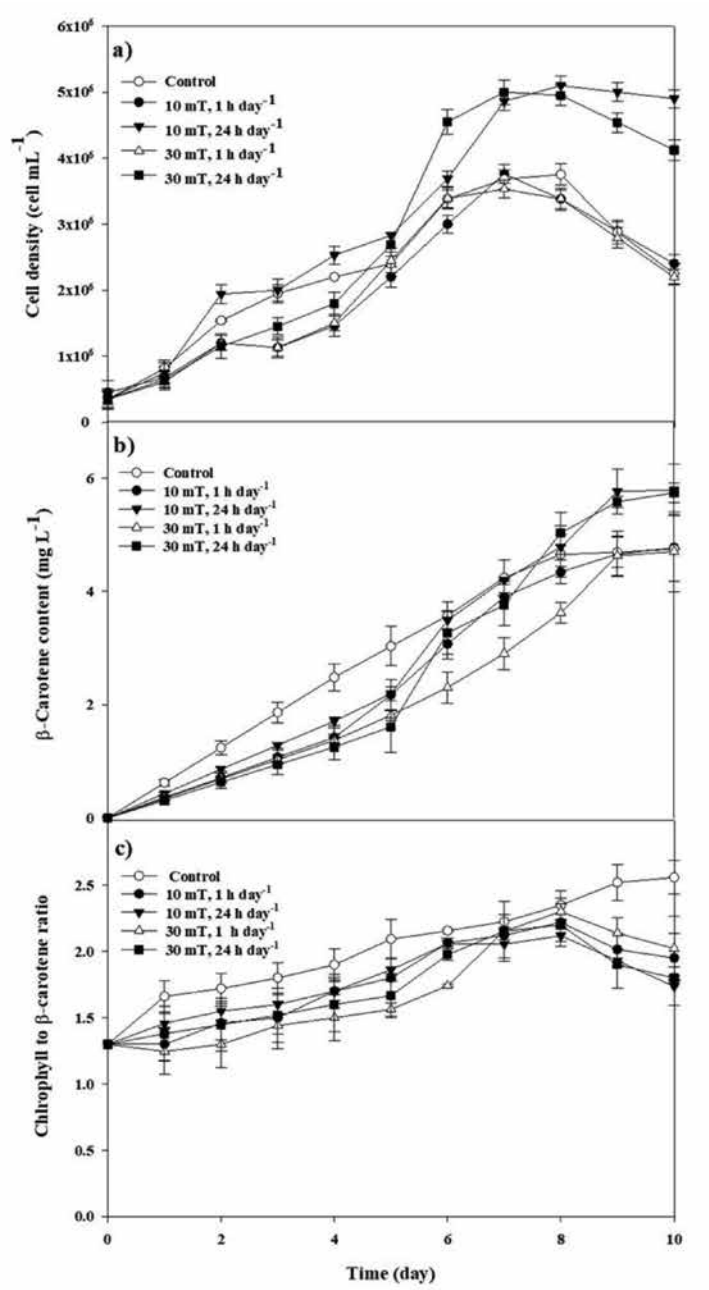

Fig. 5. Effect of different SMF intensity and exposure time a) cell density, b) β-carotene content, and c) chlorophyll to β-carotene ratio in constant starch concentration (7 g. L^{-1}), Data is shown as mean ± SD (n = 3)

Table 4. Kinetic parameters for the growth of *D. salina* in Modified Johnson medium enriched by 7 g L^{-1} starch and exposure with 10, and 30 mT SMF at 1 and 24 h.

Tests	X_{max} (g L^{-1})	P_{max} (g $L^{-1}day^{-1}$)	μ_{max} (day^{-1})	t_d (day)
Control	1.16	0.11	0.31	1.86
10 mT, 1 h day^{-1}	1.23	0.11	0.31	1.86
10 mT, 24 h day^{-1}	2.17	0.21	0.36	1.71
30 mT, 1 h day^{-1}	1.03	0.09	0.30	1.90
30 mT, 24 h day^{-1}	2.18	0.21	0.36	1.71

days. According to Table 3, even at lower concentrations of starch (3.0, 4.0, and 5.0 g L^{-1}), higher growth rates (0.19, 0.20, and 0.21 day^{-1}) were achieved, respectively, in comparison with control. Meanwhile, it was observed more β-carotene production (6 mg L^{-1}) in the media neutralized by 6.0 and 7.0 g L^{-1} of starch rather than control (4.8 mg L^{-1}) (Fig. 4b). Additionally, chlorophyll to β-carotene ratio did not differ significantly, in the studied media (Fig. 4c).

SMF effect on mixotroph cultivation

The effect of SMFs in various intensities and exposure times was investigated in a Modified Johnson medium enriched by starch. Implementation of 1 h day^{-1} by 10 and 30 mT magnetic treatment has obtained cell density (3.81×10^6 cells mL^{-1}), specific growth rate (0.31 day^{-1}), and β-carotene concentration (4.6 mg L^{-1}) similar to control (Fig. 5a, Table 4). On the other hand, 24 h day^{-1} SMF treatment had almost increased cell density up to 25% (5.11×10^6 cell m L^{-1}), specific growth rate up to 16% (0.36 day^{-1}) and also β-carotene content up to 26% (5.8 mg L^{-1}) over control (3.81×10^6 cells mL^{-1}, 4.6 mg L^{-1}). As displayed in Fig. 5c, there was no significant disparity between chlorophyll to the β-carotene ratio in 10 and 30 mT SMFs treatment, by the way, this ratio in samples treated by a magnet (1.7) was lower than in control samples (2.5). *Chlorella* spp. and *Spirulina* spp. were exposed to 10 to 60 mT SMFs and biomass

Table 5. Biochemical composition of *D. salina* at different carbon sources.

Nutrient	Total carbohydrate (mg g^{-1} Dw)	Total lipid (mg g^{-1} Dw)	Total protein (mg g^{-1} Dw)	Antioxidant activity (%)
Control	90.93 ± 9.23	296.35 ± 11.04	271.32 ± 19.55	51.70 ± 3.16
Glucose 0.5 M	115.20 ± 6.71	313.15 ± 13.82	330.00 ± 12.64	13.76 ± 1.49
Glucose 0.9 M	203.2 ± 9.94	312.50 ± 15.45	384.00 ± 8.40	11.64 ± 0.97
Fructose 0.1 M	152.80 ± 8.89	238.15 ± 10.83	326.45 ± 9.14	21.16 ± 0.74
Fructose 0.4 M	141.34 ± 5.81	221.35 ± 11.13	391.20 ± 22.49	40.74 ± 2.02
Sodium acetate 45 mM	133.87 ± 9.98	233.20 ± 16.27	321.67 ± 15.53	25.93 ± 2.2
Sodium acetate 67 mM	155.47 ± 4.71	237.62 ± 17.34	482.45 ± 12.21	48.15 ± 1.98
Sodium malonate 45 mM	79.73 ± 8.86	287.41 ± 15.00	248.41 ± 24.31	29.62 ± 0.38
Sodium malonate 67 mM	72.80 ± 6.41	233.22 ± 13.31	244.84 ± 18.89	30.16 ± 2.37
Glycerol 5 mM	17.67 ± 9.79	354.75 ± 13.39	279.60 ± 22.91	22.22 ± 1.49
Glycerol 200 mM	24.27 ± 7.70	435.03 ± 11.70	266.40 ± 12.64	35.98 ± 3.36
Starch 0.5 g L^{-1}	102.20 ± 10.55	306.58 ± 14.83	271.20 ± 12.72	12.69 ± 2.11
Starch 5.0 g L^{-1}	337.60 ± 9.92	210.23 ± 9.19	140.80 ± 14.49	27.46 ± 2.37

Table 6. Biochemical composition of *D. salina* at different concentrations of starch.

Starch Concentration (g L^{-1})	Total carbohydrate (mg g^{-1}Dw)	Total lipid (mg g^{-1}Dw)	Total protein (mg g^{-1}Dw)	Antioxidant activity (%)
0.0	55.20 ± 2.55	334.48 ± 15.62	295.52 ± 3.73	50.43 ± 3.22
3.0	102.40 ± 7.43	313.68 ± 18.52	194.51 ± 7.81	24.78 ± 1.84
4.0	136.04 ± 3.51	259.48 ± 22.2	180.16 ± 5.24	27.83 ± 1.99
5.0	168.40 ± 7.67	258.13 ± 4.41	170.20 ± 6.94	26.96 ± 2.07
6.0	206.40 ± 9.79	257.04 ± 18.72	164.20 ± 2.69	31.74 ± 1.61
7.0	300.40 ± 3.65	274.26 ± 15.8	132.53 ± 6.24	30.43 ± 1.76

Table 7. Biochemical composition of *D. salina* in Modified Johnson medium enriched by 7 g L^{-1} starch and exposure with 10, and 30 mT SMF at 1 and 24 h

Tests	Total carbohydrate (mg g^{-1}Dw)	Total lipid (mg g^{-1}Dw)	Total protein (mg g^{-1}Dw)	Antioxidant activity (%)
Control	348.84 ± 3.42	319.71 ± 19.4	148.60 ± 2.33	29.57 ± 0.65
10 mT, 1 h day^{-1}	302.92 ± 3.09	315.87 ± 29.03	188.40 ± 2.42	36.09 ± 0.79
10 mT, 24 h day^{-1}	338.40 ± 8.97	206.93 ± 19.82	223.2 ± 1.95	18.07 ± 1.53
30 mT, 1 h day^{-1}	289.88 ± 9.69	153.83 ± 9.29	206.41 ± 2.54	4.35 ± 0.31
30 mT, 24 h day^{-1}	348.00 ± 6.03	116.65 ± 5.49	226.8 ± 0.43	8.26 ± 0.22

productivity was enhanced by up to 100% which is comparable to the present study (Table 4).

Fig. 5. Effect of different SMF intensity and exposure time a) cell density, b) β-carotene content, and c) chlorophyll to β-carotene ratio in constant starch concentration (7 g. L^{-1}). Data is shown as mean ± SD (n = 3).

Biochemical compositions

According to Table 5, glucose, fructose, sodium acetate, and 5 g L^{-1} starch have raised carbohydrate content up to 337.60 mg g^{-1} DW. Glucose, glycerol, and 0.5 g L^{-1} starch have enhanced lipids in cells to 435.03 mg g^{-1} DW. Ceron Garcia et al. (Ceron Garcia et al., 2006) neutralized the cultivation of *Phaeodactylum tricornutum* with glycerol and fructose and their results showed an increase in lipid content. Protein has been increased by all the carbon sources except for glycerol and 5.0 g L^{-1} starch which decreased by 50%. In comparison, Velu et al. enriched *Dunaliella tertiolecta* medium with various carbon sources then it was shown that protein and carbohydrate content increased and lipid decreased by mixotrophic cultivation (Velu et al., 2015). Antioxidant activity experiments have indicated that all carbon sources had less scavenging rather than control. In another study, Chavoshi et al. (Chavoshi and Shariati 2019) enhanced lipid concentration in *D. salina* by adding glucose and acetate to a medium that verified this study.

Total carbohydrate content was increased up to 300.40 mg g^{-1} DW by raising starch concentration in the media. Lipid content did not differ significantly and total protein decreased slightly 50% in comparison with control. Antioxidant activity has shown less activity in all starch concentrations rather than control (Table 6). The antioxidant scavenging of carotenoids depends on the oxygen tension present in the system. At low partial pressures of oxygen, β-carotene was found to inhibit oxidation therefore by increasing cell density partial pressure of oxygen was raised in bottles, and β-carotene was degraded (Stahl and Sies, 2003). There was no similar study to this section so any comparison has not carried out. Besides, SMF has decreased lipid concentration and increased protein in *D. salina* but it did not affect carbohydrate content. SMF treatment on *Chlorella* spp. decreased carbohydrate content and increased lipid amount while the protein content did not change (Bauer et al. 2017; Deamici et al., 2016a; Small et al., 2012). Lipid content and protein concentration of *Spirulina* spp. were increased and decreased, respectively, while the carbohydrate content did not same

profile (Deamici et al., 2016b; Shao et al. 2018; Veiga et al., 2020). Overally, based on Table 7, it can be concluded mixotrophic growth alongside SMF treatment would stimulate the biosynthesis of biochemical molecules including carbohydrates, lipids, and proteins. The difference in biochemical composition pattern in the microalgae has several reasons such as the kind of species, metabolic pathway, and the consumption of available nutrients.

Discussion

Carbon source screening

Growth profile

Despite of control, Glucose showed an inhibition, and this was in contrast with Chavoshi et al. results that reported cell density enhanced by increasing glucose concentration (20–100 mM). Sodium acetate at a low concentration (0.045 M) had better performance during the exponential growth phase rather than its high concentration (0.067 M), This goes by Chavoshi et al. (Chavoshi and Shariati, 2019) that manifested increasing cell density in higher acetate concentration (50–200 mM). Mojaat et al. (Mojaat et al. 2008) had reached 1.4×10^6 and 1.1×10^6 cell mL^{-1} on 6–days in sodium malonate and sodium acetate media, respectively. Even though the gained cell density for sodium malonate (4×10^6 cell mL^{-1})and sodium acetate (5×10^6 cell mL^{-1}) were more than Mojaat et al. (Mojaat et al. 2008) in 6–days (with same inoculum 7.5×10^5 cell mL^{-1}) but there was not observed any positive effect by the addition of sodium malonate on cell density compared to control, (Fig. 1d). In contrast to Garcia et al. (Garcıa et al., 2005), glycerol had no positive effect on the growth of *D. salina*. Garcia et al. (Garcıa et al., 2005) exhibited that the biomass productivity of *Phaeodactylum tricornutum* was enhanced in mixotrophic conditions by the addition of glycerol. Glycerol is a by-product of *D. salina* and the extra addition of this compound to the medium increases its intracellular concentration the cells will be damaged and the growth will be inhibited followed by the reverse-osmosis effect. At last, a high concentration of starch (5 g L^{-1}) has the most effectiveness on cell density (7.2×10^6 cells mL^{-1}) 20% higher than the control in 10 days (Fig. 1f) that corresponded with the results of Garcia et al. (Garcıa et al., 2005) that demonstrated starch enhances cell density of *Phaeodactylum tricornutum* up to 20%. Recently, He et al.(He et al. 2020) reported the new glycerol synthesis pathway includes 6 enzyme-catalyzing steps to convert starch to glycerol. Hence, it has been conceptualized that starch provides more glycerol and has a positive influence on *D. salina* growth due to its osmotic effect and survival of the microalgae in hypersaline conditions (Chen et al., 2009). Although the cell density in starch (10–days) enriched medium was more than sodium acetate (9–days), the maximum specific growth rate at 0.067 M of sodium acetate and 0.5 g L^{-1} of starch was nearly equal (0.2 day^{-1}).

β-carotene production and chlorophyll to β-carotene ratio (CBR)

Another important factor to estimate the effect of mixotrophic growth (carbon source addition) in comparison to photoautotrophic is the production of β-carotene and

chlorophyll to β-carotene ratio (CBR). Since the biosynthesis of β-carotene is a complicated process, it is coordinated with the biogenesis of proteins of the photosynthetic apparatus and chlorophylls (Bohne et al. 2002). Not only production of β-carotene is desirable, but also its purity from other lipophilic materials like chlorophylls that could be co-extracted with β-carotene is preferred too.

The amount of β-carotene in the medium containing fructose was less than the control, and CBR was lower in comparison with the control. This may be a result of low cell density and this goes with Garcia et al. (Ceron Garcia et al., 2006) results that manifested β-carotene content had a direct relationship with cell density.

Regarding sodium salts, the outcomes of the present work are comparable to Mojaat et al. (Mojaat et al. 2008) who achieved 3.5 mg L^{-1} and 18 mg L^{-1} β-carotene in sodium acetate and sodium malonate added medium, respectively, nearly after 6–days.

Starch demonstrated the most positive effect on the growth and β-carotene content, as well as minimum CBR. Hence, it was chosen for further optimization trials. Glucose and sodium acetate had relatively higher cell density and almost the same β-carotene content in the second and third place but the gained CBR in glucose enriched medium was less than sodium acetate medium. Since there were not observed any similar studies in our literature review, maybe different initial inoculums resulted in more cell number and β-carotene content.

SMF effect on mixotroph cultivation

According to Pazur et al (Pazur et al., 2007), there is the effect of ‘‘window” on biological systems in the MF, application changes don’t occur linearly, and cell concentration is increased only in specific intensities. This may be the result of many reasons such as the capability to cause changes in the orientation of biomolecules, in DNA synthesis, modify the flow of ions across the plasma membrane, biomembranes changes (lipid and plasma), and modification in the rate of cellular reproduction (Bauer et al., 2017; Chu et al., 2020; Deamici et al., 2016a; Han et al., 2016; Shao et al., 2018; Small et al., 2012).

D. salina was successfully grown in a mixotrophic culture. Starch optimal concentration was 7.0 g L^{-1} supporting *D. salina* heterotrophic cultivation by a 25% increment in algal biomass and β-carotene production. It was found out that the intensity of the SMF field had no significant effect on cell growth but longer exposure time made cells doubling faster rather than shorter ones and higher β-carotene accumulation consequently. The outcomes of this research can be used in aquaculture to enhance algal production. Further study may contain higher SMF intensities to algal culture with different exposure times to find the optimum condition. The examination of the effect of consuming carbon wastes of a β-carotene metabolic pathway in *D. salina* on the growth condition and β-carotene production.

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