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Milking of *Dunaliella bardawil* as a biotechnological process for continuous production and extraction of β-carotene in a two-phase system

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ABSTRACT

Algal milking has been established as a biotechnological process for the continuous extraction of β-carotene from the *Dunaliella bardawil* in two-phase systems. The effect of limited nitrogen, high light intensity, and their combination on the production and extraction of β-carotene was followed. The light intensity of 15000 lux was the best stress factor for β-carotene production. A short-term milking experiment for 15 days at 15000 lux and different mixing rates 100, 120, and 150 rpm was performed. The results indicated that the highest total β-carotene production (intracellular and extracellular) was recorded at 150 rpm and 15000 lux with 20% v/v dodecane as a biocompatible solvent. In a preliminary experiment, the light intensity 2000 lux stimulated the cells to recover rapidly and complete the milking for a longer time than the recovery at 15000 lux. In a long-term milking, the experiment consists of ten cycles β-carotene was produced and extracted at 15000 lux and 150 rpm for two days and the cells were recovered at 2000 lux for three days, the process was continued for fifty days. At the end of the experiment, some cells remain alive and were continuously milked for β-carotene. Finally, the total productivity of β-carotene was 58.01 mg/l and the extraction efficiency was about 64%.

INTRODUCTION

Dunaliella is a green alga to be survived it synthesizes high concentrations of β-carotene to protect against the intense light **(Rad *et al.*, 2011; Ben-Amotz, 2019)**. The β-carotene is commercially produced from algae by culturing and stressing the algae in open ponds **(Ben-Amotz, 1995; Borowitzka, 1999; Borovkov *et al.*, 2020)**. β-carotene is widely utilized as a provitamin A, antioxidant, coloring agent, in the food, and pharmaceutical. Recently, the preparation of β-carotene from natural sources became of great importance in the industry **(Hejazi and Wijffels, 2004; Torregrosa-Crespo *et al.*, 2018)**. Firstly, to achieve high biomass, algae are grown, then cells are stressed and β-carotene is produced. Stress is accomplished by increase the light intensity or by increasing the salt concentration. High levels of β-carotene are accumulated, then the biomass is harvested and further processed **(Kleinegris *et al.*, 2011a)**. The high hydrophobicity of β-carotene makes it a good candidate for aqueous/organic biphasic

photoproduction **(Hejazi *et al.*, 2003; León *et al.*, 2003; Hosseini and Shariati, 2009)**. To counteract the poor productivity in high-value components of algal cultures, so the milking of these algal cultures is proposed, where the cell mass is reused for continuous production **(Hejazi and Wijffels, 2004; Khan *et al.*, 2018)**. Milking has already been reported in *Dunaliella salina* for carotenoid compounds in two-phase bioreactors **(Hejazi and Wijffels, 2004)**. HPLC analysis for the pigment content of the organic phase indicates that the β-carotene extracted is highly purified **(Hejazi *et al.*, 2003)**. Understanding the mechanism of extraction is not only useful for the design of the process and its scale-up but would be also useful in applying the process for other products.

The Milking was a successful process in β-carotene production from microalgae **(Hejazi *et al.*, 2004 a, 2004 b; Kleinegris *et al.*, 2011c; Mojaat *et al.*, 2008)** and glycerol production **(Senousy *et al.*, 2012)**. Milking diatoms for sustainable energy is reported by **Ramachandra *et al.* (2009)**. Besides, lipid extraction from *Botryococcus braunii* in a biphasic bioreactor was detected by **Zhang *et al.* (2011); (2013)**. **Hejazi and Wijffels (2004); Miazek *et al.* (2017)** mentioned that a good understanding of the extraction mechanism and path of the target product must be a part of the effective milking process. **(Ginzburg *et al.*, 1999; Liang *et al.*, 2017)** showed that *D. salina* cells can do endo-and exocytosis continuously. The cell membrane makes small vesicles during this process, which allow the absorption and then release of the medium components. The general use of the milking process would promote the commercialization and development of micro-algal biotechnologies. Therefore, the main objectives of this study are determination of optimum conditions for the production of β-carotene from *D. bardawil* cells under different stress conditions, and milking *D. bardawil* cells to produce β-carotene with biocompatible solvent (dodecane) in a two-phase system.

MATERIALS AND METHODS

Dunaliella bardawil was the tested organism of this study and it is isolated from El-Bardawil Lake on the northern coast of Arish, North Sinai Governorate, Egypt. It was grown in batch cultures in MH nutrient medium **(Loeblich, 1982)** at pH 7.5, temperature was 25^0C ±1 and light intensity of 37 µmol photons $m^{-2}s^{-1}$ (2000 lux) by fluorescent lamps. Stock axenic *Dunaliella* cultures (5 ml) were inoculated into 45 ml fresh nutrient media in 250 ml Erlenmeyer flasks under controlled growth conditions. The initial algal inoculum was 5×10^6 cell/ml (cells in the exponential growth phase). Three replicates were used for each treatment. Cells were counted by Sedqwick Rafter cell. Chlorophyll (a) was determined by using 80% acetone according to **(Arnon, 1949; APHA *et al.*, 2005)**. The amount of β-carotene was measured photometrically using a molar extinction coefficient ε 450 nm = 134,500 L mol^{-1} cm^{-1} **(Rabbani *et al.*, 1998)**, and the values of β-carotene expressed as mg/l and calculated for pg/cell

EXPERIMENTAL DESIGN

1- In the first experiment, 20% (v/v) dodecane was added to the algal culture, then the culture incubated at 15000 lux and different mixing rates (100, 120, and 150 rpm). Every

three days chlorophyll a, cell count, intracellular, and extracellular β-carotene contents were measured until the end of the experiment (15 days).

2- The second experiment was carried out for five days to demonstrate the effect of the limited nitrogen (0.1 g/l KNO_3), high light intensity (15000 lux), and their combination as stress factors on the cells for the β-carotene production and extraction. The effect of the different stress factors was tested on the cells for one day (1^{st} stage), then the stressed cells were subjected to the two-phase system (aqueous phase, Loeblich medium and organic phase of dodecane) at 150 rpm in the next day for β-carotene extraction (2^{nd} stage). Then, the cells were allowed to recover at 2000 lux for the next three days (3^{rd} stage), after the removal of dodecane which contains the extracted β-carotene then it was kept in the dark to be reused again in the next extraction step and to prevent light-induced β-carotene degradation. Chlorophyll (a) and intracellular β-carotene were detected in each step whereas extracellular β-carotene was measured only after the extraction step (2^{nd} stage).

3- The third experiment was established for selecting the optimum conditions required for the milking of β-carotene from *D. bardawil.* The milking of the cells was done for three cycles and each cycle was five days. The milking of the cells was carried out in two separate steps, the production and extraction step (IA or IIA) for two days at 15000 lux in the presence of the two-phase system, the organic (20% v/v dodecane) and the aqueous phase, followed by the recovery step for three days, where the solvent was removed from the aqueous phase at the beginning of each recovery stage. The cells were subjected to 15000 lux in treatment I (IB) and 2000 lux in treatment II (IIB), so the recovery step was different in the two treatments.

Finally, a long-term milking experiment was designed for a high recovery yield of β-carotene depending on the above experimental results. The extraction phase and the recovery phase were separated. Ten milking cycles were done in three replicates, each cycle was five days and divided into two steps. Two days for production and extraction of β-carotene at light intensity 15000 lux and mixing rate 150 rpm (milking step). The extraction was followed by the recovery of cells for 3 days at light intensity 2000 lux in the absence of solvent (recovery step). To preserve chlorophyll a and cell number at reasonable levels to help the cells complete the milking for a longer time, it was necessary to dilute the aqueous phase by Loeblich medium after each cycle as a substitution with the samples taken during the experiment to keep the volume of the medium constant.

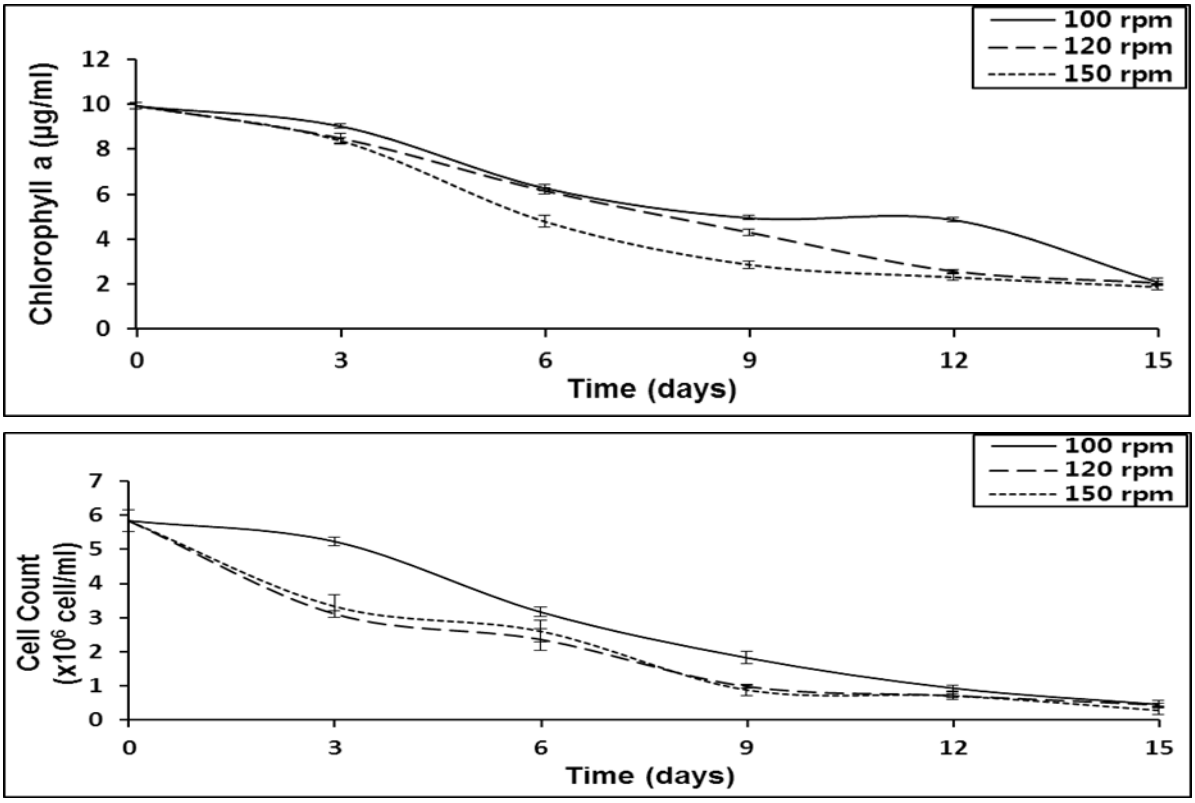
STATISTICAL ANALYSIS

The data were analyzed by one-way analysis of variance (ANOVA) using SPSS statistical package version 20. A comparison of the main effects was performed using a significant level of $P < 0.05$ by Duncan's test of homogeneity. All data were represented by average of three replicates ± SD **(Dytham, 1999)**.

RESULTS

1. Effect of different mixing rates on growth, continuous β-carotene production and extraction by *D.* b*ardawil* in a two-phase system

A two-phase experiment (Fig.1) was carried out for determining the effectiveness of β-carotene extraction from the microalga *D. bardawil* at different mixing rates (100, 120 and 150 rpm). The data explained that increase in mixing rate increases the extraction efficiency, where the highest level of extracellular β-carotene was obtained at 150 rpm. However, the level of intracellular β-carotene within the algal cells did not greatly affected by the different applied mixing rates. The results in Figure (1) revealed that chlorophyll a and cell count decreased drastically with the time at all mixing rates, so the experiment could not be run for more than 15 days. The minimum levels of chlorophyll (a) and cell count were detected at 150 rpm.



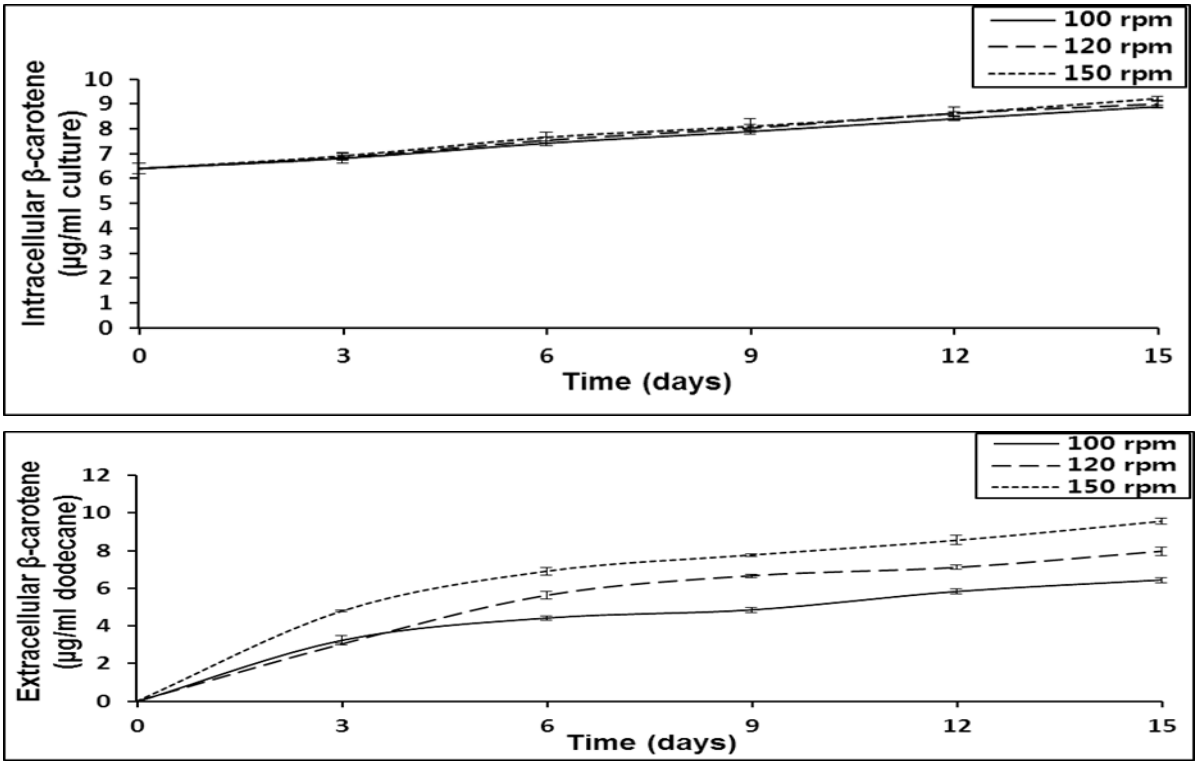


Fig. 1. Effect of different mixing rates on growth, β-carotene continuous production, and extraction by *D. bardawil* grown for 15 days at 15000 lux in a two-phase system. Data are average of three replicates; each value represents the mean ± SD.

2. Effect of limited nitrogen, high light intensity, and their combination on the β-carotene production and extraction from *D. bardawil* in a two-phase system.

This experiment was performed to investigate the most effective way to stress and milk *D. bardawil* cells. Our results in Figure (2) reported that all the stress factors used like the limited nitrogen (0.1 g/l KNO_3), light intensity at 15000 lux, and their combination increased β-carotene content inside the algal cells.

The highest values of intracellular and extracellular β-carotene after the extraction step were recorded by the cells were previously stressed by the light intensity 15000 lux. Also, the present results indicated that the highest levels of chlorophyll (a), cell count, and intracellular β-carotene after recovery step at 2000 lux were recorded by the cells were previously stressed by the light intensity 15000 lux.

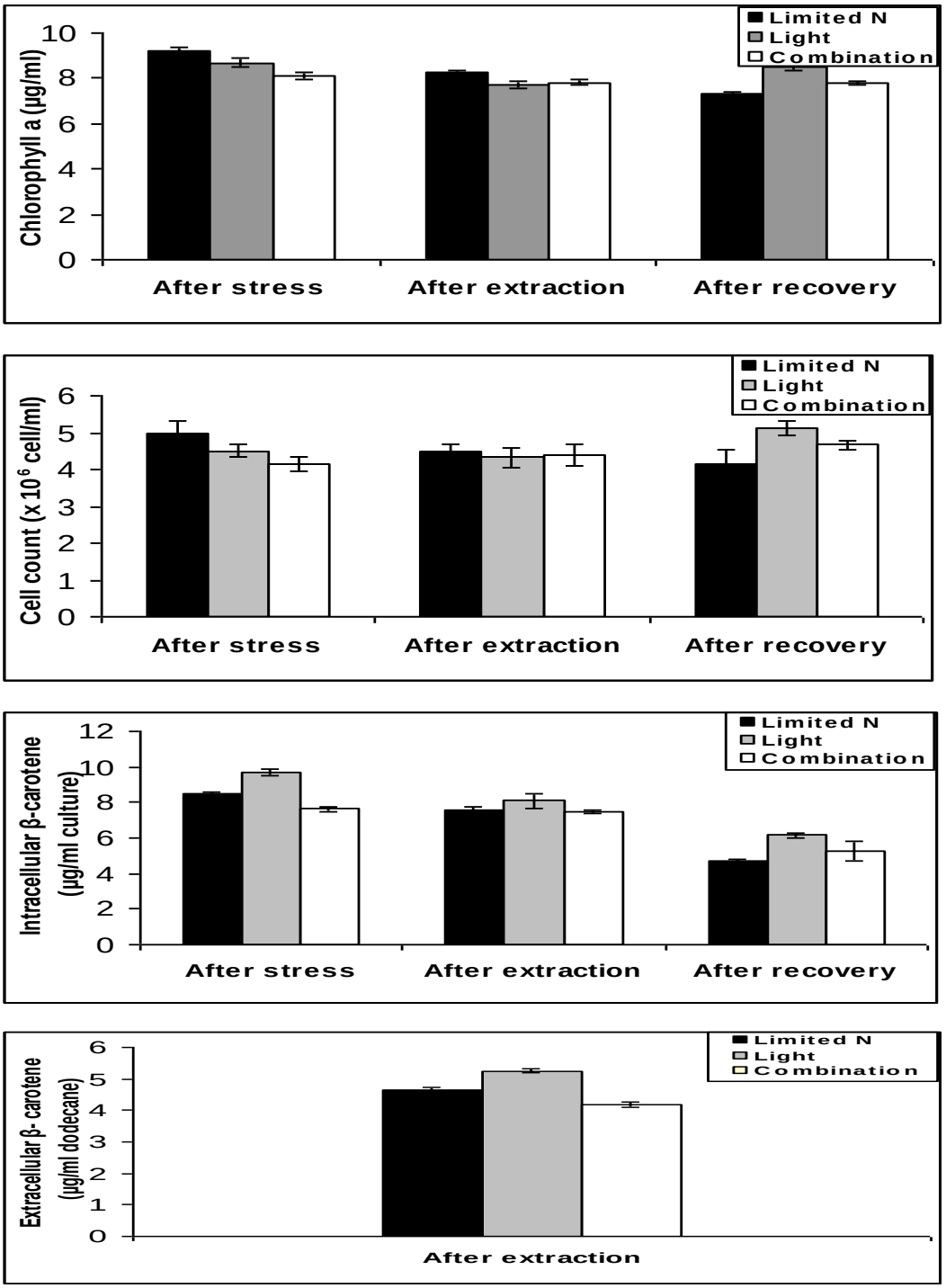


Fig. 2. Effect of limited nitrogen, high light intensity, and their combination on the production and extraction of β-carotene from *D. bardawil* using dodecane (20% v/v) at 150 rpm for 5 days. Initials: chlorophyll (a) 9.90 µg/ml, $5.5x10^6$ cell/ml and β-carotene 6.40 µg/ml.

3. Preliminary experiment to select the optimum milking conditions for the production and extraction of β-carotene in a two-phase system.

The presented data in Figure (3) revealed a sharp decrease in the values of chlorophyll (a) in the treatment I (recovery at 15000 lux) than that in the treatment II (recovery at 2000 lux) after the end of the three milking cycles.

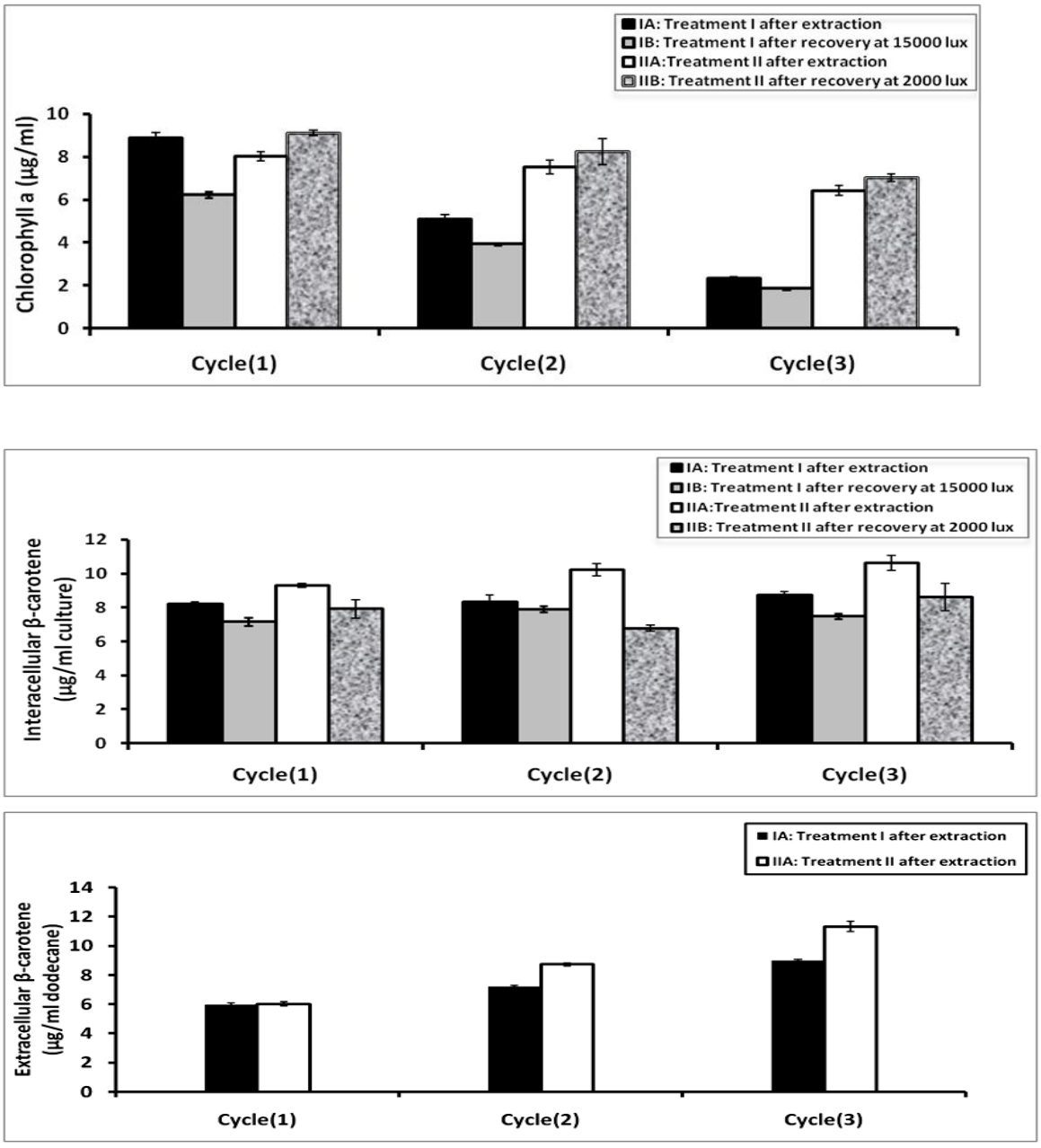


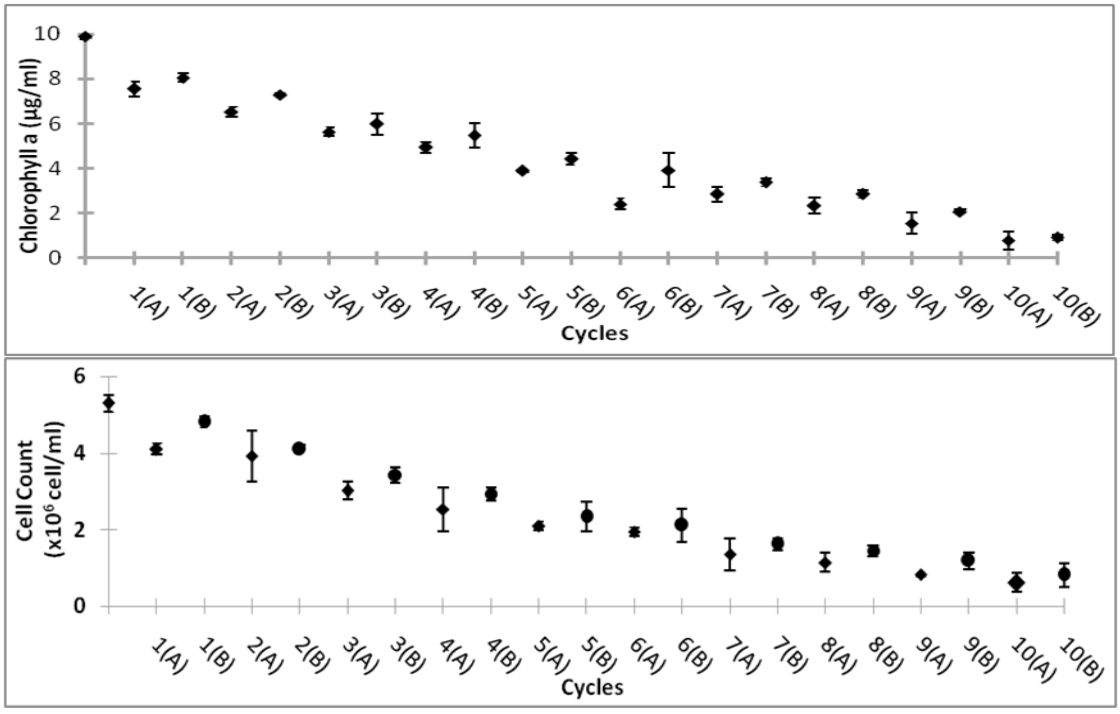
Fig. 3. Preliminary experiment to select the optimum milking conditions for β-carotene production and extraction from *D. bardawil* in 20% (v/v) dodecane at 150 rpm. N.B: Each cycle was five days (A+ B). IA, IIA: is the same stage in treatment I and treatment II, which includes the production and extraction of β-carotene for 2 days at 15000 lux in the presence of a solvent.
IB: Cell recovery stage for three days at 15000 lux in the absence of the solvent.
IIB: Cell recovery stage for three days at 2000 lux in the absence of the solvent.

Results in Figure (3) showed an increase in the levels of intracellular and extracellular β-carotene in the treatment II (recovery at 2000 lux) than that in the treatment I (recovery at 15000 lux) after the end of the three milking cycles. The extracellular β-carotene accumulated gradually in the dodecane solvent during the time of the experiment, until it reached the highest values at the end of the third cycle 11.33, 9.01 µg/ml for the treatment II and treatment I respectively.

4. Long-term milking of *D. bardawil* for β-carotene production and extraction in the two-phase system

The results represented in Figure (4) explained that the levels of chlorophyll a and cell count gradually decreased during the ten milking cycles. Also, there is decrease in the levels of chlorophyll (a) and cell count in each cycle, after each production and extraction stage. On the contrary, the presented data in Figure (4) showed an increase in the chlorophyll a and cell count levels after the recovery stage in comparison to those levels after each extraction stage.

This experiment indicated also a remarkable increase in the values of intracellular and extracellular β-carotene with the time. Also, an increase in the levels of intracellular and extracellular β-carotene after each production and extraction stage was observed during the experiment. The results in Figure (4) revealed that the cell recovery stage (at 2000 lux) after each production and extraction stage kept the cells alive for milking for 50 days. The extracted β-carotene concentration in the organic phase (dodecane) was 37.19 mg/l after ten cycles, with total β-carotene production being 58.01 mg/l at the end of the experiment. The total productivity of β-carotene 5.80 mg/l per cycle, where each cycle was 5 days (i.e 1.16 mg/l/day). The β-carotene content of the *D. bardawil* cells used in our milking experiment reached 34.7 pg/cell at the end of the milking experiment.



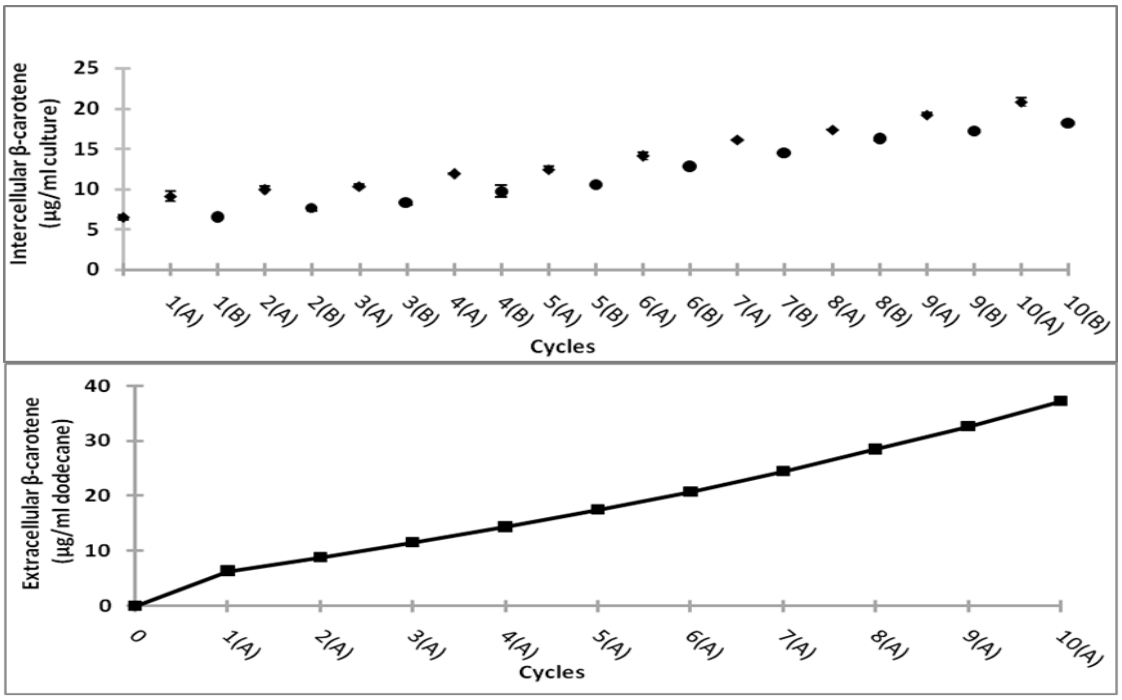


Fig. (4). Long-term milking of *Dunaliella bardawil* for the production and extraction of β-carotene in 10 cycles for 50 days. **N.B.:** Each cycle was five days (A+B). **A:** After two days of production and extraction at 15000 lux and150 rpm in presence of 20% (v/v) dodecane solvent. **B:** After three days of cell recovery at 2000 lux in the absence of the dodecane solvent.

DISCUSSION

The presented data explained the highest value of the total β-carotene production (intracellular + extracellular) was recorded at mixing rate 150 rpm (18.78 µg/ml), which is in line with **Hejazi *et al.* (2003)**. In previous works by **Frenz *et al.* (1989 a, b); Sim *et al.* (2001)** a low extraction of hydrocarbon was obtained from *Botryococcus braunii* due to insufficient mixing. Improving the mixing between the aqueous phase and organic solvent resulted in a high extraction of hydrocarbon because of increasing the cells' contact with the organic phase. Earlier research by **Miazek *et al.* (2017)** has shown that *Dunaliella* alga is biologically compatible with solvents with logs P(oct) > 5. Dodecane has been selected as the most suitable biocompatible solvent for β-carotene extraction as compared to other organic solvents like tetradecane, hexadecane, and ethyl oleate **(Shabana *et al.*, 2016)**. The presence of the dodecane in the culture medium can lead to the production of β-carotene. The least values of chlorophyll (a) and cell count were recorded at 150 rpm. In agreement with our results **León *et al.* (2003)**, who mentioned that the mixing of the organic and aqueous phase will increase extraction, however, it can also increase organic solvent damage and thus decrease the growth parameters like chlorophyll (a) and cell count. Our results seemed to be in contrary to the results obtained by **Hejazi *et al.* (2003)** and consistent with the results of **Kleinegris *et al.* (2011a)** who concluded that the cell death which is due to the toxicity of the dodecane was the mechanism that resulted in the extraction process and not the exocytosis as reported by **Hejazi *et al.* (2004b)**. There are numerous reasons why dodecane is toxic to cells, such as cell membranes disruption and restricted access to nutrients by covering the cell with solvent **(Kleinegris *et al.*, 2011b).**

All the applied stress factors increased β-carotene production inside the algal cells. This is in agreement with several studies **(Phadwal and Singh, 2003; Ben- Amotz and Avron, 1989; León *et al.*, 2003; Yokthongwattana *et al.*, 2005; Xu and Harvey, 2019)**. By comparing the effect of different stress factors used in this experiment the data revealed that light stress increased β-carotene production and extraction more than that by the other stress factors **(Borovkov *et al.*, 2020)**. The highest values of intra and extracellular β-carotene after extraction step were recorded by the cells were previously stressed by the light intensity 15000 lux. **Cifuentes *et al.* (2003); Çelekli and Dönmez (2006); Kleinegris *et al.* (2010)** mentioned that β-carotene content was increased and its rate of extraction was increased by increasing the exposure to the light. Recovering of the cells was done in this experiment at low light intensity 2000 lux since at this low light intensity the highest growth of *Dunaliella* was obtained **(Goyal, 1989)**. This experiment showed that the best stress factor for cell recovery at 2000 lux was the light stress at 15000 lux. Therefore, it was chosen for the next milking experiments. Another advantage for selecting the light stress in the next experiments, which was reported by **Kleinegris *et al.* (2010); Xu and Harvey (2019)**, where he found that the ratio of 9-cis/all-trans β-carotene improved in the event of high light intensity was applied for stressing the cells, while by using other stress factors, this effect was less obvious.

The results of present study indicated a decrease in the values of chlorophyll (a) in the treatment I (recovery at 15000 lux) than that in the treatment II (recovery at 2000 lux) after the end of the third milking cycle. This indicates the negative effect of the continuous exposure of the cells to the high light intensity (15000 lux) on the chlorophyll (a) level, thus the cells can not complete the milking for a longer period. These results are consistent with **Bouterfas *et al.* (2006)**. The chlorophyll (a) content decreased after the extraction step in treatment I and treatment II compared to the initial value, but the decreasing was in treatment I more than that in treatment II, which is due to that some of the cells die due to the direct contact with the solvent, but other cells remain alive in the aqueous phase **(Fiedler *et al.*, 2007)** and therefore, the recovery of cells under low light intensity 2000 lux in treatment II helped the cells to restore their viability and maintain their chlorophyll (a) content at relatively high levels more than the cells in treatment I, which recovered at 15000 lux. In this way, the recovered cells at 2000 lux (treatment II) can be reused again for the production and extraction of β-carotene. Our results are in agreement with previous work by **An *et al.* (2004); Fiedler *et al.* (2007); Kang and Sim (2008)**. Consequently, we carried out a long-term milking experiment by applying the milking conditions of the treatment II (the recovery was at 2000 lux). We proposed in this experiment to separate the cell recovery and growth from the production and extraction of the β-carotene according to **Kleinegris *et al.* (2011 b)**, who reported that a combination of cell growth and product production during two-phase extraction reveals little potential. Therefore, the milking can be done as a periodical process in which cells are cultivated cyclically and then milked.

The use of microalgal milking could reduce the issue of low productivity in the manufacturing of microalgal products. Moreover, this method reduces extraction and purification costs **(Hejazi and Wijffels, 2004; Tan *et al.*, 2020)**. *Dunaliella* is used as an attractive candidate for milking **(Barzegari *et al.*, 2010)**. The advantage of the milking of microalgae that it does not require destroying all of the cells to extract the desired product, which in contrast to existing commercial processes. Many cells were destroyed

due to contact with the organic solvent and death was overcome by cell growth **(Kleinegris *et al.*, 2011 c)**. Therefore, a continuous extraction process should balance cell growth and cell death. The long-term milking experiment used in our study with reasonable biomass levels and increased extracted β-carotene levels could last more than seven weeks, this is in agreement with **Hejazi *et al.* (2004 a)**.

The decrease in the levels of chlorophyll (a) and cell count after each production and extraction stage, was due to the exposure of the cells to the effect of high light stress (15000 lux) and the organic solvent **(Miazek *et al.*, 2017)**. The chlorophyll content of the cells decreases when the cells are under stress **(Yokthongwattana *et al.*, 2005)**. **Çelekli and Dönmez (2006); Xu *et al.* (2016)** reported that cell number decreased when light increased. In contrast, the increase in the chlorophyll (a) and cell count levels after each recovery stage may be due to the decrease in the light intensity (2000 lux). Similarly, **Masuda *et al.* (2002)** mentioned that more chlorophyll is formed in the cells to obtain enough light energy in the low light intensity. The elevated intracellular β-carotene levels during the experiment are attributed to the high light stress effect (15000 lux), which stimulates the cells to accumulate β-carotene **(Elleuch *et al.*, 2020)**. However, the decrease in the values of intracellular β-carotene after each recovery stage is due to the decreasing of the light intensity from 15000 lux to 2000 lux in the recovery stage. This is consistent with the results reported by **Hejazi and Wijffels (2003)**. The increase in levels of extracellular β-carotene after each production and extraction stage may be due to the increase in the light exposure **(Kleinegris *et al.*, 2010)** and the influence of the organic solvent (dodecane), which may induce the β-carotene production pathway, which is in agreement with **Hejazi *et al*. (2003); Kleinegris *et al.* (2011b) .**

The application of the cell recovery stage after each production and extraction stage kept the continuity of the production and extraction of β-carotene from the cells, improved recovery yield of β-carotene also kept the cells viable for milking for a longer time. **Hejazi *et al.* (2002), (2003), (2004 a, b)** has carried out several experiments in two-phase of bioreactors on the milking of β-carotene from the marine alga *D. salina*. They recorded that the β-carotene has been milked continuously for more than 47 days from the cells of *D. salina* and that cells stayed viable with very slow cell growth. The β-carotene was not continually milked from cells of *D. bardawil* in our milking experiment, where we followed the milking stage (production and extraction) by the recovery stage in each cycle. By carrying out this milking process regularly (at least 10 cycles), thus at the end of the experiment (50 days) we were able to obtain significant amounts of β-carotene. In previous work by our group, the extracted β-carotene by this milking method showed that it was formed of two isomers 9-cis-β-carotene and all-trans-β-carotene in a ratio of 1.13. This natural β-carotene was found to be able to improve anti-inflammatory activity and a good anti-cancer drug **(Badr *et al.*, 2014)**. The concentration of the extracted β-carotene in the organic phase (dodecane) was 37.19 mg/l after ten cycles and the total β-carotene production was 58.01 mg/l at the end of the experiment. The system of **Hejazi *et al.* (2004 a)** had a β-carotene extraction efficiency of more than 55%, whereas our milking experiment had more than 64% extraction efficiency. In comparison, **Borowitzka (1999); Hejazi *et al.* (2004 a)** systems had volumetric productivity of 0.1 and 2.5 mg/l/day respectively. Our milking experiment, however, led to total productivity of β-carotene 5.80 mg/l per cycle, where each cycle was 5 days (i.e 1.16 mg/l/day). This comparison reveals that our volumetric productivity is 12 times higher than that of

Borowitzka. In contrast, it was 2 times lower than that of Hejazi. Previous work by **Hejazi *et al.* (2004 a)** mentioned that the β-carotene content of the *D. salina* cells exceeded 51 pg/cell at the end of the milking process. That is nearly double the amount of the industrial production level recorded by **Ben-Amotz (1995)** While, the β-carotene content of the *D. bardawil* cells used in our milking experiment reached 34.7 pg/cell at the end of the milking experiment. As a result, this is almost 1.4 times greater than the industrial production level of **Ben-Amotz (1995)**.

CONCLUSION

In general, the study results suggest that the proposed approach can be recommended for commercial, simple, economical β-carotene production and extraction from *D. bardawil*. Moreover, there is no cell harvest and destruction of all cells, were in the presence of an organic solvent, cells tend to form β-carotene for a long time. The process scale-up possibility will be studied in the presence of bio-reactors in specific.

Declaration of Competing Interest

The author has declared no conflict of interest.

(i) It has not been published and it is not under consideration for publication elsewhere.

(ii) It does not contain plagiarized material.

(iii) It has been approved by all the authors and their institutions.

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