Moroccan Journal of Chemistry
ISSN : 2351-812X

Mor. J. Chem., 2023, Volume 11, Issue 01, Page 51-60
https://revues.imist.ma/index.php/morjchem

Effects of Sodium Bicarbonate and photoperiod on Cell Growth and morphology of *Isochrysis galbana*

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Received *25 Oct 2022,*
Revised *18 Dec 2022,*
Accepted *24 Dec 2022*

Citation*: Tahiri S., El Aamri F., Loulad H., Idhalla M., Elmtili N. (2023) Effects of Sodium Bicarbonate on Cell Growth and morphology of Isochrysis galbana, Mor. J. Chem., 14(1), 51-60. Doi: https://doi.org/10.48317/IMIST.PRSM/morjchem-v11i1.36837*

Abstract: Microalgae play a vital role in many aquaculture feed application processes. Maintaining a microalgae production facility has been estimated to account for an average of 30 % and up to 60 % of the total budget of aquaculture hatcheries, despite several research programs and global efforts to reduce production costs of algal biomass. The use of bicarbonate as carbon inorganic to produce microalgae biomass for bivalve hatcheries was proposed as an alternative to reduce this cost. The focus of this investigation is characterization of the interaction of bicarbonate-based microalgae cultivation and photoperiod on the growth rate and production of brown microalgae *Isochrysis galbana*. The salt was provided to the cultures at the final concentration from 0.5 to 2.5 g L^{-1}. Concerning photoperiod, two cycles of light:dark (6:18 and 12:12) were studied under light intensity at 160 µmol m^{-2} s^{-1}. The growth rate of *Isochrysis galbana* showed values significantly higher in the culture supplemented with 0.5 and 1 g L^{-1} of $NaHCO_3$ respectively under cycles 6L:18D and 12L:12D. Bicarbonate administration leads to a significant increase in cellular size at the stationary phase, probably related to starch or lipid accumulation. This study proved that the addition of bicarbonate is a viable strategy to enhance the production of microalgae and reduce production costs.

Keywords: Microalgae; Sodium bicarbonate; *Isochrysis galbana*; Photoperiod

1. Introduction

Microalgae are aquatic unicellular microorganisms and autotrophs with extremely high photosynthetic efficiency, a rapid and high reproduction rate, and have a biochemical composition rich in proteins (30-50 %), carbohydrates (20-40 %), and lipids (8-15 %) under normal conditions (Cardoso *et al.*, 2011, Ho *et al*., 2013). Among the marine brown microalgae, *Isochrysis galbana* (Parke, 1949), the organism studied here, is a *Haptophyceae*, characterized by its ovoid shape with two flagella, very widespread in the aquaculture environment, mainly in hatcheries, is commonly used as a food source at all stages of bivalve growth, due to their high content of lipids, proteins, and other nutrients supplements. Therefore, their mass production and good quality in the hatchery are mandatory to succeed and optimize the spat production process. Since this production of microalgae represents more than 40 % of the overall cost of hatchery spat production (Helm and Bourne, 2006). In 1998, the value

of microalgae production is estimated at 34 million US$ / year for world aquaculture production of bivalves and shrimps (Duerr *et al.,* 1998). The main constraint on the production of microalgae for aquaculture is related to costs. Based on the study by Coutteau and Sorgeloos (Coutteau and Sorgeloos *et al.,* 1992), maintaining a microalgae production facility accounts for an average of 30 % and up to 60 % of the total budget of aquaculture hatcheries and nurseries, despite several global efforts to reduce the costs of algal biomass production. The use of bicarbonate; an alkaline substance (Elmahadi *et al.,* 2020); as inorganic carbon instead of carbon dioxide to produce microalgae biomass for bivalve hatcheries has been proposed as an alternative to reduce this cost. More, the addition of artificial light is the easiest strategy for significantly reducing production costs (by 33 %) in all small-scale systems modeled (Oostlander *et al.,* 2020). Optimization of the yield and the production of microalgae has become essential, especially by adjusting the main environmental factors influencing the growth and quality of microalgae cultures, in particular, the nutrients. This will maintain production costs at reasonable levels. According to Guiheneuf *et al.* and Pal *et al.* (Guiheneuf *et al.,* 2008, Pal *et al.,* 2011) environmental factors governing microalgal growth and biochemical composition include the availability of light intensity, nutrients, carbon source, pH, temperature and salinity.

Most previous work has reported that carbon affects cell growth and the accumulation of macromolecules, such as lipids and proteins of many species. The algae use sodium bicarbonate ($NaHCO_3$) as an external source of carbon for photosynthesis (Munoz and Merrett, 1989, Beer, 1994, Nimer *et al.,* 1997). Until now, no studies have reported the combined effects of the photoperiod and the carbon source on the growth and the cell morphology of *Isochrysis galbana*.

The objective of this study is to conduct a study on the effect of adding different concentrations of sodium bicarbonate and photoperiods on the growth performance and cell morphology of *Isochrysis galbana*.

2. Methodology

2.1 Strain and culture medium

The experiment was carried out with the marine microalgae *Isochrysis galbana*, at the level of the phytoplankton unit within the Shellfish Farming Station, National Institute of Fisheries Research (INRH), Amsa, Morocco. The culture medium used in this study is that of Guillard F/2 (Guillard, 1975). It is a common culture medium, widely used for the growth of algae. Sodium bicarbonate ($NaHCO_3$) was dissolved in seawater filtered through a 0.2 µm filter at six different concentrations (0, 0.5, 1, 1.5, 2 and 2.5 g L^{-1}), in which group 0 (without additional $NaHCO_3$) was used as a control group. The flasks were autoclaved at 121 °C for 20 min.

Isochrysis galbana, is a unicellular, photosynthetic, golden brown, euryalin flagellate (Class Prymnesiophyceae, division Chrysophyta). This golden-brown marine microalga has been frequently used in aquaculture, mainly for feeding mollusc larvae, fish and crustaceans in the early stages of their growth, due to its good nutritional characteristics and digestibility (Photo 1).

2.2 Microalgae culture

The *Isochrysis galbana* strain was cultured in 500 mL sterile erlenmeyer flasks with 300 mL of filtered seawater supplemented with sodium bicarbonate ($NaHCO_3$) at different concentrations and enriched with the F/2 medium, in sterile conditions. The initial density was adjusted to 1×10^6 cells mL^{-1}. The experimental Erlenmeyer flasks were maintained at 24 ± 1 °C for 16 days to generate a culture in the stationary growth phase. In addition, these erlenmeyer flasks are stored under two

different photoperiods 12:12 and 6:18 (L: D) at a white light intensity set at 160 µmol m^{-2} s^{-1}. These were maintained using a time programmer for the duration of the experiment.

The experiment was performed in triplicate, and stirring was performed by shaking the erlenmeyers flasks three times every day to gas exchange and mixing the culture. The samples were collected for analysis daily by sterile pipets, under the hood and after homogenization.

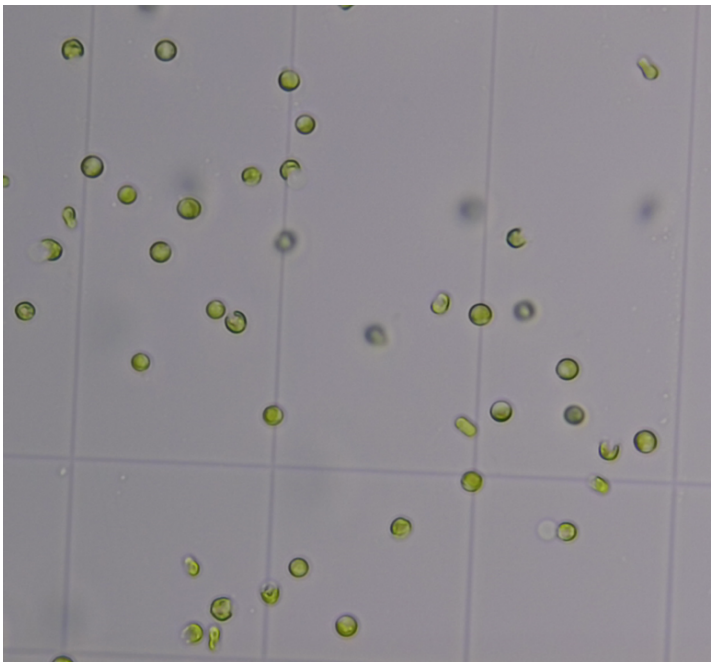

Photo 1: *Isochrysis galbana* (x40)

2.3 Analytical methods

The methodology adopted for monitoring the evolution of the *Isochrysis galbana* strain will be carried out with the following parameters:

Cell density

The cell concentration (NC) of *Isochrysis galbana* cultivated under different concentrations of sodium bicarbonate and two photoperiods was evaluated by daily counting with a counting chamber (Malassez) after immobilization of the cells with Lugol's solution, under a binocular light microscope (objective 40). They were calculated using the following equation (1):

$$NC = \frac{Number\ of\ cells\ counted}{Number\ of\ squares\ taken\ into\ account} \times 10^{-5} \qquad (1)$$

Cell density is expressed as the number of cells per milliliter.

Growth rate

The growth rate (µ) of *Isochrysis galbana* was calculated between two different values of cell concentration n_0 and n_1, according to the formula (2) used by Mohanadoss (Mohanadoss and Mohd Fadil, 2013):

$$\mu = \frac{ln(NC_1/NC_0)}{t_1 - t_0} \qquad (2)$$

µ: specific growth rate (day^{-1})

NC_0: Cell density at the beginning of a batch run

NC_1: Cell density at the end of a batch run
t_0: duration at the beginning of a batch run
t_1: duration at the end of a batch run (day)

Division rate and Doubling time

The division rate (K) (3) per day and the doubling time (D) (4) was calculated using the following equations.

$$K = \frac{\mu}{\ln(2)} \quad (3)$$

$$D = \frac{1}{K} \quad (4)$$

Cell morphology

The biometric data of *Isochrysis galbana* was obtained using images captured by a light microscope (Nikon) at 40x magnification. The sizes of cells were determined by analyzing the micrographs of typical samples using Image J. using images captured by the camera of a Nikon Eclipse E200 microscope (Nikon Corporation, Tokyo, Japan) with the Nikon Ds-Fi2 digital camera at 40x magnification. This operation is done every two days and the processing of these images was carried out using image processing software (image J) which give the possibility to determine the dimensions of each microalgae cell.

Biomass

The dry biomass of *Isochrysis galbana* was measured after centrifugation of 1.5 mL of culture (Eppendorfs) at 15000 rpm for 30 min. The cells were washed with distilled water to remove salts, then the pellets were dried at 65 °C in the oven to constant weight, then weigh to find the weight of samples per 1.5 mL.

pH

was determined using pH-meter.

2.4 Statistical Analysis

The experiment was performed in triplicate and the results were expressed as mean values and standard deviation. Statistical differences were obtained by analysis of variance (ANOVA) ($p < 0.05$).

3. Results and Discussion

The production of microalgae is an important factor in bivalve hatcheries. The optimization of their culture means choosing the species that generate rapid growth for mass cultivation, and that are also easily ingested and digested by bivalves (Brown, 2002). Studying, optimizing and controlling growing conditions can maximize algal biomass production rates. Several recent studies on the effects of bicarbonate in the culture of microalgae aimed to improve algal biomass production or functional molecule content (Srinivasan *et al*., 2018, Kim *et al*., 2019, Zhang *et al*., 2019, Salbitani *et al*., 2020).

The results, as presented in **Table 1** illustrate the growth characteristics of the species *Isochrysis galbana* in the six different concentrations of sodium bicarbonate from 0 to 2.5 g L^{-1} and two photoperiods 6L:18D and 12L:12D, for 16 days.

Table 1. The growth characteristics of *Isochrysis galbana* were measured over a 16-day culture period at different Bicarbonate concentrations.

Photoperiod	Traitement $NaHCO_3$ (g L^{-1})	NC (10^6 cell mL^{-1})	Biovolume (μm^{-3})		μ (day^{-1})	K (day^{-1})	D (day^{-1})	Biomass Productivity (g L^{-1} day^{-1})
			Ex	St				
6/18	C	2.28	18.85	48.01	0.08	0.11	8.88	0.15
	0,5	8.90	17.01	49.73	0.16	0.24	4.24	0.04
	1	4.02	26.47	61.14	0.12	0.17	5.83	0.42
	1,5	5.47	28.99	45.54	0.13	0.18	5.51	0.22
	2	2.04	25.83	52.43	0.05	0.07	13.91	0.31
	2,5	4.54	30.29	62.73	0.14	0.20	5.00	0.12
12/12	C	2.84	32.40	25.80	0.07	0.09	10.61	0.12
	0,5	3.30	23.96	29.87	0.06	0.08	11.79	0.05
	1	8.82	41.70	95.19	0.14	0.21	4.85	0.09
	1,5	6.81	33.48	41.78	0.13	0.18	5.51	0.10
	2	3.35	32.55	42.41	0.08	0.11	8.84	0.22
	2,5	1.87	46.15	31.79	0.03	0.04	24.29	0.68

(***NC:*** *cell concentration;* ***Ex:*** exponential phase; ***St:*** stationary phase; ***μ:*** growth rate; ***K:*** division rate; **D**: doubling time; **C**: control)

These results showed a positive effect of using sodium bicarbonate ($NaHCO_3$) as a supplement in the production of the *Isochrysis galbana* strain, and that it can be an efficient inorganic carbon source that gives the yields identical to those of the carbon dioxide (CO_2) supplement, a study by Umetani *et al.* (2021) also reported that bicarbonate can give healthy growth and comparative product yields as carbon dioxide, the low solubility of the latter in water may lead to inhibition of growth (Giordano *et al.,* 2005, Aishvarya *et al*., 2012). While sodium bicarbonate has greater solubility than carbon dioxide (Hsueh *et al.,* 2007) and could be an alternative source of inorganic carbon for the culture of microalgae. Moreover, the carbon dioxide gas is expensive to transport and store in hatcheries, while the bicarbonate salts can easily be transported and stored. Wood *et al.,* (1999) and Wen and Chen (2003) reported that the carbon source affects the growth and fatty acid composition of many species.

The photoperiod is the alternation of periods of light and darkness, which also has an impact on photosynthetic activity and therefore on the metabolism of microalgae. The light duration per day is also an important factor in the conduct of microalgal culture. Changes in photoperiod impose changes in cellular content (biochemical compositions) such as proteins, carbohydrates and lipids (Tzovenis *et al.,* 1997, Price *et al.,* 1998, Fabregas *et al.,* 2002) and consequently it affects cell concentration. The conditions of light intensity are the main factors affecting the cellular physiology of the microalgae (Khoeyi *et al.,* 2012), and each species of microalgae reacts differently to the photoperiod and the light intensity (Richmond, 2004). An insufficient amount of light intensity can therefore lead to a lower growth rate, on the other hand, at too high a light intensity, photoinhibition becomes an equally important problem (Wahidin *et al.,* 2013). It is therefore essential to find the optimal conditions of the photoperiod for each species of microalgae to improve its growth performance.

In addition, these results also showed that under a 6L:18D photoperiod, the maximum values of the cell concentration, the growth rate, the division rate and the doubling time were observed at the same level of sodium bicarbonate concentration which is 0.5 g L^{-1}, and the same for the photoperiod 12L:12D photoperiod with a concentration of 1 g L^{-1} of sodium bicarbonate, In contrast to other parameters such as cell morphology and biomass. The following figure illustrates the monitoring of the evolution of the cell concentration of *Isochrysis galbana* in the six different concentrations of

sodium bicarbonate from 0 to 2.5 g L^{-1} and two photoperiods 12L:12D (**Figure 1a**) and 6L:18D (**Figure 1b**), in which the culture medium containing no carbon source represents the control, for 16 days.

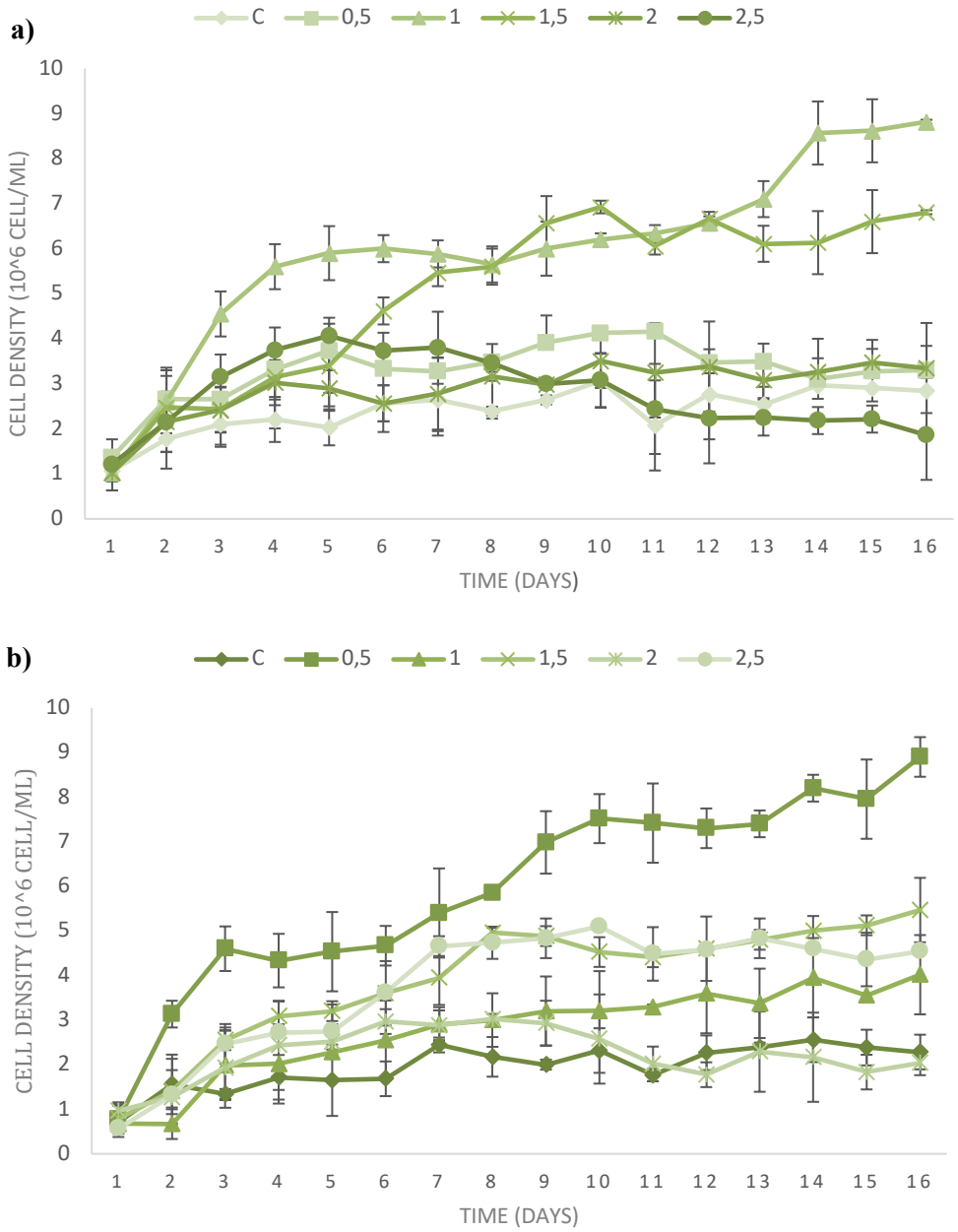

Figure 1. The cells density of the *Isochrysis galbana* culture supplemented with different concentrations of $NaHCO_3$ and in two photoperiods: **a)** 12L:12D and **b)** 6L:18D**.**

On day 16, and the photoperiod 12L:12D (**Figure 1a**), the growth values were significantly ($p < 0.05$) higher in the culture supplemented with 1 g L^{-1} of $NaHCO_3$ with a cell density of 8.82 ± 0.16 10^{+6} cell mL^{-1}, followed by the culture supplemented with 1.5 g L^{-1} of $NaHCO_3$ with 6.81 ± 0.03 10^{+6} cell mL^{-1}, compared to the control culture 0 g L^{-1} (2.84 ± 0.01 10^{+6} cell mL^{-1}) while no statistical difference was observed between the other experimental conditions (0.5 g L^{-1}; 2 g L^{-1} and 2.5 g L^{-1} of $NaHCO_3$ with densities of 3.30 ± 0.04 10^{+6} cell mL^{-1}; 3.35 ± 0.01 10^{+6} cell mL^{-1} and 1.87 ± 0.01 10^{+6} cell mL^{-1}, successively). Indeed, from the third day, the cultures supplemented with 1 g L^{-1} of $NaHCO_3$ significantly increase the value of the cell density (4.55 ± 1.01 10^{+6} cell mL^{-1}) compared to the control (2.10 ± 0.14 10^{+6} cells mL^{-1}). As shown in **Figure 1b**, with a 6L:18D photoperiod, the cell density at 0.5 g L^{-1} of $NaHCO_3$ (8.90 ± 0.03 10^{+6} cell mL^{-1}) was significantly higher, which was double that of

the concentration obtained by adding 1 g L^{-1} of sodium bicarbonate (4.02 ± 0.08 10^{+6} cell mL^{-1}). The final cell densities were significantly ($p < 0.05$) higher in the cultures treated with $NaHCO_3$ compared to the controls. Also, as illustrated in **Table 1**, the specific growth rate of *Isochrysis galbana* varied with the concentration of $NaHCO_3$, confirming that the growth rate and the cell productivity can be improved by adding inorganic carbon (Nunez and Quigg, 2015). Considering the photoperiod 6L:18D, the highest growth rate was determined by 0.5 g L^{-1} of $NaHCO_3$ (0.16 day^{-1}), while for the second photoperiod 12L:12D, the best growth rate was recorded at the level of the concentration 1 g L^{-1} of $NaHCO_3$ (0.14 day^{-1}). Regarding the division rate, at the level of the 12L:12D cycle, the highest division rate was recorded at the level of the culture added with 1 g L^{-1} of $NaHCO_3$ with a value of 0.21 divisions per day, while for 6L:18D, the value maximum was recorded in the treatment of 0.5 g L^{-1} of $NaHCO_3$ with 0.24 divisions per day. The minimum doubling time recorded in *Isochrysis galbana* in this study was 4.24 day^{-1} with 0.5 g L^{-1} of $NaHCO_3$ under a photoperiod of 6L:18D, followed by 4.85 day^{-1} with 1 g L^{-1} of $NaHCO_3$ under a photoperiod of 12L:12D.

Regarding the cell morphology, in general, the variations in biovolume according to the two photoperiods were not significant. For the photoperiod 12L:12D, the maximum value of 46.15 μm^3 was observed at the level of the treatment with of 2.5 g L^{-1} of $NaHCO_3$ during the exponential phase, also 95.19 μm^3 was recorded at the level of 1 g L^{-1} of $NaHCO_3$ for the stationary phase. For the photoperiod 6L:18D, the maximum value of 30.29 μm^3 was recorded during the exponential phase, and also 62.73 μm^3 during the stationary phase at the level of the treatment with 2.5 g L^{-1} of $NaHCO_3$.

Concerning the productivity of the biomass, under a photoperiod of 12L:12D, the biomass productivity at 2.5 g L^{-1} of $NaHCO_3$ (0.68 g L^{-1} day^{-1}) was significantly higher ($p < 0.05$) than cultures with a lower addition of sodium bicarbonate. For the photoperiod 6L:18D, the maximum value 0.42 g L^{-1} day^{-1} was recorded at the level of 1 g L^{-1} of $NaHCO_3$. Throughout our experiment, the addition of $NaHCO_3$ at different concentrations did not cause any significant change in the pH of the medium. The final pH of the cultures was recorded between 9.2 and 9.6 **(Table 2).** This indicates that *Isochrysis galbana* can resist high concentrations of bicarbonate.

Table 2. Shows the pH value of the culture of Isochrysis galbana supplemented with different concentrations of $NaHCO_3$ and in two photoperiods

Photoperiod	Traitement $NaHCO_3$ (g L^{-1})	pH
12L:12D	C	9.29 ± 0.32
	0.5	9.34 ± 0.41
	1	9.27 ± 0.25
	1.5	9.30 ± 0.07
	2	9.46 ± 0.12
	2.5	9.32 ± 0.26
6L:18D	C	9.47 ± 0.06
	0.5	9.48 ± 0.17
	1	9.44 ± 0.19
	1.5	9.45 ± 0.19
	2	9.50 ± 0.15
	2.5	9.64 ± 0.27

The majority of microalgae are alkalophilic and develop in culture media with a pH between 7 and 10.5. According to Bougaran *et al.* (2003), the growth of *Isochrysis galbana affinis Tahiti* is achieved at an average value of 7.2 to 7.8, and it is inhibited as soon as the pH exceeds 8.75 (Kain and Fogg, 1958). According to our results, the final pH of the cultures was recorded between 9.2 and 9.6. The addition of sodium bicarbonate did not cause a significant change in the pH of the medium. This indicates that the culture of *Isochrysis galbana* can resist high concentrations of bicarbonate. In general, the results show that the concentrations of 1 and 0.5 g L^{-1} of $NaHCO_3$ under photoperiods of 12L:12D and 6L:18D, respectively, seem the most optimal, and have significant effects on cell concentration and morphology of *Isochrysis galbana*. This is in agreement with other authors who have demonstrated that the strain *Chlorella vulgaris* has an optimal level of addition of 1 g L^{-1} of $NaHCO_3$ for biomass production (De Farias Silva, 2016, Mokashi, 2016), *Tetraselmis suecica* and *Nannochloropsis salina* (White *et al.,* 2012), In another study, the addition of 1 g L^{-1} of $NaHCO_3$ in *Chlorella vulgaris* produced higher chlorophyll biosynthesis (Kong *et al.,* 2011).

Conclusion

The results demonstrated that the photoperiod and the addition of sodium bicarbonate have significant effects on the production of the *Isochrysis galbana* culture.

Indeed, the concentration of 1 g/L of $NaHCO_3$ under a photoperiod of 12L:12D, moreover the concentration of 0.5 g/L of $NaHCO_3$ under a photoperiod 6L:18D, seems the most optimal and made it possible to obtain the cell concentrations the best higher with a low production cost in *Isochrysis galbana* culture.

This study proved that adding sodium bicarbonate is a viable strategy to improve microalgae production and can significantly reduce production costs.

Disclosure statement: *Conflict of Interest:* There are no conflicts of interest.
Compliance with Ethical Standards: This article does not contain any studies involving human or animal subjects.

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