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RESEARCH ARTICLE

Water compatibility of M'sila region (South- East Algeria) for cultivation of *Spirulina* & evaluation of methods of extraction of Phycocyanin and investigation of stability by β-Cyclodextrin

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ABSTRACT

Spirulina (*Arthrospira platensis*) is a microalga that has existed for more than three billion years. It belongs to the family of cyanobacteria. It is extraordinary, rich in nutrients such as proteins, carbohydrates, lipids, vitamins, and minerals. In addition, *Spirulina* is rich in phycocyanin, a blue protein pigment. It can be used as a dye in food, pharmacology, and cosmetics. Our work focused first on the compatibility of the waters of M'sila region (southeast Algeria) for the cultivation of *Arthrospira platensis*. Then, we evaluated several methods of extracting phycocyanin to be able to determine the optimal method in terms of yield. We have succeeded in developing a new extraction method coupled with maceration in a glycerol-water mixture with a molar ratio (8/2) assisted by ultrasound with a yield of 52.93 mg/g. The excellent results obtained may be due to the salinity of the waters of the region, to the used nutrient culture medium and/or the climate change of the region. We have formed an inclusion complex between phycocyanin and β-Cyclodextrin to keep it and make it more stable. The encouraging results allow Algeria to gain a foothold on the world market as a producer of blue gold.

Keywords: β-Cyclodextrin; Cultivation; Extraction-phycocyanin; *Spirulina*; Water-salinity.

INTRODUCTION

Spirulina is a filamentous cyanobacterium. It is part of a particularly interesting bacterium called *Spirulina platensis* (or *Arthrspira platensis*) (*A. platensis*), better known under the name blue-green algae (Jourdan, 1993, 2015; Kulkarni and Chavan, 2020). It has many benefits such as antioxidant properties, participates in the good functioning of the immune system, and helps in weight (Gad et al., 2011). Its high protein content makes these algae a superfood (Martelli et al., 2014; Lupatini et al., 2017; Gad et al., 2011). It set about producing oxygen, conquering and modifying our atmosphere, thus allowing the emergence of life forms (Paniagua et al., 1993; M'Baye et al., 2011; Vo et al., 2015; Furmaniak et al., 2017). For several decades, *A. platensis* has been the subject of rediscovery by scientists, both for its proven nutritional properties, mainly proteins (60 to 70%) because it is richer in protein than meat (Mazokopakis et al., 2014; Jung et al. 2019), omega-6 (20 to 25%), carbohydrates (15% to 20%), rich in minerals (mainly trace elements), 11% lipids as well as vitamins including carotene 13% vitamin B12 (Dagnelie et al., 1991). Furthermore, *A. platensis* is rich in vitamin E (which is a powerful antioxidant) (Xue et al., 2002; Tang and Suter, 2011). *A. platensis* is thus extremely rich in β-carotene (Terao 1989), amino acids (Saranraj and Sivasakthi, 2014) and bio-crude oil (Karkos et al., 2011). Besides its many interests, these microalgae have been the subject of numerous studies in several countries.

The high cost of mineral elements used in mass cultivation of these algae remains a serious problem and the need to use a natural mineral source is imposed; the waters of M'sila region may be an adequate solution given their richness in mineral elements.

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In addition, the therapeutic value of *A. platensis* thanks to its natural pigments called phycobiliproteins (Zaviel et al., 2018), and their beneficial potential, have been experimentally proven in vitro and in *vivo* to treat more pathologies (Falamas et al., 2020). Phycobiliproteins are water-soluble compounds made up of molecules, the most interesting of which is Phycocyanin (PC) (Fig. 1) (Sall et al.,1999; Jensen et al., 2016; Rahman et al., 2016). Indeed, several epidemiological studies have demonstrated the beneficial effect of PC on human health through its various properties: anti-inflammatories (Romay et al., 1998), antioxidants (De la Coba et al., 2009), immunostimulants (Li et al., 2010), anti-diabetics (Ou et al., 2013), atheroprotective (Strasky et al., 2013), anti-carcinogens (Gardeva et al., 2014) and cosmetics (Saini et al., 2021), etc.

According to the literature, most of the reported works were focused on extraction and purification methods development of PC, including: freeze/thaw cycles (Zhang and Chen 1999), sonication (Mogany et al., 2018), aqueous biphasic extraction (Wu et al., 2014), extraction by the use of solvents (phosphate buffer) (Chen et al., 2006), ultrasound-assisted extraction (Le Guillard et al., 2015 and Hadiyanto et al., 2016), maceration of materials in glycerol or a water/glycerol mixture (Pottecher, 2014), microwave-assisted extraction as a source of heating of the solvent-matrix solution (Balasubramanian et al., 2011), accelerated solvent extraction (Hoshino et al., 2017) and extraction assisted by the pulsed electric field (Amiali et al., 2006). These processes are generally long, tedious, and require large amounts of organic solvents. Moreover the achievement yields are low (Marzorati et al., 2020). Extraction and purifying methods on PC at an affordable cost, without degradation of its properties is a challenge for most researchers (Oliveira et al., 2009; Mata et al., 2010). The stated objective is the choice of methods of extraction, purification, and stabilization of PC that makes the extraction more efficient, lower cost, lower risk, environmentally friendly, and with good yields.

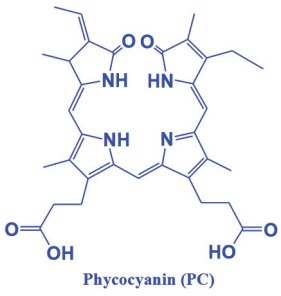

Fig 1. Chemical structure of phycocyanin (PC) of *A. platensis* (redrawn on the basis of Kim et al., 2018).

Our work consists first, in the introduction of these microalgae in Algeria and particularly in arid or semi-arid zones (M'sila). This micro-organism naturally proliferates in lakes where all conditions are met: alkaline pH (8.8-10), relatively high temperature (35-37°C), and fairly important light intensity. One of the most important parameters for the culture of *A. platensis* is the quality of the water used in the culture medium. After the climate discovery of the region and its salinity water resources, we studied the water compatibility for the cultivation of *A. platensis* to define the optimal conditions for the growth of cultivated in this region. Subsequently, we succeeded in developing a new method coupled with maceration in a glycerol/water mixture with a molar ratio (8/2) assisted by ultrasound for PC extraction.

However, the PC is a very unstable pigment because it is sensitive to light, temperature, and the basic environment where it degrades quickly, which makes its use difficult and limited. For this reason, we have studied its stability with the addition of a stabilizer. Among the stabilizers that exist, we have chosen β-Cyclodextrin (β-CD), it is a natural, water-soluble molecule (Shah et al., 2015), biocompatible (Stojanov et al., 2012), biodegradable (Chen et al., 2020) and non-toxic (Anderson et al., 2005) thus their use in fields ranging from pharmaceutical (Shao et al., 2019) to medicine (Zhang et al., 2010) and agriculture (Balogh et al., 2008). β-CD is already widely used in the pharmaceutical sector, mainly as a solubilization adjuvant to increase stability and/or improve solubility (Zidane et al., 2019; Bouleghlem et al., 2020) and it can protect certain active ingredients from photodegradation (Popielec and Loftsson, 2017).

MATERIALS AND METHODS

Chemicals and Reagents

Glycerol, for molecular biology, ≥99.0%, and phosphate buffer were provided by Sigma-Aldrich. β-CD (β-CD ≥ 97%) was provided by Roquette Frères (Lestrem, France), it was recrystallized twice in distilled water, filtered, and dried for 24 h at 100°C under vacuum before use.

Materials

Temperature was maintained at an optimum value of 30°C using adjustable thermostats; Lighting with solar radiation during the day and at night by a fluorescent white light of the brand digital luxmeter (TES-1330A) with a light intensity of 6000 Lux; Electrical conductivity (EC) and salinity were measured using a conductivity meter (ProfiLine Multi 1970i multiparameter); Absorption spectrum was also determined by scanning the sample in the range of 300-750 nm by using Specord 200 spectrophotometer (Analytik Jena, Germany). Observation was ensured by a Microscope Paralux Bio 400. Centrifugation was performed by using Centrifuge

type (8 x 15mL Cap, 5000 rpm, 115VAC). PC extraction using an ultrasonic bath (1000 W, model BT90H) combined with an ultrasonic generator at 170 kHz (Ultrasonic Power Corporation), the inclusion complex (β-CD: PC)1/1 was prepared by a freeze dryer (Christ Alpha 2-4 LSC plus, Osterode, Germany).

Description of the study area

The city of M'sila is part of the HODNA watershed, it occupies a privileged position in the central part of northern Algeria as a whole, it is part of the central highlands region and extends over an area of 18175 km^2, at a distance of approximately 250 km south-east of Algiers. Regarding the geographical location, the region of M'sila is in latitude 35°42'07"N and longitude 4°32'50"E, at an altitude of about 500 m. It is characterized by a large agricultural area, mainly including the cultivation of cereals, market gardening, and arboriculture. The territory of the province constitutes a hinge zone of transition between the two great mountain ranges, which are the Tellian Atlas and the Saharan Atlas. M'sila station is characterized by a seasonal regime; this means that autumn is the rainiest season, while summer is the driest season (DAS 2018). The average monthly temperature of the year is 23.3°C. It was recorded an average of 31.2°C, in the hottest month (July), and an average of 7.7°C for the colder month (January). It is worth noting that the months of (December and January) are the least sunny ones with a value equal to 6.9 h/day and an overall radiation intensity on a horizontal surface of 5428 kWh/m^2 (DAS 2018).

Strain and cultivation conditions

We used *A. platensis* grown on a farm located in northwest Algeria, which is an *A. platensis* native of Peru, species of *A. platensis* (CCC540) type *Paracas*. The strain received was 1,5L with an estimated concentration of 0.2 g/L. The volume was put in a sterilized bottle of 5L containing 3L mineral water. The stopper was left open and the bottle was shaken occasionally all along the way (631.3 Km).

Growth measurement and evolution

The measurement of the growth of *A. platensis* was carried out using a spectrophotometer and was calculated according to Equation 1 (Madkour and Kamil, 2012 and Tatang et al., 2020). Cell density was determined using an optical microscope and a Nageotte cell for counting. The purity and identification of the culture were done systematically using the optical microscope. Currently, there are several methods to quantify this cell growth. Among these methods, the most used were the counting of cells under an optical microscope and the measurement of the optical density by spectrophotometry.

However, these measuring instruments remain relatively expensive. We used a method of measuring the concentration of biomass adapted to *A. platensis*; simple, economical, and efficient. The procedure was done after membrane filtration, before being dried for 3 days at 30°C; the fresh biomass was washed three times with distilled water, and then neutralized with sodium chloride (10%) to remove all the salts of the culture medium. Finally, the dry weight (g/L) in biomass was obtained by correlation with the optical density (OD) at 680 nm according to Equation 2 (Hu 2004).

A. platensis culture

The *A. platensis* culture was initially inoculated with a cell concentration of 8.10^5 cells/mL, corresponding to a concentration of 6 mg/L (Dorothy et al., 2015), the strain was divided into four equal parts (test 1, test 2, test 3, and test 4) (Table 1), in order to avoid losing the entire culture, to investigate the culture media and choose the best medium (it was carefully monitored and exposed to different conditions). The used water was taken from tap water which was used as substrate to prepare the culture media (2, 3, and 4) with the addition of low amounts of other nutrients and without NaCl compared to the culture medium of Zarourk (1966) and Jourdan (2006).

Harvest of *A. platensis*

The *A. platensis* harvest was done early in the morning to avoid sunshine which could spoil the crop. The water in the basins was then pumped and then filtered twice (60 μm filters) in order to obtain a wet *A. platensis* paste, washed with fresh water, filtered through a very fine mesh (approximately 10 μm) and the biomass has been dried in the shade and at low temperature to a humidity content of 10%. After the drying step, dry *A. platensis* becomes hard and brittle. It was then grounded using a mixer to obtain a powder. The obtained powder was weighed to have the mass of the dry biomass, so the rate of drying is obtained by the ratio of fresh weight/dry weight. The dry *A. platensis* was put in well-sealed boxes, placed in a dry place, and protected from light.

Table 1: The composition of the tests (1, 2, 3 and 4) medium

Culture medium	Test 1	Test 2	Test 3	Test 4
Water	Distilled (3L)	3L*	3L*	3L*
Agitation	Stirring	Stirring	Stirring	Stirring
Strong lighting	Day/Night	Day	Day/ Night	Day/Night
Temperature	30°C	30°C	30°C	30°C
pH	9.5	9.5	9.5	9.5
$NaNO_3$	5 g/L	5 g/L	1.5 g/L	1.5 g/L
CH_4N_2O	2 g/L	2 g/L	0.2 g/L	0.2 g/L
$NaHCO_3$	10 g/L	10 g/L	10 g/L	10 g/L
$NH_4H_2PO_4$	1 g/L	1 g/L	0.1g/L	0.1g/L
NaCl	0.1g/L	0.1g/L	-------	0.1g/L

*Water of M'sila region.

Determination of the rate of humidity

The water content was determined using 200 mg of the obtained *A. platensis* powder sample which was dried at 103°C in an oven until a constant weight was obtained. The difference between the two weighings before and after drying makes it possible to calculate the water content.

Determination of *Chlorophyll a* (*Chl a*) content

Chlorophyll a is the primordial pigment that participates in photosynthesis (Choi and Lee, 2018). The concentration of *chlorophyll* was determined according to Equation 3 (Bennett and Bogorad, 1973) by measuring optical density at 663 nm and 750 nm using a spectrophotometer.

Phycocyanin extraction methods

This study aims to compare different methods and the development of a new method of PC extraction. The extracts of the PC were obtained using physical methods (freezing/thawing, ultrasound), chemical methods (glycerol/water), and a physico-chemical method (assisted by ultrasound which consists of maceration in the mixture (Glycerol/Water).

Water extraction

A. platensis powder (2g) was added to 250 mL of water. The mixture was prepared in the dark for 10 days at 25°C. The solution obtained undergoes decantation and then centrifugation (5000 rpm at 0°C). We took the supernatant and diluted it by a factor of about 100 with distilled water (M'Baye and Bassene, 2011).

Extraction by freezing and thawing cycles

We have put 2g of dry *A. platensis* in 5.10^{-2}M phosphate buffer at pH = 6.9 for 2 days. The solution undergoes freezing (-10°C) and thawing cycles. The operation was repeated twice, in 24 hours (Mogany et al., 2018).

Ultrasound-assisted extraction

We have introduced 2g of *A. platensis* in 250 mL of phosphate buffer (5.10^{-2}M, pH = 6.9). The solution was put under the action of ultrasound for 45 min followed by centrifugation (5000 rpm at 0°C). The supernatant was sampled and 100 mL of buffer was re-added to the base and returned to ultrasonic action for 2-3 min, then centrifuged to take the supernatant again and mix it with the first one and measure the absorbances at 615 nm and 652 nm (Le Guillard et al., 2015).

Extraction by maceration in the glycerol/distilled water mixture of (Glycerol/Water) 4/6

2g of *A. platensis* obtained was mixed in glycerol/distilled water (4/6). It was left to macerate for 7 days at room temperature in the dark. Filtration is carried out by a food-compatible filter (Nylon bag 330×500 filters 50 microns) (Pottecher 2014).

Extraction by maceration in a mixture of glycerol/water assisted by ultrasound (new method)

To 2g of *A. platensis* powder obtained, we have added a mixture of glycerol/water at different molar ratios (0/10, 1/9, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2, 9/1 and 10/0). The solution is placed under the action of ultrasound for 20 min. The experiment was carried out between 20 and 25°C with air humidity between 50 and 60%. The water-soluble part has been recovered, which is an extract rich in PC. The yield of PC extraction was measured using a spectrophotometer.

Analytical procedures

$$[\,]_{biomass} = 0.7.OD_{56} \quad (1)$$

Or; $[\,]_{biomass}$ is the biomass concentration (g/L); OD is the optical density with 1 unit of absorbance representing 0.7g/L of *A. platensis*.

$$\text{Dry Weight} = 0{,}7247.\ OD_{680} + 0{,}0044 \quad (2)$$

$$Chla = \frac{(OD_{663nm} - OD_{750nm}).106.V_1(mL)}{89.V_2(mL)} \quad (3)$$

Or V_1: Volume of solvent (in mL) and V_2: Volume of the sample (in mL). The extraction biomass concentration [PC], yield, and purity of phycocyanin (P_{PC}) were measured using a spectrophotometer. The optical density was measured at 615nm, 652nm, 620nm, and at 280nm. The phycocyanin concentration in mg/mL was calculated from the optical densities at 652nm and 620nm, according to Equation 4 (Bennett and Bogorad, 1973; Silveira et al., 2007).

$$[PC] = \frac{OD_{615} - 0.474(OD_{652})}{5.34} \quad (4)$$

The purity of phycocyanin is deduced from Equation 5 (Bennett and Bogorad, 1973).

$$P_{PC} = \frac{Abs_{620}}{Abs_{280}} \quad (5)$$

With: Abs at 620 nm indicating the concentration of PC and Abs at 280 nm indicating the total concentration of proteins. The extraction yield was calculated using Equation 6 (M'Baye and Bassene, 2011).

$$\text{Yield} = \frac{[PC].V}{DB} \quad (6)$$

Where, [PC] is the PC concentration (mg/mL), V is the volume of solvent (mL), DB is the dried biomass (g) (M'Baye and Bassene, 2011).

Phycocyanin stability studies

Formation of a new complex between PC and β-CD

The preparation of the inclusion complex between β-CD and PC with a molar ratio (1/1) was carried out by freeze-drying; to an aqueous solution of β-CD (15µM), a quantity of PC was added. The mixtures were stirred at 150 rpm at low temperature for 5 h then filtered through an organic membrane. The resulting suspension was frozen (-10°C) and then lyophilized using a freeze-dryer until the water is completely removed. The recovered powder is considered as the solid inclusion complex.

Forced degradation of phycocyanin in the presence of β-Cyclodextrin

The complex was dissolved at approximately 1 mg/mL in distilled water before being exposed for 2 hours to the following degradation conditions: acid degradation (pH 1-8), basic degradation (pH 9-12), photostability (7500 Lux,1.1 W/m^2, 250-785 nm), thermodegradation 30-90°C. The concentration of undegraded PC was evaluated against a solution stored at 25°C and in the dark.

RESULTS AND DISCUSSION

Study and characterization of the water region used

The cultivation of *A. platensis* is subject to the influence of several physical or biological environmental parameters which are dependent on the intrinsic characteristics of the algal species and the geometry of the production system. These parameters affect not only photosynthetic activity and biomass productivity, but also the physiological and metabolic behavior of *A. platensis* in culture. These are light, temperature, pH, water salinity, nutrients, etc. Analysis of different parameters of water in the M'sila region is presented in Table 2. Water samples from the study area have pH values ranging from 8.6 to 9.8, indicating that the water in the region is slightly alkaline (Table 2).

The concentration of calcium and magnesium is acceptable and exceeds the concentrations in the culture medium Zarrouk (1966) and the concentration limits reported by Jourdan (2006). The water coming from the infiltration zone has a carbonate facie, and then along the flow towards the south of the region, it gradually changes to a chlorinated, sulfated calcium and magnesium water in relation to the dissolution of the salt formations of the land and salt lake. The presence of nitrates is linked to the agricultural activity of this region. This is to be put in connection with the carbonate formations and the salt formations of Chott HODNA (Amroune et al., 2017). The results, from the study of the parameters, allow us to better understand the particularities of the interaction of the elements composing the water of the region used for the culture medium and the impact on the growth of *A. platensis*. From Table 2 the minimum air temperature was 28-35°C, so it was within the standards compared to the optimum reported in the literature (Richmond 1983) for the good growth of *A. platensis*.

Table 2: Physicochemical analysis of water in the M'sila region (adapted on the basis of Mahmoudi and Yakhlef 2015, Belhadj et al 2017).

Parameters	Jan	May	Oct
T(average)[a]	7.7	32.8	28.6
pH	8.6	9.8	9.2
Salinity[b]	12.90	15.55	15.81
O_2[c]	84.20	86.66	85.3
EC[d]	3.600	3.586	3.544
Ca^{2+}	436	452	432
Mg^{2+}	222	228	221
Na^{+}	550	554	555
K^{+}	9	8	9
Cl-	840	845	836
SO_4^{2-}	1394	1388	1388
HCO_3^{-}	353	350	353
NO_3^{-}	89	101	100
NO_2^{-}	> 0.02	> 0.1	> 0.1
Fe	17.2	20.0	18.5

[a]T (°C), [b]Salinity (PSU), [c]Dissolved oxygen O_2 (in%), [d]Electric Conductivity (mS /cm) and the chemical element in (mg/L).

Observations with the naked Eye

Observation of the culture medium with the naked eye is the means of monitoring the development of the culture, according to Jourdan (Jourdan 2015). It made it possible to identify possible problems such as coloring and discoloration of certain media (Jourdan 2015). From Fig. 2, (Test 4) gave a healthy green-colored culture. From test 3, the crop turns grayish-yellow, because it is exposed to strong sunlight, the yellowing of the culture medium is followed by the appearance of yellow foams and accompanied by a strong odor of ammonia. This means that there is an excess of urea in the medium. Test 1 is colorless; the problem is related to the quality of water used, or to the presence of other microorganisms that are harmful to *A. platensis*. The culture color turned green from blue-green characterizes cyanobacteria. This phenomenon is due to the depletion of nitrogen in the culture medium (Goksan and Zekruyaoulu, 2007), and when there is a nitrogen deficiency in the medium, PC is used as a nitrogen source, which is responsible for the characteristic blue-green color which confirms that this crop is too shaded (Test 2) and this color is due to the extracellular pigments of *A. platensis* (Menegol et al., 2017). In addition to the nitrogen deficit, the high light intensity, and thus the high temperature during the experiment most

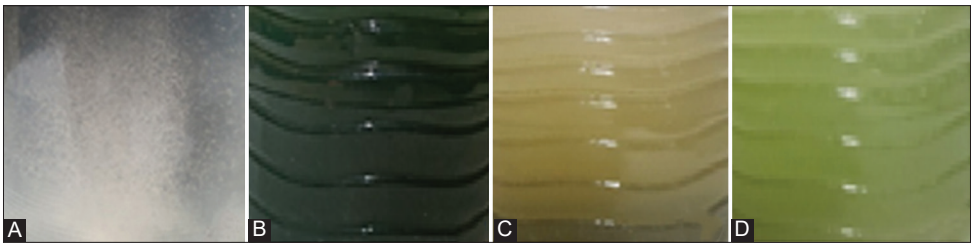

Fig 2. Observations with the naked eye of culture media: (A) (Test 1), (B) (Test 2), (C) (Test 3) and (D) (Test 4).

likely led to the color change, as in our case, these results are confirmed by the literature (Zhu et al. 2020; Trabelsi et al., 2009). The modification of the morphology of *A. platensis* may be due to the variation of several parameters, which are the concentration of nitrogenous elements such as $NaNO_3$, CH_4N_2O, $NH_4H_2PO_4$, and $NaHCO_3$, the concentration of bicarbonate, the concentration of NaCl, day/night lighting cycles, the effect of the composition of culture media (concentration of monoammonium phosphate) (Test 1 and 4), alkaline pH and finally salinity.

According to our observations, the medium of (Test 4) is the most suitable for cultivation and good growth of *A. platensis*. Therefore, water from the M'sila region replaced the lack of NaCl and the small amounts of other nutrients. To increase the growth, this time we used a greenhouse basin (150 ×75 × 22 cm, 180 L) (Fig. 3), using the culture medium used in test 4, the nutrients were added every 4 to 5 days and *A. platensis* was cultured for more than three weeks.

Physico-chemical analysis of culture media (the Basin)

Temperature

The daily average temperature of air measurements during the cultivation period was 30°C. The minimum temperature was 25°C while the maximum was 35.5°C. The average water temperature during this experiment increased from 20 to 32°C, it was on average 29.17°C, which is practically identical to that of the ambient air temperature. However, it has a positive effect on the growth of *A. platensis* and the production of biochemical constitutions.

The pH

We noticed that the pH of the medium was 9.55. The pH is slightly basic, given that *A. platensis* was carried out in an alkaline medium; our results are in accordance with those obtained in the literature (Zarrouk 1966 and Jourdan 2006). The increase or decrease in pH corresponds to the carbonate-bicarbonate system (CO_3^{2-}, HCO_3^-). Bicarbonate ions are assimilated by *A. platensis* and subsequently converted into carbon dioxide and carbonate.

Carbon dioxide is used in photosynthesis and carbonate is excreted in the medium. Therefore, the increase in pH is due to the shift of the carbonate-bicarbonate balance towards carbonate, following the reaction schemes below:

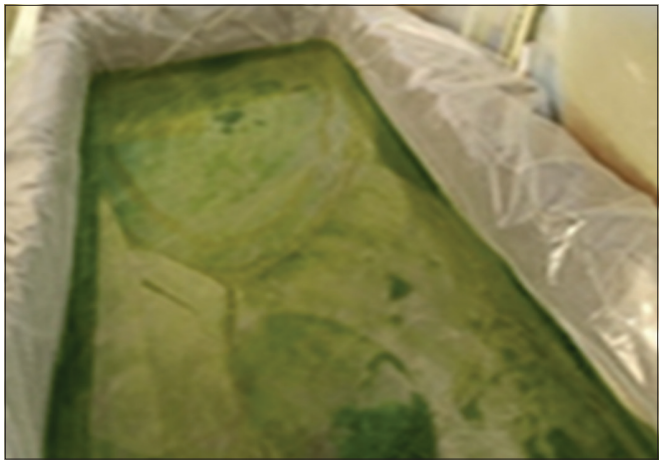

Fig 3. Basin (150 × 75 × 22 cm) of 180L just after the inoculation of the *A. platensis*.

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$
$$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+$$

The regular increase in the salinity in the medium is due to the evaporation of water during this period and to compensate this, water has been added every two days, provided that it does not exceed the optimal zone of development, which is between 22 and 62 grams of salt per liter. The initial difference is due to the chemical nature of water in the area. Then, it is the evaporation that will increase it during the experiment period.

The light

During the cultivation period of *A. platensis*, the incident short-wave solar radiation per square meter was between 6.9 - 7.8 kWh.

The rate of humidity

The humidity level, which represents the water content in *A. platensis* powder, is measured as a percentage of water relative to its dry weight. We found a humidity level of 7.58%. For the ordinary culture medium studied, the moisture content is less than 10%, a recommended condition for the long-term storage of the powders of these microalgae (Richmond 1983). This value is very close to that presented in the literature (Ravelonandro et al.,

2011; Valencia-Hernandez et al., 2021), so this condition is recommended for the storage of dry *A. platensis* for a long period.

Microscopic observation

Microscopic observation of the samples allows a more objective judgment of the state of health of the environment because it makes it possible to observe the appearance of the filaments and to identify the organisms that can harm the environment. According to Fig. 4 and concerning the appearance of the filaments, several cases may arise: broken filaments, which can be caused either by a sudden agitation of the culture medium, or by an excess of light, or by a lack of potassium.

Regarding biomass productivity, various authors have reported that, in microalgae crops, nutritional status is the main factor that can affect growth and productivity (Trabelsi et al., 2009; Keymer et al., 2014). Growth rate is expressed in (g/L) dry weight (Fig. 5) (Left). For an ordinary culture medium, the maximum biomass concentration and cell density are 1.40 g/L and $5.04.10^7$ cells/mL, respectively. These results are consistent with those reported in the literature (Chi et al., 2016; Cheng et al., 2018).

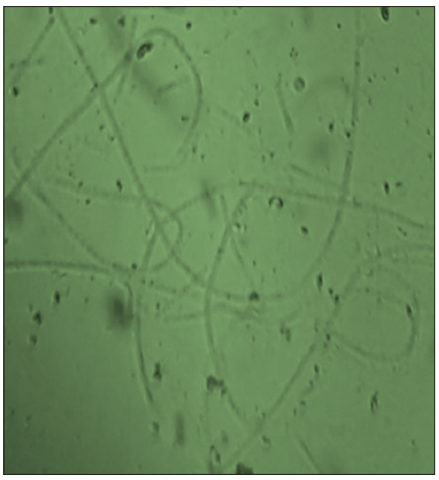

Fig 4. Optical microscope observation of *A. platensis*

The weight of dry biomass changes linearly as a function of optical densities. (Fig. 5) (Right). The fit between dry weight and optical density (Equation 2) was obtained at 680 nm (OD_{680}) with an $R^2 = 0.9990$. This method which uses dry weight is more important than the method using wet mass (Moraes et al., 2018). Calculation results of total expected biomass can be measured in seconds. The experiment is easy to perform and inexpensive.

Study of the growth of *A. platensis* by swab seeding

For the preparation of the stock suspension, we aseptically prepared a solution having a dilution of 1/10 (1 g of sample to which 9 mL of physiological water was added). Different decimal dilutions are used (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) for the analysis and after a few hours of incubation at 25°C, colonies visible to the naked eye begin to form. From one Petri dish to another, the concentration of *A. platensis* ranges from a high concentration in the Petri dish with the dilution highest (10^{-4}) to a low concentration in the Petri dish with the dilution weakest (10^{-2}). During the incubation process, *A. platensis* multiplies exponentially; we can say that this ordinary medium is very suitable for growing *A. platensis* because it contains all the necessary elements for growth. (Fig. 6). These results can be used by stating that the harvest is a weight factor in the growth and that ripe *A. platensis* should not be left in the medium for too long. If the concentration of *A. platensis* is low, the growth will be faster, the same results obtained in the literature (Khannapho et al., 2021).

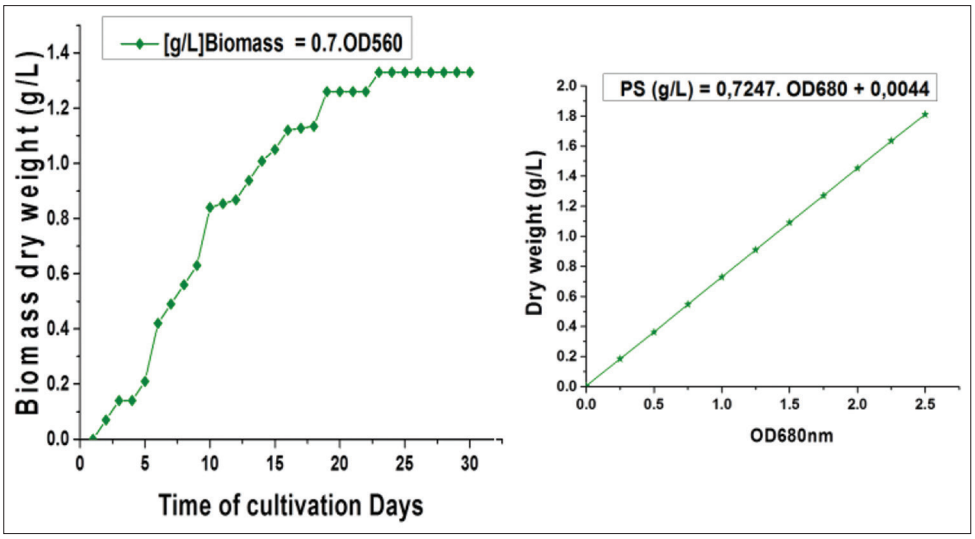

Fig 5. (left) Growth of *A. platensis* in ordinary culture medium for biomass estimation (g/L); (right) Evolution of dry weight (g/L) as a function of optical density (OD680nm)

Determination of the content of *Chl a*

The amount of *Chl a* observed in this study is presented in Fig. 7. We found that the *Chl a* content remained almost stable until the 5th day, from which it gradually increased over time. The concentration of *Chl a* is 17.15 mg/L. Comparison of our results with the results cited in the literature, the *Chl a* content depends on the culture media, environmental factors, the region, and the culture medium (Danesi et al., 2011; Marzorati et al., 2020). *A. platensis* contains *Chl a*, typical in plants. This pigment is of interest in the food and pharmaceutical industry.

Optimization of the maceration extraction method assisted by ultrasonic (new method)

The optimization of the maceration extraction assisted by ultrasonic in the mixture (glycerol/water) was carried out at different molar ratios of glycerol and distilled water. The results of the different molar ratios of the eleven tests, as well as the PC concentration and the degree of PC purity, are shown in Table 3.

Extraction of phycocyanin

These classical methods have a single advantage, which is being easy to manipulate no more. But our approach consists of modifying and/or coupling two methods very well known in the experimental plan: ultrasound-assisted extraction (Le Guillard et al., 2015) and maceration in glycerol (Pottecher 2014) (see in Table 4).

The extraction was carried out at different molar ratios of glycerol and water (0/10, 1/9, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2, 9/1, and 10/0). According to Table 3, the maximum PC extraction yield is obtained in case 8/2 (52.93 mg/g) (9th test). On the other hand, a decrease in the extraction yield is observed in the case of the use of 10/0 (100% glycerol) (11th test), which demonstrates the solubility of PC in glycerol in the presence of water. The solvent combining glycerol and water is therefore very effective in extracting PC. The different molar ratios of water allow the dilution of glycerol, and therefore decrease its viscosity and increase its polarity and therefore its power of extraction. To find out the effectiveness of our new method, we compared our results with conventional and unconventional methods (extraction with water, extraction by Freezing/Thawing cycles, extraction assisted by Ultrasound and extraction by maceration in the mixture (Glycerol/Water) 4/6.

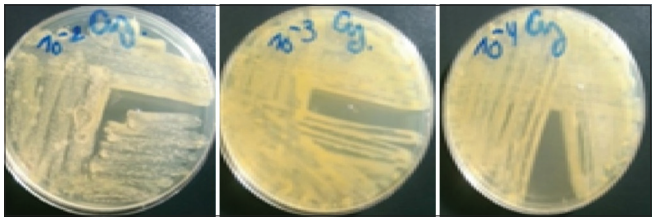

Fig 6. Result of seeding by swabbing

Table 4, shows the phycocyanin concentrations, the degree of purity and the duration of time obtained by the conventional extraction with water, the extraction by freeze/thaw cycles, extraction assisted by ultrasound, extraction by maceration in the mixture (Glycerol/Water) 4/6 and extraction by maceration in a mixture (Glycerol/Water) 8/2 assisted by ultrasound.

From Table 4, the PC concentration of dry *A. platensis* varies between 0.08-3.93mg/mL, PC purity varies between 0.25-1.10 and the time varies between 20 min and 10 days. Ultrasonic assisted extraction (by new method), which consists of maceration in the mixture (Glycerol/Water) 8/2, gave higher concentrations of PC, a very short time compared to other methods and the degree of purity is 0.79. This treatment will therefore require many long purification steps, to reach a level of food-grade purity equal to 7, but according to the literature, this indicative value allows us to affirm that this extraction is of the food order according to Rito-Palomares et al., (2001), PC preparations with a degree of purity less than 0.7 are suitable for food-grade, and those with a degree of purity in the range 0.7 to 3.9 are of reactive grade (Rito-Palomares et al., 2001). These differences in the values of the PC concentration and the degree of purity may be due to the conditions of the extraction routes used, the solvents used, the mode of purification followed, the drying means, and/or the presence of impurities inherent in this type of non-selective treatment (Moraes et al., 2011; Rito-Palomares et al., 2001). Our results for the PC concentration via the newly proposed method reached 3.93 mg/mL and these are in relation to the literature (Gupta and Sainis 2009; Akimoto et al., 2012; García et al., 2021). The results showed that the purity of the PC, using the classical separation method with ordinary water, was very low (0.25). The latter causes the bursting of the *A. platensis* cells due to the effect of the centrifuge which has been carried out (5000 rpm at 0°C) and consequently, the extrusion of all the intracellular

Table 3: Optimization of the extraction of PC by maceration in a mixture (glycerol/water) assisted by ultrasound

	1	2	3	4	5	6	7	8	9	10	11
(Glycerol/Water)	0/10	1/9	2/8	3/7	4/6	5/5	6/4	7/3	8/2	9/1	10/0
[PC] (mg/mL)	0.02	0.15	1.5	2.7	2.94	2.98	3.01	3.59	3.93	2.90	2.20
Abs620/Abs280	0.11	0.23	0.32	0.42	0.55	0.60	0.70	0.75	0.79	0.98	1.00
Yield (mg/g)	0.26	2.02	20.20	36.36	39.59	40.13	40.53	48.35	52.93	39.05	29.62

elements (Soni et al., 2019; Safaei et al., 2019). The release of the PC is directly related to cell disruption, but *A. platensis* has strong multi-layered cell walls, making the extraction procedure difficult (Patel et al., 2005). In this case, the yield is moderate and/or low in some cases, depending on the technique used. On the other hand, the extraction by freezing/thawing cycles gave higher purity values than the other methods (1.10), but the phycocyanin concentration [PC] is very low (0.08mg/mL) compared to the other methods. According to the literature, the extraction purity ranges from 0.40-2.0 with PC extraction ranges from 0.09-3.73 mg/mL (Kumar et al., 2014). In the following, we have grouped the yield of PC in mg/g, according to the different techniques used to distinguish the best method of extraction of PC. From Fig 8, extraction by maceration in the mixture (Glycerol/Water) 8/2 ultrasound-assisted, proved to be the most efficient method, with a good yield is 52.93mg/g and a PC concentration of 3.93 mg/mL higher than ultrasound-assisted extraction alone (the most frequent method used) (Yield = 4.71 mg/g and [PC] = 0.35 mg/mL) and/or by extraction by maceration in the mixture (Glycerol/Water) 4/6, with an extraction yield (Yield = 38.81 mg/g and [PC]= 2.88 mg/mL). We suggest that this increase is mainly due to *A. platensis* which favors the extraction by glycerol mixed with water (8/2) and could be related to the culture conditions, or to the PC content (52,93%). This result is original, it is better compared to those cited in the literature and those reported by Marzorati (2020) and Minchev et al., (2021); as well as by Pez et al., (2021) and Khandual et al., 2021, Aoki et al., (2021).

It was concluded that the coupling procedure between the two methods improved the yield of PC extraction. In addition, it is the only extraction method that is coupled between ultrasound and maceration in the mixture (Glycerol/Water), it is simple, very short, fast, and efficient and it gives a good yield and better value for the PC concentration. In addition, this green chemistry technique only requires glycerol, as we know that glycerol is naturally present in all animal or vegetable oils and fats. It is also used in the composition of many cosmetic, food, and pharmaceutical products. Finally, we succeeded in resuming the development of a new extraction method coupled with maceration in a glycerol/water mixture with a molar ratio (8/2) assisted by ultrasound.

Table 4: Different methods for PC extraction

Extraction mode	[PC][b]	P_{PC}[c]	Time
Extraction with water	0.24	0.25	10 Days
Extraction by freezing/thawing cycles	0.08	1.10	4 Days
Ultrasonic assisted extraction	0.35	0.84	45 min
Extraction by maceration in the mixture $4/6_a$	2.88	0.58	7 Days
Extraction by maceration in a mixture $8/2_a$ assisted by ultrasound	3.93	0.79	20 min

[a]Mixture of the (Glycerol/Water); [b]The concentration of phycocyanin (mg/mL); [c]Phycocyanin purity (Degree of purity).

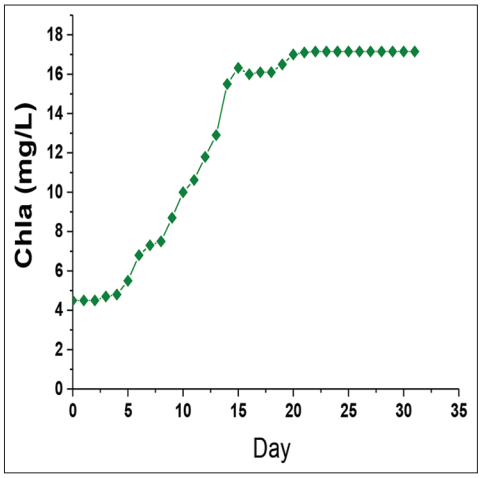

Fig 7. Evolution of the growth of *Chl a* content as a function of time

RESULT OF PHYCOCYANIN STABILITY

Formation of the inclusion complex (β-CD: PC) 1/1

The inclusion complex (β-CD: PC) obtained in solid form with an encapsulation efficiency of around 75%, indicates that losses are negligible during the lyophilization process. This technique is one of the most recommended methods for encapsulating guest thermolabile, light-sensitive, and unstable in aqueous solutions at acidic and/or basic pH (Contreras et al., 2014; Zidane et al., 2019; Bouleghlem et al., 2020).

Forced degradation of phycocyanin in the presence of β-CD

Following the results found, we studied the stability of PC in the presence of β-CD under conditions of forced degradation; the results indicated that the color of phycocyanin was not altered, under the tested experimental conditions, by the light used, under the conditions of the photostability study in the presence of β-CD, the color of the PC did not change, but in the absence of β-CD

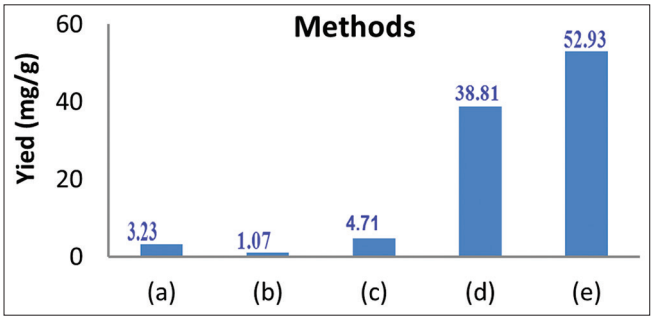

Fig 8. Phycocyanin extraction yield (in mg/g) using different extraction techniques: (a) Water extraction. (b) Extraction by Freezing/Thawing cycles. (c) Ultrasonic assisted extraction. ((d) Extraction by maceration in a mixture ($C_3H_8O_3/H_2O$) 4/6. (e) Extraction by maceration in a mixture ($C_3H_8O_3/H_2O$) 8/2 assisted by ultrasound.

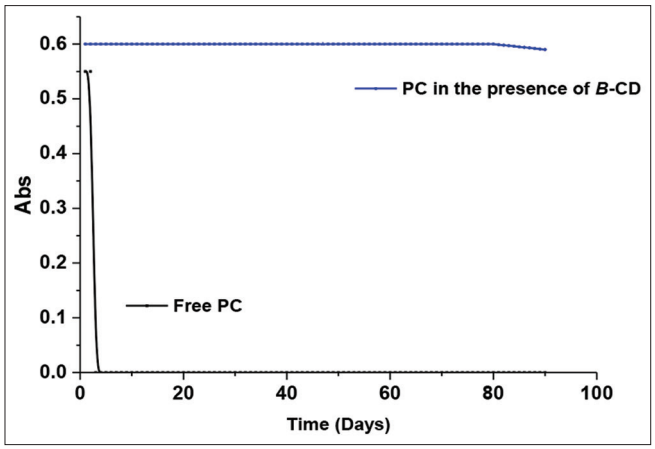

Fig 9. Study of the photostability of a free PC and in the presence of β-CD: Absorbance as a function of time

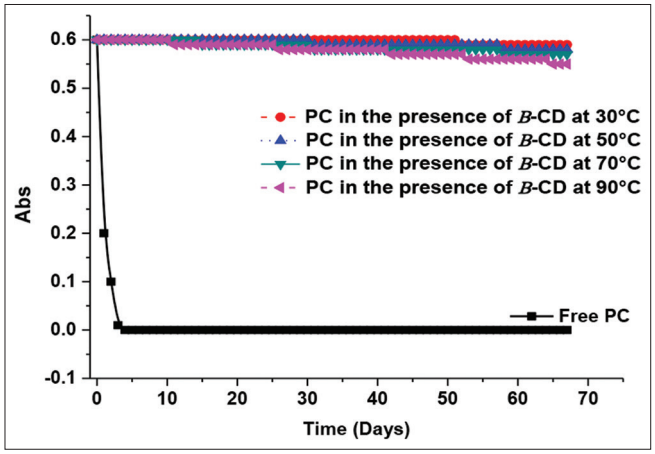

Fig 10. Stability of PC in the presence and/or absence of β-CD at different temperatures: Absorbance as a function of time

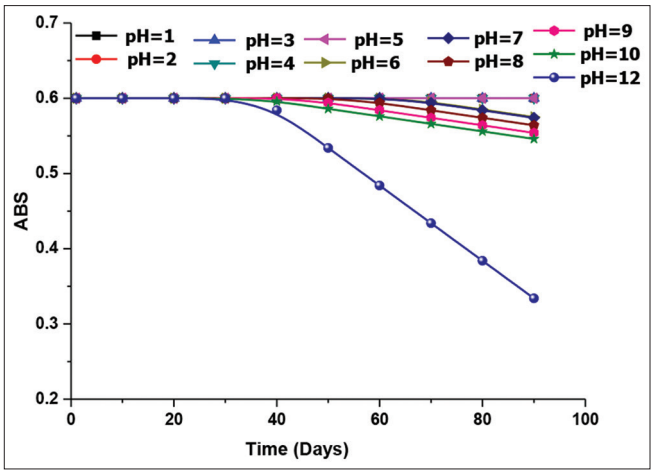

Fig 11. Stability of PC in the presence of β-CD at different pH: Absorbance as a function of time

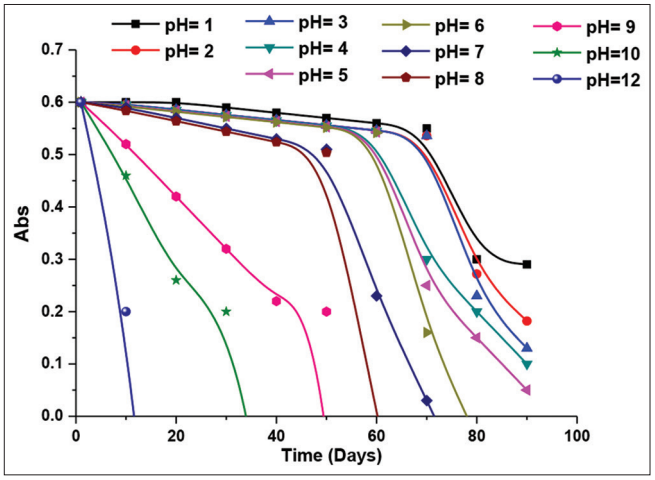

Fig 12. Stability of PC in the absence of β-CD at different pH: Absorbance as a function of time

we noticed a degradation in the second day (Fig. 9). The study of the thermodegradation of PC in the presence and/or absence of β-CD in the range of 30°C to 90°C, is presented in Fig. 10. PC in the absence of β-CD lost its color in the first days. On the other hand, and in the presence of β-CD the color was stable for a long time even at high temperatures. For the study of the degradation in acidic pH of 1 to 8 at room temperature, the PC in the presence of β-CD was stable for a long period (more than 80 days) (Fig. 11), but in the absence of the β-CD, the PC was stable for 40 days that's all. And in the basic range of 8 to 12, it was observed that PC in the absence of β-CD lost its color gradually with the change in pH from 9 to 12 after 40 days (Fig. 12), indicating that it is very unstable at very basic pH. In contrast, when using the PC in the presence of a β-CD in a very basic medium, the color change of the pigment was not observed.

So, we can conclude that there is no change in color, which explains that the PC in the presence of a β-CD is stable in the very basic medium. However, this study showed that the presence of the β-CD regardless of the medium used, and regardless of the temperature, and regardless of the intensity of light, protected the PC against degradation for a long time.

CONCLUSION

The present study has shown that the climate of M'sila region is strongly recommended for the cultivation of *A. platensis*. The results of the physicochemical characteristics of water in the region show that it has the qualities required to cultivate the *A. platensis*, because its natural salinity, sunshine, the temperature of 29.17°C, as well as the naturally basic (pH = 9.55) remain within the optimum growth interval of *A. platensis* and the humidity rate 7.58%, this value is less than 10%, it is a recommended condition for the long-term storage of this microalgae powder. So this region is well placed to harness this solar energy for the cultivation of microalgae. The obtained

yield of the PC was 52.93 mg/g. This value is higher than those reported by literature. The evaluation of different extraction methods has shown that a better extraction yield was achieved by maceration in a mixture (glycerol/water) assisted by ultrasound ([PC] = 3.93 mg/mL and P_{pc} = 0.79). By the proposed extraction method. We have also shown that the concentration, degree of purity, duration and yield of a PC can vary depending on the suitable extraction method. However, we can say that we have succeeded in developing a new extraction method giving the best yield. The study of the stability of PC by the formation of a new inclusion complex (β-CD: PC)1/1 by lyophilization, tests of forced degradation (light, temperature, pH (acid and alkaline)) PC in the presence of β-CD, gave promising results. Up to now, no inclusion complex (β-CD: PC) study has been described in the literature. The complexation via β-CD has made it possible to stabilize the PC with a food quality. This study suggests that the inclusion complex could be considered as an innovation tool for the pharmaceutical formulation optimization since the encapsulation has effectively retained all of the physicochemical properties of PC.

Author's contributions

Zidane, S. Conceptualization, methodology, investigation, writing - original draft, data curation, validation, writing – article and editing, supervision; Bouleghlem, H. Proposal of extraction methods, Investigation, data, validation, writing – participate in the writing of the original draft, revision of the final version; Mohamed Seghir, A. Import *spirulina*, cultivated and controlled the growth of *spirulina*, designed experiments and participated in laboratory work; Benkhirddine, I.B and Ben-Ammar, L. Cultivated and controlled the growth of spirulina, participated in laboratory work. All authors have read and approved the final manuscript.

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