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Original Article

Outdoor Cultivation of *Spirulina platensis* for Mass Production

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Abstract

In the present study, the blue-green alga *Spirulina platensis* (NRC) was used for mass production under outdoor cultivation in three open ponds with a final capacity of 75 m^3 net cultivation volume. Subculturing was performed within sequences and gradual volumes till 1,200 L open plate photobioreactor. The first and second ponds (30 cm depth) were used for the actual continuous production, while the third pond (80 cm depth) was used as a continuous inoculum supplier. In spite of low turbulence of the third pond due to high depth, all ponds had the same mechanical specification concerning paddle wheel structure and turbulence rate (16 rpm). A final nutrient concentration was employed based on Zarrouk medium by commercial grade compounds with some modifications. The nutrition was performed for the third pond by extra supplementation of extra doses of macro and micro-nutrients during the production period and dilution took place when culture was transferred to production ponds (first and second). Each production pond was harvested every 48 hours and the remainder water was return again into the third pond. The harvested pond yielded about 40 kg per day of fresh algal weight containing about 85% moisture on a dry weight basis. The results proved that using urea as nitrogen and carbon source with corn steam liquor instead of sodium nitrate and low bicarbonate, reduces production cost and supports growth medium by an adequate amount of carbon dioxide on the expense of the luxury use of sodium bicarbonate (16.8 $g.l^{-1}$). Chemical analysis of the produced biomass showed 58-62% crude protein, 6-8% of ether extract and 8-11% of total carbohydrates. *S. platensis* contained total essential amino acids (131.3 mg/g), with a predominance of arginine followed by glutamic acid, leucine and phenylalanine.

Keywords: chemical composition, mass production, open ponds, productivity, *Spirulina*

Introduction

The approach of industrial cultivation of algae is to be considered of national importance, as a unique source of bioactive phytonutrients, food additives, fodder, biofuel and other pharmaceutical components. In the last decades, several researches work have revealed that algae are a potential source of new bioactive compounds of pharmaceutical importance including antioxidants, natural pigments and food additives (Talero *et al*., 2015; Yan *et al*., 2016; Chew *et al*., 2017; El-Shouny *et al*., 2017).

Several microalgae species were introduced for mass production, such as biodiesel production (El-Sheekh *et al*., 2013; Abomhra *et al*., 2014; El-Sheekh *et al*., 2017), wastewater treatment (Abou-Shanab *et al*., 2014), fish feeding (El-Sheekh *et al*., 2014), antimicrobial production (El-shouny *et al*., 2016). The commercial-scale culture of microalgae generally requires the ability to economically produce large quantities of algal biomass that requires culture volumes of 10,000 to greater than 1,000,000 liters (Borowitzka, 2005). Almost all commercial-scale culture is currently in open-outdoor ponds including *Spirulina* spp. (Belay, 1997; El Shimi *et al*., 2015; Mobin and Alam, 2017); *Dunaliella* (Schlipalius, 1991; Ben-Amotz 1999; Raja *et al*., 2007; Mobin and Alam, 2017); *Chlorella* spp. (El-Sayed, 1999; El-Sayed *et al*., 2011; Mobin and Alam, 2017; Schreiber *et al*., 2017); *Haematococcus* (Bubrick, 1991; Olaizola, 2000; Li *et al*., 2011; Panis and Carreon, 2016; Mobin and Alam, 2017); *Porphyridium* sp. (Merchuk *et al*., 2000); *Phaeodactylum tricornutum* (Reis *et al*., 1996; Acien Fernandez *et al*., 2003) and *Scenedesmus* sp. (El-Sayed *et al*., 2001; El-Sayed, 2007; El-Sayed *et al*., 2008; Abomohra *et al*., 2014; Dong *et al*., 2016; El-Sheekh *et al*., 2017).

The microalgae are expensive to produce, thus different systems have been designed for the growth and handling of microalgae on a large scale (Gudin and Chaumont, 1980; Weissman *et al*., 1988; Richmond, 2004; Tredici, 2004; El-Sayed, 2007). Within the open systems, the best choice seems to be the open shallow pond, made of leveled raceways 2-10 m wide and 15-30 cm deep, running as simple

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loops or as meandering systems. Each unit covers an area of several hundreds to a few thousand square meters. Turbulence is usually provided by rotating paddle wheels, which create a flow of the algal suspensions along the channels at a rate of 0.2-0.5 $m.s^{-1}$.

The habitat of *Spirulina platensis* ranges from sea, brackish, fresh-water and in some cases lake water. The worldwide production of *Spirulina* has been increasing since 1980; currently, up to 3,000 t have been produced (Spolaore *et al*., 2006).

The adequate supply of carbon dioxide is critical and it is usually controlled through a pH-stat, so warranting both provisions of carbon and optimum pH of the culture simultaneously. Carbon could be assimilated through different chemicals including carbonate and organic acids with a special care to avoid the alkalization of growth media. Urea, a largely used nitrogen source, can also be a proper carbon supplier instead of highly priced nitrogen source like nitrate (El-Sayed *et al*., 2001).

Open pond culturing is cheaper than cultivation in closed photobioreactors, but is limited to a relatively small number of algae species. Furthermore, commercial outdoor cultivation is generally restricted to tropical and subtropical zones in regions of low rainfall and low cloud cover. Although most microalgae require light and carbon dioxide, they are very diverse in their other environmental requirements (Borowitzka, 1999).

Different research studies interpret the algae worldwide production; however, all of the production inputs are available and most of these refer to the nutrition, the knowledge, and the cost. The current work was achieved in order to modify the cultivation technique of *Spirulina platensis* to minimize production costs through decreasing the cultivation period, using low-cost cultivation medium, as well as increasing the harvesting frequency.

Materials and Methods

Alga and growth conditions

Spirulina platensis was isolated from the brackish Egyptian water in Wady El-Natron district, El-Behara Governorate (30.58333°N 30.33333°E). Isolation was performed by dilution technique and the alga was maintained in Zarrouk nutrient medium (Zarrouk, 1966) containing ($g.l^{-1}$) 2.5 $NaNO_3$, 16.8 $NaHCO_3$, 0.5 K_2HPO_4, 1.0 K_2SO_4, 1.0 NaCl, 0.04 $CaCl_2$, 0.08 Na_2EDTA, 0.2$MgSO_4 \cdot 7H_2O$, 0.01$FeSO_4 \cdot 7H_2O$ and 1.0 ml of A5 micronutrient solution.

Inoculum preparation

The inoculum was operated through different growth containers. The first inoculation was done by injection of pure alga into 250 ml washing bottle containing 200 ml of sterilized growth medium aerated by free oil air stream with the continuous illumination of 120 μ*.e*. When indoor-growth reached the maximum, dilution and scaling up of cultivation volume was performed. Laboratory growth was performed using fully transparent Plexi-glass cylinders (100 cm length x 75 mm diameter with 5 mm in thickness) containing 2.5 L of algal broth (Battah *et al*., 2013).

The entire grown alga was then transferred to vertical fully transparent photobioreactor (200 L). Intermediated growth was performed within 1,200 L open plate photobioreactor. The technical specification is listed in Tables 1 and 2, respectively Figs. 1 and 2. Outdoor cultivation started by transferring the alga to the open plate volume of the first open pond.

After 9 days when the first open pond reached 15 m^3 and received inoculum (1,000 L), growth solution height was adjusted at about 10 cm to give about 5 m^3 of growth volume. Dilution by tap water and macronutrients was employed till the full volume pond capacity reached 15m^3 (El-Sayed *et al*., 2001).

The piping system allowed the gravity transfer to the second pond and dilution took place as mentioned before. About 60% of both ponds volume was transferred to the third pond and continuous dilution till 45 m^3 was achieved.

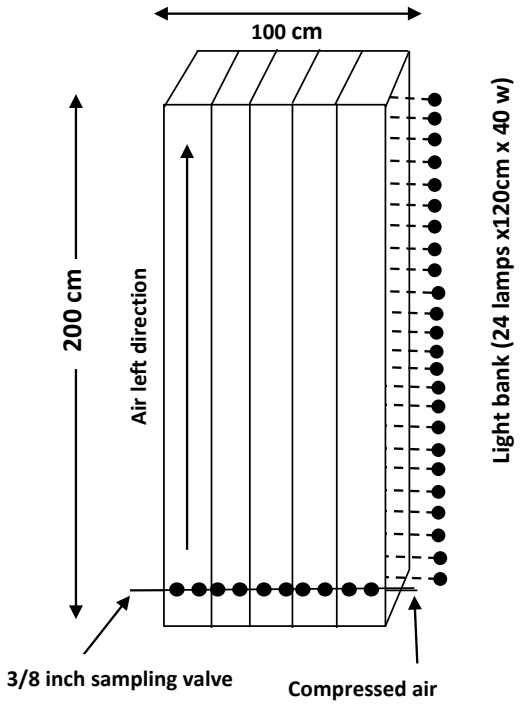

Fig. 1. Open sheet (200 L) photobioreactor

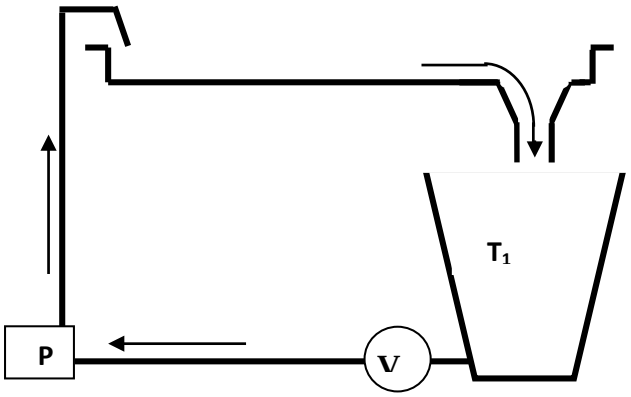

Fig. 2. Open plate (1,000 L) for outdoor sub-culture. T= 1,000 L capacity tank; V= 75 mm valve; P= 2hp electric pump

Table 1. Technical specifications of open sheet (indoor bioreactor)

Item	Specification
Unit height (cm)	200
Unit width (cm)	100
Unit depth (cm)	10 cm
Sheet thickness (mm)	9 mm
Unit volume (L)	200
Unit surface area (cm^2)	$4x10^4$
Wilding material	Epoxy
Needed land length (m)	1.4
Needed land width (m)	0.6
Needed total area (m^2)	0.84

Table 2. Technical specifications of open plate unit

Item	Specification
Plate area (m^2)	7.42
Tank surface (m^2)	1.36
Out let (cm)	0.63 diameter
exposed area (m^2)	8.78
Pump power (hp)	2.0
Flow rate ($m^3.h^{-1}$)	60.0
Pip system (mm)	75
Suspension height (cm)	5-10

Outdoor nutrition

Based on Zarrouk medium, a nutritional modification was done to meet the minimization of production cost during the outdoor stage. Outdoor nutrition was employed using urea as a nitrogen source instead of sodium nitrate at a final concentration of 1.8 $g.l^{-1}$ that represented two-fold of nitrogen content of sodium nitrate, aiming to increase the carbon feeding. In addition, corn steam liqueur was added at 0.7 $g.l^{-1}$ to increase organic carbon content with low $NaHCO_3$ use (El-Sayed *et al*., 2015). Phosphorus was supplemented from 0.2 $ml.l^{-1}$ of commercial phosphoric acid (84% P_2O_5), while potassium was added as 1.0 $g.l^{-1}$ of a commercial potassium sulfate (52% K_2O). Sodium chloride at 1.0 $g.l^{-1}$ was added. Sodium bicarbonate was minimized to be 5.0 g $.l^{-1}$ from commercial grade with 0.025 $g.l^{-1}$ ferrous sulfate. Re-cycling water after harvesting minimized some of the required nutrients mainly sodium chloride. Acid reaction was adjusted using commercial potassium hydroxide.

Pond structure and culture technique

Pond structure determines the net produced mass as tested, in this case with both *Scenedesmus* sp. (El-Sayed, 2004) and *Spirulina platensis*. Old pond structure which consisted of three open ponds with 15 m^3 per each (El-Sayed, 2007) allowed the weekly harvesting, one time of each pond.

Routine work (NRC) was run by weekly harvesting of one pond to obtain about 15 kg of fresh biomass. The current structure allowed the daily harvesting of at least one pond (15 m^3). Net obtained biomass was found to be depending on the growth potential and the obtained dry weight per liter ($g.l^{-1}$). Here, the third pond height was raised to be 80 cm highest. As the first pond was obtained, the underground piping system allowed the re-completing of culture volume by opening the connection valves tubing pipes pots phenomenon. By the next day, the second pond was harvested by the same technique. The drain of every batch was re-cycled again to the third pond (80 cm depth).

As the first pond reached their maximum growth (Ca; 1.0 $g.l^{-1}$), a 75 mm of polyethylene valve was opened to transfer half of the pond volume into the second pond. Dilution was performed to complete the third pond which contained 45 m^3 of culture volume. The remainder water of the first pond harvesting was immediately turned from centrifuge pipe to the third pond. Then, the first pond was re-filled by a new inoculum from the third pond to re-grow again.

By the second day, all of this procedure was repeated with the second pond. Routine processes were followed day by day and the harvesting frequency becomes conjugated for each pond. The third pond actually acted as a large and continuous inoculum supplier. Most of the afore-mentioned transfer processes were energy free, using gravity and tubing pipes pots.

Paddle wheel and power stirring

A new shape of the paddle was performed to enhance the turbulence, maintenance and power consumption. As shown in Fig. 3, the minimum weight of stirring wheal was put in consideration. Thus, most of the paddle parts were made from hard aluminium plate joint by stainless steel pins screw. A 0.75 hp gear motor with 16 rpm was used to perform the turbulence instead of 1.5 hp.

Harvesting and water recycling

Methods of algal cultures harvesting widely varied due to cultivation method and cultivated strain. Isolation by gravity methods is universally done in the presence of fluctuation agents like aluminium sulfate which changes the media reaction, especially with *Spirulina* cultures. In the present case, the addition of ferrous sulfate led to increasing of colony size, which in turn increased the separating efficiency.

One worldwide harvesting method is to use the continuous separating apparatus namely self-cleaning. In the present study, continuous harvesting was done (Westevalia Separator centrifuge 15,000 $l.h^{-1}$). The algal slurry was pumped into the reservoir tank to allow the continuous operation through the 75 mm plastic valve. Drain water was re-cycled to the large pond 45 m^3.

Pond maintenance

Pond maintenance included growth volume, daily cleaning, turbulence and nutrition. Growth volume was adjusted at the first time of inoculation to be equivalent to growth rate, by dilution with tap water and proper nutrients. Completing growth volume to the pond full capacity was done as mentioned before. The main consideration for pond maintenance was to clean the pond margins, that was proposed by high growth rate to prevent the growth of larva which may destroy culture.

Table 3. Technical specifications of different open ponds used

Item	Specification
Unit depth (P1&P2)	30 cm
Unit depth (P3)	80 cm
Unit length	30 m
Unit width	2.5 m
Unit volume (P1&P2)	15 m^3
Unit volume (P3)	45 m^3
Surface area	75 m^2
Gear motor	0.6hp

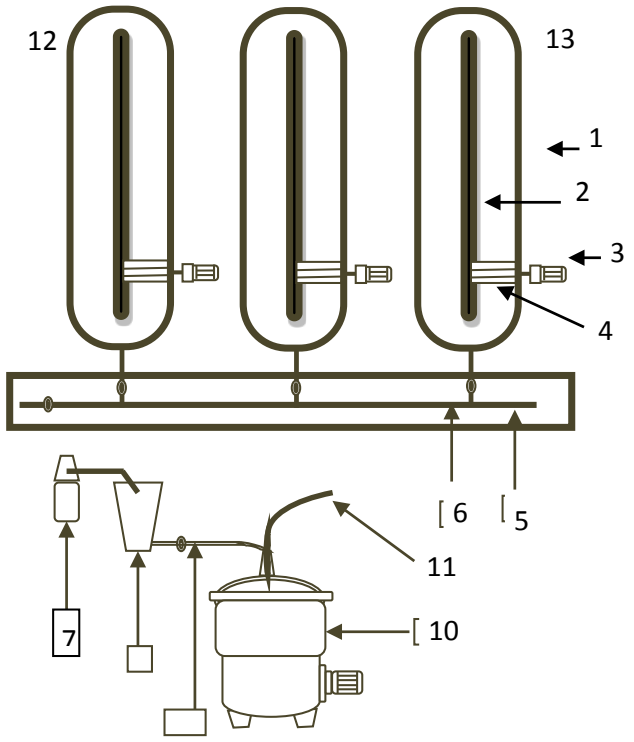

Fig. 3. Unit structure and process of *Spirulina platensis* production (NRC-Egypt). 1- Outer pond wall, 2- Middle separator wall, 3- Gear Motor, 4- Paddle wheal 5- Piping tunnel. 6- 75 mm Pipe, 7- Electric pump, 8- 500 L tank, 9- Feeding pipe, 10- Continuous centrifuge, 11- Drain way to the third pond (to recycle), 12- Pond 3 with 45 m^3 growth volume, 13- Pond with 15 m^3 growth volume

Results and Discussion

Effect of growth unit

Variable growth patterns were observed as *Spirulina* was cultivated within different growth units. The differences in growth yield could be attributed to the efficiency of light harvesting. Plexi-glass cylinders (2.5 L) represented the most efficient growth, where 1.44 g/l.d was obtained by the day 12. Meanwhile, the obtained yield was found to be inverse to the unite volume, where 1.39, 1.21 and 1.09 g/l.d were obtained with an open sheet (200 L, open plate (1,200 L) and open pond (15,000 L) respectively (Fig. 4). Otherwise, surface area exposed to light was the major effective factor in light harvesting, besides agitation. Air agitation seemed to be more efficient. These results are in agreement with that obtained by Ravelonandro *et al*. (2011) who found that agitation of the culture, medium salinity (ranging from 13 to 35 g L^{-1}) and CO_2 addition (ranging from 0 to 2%, v/v) enhanced the growth and protein content of *Spirulina platensis*.

The differences on the obtained yield could be imputed to the relation between both growth surface area and growth volume (Table 4). The effect of growth unit in concern technical proprieties including shape and volume on algal growth yield was early described.

The high variation between indoor growth units (Plexi-glass cylinders and open sheet), in concern with volume and growth area (Table 4) seemed to be ineffective due to the long growth column of the open sheet (2.0 m)with high aeration and light bank.

Open plat photobioreactor was the most proper growth unit with sufficient growth biomass, but highly consumed power (2.0 hp). In this regard, El-Sayed (2007) reported that mineral nutrition and electric power including light, aeration, agitation, pumping and harvesting represented the main category of algal cost production. The maximum power consumption is very closely related to culture volume especially during indoor growth and inoculum preparation.

Effect of pond structure

The growth of the first and second ponds possessed the normal vegetative growth even in a short growth period (48 hours) and growth was sharply increased after each transferring and dilution process. On the contrary, the third pond represented a low growth rate during the whole production period that took a long time (6 months). The

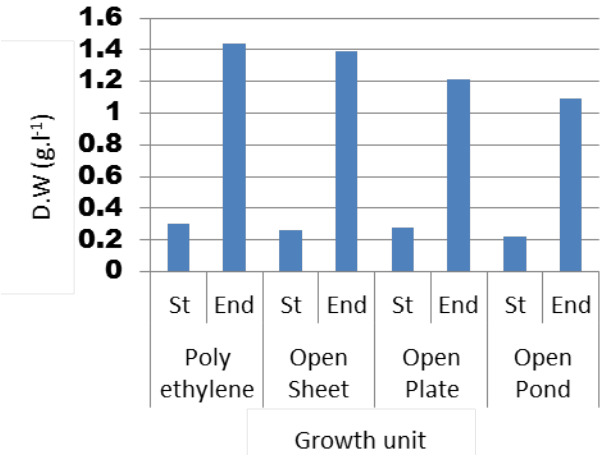

Fig. 4. Dry weight (g.l^{-1}) of *Spirulina platensis* grown within different growth units

Table 4. Technical specifications of different growth units affecting growth yield of *Spirulina platensiss*

	PE	OSP	OPP	OP
G.V (L)	2.5	200	1,200	15,000
G.A (M^2)	0.0056	4.4	9.53	75
Ratio	446.63	45.5	125.92	200
Y (g)	1.44	1.39	1.21	1.09
U.Y (g)	2.88	278	1210	16350

PE =polyethylene; OSP=open sheet photobioreactor; OPP= open plate photobioreactor and OP = open pond; G.V (L)= growth volume; G.A (M2)= growth area; Y (g)= yield per gram and U.Y (g)= unit yield per gram

lack of growth within the third pond could be referred to its structure in concern pond height (80 cm). Increasing the pond height altered algal growth due to the altering of light penetration that reduced the photosynthetic activity. The poor growth within this pond and surviving could be attributed to the rough painting of pond that enhances immobilization (El-Sayed, 2010) and rough scrubber (Craggs *et al*., 1995) phenomenon. Wang (1974) stated that high turbidity conditions retard algal growth, a light-inhibition effect. In some cases, however, the particulate matter appeared to stimulate algal growth. Former growth within the equal pond volume represented different growth pattern. Increasing one pond height to 80 cm led to massive yield increases and about 40 kg of fresh biomass were daily obtained. Thus, growth yield differed when calculated as yield per gram to surface area. Comparing with traditional pond use, increasing pond height led to increasing the harvesting frequency by 30 times per month instead of 12-15 times. The third pond deep with low turbulence only allowed the movement of daughter cells with low shape and weight, while mother cells that had larger cell size and high weight due to cell division inhibition settled down. When pond volume was transferred to the harvested pond, gravity obligated mother cells that deeply settled, to move quickly to the empty pond. Then, diluted medium allowed the fast cell division and fast growth.

Nutrition

Nutrition plays two important roles in cell multiplication and biomass production. The first role is the routine nutritional regime that allows the proper growth based on the cultivated algae. The current nutritional regime was carried out through the application of commercial fertilizers containing NPK plus micronutrients (El-Sayed *et al*., 2001). Using of urea as a nitrogen and carbon source with corn steam liquor instead of sodium nitrate and low bicarbonate, reduces production cost and supports growth medium by an adequate amount of carbon dioxide on the expense of the luxury use of sodium bicarbonate (16.8 $g.l^{-1}$).

These results are in agreement with those obtained by Soni *et al*. (2017) who stated that *Spirulina* grown under urea and cheap carbon and nitrogen sources cultivated in an open raceway pond gave a high biomass and protein content. The second reason in this topic is the effect of hyper nutritional doses given to the third pond. Hypersalinity altered algal growth through the retarding of cell division and cells become larger in their shape. In disagreement with the current results, Ravelonandro *et al*. (2011) found that *Arthrospira* (*Spirulina*) *platensis* showed higher specific growth rate (μmax) and protein content for lower salinity.

As alga was transferred to the production pond, dilution and proper turbulence due to less height (30 cm) increased the illumination and photosynthetic efficiency. In addition, dilution decreased the hypersalinity effect and self-shading of algal cells. *Spirulina* was early classified as brackish alga; accordingly, it has a wide range of salinity in which making the hyper salinization of the incubation pond nearly to be safe.

Water recycling

The remainder water after pond harvesting was discarded by the old and routine cultivation (NRC); such water was return again to the third pond. The initial of this water contained some algal cells and different biologically active growth promoters, including amino and organic-acids, phytohormones, nutrients and other cell components excreted through cell division or dying cell.

Chemical composition

Chemical analysis of mass-produced *Spirulina platensis* are shown in Table (5) and are relatively in agreement with the commonly listed analysis, where Richnond (1988) mentioned that *Spirulina* composed of 50-70% protein and about 15% of carbohydrates. Furthermore, Hassan *et al.* (2015) reported that *Spirulina* protein ranged from 36.6 to 51.65% due to growth conditions. In addition, *Spirulina* stress and vegetative had the highest values of ash, fat and fiber (12.08 and 10.4%, 12.8 and 6.9%, and 6.3 and 5.4%, respectively).

Lupatani *et al*. (2017) reported that the protein content of *Spirulina* varies between 460–630 g kg^{-1}, dry matter basis, depending on growing conditions. These levels are quite exceptional, even among microorganisms. Ali and Saleh (2012) stated that *Spirulina* chemical composition includes proteins (50-70%) and carbohydrates (15-25%).

The results showed that vegetative *S. platensis* contained lightly higher total essential amino acids (131.3 mg/g), compared with *S. platensis* stress (107.8 mg/g). Arginine was the most common aminoacid of both, the dry matter of *S. platensis* (vegetative and stress) followed by glutamic acid. Leucine was the most abundant essential aminoacid for both grown algae as indicated in Table 2, followed by phenylalanine. *S. platensis* (vegetative and stress) is rich in unsaturated fatty acids. Long-chain and poly-unsaturated fatty acids represent the maximum of stress grown algae, compared with those grown under vegetative growth conditions, making it for a future use in the food industry and functional foods. The important fatty acids like linoleic acid and linolenic acid are present in dry biomass of *Spirulina* as 19.2 and 22.3%, respectively, for stress grown material (Hassan *et al*., 2015).

Productivity and annual yield

When the first pond reached the maximum growth (approximately 1.0 $g.l^{-1}$), harvesting was performed by continuous centrifugation. Dry weight, pigments content and other biochemical analysis including crude protein, lipid content, total carbohydrates and minerals were determined. In agreement with the hereby results, Benavides *et al*. (2017) obtained the highest biomass productivity of *Spirulina* over 20 $g\,m^{-2}\,d^{-1}$ in a thin-layer cascade (TLC) at the optimal temperature.

Table 5. Chemical composition of the outdoor produced *Spirulina platensis*

C.P	E.E	T.C	Chl a	Car.	S.C	Phyc	Fibers	Ash	Mois
58.4	6.3	10.4	1.2	0.14	0.08	2.3	6.02	8.9	7.2

C.P= crude protein; E.E= ether extract; T.C= total carbohydrate; Chl a= chlorphyll a; Car.= carotenes; S.C=secondary carotenoids; Phyc= phycocyanine and Mois= misture

Per one pond with 15 m^3 capacity, the obtained biomass was 50 kg fresh weight every 48 hours. Regarding the moisture content (95%), the net dried biomass could be accounted as 2.0 kg (on the average per day). Thus the estimated yield per the current cultivated area (225 m^2) was in approximately 1, 200 kg/year. In mass algae cultivation, the actual cultivated area is 3,800 m^2 of each feddan. Remarkably, the expected yield per feddan is not less than 20 tons per year. In a 10 month industrial trial in 450 m^2 ponds, Jiménez *et al*. (2003) obtained a production of *Spirulina* equivalent to 30-32 metric tons of dry powder per hectare per annum.

Conclusions

In spite of nutritional aspects that determine the production potential of different algae species, supplying the production ponds by a ready-made inoculum with proper technical aspects of turbulence and dilution could increase the production yield of open pond grown algae.

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