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Rational design of a culture medium for the intensification of lipid storage in *Chlorella* sp. Performance evaluation in air-lift bioreactor

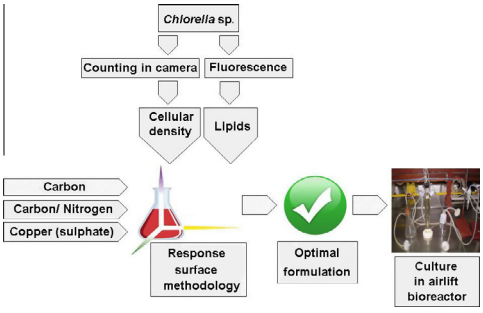
Pablo C. Giordano [a,b], Alejandro J. Beccaria [b,*], Héctor C. Goicoechea [a,*]

[a] Laboratorio de Desarrollo Analítico y Quimiometría (LADAQ), Cátedra de Química Analítica I, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, CC 242 (3000), Santa Fe, Argentina
[b] Laboratorio de Fermentaciones (LFF), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, CC 242 (3000), Santa Fe, Argentina

HIGHLIGHTS

- Better results were obtained in the factors selection stage by GA.
- RSM allowed obtaining reliable models that could fit the responses.
- The composition of a medium to maximize lipid storage in *Chlorella* sp. was defined.
- Enzymatic hydrolysate improved lipid storage in *Chlorella* sp.
- Culture in an air-lift bioreactor is viable.

GRAPHICAL ABSTRACT



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ABSTRACT

An optimal medium to culture *Chlorella* sp., microalgae capable of storage intracellular lipids was obtained. This culture medium consists of a saline base plus carbon-energy and nitrogen sources. Significant factors exerting influence on the culture parameters were selected. Then, by applying response surface methodology coupled to desirability function, an optimal formulation, specific for the heterotrophic growth of *Chlorella* sp. that allows maximizing lipid concentration was obtained. During the experimental verification, the possibility of replacing commercial glucose by hydrolysates obtained from lignocellulosic materials was evaluated. Biochemical hydrolysate of corn bran allowed obtaining important improvements in lipid concentration. Finally, the optimal formulation was evaluated in an air-lift bioreactor performing a fed-batch culture. Culturing the strain in these conditions allowed rising lipid concentrations.

1. Introduction

Biodiesel is chemically formed by the transesterification of short-chain fatty acids with short chain monoalcohols like methanol or ethanol. Fatty acids are obtained, mainly from vegetable oils and animal fats, which constitute the raw material for the production of this biofuel. Biodiesel has many advantages such as renewability, biodegradability, non toxicity, zero net emission of carbon dioxide or sulphur to the atmosphere (Vicente et al., 2004).

In the biodiesel production processes, raw material is represented mainly by oleaginous vegetable crops, which contribute to the ecological consolidation of this biofuel since plants fix atmospheric carbon dioxide in order to use it as a nutrient. However, there are many problems related to the distraction of nutritious resources and the impoverishment of farmlands.

* Corresponding authors. Address: Laboratorio de Desarrollo Analítico y Quimiometría, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Paraje "El Pozo", Ruta Nacional N° 168 S/N, S3000ZAA Santa Fe, Argentina. Tel.: +54 342 4575206x190; fax: +54 342 4575 221.
E-mail addresses: abeccari@fbcb.unl.edu.ar (A.J. Beccaria), hgoico@fbcb.unl.edu.ar (H.C. Goicoechea).

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One alternative to the use of oleaginous vegetables for the production of biodiesel is the employment of oleaginous microorganisms, like microalgae, which have high biomass production, high photosynthetic efficiency, fast grow, capacity to adapt to different environments, and do not require farmlands, in contrast with different oleaginous vegetable crops.

Chlorella is a microalgae gender which can grow photoautotrophically, mixotrophically or heterotrophically, and its fatty acid profile, like other microalgae, is suitable for biodiesel production (Feng et al., 2011). Photoautotrophic growth is considered as the most desirable method for growing microalgae, in which the sunlight is employed as an always available and free energy source (Azma et al., 2011; Bellou and Aggelis, 2012). Moreover, the photosynthetic efficiency of microalgae is substantially higher than that of plants (Bitaubé Pérez et al., 2008). However, autotrophic growth presents two main problems: the first one is the light supply, since as the culture gets denser, light penetration reduces drastically, lowering biomass productivity and lipid content; and the second one are the challenges in the design of culture systems (photobioreactors), which has not been resolved until now in order to reach commercial productions in the biofuels area (Cheng et al., 2009).

In comparison with the photoautotrophic culture, the heterotrophic one allows the microalgae to accumulate a higher proportion of lipids in less time, which makes the scale up easier. Interestingly, the intracellular lipid production is critically affected by the carbon/nitrogen ratio (Cheng et al., 2009).

Regarding the process economy, the carbon source represents the 80% of the total cost of the culture medium (Cheng et al., 2009). There are two main tendencies to reduce the cost that implies the formulation of a heterotrophic culture: the first one is the replacement of glucose for cheaper carbon sources, which may be obtained from waste materials, produced in large quantities by diverse industries all around the world. The second tendency is the use of mixotrophic cultures, in which photosynthesis is the main energy source, but both an organic compound and carbon dioxide are essential. There is a subtype of mixotrophy called *anphitrophy* in which organisms are capable of living autotrophically or heterotrophically, depending on the concentration of the organic compounds and the available light intensity (Mata et al., 2010). These strategies would allow biodiesel to rise as a renewable energy source capable of competing economically with fossil diesel.

In the present study, the composition of a culture medium for *Chlorella* sp. was defined. Its composition was based upon the Bold 3 N medium. Taking into account that this medium is specific for the growth of *Chlorella* in autotrophy, an organic carbon source was added to the original formulation.

The first step consisted in evaluating the influence of different factors on biomass growth and lipid accumulation through the application of a Plackett–Burman design and a genetic algorithm based routine (Giordano et al., 2011). After, in the optimization step, by the application of response surface methodology, it was possible to obtain a culture medium formulation that guarantees the maximization of lipid accumulation.

Later, the culture medium formulation was evaluated by replacing glucose for carbon sources obtained from the hydrolysis of waste materials which were studied in a previous published work (Giordano et al., 2013), obtaining significant rises in lipid accumulation, regarding the formulation containing glucose, fact that confirms the viability of replacing traditional carbon sources by others that are not conventional and have low cost.

Finally, the obtained formulation was evaluated in an air-lift bioreactor obtaining remarkable results. The cost of manufacture is one of the major bottlenecks in the large scale biofuels production (Meng et al., 2009). Liang (2013) identified the carbon-energy sources and bioreactor design as two of the major areas linked to microalgae biofuel cost. In air-lift bioreactors, the power requirement comes only from the air supply with a low energy input compared to the traditional stirred tank bioreactors.

2. Methods

2.1. Microalgae strain

Chlorella sp. (UTEX 1822) strain was acquired from the *Culture Collection of Algae*, Texas University (Austin, Texas, EE.UU.). The strain was grew autotrophically and axenically in Bold 3 N medium and stored at 20 °C in a growth chamber with photoperiod (13.7 $\mu Em^{-2}\,s^{-1}$ of luminic intensity) of 18 h of light: 6 h of darkness, renewing the culture each 15 days.

2.2. Reagents and culture media design

To evaluate the influence of different factors in lipid accumulation, a Plackett–Burman design (PB) was built. After that, and in order to obtain an optimal combination of the most important factors, a central composite design (CCD) was constructed. The culture media combinations suggested by these designs were obtained combining different volumes of the corresponding stock solutions and then, adding distilled water until a volume of 10 mL was reached. The pH was adjusted at 6.2 adding NaOH 1 mol L^{-1} or HCl 1 mol L^{-1}, when it was necessary. Then, the media were sterilized at 121 °C for 15 min.

All culture media suggested by the mentioned experimental designs were composed of a saline base similar to that of the Bold 3 N medium (in g L^{-1}): $CaCl_2.2H_2O$ 0.025, $MgSO_4.7H_2O$ 0.074, K_2HPO_4 0.075, KH_2PO_4 0.175, NaCl 0.025 and 6 mL^{-1} of trace metals solution, whose composition was (in g L^{-1}): $Na_2EDTA.2H_2O$ 0.750, $FeCl_3.6H_2O$ 0.097, $MnCl_2.4H_2O$ 0.041, $CoCl_2.6H_2O$, 0.002 and $Na_2MoO_4.2H_2O$ 0.004. With the aim of fulfil the formulations suggested by the experimental designs, $ZnSO_4$, $CuSO_4$, carbon source (glycerol or glucose) and nitrogen source (KNO_3 or yeast extract) were added to the saline base. All reagent solutions were prepared by dissolving the corresponding solids in distilled water, with the exception of glycerol, which was prepared diluting a volume of it in distilled water.

2.3. Inoculum preparation and culture conditions

Inoculum culture consisted in 9 mL of sterile Bold 3 N medium and 1 mL of microalgae culture grown at 20 °C, all contained in a 50 mL *T* flask. After 10 days of incubation at 20 °C, Erlenmeyer flasks containing each 10 mL of culture medium corresponding to the combinations suggested by PB or CCD, were inoculated with an aliquot obtained from the inoculum culture so as to obtain an initial density of 5×10^5 or 1×10^6 cells mL^{-1}, according to the designs. Then, all cultures were incubated at 30 °C, in static or agitated (150 rpm) conditions, according to the designs. The culturing period lasted 10 days.

In a further step, cultivation in an air-lift bioreactor was performed. The external tube was set as the downcomer, with a diameter of 3.8 and 35.5 cm height; the internal tube was set as the riser, with a diameter of 2.8 and 21.0 cm height; the sparger was set at 2.5 cm from the reactor base. The bioreactor contained 300 mL of culture medium (liquid height: 29 cm); at days 5 and 13, 25 and 40 mL of fresh culture medium were fed, respectively. Oxygen was supplied through a rising air flow, which was initiated at 0.33 vvm and reached to 0.66 vvm. Besides the sparger port, the bioreactor had three other ports. One of them was the air outlet, which was bubbled in a 5% V/V bleach solution; the second one was the inoculation-feeding port and the last one, the sampling

port. Temperature was controlled at 26 ± 2 °C. The culture lasted 15 days and samples were collected each 2 days to determine glucose and lipid concentrations, and cellular density. The complete system employed, including the air-lift bioreactor to culture the microalgae consisted of a recipient containing bleach solution, air-lift bioreactor, feeding and sampling systems. The bioreactor was covered with aluminium foil to achieve the darkness for heterotrophic culture condition.

In a previous stage, before the culture, the bioreactor was characterized determining mixing time (t_m) and the volumetric mass transfer coefficient ($K_L.a$) at flow speeds from 50 to 400 mL min^{-1}. For the determination of $K_L.a$, the gassing-out method was employed (Wise, 1951), which consists in determining the dissolved oxygen concentration present in the liquid during aeration through the sparger as a function of the time, after degassing it with a nitrogen flow. A sensor for dissolved oxygen (Hamilton, Bonadz, Swiss) was employed for this determination.

Eq. (1) is a mathematical representation of oxygen transfer rate:

$$\mathrm{OTR} = dC/dt = K_L.a \times (C^* - C) \quad (1)$$

where OTR stands for oxygen transfer rate from gas phase to liquid phase; K_L is the mass transfer coefficient; a represents the interfacial area; C^* is the saturating oxygen concentration present in the liquid phase in equilibrium with gas phase; and C is the oxygen concentration present in the liquid at a determined moment.

Eq. (2) is obtained by solving Eq. (1):

$$\ln[(C^* - C)/(C^* - C_0)] = -K_L.a \times t \quad (2)$$

Then, when plotting $\ln[(C^* - C)/(C^* - C_0)]$ as a function of time (t), a straight line is obtained which slope is $K_L.a$.

In what respects to mixing time, it was determined filling the bioreactor with H_2SO_4 6 mM (pH of 2.2). Once the aeration was initiated, NaOH 2 M (2.4 mL) was added so as to raise the pH up to a value near to 12.2. Alkali injection times were of 1 s. Mixing time was defined as the time employed by the system to raise pH from 2.2 up to 12.2.

2.4. Analytical

Once the culture period was concluded, biomass was spectrophotometrically quantitated by determining optical density at 682 nm ($OD_{682\ nm}$). After, the value obtained was correlated with a cellular density value through the construction of a calibration curve.

In a further step, lipid concentration was fluorimetrically obtained by employing Nile Red dye. This dye operates as a liposoluble fluorescent probe because when it interacts with hydrophobic molecules (lipids, lipoproteins or proteinic hydrophobic domains), characteristic excitation and emission spectra are obtained (Chen et al., 2011).

The protocol for the dosage of lipid concentration consisted in adding 10 μL of dye solution (1 μg mL^{-1}) to 700 μL of cellular suspension, and after 2 min the emission was registered. The excitation and emission wavelengths were 530 and 586 nm, respectively. The emission intensity of a blank test was subtracted to each sample fluorescence intensity value obtained. All determinations were done by triplicate. Sunflower oil diluted in a water: isopropanol 80:20 V/V was used as a standard in order to build a calibration curve (range of linearity: 0–1000 ppm) which allows to relate lipid concentration and fluorescence intensity.

Glucose concentration was determined with a HPLC (Shimadzu, Kyoto, Japan) equipped with a refraction index detector RID-10A (Shimadzu, Kyoto, Japan) and an Aminex HPX-87C (250 × 4 mm) (Bio-Rad, Hercules, CA, EE.UU.) column operating at 67 °C with ultrapure water as mobile phase, flow speed was 0.5 mL min^{-1}. Injection volume was 20 μL, and both samples and glucose standards (prepared *in situ* at the moment of performing the analysis) were filtered through 0.45 μm membranes (Millipore, Billerica, USA). This methodology was optimized and carried out at the Laboratorio de Microbiología, Facultad de Química de la Universidad de la República, Montevideo, Uruguay.

Specific growth rate (μ) corresponding to the exponential growth phase was calculated according to Eq. (3):

$$\mu = \frac{(\ln X_2 - \ln X_1)}{(t_2 - t_1)} \quad (3)$$

where X_2 and X_1 are cellular densities (cells mL^{-1}) obtained at times t_2 y t_1, respectively.

With the aim of determining cell specific oxygen uptake (qO_2) corresponding to *Chlorella* sp. strain employed in this study, such strain was heterotrophically cultured in the optimal culture formulation for 72 h. After, oxygen concentration present in the culture (isolated from the outer atmosphere) was determined at regular periods of time. Then, a curve to relate oxygen concentration and time was built, and the curve slope (Q) was obtained. qO_2 was calculated according to Eq. (4):

$$qO_2 = 0.079\,Q\,X^{-1} \quad (4)$$

where X is the cell concentration (cells L^{-1}) employed and 0.079 (ppm%$^{-1}$) a conversion factor. This factor was obtained from Eq. (5) (Pirt, 1975):

$$C^* = 14.16 - 0.3943\,T + 0.007714\,T^2 - 0.0000646\,T^3 \quad (5)$$

where C^* is the dissolved oxygen concentration at saturation conditions, expressed as ppm, and T is temperature expressed in degrees Celsius (26.7 °C in this study).

For the days in which sampling was done, corresponding dissolved oxygen concentration present in the air-lift reactor was calculated by employing Eq. (6):

$$C = C^* - \left(\frac{qO_2 X_t}{K_L.a}\right) \quad (6)$$

where qO_2 is the oxygen consumption specific speed and X_t is the cellular density at t time.

3. Results and discussion

3.1. Factors screening phase

In order to evaluate the incidence of different factors on lipid accumulation by *Chlorella* sp. cells, a PB consisting in 12 experiments was built. Classical analysis and that based on genetic algorithms were implemented once the values of the responses were obtained: lipid concentration (L) and cellular density (X) (Giordano et al., 2011).

The significance of eight different factors: initial cellular density (X_0), stirring (S), nitrogen source type (NS), carbon source type (CS), carbon source/nitrogen source ratio (CN) (grams of carbon source/grams of nitrogen source), and carbon source (CC), $ZnSO_4$ (Zn) and $CuSO_4$ (Cu) concentrations, was evaluated. Table 1 shows the twelve experiments corresponding to PB and the obtained values for L and X.

The number of experiments given by a PB is not enough to solve the complete system of equations corresponding to more than four factors. Then, the classical analysis may estimate the parameters corresponding to main factors which may be confounded with the terms corresponding to factors interactions. This fact could be translated in potentially incorrect results concerning coefficients significance. For details of this problem see the work published by Giordano et al., 2011.

Table 1
Plackett–Burman design built to evaluate the factors significance in *Chlorella* sp. cultures.

Run	Factors								Responses	
	X_0 ($\times 10^6$ cells mL^{-1})	S	NS[a]	CS[b]	CN	CC (g L^{-1})	Zn (mg L^{-1})	Cu (mg L^{-1})	L (ppm)	X ($\times 10^6$ cells mL^{-1})
1	0.50	0	YE	Glc	10.00	5.00	0.20	0.10	15.00	10.25
2	1.00	150	PN	Glc	10.00	5.00	1.00	0.10	30.55	64.38
3	0.50	0	PN	Glc	20.00	25.00	0.20	0.30	33.18	13.80
4	1.00	150	YE	Gro	20.00	25.00	0.20	0.10	17.91	2.89
5	0.50	150	PN	Glc	20.00	25.00	1.00	0.10	34.37	20.95
6	1.00	0	PN	Gro	10.00	25.00	1.00	0.30	15.40	1.04
7	1.00	150	YE	Glc	10.00	25.00	0.20	0.30	16.92	0.80
8	0.50	150	PN	Gro	10.00	5.00	0.20	0.30	11.47	0.33
9	1.00	0	PN	Gro	20.00	5.00	0.20	0.10	10.69	0.87
10	0.50	150	YE	Gro	20.00	5.00	1.00	0.30	9.59	1.16
11	1.00	0	YE	Glc	20.00	5.00	1.00	0.30	14.69	11.68
12	0.50	0	YE	Gro	10.00	25.00	1.00	0.10	10.41	1.81

[a] YE: yeast extract, PN: potassium nitrate.
[b] Glc: glucose, Gro: glycerol.

When applying quadratic least squares (QLS), the models terms were obtained, and then the root mean square error for QLS analysis (RMSE0) was calculated for each of the responses. In the case of the analysis based in genetic algorithms (GA), an initial population was built, root mean square error for GA analysis (RMSE) for each response was calculated, models were hierarchisized and the best of them was obtained. Finally, the values of determination coefficients (R^2) were compared so as to decide which of both analyses originated a model which better explains the variability in the responses.

Following, it is described and compared data obtained by applying both classical analysis and that based upon GA in what refers to the significance of the studied factors.

Regarding to X response, by applying classical analysis, only CS was significant with a p-value of 0.066 exerting a negative effect. R^2 for this model resulted to be 0.298, indicating a very poor fit. However, by GA based analysis, three factors and one interaction were significant: NS ($p = 0.049$), CS ($p = 0.042$) and Cu ($p = 0.046$) had a negative effect over the response while the interaction CS/CC ($p = 0.089$) evidenced a contrary effect. R^2 was 0.876, then an 88% of system variability could be explained by the model, representing an improvement of 193.9% compared to the QLS model.

Turning the attention to L response, through classical analysis, NS ($p = 0.014$), CS ($p = 0.003$) and CC ($p = 0.059$) were found as significant factors. Both NS and CC exerted a positive effect over the response, while CS generated the opposite effect. The R^2 corresponding to the model was 0.801, representing a very good fit of the response.

On the other hand, when the GA based analysis was applied to the data corresponding to L response, NS (negative effect), CS (positive effect) and CC (positive effect) resulted to be significant with p-values of 1.6×10^{-4}, 2×10^{-5} and 0.008, respectively. Moreover, there were two interactions which also were significant: NS/CS ($p = 6.6 \times 10^{-4}$) and CN/CC ($p = 0.077$), exerting negative and positive effects over the response, respectively. The value of R^2 corresponding to the model resulted to be 0.978, improving 22.1% the response fit, in comparison with the classical analysis.

Consequently, if factors selection would have been done only by means of the classical analysis, Cu would have not been considered as significant. Furthermore, in the case of X response, the fit obtained by the application of classical analysis was very poor since it was only CS identified as significant. The analysis based in GA allowed identifying, besides CS, NS and Cu as significant factors, 1 interaction between CS and CC which was also significant.

In the case of L response, the information obtained after the application of any of both analysis allows to obtain similar conclusions to those obtained in the case of X response since the same three factors were identified as significant, although the addition of two significant interactions allowed obtaining a higher determination coefficient if the analysis is performed with the GA based analysis.

Considering the significance of factors, initial cellular density did not have significant effect over none of the responses. Then, at least for 10 days of incubation, half an order of difference in the inoculum does not have major influences on the responses values.

Stirring resulted to be another factor that has no influence over none of the responses. This could be explained by the fact that the culture volume employed in this study avoided that physicochemical gradient was significant (Wu and Shi, 2007).

The third factor that also did not show influence over the responses was zinc sulphate concentration. Huang et al., 2009 described that a $ZnSO_4$ concentration between 0.6 and 1.0 mg L^{-1} allowed obtaining an important lipid accumulation in *Chlorella vulgaris* and *Chlorella pyrenoidosa* cells. In this study, that effect was not evident at least for the range between 0.2 and 1.0 mg L^{-1}, which could be explained by the fact that different media formulations were used, in comparison with the study purchased by Huang et al., 2009.

The type of nitrogen source exerts a negative effect over both responses, indicating that both lipid storage and biomass growth are favored when the nitrogen source present in the culture medium is yeast extract and not potassium nitrate. Xiong et al., 2008 obtained similar results when formulating culture media to grow *Chlorella prototecoides* with different inorganic (KNO_3 and NH_4NO_3) and organic (urea, yeast extract and glicine) nitrogen sources. Analogously, Li et al., 2011 obtained a higher lipid storage and biomass growth by including casein as nitrogen source in the formulation of a culture medium for *Chlorella minutissima*, when compared with other nitrogen sources such as urea, KNO_3 and $(NH_4)_2SO_4$. This preference for yeast extract at the expense of other nitrogen sources may be the fact this nitrogen source provides several compounds like vitamins, aminoacids and trace elements which are useful for microalgae (Losen et al., 2004).

The kind of carbon source also exerts significant negative effects over both responses. Then, lipid and biomass generation are more favored by culture media formulations containing glucose instead of glycerol. The carbon source mostly used for the growth of microalgae is glucose (Chen, 1996). Azma et al. (2011) evaluated glucose and sodium acetate as carbon sources for culturing *Tetraselmis suecica*, obtaining better results with glucose, while Isleten-Hosoglu et al., 2012 developed specific culture media for *Chlorella saccharophila* in which the carbon source was composed of different proportions of glucose and glycerol, and confirmed that

glucose was better than glycerol, supporting the results obtained in this study. This suggests that microalgae cells prefer glucose at the expense of other carbon sources, which is one of the most important components in lipid synthesis (Huang et al., 2009).

In the case of *L* response, interaction NS/CS was significant, and this could be explained by the fact that both carbon and nitrogen have a very important incidence in lipid storage. Then, by varying the quality of the sources of these macronutrients, it may be expected that lipid concentration could be affected (Li et al., 2008).

Cu factor was only significant in the case of *X* response, having a negative effect over it. This effect may be explained taking into account that at the evaluated concentrations, copper can be toxic for microalgae cells (Cordero et al., 2005). Contrary to what was described by Huang et al. (2009), who described an increment in lipid storage in *C. vulgaris* and *C. pyrenoidosa* cells when increasing $CuSO_4$ concentration in the culture medium from 0.1 to 0.3 mg L^{-1}, that effect was not observed in this study.

CC factor and its interaction with CN were significant for *L* response, while its interaction with CS was significant for *X* response, exerting positive effects in all cases. It is expected that carbon source concentration has a positive effect since microalgae employ such source to synthesize biomass and lipids (Cheng et al., 2009). In another similar study, Feng et al. (2005) found that a rise in glucose concentration has a positive influence on lipid storage by *Chlorella* sp. cells. As it was stated previously, both carbon and nitrogen have a highly incidence on lipid storage, and that is why CN factor significantly influences over lipid concentration. Both Cheng et al. (2009) and Turcotte and Kosaric (1989) have described that a high carbon-to-nitrogen ratio allows obtaining high lipid concentrations. Moreover, a low nitrogen concentration in the medium activates lipid storage in cells and contributes to their accumulation during the growth stationary phase (Turcotte and Kosaric, 1989).

As a conclusion, the factors to be taken into account in the optimization phase are carbon-to-nitrogen ratio, and the concentrations of both carbon source and copper sulphate. Even though both the type of nitrogen source and the type of carbon source resulted to be significant, in the optimization step they will be fixed at their low values, owing to they are categorical factors. Then, the nitrogen source to be employed will be yeast extract and the carbon source will be glucose. This is due to the fact that both factors exert negative effects over both responses. As regards the factors that resulted to be non significant, they will also be fixed at their low levels, meaning that initial cellular density will be 5×10^5 cells mL^{-1}, cultures will not be stirred and the zinc sulphate concentration will be fixed at 0.2 mg L^{-1}.

3.2. Optimization stage

Once being identified the significant factors in *Chlorella* sp. cultures, a CCD consistent in 20 experiments was built. The factors included in this design were the following: carbon source concentration (5–50 g L^{-1}), carbon source/nitrogen source ratio (10–50) and copper sulphate concentration (0.0–1.0 mg L^{-1}).

All cultures were developed in static conditions for 10 days, with an initial cellular density of 5×10^5 cells mL^{-1}, glucose as carbon source, yeast extract as nitrogen source and 0.2 mg L^{-1} of zinc sulphate.

Table 2 shows the 20 factors combinations suggested by the central composite design and final values of *L* and *X* responses, obtained for each of the combinations.

Previous to the data analysis by means of QLS, the value of cellular density corresponding to experiment number 8 was excluded from further analysis since it was detected as an outlier by the application of a DFFITS (*difference between fitted values test*) test. This test computes a standardized value (it can be seen as a certain number of standard deviation units) to measure the influence that each point exerts over the predicted value. When this standardized value exceeds a certain limit, it is awarded to the fact that the experiment has a disproportionate influence over the model, suggesting that this value, in particular, is atypical (Myers and Montgomery, 1995).

Table 2
Central composite design built to find the optimal conditions for lipid storage in *Chlorella* sp. cultures.

Run	Factors			Responses	
	CC (g L^{-1})	CN	Cu (mg L^{-1})	*L* (ppm)	*X* ($\times 10^6$ cells mL^{-1})
1	40.88	41.89	0.20	40.09	25.00
2	27.50	10.00	0.50	18.15	24.59
3	27.50	30.00	0.50	24.76	16.59
4	14.12	18.11	0.20	23.38	28.27
5	27.50	30.00	0.00	37.04	20.47
6	27.50	30.00	1.00	37.34	14.29
7	14.12	41.89	0.20	18.67	21.11
8	40.88	41.89	0.80	37.51	12.90
9	27.50	30.00	0.50	20.73	20.13
10	14.12	41.89	0.80	26.32	23.05
11	27.50	30.00	0.50	16.20	16.86
12	5.00	30.00	0.50	13.39	11.79
13	40.88	18.11	0.20	22.78	13.75
14	27.50	30.00	0.50	21.15	17.38
15	27.50	50.00	0.50	30.92	16.56
16	50.00	30.00	0.50	28.67	16.49
17	40.88	18.11	0.80	28.65	15.79
18	14.12	18.11	0.80	12.34	23.23
19	27.50	30.00	0.50	19.46	21.04
20	27.50	30.00	0.50	19.49	17.28

Thus, the obtained results were: both responses were adjusted with cubic models, whose associated probability values were $<1 \times 10^{-4}$ and 0.013, for *L* and *X* responses, respectively. Concerning the lack of fit, in none of the 2 cases, was significant, indicating the high reliability of the models, and that they may be employed for predictions. Eqs. (7) and (8) are mathematical representations of both models:

$$y_1 = 63.82 - 1.17x_1 - 1.18x_2 + 0.05x_1x_2 + 62.23x_3^2 - 0.07x_1x_2x_3 \quad (7)$$

$$y_2 = 132.50 - 7.05x_2 - 44.41x_3 + 2.05x_1x_3 + 1.74x_2x_3 - 0.09x_1x_2x_3 - 3x10^{-3}x_1x_2^2 \quad (8)$$

where y_1 and y_2 are *L* and *X* responses, respectively, and x_1, x_2 and x_3 are CC, CN and Cu factors, respectively. Only significant factors have been included in the equations. R^2 values obtained were 0.941 and 0.833 for *L* and *X* responses, respectively, indicating that the models explain a high percentage of the system variability. Table 3 resumes the exposed results, obtained by applying ANOVA to the data rendered by the experimental design developed.

By analyzing the mathematic models, it can be checked that the three tested factors were significant for both responses. By looking at Fig. 1 it may be elucidated the individual and mutual effects of factors over the responses. As it is shown in Fig. 1, when Cu is 0 mg L^{-1}, cellular density rises when CC increases, but this effect is reverted when Cu rises. A similar behaviour was observed with CN when varying CC, since when this factor takes lower values, cellular density reaches its maximum for extreme values of CN, but when CC rises, cellular density only increases for medium values of CN. Concerning lipid concentration, the three factors have positive effects over the response, meaning that at higher concentrations of Cu and CC, and higher values of CN, lipid concentration is expected to reach the maximum value.

Table 3
Statistical results obtained by applying an ANOVA test to the data.

Statistical parameter	L (ppm)	X ($\times 10^6$ cells mL^{-1})
Adjusted model[a]	Cub	Cub
p-Model[b]	$<1 \times 10^{-4}$ (S)	0.013 (S)
p-Lack of fit[c]	0.602 (NS)	0.174 (NS)
R^2	0.941	0.833

[a] Cub: cubic.
[b] S: significant.
[c] NS: non-significant.

The marked effect of copper sulphate concentration over lipid concentration may be explained by the fact that copper is a cofactor of the enzymes which are responsible for lipid synthesis (Huang et al., 2009). The negative effect that copper sulphate concentration exerts over cellular density may be due to a toxic effect caused by this metal over microalgae cells (Cordero et al., 2005).

Both carbon concentration and carbon source/nitrogen source ratio exert positive effects over lipid concentration, which is in agreement with the results reported by Cheng et al., 2009 and Turcotte and Kosaric, 1989, who described that a high carbon-to-nitrogen ratio derives in a lack of nitrogen, which allows obtaining higher concentrations of lipids. Moreover, carbon is one of the raw materials for lipid synthesis.

Regarding cellular density, carbon source concentration and carbon source/nitrogen source ratio, not only have individual effects but they also interact between them to influence the response value. Copper sulphate concentration, as well, participates in interactions, but, depending on the factor level, the effect of other factors varies. It is worth to mention that cellular growth depends on the balance between carbon and nitrogen, since both elements are necessary for the formation of algal material and to maintain the cellular division rate; at high and low concentrations they may cause the cessation of growth because of a high osmotic pressure or nutrients scarcity, respectively (Azma et al., 2011; Cheng et al., 2009).

The next step was the application of the global desirability function (D) in order to obtain the optimal combination of factors values that guarantee the fulfilment of the requirements set. Table 4 resumes the used criteria at the time of applying the desirability function. It was tried to minimize the cellular density so as the nutrients were destined mainly to lipid generation. As a result, the optimal combination suggested was: CC 33.30 g L^{-1}, CN 33.10 and Cu 1.00 mg L^{-1}, and the corresponding predicted values for both responses were: L 40.88 ppm and X 1.41×10^7 cells mL^{-1}, with $D = 0.944$.

With these results, it can be concluded that the complete formulation of the culture medium that would guarantee a good lipid

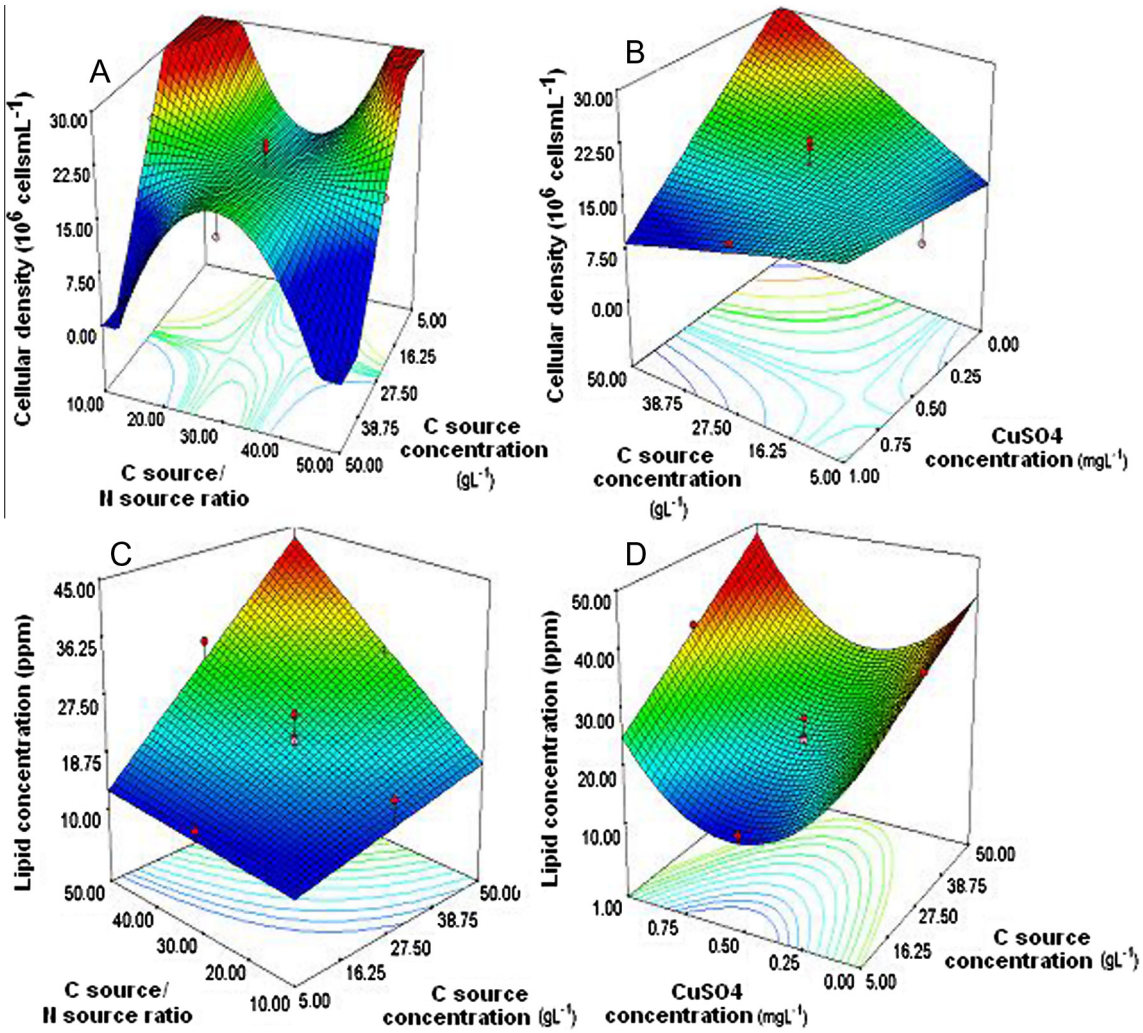

Fig. 1. Response surfaces corresponding to cellular density and lipid concentration as a function of the carbon source/nitrogen source ratio and the concentration of carbon source (A and C, respectively), and as a function of concentrations of carbon source and copper sulphate (B and D, respectively). For all figures, the third factor was kept constant in its medium value.

Table 4
Criteria used in the optimization of multiple responses.

Factors and responses	Optimization criterion	Lower limit	Upper limit
CC (g L^{-1})	In range	5.00	50.00
CN	In range	10.00	50.00
Cu (mg L^{-1})	In range	0.00	1.00
L (ppm)	Maximize	11.79	28.27
X ($\times 10^6$ cells mL^{-1})	Minimize	12.34	40.09

storage would be the following (in g L^{-1}): glucose 33.25, yeast extract 1.00, NaCl 0.43, KH_2PO_4 0.175, K_2HPO_4 0.075, $MgSO_4$ 0.036, $CaCl_2.2H_2O$ 0.025, plus $ZnSO_4$ 0.20 mg L^{-1}, $CuSO_4$ 1.00 mg L^{-1} and 6 mL L^{-1} of a trace metals solution.

3.3. Experimental verification

The optimal factors combination was experimentally evaluated. The microalgae strain was cultured in three culture media which composition corresponded to the optimal formulation described in the previous section. In the same way, the microalgae was cultured, by triplicate, in four different additional culture media, which differed from the optimal formulation that instead of including commercial glucose as a carbon source, they included acid or enzymatic hydrolysate of two lignocellulosic materials: corn and wheat bran. The methods to hydrolyze these materials were developed and studied in a previous work (Giordano et al., 2013). This was developed in order to evaluate if it is viable to replace commercial glucose by non conventional glucose, which does not generates conflicts with human feed issues. The formulations that contained acid and enzymatic hydrolysate of wheat bran were named as AHWB and EHWB, respectively, while the ones containing acid and enzymatic hydrolysates of corn bran were named as AHCB and EHCB, respectively. Table 5 resumes the predicted (only for the optimum formulation) and experimental values for lipid concentration and cellular density.

From the experimental data obtained with these experiments, it can be concluded that the combination predicted as optimum through the application of the desirability function effectively allows maximizing the lipid storage in *Chlorella* sp. cells. Moreover, it can be confirmed the suitability of the obtained models to describe the studied process, mainly for L response.

If the attention is turned to the culture media in which the carbon source was a hydrolysate instead of glucose, similar results were obtained except in one case. In AHWB, EHWB and AHCB, lipid concentrations between 7.7 and 11.7 ppm were obtained, i.e. 67–78% less than the optimum formulation, and the obtained cellular densities represent 8–24% of that obtained in the optimum formulation. In the case of media formulated with acid hydrolysates, is likely that they contain cellular growth inhibiting compounds like 5-hydroxymethilfurfural and furfural, generated during the hydrolysis process, as a consequence of glucose and xylose degradation, respectively (Sun and Cheng, 2002).

The only exception was the EHCB medium, for which 90% more biomass and 372% more lipids were obtained. This fact confirms that the replacement of glucose for enzymatic hydrolysate of corn bran, not only is viable but also it allows improving significantly the lipid storage in *Chlorella* sp. cells. Yan et al., 2011 also reported improvements both in algal biomass and lipid storage when culturing *C. prototecoides* cells in media containing enzymatic hydrolysate of sweet sorghum juice, residual molasses and corn powder, respectively. In addition, Cheng et al., 2009 informed that the replacement of glucose for sugar cane juice hydrolysate was viable in culture media for *C. prototecoides*, since they obtained similar concentrations both of biomass and lipids. This could be due to the fact that enzymatic hydrolysates contain beneficial compounds for microalgae cells such as proteins and aminoacids, among others. Nevertheless, for the case of EHWB, the inverse effect was observed.

3.4. Culture developed in an air-lift bioreactor

Once the predicted values were verified for both responses, the *Chlorella* sp. strain was cultured in an air-lift bioreactor, which was characterised. Fig. 2 shows the evolution of t_m and $K_L.a$ as a function of air flow (50, 100, 200 and 400 mL min^{-1}). It can be observed that when the air flow rises, so does the $K_L.a$ value, while the mixture time diminishes, as it was expected.

The culture medium employed was the optimum formulation obtained, and the total culturing time was 15 days, taking a sample each 48 h in order to perform glucose, lipids and biomass quantitation. Two feeds with sterile medium were performed in order to re-establish the original culture volume (300 mL): at days 5 (25 mL) and 13 (40 mL). Fig. 3 shows the evolution of cellular density and the concentrations of glucose, lipids and dissolved oxygen as a function of time for the culture of *Chlorella* sp. developed in the air-lift bioreactor (blue arrows indicate sterile culture medium feeds).

As it can be seen in Fig. 3, the microalgae grew exponentially until day 5, the phase being characterized by a specific growth rate of 0.73 d^{-1}, value that is very close to 0.74 d^{-1} obtained by Ip and Chen (2005) for *Chlorella zofingiensis* grown heterotrophically in a culture medium containing glucose, but substantially lower than 0.92 d^{-1}, informed by Shi et al. (2002) for *C. prototecoides* grown in similar conditions. The value for μ obtained in this study resulted to be higher than that informed by Bhosale et al. (2006) (0.25 d^{-1} for *C. prototecoides*) and Chiu et al. (2008) (0.23 d^{-1} for *Chlorella* sp.) for autotrophic cultures, but lower than that informed by Ip et al. (2004) (1.03 d^{-1} for *C. zofingiensis* grown in medium containing glucose) for a mixotrophic culture.

At the end of the exponential growth phase, a cellular density value of 1.8×10^7 cells mL^{-1} was reached, which represents a 14% less than what was obtained in cultures developed in Erlenmeyer flasks. Then, on the one hand, at least during the batch phase corresponding to the culture developed in an air-lift bioreactor, the reduction of cellular density was achieved. On the other hand, lipid concentration increased from day 1 to day 3, being constant at day 5, moment at which the first feed was made with sterile medium. Although the nutrients concentrations increased,

Table 5
Predicted and experimental responses values for the tested media. Values in brackets stand for standard deviations.

Culture medium formulation	L (ppm)		X ($\times 10^6$ cells mL^{-1})	
	Predicted	Experimental	Predicted	Experimental
Optimal	40.9	35.7 (2.0)	18.1	21.5 (2.7)
AHWB	–	9.8 (0.7)	–	5.2 (0.4)
EHWB	–	7.7 (2.4)	–	4.3 (1.2)
AHCB	–	11.8 (0.1)	–	1.6 (0.3)
EHCB	–	168.5 (33.4)	–	40.8 (2.2)

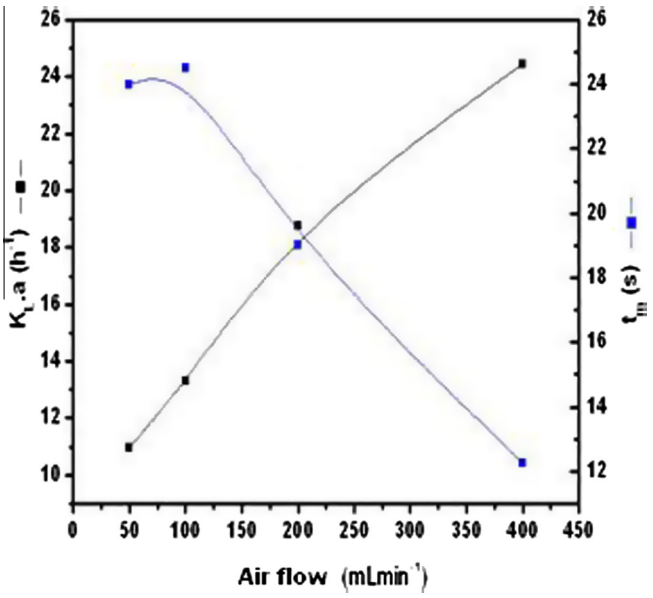

Fig. 2. Evolution of t_m and $K_L.a$ as a function of the air flow, obtained in the characterization of an air-lift bioreactor.

microalgae cells entered in a stationary phase and the cellular density registered a slight increment of 2.4×10^7 cells mL^{-1} between days 5 and 13, but lipid concentration increased from 22 to 75 ppm, representing 110% increase in respect to that obtained in 10 mL static cultures. From day 11, lipid concentration declines, probably due to that microalgae starts to consume them, which is supported by the fact that between days 7 and 9 glucose concentration does not decline while lipid concentrations does. Both stationary phase and the decline in lipid concentration are not reverted though a second feed made at day 13.

One possible explanation for the higher lipid storage at the beginning of the stationary phase could be the nitrogen deficiency or limitation, which may slow the synthesis rate of essential cellular structures like proteins and nucleic acids. Then, the incorporated carbon would be most converted into lipids (Turcotte and Kosaric, 1989; Cheng et al., 2009).

During the culture development, dissolved oxygen concentration estimated was never zero, consequently, it never existed an anaerobiosis environment in any moment that could limit the microorganism growth. Moreover, supposing that dissolved oxygen concentration had been the only limiting substrate for growth, the maximum reachable cellular density would have been 1.03×10^8 cells mL^{-1} (value obtained by making zero the term C in Eq. (6)). However, the maximum value obtained for this parameter was 3.98×10^7 cells mL^{-1} at day 13, meaning that growth limitation did not occur as a consequence of dissolved oxygen deficiency, but for another reason, maybe for example, due to the generation of some compound that could be toxic for *Chlorella* sp. cells.

4. Conclusion

The developed medium could be a promising formulation for culturing *Chlorella* with the aim of obtaining high lipid concentrations. In addition, it was successfully scaled in an air-lift bioreactor, and copper sulphate concentration was not set as a constant, like in other works, and it was demonstrated that it exerts significant influence on lipid storage.

The enzymatic hydrolysate of corn bran allowed obtaining significant improvements both in lipid storage and algal biomass generation, confirming the viability of replacing traditional carbon sources by other non conventional and of very low cost, which positively insides in process economy.

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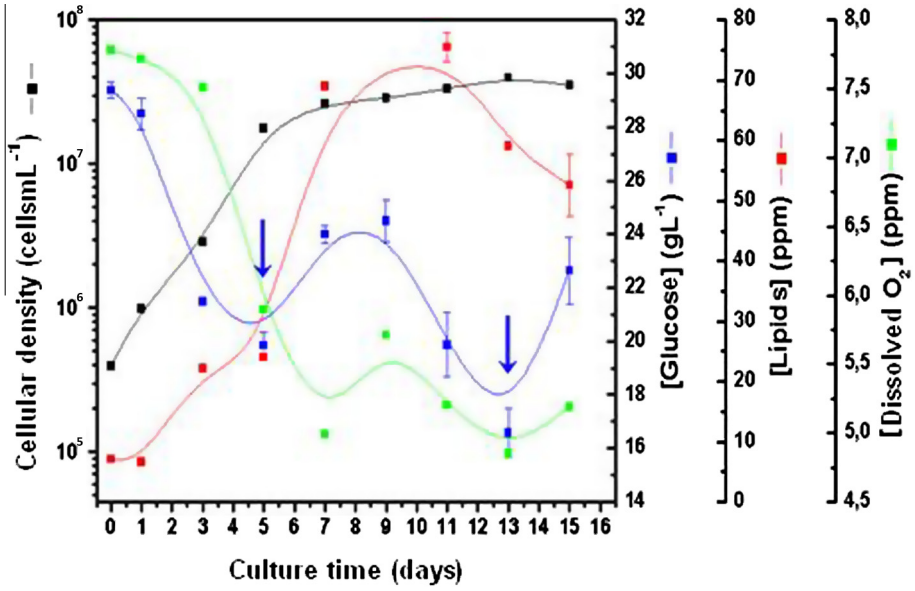

Fig. 3. Evolution of cellular density and of glucose, lipids and dissolved oxygen concentrations, as a function of culturing time, for a *Chlorella* sp. culture developed in an air-lift bioreactor. Blue arrows indicate sterile culture medium feed.

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