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ORIGINAL ARTICLE

Accurate method for rapid biomass quantification based on specific absorbance of microalgae species with biofuel importance

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Significance and Impact of the Study: An accurate, rapid and specific methodology for biomass measurements based on spectrophotometry of three microalgae species with relevance in biofuel production was established. Also, the specific wavelength for maximum absorbance was identified for the studied species. The results could enhance the efficiency of microalgae cultures and also show the potential to be established in other species with aquaculture relevance. In addition, the predictive model showed high accuracy in a wide-ranging cell densities providing a methodology with low operational cost and high potential for automation; indeed, an easy-to-use software was developed for practical use of the predictive models.

Keywords
microalgae, biofuels, modelling, optimisation, rapid methods.

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Abstract

The development of microalgae culture technology has been an integral part to produce biomass feedstock to biofuel production. Due to this, numerous attempts have been made to improve some operational parameters of microalgae production. Despite this, specialized research in cell growth monitoring, considered as a fundamental parameter to achieve profitable applications of microalgae for biofuels production, presents some opportunity areas mainly related to the development of specific and accurate methodologies for growth monitoring. In this work, predictive models were developed through statistical tools that correlate a specific micro-algal absorbance with cell density measured by cell count (cells•per ml), for three species of interest for biofuels production. The results allow the precise prediction of cell density through a logistic model based on spectrophotometry, valid for all the kinetics analysed. The adjusted determination coefficients (r^2_{adj}) for the developed models were 0·993, 0·995 and 0·994 for *Dunaliella tertiolecta*, *Nannochloropsis oculata* and *Chaetoceros muelleri* respectively. The results showed that the equations obtained here can be used with an extremely low error (≤2%) for all the cell growth ranges analysed, with low operational cost and high potential of automation. Finally, a user-friendly software was designed to give practical use to the developed predictive models.

Introduction

Microalgae represent a suitable alternative feedstock for biofuels production, mainly because this material presents convenient properties, such as high caloric value, elevated yields, low viscosity and even the potential to generate coproducts such as phytochemicals. Other relevant microalgae property is their high lipid yield, which allows the production of liquid biofuels like biodiesel (Prabakaran and Ravindran 2011; Liu *et al.* 2013; Caguimbal *et al.* 2019).

Among all the microalgae species, the present study focused on *D. tertiolecta*, *N. oculata* and *C. muelleri*, mainly because of their wide availability, high biomass accumulation rates, adequate growth in saline water, higher oil yields and their successful cultivation at large scale (Tang *et al.* 2011; Moazami *et al.* 2012; Wei *et al.* 2013; El-Arrousi *et al.* 2015; Lin and Wu 2017).

Although microalgae are a promising raw material for obtaining biofuels and other value-added products, their culture involves several factors to take into account; these parameters present high influence in microalgae technology success and commercial viability (Kim *et al.* 2005; Wang *et al.* 2015; Caguimbal *et al.* 2019). One of these factors is the monitoring and cell growing control; which despite the efforts made, still presents opportunity areas, mainly regarding adequate measuring, wavelengths selection and the establishment of specific standard curves for each microalgae species and/or specific culture conditions (Lee *et al.* 2015; Santos-Ballardo *et al.* 2015). An adequate cell growth monitoring is useful to estimate the maximum productivity in microalgae cultivation, to achieve a sustained harvest management and also allows the designing, monitoring, modelling and optimizing the microalgae cultivation systems (Kenny and Flynn 2016; Su *et al.* 2016). Until now, the most common methods for measuring cell growth are the dry weight direct determination and through cell counting using a haemocytometer; despite their high efficiency, these approaches result in a tedious and time-consuming task which directly affects the commercial viability (Ribeiro-Rodrigues *et al.* 2011).

Due to this, some efforts have been realized in order to search easier, simpler, specific and faster micro-algal biomass measuring methods; the main attempts have been based on developing predictive models using different approaches such as volatile suspended solids, optical density (OD), total suspended solids, turbidity and wet weight measurements (Santos-Ballardo *et al.* 2015; Su *et al.* 2016). Sarrafzadeh *et al.* (2015) compared the traditional dry weight determination technique, with some alternative biomass measuring methods such as flow cytometry and indirect methods like dielectric permittivity and the use of OD; they concluded that OD can provide a simple, accurate and rapid way for measuring the cell concentration of microalgae using only a spectrophotometer adjusted to a specific light wavelength. The OD method offers advantages, such as not requiring expensive equipment or highly trained personal, and also is easily adaptable to automated measurement systems (Ribeiro-Rodrigues *et al.* 2011; Su *et al.* 2016).

Although spectrophotometry is an especially useful and practical methodology, it presents some disadvantages, such as the interference of pigment on absorbance measurement, besides, the OD measurement could be affected for sample concentration and culture time (Griffitths *et al.* 2011; Su *et al.* 2016). Due to this, the developing of predictive mathematical models had been proposed as a good option to do an efficient analysis on microalgae cell growth (Baird 2003; Zhang *et al.* 2017).

The aim of the present work was the establishment of specific predictive models for estimating cell density of some key microalgae species used for biofuel production based on spectrophotometric absorbance. Finally, a method for micro-algal biomass measurement was developed for all the species analysed, with remarkable characteristics in quickness, versatility, reliability, simplicity, stability and efficiency, showing high possibilities for automation.

Results and discussion

Microalgae maximum absorbance

The light absorbance patterns for each microalgae species were scanned from 550 to 800 nm; thus, the maximum absorbance obtained for *D. tertiolecta, N. oculata* and *C. muelleri* were 681, 682 and 679 nm respectively (Fig. 1).

The results obtained are similar to previous reports, where OD was used for determining cell growth of some microalgae species, and the wavelength used ranged from 630 to 750 nm (Griffitths *et al.* 2011; Hao *et al.* 2011; Almomani and Örmeci 2018). For example, Balduyck *et al.* (2015) analysed the emission spectrum between 400 and 800 nm for *Tisochrysis lutea* and *N. oculata*, showing a relative high peak approximately at 570 nm and a second one between wavelengths 620 and 670 nm, for both microalgae species. Besides, Su *et al.* (2016) used a wavelength of 660 nm to analyse the cell growth of five microalgae species and recognized a high correlation between OD and cell density.

Usually, in standard assessments of micro-algal growth kinetics analysed by spectrophotometry, the use of wavelengths within the maximal absorbance range of chlorophyll a (400–460 and 650–680 nm) is recommended; regarding this, Jia *et al.* (2015) designed a sensor for measuring accurately microalgae biomass concentration based

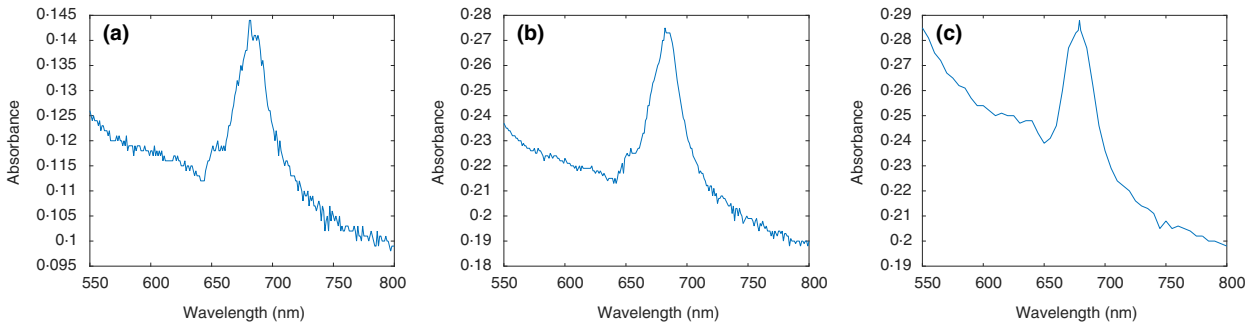

Figure 1 Light absorbance patterns scanned from 550 to 800 nm for different microalgae species. (a) *Dunaliella tertiolecta*; (b) *Nannochloropsis oculata* and (c) *Chaetoceros muelleri*. nm = nanometre.

on OD measurement, using 650, 685 and 780 nm wavelengths; the growth rates calculated at each wavelength were considered good indicators for monitoring microalgae growth transitions and for disturbances detection into the culture system (e.g. light intensity changes, water addition, rain, and harvesting). It is important to remark that the optimal wavelength could present variations depending on the microalgae species used and specific culture conditions (Griffitths *et al.* 2011).

In the present study, the maximum absorbance was measured at different cellular growth stages and diverse cell concentrations, finding the same results in all experiments (data not shown). The variability on the maximum absorbance for different microalgae species has been reported previously, and this behaviour could be explained due to the accumulation of different types and contents of intracellular pigments, such as phycobiliproteins, carotenoids and chlorophyll, which could generate an effect on the cell absorbance (Wellburn 1994; Bricaud *et al.* 1998).

Growth performance of microalgae species

Table 1 shows the parameters for cell growth performance obtained in the growth kinetics for each microalgae studied: maximum cell density (measured by two methods), specific growth rate (μ) and duplication time (Δt). The maximum cell density was achieved at the end of the stationary phase.

Growth parameters from similar species to the studied in the present work have been already reported. For *Dunaliella tertiolecta* grown under nutrient variations, the reported values ranged within 0·43–0·87 day^{-1} and 0·79–1·58 day, for μ and Δt respectively (Chen *et al.* 2011); furthermore, El-Arrousi *et al.* (2015) reported μ: 0·34 day^{-1} and Δt: 2 days for the same microalga under auxin treatment coupled with salt stress. Also, values of μ: 0·43–0·64 day^{-1} and Δt: 1·08–1·61 days for *Dunaliella salina* were reported using modelling growth and different nutritional conditions (Santos-Jesus and Maciel-Filho 2010). Regarding *Nannochloropsis* strains, the specific growth rates varied dramatically among different previous reports ranging from μ: 0·004 to 3·3 day^{-1} and Δt: 0·43 to 173·28 days for different *Nannochloropsis* species (Ma *et al.* 2014; Xu and Boeing 2014). Furthermore, for *N. oculata*, Yokose *et al.* (2009) reported a range from μ: 0·06 to 0·28 day^{-1} and Δt: 2·47 to 11·55 days for cultures developed under different growth conditions.

Related to the *Chaetoceros* genus, values ranging μ: 0·86–1·82 day^{-1} and Δt: 0·38–0·8 days have been reported for *Chaetoceros gracilis* (Tokushima *et al.* 2016); meanwhile, *Chaetoceros salsugineum* obtained from an estuary in Japan reached μ: 12·96 day^{-1} and Δt: 0·053 days under optimal conditions (Ichimi *et al.* 2012); and for *Chaetoceros calcitrans*, Phatarpekar *et al.* (2000) reported growth rate parameters of μ: 0·225–0·665 day^{-1} and Δt: 1·042–3·080 days.

The results obtained in the present study are near the ranges previously reported; therefore, an adequate performance in growth kinetics could be assumed; and due to this, these data were used to obtain predictive models. Furthermore, differences within the analysed species were observed; these comparisons must be approached with caution because the micro-algal cell growth depends on many parameters, such as the culture parameters (temperature, pH, media composition and gas flow rate), the photobioreactor design (closed, open ponds, air lift, etc.) and also the physiological demands for the microalgae strains (Ma *et al.* 2014; Santos-Ballardo *et al.* 2016).

Predictive models

The results obtained showed that the specific growth kinetics developed using both methods (absorbance at a particular wavelength and cell counting) showed a high positive correlation, with values of Pearson coefficients (r)

Table 1 Growth performance of microalgae species

	(Max *D*) (cells•per ml)	(Max Abs)* (a.u.)	Days for (max *D*) (d)	μ† (d^{-1})	Δt† (d)	μ‡ (d^{-1})	Δt‡ (d)
Dunaliella tertiolecta	$2{\cdot}1 \times 10^6 \pm 1{\cdot}15 \times 10^5$	0·187 ± 0·002	18	0·221	3·133	0·196	3·542
Nannochloropsis oculata	$8{\cdot}26 \times 10^6 \pm 5{\cdot}12 \times 10^5$	0·179 ± 0·009	21	0·183	3·785	0·182	3·808
Chaetoceros muelleri	$2{\cdot}49 \times 10^6 \pm 5{\cdot}58 \times 105$	0·164 ± 0·002	17	0·185	3·743	0·178	3·885

(Max *D*): maximum density; (max Abs): maximum absorbance; a.u.: arbitrary units; d: days; ml: milliliter; μ: specific growth rate; Δt: duplication time.

*Values obtained using the specific wavelength for each microalgae.

†Values obtained from the growth kinetics developed using cell counting.

‡Values obtained from the growth kinetics developed using absorbance.

of 0·970, 0·983 and 0·977, for *D. tertiolecta*, *Nannochloropsis oculata* and *C. muelleri* respectively. Besides, the adjusted determination coefficients (r^2_{adj}) for the developed models ranged from 0·993 to 0·994; Table 2 shows the calculated regression coefficients for the predictive models.

Some authors reported remarkably high positive Pearson correlations between cell counting through haemocytometer (cells•per ml) and spectrophotometric absorbance of some microalgae species (Pavia-Gómez *et al.* 2015; Santos-Ballardo *et al.* 2015; Sarrafzadeh *et al.* 2015). This association could be explained due to the relatively high pigment content in the micro-algal cells (including different chlorophylls types and some carotenoids). Furthermore, the absorbance of some pigments is higher in specific range of the light spectrum, this is important to choose the wavelength with the maximum absorbance range of the cellular pigments (Lee *et al.* 2013). Moreover, previous reports showed high correlations between the cell density (cells•per ml) and diverse microalgae characteristics, such as pigment content (including chlorophyll *a*, *b* and *c*, or some carotenoids), inorganic particles and also intracellular carbohydrates and/or lipids (Griffitths *et al.* 2011; Balduyck *et al.* 2015; Mansouri 2016; Su *et al.* 2016; Almomani and Örmeci 2018).

The predictive models for cell counting (cells•per ml^{-1}) were developed (using a logistic model) as a function of absorbance (Eqn 4). After solving the obtained equations, the following predictive models were obtained for each microalgae species (using the specific wavelength determined) with Eqn (1):

$$x = \left(\sqrt[p]{\left(\left(\frac{A_1 - A_2}{y - A_2} \right) - 1 \right)} \right) x_0 \qquad (1)$$

This model was valid for all the measured absorbance. At low and high cell densities, predictive values showed slight deviations from experimental ones (Fig. 2). Furthermore, the percentile deviations (δ_p) for the fitted models represented in absolute value are shown in the Fig. 3.

The δ_p was lower than 0·2% for the following ranges: from $0{\cdot}1 \times 10^6$ to $2{\cdot}1 \times 10^6$ cells•per ml for *D. tertiolecta (*which represents all the cell growth kinetic*)*; from $0{\cdot}7 \times 10^6$ to $8{\cdot}26 \times 10^6$ cells per•ml for *N. oculata* and from $0{\cdot}3 \times 10^6$ to $2{\cdot}49 \times 10^6$ cells per•ml for *C. muelleri*. In general, the models generated worked efficiently in all the ranges of the microalgae kinetics developed, showing a very low percentile deviation (≤2% in the worst situation). It means that within all the micro-algal density ranges analysed, the cell counting (cells per ml) can be accurately estimated using absorbance data measured at the specific wavelength for each microalgae species used.

Moreover, some authors developed operative equations to estimate cell density using spectrophotometric absorbance for some microalgae species. For example, for *Isochrysis galbana*, a direct spectrophotometric method to determine cell density was developed using a linear model, obtaining R^2 from 0·9586 to 0·9981 using different

Table 2 Regression coefficients for predictive models

	A_1	A_2	x_0	p	rmse	Chi-sqr	r^2_{adj}
Dunaliella tertiolecta	−0·036	0·267	$5{\cdot}621 \times 10^5$	0·817	$29{\cdot}718 \times 10^{-5}$	$2{\cdot}122 \times 10^{-5}$	0·993
Nannochloropsis oculata	0·024	0·193	$40{\cdot}368 \times 10^5$	2·236	$27{\cdot}111 \times 10^{-5}$	$1{\cdot}595 \times 10^{-5}$	0·994
Chaetoceros muelleri	0·020	0·223	$16{\cdot}223 \times 10^5$	2·167	$22{\cdot}097 \times 10^{-5}$	$1{\cdot}473 \times 10^{-5}$	0·995

*A*1, *A*2, x_0 and p are regression coefficients for Equations (1 and 4).

rmse is the root mean square error.

Chi-sqr is the reduced Chi-square.

r^2_{adj} is the adjusted determination coefficient.

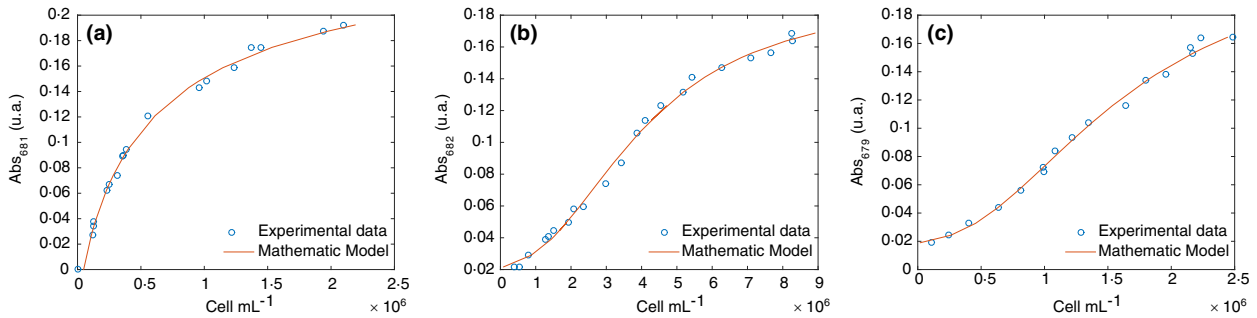

Figure 2 Relationship between cell counting and absorbance for different microalgae species. White dots represent the experimentally measured values, and fitted curve represents the theoretical values calculated using the developed mathematic models. (a) *Dunaliella tertiolecta*; (b) *Nannochloropsis oculata* and (c) *Chaetoceros muelleri*. Abs = absorbance; ml = millilitre; a.u. = arbitrary units.

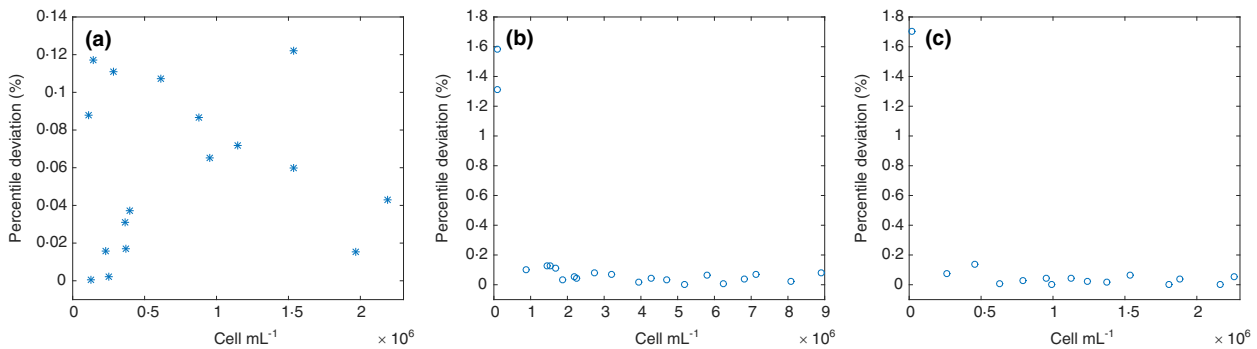

Figure 3 Percentile deviation for the cell density (cells per•ml) proposed models as function of absorbance registered experimentally on microalgae cultures. (a) *Dunaliella tertiolecta*; (b) *Nannochloropsis oculata* and (c) *Chaetoceros muelleri*. ml = milliliter.

wavelengths (Pavia-Gómez *et al.* 2015). Also, Almomani and Örmeci (2018) developed a method to determine microalgae concentration using the first derivative of absorbance for *Chlorella vulgaris*; they found a strong linear association ($R^2 > 0{\cdot}95$) between cell concentration and absorbance at 695 nm. It is important to remark that for percentile error reduction, the linear models can only be used during the exponential growth phase, which limits most of the predictive methods developed to a short range of use.

Furthermore, Griffitths *et al.* (2011) developed two linear models for three microalgae species (*Scenedesmus* sp., *Spirulina platensis* and *Nannochloropsis* sp.), the first model uses an absorbance of 680 nm, showing average error between 10 and 19% until the 14th day. To diminish the deviations, a second model was developed using the bleached cells (after chlorophyll remove) and absorbance at 750 nm, showing 5–16% of average error for the same culture time; although a slight reduction in the average error was achieved, the cost and effort increments related to removing the cellular pigment represent a disadvantage of the developed technique. Besides, Santos-Ballardo *et al.* (2015) developed a predictive method using nonlinear models for four microalgae species, showing percentile errors between 1 and 22·5%; the developed models are useful for a wide range of the cell growth but are not suitable for low cell densities.

In the present work, efficient models were achieved for predicting practically all the cell densities reported in the growth kinetics with a very low percentile deviation, including the lowest and the highest values; also the predictive methods developed do not require specific actions to remove pigments to assure the accuracy, which represents a time reduction for the measurements.

Additionally, for technical implementation of the mathematical models established (Table 2), a graphical user interface was developed using Matlab® (Code program S1). This interface allows to the potential users a simple way to monitoring these specific microalgae. The program uses the absorbance values obtained experimentally to calculate the cell density (cells•per ml) directly. The data

calculated are shown numerically and are represented in a graph for each microalgae species (Fig. S1). The interface use is very simple and allows to estimate the number of cells•per ml from the absorbance quickly and accurately.

As conclusion, an accurate, fast and specific methodology for cell density measurements based on spectrophotometry was established for three microalgae species with high importance in biofuel production. The specific wavelength for the maximum absorbance was established for each studied species. In addition, the developed predictive models showed high accuracy in a wide range of cell densities, generating methodologies with low operational cost and high potential for automation. Besides, this tool for cell counting could enhance the efficiency of cultures of these microalgae species and might also be assessed in other species.

Material and methods

Microalgae material

Dunaliella tertiolecta, *N. oculata* and *C. muelleri* were obtained from the microalgae collection of the Bioenergy Laboratory of Universidad Politécnica de Sinaloa, and gently donated by the Acuario Mazatlán, Mexico.

Microalgae culture conditions

The medium reported by Guillard and Ryther (1962), with artificial seawater (average salinity and pH between 32 and 33 g l^{-1} and 7·5 and 7·9 respectively), was used for microalgae cultivation. Cultures were grown at 21 ± 2°C in 19 l plastic containers. Using a blower (ELITE 802^{TM} 1·5 hp), an average flow of 40 cm^3 s^{-1} of filtered air was supplied continuously through a 1 μm cut-off cartridge. Microalgae cultures were subjected to a constant light condition (24 h). Illumination was provided by fluorescent lamps (Phillips F48T8/TL865, 30 W, Phillips, AE Eindhoven, Netherlands), with an intensity of 6000–6500 lux, generating an active radiation of 120–130 μmol photons m^{-2} s^{-1}.

Maximum absorbance determination

The maximum absorbance (A_{max}) was determined by scanning sample cultures between 550 and 800 nm, using a UV-visible spectrophotometer (Genesys 10S UV-VIS, Thermo Fisher Scientific, Waltham, MA, USA). For each microalgae species, the A_{max} value was used to perform the cell growth kinetics through the spectrophotometric method (SM) (Ribeiro-Rodrigues *et al.* 2011; Santos-Ballardo *et al.* 2015).

Cell growth kinetics

Each microalgae species analysed was grown separately and cell growth was analysed by daily measurements performed by triplicate, until cultures reached their senescence phase. Two analytical methods were used: (i) the SM, developed by measuring the absorbance of cell suspensions using a UV visible spectrophotometer (Mikschofsky *et al.* 2009), and (ii) cell counting by using a haemocytometer (Neubauer chamber, Labcon, Petaluma, CA, USA) and microscope (Labomed Fremont, CA, USA). Microalgae density as cell number per millilitre (cell•per ml) was calculated (Godoy-Hernández and Vázquez-Flota 2006). Finally, Pearson correlation (r) between both analytical methods was analysed (using OriginPro^{TM}8.0 software), it was applied to determine the association between the kinetics developed, and to estimate the strength of this relationship (Schober *et al.* 2018).

Cell growth efficiency

Using the growth kinetics, the specific growth rate (Eqn 2) and duplication time (Eqn 3) were calculated for the studied microalgae (Santos-Ballardo *et al.* 2015):

$$\text{Specific growth rate}: \mu = \frac{\ln N - \ln N_0}{t} \quad (2)$$

$$\text{Duplication time}: \Delta t = \frac{\ln 2}{\mu} \quad (3)$$

Wherein: μ, specific growth rate; Δt, duplication time; N_0, initial biomass concentration; N, final biomass concentration and t, time (days).

Predictive models

The adjusted determination coefficient (r^2_{adj}) was obtained for absorbance (y) and cell counting (where x is cells•per ml) data, using OriginPro® 8.0 software. Predictive models were developed between SM and cell counting using nonlinear regression, specifically a logistic model, as shown in Eqn (4):

$$y = A_2 + \frac{(A_1 + A_2)}{1 + \left(\frac{x}{x_0}\right)^p} \quad (4)$$

where A_1, A_2, x_0 and p are regression coefficients estimated by a regression analysis using the OriginPro® 8.0 software.

For efficiency validation of the developed models for each microalgae studied, the percentile deviation (δ_p) for the predicted cell density (x_{pre}) and the experimental data during the growth kinetics were calculated using the Eqn (5) (Rosas and Riveros 1990; Santos-Ballardo *et al.* 2015):

$$\delta_p = \left(\frac{x - x_{pre}}{x}\right) \times 100 \quad (5)$$

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Author's contribution

Ambriz-Pérez Dulce Libna performed substantial contribution to conception and design of this project, analysis of data, also drafting and reviewed the manuscript, apart from, approval of the final submitted version. Orozco-Guillen Eber Enrique performed substantial contribution to the analysis of data, also critically reviewed the manuscript and approved the final submitted version. Galán-Hernández Néstor Daniel performed substantial contribution to the analysis of data, also critically reviewed the manuscript and approved the final submitted version. Luna-Avelar Karla Denisse performed substantial contribution to the acquisition of data, also critically reviewed the manuscript and approved the final submitted version. Valdez-Ortiz Angel performed substantial contribution to the analysis of data, also critically reviewed the manuscript and approval the final submitted version. Santos-Ballardo David Ulises performed substantial contribution to conception and design of the project, analysis of data, also drafting and reviewed the manuscript, final approval and uploaded the final submitted version.

Availability of data and material (data transparency)

Authors agree to make their data available upon reasonable request.

Code availability (software application or custom code)

The Matlab® code and image of the programme developed are provided at the end of the document as supplementary information.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Microalgae biomass quantification.

Code S1. Code program S1.