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How to combine CO_2 abatement and starch production in *Chlorella vulgaris*

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A B S T R A C T

Microalgae production has gained attention in recent years as promising systems for CO_2 abatement as well as a source of proteins, pigments, vitamins, lipids, and carbohydrates. Particularly, starch can be used for bioethanol production in a well-established fermentative process. The aim of this work was to maximize and model biomass productivity and CO_2 assimilation in continuous cultures of *Chlorella vulgaris*. The following culture parameters were studied: dilution rate, pH, temperature, light intensity, and nitrogen supply. The proposed model ($r^2 = 0.95$) predicted a maximum biomass productivity of $0.7\,g\,L^{-1}\,d^{-1}$ and CO_2 assimilation of $1.3\,g\,L^{-1}\,d^{-1}$. The experimental data agreed with these predictions, resulting in a maximum biomass productivity of $0.67\,g\,L^{-1}\,d^{-1}$ (resulting in a CO_2 assimilation of $1.23\,g\,L^{-1}\,d^{-1}$). In addition, the starch content was determined, and the results were used as input into a second model, which aimed at predicting starch accumulation during CO_2 abatement processes ($r^2 = 0.84$). This second model predicted a daily and continuous production of biomass with a maximum starch content of $0.25\,g\,g^{-1}\,d^{-1}$ (25% dcw), but under different culture conditions than those found for maximizing biomass productivity and CO_2 assimilation. The maximum starch content experimentally determined was $0.2\,g\,g^{-1}\,d^{-1}$ (20% dcw). Thus, to implement a biological system for CO_2 abatement coupled to starch accumulation, it is necessary to find a compromise between these two processes. Hence, although yield in both processes would be reduced, a simultaneous process for CO_2 mitigation and starch production would be feasible.

1. Introduction

The atmospheric concentration of greenhouse gases has increase radically since industrial revolution [1]. Specifically, atmospheric CO_2 concentration has risen from 280 ppm in pre-industrial era to 400 ppm in 2015 [2]. One of the proposals to reduce the atmospheric level of CO_2 is the bio-mitigation by microalgae. Microalgae are promising organisms for sustainable production of food, feed, materials, chemical and fuels [3]. Certainly, one of the most attractive features is their capability to storage large amounts of high-energy compounds such as lipids and carbohydrates via photosynthetic CO_2 assimilation. These compounds can later be used as raw materials to produce biofuels [4]. Triacylglycerols (TAGs) are usually the first target on microalgae biofuels research, since they can be used to produce biodiesel [5]. Nevertheless, more attention is being paid to bioethanol derived from starch, which might be more economically feasible than the one derived from land crops [6]. Biofuels derived from microalgae provide several advantages over land crops: they do not compete with arable land, offer high productivities and they can be cultivated in wastewater [7]. However, large scale biofuel production based on microalgae is still not commercially feasible [8,9]. Several strategies, such as the use of flue gases as carbon source or mixotrophic production have been suggested in order to beat these constraints during commercialization [10–12].

Chlorella vulgaris is a well-known eukaryotic microalgae, with promising applications for food, feed and pigment production [13]. However, it is necessary to first gather knowledge on *C. vulgaris* physiology to maximize its autotrophic production in order to predict and enhance biomass productivity and CO_2 fixation rate. For this reason, the development of growth models is a promising tool to understand and increase the yields of the cultures. Among possible strategies for developing models, the “single parameter optimization” approach was selected based on our previous work [14]. The main goal of the present research was to maximize and model biomass productivity and CO_2 assimilation in continuous cultures of *Chlorella vulgaris*. However, we

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consider important to give a use to the generated biomass. Among all compounds present in microalgal biomass, we focused on starch as raw material for ethanolic fermentation. This selection was done by considering starch a co-product of CO_2 abatement and not as the main product of microalgal cultivation. Thus, starch content was determined during the experiments of maximization of biomass productivity and CO_2 assimilation. In a second step, a predictive model was defined for starch accumulation during CO_2 assimilation in autotrophic continuous cultures of *C. vulgaris*.

2. Materials and methods

2.1. Microorganism and culture medium

Chlorella vulgaris UAM 9.11 was grown in Arnon medium [15], which was selected based on previous works of our group [16]. The medium was supplied with different nitrate concentrations (1, 5, 10 and 20 mM $NaNO_3$), depending on the experiment.

2.2. Culture conditions

Chlorella vulgaris was cultivated photo-autotrophically using indoors glass bubble column photo-bioreactors (0.07 m diameter, 0.5 m height) with a working volume of 1.8 L. Photo-chemostat mode was selected as a continuous regime during the light hours. Thus, a continuous dilution of the cultures occurred only during the illuminated hours of the day. A screening of the dilution rates was performed, ranging from 0.2 to 1.1 d^{-1}. The photo-bioreactors were illuminated by six white-light lamps controlled by an automated system simulating a solar sine cycle (12 h light: 12 h dark) and providing a maximum irradiance on the reactor surface that was selected as 1000, 2000 and 3000 $\mu mol_{PAR}\, m^{-2}\, s^{-1}$, depending on the experiment. The photo-bioreactors were equipped with a gas inlet (bottom), a gas outlet (top), a fresh medium inlet, a harvest valve and a port for pH probe. PH was controlled by addition of CO_2 in the airstream on demand according to a set-point, which was also screened (from 6.5 to 9). Aeration rate was kept constant at 33 L (L culture^{-1}) h^{-1} in all photo-bioreactors using a 0.22 μm pore size sparger. The temperature in the photo-bioreactors was controlled by a water jacket and a range of different values was also screened (15 to 35 °C). A steady state was achieved after harvesting a minimum of 3 times the whole volume of the photo-bioreactor and determining at least 5 times the same biomass concentration in 5 non-consecutive days. When steady state was achieved, samples were collected for analytical determinations, always at the same hour of the light cycle (solar noon). All the experiments were done in biological replicates (n = 3).

2.3. Analytical determinations

Biomass was harvested by centrifugation, 10 min at 1500*g*, rinsing with ammonium formate (1%) to remove salts, lyophilized (Virtis Sentry) and stored at −20 °C for posterior biochemical analysis. All analyses were done in triplicates.

For estimation of daily growth, algal biomass concentration was determined as dry cell weight (dcw) and total organic carbon (TOC) [1]. For dcw measures, 0.45 μm pore glass microfiber Whatman GF/C filters were used. Pre-weighted filters containing washed cells were dried in an oven at 80 °C for 24 h before weighing on a precision scale. The total organic carbon concentration in the culture was measured using a TOC analyser (Shimadzu V-$_{CPH}$). In addition, the total organic carbon was used to determine CO_2 fixation rates as described in Eq. (5). TOC-estimated biomass was calculated according to Eq. (2), which integrates the total organic carbon in the culture and the percentage of carbon present in the biomass. Carbon percentage of biomass was previously determined by an elemental analyser (CHNS-O THERMO, FLASH-EA 1112 Series). TOC was measured in the supernatant to discriminate carbon present in the excreted compounds from carbon present in biomass. A modification of the spectrophotometric protocol described by Lin [17] was developed to determine starch content. Lyophilized samples (5 mg) were rinsed in 1 mL of chloroform:methanol solution (2:1 v/v) together with 1 mL of 0.5 mm diameter beads and placed in a bead beater(BioSpec). Following, centrifugation was performed (4500 rpm, 5 min), discarding the supernatant. Starch, present in the pellet, was solubilized (KOH 0,2 M; 100 °C; 30 min), followed by a pH readjustment (pH 5) using acetic acid (1 N). Free glucose residues were obtained by α-amilase and amiloglucosidase (0,2 U μL^{-1} CH_3COONa 0,1 M pH 4.5 y 0,03 U μL^{-1} CH_3COONa 0,1 M pH 4.5 respectively). Free glucose valorisation was done by hexo-kinase (1 U μL^{-1} HEPES 100 mM pH 7.7) and glucose 6 phosphate de-hydrogenase (2,5 U μL^{-1} HEPES 100 mM pH 7.7). Thus, the increase of absorbance (λ = 340 nm) of each sample indicated the content of glucose, therefore starch. The content was referred to a standard curve with different starch dilutions treated in the same way as the samples.

2.4. Measurements and calculations of irradiance

Maximum incident PAR irradiance (I_{max} $\mu mol_{PAR}\, m^{-2}\, s^{-1}$) was measured as photosynthetically active irradiance (PAR) directly emitted by the lamps (4π quantum scalar irradiance sensor QSL-100, Biospherical Instrument, San Diego, CA). The sensor was placed inside the empty photo-bioreactor before inoculation (without cells).

Average PAR irradiance, I_{av} (1000, 2000 and 3000 $\mu mol_{PAR}\, m^{-2}\, s^{-1}$), defines the PAR irradiance available for each cell inside the culture once a steady state is achieved (i.e., the remaining light available after reflection by diffusion and shading). Average PAR irradiance (I_{av}) was calculated as a function of I_{max}, light path (p), biomass concentration (C_b) and the extinction coefficient of the biomass (K_a) as described by Molina-Grima [18] and shown on Eq. (1):

$$I_{av} = \frac{I_{max}}{p \times C_b \times K_a}\left(1 - e^{(-p \times C_b \times K_a)}\right) \quad (1)$$

where I_{max} was different depending on the experiment (3000/2; 2000/2 or 1000/2 $\mu mol_{PAR}\, m^{-2}\, s^{-1}$). The light path (p) was 0.07 m, C_b was the biomass concentration in steady state, and the extinction coefficient (K_a) was experimentally calculated for *Chlorella vulgaris* as 0.6 $m^2\, g^{-1}$.

2.5. Numerical methods

Different culture parameters were determined using the following equations:

1. Organic carbon in biomass:

$$C_{organic\ in\ biomass}\ (g\ L^{-1}) = TOC_{culture}\ (g\ L^{-1}) - TOC_{supernatant}\ (g\ L^{-1}) \quad (2)$$

2. Biomass concentration:

$$Biomass\ concentration\ (g\ L^{-1}) = C_{organic\ in\ biomass}\ (g\ L^{-1}) * \%C_{elemental\ analysis} \quad (3)$$

3. Biomass productivity:

$$Biomass\ productivity\ (g\ L^{-1} d^{-1}) = Biomass\ concentration\ (g\ L^{-1}) * time\ (g\ L^{-1}) \quad (4)$$

4. CO_2 fixation rate:

$$CO_2\ fixation\ rate\ (g\ L^{-1} d^{-1}) = \Delta C_{organic\ in\ biomass}\ (g\ L^{-1} d^{-1}) * (44/12) \quad (5)$$

5. Specific Nitrogen Input (SNI):

$$SNI = \frac{([NaNO_3] * D)}{C_b} \quad (6)$$

The specific nitrogen input (SNI) was determined according to Eq. (6) once steady state was achieved and in order to normalize the cell's nitrogen supply in continuous cultivation. Here, SNI (mmol $NaNO_3$ g biomass^{-1} d^{-1}) is determined by the concentration of $NaNO_3$ supplied in the medium (mM); where D is the dilution rate (d^{-1}) and C_b refers to the biomass concentration (g L^{-1}) in the reactor in steady state.

2.6. Experimental design

We used a single parameter optimization design to optimize biomass productivity and therefore CO_2 assimilation in *Chlorella vulgaris* continuous cultures. This optimization was later used to formulate a model based on different culture parameters. Hence, a sequential study of culture parameters was performed (i.e. pH, dilution rate, temperature, light intensity and nitrogen supply): the optimal value of biomass productivity and CO_2 assimilation found for one parameter was fixed as constant in the following case of study. Afterwards, starch accumulation was modelled considering all studied parameters.

2.7. Statistical analysis

A one-way ANOVA was used to evaluate the optimization experiments. Tukey's test was used as *post-hoc* test for comparison of means. For both tests the level of significance was kept at 0.05. Stat-graphics Centurion XV and Sigma-Plot 12 software were used for statistical analysis. Linear regression was used to assess the fit of the model to experimental data. The result of the linear regression was indicated by the coefficient of determination (r^2), which is present in the text when necessary.

A model was developed to estimate biomass productivity and CO_2 fixation rate in continuous cultures of *C. vulgaris*. This model was based on the Arrhenius equation, with the addition of mathematical propositions from Luedeking and Piret [16] and Molina-Grima et al. [15]. The development of the model is shown in the section of results.

3. Results

In the following section, we described all the experiments performed to optimize biomass productivity and CO_2 fixation rate of continuous cultures of *C. vulgaris*

A. Effect of pH on biomass productivity, CO_2 fixation rate and starch accumulation

In the experiments described in Fig. 1A (Culture conditions: dilution rate 0.4 d^{-1}, temperature 25 °C, I_{max} 3000 µmol$_{PAR}$ m^{-2} s^{-1}, 20 mM NO_3^-), we analysed biomass productivity, CO_2 fixation and starch accumulation under pH values ranging from 6.5 to 9.

Chlorella vulgaris tolerated a broad pH range, with no statistical effect on biomass productivity or CO_2 fixation (0.5 g L^{-1} d^{-1} and 1 g CO_2 L^{-1} d^{-1} respectively) in the range of 6.5 to 8. At pH values of 8.5 and 9, both biomass productivity and CO_2 fixation rate decreased 20% and 65%, indicating that pH had an effect on biomass productivity and CO_2 assimilation ($p < 0.05$). A pH of 7.5 was selected as optimal for the following experiments once it was the middle point in the pH range where cultures showed the highest biomass productivity and CO_2 fixation rate. In this way, it was guaranteed to perform always the culture in the optimal pH range in case of possible failures of the bioreactor.

Starch content was determined once a steady state was achieved (Fig. 1B). It increased under either acid or alkaline pH values, showing a max content when pH was 8.5 (14% dcw).

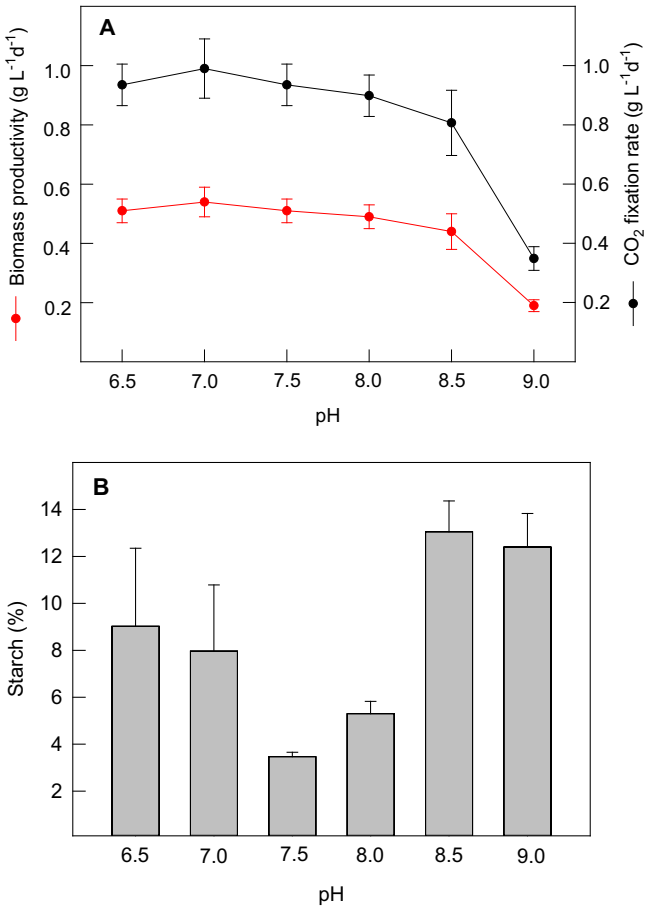

Fig. 1. A) Influence of pH on biomass productivity and CO_2 fixation rate. Culture conditions: Dilution rate 0.4 d^{-1}, temperature 25 °C, I_{max} 3000 µmol$_{PAR}$ m^{-2} s^{-1}, 20 mM NO_3^-. B) Starch accumulation in biomass in the same culture conditions as A. (Error bars correspond to standard deviation, n = 3).

B. Effect of temperature on biomass productivity, CO_2 fixation rate and starch accumulation

Once pH 7.5 was selected as optimal, a range of temperatures (from 15 to 35 °C) was evaluated in the experiments described in Fig. 2A (Culture conditions: dilution rate 0.4 d^{-1}, pH 7.5, I_{max} 3000 µmol$_{PAR}$ m^{-2} s^{-1}, 20 mM NO_3^-).

The highest biomass productivity (0.5 g L^{-1} d^{-1}) and CO_2 fixation rates (1 g CO_2 L^{-1} d^{-1}) were found in the range 15–25 °C. No statistical difference was found in this range of temperatures. At 30 °C, both parameters were reduced to 50%. Higher temperatures (35 °C) were not tolerated by this microalga and the cultures collapsed. ANOVA indicated that there was an effect of temperature on biomass productivity and CO_2 fixation rate ($p < 0.05$). The temperature of 20 °C was selected as optimal for the following experiments since it was the middle point in the temperature range where cultures showed the highest biomass productivity and CO_2 fixation rate. Like pH, this selection guaranteed to perform always the culture in the optimal temperature range in case of possible failures of the bioreactor.

Regarding starch content, the highest accumulation was observed at 30 °C (8% of dcw) (Fig. 2B).

C. Effect of dilution rate on biomass productivity, CO_2 fixation rate and starch accumulation

Once both optimal pH and temperature were selected (7.5 and 20 °C), the effect of dilution rate (from 0.2 to 1.1 d^{-1}) was evaluated.

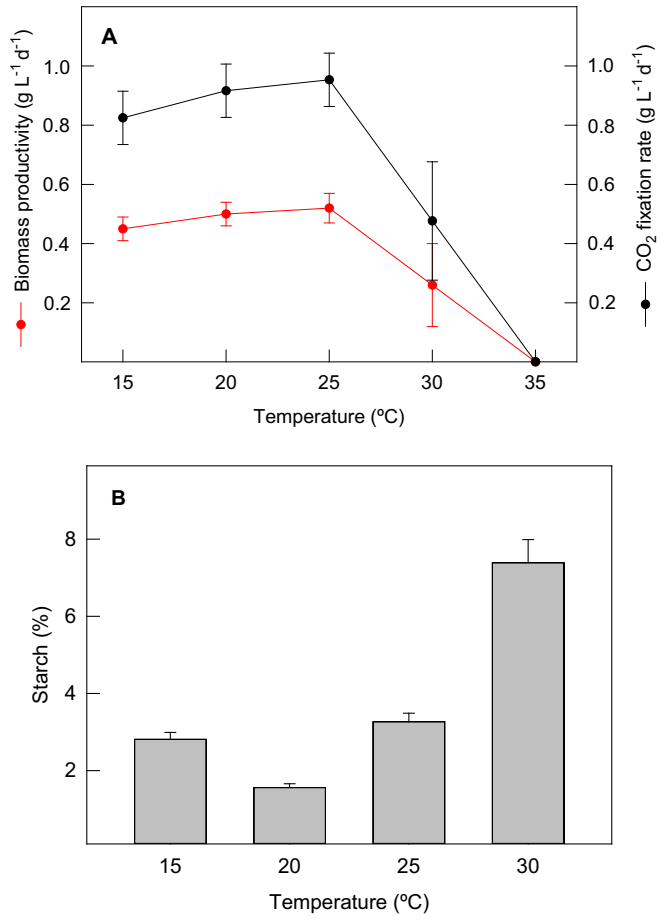

Fig. 2. A) Influence of temperature on biomass productivity and CO_2 fixation rate. Culture conditions: Dilution rate 0.4 d^{-1}, pH 7.5, I_{max} 3000 $\mu mol_{PAR}\ m^{-2}\ s^{-1}$, 20 mM NO_3^-. B) Starch accumulation in biomass in the same culture conditions as A. (Error bars correspond to standard deviation, n = 3).

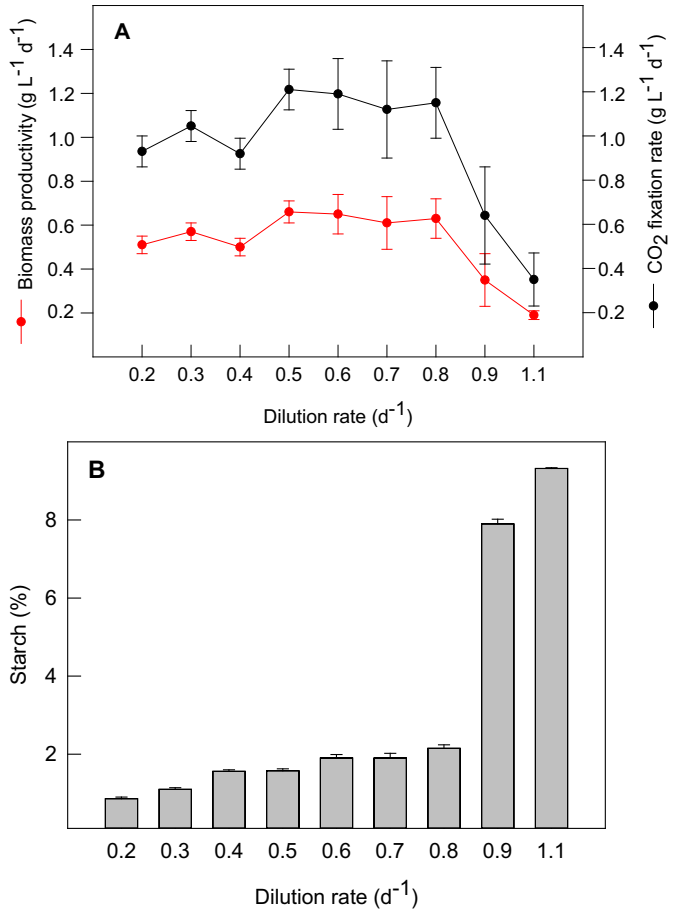

Fig. 3. A) Influence of dilution rate on biomass productivity and CO_2 fixation rate. Culture conditions: temperature 20 °C, pH 7.5, I_{max} 3000 $\mu mol_{PAR}\ m^{-2}\ s^{-1}$, 20 mM NO_3^-. B) Starch accumulation in biomass in the same culture conditions as A. (Error bars correspond to standard deviation, n = 3).

Thus, culture conditions were: temperature 20 °C, pH 7.5, I_{max} 3000 $\mu mol_{PAR}\ m^{-2}\ s^{-1}$, 20 mM NO_3^- (Fig. 3A). The highest biomass productivity was found to be 0.7 $g\ L^{-1}\ d^{-1}$ and was found in the 0.5–0.8 d^{-1} range. At lower dilution rates, biomass productivity and CO_2 fixation showed a reduction of 20%. Nevertheless, at higher dilution rates both biomass productivity and CO_2 fixation rate were 50 to 75% reduced. ANOVA indicated that there was an effect of dilution rate on biomass productivity and CO_2 fixation ($p < 0.05$). The value 0.5 d^{-1} was selected as optimal for the following set of experiments, because this dilution rate required less volume of fresh medium, thus saving nutrients.

Starch accumulation in biomass was trigged when high dilution rates were used (8 and 10% dcw when D = 0.9 and 1.1 d^{-1} respectively) (Fig. 3B).

D. Effect of light intensity on biomass productivity, CO_2 fixation rate and starch accumulation

The next parameter to be analysed was light intensity (1000, 2000 and 3000 $\mu mol_{PAR}\ m^{-2}\ s^{-1}$). The culture conditions were: temperature 20 °C, pH 7.5, dilution rate 0.5 d^{-1}, 20 mM NO_3^- (Fig. 4A). The highest irradiance resulted in the highest biomass productivity and CO_2 fixation rate. Mid irradiance resulted in a 35% decrease in yield, while the lowest output (−60%) was achieved at the lowest irradiance. Light intensity showed an effect on biomass and CO_2 fixation rate (ANOVA, $p < 0.05$). However, the increase of light intensity showed a negative effect on starch accumulation. The highest content was 10%dcw, achieved at 1000 $\mu mol_{PAR}\ m^{-2}\ s^{-1}$, (Fig. 4B).

E. Effect of different nitrate concentration supply on biomass productivity, CO_2 fixation rate and starch accumulation

The last parameter was $NaNO_3$ supply in the medium. Different nitrate concentrations were used: 1, 5, 10 and 20 mM. The culture conditions were: temperature 20 °C, pH 7.5, dilution rate 0.5 d^{-1}, I_{max} 3000 $\mu mol_{PAR}\ m^{-2}\ s^{-1}$. The highest biomass productivity and CO_2 fixation rate (0.65 and 1.3 $g\ L^{-1}\ d^{-1}$ respectively) were reached when the cultures were supplied with 20 mM $NaNO_3$ (Fig. 5A). When the cultures were supplied with 5 and 10 mM $NaNO_3$, similar yields were obtained. However, biomass productivity and CO_2 assimilation decreased significantly (0.12 g and 0.25 $L^{-1}\ d^{-1}$, respectively) when the culture was supplied with 1 mM $NaNO_3$. The results of the ANOVA showed that $NaNO_3$ concentration affected both biomass productivity and CO_2 assimilation ($p < 0.05$).

The content of starch was reduced when the culture was supplied with high nitrate concentration. The content of starch in the biomass increased to 20%dcw when the cultures were supplied with 1 mM $NaNO_3$(Fig. 5B).

F. Modelling biomass productivity and CO_2 fixation rate in continuous cultures

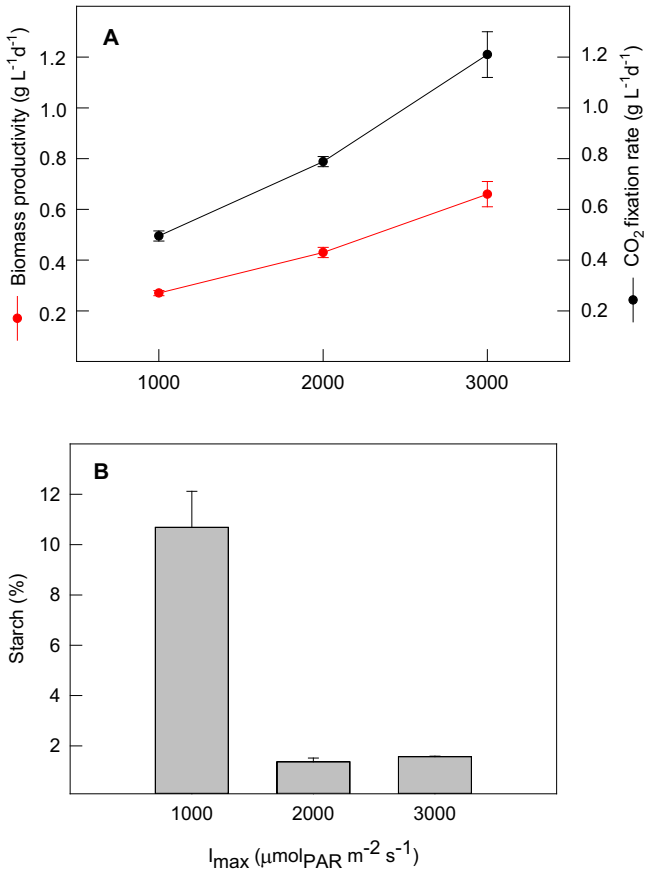

Fig. 4. A) Influence of max light intensity (I_{max}) on biomass productivity and CO_2 fixation rate. Culture conditions: temperature 20 °C, pH 7.5, Dilution rate 0.5 d^{-1}, 20 mM NO_3^-. B Starch accumulation in biomass in the same culture conditions as A. (Error bars correspond to standard deviation, n = 3).

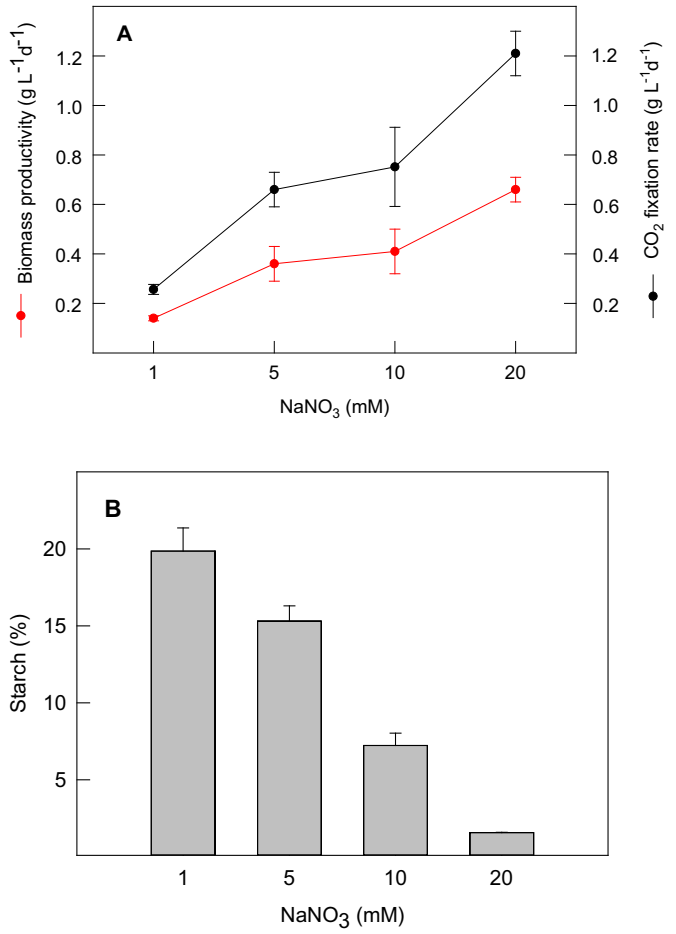

Fig. 5. A) Influence of $NaNO_3$ supply on biomass productivity and CO_2 fixation rate. Culture conditions: temperature 20 °C, pH 7.5, dilution rate 0.5 d^{-1}, I_{max} 3000 $\mu mol_{PAR}\, m^{-2}\, s^{-1}$. B) Starch accumulation in biomass in the same culture conditions as A. C) starch accumulation during light hours in the same culture conditions. (Error bars correspond to standard deviation, n = 3).

Once all culture parameters were combined, the statistical analysis indicated that biomass productivity and CO_2 fixation rate were influenced by averaged irradiance (I_{av}), temperature, specific nitrogen input (SNI) and pH (ordered by importance).

All parameters were adjusted to Eq. (7) by nonlinear regression ($r^2 = 0.95$), with the following constants: $\mu_{max} = 1{,}2\ d^{-1}$; n = 1,5; $I_k = 35{,}5\ \mu mol_{PAR}\, m^{-2}\, s^{-1}$; A1 = $3{,}67{\cdot}10^5\ d^{-1}$; $E_{a1} = 3{,}22{\cdot}10^4\ J\ mol^{-1}$; $A_2 = 2{,}97{\cdot}10^{12}\ d^{-1}$; $E_{a2} = 7{,}3{\cdot}10^4\ J\ mol^{-1}$; $k_{SNI} = 2{,}15\ mmol\ g^{-1}\ d^{-1}$; $A_3 = 4{,}97\ d^{-1}$; $E_{a3} = 4{,}81\ J\ mol^{-1}$; $A_4 = 3{,}27{\cdot}10^5\ d^{-1}$; $E_{a4} = 109{,}4\ J\ mol^{-1}$.

$$\mu(I_{av}, T, SNI, pH) = \left[\frac{\mu_{max} * I_{av}^n}{I_k^n + I_{av}^n}\right] * \left[A_1 * e^{\left(\frac{E_{a1}}{RT}\right)} - A_2 * e^{\left(\frac{E_{a2}}{RT}\right)}\right] * \left[\frac{SNI}{K_{SNI} + SNI}\right] * \left[A_3 * e^{\left(\frac{E_{a3}}{pH}\right)} - A_4 * e^{\left(\frac{E_{a4}}{pH}\right)}\right] \quad (7)$$

In Eq. (7), $\mu(I_{av},T,SNI,pH)$ represents the growth rate influenced by light average (I_{av}), temperature (T), specific nitrogen input (SNI) and pH. The first element of the equation represents the effect of light on growth. μ_{max} (d^{-1}) represents the maximum specific growth; I_{av} is the average irradiance ($\mu mol_{PAR}\, m^{-2}\, s^{-1}$); n is a shape factor to describe the transition from weak to strong illumination and I_k ($\mu mol_{PAR}\, m^{-2}\, s^{-1}$) is the light saturation constant that defines the light affinity of this microalga and corresponds to I_{av} where $\mu = \mu_{max} / 2$.

The second element of the equation represents the effect of temperature (T) on growth rate. The Arrhenius equation was used to fit the experimental data. A_1 represents the positive effect of temperature on growth until 20 °C, whereas A_2 represents the negative effect at high temperatures. The energy activation for both parameters is defined by E_{a1} and E_{a2}. R is the universal gas constant.

The third element represents the effect of nitrogen availability on growth rate determined as specific nitrogen supply (mmol $g^{-1}\ d^{-1}$) and influenced by a semi-saturation constant, K_{SNI} (mmol $g^{-1}\ d^{-1}$).

The forth element describes the effect of pH on growth rate. A_3 represents the positive effect of pH on the growth until the value of 8, whereas the negative effect of a pH higher than 8 is represented by A_4. E_{A3} and E_{A4} represent the energy activation for both parameters, while *e* represents the mathematical constant.

G. Surface response plot for biomass productivity and CO_2 fixation rate

Once the model was proposed, it was possible to generate a surface response plot for the biomass productivity and CO_2 fixation rate under different culture conditions (Fig. 6A and B). Under optimal growth conditions (pH 7.5; temperature 20 °C; 0.5 d^{-1}; I_{max} 3000; 20 mM NO_3^-), the model predicts a biomass productivity of 0.6–0.7 g $L^{-1}\ d^{-1}$, very similar to experimentally determined values (0.67 g $L^{-1}\ d^{-1}$). Therefore, under these conditions cultures would assimilate 1.3 g CO_2 per liter and day.

H. Starch accumulation model in continuous cultures

The main scope of this study was the optimization of culture conditions to maximize biomass productivity and CO_2 fixation rate. In addition to this, starch was determined during the same experiments to

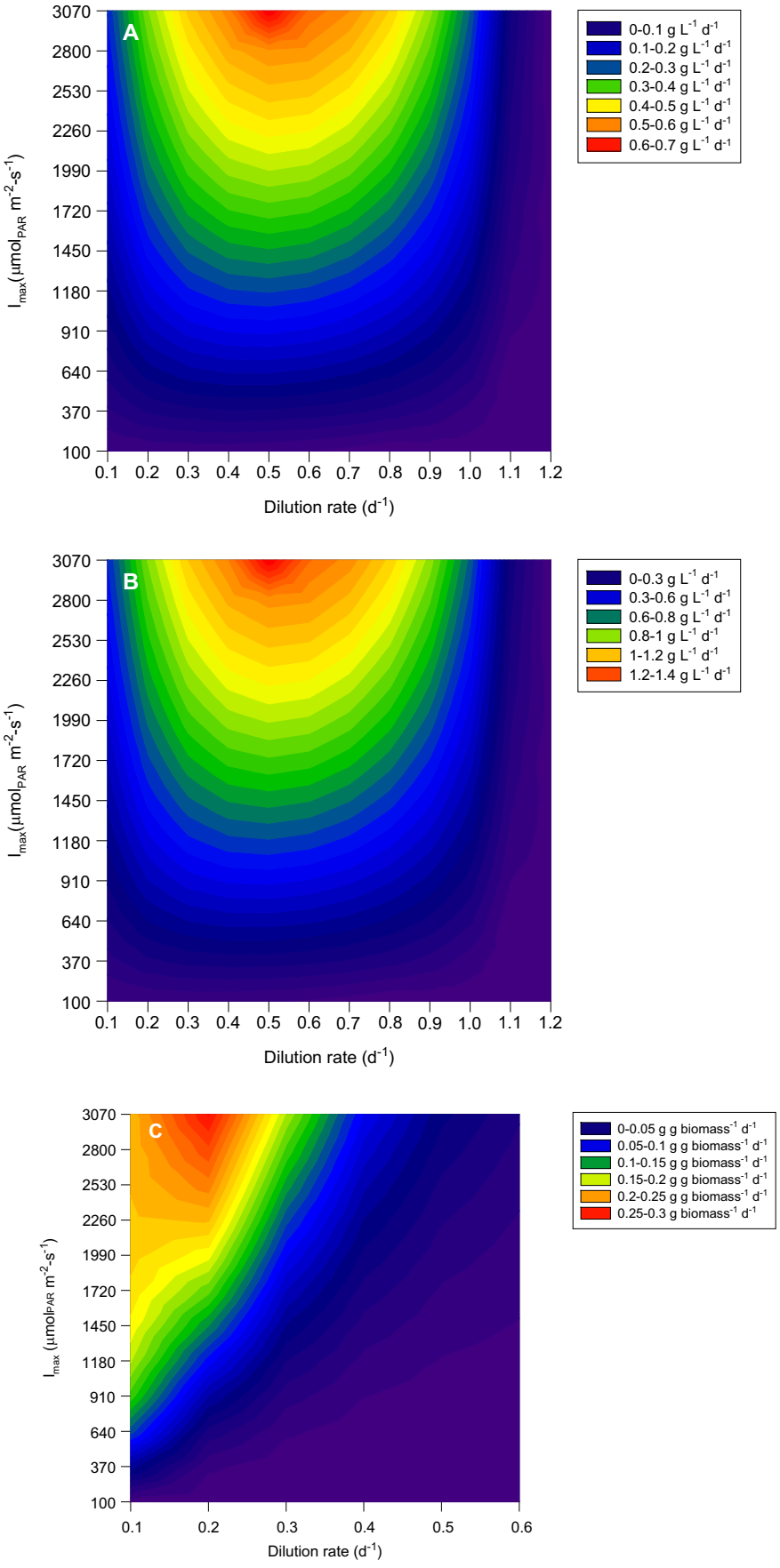

Fig. 6. A) Prediction of biomass productivity according to the proposed model. Culture conditions: temperature 20 °C, $NaNO_3$ 20 mM, pH 7.5. B) Prediction of CO_2 assimilation under the same conditions as A. C) Prediction of starch accumulation according to the proposed model. Culture conditions: 20 °C, pH: 7.5, I_{max} 3000 $\mu mol_{PAR}\, m^{-2}\, s^{-1}$.

model its accumulation. Starch accumulation was mainly influenced by dilution rate and specific nitrogen input (SNI). The data were adjusted to the equation proposed by Luedeking and Piret [19] (Eq. (8)). The accumulation of starch in biomass (Q_p) was influenced by the summative effect of two factors; First, the production of starch linked to the primary metabolism, $Y_{p/x}$ (g starch g biomass^{-1}), depends on growth rate (μ). The second factor (β; g starch g biomass^{-1}) represents the accumulation of starch under stress conditions such as nitrogen depletion.

$$Q_p = Y_{p/x} * \mu + \beta \quad (8)$$

However, the β factor depends on dilution rate and SNI. Thus, Eq. (8) might be extended to Eq. (9) where K_c and K_i represent the semi-saturation and the inhibition constants regarding SNI, while a, b, and m are geometric parameters. The parameters were adjusted by nonlinear regression ($r^2 = 0.84$) as follows: $Y_{p/x} = 0.016$ g g^{-1}; a = 0.00005772; b = 3.18; $\beta_{max} = 3.4$ mg starch g biomass^{-1} d^{-1}; $K_i = 0.3$ mmol $NaNO_3$ g^{-1} d^{-1}; $K_c = 1.7$ mmol NO_3^- g^{-1} d^{-1}; m = 1.72.

$$Q_p = [Y_{p/x} * \mu] + [a * e^{b*D}] + \left[\frac{\beta_{max} * (SIN * k_i)^m}{[(SIN * k_i) + (k_s * k_i) + SIN^2]^m} \right] \quad (9)$$

I. Surface response plot for starch accumulation

According to the model, a maximum starch accumulation of 0.25 g starch g biomass^{-1} is predicted when D = 0.2 d^{-1}, $NaNO_3$ = 1 mM, and $I_{max} = 3000$ μmol$_{PAR}$ m^{-2} s^{-1} (Fig. 6C). However, in these conditions the biomass productivity and CO_2 assimilation would decrease to 0.2 and 0.37 g L^{-1} d^{-1}.

4. Discussion

Modelling is an important tool to understand and improve operational procedures of microalgae production [20]. The main scope of this work was the optimization of the culture parameters to maximize the autotrophic growth and therefore the CO_2 assimilation of *C. vulgaris* in continuous cultures. Our results were used to develop a model to simulate the response of *C. vulgaris* to different culture conditions and consequently to define the optimal ones. For this purpose, photo-chemostat (dilution occurs only during the light hours) was chosen because of its advantages when compared with other operational processes such as batch or semi-batch. These advantages are: a constant daily biomass productivity with similar biochemical composition and a constant harvesting volume in steady state [21]. Thus, the specific effect of each culture parameter on *C. vulgaris* performance was individualized and this allowed us to generate an accurate growth model. Moreover, starch accumulation during this optimization was determined and used to generate a model to predict the pattern of starch accumulation in CO_2 abatement process. The optimization process of this model followed the same approach as the previous model, i.e., “single parameter optimization” [14].

pH was the first studied parameter, as the capability to tolerate a broad range of pH values is a key feature of microorganisms in CO_2 abatement process [22]. pH can modify the $CO_3^{2-}/HCO_3^-/CO_2$ equilibrium in the culture, affecting the CO_2 availability for the algae [23]. Our results verified that *C. vulgaris* showed a wide tolerance to pH variations (from 6 to 9). The optimal value found in this work was in the range from 6.5 to 8.5, although pH 7.5 was selected as set-point for the rest of the experiments as being considered the middle value of the range where *Chlorella* showed the highest biomass productivity. This result is comparable to those published by Rincón et al. [24], who found that the optimum pH in *C. vulgaris* biofilm cultivations varied from 6.4 to 7.5, depending on the light incidence in the bioreactor. On the other hand, it has been described that *C. sorokiniana* tolerates extremely alkaline pH values (pH > 10) [25], highlighting the good tolerance of *Chlorella* species to wide pH changes.

The following parameter was temperature. The control of this parameter represents one of the most costly factors from a large-scale cultivation point of view [26]. Additionally, it influences the microalgal biochemical composition [27]. Although *C. vulgaris* could tolerate temperature variations from 15 to 30 °C, the optimum determined in this work was in the range from 15 to 25 °C. Like pH, the middle value, 20 °C, was selected for next experiments. The optimal temperature found in this research was lower than those published by Wang et al. or Huesemann et al. [28,29]. These publications described that some *Chlorella* species dwell in the range from 25 to 45 °C. However, recent studies have reported that *C. vulgaris* in the range from 20 to 28 °C [30,31], similarly to our results. Although *C. vulgaris* might not be classified as a psychrophilic microalgae as some diatoms [32], its adaptability to relatively low temperatures must be considered as a promising feature for cold climate cultivations (where the control of temperature in the photo-bioreactors represents a substantial part of the costs [33]).

Following, we studied the effect of dilution rate. This parameter determines the cell growth rate because it influences directly nutrient and light availability; therefore, having a direct impact in the biochemical composition. A steady state is achieved once both rates are equal (D = μ) [34]. Our data suggested that *C. vulgaris* possesses a resilient growth, adapting to different dilution rates. *C. vulgaris* showed a maximum productivity in a wide range of dilution rates, from 0.5 to 0.8 d^{-1}; almost two times the optimal dilution rate found by Tang et al. for *Chlorella minutissima* [35]. This capability of adapting its growth to different dilution rates has been reported in previous studies although in a smaller range of dilution rates [36,37]. Matos et al. reported the maximum biomass productivity (0.12 g L^{-1} d^{-1}) at 0.3 d^{-1}, lower than our result (0.56 g L^{-1} d^{-1}) at the same dilution rate. On the other hand, D-H Cho et al. determined a maximum biomass productivity of 1 g L^{-1} d^{-1} at 0.75 d^{-1}, rendering an increase of 33% regarding the best yield of our work (0.67 g L^{-1} d^{-1}). Most likely, these differences are caused by using different culture mediums and temperature/pH set-points. Regardless, our results suggested the similar trend described in the above-mentioned publications: lower dilution rates result in lower biomass productivities (although higher biomass concentrations). Other operational regimes such as batch or semi-continuous have been tested for this microalga in other studies but rendering lower yields (0.3 g L^{-1} d^{-1}) [38]. Regarding other microalgal species cultivated photo-autotrophically in continuous fed as *Scenedesmus*, *Chlorococcum* or *Pseudokirchneriella* [14,39], *Chlorella* might be defined as a fast growing and efficient CO_2 assimilation microorganism. Considering our data, *C. vulgaris* might be a candidate for a fast turn-over process thanks to its capability of adapting its growth to fast dilution rates. In our work, the lowest dilution rate (0.5 d^{-1}) was chosen for the following experiments. This was done to handle less volume of medium and therefore simulate large-scale strategies, saving nutrients consequently.

The incident irradiance on reactor's surface was also evaluated. We tested a range of light intensities that embraced the maximum irradiances that typically can be found outdoors at different locations and/or environmental situations (from low intensities typically found in northern latitudes, 1000 mol$_{PAR}$ m^{-2} s^{-1} to extremely high light intensities found in more meridional locations, 3000 mol$_{PAR}$ m^{-2} s^{-1}) [14,34,40]. However, as San Pedro et al. showed [41], the effect of light might be considered as light availability (I_{av}) and not just as the light irradiance that impinges on the reactor's surface. I_{av} is defined as the average light that each cell receives and it is strongly influenced by the imposed dilution rate (which affects directly the biomass concentration in the culture) [42]. Indeed, Cuaresma et al. [43] and Molina Grima et al. [44] showed the direct relationship between dilution rate and light availability. Our results are in line with these researchers, showing that light availability is the major factor that influences microalgal cultures [45]. *C. vulgaris* rendered the highest biomass productivity, and therefore CO_2 assimilation, at the highest light availability. Similar to other Chlorella species, *C. vulgaris* showed a good adaptability to a wide range of irradiances [43,46]. Nevertheless, understanding which light

intensity causes cellular photoinhibition is as equally important as determining the optimum irradiance. Although some publications report damages on *Chlorella* photosynthetic apparatus at lower light intensities (from 100 to 500 $\mu mol_{PAR}\, m^{-2}\, s^{-1}$) [47–49], our data indicated that the cells did not suffer photo-inhibition under the light regime used in our research. The excess of light is the major responsible for photo-inhibition but there are other factors that have also a direct effect (such as nitrogen depletion) [50]. Thus, the observed differences between the results from other research groups and our results might be attributed to the effect of nitrogen depletion, which typically occurs in batch mode. The dilution rate used in the experiments for studying the effect of the different irradiances ($0.5\, d^{-1}$) ensured a sufficient nutrient supply, avoiding situations of nitrogen starvation. Hence, we can state that *C. vulgaris* did not experience photo-inhibition under high irradiance in our experiments. This robustness to deal with high light intensities makes *C. vulgaris* a suitable candidate for large-scale outdoors cultivation [51].

The last parameter to study was the nitrogen supply. This parameter has a strong influence in the performance of the microalgal cultures [41]. Generally, C flux is driven to the production of lipids and carbohydrates in N-depleted situations, impacting culture yields and the biochemical composition of biomass [52,53]. On the other hand, nitrogen supply is considered one of the culture parameters that increase the most the production costs [54,55]. Thus, we considered important to study deeply how nitrogen supply affects the culture of *C. vulgaris*. Like irradiance, it was necessary to normalize the effect of N in biomass productivity and CO_2 assimilation. Therefore, the specific nitrogen input (SNI) parameter was considered [41]. Our data indicated that the higher nitrogen input, the better *C. vulgaris* performed. This confirmed that an excess of nitrogen supply does not lead to growth inhibition. However, our results show that *C. vulgaris* needs more nitrogen input than other microalgal species such as Nannochloropsis, Rhodomonas, Isochrysis or Choricystis to render the maximum biomass productivity [56,57]. Notwithstanding, we consider that this characteristic must be considered when using this microalga in wastewater treatments [58–60].

Once all the parameters were optimized, the results were used to define a model for biomass productivity (and therefore CO_2 fixation as *C. vulgaris* was cultivated auto-phototrophically). The literature is extensive regarding growth models for *Chlorella* species, with the majority related to batch cultures [61–64]. The ones under continuous mode only include a few culture parameters such as light or temperature [65,66]. The growth model presented in this research represents a comprehensive approach, as it includes five different culture parameters, achieving a good fit to the experimental data ($R^2 = 0.95$). Like other growth models for species as *Scenedesmus* or *Nannochloropsis*, our results confirmed that irradiance and temperature are the main parameters that affect the microalgal performance [14,41], followed by nitrogen supply and pH. The growth model allowed us to combine the optimum culture parameters that resulted in maximum yields. Thus, the model predicts that *C. vulgaris* can produce up to 0.7 g biomass $L^{-1}\, d^{-1}$ under optimal conditions (meaning 1.3 g $CO_2\, L^{-1}\, d^{-1}$). Hai-Xing Chang et al. [66] and Bechet et al. [65] described theoretical growth models for *C. vulgaris* as well, predicting a CO_2 assimilation rates of 1.6 and 1.8 g $L^{-1}\, d^{-1}$ respectively. However, these models were obtained from cultures under 24 h of continuous light, conditions that are not realistic when compared with outdoor cultivations. Regarding light intensities and photo-period, the range of irradiances used in our research simulated light conditions that typically are found outdoors [34] and therefore is more applicable to large-scale cultivation. The model predicted that *C. vulgaris* shows a slightly better yields under similar light intensities than *Scenedesmus vacuolatus*, strain previously modelled for CO_2 abatement purposes by our group [14].

As a secondary aim, the starch content was determined during the optimization phase. Our goal was to combine starch production with maximum biomass productivity and CO_2 assimilation. Certainly, from an economic point of view, the use of starch as a raw material for production of bioethanol, bioplastics, chemicals or animal feed might be only justified in the biorefinery framework [68,69] and not as the ultimate and single product of *C. vulgaris*.

Starch is considered a linking factor between primary and secondary metabolism [70]. Hence, starch is characterized by a fast metabolic turn-over, providing a fast energy source through respiration during dark periods, while lipids are used to provide energy in situations of long-term stress [67]. Starch production occurs during primary metabolism, however its accumulation mainly happens during stress conditions that affect the microalgal growth, especially nitrogen depletion [71]. It represents the most abundant storage polysaccharide in *C. vulgaris* [72]. A wide range of starch contents (from 16 to 55% dcw) in different microalgal species has been reported from a myriad of heterogeneous culture conditions [73] also including mixotrophic approaches [74].

In this work, it has been demonstrated that nitrogen availability (SNI) and dilution rate (D) are the parameters that show the major influence on starch accumulation in continuous cultures. Light is described as the culture parameter that shows a powerful influence in starch production. Indeed, Brányiková et al. reported that starch synthesis is triggered by high light intensities in microalgae as a result of an increase in the photosynthetic process [75]. In addition, it has been proposed that an increase in starch content happens at the end of the light period in *Acutodesmus obliquus* cultures [76], highlighting the relationship between light and starch production. However, it is important to differentiate synthesis from consumption: our results refer to the stored starch and not to de novo starch or used for growth and proliferation. We hypothesize that under high light intensities more starch is used for growth, thus less accumulation happens.

Our results suggested that the culture conditions that lead to a high biomass productivity (i.e. high values of pH, temperature, dilution rate, irradiance and $NaNO_3$ supply), also lead to low starch accumulation. This trend is caused by the use of starch in microalgal growth and proliferation. Indeed, it has been reported that starch is used for cell division in *Neochloris oleoabundans* [77]. Behrens et al. [78] found a maximum starch accumulation in *C. vulgaris* batch cultures in a slightly alkaline pH (7.5–8). Also, Dammark et al. (2018) [79] predict that *Tetraselmis* sp. can accumulate 0.64 g starch g $biomass^{-1}$ (64% dwc) at pH 7. However, these results are under batch and constant light (24 h) cultivations. Typically, nutrients and light depletion occurs at the end of stationary phase in batch cultures, affecting directly the storage of energy compounds [80].

The effect of temperature on starch accumulation was studied during the 70's and 80's [81]. It was shown that extreme temperatures affect positively the starch accumulation because of blocking algal division. However, this trend stopped when temperature was high enough to inhibit photosynthesis and to be lethal. Our data agree with those results, showing that higher temperature lead to higher starch accumulation. Other strategies to increase the starch content have been considered. Not only macronutrients, such as phosphorus and sulphur starvation might be used [82,83], but also micronutrients starvation as iron, manganese or zinc [84], or inhibition of nuclear DNA replication and cytoplasmic proteo-synthesis [81,85]. These findings are in line with our data: culture conditions that affect growth increase the starch accumulation in microalgal biomass.

There are some models on starch accumulation in *C. vulgaris* published but only for batch cultures and not combining starch accumulation to CO_2 abatement [56,57]. These publications focused on different culture parameters than ours, specifically on nutrient composition or mixotrophic cultivations. Other studies report higher starch accumulation than ours, reporting up to 67% dcw [75,86]. However, these results correspond again to batch cultures, where the effect of different stress factors, such as shadowing or nutrient depletion (specifically nitrogen starvation), cannot be easily quantified.

5. Conclusions

The goal of this work was to evaluate the culture conditions to maximize biomass productivity and CO_2 assimilation in continuous cultures of *C. vulgaris*. These results were used to define a growth model. In addition to this, the accumulation of starch in biomass during this optimization was modelled. The optimal conditions for enhancing the biomass productivity and hence the rate of CO_2 fixation were as follows: temperature 20 °C, pH 7.5, $D = 0.5\,d^{-1}$; max light intensity of $3000\,\mu mol_{PAR}\,m^{-2}\,s^{-1}$ and nitrogen supply at 20 mM $NaNO_3$; similar conditions to those predicted by the growth model. In these conditions, *C. vulgaris* produces $0.67\,g\,biomass\,L^{-1}\,d^{-1}$ (corresponding to a CO_2 fixation rate of $1.23\,g\,L^{-1}\,d^{-1}$). Regarding the model for starch accumulation, it predicted a starch accumulation lower than 5% in the optimal conditions for growth (predictions in line with experimental data). Thus, if the objective is to generate a starch-rich biomass linked to CO_2 abatement, dilution rate and SNI must change to the following values: $D = 1.1\,d^{-1}$ and $SNI = 1\,mmol\,NaNO_3\,g\,biomass^{-1}\,d^{-1}$. In these culture conditions, it is possible to have a continuous and daily production of biomass with high content in starch (25%), but biomass productivity and CO_2 fixation would decrease ($0.2\,g\,L^{-1}\,d^{-1}$ and $0.37\,g\,CO_2\,L^{-1}\,d^{-1}$ respectively). Hence, to implement a biological system for CO_2 abatement coupled to starch accumulation, it is necessary to find a compromise between these two processes. Although yields in both processes would be reduced, a simultaneous process for CO_2 mitigation and starch production could be feasible.

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Authors' contributions

García-Cubero: Conception, experimental design and set-up, experimental work, analysis and interpretation of the data, statistical analysis of the data, drafting of the manuscript and final approval of the manuscript. Moreno-Fernández: experimental design and set-up, critical revision of the manuscript and final approval of the manuscript. Acién-Fernández: analysis and interpretation of the data, statistical analysis of the data, critical revision of the manuscript and final approval of the manuscript. García-González: Obtaining of funding, conception, experimental design, analysis and interpretation of the data, critical revision of the manuscript and final approval of the manuscript.

Conflict of interest

The authors declare no conflict of interest.
No conflicts, informed consent, human or animal rights applicable.

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