Bioresource Technology 212 (2016) 207–216

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

A novel culture medium designed for the simultaneous enhancement of biomass and lipid production by *Chlorella vulgaris* UTEX 26

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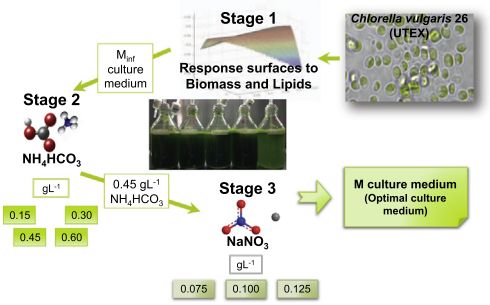
HIGHLIGHTS

- A new culture medium for *Chlorella vulgaris* UTEX 26 was designed.
- Biomass and lipid concentrations were increased.
- The new culture medium can be used to perform recycling cultures.
- Main fatty acids from *Chlorella vulgaris* UTEX 26 were C16 and C18.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 January 2016
Received in revised form 16 March 2016
Accepted 11 April 2016
Available online 13 April 2016

Keywords:
Chlorella vulgaris
Response surface method
Culture medium
Ammonium bicarbonate
Fatty acids

ABSTRACT

A novel culture medium to enhance the biomass and lipid production simultaneously by *Chlorella vulgaris* UTEX 26 was designed in three stages of optimization. Initially, a culture medium was inferred applying the response surface method to adjust six factors [$NaNO_3$, NH_4HCO_3, $MgSO_4{\cdot}7H_2O$, KH_2PO_4, K_2HPO_4 and $(NH_4)_2HPO_4$], which were selected on the basement of BBM (Bold's Basal Medium) and HAMGM (Highly Assimilable Minimal Growth Medium) culture media. Afterwards, the nitrogen source compound was optimized to reduce both, ammonium and nitrate concentrations. As result of the optimization process, the proposed culture medium improved 40% the biomass (0.73 g L^{-1}) compared with the BBM medium and 85% the lipid concentration (281 mg L^{-1}), with respect to HAMGM medium. Some culture media components concentrations were reduced up to 50%. Gas chromatography analysis revealed that C16:0, C18:0, C18:1, C18:2 and C18:3 were the major fatty acids produced by *C. vulgaris* UTEX 26.

1. Introduction

Microalgae production offers the potential to produce bioenergy as well as high value compounds, wastewater treatment, nitrogen fixing and CO_2 mitigation (Hadj-Romdhane et al., 2012; Mendoza et al., 2013). Microalgae have been taken into consideration as a feedstock for renewable biofuels production, such as bioelectricity, methane produced by anaerobic digestion of the algal biomass, biohydrogen produced under anaerobic conditions, bioethanol (sugar fermentation) and biodiesel derived from microalgal oil (Hadj-Romdhane et al., 2012; Aguirre et al., 2013; Fon Sing et al., 2013; Ramanna et al., 2014).

For large-scale microalgae culture, open ponds or raceways are used by their low cost (Fon Sing et al., 2013). However, to improve the microalgae production in this kind of culture system, it is necessary to use an adequate culture medium selected for every microalgae species, different culture strategies (continuous or sequenced batch) and to recycle the wasted culture medium.

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http://dx.doi.org/10.1016/j.biortech.2016.04.051
0960-8524/

Conventional culture media are not specific to a particular microalgae. The Bold's Basal Medium (BBM) is widely used for various kinds of microalgae (Blair et al., 2014). Meanwhile, the Highly Assimilable Minimal Growth Medium (HAMGM) was designed by Hadj-Romdhane et al. (2012) for *Chlorella vulgaris* culture in an air-lift photobioreactor (PBR) in continuous regimen and with the objective of recycling the wasted culture medium. Microalgal growth in raceway, exhibits low biomass productivity compared with the obtained in an air-lift PBR (Fon Sing et al., 2013), thereby, the nutrients of HAMGM medium could be in excess when the culture is performed in raceway. The main differences between BBM and HAMGM culture media are the source and amount of nitrogen and carbon. BBM medium contains 0.25 g L^{-1} of $NaNO_3$ as nitrogen source, while HAMGM medium contains 1.099 g L^{-1} of NH_4HCO_3 as nitrogen and carbon source.

Microalgal cells consist mainly of carbohydrates, proteins and lipids, but their biochemical composition varies among species. Under optimal growth conditions, unicellular microalgae synthesize protein to maintain cell growth while carbohydrate and lipid are present in lower quantities (Das et al., 2011). An optimal medium formulation is also critical to ensure sufficient and stable supply of nutrients to attain maximal growth rate, and ultimately to produce biofuels at higher efficiencies (Schenk et al., 2008).

Several studies have been focused on modifying the concentrations of conventional culture media components (Blair et al., 2014) to increase biomass and/or lipid production. Fertilizers and organic wastes have been reported as alternative nitrogen and carbon sources (Giordano et al., 2014). Recently, microalgae growth in wastewater for biofuels production has taken greater importance (He et al., 2013; Wu et al., 2013; Ramanna et al., 2014). Moreover, statistical models and response surfaces plots have been applied to understand the correlations among several factors such as temperature, light intensity, nitrogen nutrient content and carbon dioxide concentration that affect the growth rate and lipid content in microalgal species (Chen et al., 2014). However, most of these studies have been conducted in flasks or small photobioreactors, therefore, the reports about the application of these culture media to large scale cultures are still limited.

In the present study, a new culture medium was designed to improve the biomass and lipid concentrations from *C. vulgaris* UTEX 26. The medium composition was initially based on both, BBM and HAMGM culture media. Afterwards, the initial concentrations of the nitrogen source were modified to determine the effect on the production of biomass and lipids.

2. Materials and methods

2.1. Microalgae strain and growth conditions

C. vulgaris UTEX 26 strain was acquired from the *Culture Collection of Algae*, Texas University (Austin, Texas, EE.UU.). Prior to use it, *C. vulgaris* UTEX 26 was grown autotrophically and axenically in BBM medium in a 1 L PBR with a working volume of 0.9 L, and bubbled with air at a constant flow rate of 0.5 vvm. The PBR was illuminated with light intensity of 180 μmol photons m^{-2} s^{-1} on the surface of the reactor by using cool white fluorescent tubes placed by one side of the PBR, with a photoperiod of 12 h of light: 12 h of darkness and the temperature was maintained at 24 ± 1 °C. The growth conditions were the same for all the experiments.

2.2. Novel culture medium design

The optimization strategy proposed for obtaining the new culture medium was divided in three stages (Fig. 1). For the first one, the response surface method (Zamudio-Pérez et al., 2014) was applied in order to obtain the best combination of factors considered in this experiment. Two response surfaces were generated for each one of the 15 combinations of factors, which in turn were obtained from the combination in pairs of 6 factors (Table 1). The culture medium (M_{inf} medium) that resulted from this stage was not appropriate to elaborate a conclusion on the nitrogen source and, it was used to initiate the second stage, in which a new NH_4HCO_3 concentration was established. In the third stage, the $NaNO_3$ concentration was modified to obtain the novel culture medium (M medium) proposed in this study.

2.2.1. Selection of the macronutrients concentration by response surface method

For the design of the new culture medium, the composition of both BBM and HAMGM culture media were considered, a response surface method was established in the first stage. The experimental design involved six factors named $NaNO_3$ (F1), NH_4HCO_3 (F2), $MgSO_4{\cdot}7H_2O$ (F3), KH_2PO_4 (F4), K_2HPO_4 (F5) and $(NH_4)_2HPO_4$ (F6), all of them with five levels (Table 1), and therefore, five different culture media (M1, M2, M3, M4 and M5) were obtained (Taguchi-like experimental design). Each level was chosen because this one generated definite response surfaces for all pair of factors (15 combinations). For every pair of factors, a response surface was made for each response variable. Biomass and lipid concentrations were proposed as the response variable. These ones were determined on days 8, 12, 16 and 20 of culture.

An average of the trace metals concentrations of the BBM and HAMGM culture media was used as the trace metals concentration for the five culture media. The pH was adjusted at 7 by adding 1 N NaOH or 2 N HCl solutions when necessary. Culture media were sterilized at 121 °C for 15 min, except for NH_4HCO_3 and $(NH_4)_2HPO_4$, which were sterilized by filtration with 0.22 μm membrane filters. The batch experiments started up with a biomass concentration of 0.02 g L^{-1}.

2.2.2. Effect of the concentration and nitrogen sources

In the second stage, NH_4HCO_3 concentration was varied at four different levels (0.15, 0.30, 0.45 and 0.60 g L^{-1}), without $NaNO_3$ addition. Biomass and ammonium (NH_4^+) concentrations, as well as pH were determined on days 0, 4, 8, 10, 12, 14 and 16 of culture. The lipid concentration was determined on the sixteenth day of culture. The initial biomass concentration in the batch experiments was of 0.012 g L^{-1}.

In the third stage, three different levels of $NaNO_3$ (0.075, 0.100 and 0.125 g L^{-1}) were tested, with the concentration of NH_4HCO_3 previously established. $NaNO_3$ was added on the eighth day of culture because nitrate (NO_3^-) could not be consumed due to the presence of ammonium in the reactor before that moment. Biomass concentration was determined on days 0, 4, 8, 12, 14, 16 and 20 of culture. The lipid concentration was determined on the sixteenth and twentieth days of culture.

Ammonium and nitrate concentrations, as well as pH were also determined during the experiments. The initial biomass concentration in the batch experiments was of 0.012 g L^{-1}. The obtained culture medium was named M medium and it was compared with the HAMGM and BBM culture media.

2.3. Analytical methods

2.3.1. Biomass determination

Dry biomass was determined gravimetrically. The sample was filtered through a glass microfiber membrane (Ahlstrom, 4.7 cm diameter, 0.7 μm pore size) to constant weight. Afterwards, the membrane was dried at 60° C for 48 h in a digital oven Riossa ECF-91, after drying it was cooled down in a desiccator and weighed. The dry biomass was determined by weight difference.

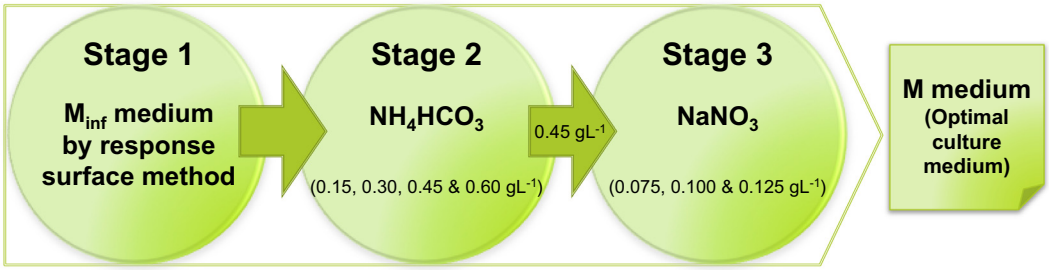

Fig. 1. Optimization strategy proposed to obtain a novel culture medium to improve the production of biomass and lipids from *Chlorella vulgaris* UTEX 26.

Table 1
Experimental design to evaluate the factors effect on *Chlorella vulgaris* UTEX 26 cultures, biomass and lipid concentrations on day 16.

Culture media	F1 $NaNO_3$	F2 NH_4HCO_3	F3 $MgSO_4{\cdot}7H_2O$	F4 KH_2PO_4	F5 K_2HPO_4	F6 $(NH_4)_2HPO_4$	Responses	
	g L^{-1}						Biomass (g L^{-1})	Lipids (mg L^{-1})
M1	0	1.099	0.075	0.073	0	0.164	0.49	137.8
M2	0	0.769	0.159	0.165	0.075	0.139	0.56	243.3
M3	0.125	0.550	0.131	0.124	0.038	0.082	0.58	217.8
M4	0.250	0.440	0.120	0.081	0.015	0.033	0.61	142.2
M5	0.250	0	0.187	0.175	0.075	0	0.39	134.4

The specific growth rate (μ) corresponding to the exponential growth phase was calculated according to:

$$\mu = \frac{X_1 - X_0}{t_1 - t_0} \quad (1)$$

where: X_1 represents the dry biomass (g L^{-1}) at time t_1 (d), and X_0 represents the initial concentration (g L^{-1}) at time t_0 (d).

2.3.2. Determination of pH, ammonium and nitrate

The pH determination was performed using a potentiometer Jenway model 3520 and an electrode glass body.

Ammonium concentrations were determined colorimetrically (APHA-AWA-WPCF, 1992). All the analyses were carried out with the culture supernatant obtained after the centrifugation of the sample at 3823*g* for 10 min (Hermle Z306, Germany).

The nitrate concentration in the medium was determined colorimetrically (modified of Black, 1995) by using 0.5 mL of culture supernatant obtained after centrifugation of the sample at 3823*g* for 10 min, then it was placed in a test tube and dried at 100 °C for 12 h in an oven Riossa H62. A 0.5 mL portion of diphenylsulfonic acid was added to the dry sample followed by the addition in four pulses of 2.2 mL of 12 N KOH solution during constant mixing. A sample of 100 µL of supernatant mixed with 900 µL of distilled water was read at 410 nm using a spectrophotometer GENESYS 10S UV–Vis (Thermo Scientific, USA). The nitrate concentration was calculated with a standard curve of the culture medium prepared within a range of 0–500 mg L^{-1} of NO_3^- as $NaNO_3$.

2.3.3. Cell disruption, lipid extraction and quantitative lipid determination

A sample of 30 mL of microalgae culture was centrifuged at 3823*g* for 15 min (Hermle Z306, Germany). The cell pellet was hydrolyzed with 5 mL of 0.4 N HCl solution at 90 °C in a dry bath for 2 h. Subsequently, the sample was cooled and 10 mL of isopropanol were added and shaken at 250 rpm for 1 h at room temperature, afterwards, 15 mL of hexane were added and shaken at 250 rpm for 1 h at room temperature. Then, 5.0 mL of distilled water were added to disrupt the phases. Then the mixture was placed in a separated funnel and the upper layer (oil extract) was recuperated. The oil extract was filtered through a glass microfiber membrane (Ahlstrom, 2.5 cm diameter, 0.7 µm pore size) in a column with anhydrous sodium sulfate, and then recovered in a vial at constant weight. The solvent was evaporated in a dry bath at 45 °C for 48 h. The weight of the total lipids obtained from each sample was determined by weight difference. The samples were analyzed in triplicate.

2.3.4. Fatty acids methyl esters (FAMES) determination

The lipid composition was determined as FAMES by gas chromatography after direct lipid transesterification. The total lipids extracts (Section 2.3.3) were transferred to a tube with screw cap and 1 mL of 0.5 N methanolic HCl solution was added. The sample was heated in a dry bath at 80 °C for 2 h, then 200 µL of distilled water were added to terminate the reaction (Ortega et al., 2004). After cooling at room temperature, 1 mL of hexane was added and mixed, then the upper layer (fatty acids methyl esters) was collected for analysis. FAMES were detected by gas chromatography in a Clarus 500 GC (Perkin Elmer Precisely, USA) equipped with a flame-ionization detector and a 30 m long capillary column (AT^{TM} – WAX of 30 m × 0.25 mm ID × 0.20 µm film thickness). The temperatures of injector and detector were maintained at 230 and 250 °C, respectively. The oven temperature was initially set at 140 °C for 5 min and increased at a rate of 8 °C min^{-1} until 250 °C for 15 min. Helium was used as the carrier gas at a flow rate of 20 cm s^{-1}. Standard curves of individual FAME were set by using analytical standard FAMES (Sigma, USA). For each measurement, 1 µL of the sample was injected into the GC.

2.4. Data analysis

All the experiments were carried out in triplicate. The response surfaces were obtained with MATLAB R2013a (8.1.0.604) program. The results for the adjustment of the nitrogen source and the comparison of culture media were subjected to an ANOVA analysis ($p < 0.05$) to identify the relative significance of every treatment.

3. Results and discussion

3.1. Selection of the macronutrients concentration

Generally, high concentrations of biomass and lipids were found on opposite sides of the response surfaces; however, on

day 16, regions of high biomass and lipid concentrations were found in the center of the response surfaces, which correspond to intermediate values for each factor (Figs. 2 and 3). Additionally, combinations of F1 with other factors allowed inferring concentrations for every factor to obtain high biomass and lipid concentrations (Figs. 2 and 3). The values for every factor for both high concentrations of biomass and lipids were found just on day 16 of culture. Only the analysis of response surfaces for this day was described.

On day 16, the region of high concentration of biomass corresponded to a range of 0.55–0.61 g L^{-1} and was observed as a band across the response surface. This region was favored by all the concentrations of $NaNO_3$, intermediate concentrations of NH_4HCO_3 and $MgSO_4{\cdot}7H_2O$, and intermediate and lower concentrations of KH_2PO_4, K_2HPO_4 and $(NH_4)_2HPO_4$ (Figs. 2 and 3). However, a small region of maximum biomass concentration was favored by an increase of $NaNO_3$ concentrations and a decrease of all the others factors. With the M2, M3 and M4 culture media, high biomass concentrations were obtained (Table 1), which were 164% higher than the biomass concentration from *Chlorella sorokiniana* cultured in wastewater with sixfold higher nitrogen concentration (Ramanna et al., 2014) than the included in the M4 culture medium. The M4 culture medium, with μ of 0.207 d^{-1}, produced the highest biomass with 0.61 g L^{-1}.

On the other hand, the M5 medium, with $NaNO_3$ as the only source of nitrogen showed the lowest biomass concentration from *C. vulgaris* UTEX 26 (0.39 g L^{-1}). This result may be due to the absence of HCO_3^-, unlike of the other culture media, and due to the intracellular conversion of NO_3^- via the nitrate reduction pathway to nitrite (NO_2^-), followed by nitrite reduction to NH_4^+ catalyzed by nitrite reductase and ferredoxin. These two reactions require NADH, and a decrease in cellular NADH would minimize the production of intracellular lipids, proteins and chlorophyll formation (Ramanna et al., 2014).

The region where high lipid concentration (215–243 mg L^{-1}) was obtained on day 16 of culture was favored by low $NaNO_3$ concentrations and high concentrations of all the other factors (Figs. 2 and 3). For this region, lipid concentrations of 243.3 and 217.8 mg L^{-1} were obtained with the M2 and M3 culture media, respectively. These lipid concentrations were threefold higher than those obtained with *Chlorella* sp. MIC-G4 grown in BBM medium under stirring conditions for 8 days with a nitrogen source and for another 8 days without a nitrogen source (Ratha et al., 2013).

For the culture medium inferred (M_{inf}) from the response surfaces, factor concentrations located in the center of the response surfaces domain were chosen in order to guarantee both high biomass and lipid concentrations. The formulation of M_{inf} medium is the following (g L^{-1}): $NaNO_3$ 0.125, NH_4HCO_3 0.600, $MgSO_4{\cdot}7H_2O$ 0.135, KH_2PO_4 0.130, K_2HPO_4 0.040, $(NH_4)_2HPO_4$ 0.100, $CaCl_2{\cdot}2H_2O$ 0.025, Na_2EDTA 0.0375, $FeSO_4{\cdot}7H_2O$ 0.0037, $ZnSO_4{\cdot}7H_2O$ 0.0099, H_3BO_3 0.0086, $MnCl_2{\cdot}4H_2O$ 0.0019, $CuSO_4{\cdot}5H_2O$ 0.0012, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.0007 and $CoCl_2{\cdot}6H_2O$ 0.0006.

In the M1, M2, M3 and M4 culture media, the nitrogen sources were in excess. The NH_4^+ uptake was close to 50% in M1 and M2 culture media whereas it was complete in the M3 and M4 culture media. On the other hand, the NO_3^- uptake was low (2% and 15%, respectively) in the M3 and M4 culture media.

In all the M culture media, the phosphorus concentration was in excess (data not shown), however, under nitrogen deficiency, it has been reported that phosphorus assimilated by microalgal cell is

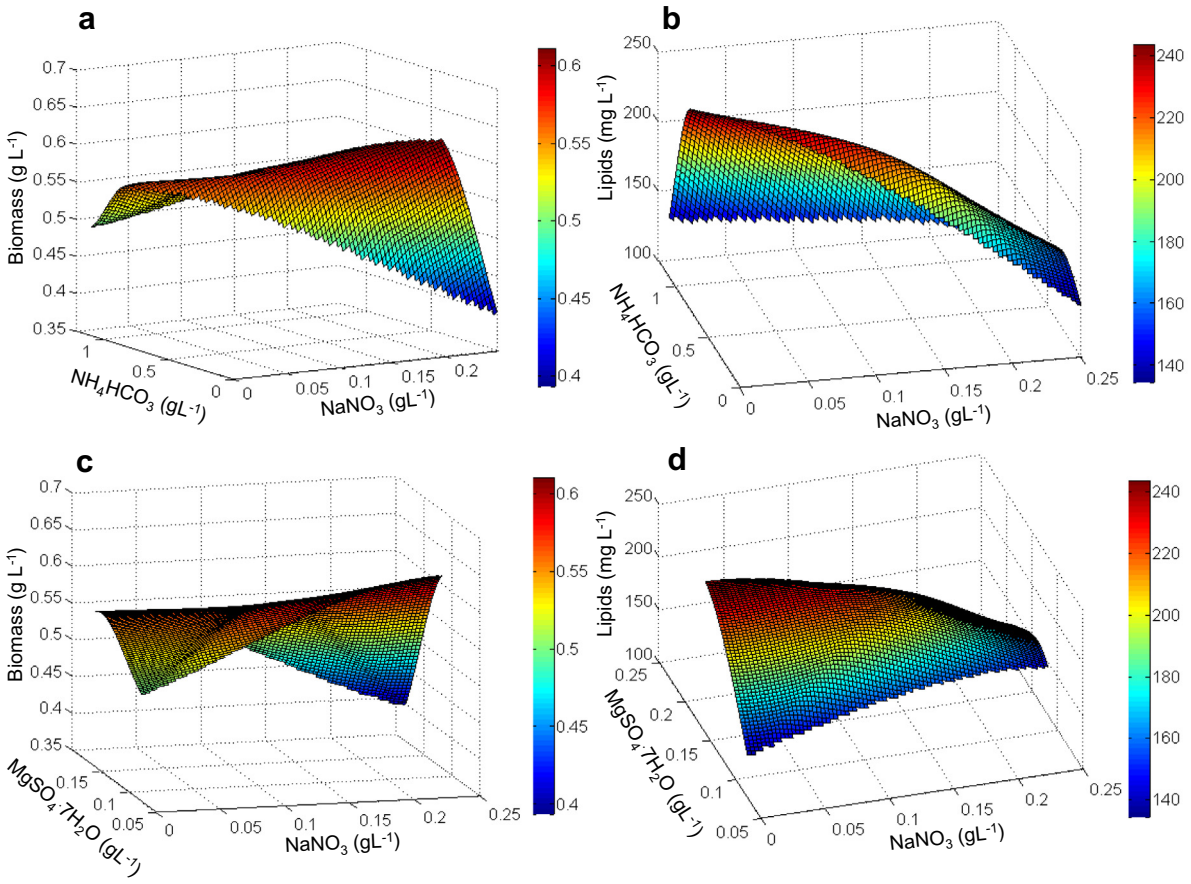

Fig. 2. Response surfaces on day 16. (a) Biomass as a response of the $NaNO_3$ and NH_4HCO_3 concentrations. (b) Lipids as a response of the $NaNO_3$ and NH_4HCO_3 concentrations. (c) Biomass as a response of the $NaNO_3$ and $MgSO_4{\cdot}7H_2O$ concentrations. (d) Lipids as a response of the $NaNO_3$ and $MgSO_4{\cdot}7H_2O$ concentrations.

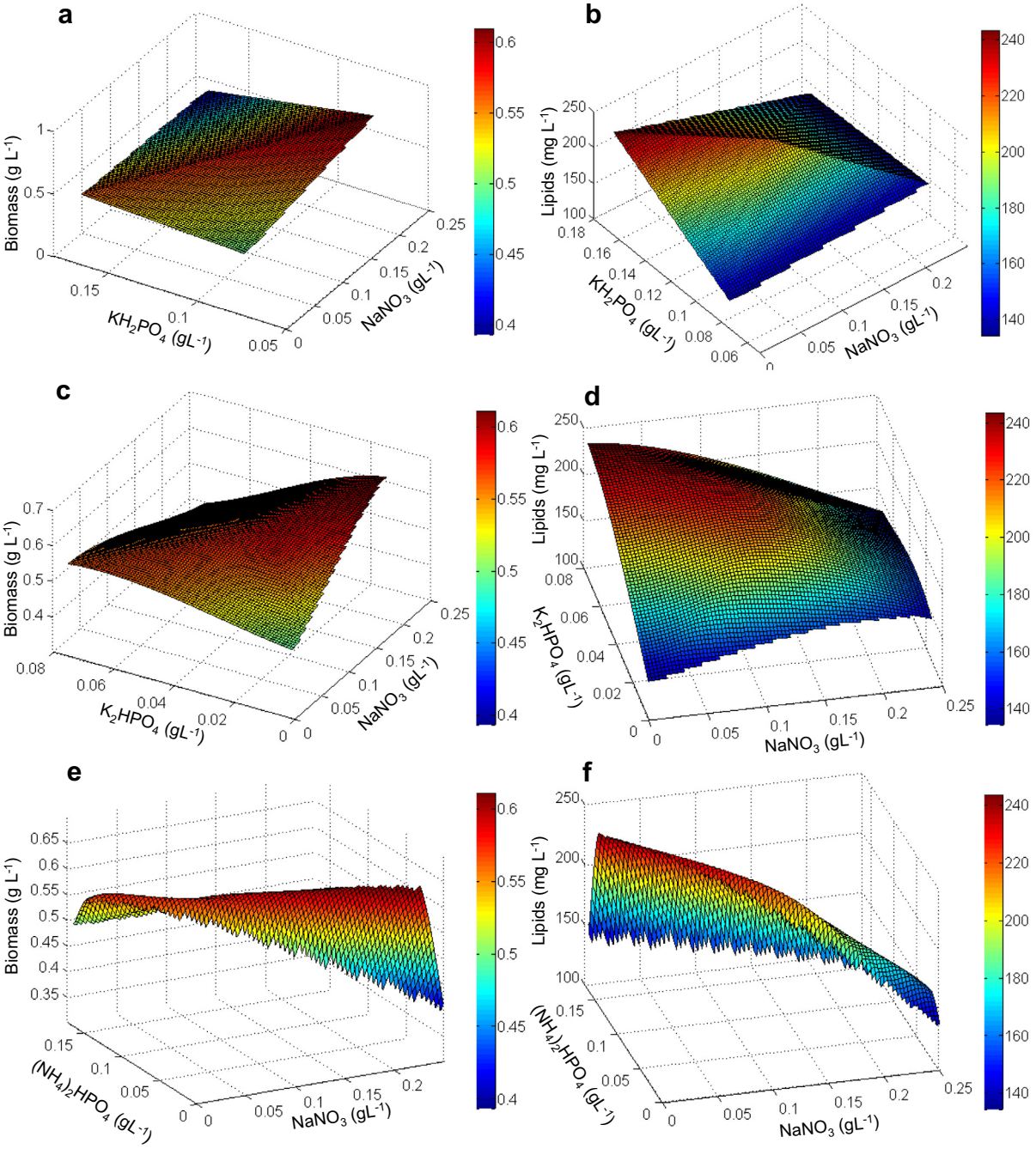

Fig. 3. Response surfaces on day 16. (a) Biomass as a response of the $NaNO_3$ and KH_2PO_4 concentrations. (b) Lipids as a response of the $NaNO_3$ and KH_2PO_4 concentrations. (c) Biomass as a response of the $NaNO_3$ and K_2HPO_4 concentrations. (d) Lipids as a response of the $NaNO_3$ and K_2HPO_4 concentrations. (e) Biomass as a response of the $NaNO_3$ and $(NH_4)_2HPO_4$ concentrations. (f) Lipids as a response of the $NaNO_3$ and $(NH_4)_2HPO_4$ concentrations.

used to synthesize enzymes for the lipid synthesis, DNA, RNA, ATP and intermediate metabolic products (Chu et al., 2013).

The results obtained in the first stage allowed the diminishment of the high concentrations of nutrients from the HAMGM and BBM culture media. However, the nitrogen sources were in excess.

3.2. Screening nitrogen sources

Nitrogen is available in several forms, the nitrogen speciation that is supplied to the microalgae may affect the cellular composition by altering the lipid content and fatty acid composition, as well as the growth rate and culture stability (Borowitzka and Moheimani, 2013; Ramanna et al., 2014).

The most appropriate nitrogen source and concentration must be identified for each microalgal specie. In order to investigate what the optimal concentration for the nitrogen source for cell growth and lipid accumulation is while considering an excess of the source of nitrogen in M_{inf} medium, four levels (0.15, 0.30, 0.45 and 0.60 g L^{-1}) of NH_4HCO_3 were tested without $NaNO_3$ and the next step was the application of three levels of $NaNO_3$ (0.075, 0.100 and 0.125 g L^{-1}) with the concentration of NH_4HCO_3 previously established. Based on these experiments, the effects of decreasing the nitrogen sources concentration and the simultaneous addition of NH_4HCO_3 and $NaNO_3$, on production of biomass and lipids from *C. vulgaris* UTEX 26, were evaluated.

3.2.1. Selection of ammonium bicarbonate concentration

An increase in NH_4HCO_3 concentration exerted a positive influence in *C. vulgaris* UTEX 26 growth (Fig. 4). All treatments completed the exponential growth phase after 12 days of culture. On day 12, a 70% and 80% of NH_4^+ available was consumed (Fig. 4) and then the pH was between 4.0 and 5.0 from that point to the end of culture period. The highest biomass concentration (0.70 g L^{-1}) was achieved on 16 days of culture with 0.45 and 0.60 g L^{-1} of NH_4HCO_3, with a μ of 0.329 and 0.332 d^{-1}, respectively. The lowest biomass concentration (0.47 g L^{-1}) was obtained with 0.15 g L^{-1} of NH_4HCO_3. Only in the culture medium with 0.15 of NH_4HCO_3, NH_4^+ was completely consumed, while in the other three treatments, more than 85% of NH_4^+ was utilized. These results indicate that the microalgal growth was proportional to the amount of nitrogen in the culture medium. Similarly, Blair et al. (2014), found twofold more biomass concentration of *C. vulgaris* grown with 0.25 g L^{-1} than with 0.062 g L^{-1} of $NaNO_3$. On the other hand, in the treatment with 0.45 g L^{-1} of NH_4HCO_3 as inorganic nitrogen and carbon source (additional to the CO_2 provided by the aeration), biomass production was 150% higher than 0.28 g L^{-1} of *Auxenochlorella protothecoides* KRT1009 produced by Bohutskyi et al. (2014) in a phototrophic culture, with an excess of CO_2 as carbon source and a nitrogen concentration 65% higher than the provided by 0.45 g L^{-1} of NH_4HCO_3.

The highest lipid concentrations, 153 and 132 mg L^{-1}, on day 16 were obtained with 0.30 and 0.45 g L^{-1} of NH_4HCO_3, respectively, and there was no significant difference between both NH_4HCO_3 concentrations. Therefore, a concentration of 0.45 g L^{-1} of NH_4HCO_3 treatment was chosen for the third stage. The lowest lipid concentration (93 mg L^{-1}) was obtained with 0.60 g L^{-1} of NH_4HCO_3. This lipid concentration was 50% higher than lipid concentration obtained with *A. protothecoides* KRT1009, which was produced by Bohutskyi et al. (2014) in a photoautotrophic culture, with an excess of CO_2 and a nitrogen concentration 23% higher than the provided by 0.60 g L^{-1} of NH_4HCO_3.

3.2.2. Selection of sodium nitrate concentration

The three different concentrations of $NaNO_3$ (0.075, 0.100 and 0.125 g L^{-1}) did not exert any effect on biomass concentration, and there was no significant difference with the M medium, which contains 0.45 g L^{-1} of NH_4HCO_3 as the unique nitrogen source (Fig. 5a). On day 12, 86% of NH_4^+ available was consumed in all treatments (Fig. 5b), then, the pH was decreasing until 4.0 from that point to the end of the culture period. The μ was 0.225 d^{-1} for all the treatments. At the end of the culture the biomass concentration was 0.72 g L^{-1}.

$NaNO_3$ was added on day 8, when 42% of the available NH_4^+ had been consumed (Fig. 5b); however, at the end of the culture there was not NO_3^- uptake. Similar results were found by Wu et al. (2013) and Ramanna et al. (2014), these authors described that if the nitrogen source in the culture medium was comprised of both, NH_4^+ and NO_3^-, the NH_4^+ consumption is favored over NO_3^-. The NH_4^+ uptake does not require a redox reaction for its assimilation and thus it needs less energy. The NH_4^+ is directly absorbed by passive diffusion and accumulated to form amino acids (Liu et al., 2015). The interaction between NO_3^- and NH_4^+ uptakes indicated that NH_4^+ itself does not inhibit the NO_3^- uptake, but a product of NH_4^+ assimilation causes a rapid and reversible inactivation of NO_3^- transport (Wu et al., 2013).

In regards of lipid concentration, on day 16 of culture, there was no significant difference among treatments and M medium, which reached an average lipid concentration of 160 mg L^{-1}. Nevertheless, on day 20, the M medium showed the highest lipid concentration (281 mg L^{-1}), which was significantly different ($p < 0.05$) from

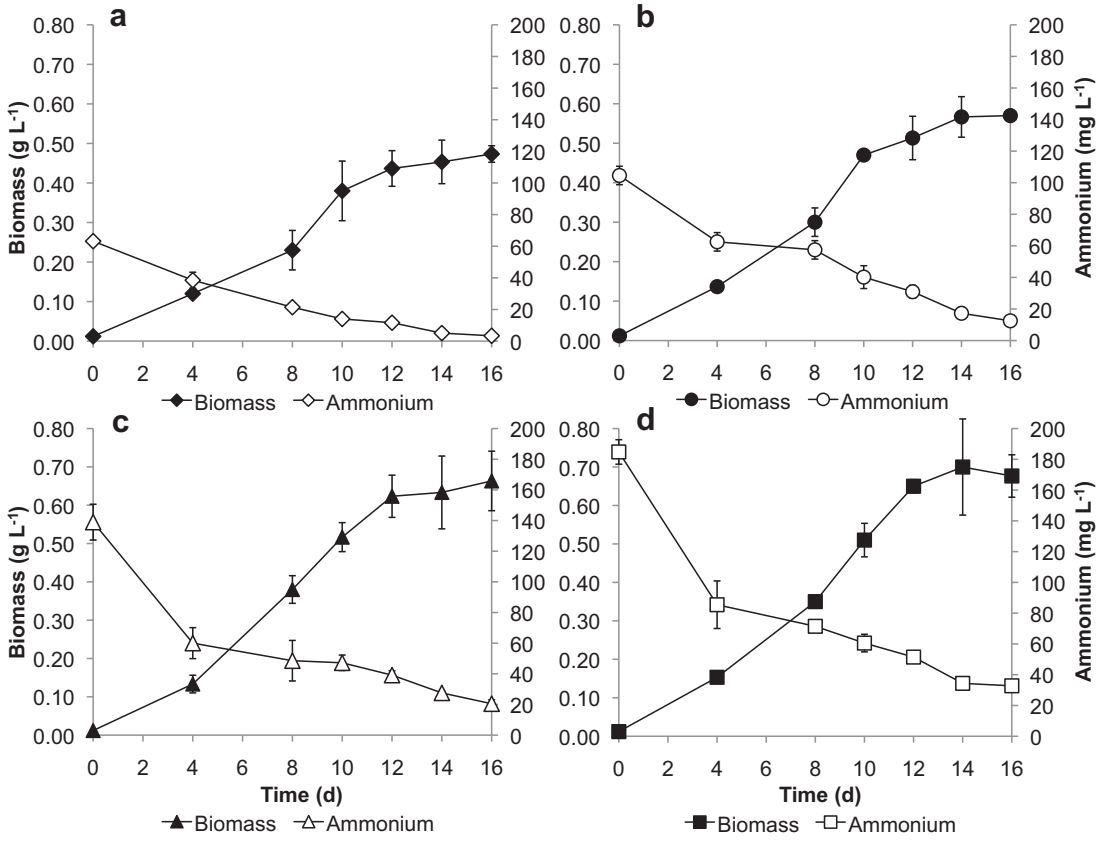

Fig. 4. Profiles of biomass and ammonium concentrations as a function of culture time for *Chlorella vulgaris* UTEX 26 grown in the culture media with NH_4HCO_3. (a) Treatment with 0.15 g L^{-1} of NH_4HCO_3. (b) Treatment with 0.30 g L^{-1} of NH_4HCO_3. (c) Treatment with 0.45 g L^{-1} of NH_4HCO_3. (d) Treatment with 0.60 g L^{-1} of NH_4HCO_3.

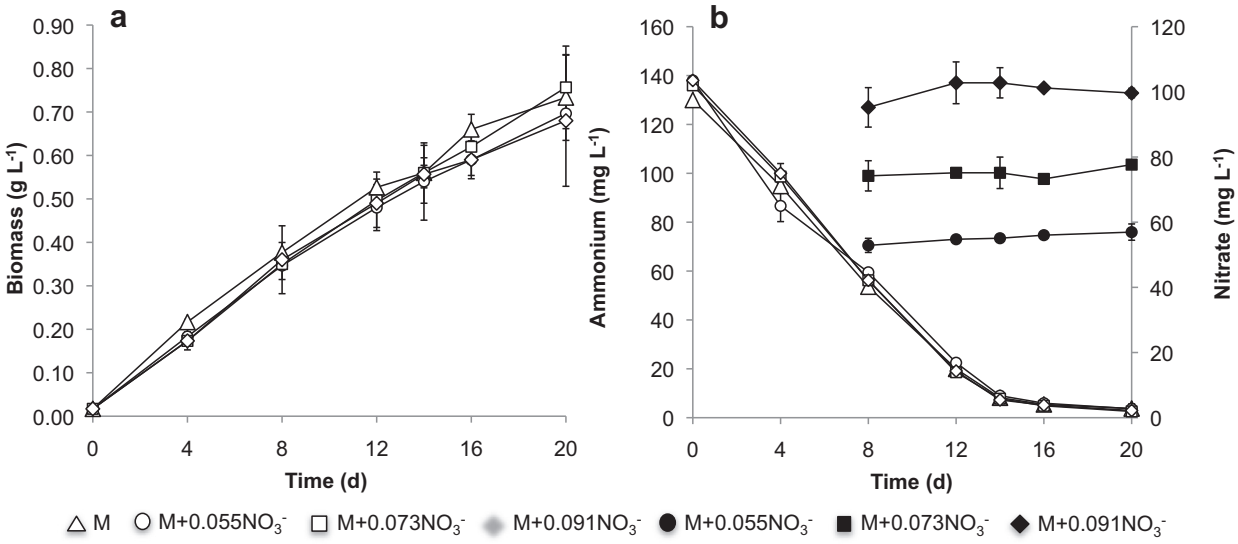

Fig. 5. (a) Profiles of biomass concentration as a function of culture time for *Chlorella vulgaris* UTEX 26 in the culture media with NH_4HCO_3 and $NaNO_3$. (b) Profiles of ammonium (white symbols) and nitrate (black symbols) concentrations as a function of culture time for *Chlorella vulgaris* UTEX 26 grown in the culture media with NH_4HCO_3 and $NaNO_3$.

the other treatments. The increase of lipids from *C. vulgaris* UTEX 26 on day 20 in M medium was due to a nitrogen limitation during four days (Fig. 5b). It has been reported that nitrogen stress conditions are effective to induce neutral lipid accumulation within microalgal cells (Adams et al., 2013; Griffiths et al., 2014; Singh et al., 2014). Nitrogen limitation results in cellular changes such as: reduction of the cellular content of the thylakoid membrane, acyl hydrolase activation and induction of phospholipid hydrolysis (Xin et al., 2010). These changes lead to an increase in intracellular fatty acid acyl-CoA while nitrogen limitation activates diacylglycerol acyltransferase, which converts acyl-CoA to triacylglycerides (TAGs) (Takagi et al., 2000). Consequently, nitrogen deficiency ultimately leads to an increase in lipid and TAG content in microalgal cells (Hu et al., 2008; Schenk et al., 2008; Xin et al., 2010).

Lipid concentration from *C. vulgaris* UTEX 26 on day 20 in M medium was 52% higher than the treatments with NH_4^+ and NO_3^-, which presented a higher nitrogen concentration than the M medium. Similar results were reported by Ramanna et al. (2014), they found that an increase in the urea concentration led to a decrease of lipid concentration when *C. sorokiniana* was cultured.

3.3. Comparison of M medium with the HAMGM and BBM culture media

The results of biomass and lipid production from *C. vulgaris* UTEX 26 achieved with the M medium were compared with the results obtained with two conventional culture media (BBM and HAMGM). For our culture conditions, M medium does not contain an excess of nitrogen as HAMGM medium, and if required, the M medium could be recycled because it does not contain any poorly assimilated ions (Na^+ or Cl^{-1}) by *C. vulgaris* (Hadj-Romdhane et al., 2012).

The M medium achieved a positive effect on *C. vulgaris* UTEX 26 growth (Fig. 6a). From the fourth day of culture, biomass concentration was twofold higher than in the HAMGM and BBM culture media. Significant differences ($p < 0.05$) between M and BBM culture media continued until the end of the microalgal culture. On the other hand, from the twelfth day until the end of the microalgal culture, no differences between M and HAMGM culture media were observed. On day 16, all the available sources of nitrogen of the culture media had been consumed, except for HAMGM medium (Fig 6b). *C. vulgaris* UTEX 26 cultivated in M medium exhibited a μ of 0.23 d^{-1} and a biomass concentration of 0.66 g L^{-1}; which was 61% higher than the biomass obtained with BBM medium and threefold higher than the biomass concentration of *C. sorokiniana* cultured by Ramanna et al. (2014), in wastewater with a nitrogen concentration ninefold higher than the included in the M medium.

The highest biomass concentration was obtained on day 20 of culture at 0.73 g L^{-1} with M medium. This result was 45% higher than the biomass concentration of *C. vulgaris* obtained by Yeh et al. (2010), a work in which 152% more carbon source was utilized in comparison to the M medium. Furthermore, it has been reported by Mandalam and Palsson (1998) and Blair et al. (2014) that addition of major elements (nitrogen, phosphorus or magnesium) can increase the overall capacity of a culture medium to produce biomass, however, with the culture conditions used in this work the result were different. The HAMGM medium contains a nitrogen and carbon source 146% higher than the M medium and there was no significant difference for biomass production between the M and HAMGM culture media. Moreover, Griffiths et al. (2014) used 0.17 g L^{-1} of NO_3^- (0.038 g L^{-1} of nitrogen) and they achieved a biomass concentration of 1.4 g L^{-1} on day 20 of culture, which was 1.9-fold higher than biomass obtained with M medium. Nevertheless, considering the molecular formula of *C. vulgaris* ($CH_{1.776}O_{0.459}N_{0.148}S_{0.006}P_{0.010}$) reported by Hadj-Romdhane et al. (2012), the biomass concentration reported by Griffiths et al. (2014) could not be confirmed stoichiometrically with the added nitrogen. This amount of biomass could be achieved because the microalgal culture was supplied with air enriched with 0.29% CO_2 and stressed 18 days under nitrogen starvation. It has been established that CO_2 stimulates the lipid production (Moheimani, 2013).

Regarding lipid concentration, there was no significant difference among the productions obtained on day 16 with M, HAMGM and BBM culture media (Fig. 6c). The M culture medium obtained a lipid concentration of 160 mg L^{-1}.

At the end of the culture (on day 20), the highest lipid concentration was obtained with M medium (281 mg L^{-1}), which was significantly different ($p < 0.05$) from HAMGM and BBM culture media. The increase of lipid production in M medium could be due to the four days of nitrogen starvation after 16 days of culture.

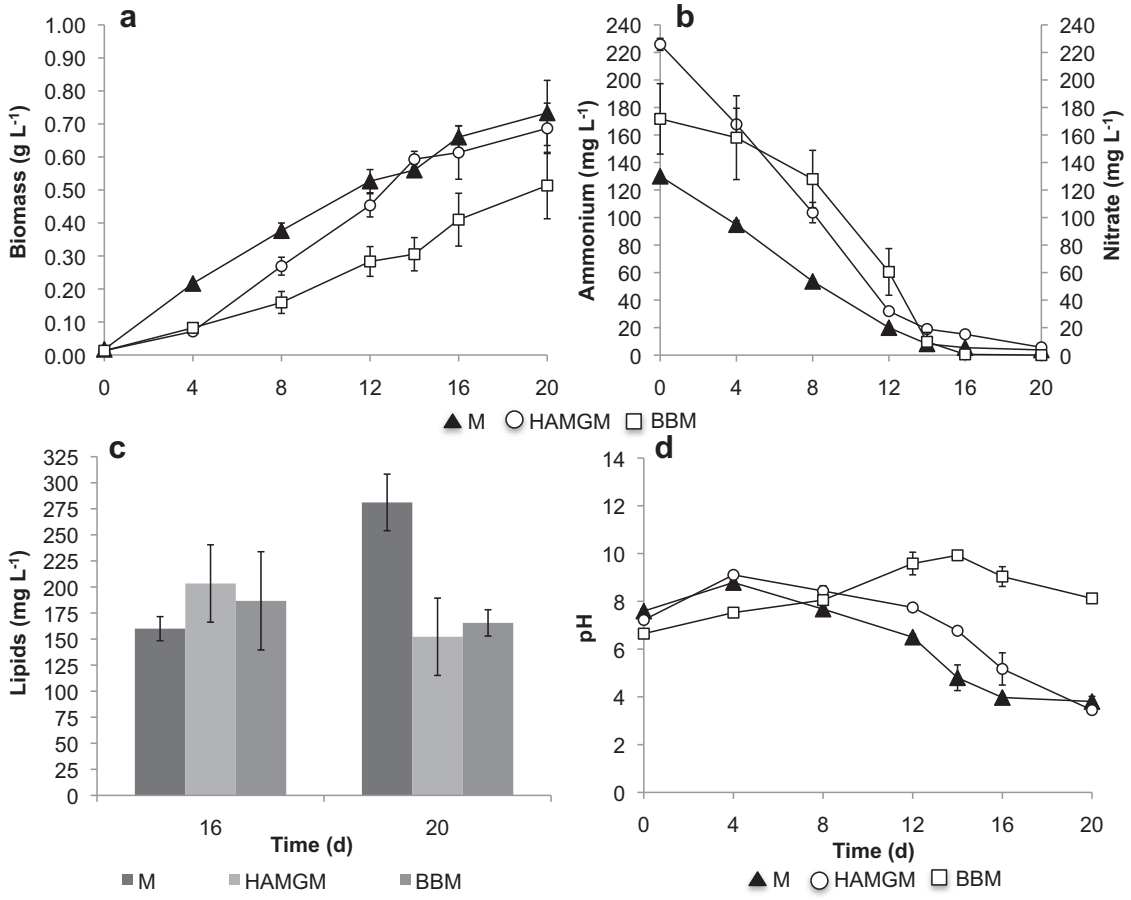

Fig. 6. *Chlorella vulgaris* UTEX 26 culture in the M, HAMGM and BBM culture media. (a) Profiles of biomass concentration as a function of culture time. (b) Profiles of the ammonium and nitrate concentrations as a function of culture time. (c) Lipid concentrations on day 16 and 20 of culture. (d) Profiles of pH variation as a function of culture time.

Widjaja et al. (2009) reported the factors that influence lipid production on *C. vulgaris* and their results suggested that the lipid concentration was higher on day 20, as a result of nitrogen starvation after day 15. The lipid productivity achieved in M medium (30.2 mg L^{-1} d^{-1}) was 2.5-fold higher than lipid productivity of *Chlorella protothecoides* UTEX 250, which was cultivated under heterotrophic conditions for 11 days and then it was maintained under autotrophic conditions for five days (Cheng et al., 2013). Griffiths et al. (2014) estimated that the starting NO_3^- concentrations to achieve maximum lipid concentration and lipid productivity, in *C. vulgaris* UTEX 395 batch cultures, were 0.305 and 0.241 g L^{-1}, respectively, whose nitrogen concentrations were only 14% y 32% lower than nitrogen concentration in M medium (0.079 g L^{-1} nitrogen). Moreover, they obtained the highest lipid concentration with 0.17 g L^{-1} of NO_3^-, on day 20 of culture and the lipid productivity was 19% higher than the lipid productivity achieved in this work with M medium. This could be justified due to the high average growth rate obtained in an air-lift PBR with enriched air with 0.29% CO_2, which was sparged at 2 L min^{-1}.

The extra CO_2 dissociated in solution as HCO_3^- may exert a dual effect by augmenting microalgal growth as well as providing an excess of carbon flux towards lipid production (Moheimani, 2013). So that, in M medium, 0.45 g L^{-1} of NH_4HCO_3 was an effective nitrogen and carbon mineral source to increase lipid production, with the culture conditions established in this work. Adams et al. (2013) performed cultures of *C. vulgaris* in an air-lift PBR with a carbon source (CO_2) six hundred times higher than the contained in M medium and a similar nitrogen concentration reaching 48% of lipids in biomass, only 10% greater than the obtained in this work with M medium. If the M medium is enriched with CO_2, biomass and lipid production could be enhanced, but the costs of microalgal production are increased too.

The pH decreased to 4.0 in the M and HAMGM culture media (Fig. 6d), a point in which more than 90% of available NH_4^+ had been consumed. Conversely, the BBM medium maintained an alkaline pH (7.5–10.0).

The HAMGM medium contains a NH_4HCO_3 concentration 146% higher than the M medium. Moreover, HAMGM medium has seven compounds in excess and is 42% more expensive compared with M medium. The production costs for both M and BBM culture media are similar, however, BBM medium has six compounds in excess and does not contain any bicarbonate source with respect to M medium. The addition of some carbon source to BBM medium increases the production costs. Specifically, addition of $NaHCO_3$ to BBM medium, with equivalent carbon concentration to M medium, increases the cost of BBM medium 12%. Therefore the M medium is a cheaper option.

3.3.1. Fatty acid profiles

Environmental conditions as well as nutrient concentrations and microalgal physiological state affect the degree of saturation in microalgal lipids (Chia et al., 2013; Cho et al., 2015). The fatty acid carbon chain length and unsaturation degree are needed to determine biodiesel specifications. High levels of unsaturated fatty acids influence biodiesel properties such as iodine value, cetane number, oxidative stability, cold flow and viscosity, which will

Table 2
Fatty acid profiles of *Chlorella vulgaris* UTEX 26 grown in the M, HAMGM and BBM culture media on days 16 and 20 of culture (% of total FAMES).

Fatty acid	M		HAMGM		BBM	
	16	20	16	20	16	20
C15:0	–	–	–	–	–	11.5
C16:0	30.2	38.3	34.7	37.6	43.4	61.8
C16:1	–	–	–	–	2.8	–
C18:0	21.3	15.2	14.4	8.1	19.2	16.2
C18:1	3.3	4.3	7.4	7.3	7.7	10.5
C18:2	7.8	9.6	8.5	11.1	3.9	–
C18:3n3	37.4	32.6	35.0	35.9	23.0	–
C15–C18	100	100	100	100	100	100
Unsaturated	48.5	46.5	50.9	54.3	37.4	10.5
Saturated	51.5	53.5	49.1	45.7	62.6	89.5

cause that the resulting biodiesel is off specification (Schenk et al., 2008; Meng et al., 2009; Ramanna et al., 2014). A blend of polyunsaturated fatty acids (PUFA), monounsaturated fatty acid (MUFA) and saturated fatty acids (SFA) in a balanced proportion may produce biodiesel with the best properties (Singh et al., 2014).

Gas chromatograph analysis of lipids extracted from *C. vulgaris* UTEX 26 grown on M, HAMGM and BBM culture media showed a fatty acid profile that consisted of fatty acids with carbon chains ranging from C15:0 to C18:0 (Table 2) on days 16 and 20 of culture. The majority of the fatty acids were C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid) and C18:3 (linolenic acid), which are more suitable for obtaining ideal biodiesel properties (Cho et al., 2015; Jusoh et al., 2015). Ramanna et al. (2014) found that *C. sorokiniana* grown in wastewater also showed a similar fatty acid profile with the majority of fatty acids comprising of carbon chain lengths of C14–C18.

The M and BBM culture media showed a clear increase of SFA under nitrogen limitation on day 20 of culture; however, FAMES in M culture medium were 84% and 190% higher than in HAMGM and BBM culture media, respectively. Additionally, the percentages of SFA obtained from this study were higher than those reported by Cho et al. (2015), who reported values ranging from 16.5% to 27% with *C. vulgaris* cultivated under different concentrations of effluent streams from a sewage sludge fermentation system.

4. Conclusions

The present study demonstrated that the M medium for *C. vulgaris* UTEX 26 improved biomass and lipid concentrations (40% and 85%, respectively) with respect to the conventional culture media. Fatty acids (C16:0, C18:0, C18:1, C18:2 and C18:3) produced by *C. vulgaris* UTEX 26 are suitable for producing biodiesel. The M medium allowed to decrease until 50% some nutrient concentrations required in the conventional culture media, moreover, medium recycling could be also possible. The M medium developed in this work is a promising alternative for microalgal culturing to obtain high concentrations of biomass and lipids in a large scale process.

Acknowledgements

The authors are grateful for the financial support provided by the Unidad Profesional Interdisciplinaria de Biotecnología of Instituto Politécnico Nacional (grant SIP 20151343), to SENER (Secretaría de Energía, grant 174627) and to CONACYT (Consejo Nacional de Ciencia y Tecnología, grant 233384). The authors would also like to thank Claudia Guerrero Barajas for her participation in reviewing the paper and the relevant comments that enhanced the final version of this manuscript.

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