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PII: S2589-014X(19)30244-0

DOI: https://doi.org/10.1016/j.biteb.2019.100354

Reference: BITEB 100354

To appear in: *Bioresource Technology Reports*

Received date: 3 October 2019

Revised date: 25 November 2019

Accepted date: 25 November 2019

Please cite this article as: D.M. Ribeiro, L.F. Roncaratti, G.C. Possa, et al., A low-cost approach for Chlorella sorokiniana production through combined use of urea, ammonia and nitrate based fertilizers, *Bioresource Technology Reports*(2018), https://doi.org/10.1016/j.biteb.2019.100354

A low-cost approach for *Chlorella Sorokiniana* production through combined use of urea, ammonia and nitrate based fertilizers

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Keywords: Microalgae, Nitrogen use efficiency, Culture medium, Predator control, Automated photobioreactors

Abstract

In this study, the use of combined nitrogen sources (urea, ammonia and nitrate) in a medium formulation is proposed as part of a low-cost approach for the cultivation of *Chlorella Sorokiniana*. The "Blue Green Nitrogen Mix" (BGNIM) medium, was able to support algal growth at similar levels of biomass productivities compared to the widely used BG11 medium varying between 47 and 50 $mgDW \cdot L^{-1} \cdot d^{-1}$. The combined use of nitrogen sources led to alterations in metabolites production. Protein abundance increased 7%, carotenoids 41%, soluble sugar 12%, alanine 370%, serine 350%, valine 180%, myo-inositol 190%, glyceric acid 230% and glutamic acid 220% when compared to BG11 standard medium. Additionally, a large pH shift was detected during the initial part of the growth curve in BGNIM cultures, opening opportunities for control of pH sensitive predators during large-scale production. Importantly, the BGNIM formulation provided a cost reduction of approximately 95% compared to BG11 standard medium.

1. Introduction

Biomass production represents an important alternative for feedstock supply for industrial processes in the context of carbon emissions mitigation. Algal biomass, in particular, stands out for its biological characteristics and biotechnological applications. The term algae is used to describe a diverse set of photosynthetic organisms containing both unicellular and multicellular species with a huge diversity of morphological and physiological characteristics (Bicudo, Carlos Eduardo de Mattos & Menezes, 2017). Algae belonging to the genus *Chlorella* are eukaryotic, unicellular, free living photosynthetic organisms with a diameter ranging from 1 to 20 µm that can be spherical or ellipsoid (Bicudo, Carlos Eduardo de Mattos & Menezes, 2017). They are cosmopolitan organisms, found in freshwater and marine environments, in soils and in the air, and can also colonize several invertebrate phyla such as Protozoa, Porifera, Platyhelminthes, Coelenterata, sponges or ascomycetes fungi in lichen (Wehr et al., 2015).

Amongst the several species reported for the genus, *Chlorella sorokiniana*, described as a "*high-temperature strain of chlorella*", has been indicated as a promising species for biotechnology processes and applications by the National Alliance for Advanced Biofuels and Bioproducts (NAABB, 2014). A number of studies have demonstrated that this species has a robust ability to produce biomass, proteins, sugars and high oil yields under a variety of conditions and can also be genetically manipulated (NAABB, 2014). *Chlorella sorokiniana* can acclimatize to artificial conditions including those involving the use of LED light sources, and automatization of aeration, temperature and pH control (Cazzaniga et al., 2014).

Considerable progress has been made in demonstrating the potential for large-scale production of microalgal biomass, but several challenges still persist to render it economically viable. The selection of more productive strains, the development of low cost culture media and more efficient photobioreactors, as well as improvements in nutrient use efficiency and photosynthetic rate optimization represent some of these challenges. Agroindustrial effluents or residues are a promising option for the formulation of culture media (Santana et al., 2017). However, variations in macro and micronutrients contents, contamination by heavy metals, as well as the presence of competitors and pathogens can cause unstable or low productivity (Christenson and Sims, 2011).

In this context, the development of fertilizer-based synthetic culture media represents an attractive alternative for lowering the production cost of algal biomass. However, whilst a number of studies have demonstrated the feasibility of this approach, such media are typically restricted to using Nitrogen, Phosphorus and Potassium (NPK) fertilizers. Furthermore, to our knowledge, no previous study using fertilizers for algae production discusses the biochemical strategy of combining three different sources of nitrogen (urea, ammonia and nitrate) while addressing possible changes in algal metabolism and biomass composition.

Nitrogen is the most important macronutrient for microalgal culture due to its requirement for the synthesis of proteins, pigments and nucleic acids. The quantity and molecular source of nitrogen (nitrate, ammonium or urea) used can be manipulated to optimize and redirect the production of biomass and compounds of interest. In particular, strategies involving nitrogen limitation generally negatively affect the photosynthetic machinery, resulting in chlorosis, and impact upon microalgal growth, and adequate supply of this macronutrient, on the other hand, tends to result in increased production of biomass and compounds of interest (Li et al., 2016).

In many different synthetic culture media, nitrate salts, particularly sodium nitrate, are typically the sole or principal source of nitrogen, as in the case of BG11 medium which is frequently used in *Chlorella* research. Nitrate uptake is carried out by a specific transporter and it is then reduced to nitrite by nitrate reductase. Nitrite is transported to the chloroplast and reduced to ammonium by nitrite reductase, which is in turn incorporated into amino acids via the GS-GOGAT cycle (Caspi et al., 2016).

Algae can also use ammonium in the medium as a source of nitrogen which is transported into the cell by the transmembrane proton gradient (Gutierrez et al., 2016). The use of ammonium as the sole source of nitrogen in culture media can result in toxicity, however this can be mitigated by using strains with high GS-GOGAT activity (Wang et al., 2019). In addition, ammonium is the main source of nitrogen in wastewater, and controlling its toxicity poses a major challenge in wastewater treatment (Yu et al., 2019).

Urea has also been reported as a promising source of nitrogen for microalgal culture. Urea is a commercially available nitrogen source used in agriculture for cultivation of crops due to its universal availability and affordability (Arumugam et al., 2013). Furthermore the urea molecule contains a higher percentage of nitrogen than other sources, it can be obtained at low cost and it has proven efficient in plant nutrition.

During its metabolism by microalgae, urea is transported into the cell by the DUR3 transporter (Pinton et al., 2016) and hydrolyzed by two enzymes, urea carboxylase and allophanate hydrolase, releasing two molecules of ammonium (NH_4^+) and one molecule of carbon dioxide (CO_2) in a process that requires ATP, Mg^{2+} and K^+ (Caspi et al., 2016).

The use of separate nitrogen sources has previously been studied and mixtures such as urea and nitrate or ammonium and nitrate have also been tested (Lin and Lin, 2011; Soares et al., 2018), however a detailed study of *Chlorella* cultivation in a mixture of the three nitrogen sources has not yet been performed. Improving our understanding of nitrogen use efficiency is a major challenge and study of the reciprocal interactions between urea, ammonia and nitrate can provide a basis for understanding use of N sources and optimize their assimilation (Pinton et al., 2016) and consequently influencing carbon assimilation (Lu et al., 2018).

In this paper we report the development of a low-cost synthetic culture media based on commercial fertilizers, using a combination of three nitrogen sources (urea, ammonia and nitrate) named "Blue Green Nitrogen Mix" (BGNIM). We hypothesized that that activating different nitrogen assimilation pathways would lead to increases in nitrogen containing compound production. Algal cultivation performance and characteristics of this medium were studied using a detailed analysis of growth in automatized photobioreactors and biomass chemical composition. The results obtained using BGNIM were compared with those obtained with BG11 standard medium, as well as with a modified version of BG11 where the original nitrogen source was replaced by urea (BGU). Finally, a comparison among media productivity and associated costs is presented.

2. Materials and methods

2.1. Microalgae strain

The microalga *Chlorella sorokiniana* Embrapa_LBA#39 (GenBank access - KM061456.1), a Tropical strain isolated from the Brazilian Savanna (Cerrado) and identified by Hadi et al. (2016), was used for a comparative study of different media. This strain has been deposited in the Collection of Microorganism and Microalgae for Agroenergy and Biorefineries of the Brazilian Agricultural Research Corporation - Embrapa (Brasília-DF) and preserved through protocols described by Fernandes et al.

(2019). Cultures were stored in liquid BG11 medium and kept in a growth chamber under constant temperature of 25ºC, with photoperiod of 12h/12h.

2.2. Culture media

Three culture media formulations for growth of *Chlorella sorokiniana* were used (Table 1), as follows: i) Blue Green 11 (BG11) as a standard medium, ii) a similar medium to BG11 in which the source of nitrogen (sodium nitrate) was replaced by the equivalent amount of nitrogen in the form of urea, hereafter referred as "Blue Green Urea" (BGU) and iii) a similar medium to the BG11 formulation based on a commercial plant fertilizer with a combination of three nitrogen sources (urea, ammonia and nitrate), respecting the elemental composition of N, P, K, Mg and Ca found in BG11 (Table 1a) - hereafter referred to as "Blue Green Nitrogen Mix" (BGNIM) - composed of 510 mg/L of Urea, 35 mg/L of Monoammonium Phosphate, 75 mg/L of Heptahydrate Magnesium Sulphate, 40 mg/L of Calcium Nitrate and 100 mg/L of Micronutrient Mix (REXOLIN, YaraTera, Brazil). All the nutrients used are presented in table 1b.

2.3. Photobioreactors

The experiments with the three media formulations were performed in parallel, using three automated photobioreactors. A schematic drawing of the apparatus is illustrated in Figure 1 and details are described as follows. Each reactor consists of a Duran GLS 80 wide neck glass bottle (with diameter of 101 mm and one liter capacity), a magnetic stirrer, an LED panel, a light sensor and a screw cap. The illumination panel is made out of a strip with 300 SMD 5050 5000K white LEDs glued inside a 200mm diameter PVC tube, providing a very homogeneous light distribution, up to a maximum illuminance of 4000 lux inside the tube.

The emission spectrum of the panel is illustrated in panel A of Figure 2. In addition to serving as a light source for cultivation, the panel was also used to indirectly measure the density of microalgae through the irradiance of the light scattered by the culture when illuminated by the panel with a specific illuminance. This irradiance is measured through a TCS34725 sensor docked outside the bottle wall. This sensor is capable of individually measuring incident irradiance at three wavelengths: 465 nm blue, 525 nm green and 615 nm red. From the absorption spectrum of *Chlorella sorokiniana* | LBA#39 it was possible to identify that 525 nm green is out of the

absorption peaks of the active pigments (see panel A of Fig. 2) and, therefore, this wavelength was used for the proxy measurement of microalgae density. The sensor reading at this wavelength was then related to the microalgae density through a calibration curve obtained with direct microalgae counting with a Neubauer chamber (see panel B of Fig. 2). Attached to the screw cap of each reactor there is a pH probe and two tubes for air inlet and air outlet.

The air circulating through the culture is provided by an air compressor connected to an air reservoir. An NDIR carbon dioxide (CO_2) sensor was placed inside the reservoir and, via feedback, triggers a solenoid valve that connects the reservoir to a CO_2 cylinder so that the CO_2 concentration inside the reservoir can be kept constant. The entire system (illumination panels, light sensors, pH sensors, air compressor and solenoid valve) is interconnected through an Arduino Mega microcontroller board and control software.

2.4. Experiments

The growth dynamics of *Chlorella sorokiniana* in the three media was carried out in parallel in the three reactors, the experiment was repeated three times to obtain independent biological replicates, all of which were initially inoculated with a similar initial concentration of 2 x 10^5 cells/ml and pH 6.8. Throughout the experiments, all three culture broths were maintained under a photoperiod of 16h/8h (with 1500 lux in the light period), temperature 28 ± 1 °C and air lifted with 2 L/min of atmospheric air containing 0.1% (v/v) CO_2 (1000 ppm). Every 5 minutes the system recorded the microalgae density and pH in each reactor. Each experiment started with the inoculum adapted to the conditions of the experiment, was carried out over 8 days and was conducted in triplicate. After 8 days the whole volume of each photobioreactor was centrifuged at 5000 g for 10 min, the recovered biomass was submerged in liquid nitrogen and then lyophilized, weighed and used to calculate algal biomass productivity.

2.5. Analysis of algal biomass composition

The analyses of algal biomass composition were performed using the lyophilized biomass from the biological triplicates of the experiment. Pigments were extracted by incubating 10 mg of algal biomass with 10 ml of acetone, containing 0.1% (v/v) butylated hydroxy toluene (BHT) overnight. Chlorophylls *a* and *b* and total carotenoid contents were determined according to Lichtenthaler and Wellburn (1983).

Carbon, nitrogen and hydrogen contents were measured from 2 mg lyophilized algal biomass samples, using a CHNS/O elemental analyzer (PE2400 series II PerkinElmer). The conversion factor 4.78, specific for microalgae, was used to estimate protein abundance from nitrogen content.

Soluble sugars were extracted with 80% ethanol, from 10 mg of biomass sample. Starch in the pellet remaining after the extraction were quantified using the enzymatic method described by Amaral et al. (2007). After removal of starch hydrolysates, 1 ml of concentrated sulfuric acid was added to the samples to hydrolyze structural sugars. Monosaccharides (trioses, pentoses and hexoses) obtained at each step were quantified by complexing the free aldehyde group with MBTH (3-methyl-2-hydrazone benzothiazolinone) and using the spectrophotometric quantification methodology described by Van Wychen and Laurens (2016) . The extraction and derivatization of the samples for metabolite profiling (amino acids and organic acids) were performed using the methanol-ribitol method (Fernie et al., 2006), using 10 mg of algal biomass, and analyzed by GC/MS.

Fatty acid profiles were determined according to Van Wychen et al. (2013), after treatment of 10 mg of biomass for transesterification with 0.2 ml of chloroform: methanol (2:1, v/v) and 0.3 ml of 0.6 M HCl in methanol heated at 85 °C in dri-block for 1 hour. After heating, 1 ml hexane was used to extract the Fatty acid methyl esters (FAMES). FAMEs were separated by gas chromatography using an Agilent Chromatograph (Agilent Technologies, California, USA), coupled with a flame ionization detector (FID) and a fused silica capillary column (100 m x 250 μm x 0.2 μm, Supelco SP). The operating parameters were set as follows: detector temperature, 260 ºC column temperature, 140 ºC for 5 minutes, programmed to increase 4 °C/min, up to 240 ºC, with a final running time of 48 minutes. The carrier gas was Helium at 1.2 $mL \cdot min^{-1}$, with injection of 1 μL sample. The retention times of fatty acids were compared to those of standard methyl esters (Sigma-Aldrich, St. Louis, MO, USA). Retention times and percentages of the peak area were calculated automatically by the ChemStation Software. Fatty acids quantifications (FA) were performed using the methyl ester of nonadecanoate acid (Sigma-Aldrich, USA) as an internal standard.

2.6. Data analysis

Specific growth rate (μ) and algal biomass productivity ($mgDW \cdot L^{-1} \cdot day^{-1}$) were calculated using the equation described by Kishi and Toda (2018) with the data of

cell number and biomass collect at the end of the experiment. The cost of the medium was calculated as the sum of the cost of each reagent amount used in its preparation. The cost for the production of 1 kg of dry algae biomass, based only on the cost of the culture medium, was calculated as the cost of one liter of medium divided by the biomass obtained in this volume. The biomass composition data were subjected to analysis of variance (ANOVA) at 5% probability followed by a Tukey test, using the software Action Stat version 3.5.

3. Results and Discussion

Cultivations were performed using automated photobioreactors, under a light/dark cycle of 16h/8h at 28ºC and constant aeration with 0.1% of CO_2 over 8 days. Figure 3 shows the growth dynamics of *Chlorella sorokiniana* cultures LBA#39 on BG11, BGU and BGNIM media, as monitored by microalgae density and by the pH of the culture over time. Both variables were recorded every 5 minutes. Each value in the time series presented in Figure 3 represents the average value of the three biological replicates of the experiment at that time.

3.1. Cell growth and biomass productivity

Biomass productivity varied between 47 and 50 $mg \cdot L^{-1} \cdot d^{-1}$, in BGNIM and BGU, respectively (Table 3). These values are below the maximum biomass production potential for *Chlorella*, estimated at 196 $mg \cdot L^{-1} \cdot d^{-1}$ (Weyer et al., 2010), but above the large-scale biomass yields reported in the literature which range from 30 to 42 $mg \cdot L^{-1} \cdot d^{-1}$ (Laurens, 2017; Weyer et al., 2010). Furthermore, the productivities observed in this study are comparable to those obtained when wastewater is used as cultivation media. Indeed, biomass productivities ranging from 12 to 68 $mg \cdot L^{-1} \cdot d^{-1}$ for *C. sorokiniana* strains cultivated in municipal treatment plant wastewater, sugarcane bagasse hydrolysate and other wastewaters have been reported (De Lourdes et al., 2017; Manzoor et al., 2019; Park et al., 2011). However, the use of biomass produced in effluents can be limited, since there is a risk of accumulation of heavy metals or potential contaminants, reducing their suitability for human, animal or plant nutrition.

Taken together, the results presented here indicate that the growth of *Chlorella sorokiniana* LBA#39 is similar in the BG11, BGU and BGNIM media (Table 2), indicating that all these three formulations provide the nutrients required to obtain equivalent biomass productivity (Table 3) during eight days of cultivation.

3.2. Changes in pH during growth in different media

The data illustrated in Figure 3 shows differences among the growth dynamics associated with the three medium formulations. Though the variations in cell densities over time follow similar patterns (Figure 3A), driven by the photoperiod of the experiment, the lag phase for the BGNIM medium is shorter, ending approximately 24h earlier than in the other two media. Moreover, while the variations in pH in BG11 and BGU are apparently driven by the photoperiod as a consequence of photosynthesis and respiration, the end of the lag phase in the BGNIM media was associated with a significant drop in pH from 7 to 2.8 over the next two photoperiods, before recovery to pH 7 over the following two photoperiods. From this point onwards the pH curve of the BGNIM formulation started follow a regular pattern similar to those obtained for the BG11 and BGU media (Figure 3B).

The use of automated photobioreactors provided continuous and precise measurements of the culture growth dynamics. This approach allowed the identification of a characteristic feature of the BGNIM medium, i.e., the strong drop in pH after the lag phase (Figure 3B), which is likely due to the electrogenic absorption of ammonium (Scherholz and Curtis, 2013). Acidification of the culture medium is caused by cell extrusion of H^+, as is also known to occur during soil acidification when plants assimilate ammonium. Indeed, such pH variation is not observed in cultures using BGU medium, which has no ammonium phosphate in its composition but urea as a nitrogen source (Figure 3B).

In the presence of different sources of nitrogen, *Chlorella* tends to absorb ammonium (NH_4) first by the ammonia (NH_3) channel transporter (Amt) releasing the H^+, causing acidification. Nitrates (NO_3^-) and nitrites (NO_2^-) are absorbed by the I and II (Nrt2 / Nar2) systems or the High Affinity Nitrate Transporter (HANT) system, while urea (CH_4N_2O) is absorbed by the Active Urea Transporter (DUR3) (Figure 4). The main route of nitrogen assimilation is GS-GOGAT and its main product is glutamine, a basic component for amino acid and protein synthesis, as can be seen in Figure 4.

In this study the transient reduction in pH did not appear to affect algal growth. Indeed, cell growth occurred in BGNIM during the period of acidification and the lag phase for this medium was shortened in comparison to BG11 and BGU, indicating that this strain of *Chlorella* appears to be tolerant to such condition. This characteristic may be advantageous when considering defense against predators that cannot resist acidification, a method described for rotifer and cladoceran control and could also

represent a method for ciliate contamination control (Ashraf et al., 2011). It is well known that culture crashes due to predator contamination pose a major threat for large-scale production of algae, especially in open ponds, and must be properly managed (Lammers et al., 2017; Ribeiro et al., 2019a).

3.3. Biochemical changes

As shown in Tables 2 and 3, no statistical difference could be observed in cell number yields, specific growth rates and biomass yield among the three media tested. Total carbohydrate and transesterifiable fatty acid yields were also similar, independent of the growth medium, whereas significant differences in the contents of nitrogen, hydrogen, proteins, soluble sugars, chlorophyll *a* and *b* and carotenoid were observed (Table 3). Algae grown on BGNIM and BGU media showed slightly higher protein, soluble sugar and carotenoid content than those grown using BG11 medium (Table 3). On the other hand, BGU cultures presented greater accumulation of chlorophyll *a* and chlorophyll *b* than BG11 and BGNIM (Table 3). No statistical difference was observed in the fatty acid profiles of *Chlorella sorokiniana* grown in the different media (Figure 5). Alpha-linolenic polyunsaturated acid (C18: 2n3) was the major component, followed by palmitic acid (C16) and Linoleic acid (C18: 2n6c) (Figure 5).

Ammonium and urea are nitrogen sources that may be toxic to microalgae, as can also be the case for vascular plants . On the other hand, if employed in appropriate concentrations, their use can lead to accumulation of biomass components of interest (Ikaran et al., 2015). Here, both urea-containing media (i.e.: BGU and BGNIM) supported the accumulation of higher concentrations of protein, soluble sugars and carotenoids, which are valuable components of algal biomass (Table 3). Proteins are one of the most useful fractions of algal biomass when considering use as animal feed and human food (Odjadjare et al., 2017).

Whilst soluble sugars were greater in the BGNIM medium, total carbohydrates and total fatty acids showed no statistical difference between the three media tested. The combination of different nitrogen sources did not significantly affect the fatty acid profile after eight days of *Chlorella sorokiniana* LBA#39 growth. Quantification and characterization of lipid contents and fatty acids profiles is recommended for understanding the pathways used by microalgae to accumulate these compounds of interest as well as to assess the potential suitability of oil for biofuel production. *Chlorella sorokiniana* LBA#39 presented similar fatty acid profiles in all three media

(Figure 5). Alpha-linolenic polyunsaturated acid (C18:2n3) was the major component of the fatty acid profile in all media (Figure 5), as seen in other reports for *Chlorella sorokiniana* grown under different aeration and/or carbon source conditions (Zheng et al., 2017).

The abundance and efficiency of photosynthetic complexes are related to the availability of different mineral nutrients and co-factors which, in addition to pigments (chlorophylls and carotenoids), also include manganese, calcium, chloride, iron, copper, and quinones. Algal biomass produced using BGNIM medium yielded lower contents of chlorophyll than using BG11 and BGU, but a higher content of carotenoids when compared to BG11. Importantly, carotenoids are amongst the most valuable compounds obtained from algal biomass (Borowitzka, 2013). *Chlorella* species represent a strong candidate for the commercial production of algal carotenoids, and in addition to their economic value, carotenoids play an important role in the robustness of microalgae to photo-oxidation (Patias et al., 2017).

The decrease in chlorophylls and increase in carotenoids in the BGNIM medium may be a result of photo-oxidation caused by photorespiration and photochemical processes that generate reactive oxygen species (ROS) causing damage to the photosynthetic apparatus (Srinivasan et al., 2018) and resulting in a decrease in carbon fixation. A parallel between oxygen uptake and nitrogen uptake has been described and such phenomenon might be reflected in the change in pigments (Table 3) and metabolic profile (Table 4) observed in culture with BGNIM medium.

3.4. Changes in metabolic profile

The metabolic profiles of *Chlorella sorokiniana* biomass produced in BG11, BGU and BGNIM culture media are presented in Table 4, where sugars, amino acids and organic acids were identified. In cultivation using BGNIM medium a higher abundance of a number of metabolites was detected when compared to BG11, as alanine increased 370%, serine 350%, valine 180%, myo-inositol 190%, glyceric acid 230% and glutamic acid 220% (Table 4).

Metabolite profiling revealed differences in concentrations of some compounds between biomass produced in the evaluated media. Increases in glutamic acid using BGNIM medium likely reflect alterations in operation of the GS-GOGAT cycle and transamination reactions as a result of the use of the three different sources of nitrogen. High levels of alanine have already been detected in *Chlorella* as a result of urea and

ammonium absorption (Baker and Thompson, 2008). Indeed, alanine is the main amino acid produced in *Chlorella* under high oxygen tension and under sulfur limiting condition, which also is connected to ammonium absorption. Connections between alanine metabolism and lipid biosynthesis have been proposed previously for *Chlorella* (Chen et al., 2017).

Increases in glyceric acid, malic acid and the amino acids serine and valine are indications of alterations in various metabolic processes in *Chlorella sorokininana* LBA#39 when grown in the BGNIM medium, particularly those associated with glycolysis and pyruvate metabolism (Cecchin et al., 2018). The use of urea may also provide additional CO_2 for algal metabolism and may activate metabolic pathways that were not activated under cultivation with only sodium nitrate as a nitrogen source. Indeed, algae are known to adopt a PEP carboxylase assimilation strategy when cultured with acetate, and expression of PEPcase genes is known to increase in high levels of CO_2 (Cheng et al., 2017). Photo-oxidation and absorption of ammonia are related to PEPcase pathway as well (Giordano et al., 2003). Ultimately, specific stable isotope labeling experiments are needed to determine the fate of the CO_2 released from urea during its breakdown, and to further investigate the effects of altered nitrogen source on cellular metabolism.

3.5. Cost and productivity evaluation

Analyses of the costs of the different media indicate that the replacement of the nitrogen source by urea (BG11→BGU) has the potential to reduce the cost of the medium by 65% (Table 5). Furthermore, when the formulation components are all derived from commercial fertilizers (BG11→BGNIM), the cost is reduced by approximately 95% (Table 5). Considering the biomass productivity determined in this study, the use of BGU medium would reduce the cost of production of 1 kg algal biomass by 64% compared to BG11 media whilst using BGNIM medium would reduce costs by 96%.

The production of biomass, proteins, carbohydrates, fatty acids, bioethanol and biodiesel per year was estimated and are shown in table 5. Importantly, while optimization of biomass production is being sought, new applications for algal biomass as source of added value products are also being identified and developed, generating a greater economic sustainability for potential algal biorefineries (Chew et al., 2017).

Finally, there are opportunities to integrate the algal biorefinery concept into existing systems (Brasil et al., 2017), with a number of authors indicating the potential of algae in the production of higher value food, feed, fertilizer, nutraceutical and oleochemical bio-products (Deprá et al., 2018; Laurens, 2017; Ribeiro et al., 2019a, 2019b). Apart from the potential of microalgae to mitigate air and water pollution, microalgae could be an important source of feedstock for achieving the Goals of Sustainable Development proposed by the General Assembly of the United Nations (2015).

4. Conclusion

Despite many recent advances in large-scale microalgal production, several challenges still persist to achieve economic viability. In this study, we proposed the use of a low-cost medium (BGNIM) which has the potential to significantly reduce algal production costs. Indeed, the use of BGNIM led to similar biomass productivity compared to the widely used, though expensive, BG11 standard medium. Furthermore, this approach slightly increased protein and carotenoid accumulation, which are valuable fractions of algal biomass, compared to BG11. Additionally, the acidification observed might be used for predator control during large-scale production, a major issue for outdoor continued cultivation.

Acknowledgements

The authors are grateful to the Universidade de Brasília (UnB), Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Brazil) and Fundação de Apoio a Pesquisa do Distrito Federal (FAPDF) for supporting this work. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

Dágon M. Ribeiro, Luiz F. Roncaratti, Gabriela C. Possa, Thomas C. R. Williams, Bruno dos S. A. F. Brasil contributed to conception, design, analysis and interpretation of the data; collection and assembly of data, article writing, and final approval of the article. Lorena C. Garcia and Letícia J. Cançado contributed to collection and assembly

of data and critical revision of the article for important intellectual content. Dágon M. Ribeiro takes responsibility for the integrity of the work as a whole.

Statement of informed consent

No applicable conflicts, informed consent, human or animal rights.

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Table 1. A) Elemental composition of the formulations tested and **B)** nutrients used, their concentration in grams per liter and their source of purchase**.**

A)

	Medium formulation		
Elemental	**BG 11 mg/L**	**BGU mg/L**	**BGNIM mg/L**
N	247.59	247.62	249.00
H	6.60	42.17	40.37
C	6.49	112.49	102.00
O	946.00	240.12	232.30
S	9.79	9.79	11.04
Fe	1.28	1.28	2.66
Cl	7.12	7.12	-
K	11.49	11.49	11.60
Mg	7.40	7.40	7.40
Na	252.60	5.40	-
P	9.10	9.10	9.42
Zn	0.05	0.05	3.38
Co	0.01	0.01	-
Cu	0.02	0.02	0.36
Mn	0.50	0.50	2.48
Mo	0.15	0.15	0.04
Ca	9.81	9.81	9.77
B	0.50	0.50	2.10

B)

Macronutrients	**Chemical Composition**	**BG 11 (mg/L)**	**BGU (mg/L)**	**BGNIM (mg/L)**
Sodium Nitrate	$NaNO_3$	1500	-	-
Urea	CH_4N_2O	-	530	-
Urea – Fertilizer	CH_4N_2O	-	-	510
Dihydrogen Potassium phosphate	KH_2PO_4	40	40	
Monoammonium Phosphate (MAP) - Fertilizer	$NH_4H_2PO_4$	-	-	35
Magnesium Sulphate Heptahydrate	$MgSO_4 \cdot 7H_2O$	75	75	-
Magnesium Sulphate Heptahydrate - Fertilizer	$MgSO_4 \cdot 7H_2O$	-	-	75
Calcium Chloride Dihydrate	$CaCl_2.2H_2O$	36	36	
Calcium Nitrate - Fertilizer	$Ca\ (NO_3)_2$	-	-	40
Sodium Carbonate	Na_2CO_3	20	20	-
Micronutrients	**Micronutrients**			
Disodium ethylenediaminetetraacetic acid dehydrate	EDTA NA_2	1	1	-
Cobalt (II) Nitrate Hexahydrate	$Co(NO_3)_2.6H_2O$	0,05	0,05	-
Sodium Molybdate Dihydrate	$Na_2MoO_4 \cdot 2H_2O$	0,39	0,39	-
Boric Acid	H_3BO_3	2,86	2,86	-
Copper (III) sulphate pentahydrate	$CuSO_4.5H_2O$	0,08	0,08	-
Manganese Chloride Tetrahydrate	$MnCl_2.4H_2O$	1,81	1,81	-
Zinc Sulphate Heptahydrate	$ZnSO_4.7H_2O$	0,22	0,22	-
Green Ferric Ammonium Citrate	$C_6H_8FeNO_7$	6	6	-
Citric Acid	$C_6H_8O_7$	6	6	-
Micronutrients – Rexolin - Fetilizer	–Micronutrientes	-	-	100

Table 2. Number of cells, specific growth rate and pH at different cell growth points.

Medium	Cell number yield (cell/ml)	Specific growth rate (μ)	pH at 0h	75h	192h
Blue Green 11 (BG11)	8,34 x 10^7 (±0.22)[a]	1,36 (±0.29)[a]	6,8	7,4	8,5
Blue Green Urea (BGU)	9,23 x 10^7 (±0.12)[a]	1,34 (±0.15)[a]	6,8	7	7,7
Blue Green Nitrogen Mix (BGNIM)	6,79 x 10^7 (±0.54)[a]	1,35 (±0.11)[a]	6,8	2,8	7,3

* Results are presented as mean ± error bars show the standard deviation of triplicate experiments (n = 3). Means followed by the same letter do not differ by One-way ANOVA followed by Tukey test at the 5% probability level ($p \leq 0.05$).

Table 3 – Biomass content, yield and compounds of interest accumulated in *Chlorella sorokiniana* grown in the different media.

***Chlorella sorokiniana*|LBA#39**	**BG11**	**BGU**	**BGNIM**
Biomass Productivity (mgDW·L^{-1}·d^{-1})	50.90 (±3.19)^{a}	48.81 (±1.74)^{a}	47.93 (±3.35)^{a}
Carbon (%)	48.57 (±0.93)^{a}	49.32 (±0.69)^{a}	49.63 (±0.47)^{a}
Nitrogen (%)	**7.43 (±0.23)^{b}**	**7.62 (±0.12)^{ab}**	**7.85 (±0.21)^{a}**
Hydrogen (%)	**8.71 (±0.22)^{b}**	**9.32 (±0.14)^{a}**	**9.33 (±0.07)^{a}**
Protein (%)	**41.67 (±1.11)^{b}**	**44.57 (±0.06)^{a}**	**44.63 (±1.04)^{a}**
Protein yield (mgDW·L^{-1}·d^{-1})	21.22 (±1.25)^{a}	21.76 (±2.07)^{a}	21.39 (±2.30)^{a}
Soluble sugars (%)	**12.68 (±0.51)^{b}**	**13.93 (±0.5)^{ab}**	**14.26 (±0.48)^{a}**
Reservoir Sugars - Starch (%)	4.55 (±0.47)^{a}	4.87 (±0.59)^{a}	4.70 (±1.08)^{a}
Structural sugars (%)	5.16 (±1.92)^{a}	3.82 (±0.72)^{a}	5.57 (±0.93)^{a}
Carbohydrate content Total (%)	22.41 (±2.70)^{a}	22.64 (±1.03)^{a}	24.55 (±1.38)^{a}
Carbohydrate yield (mgDW·L^{-1}·d^{-1})	11.41 (±1.19)^{a}	11.05 (±0.45)^{a}	11.77 (±1.29^{a}
Total FA (%)	6.50 (±0.01)^{a}	6.50 (±0.01)^{a}	6.20 (±0.01)^{a}
FA yield (mgDW·L^{-1}·d^{-1})	3.30 (±0.32)^{a}	3.20 (±0.38)^{a}	3.00 (±0.23)^{a}
Chlorophyll *a* (%)	**2.30 (±0.01)^{b}**	**2.60 (±0.04)^{a}**	**1.70 (±0.10)^{c}**
Chlorophyll *b* (%)	**1.10 (±0.01)^{b}**	**1.30 (±0.06)^{a}**	**0.60 (±0.05)^{c}**
Total Chlorophyll (%)	**3.60 (±0.02)^{b}**	**3.90 (±0.10)^{a}**	**2.30 (±0.15)^{c}**
Chlorophyll *a* / *b* ratio	**2.09 (±0.01)^{b}**	**1.97 (±0.06)^{b}**	**2.63 (±0.03)^{a}**
Carotenoids (%)	**0.43 (±0.01)^{b}**	**0.58 (±0.02)^{a}**	**0.61 (±0.01)^{a}**
Carotenoid yield (mgDW·L^{-1}·d^{-1})	**0.22 (±0.01)^{b}**	**0.28 (±0.01)^{a}**	**0.29 (±0.01)^{a}**

* Results are presented as mean ± error bars show the standard deviation of triplicate experiments (n = 3). Means followed by the same letter do not differ by One-way ANOVA with Tukey test at the 5% probability level ($p \leq 0.05$).

Table 4. Identified metabolites of *Chlorella sorokiniana* grown in BG11, BGU and BGNIM culture media.

nº	Metabolite	BG11	BGU	BGNIM
1	**Threonic acid**	**1.0 (±0.08)[a]**	**0.7 (±0.24)[b]**	**0.9 (±0.03)[a]**
2	**Alanine**	**1.0 (±0.18)[b]**	**4.5 (±0.22)[a]**	**4.7 (±0.17)[a]**
3	Beta-Alanine	1.0 (±0.04)[a]	1.0 (±0.09)[a]	1.2 (±0.63)[a]
4	Fumaric acid	1.0 (±0.02)[a]	1.0 (±0.07)[a]	0.9 (±0.44)[a]
5	**Glyceric acid**	**1.0 (±0.07)[b]**	**1.2 (±0.19)[b]**	**3.3 (±0.37)[a]**
6	Glycerol-3-phosphate	1.0 (±0.03)[a]	0.9 (±0.20)[a]	0.8 (±0.25)[a]
7	**Myo-Inositol**	**1.0 (±0.06)[b]**	**0.9 (±0.05)[b]**	**2.9 (±0.01)[a]**
8	Lumichrome	1.0 (±0.08)[a]	1.2 (±0.07)[a]	1.2 (±0.32)[a]
9	**Malic acid**	**1.0 (±0.03)[b]**	**0.8 (±0.09)[c]**	**1.1 (±0.02)[a]**
10	**Glutamic acid[+]**	**1.0 (±0.05)[b]**	**0.9 (±0.12)[b]**	**3.2 (±0.02)[a]**
11	**Sucrose**	**1.0 (±0.02)[a]**	**0.1 (±0.31)[c]**	**0.5 (±0.39)[b]**
12	**Serine**	**1.0 (±0.01)[c]**	**1.1 (±0.03)[b]**	**4.5 (±0.03)[a]**
13	Succinic acid	1.0 (±0.01)[a]	1.5 (±0.12)[a]	0.9 (±0.08)[a]
14	Threonine	1.0 (±0.01)[a]	0.6 (±0.20)[a]	0.9 (±0.38)[a]
15	**Valine**	**1.0 (±0.02)[b]**	**0.7 (±0.31)[b]**	**2.8 (±0.39)[a]**

* Results are presented as mean ± error bars show the standard deviation of triplicate experiments (n = 3). Means followed by the same letter do not differ by One-way ANOVA followed by the Tukey test at the 5% probability level ($p \leq 0.05$). + detected as pyro-glutamic acid

Table 5. Comparison between productivity and costs of media tested.

Estimates	BG11	BGU	BGNIM
Biomass Productivity ($mgDW \cdot L^{-1} \cdot d^{-1}$)	50.93	48.81	47.93
Biomass Productivity ($ton \cdot year^{-1}$)[a]	2.40	2.30	2.30
Total Protein Productivity ($ton \cdot year^{-1}$)	1.01	1.04	1.02
Total Carbohydrates Productivity ($ton \cdot year^{-1}$)	0.54	0.53	0.56
Total FA Productivity ($ton \cdot year^{-1}$)	0.15	0.15	0.14
Bio-ethanol Productivity ($L \cdot year^{-1}$)[b]	328.70	318.30	338.90
Biodiesel from FA Productivity ($kg \cdot year^{-1}$)[c]	158.90	152.30	142.60
Cost 1 L of Medium (USD)[d]	0.17	0.06	0.01
Cost for 1kg Biomass (USD)[d]	470.41	171.22	19.37

[a]Year round estimates considering 200 $m^3.d^{-1}$ with 240 working days per year, [b]Estimated based on the conversion rate of 0.6 L per kg of total carbohydrates, [c]Estimated based on the conversion rate of 1 kg of fatty acid to 1 kg of biodiesel (Cabanelas et al., 2013; Santana et al., 2017), [d]USD = United States Dollar

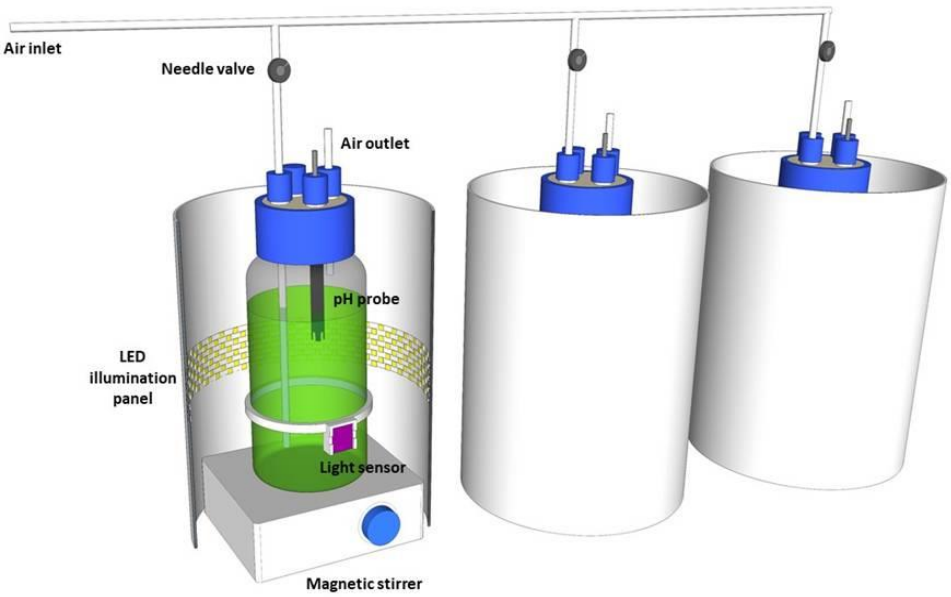


Figure 1. Schematics of the experimental apparatus. Each photobioreactor held one liter of microalgae culture in a glass bottle illuminated by a neutral white (5000 K) LED strip attached to a PVC tube. A light sensor attached to each bottle wall provides the irradiance of the light scattered through the culture. Each lid holds a pH probe and is connected to a gas line.

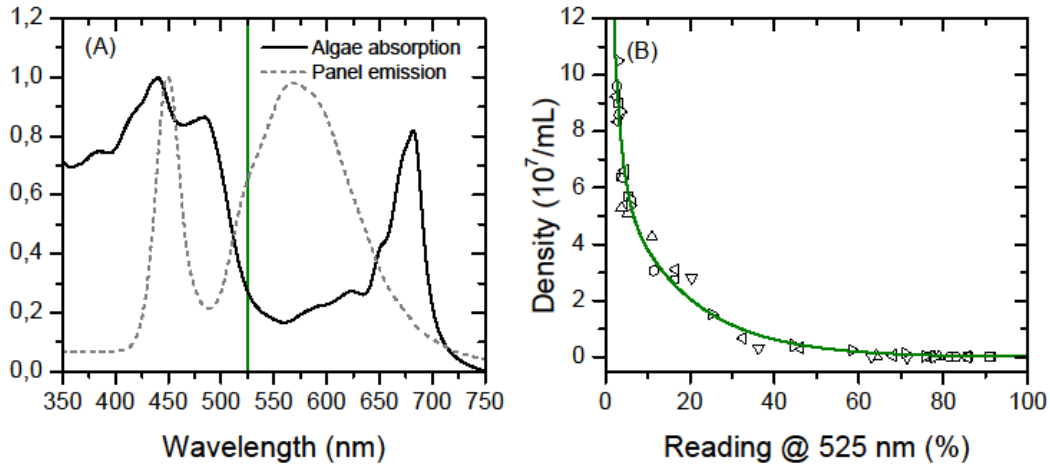


Figure 2. (A) Absorption spectrum of Chlorella sorokiniana LBA#39 (solid line), emission spectrum of the LEDs used to illuminate the culture (dashed line) and wavelength used for monitoring the grown kinetics of the cultures (vertical line @ 525nm). (B) Calibration curve used in the algae density measurements obtained with direct cell count within a Neubauer chamber. Different symbols represent cell counts taken under different experiments/reactors.

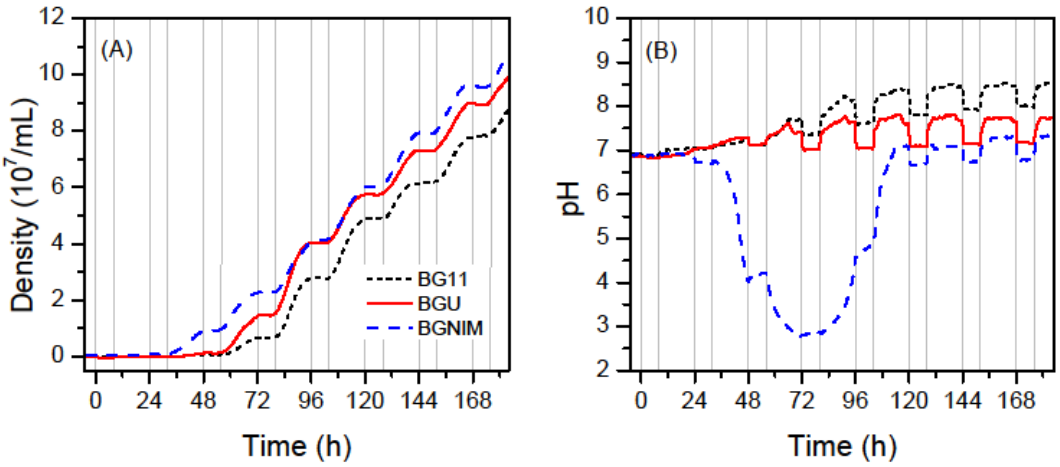


Figure 3. Growth dynamics of *Chlorella sorokiniana* in the three medium formulations. (A) Microalgae density and (B) pH value of the culture.
* Results shown are the mean of biological triplicates of the experiment (n = 3).

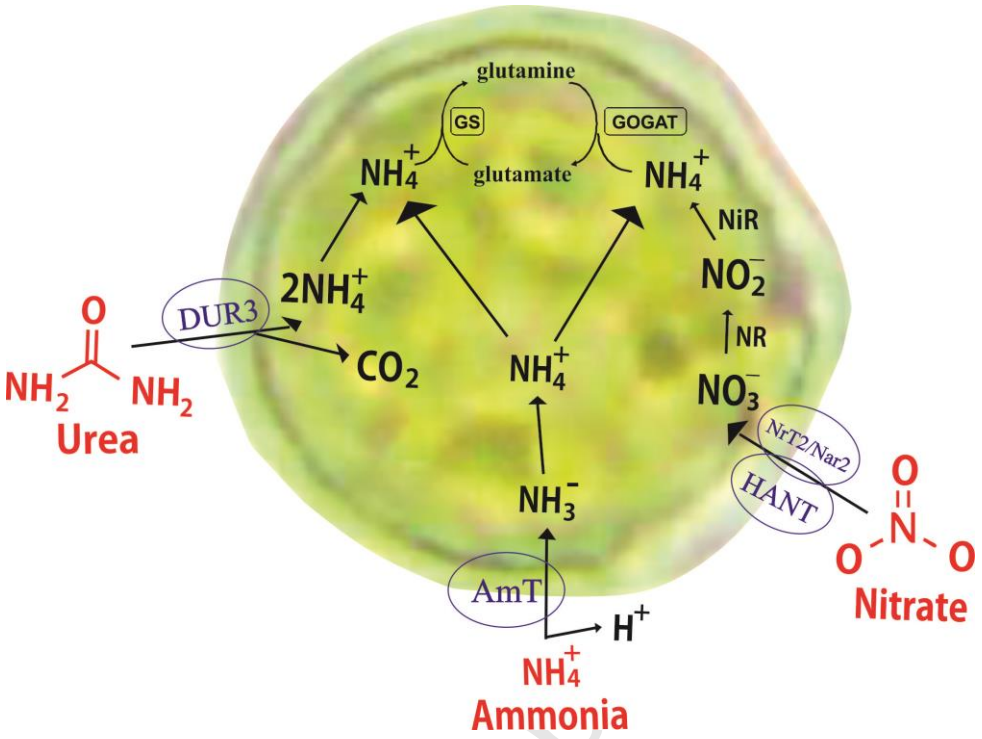


Figure 4. Different sources and nitrogen transport systems. **Amt** - Ammonia Channel Transporter, **Nrt2 / Nar2** - Nitrates and nitrites systems, **HANT** - High-Affinity Nitrate Transporter system, **DUR3** - Active Urea Transporter, **GS-GOGAT** - Glutamine / Glutamate synthase.

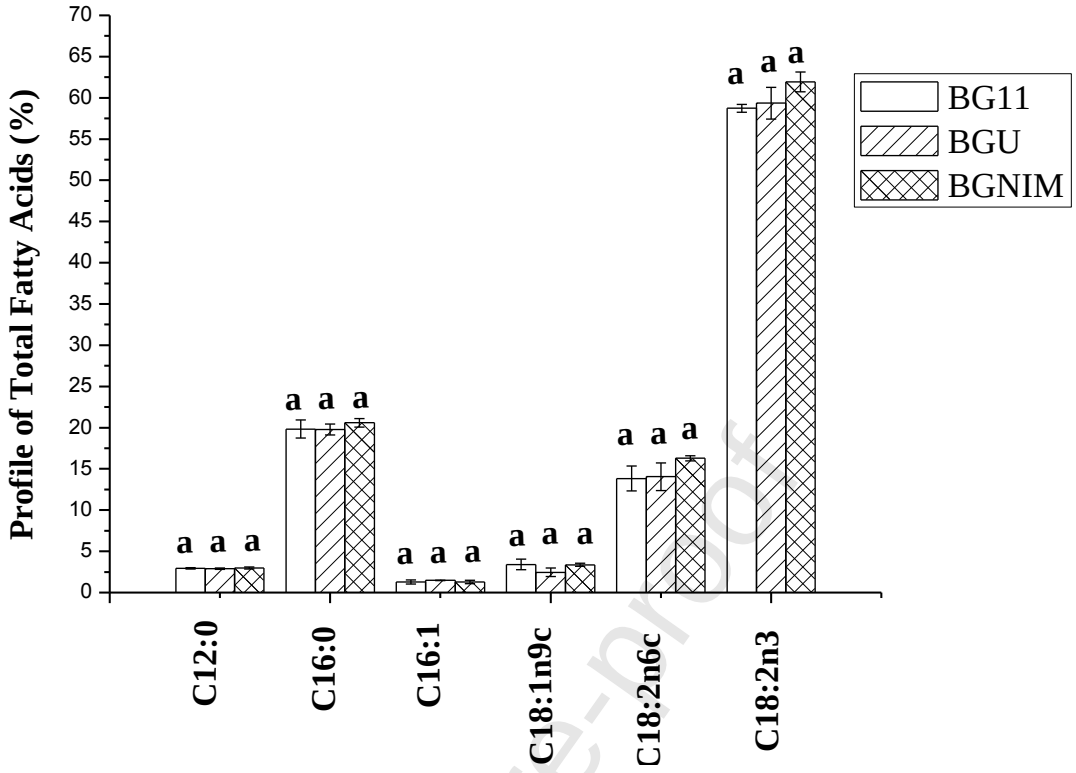
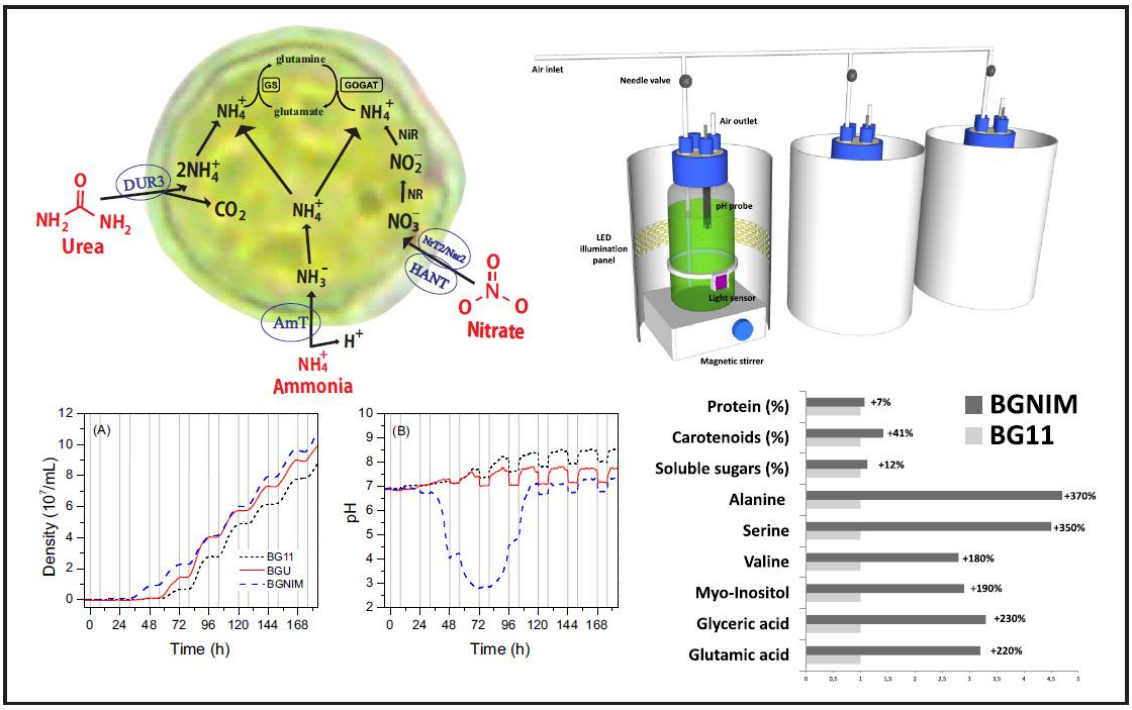


Figure 5. Profile of fatty acid methyl esters (FAMEs) from Chlorella sorokiniana grown on BG11, BGU and BGNIM culture media. All the experiments were conducted in three independent replicates (n=3). The results are presented as means of the replicates ± error bars show the standard deviation.

* Means followed by the same letter do not differ by Tukey test at the 5% probability level.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. No conflicts, informed consent, human or animal rights applicable.



Graphical abstract

A low-cost approach for *Chlorella Sorokiniana* production through combined use of urea, ammonia and nitrate based fertilizers

Highlights

- BGNIM medium presented similar productivity to standard media at a lower cost
- Increased production of high-value metabolites was observed using BGNIM medium
- The acidification observed might be used for predator control