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Enhancing Secondary Metabolites in *Chlorella sorokiniana* using alternative medium with vinasse

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Abstract: Microalgae production is expensive and demands high volumes of water and energy to meet all market demands and yet the production of secondary metabolites using alternative media needs deeper studies. Among alternative media, the use of sugar cane vinasse has gained attention for microalgae cultivation. In this work, we've compared the biomass yield and secondary metabolites of *Chlorella sorokiniana* grown in a commercial medium (Sueoka) and in alternative medium prepared with cane vinasse (0.1%) supplemented with N, P₂O₅ and K₂O (commercial formula 20-5-20 g.L⁻¹). The production of microalgae biomass grown in an alternative medium was 2.2% lower, but reached its maximum growth point 14 days faster than the commercial medium. The average level of Phenolic compounds on the commercial medium were 6.02 ± 0.13 mg GAE .g⁻¹ and increased to 15.28 ± 0.32 mg GAE.g⁻¹ when *C. sorokiniana* were grown in the vinasse medium. Same how, Flavonoids contents varied from 13.12 ± 1.33 to 72.30 ± 5.28 mg QE. g⁻¹ to commercial and vinasse medium, respectively. The maximum AOA of *C. sorokiniana* grown in vinasse medium was 88.05% at a concentration of 1500 µg.mL⁻¹ of extract, with IC₅₀ at 357.7 ± 27.35 µg.mL⁻¹. Different factors seem to induce variances on secondary metabolites synthesis, mostly stress due to QOD and ions added to the medium by vinasse. The authors recognize there are more investigations to be done, but increasing a natural and low cost pathway for flavonoids yield open up an interesting field for the bioprospection of microalgae.

Keywords: Antioxidant activity; dietary supplements; microalgal cultivation

1. Introduction

Microalgae are unicellular organisms with little or no cell differentiation but with a remarkable capacity of colony formation [1]. The green microalgae (Chlorophyta) have been widely exploited due to their biochemical properties and potential use for human, animal and aquaculture dietary supplementation. As a well-known source of secondary metabolites, specially antioxidants, the green microalgae have also a great appeal for cosmetics and pharmaceuticals and lately, many manuscripts have reported it as a promising biofuel source [2,3].

The microalgae cultivation has a wide biotechnological potential [4]. With rapid growth and high biomass production, in addition to the synthesis of secondary metabolites, its potentialities may still be favored by the regulation of their growth medium once the biochemical composition of microalgae depends on the macro and micronutrients used during medium preparation [5]. Microalgae bioactive compounds are synthesized by secondary metabolism and can be ranged among the most diverse biological activities, such as anticancer, antioxidant and antimicrobial [6, 7, 8]. These compounds can be used in isolation when extracted by specific solvents and extraction

methods, depending on their chemical affinities, or be used as in the naturally occurring pool of compounds [9].

Phenolic compounds, for example, can be found in extracts of microalgae from several taxonomic groups [10]. It is an important group from plant metabolism and have become very relevant for human health due to their characteristics particularly related to their antioxidant activity [11, 12] and pharmacological properties, such as antiviral and antimicrobial activities [13].

Several *Chlorella* species have been studied due to their anti-cancer properties. It's known that microalgae are an important source of carotenoids, carbohydrates, vitamins, unsaturated fatty acids, and other bioactive substances [14], but *Chlorella sorokiniana* is among the most interesting species considering pharmacological properties. Recent study investigated the immune response to lung cancer cells and found out as result a significant reduction of subcutaneous tumors from the ingestion of these microalgae [15]. Another study published important results where *C. sorokiniana* membrane was used to inhibit Colon carcinoma growth in mice [16]. Antioxidants properties of *C. sorokiniana* are also told to be neuroprotective, improving short-term memory [17], inhibiting cholinesterase and modulating disaggregation of β -amyloid fibrils, also known as mediators of Alzheimer's disease [4].

Another peculiarity of *C. sorokiniana* is that the species have been tested for bioremediation of xenobiotics compounds and wastewater treatments [18, 19, 20]. Moreover, the literature has several reports about its advantages as biofuel [21, 22, 23]. Such diversity of appliances and bioprospections can be also assigned to *C. sorokiniana* ease of cultivation, to a high productivity in reduced areas, to a high rate of duplication. And last but not least, *C. sorokiniana* can be grown in non-agricultural areas and use wastewater as substrate; thus microalgae seems more advantageous when compared to other oilseeds [24].

Summarizing, the cultivation of *Chlorella* can occur in a photoautotrophic, heterotrophic or myxotrophic systems [3]. In photoautotrophic system, the green microalgae obtain their energy from the light and carbon from the CO₂ in the air; by photosynthesis the microalgae also synthesize important biomolecules, such as polysaccharides, proteins and lipids [25]. When microalgae use organic compounds and carbon, both as a source of energy, it is named as a heterotrophic system; the same system can also provide light as an energy source. In a mixotrophic cultivation, microalgae can alternate their energy source, using light energy, organic compounds and inorganic compounds; as a carbon source they can use organic compounds or CO₂ [26]. Nevertheless, growing microalgae is an expensive activity, with a high water consumption and energy requirements. Thus, growing them using a residue as a complement to the culture medium is the alternative to reduce costs and save water [27].

In the present study, we prospect biotechnological uses for the biomass of *C. sorokiniana*, grown in a medium enriched with NPK and vinasse compared to the species grown in the commercial medium (Sueoka); the authors have also used the aqueous extract of microalgae to identify the presence of phenolic and flavonoid compounds, in addition to assessing the potential antioxidant.

2. Materials and Methods

2.1. Microalgae Cultivation

The experiment took place at the CPBio laboratory of the State University of Mato Grosso do Sul. *C. sorokiniana* (Chlorophyceae) strain was provided by the Andre Tosello Foundation (CTT 7727; IBVF 211-32, University of Seville, Spain).

The strain was grown for 56 days in 5 liter terephthalated polyethylene bottles, under a static and non-axenic cultivation system, with constant aeration and controlled temperature (25°C) and photoperiod (12 h light / 12 h dark). Conductivity, pH and cell density were measured during the 9-week period of cultivation.

We've tested two different media to compare the microalgae growth responses. The first medium used was the commercial formula Sueoka [28], which composition is shown in table 1.

Table 1. Composition of Sueoka Culture Medium. a) Macronutrients contents; b) Elements Hutner's Traces in 200x in 500 ml of water. * in 250 ml of water.

a) Macronutrients Sueoka Medium	
KH ₂ PO ₄	8,3 mM
K ₂ HPO ₄	5,3 mM
MgSO ₄ ·7H ₂ O	0,25 mM
CaCl ₂ ·2H ₂ O	0,133 mM
Hutner's Trace Elements	1x
NH ₄ Cl	9,35 mM
b) Hutner's Trace Elements 200 x	
EDTA-Na ₂ H ₂ O *	12,7 g
H ₃ BO ₃	2,28 g
ZnSO ₄ ·7H ₂ O	4,40 g
MnCl ₂ ·4H ₂ O	1,02 g
FeSO ₄ ·7H ₂ O	1,00 g
CoCl ₂ ·6H ₂ O	0,32 g
CuSO ₄ ·5H ₂ O	0,32 g
(NH ₄) ₆ MoO ₂₄ ·4H ₂ O	0,22 g

The second medium was prepared using 0.07 g of chemical formula N:P:K (5:20:20 g L⁻¹), diluted in 2000ml distilled water [29] and vinasse (0.1 %). NPK contents consisted of 0.14 N, 0.035 P₂O₅ and 0.14 K₂O and 10 mL L⁻¹ of sugar cane vinasse (values between 0.15-0.70 N; 0.01-0.21 P₂O₅; 1.2-2.1 K₂O; 0.13-1.5 CaO; 0.20-0.49 MgO, among other compounds found in smaller concentrations).

The biomasses from both media were collected by thermal decantation (7.0 ± 1.5°C) directly from the bottles and centrifuged at 3500 rpm for 10 minutes. The pellets were lyophilized under 0.16mbar.

2.2. Microalgae extracts

The biomass was diluted in water (1:20 v:v) and warmed until the solution reached a boiling stage. The solution was then homogenized with the aid of a magnetic mixer during one hour. After, the solution was centrifuged at 3500 rpm for 10 minutes and the supernatant was collected and lyophilized under 0.16 mbar.

2.3. Total phenolic and flavonoid contents

Total phenolic content in the extract was determined by the Folin-Ciocalteu reagent [30]. Gallic acid (2 - 100 µL) was the standard solution for the calibration curve. The absorbance of the solutions was measured at 760 nm and the level of phenol in the extract samples (100 µg mL⁻¹) was determined in triplicate by interpolation of absorbance using an analytical curve settled with a linear regression model. The equation was established in order to relate the concentration of Gallic acid and the absorbance of each reading, which enabled indirect quantification of the total phenolic content in the extract. Data were expressed in milligrams (mg) of Gallic acid per gram of extract (mg GAE g⁻¹ of extract).

The total amount of flavonoids was determined by interpolation of absorption of the extracted samples (100 µg mL⁻¹) using an analytic curve adapted from [31]. The reagent was a 2% solution of Aluminum Trichloride (AlCl₃) in methanol.

Quercetin was chosen as a standard (2 - 100 µL) to produce the calibration curve. Total flavonoids content was determined in triplicate. Absorbance was registered at 415nm against a methanol blank (spectrophotometer). Based on the linear regression model, the equation enabled the association between the Gallic acid concentrations with the absorbance of each reading, what configured an indirect quantification approach of flavonoids in the extract. Data were expressed in milligrams quercetin equivalent per gram of extract (mg QE g⁻¹of extract).

2.4. DPPH essays

Antioxidant activity of the extracts was estimated in vitro using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical capture ability (%SRL). The aqueous extract was diluted in two different concentrations (50 a 2500 $\mu\text{m mL}^{-1}$) using ethanol.

Further, DPPH aliquots were added to the samples in glass cuvettes. The absorption was measured in spectrophotometer at 517nm using 80% ethanol blank. Ascorbic acid (AA) and butylated hydroxyl toluene solution (BHT), natural and synthetic antioxidant, were used as positive controls. Absorption readings enabled calculations of the inhibition index as follows:

$$\% \text{ inhibition DPPH} = (\text{Abscontrol} - \text{Abssample}) / \text{Abscontrol} \times 100, \quad (1)$$

The IC_{50} was calculated based on the concentration necessary to capture 50% of the free radicals in the reaction.

2.5. Data analysis

Data were analyzed using the Statistica® 6.0 software and Graph Pad Prism 5.0. A variance analysis (ANOVA) was developed fixing the significance level to 95%. Student Newman Keulstests were carried out to check for significant differences between cultivation media.

3. Results and Discussion

3.1. Biomass Performance

The figure 1 shows the evolution of the biomass density during eight weeks, comparing the culture of *C. sorokiniana* in commercial medium Sueoka and in alternative medium enriched with vinasse and NPK. The biomass of *C. sorokiniana* grown in medium enriched with NPK and vinasse reached the yield peak at the 35th day with 8.9 g L⁻¹ (± 0.13) of dry mass. When microalgae were cultivated in commercial medium, the highest production was 9.1 g L⁻¹ (± 0.54) achieved within 49 days. It's important to emphasize that despite the microalgae dry biomass values being 2.2% lower in the alternative environment, the peak of algal biomass production medium was 14 days faster when compared to the commercial medium. Achieving higher biomass production in a relatively shorter time can mean savings in energy and culture media besides an increased production to industrial scale. Furthermore, in a study with *C. sorokiniana* cultivated in commercial Bold's Basal Medium (BBM) the biomass yield was estimated in 16.31 mg L⁻¹ d⁻¹ [32] and in another similar study with the same species cultivated in BBM, the authors estimated the biomass in 43.33 mg L⁻¹ d⁻¹ [23]. Both studies showed values considerable lower in comparison to those obtained in the present work.

When the algal density was considered, [33] another study estimated it in 1.01 x 10⁻⁵ ml⁻¹ in the 23th day of cultivation in mixed medium (BBM and NPK), while in our working using the alternative medium we have a density of 45 x 10⁻⁵ ml⁻¹ in the 21th day of cultivation.

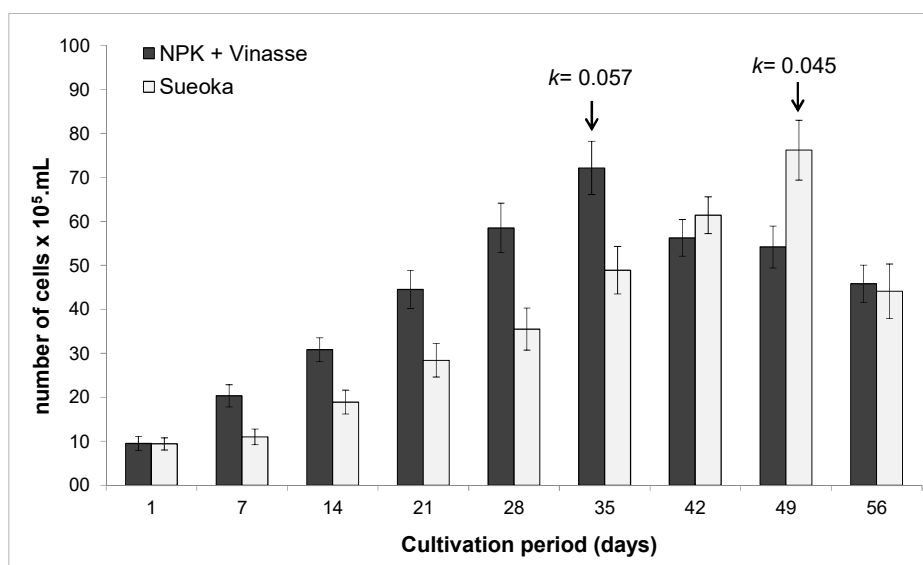


Figure 1. Average ($N = 5 \pm$ standard error) of the density of microalgae *C. sorokiniana* over eight weeks of production and exponential growth rate (k).

3.2. Total Phenolic and Flavonoid Contents

Phenols and flavonoids are the secondary metabolites more produced by microalgae when in detriment of UV exposure or under stress conditions induced by the culture medium. Those compounds are highlighted in cell protection and have an special role against the presence of harmful compounds [34, 35, 36]. Our results corroborate with the previous authors (Table 2), since microalgae biomasses were grown under UV radiation and used culture medium enriched with vinasse and NPK (EA- Cs2). It is possible the highest content of these metabolites is a *C. sorokiniana* response for cell preservation and multiplication.

Table 2. Total Phenolic and flavonoids content in the aqueous extract of *Chorella sorokiniana*. AE-Cs1 aqueous extract of *C. sorokiniana* grown in commercial formula Sueoka; AE-Cs2 aqueous extract of *C. sorokiniana* grown in medium enriched with NPK and vinasse.

	Total Phenolic (mg GAEg ⁻¹)	Flavonoids (mgQEg ⁻¹)
AE-Cs1	6,02±0,13	13,12±1,33
AE-Cs2	15,28±0,32	72,30±5,28

The highest content of total phenolic and flavonoids found in AE-Cs2 could indicate that adding organic and mineral compounds to culture medium enhance the secondary metabolism of *C. sorokiniana* and can be an advantageous practice for scale production aiming to cosmetics, pharmaceuticals and dietary industries. Usually, commercial media present important restriction concerning nutrients balance for microalgae development.

Some studies demonstrate a ratio between presence/absence of UV radiation and the addition/restriction of nutrients with the synthesis of secondary metabolites. [36] Some bioactives have variation when produced by *Chlorella minutissima* grown with different doses of nitrogen in a mixotrophic and in a photoautotrophic systems, and the authors concluded the presence of this nutrient, especially in mixotrophic conditions, increased the production of secondary metabolites which reinforces our results. Under similar conditions, we've added NPK, with no detriment to carbon sources as the sugars from the vinasse, the nutrients were assimilated by *C. sorokiniana* during the dark periods, characterizing mixotrophic cultivation.

Phenolic compounds are ubiquitous constituents in the group of phytochemicals and they exert a range of biological activities, including antioxidant activity and anti-inflammatory and

antimicrobial effects [37, 38]. Interestingly, total phenolic contents found in this study were lower than the values of flavonoids, in both extracts. A possible explanation would be the high polarity of the water which was used as a solvent in the preparation of the extracts. Water is not a good extractor of phenolic compounds [39]. Nevertheless, water can extract glycosylated compounds which are usually complexed to flavonoids and have antioxidant activity [40, 41].

[42] Studies showed that microalgae contain a wide range of flavonoids and that, therefore, they must have the enzyme pool necessary for their biosynthesis. In addition, some microalgae have shown they have a flavonoid synthesis pattern compatible to the pathway of some superior plants.[43, 44] verified the presence of precursors at the beginning of the metabolic route: cumaric acid and p-coumaric acid and another group derived from its metabolites in freshwater algae. However, literature has an insufficient data concerning flavonoids contents in microalgae and their specific function It is known they are a large group of secondary metabolites involved in cellular processes for UV protection, since signaling to pigmentation, and about its applications as a supplement in functional foods and the use by Pharmaceutical industry due to its antioxidant activity and hormone-like roles [45, 46, 47, 48, 49]. Thus, there is an urgent need for deep studies concerning the identification of these compounds as for the subclasses usually associated to microalgae, in addition to the standardization of solvent affinities in order to enhance extraction processes of these metabolites and their real functions.

3.3. Antioxidant Activity

Once we tested the presence of secondary metabolites, the *in vitro* antioxidant capacity has been assessed using the DPPH stable free radical scavenging method. The method is based on the ability of certain substances in donating a hydrogen atom to the radical, reducing it to hydrazine, and causing a color change from violet to pale yellow. This change in color is accompanied by a drop in absorbance [50]. The IC₅₀ and maximum activity values are shown in Table 3.

Table 3. DPPH radical scavenging activity in the aqueous extract of *Chlorella sorokiniana* compared to the standard antioxidants ascorbic acid (AA) and BHT. IC₅₀ concentration of the extract required to inhibit 50% of DPPH free radicals in µg mL⁻¹; AE-Cs1 aqueous extract of *C. sorokiniana* grown in commercial medium Sueoka; AE-Cs2aqueous extract of *C. sorokiniana* grown in medium enriched with NPK and vinasse.

	IC ₅₀	µgmL ⁻¹	%
AA	4,76 ± 0,29	10	88,79
BHT	49,71 ± 5,17	500	94,25
AE-Cs1	2062 ± 266,5	4000	89,14
AE-Cs2	357,7 ± 27,35	1500	88,05

Considering the unreasonable results expressed in the AE-Cs1 in comparison to the controls, we could infer that the extract of *C. sorokiniana* grown with the addition of vinasse and NPK tested a better antioxidant activity. Since the IC₅₀ value found for AE-Cs1 (2062 µg / mL) is almost five times higher than AE-Cs2 (357.7 µg / mL) and although both demonstrate a very close maximum activity, the concentration of AE-Cs1 needed to achieve this pattern is 2.6 times higher than for AE-Cs2.

[51] In one study of the antioxidant effect of marine microalgae *C. vulgaris*, found a maximum value of 85% of free radical scavenging activity with the minimum concentration of 70 mg L⁻¹ ethanol extract. A possible explanation could possibly be the low levels of metabolites found in our extracts. Some authors mentioned the correlation between phenolic compounds content and the antioxidant activity for microalgae. [35, 52] For explained that, in addition to phenolic compounds, carotenoids are also responsible for a large part of microalgae antioxidant activity. On the other hand, [53] another work show a low correlation between phenolic compounds and antioxidant activity, questioning the true role of these compounds.

Although the IC₅₀ values of the extracts are considerably high in comparison to the controls, they still have an antioxidant activity that is worth of further investigation. Notably due to the great

use of these compounds by the food industry, which aims to maintain the sensorial and nutritional quality and increase the shelf-life of products, especially lipid-based products, where the aim is to minimize rancidness and to delay the formation of toxic compounds[54]. Furthermore, the search for new natural sources of antioxidant compounds is of great value, since a large part of the synthetic substances used have toxic characteristics [55, 56].

4. Conclusions

Using vinasse and NPK, respectively as a source of carbon and nutrients to the cultivation *C. sorokiniana*, showed to improve biomass yield and enhance the synthesis of secondary metabolites, in addition of being a natural and low cost medium supplement. The contents of phenolic compounds and flavonoids, and the antioxidant activity found in the aqueous extract of *C. sorokiniana* produced in medium enriched with vinasse and NPK seems to be a viable alternative for a higher scale aiming the cosmetics, the pharmaceuticals and the dietary industry. Bioprospecting *C. sorokiniana* can generate low-cost services and products of high commercial value, thus the authors fully recommend more and deeper studies considering the species production.

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