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Enhancing secondary metabolite production by *Chlorella sorokiniana* using an alternative medium with vinasse

Avaliação da produção de metabólitos secundários por *Chlorella sorokiniana* usando meio alternativo com vinhaça

Evaluación de la producción de metabolito secundario de *Chlorella sorokiniana* usando medio alternativo con vinaza

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Abstract
Microalgae production is expensive and requires high volumes of water and energy. The use of sugar cane vinasse as an alternative medium, has gained attention for microalgae cultivation. In this study, we compared the biomass yield and secondary metabolite production by *Chlorella sorokiniana* grown in a commercial medium (Sueoka) and versus those grown in a medium prepared with cane vinasse (0.1%) supplemented with N:P:K (20-5-20 $g.L^{-1}$). Microalgae reached the maximum growth point 14 days faster in the alternative medium. Increased average phenolic compound levels and flavonoid content were found in the vinasse medium (15.28 ± 0.32 mg $GAE.g^{-1}$ and 72.30 ± 5.28 mg QE. g^{-1}, respectively) compared to that of the commercial medium (6.02 ± 0.13 mg GAE $.g^{-1}$ and 13.12 ± 1.33 mg QE g^{-1}, respectively). The maximum antioxidant activity (AOA) of *C. sorokiniana* grown in vinasse medium was 88.05% with an extract concentration of 1500 $\mu g.mL^{-1}$, and an IC_{50} of 357.7 ± 27.35 $\mu g.mL^{-1}$. Different factors, such as stress due to chemical oxygen demand (COD), and vinasse-added ions, may have induced variances in secondary metabolite synthesis. Further investigations are needed to explore natural and low cost alternatives to increasing flavonoid yield for the bioprospection of microalgae.
Keywords: Antioxidant activity; Dietary supplements; DPPH; Microalgal cultivation.

Resumo
A produção de microalgas é cara e requer grandes volumes de água e energia. O uso da vinhaça da cana-de-açúcar como meio alternativo, tem ganhado destaque para o cultivo de microalgas. Neste estudo, comparamos o rendimento de biomassa e a produção de metabólitos secundários por *Chlorella sorokiniana* cultivada em meio comercial (Sueoka) e com aqueles cultivados em meio preparado com vinhaça de cana (0,1%) suplementado com N:P:K (20-5-20 gL^{-1}). As microalgas atingiram o ponto máximo de crescimento 14 dias mais rápido no meio alternativo. Níveis médios de compostos fenólicos e conteúdo de flavonóides foram encontrados no meio de vinhaça (15,28 ± 0,32 mg $GAE.g^{-1}$ e 72,30 ± 5,28 mg $QE.g^{-1}$, respectivamente) em comparação com o meio comercial (6,02 ± 0,13 mg $GAE.g^{-1}$ e 13,12 ± 1,33 mg $QE.g^{-1}$, respectivamente). A atividade antioxidante máxima (AOA) de *C. sorokiniana* cultivada em meio de vinhaça foi de 88,05% com concentração de extrato de 1500 $\mu g.mL^{-1}$ e IC_{50} de 357,7 ± 27,35 $\mu g.mL^{-1}$. Diferentes fatores, como estresse devido à demanda química de oxigênio (DQO) e íons adicionados da vinhaça, podem ter induzido variações na síntese de metabólitos secundários. Mais investigações são necessárias para explorar alternativas naturais e de baixo custo para aumentar a produção de flavonoides para a bioprospecção de microalgas.
Palavras-chave: Atividade antioxidante; Suplementos dietéticos; DPPH; Cultivo de microalgas.

Resumen

La producción de microalgas es cara y requiere grandes volúmenes de agua y energía. El uso de la vinaza de caña de azúcar como medio alternativo ha ganado atención para el cultivo de microalgas. En este estudio, comparamos el rendimiento de biomasa y la producción de metabolitos secundarios de *Chlorella sorokiniana* cultivada en un medio comercial (Sueoka) y los cultivados en un medio preparado con vinaza de caña (0.1%) suplementado con N:P:K (20-5-20 gL^{-1}). Las microalgas alcanzaron el punto de crecimiento máximo 14 días más rápido en el medio alternativo. Se encontraron mayores niveles promedio de compuestos fenólicos y contenido de flavonoides en el medio de vinaza (15,28 ± 0,32 mg $GAE.g^{-1}$ y 72,30 ± 5,28 mg $QE.g^{-1}$, respectivamente) en comparación con el del medio comercial (6,02 ± 0,13 mg $GAE.g^{-1}$ y 13,12 ± 1,33 mg $QE.g^{-1}$, respectivamente). La actividad antioxidante máxima (AOA) de *C. sorokiniana* cultivada en medio vinaza fue del 88,05% con una concentración de extracto de 1500 $\mu g.mL^{-1}$ y una IC_{50} de 357,7 ± 27,35 $\mu g.mL^{-1}$. Diferentes factores, como el estrés debido a la demanda química de oxígeno (DQO) y los iones añadidos de vinaza, pueden haber inducido variaciones en la síntesis de metabolitos secundarios. Se necesitan más investigaciones para explorar alternativas naturales y de bajo costo para aumentar el rendimiento de flavonoides para la bioprospección de microalgas.

Palabras clave: Actividad antioxidante; Suplementos dietéticos; DPPH; Cultivo de microalgas.

1. Introduction

Microalgae are unicellular organisms that exhibit little or no cell differentiation but have a remarkable capacity for colony formation (Ohse et al. 2008). Green microalgae (Chlorophyta) possess a wide range of biochemical properties, and are, therefore, used as human, animal, and aquaculture dietary supplements. As well-known sources of secondary metabolites, especially antioxidants, green microalgae are also being increasingly used in cosmetics and pharmaceutics. Recently, many studies have reported that microalgae are promising biofuel sources (Skjånes et al. 2013; Zhang et al. 2013).

Microalgae cultivation also has many biotechnological applications (Olasehinde et al. 2019). With rapid growth and high biomass production, in addition to the synthesis of secondary metabolites, their potentialities can be customized by regulating their growth medium, as the biochemical composition of microalgae depends on the macro- and micronutrients incorporated during medium preparation (Plaza et al. 2009). Some bioactive compounds produced by secondary metabolites in microalgae, which have the most diverse biological activities, including anticancer, antioxidant, and antimicrobial activities (Katharios et al. 2005; Parisi et al. 2009; Chu 2012). These compounds can be isolated using specific solvents and extraction methods, depending on their chemical affinities, or may be used as a naturally occurring pool of compounds (Mariutti & Bragagnolo 2007).

Phenolic compounds, for example, may be found in extracts of microalgae from several taxonomic groups (Scholz & Liebezeit 2012). It is an important compound group in plant metabolism and has become very relevant for human health applications because of its characteristics, particularly those related to their antioxidant activity (Vendramini & Trugo 2004; Cha et al. 2010) and pharmacological properties, such as antiviral and antimicrobial activities (Abd El-Baky et al. 2008).

Microalgae are known to be an important source of carotenoids, carbohydrates, vitamins, unsaturated fatty acids, and other bioactive substances (Borowitzka 2013). Several *Chlorella* species have been studied for their anti-cancer properties; *Chlorella sorokiniana* is among the most interesting ones being studied, considering its known pharmacological properties. A recent study investigated the improved immune response to lung cancer cells and found a significant reduction in subcutaneous tumors upon ingestion of these microalgae (Lin et al. 2017). Another study published important results where the *C. sorokiniana* membrane was used to inhibit colon carcinoma growth in mice (Ishiguro et al. 2020). Antioxidant properties of *C. sorokiniana* are also found to be neuroprotective, and are known to improve short-term memory (Morgese et al. 2016), inhibit cholinesterase activity, and modulate disaggregation of β-amyloid fibrils, which are the mediators of Alzheimer's disease (Olasehinde et al. 2019).

C. sorokiniana has also been tested for bioremediation of xenobiotic compounds and wastewater treatments (Shen et al. 2018; Sutherland & Ralph 2019; Chen et al. 2020). Moreover, there are several reports about its advantages as biofuel (Li et al. 2013; Choi et al. 2019; Menegazzo et al. 2020). The diverse applications and bioprospections of this species

can also be attributed to its ease of cultivation, exhibiting high productivity and high rate of duplication. Last but not least, *C. sorokiniana* can be grown in non-agricultural areas using wastewater as its substrate. Thus, microalgae seem to be more advantageous than other oilseeds (Wu et al. 2012).

In summary, the cultivation of *Chlorella* can occur in photoautotrophic, heterotrophic, or myxotrophic systems (Zhang et al. 2013). In photoautotrophic systems, green microalgae obtain their energy from light and carbon from the CO_2 in the air. Through photosynthesis, the microalgae also synthesize important biomolecules, such as polysaccharides, proteins, and lipids (Huang et al. 2010). A heterotrophic system allows for the usage of organic compounds as energy source; the same system can also provide light as an energy source. In a mixotrophic system, microalgae can alternate the usage of light energy, organic compounds, and inorganic compounds as energy sources; they can use organic compounds or CO_2 as carbon sources (Jiang et al. 2011). Despite having varied cultivation systems, growing microalgae is still expensive, with high water and energy requirements. Thus, growing them using a residue as a complement to the culture medium can be an alternative to reduce costs and save water (Pires et al. 2013).

In this study, we investigated the benefits of *C. sorokiniana* biomass grown in a medium enriched with nitrogen, phosphorus, and potassium (NPK) and vinasse compared to those grown in a commercial medium (Sueoka). We also identified the presence of phenolic and flavonoid compounds in the microalgal aqueous extract, in addition to assessing its potential antioxidant activity.

2. Methodology

This study has a laboratory character and quantitative-experimental methodology (KOCHE 2011). It was carried out in a systematic way that can be verifiable and reproducible (PEREIRA et al. 2018), characterization of the production of metabolites of metabolites in microalgae C. sorokiniana biomass in commercial medium Sueoka and in alternative medium NPK + vinasse, looking for means with greater cost benefit.

2.1 Microalgae Cultivation

The experiment was conducted at the CPBio laboratory of the State University of Mato Grosso do Sul. The *C. sorokiniana* (Trebouxiophyceae) 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-L terephthalated polyethylene bottles under a static and non-axenic cultivation system, with constant aeration, controlled temperature (25 ^{0}C) and controlled photoperiod of 2.500 LUX provided by white fluorescent lamps (12 h light / 12 h dark). Conductivity, pH, and cell density were measured during the 9-week period of cultivation.

We tested two different media to compare microalgae growth responses. The first medium used was the commercial formula Sueoka (Sueoka 1960), the composition of which is shown in Table 1.

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Table 1. Composition of Sueoka Culture Medium. a) Macronutrient content; b) Hutner's Trace Elements (200x) in 500 mL of water. * Composition in 250 mL of water.

a) Macronutrients Sueoka Medium	
KH_2PO_4	8.3 mM
K_2HPO_4	5.3 mM
$MgSO_4 \cdot 7H_2O$	0.25 mM
$CaCl_2 \cdot 2H_2O$	0.133 mM
Hutner's Trace Elements	1x
NH_4Cl	9.35 mM
b) Hutner's Trace Elements (200 x)	
EDTA-Na·$2H_2O$ *	12.7 g
H_3BO_3	2.28 g
$ZnSO_4 \cdot 7H_2O$	4.40 g
$MnCl_2 \cdot 4H_2O$	1.02 g
$FeSO_4 \cdot 7H_2O$	1.00 g
$CoCl_2 \cdot 6H_2O$	0.32 g
$CuSO_4 \cdot 5H_2O$	0.32 g
$(NH_4)_6MoO_{24} \cdot 4H_2O$	0.22 g

Source: Sueoka (1960).

As shown in table 1, Sueoka medium is chemically formulated as macronutrients and micronutrients and is widely used for the production of microalgae.

The second medium was prepared using 0.07 g of a chemical mixture comprised of nitrogen, phosphorus, and potassium (N:P:K ratio of 20:05:20 g L^{-1}), diluted in 2000 mL distilled water (Sipaúba-Tavares & Rocha 2003) and vinasse (0.1 %). NPK contents consisted of 0.14 g L^{-1} N, 0.035 g L^{-1} P_2O_5 and 0.14 g L^{-1} K_2O. Sugar cane vinasse consisted of values between 0.15-0.70 g L^{-1} N; 0.01-0.21 g L^{-1} P_2O_5; 1.2-2.1 g L^{-1} K_2O; 0.13-1.5 g L^{-1} CaO; 0.20-0.49 g L^{-1} 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 were centrifuged at 3500 rpm for 10 min. The pellets were lyophilized at 0.16 mbar.

2.2 Microalgae extracts

The biomasses were diluted in water (1:20 v:v) and were warmed until the solutions reached the boiling stage. The solutions were then homogenized with the aid of a magnetic mixer for 1 h. The solutions were centrifuged at 3500 rpm for 10 min and the supernatants were collected and lyophilized under 0.16 mbar.

2.3 Total phenolic and flavonoid contents

The total phenolic content in each extract was determined using the Folin-Ciocalteu reagent (Meda et al. 2005). Gallic acid (2–100 µL) was the standard solution for the calibration curve. The absorbance values of the solutions were measured at 760 nm, and the phenolic levels in the extracts (100 µg mL^{-1}) were determined in triplicates by interpolation of absorbance

readings by interpolation of absorbance readings via linear regression. Data were expressed in milligrams (mg) of gallic acid per gram of dry extract (mg GAE g^{-1} of extract).

The total amount of flavonoids from each extract was determined by interpolation of the absorption of the extracted samples (100 µm mL-1) using an analytic curve adapted from a previous study (Liberio et al. 2011). The reagent used was a 2% solution of aluminum trichloride ($AlCl_3$) in methanol.

Quercetin was chosen as the standard (2 – 100 µL) to produce the calibration curve. Total flavonoid content was determined in triplicates. Absorbance was measured at 415 nm against a methanol blank (spectrophotometer). Data were expressed in mg quercetin equivalent per gram of dry extract (mg QE g^{-1} of extract).

2.4 DPPH essays

The antioxidant activity of each extract was estimated *in vitro* based on 2.2-diphenyl-1-picrylhydrasyl (DPPH) free radical capture ability. Each aqueous extract was diluted in two different concentrations (50, 2500 µm mL^{-1}) using ethanol.

Further, DPPH aliquots were added to the samples in glass cuvettes. The absorption was measured using a spectrophotometer at 517 nm; an 80% ethanol solution was used as blank. Ascorbic acid (AA) and butylated hydroxyl toluene solution (BHT), natural and synthetic antioxidants, respectively, were used as positive controls. Inhibition indices were calculated with the absorption readings using Equation 1:

$$\% \text{ Inhibition } DPPH = (Abscontrol - Abs\text{sample}) / Abscontrol \times 100, \quad 1)$$

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

2.5 Data analysis

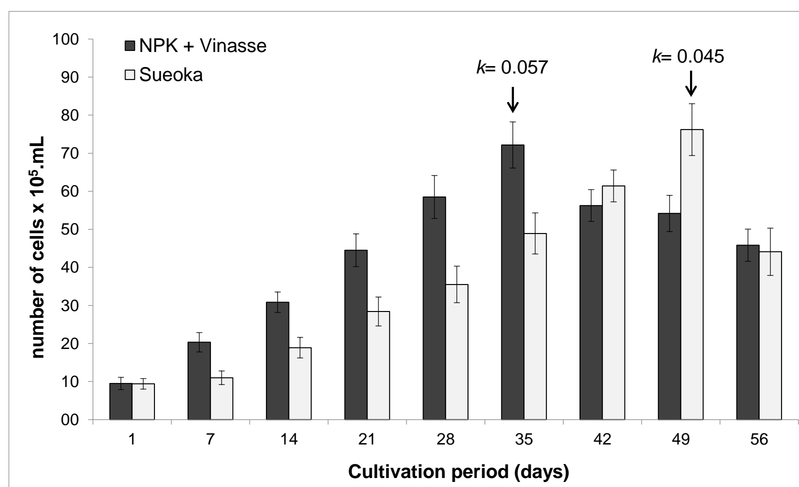
Data were analyzed using the Statistica® 6.0 software and Graph Pad Prism 5.0. A variance analysis (ANOVA) was used, with a significance level of 95%. Student Newman Keulstests were carried out to check for significant differences ($p>0.05$) between cultivation media.

3. Results and Discussion

3.1 Biomass Performance

Figure 1 shows the evolution of biomass density of *C. sorokiniana* over eight weeks in both the commercial medium Sueoka and the alternative medium enriched with vinasse and NPK. The biomass of *C. sorokiniana* grown in the alternative medium reached the yield peak on the 35th day with 8.9 g (± 0.13) of dry mass. The highest dry mass yield in the commercial medium, on the other hand, was 9.1g (± 0.54), which was achieved on the 49th day of culture.

Figure 1. Average (N = 5 ± standard error) densities of microalgae *C. sorokiniana* over eight weeks of production. The figure also shows the exponential growth rate (k).



Source: Authors.

It is important to emphasize that despite the microalgae dry biomass values being 2.2% lower in the alternative environment, the peak in the algal biomass production medium was observed 14 days faster than that in the commercial médium (Figure 1). Achieving higher biomass production in a relatively shorter time indicate less energy expenditure and decreased amounts of culture media needed, in addition to increased production at an industrial scale.

In a study with *C. sorokiniana* cultivated in commercial *Bold's Basal Medium (*BBM), the biomass yield was estimated to be 16.31 mg L^{-1} d^{-1} (Kobayashi et al. 2013). In another similar study with the same species cultivated in BBM, the authors estimated the biomass to be 43.33 mg L^{-1} d^{-1} (Menegazzo et al. 2020). Both studies showed considerably lower values than those obtained in the present study.

Another study estimated algal density of 1.01×10^{-5} ml^{-1} on the 23th day of cultivation in mixed medium (BBM and NPK) (Ribeiro et al. 2019), whereas a density of 45×10^{-5} ml^{-1} was obtained on the 21th day of cultivation in our alternative medium.

3.2 Total Phenolic and Flavonoid Contents

Phenols and flavonoids are secondary metabolites produced by microalgae when exposed to UV light or when subjected to stressful conditions in culture medium. These compounds are primarily utilized for cell protection, as they exhibit special protective roles in the presence of harmful compounds (Goiris et al. 2015; Safafar et al. 2015; Aremu et al. 2015) Our results corroborate those of previous studies (Table 2), as microalgae biomasses were grown under UV radiation using culture medium enriched with vinasse and NPK (AE-Cs2). It is possible that the production of these metabolites was highly increased in *C. sorokiniana* for cell preservation and multiplication.

Table 2. Total, Phenolic and flavonoids content found in the aqueous extracts of *Chlorella 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

Source: Authors.

As can be seen in table 2, the total number of phenolics present in the medium of cultivation with alternative medium is more than twice as much as the microalgae cultivated in commercial medium. Total flavonoids also show a significantly higher amount of biomass grown in an alternative medium than cultivated in a commercial medium.

The highest total phenolic and flavonoid contents found in AE-Cs2 may indicate that the addition of organic and mineral compounds to the culture medium enhances the secondary metabolism of *C. sorokiniana* and may be an advantageous practice for larger scale production in the fields of cosmetics, pharmaceutics, and dietary industries. Usually, commercial media present important restrictions on nutrient balance for microalgae development.

Some studies have demonstrated the relationships between the presence/absence of UV radiation and the addition/restriction of nutrients with the synthesis of secondary metabolites. Production of some bioactives vary when *Chlorella minutissima* is grown with different doses of nitrogen in mixotrophic and in photoautotrophic systems; the authors concluded that the presence of this nutrient, especially in mixotrophic conditions, increases the production of secondary metabolites, which reinforces our results (Aremu et al. 2015). Under similar conditions, we added NPK, with no detriment to carbon sources, as the sugars from the vinasse, and the nutrients were assimilated by *C. sorokiniana* during dark periods, thereby characterizing mixotrophic cultivation.

Phenolic compounds are ubiquitous phytochemicals and exert a range of biological activities, including antioxidant, anti-inflammatory, and antimicrobial effects (Hajimahmoodi et al. 2010; Raposo & Morais 2015). Interestingly, the 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 used as a solvent for the preparation of these extracts. Water is not a good extractor of phenolic compounds (Vizzoto & Pereira 2011). However, water can extract glycosylated compounds that are usually complexed to flavonoids and have antioxidant activity (Martínez-Flórez et al. 2002; Havsteen 2012).

Studies have shown that microalgae contain a wide range of flavonoids; therefore, they must have the enzyme pool necessary for flavonoid biosynthesis (Goiris et al. 2014). In addition, some microalgae have shown that they have a flavonoid synthesis pattern compatible with the pathway of some superior plants. This is verified by the presence of precursors at the beginning of the metabolic route: coumaric acid, p-coumaric acid and another group derived from its metabolites in freshwater algae (Kledjus et al. 2009; Onofrejová et al. 2010). However, there is insufficient data regarding flavonoid contents in microalgae and their specific function. Flavonoids are a large group of secondary metabolites involved in cellular processes associated with UV protection and cellular signaling to promote pigmentation; they are used in functional foods and have been explored by the pharmaceutical industry owing to their antioxidant activity and hormone-like roles (Stafford 1991; Koes et al. 2005; Markham & Andersen 2006; Agati & Tattini 2010; Buer et al. 2010). Thus, there is an urgent need for further studies concerning the identification of these compounds as subclasses that are usually associated with microalgae, in addition to the standardization of solvent affinities to enhance the extraction processes of these metabolites and their real functions.

3.3 Antioxidant Activity

Once we confirmed the presence of secondary metabolites, the *in vitro* antioxidant capacity was assessed using the DPPH stable free radical scavenging method. This method is based on the ability of certain substances to donate a hydrogen atom to the free radical, reducing it to hydrazine, which causes a color change from violet to pale yellow. This color change is accompanied by a drop in absorbance (Alves et al. 2010). The IC_{50} and maximum activity values are shown in Table 3.

Table 3. DPPH radical scavenging activity in the aqueous extracts of *Chlorella sorokiniana* compared to the standard antioxidants ascorbic acid (AA) and BHT. IC_{50}: concentration of each 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-Cs2: aqueous 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**

Source: Authors.

Considering the unreasonable results expressed in AE-Cs1 in comparison to the controls, we could infer that the extract of *C. sorokiniana* grown with the addition of vinasse and NPK showed better antioxidant activity, as can be seen in the table 3. Since the IC_{50} value of AE-Cs1 (2062 µg/mL) was almost five times higher than that of AE-Cs2 (357.7 µg / µg/mL), and although both demonstrate a very close maximum activity, the concentration of AE-Cs1 needed to achieve this pattern was 2.6 times higher than that of AE-Cs2.

In one study, the antioxidant effect of marine microalgae *C. vulgaris* was found to have a maximum value of 85% of free radical scavenging activity with a minimum concentration of 70 mg L^{-1} ethanol extract (Vijayavel & Anbuselvam 2007). A possible explanation for these differences could be the low levels of metabolites found in our extracts.

Some authors have mentioned the correlation between phenolic compound content and the antioxidant activity of microalgae. In addition, carotenoids are also found to be responsible for a large part of microalgae antioxidant activity (Safafar et al. 2015; Goiris et al. 2012). On the other hand, Li et al. (2007) in another study showed a low correlation between phenolic compounds and antioxidant activity. These conflicting results lead us to questioning the true role of these compounds.

Although the IC_{50} values of the extracts are considerably higher than those of the controls, they still have antioxidant activities that are worth further investigations. These compounds are greatly used by the food industry, to maintain the sensorial and nutritional quality, and to increase the shelf-life of products, especially lipid-based ones. To prolong shelf-life, rancidness should be minimized and the formation of toxic compounds should be delayed (Maisuthisakul et al. 2007). Furthermore, the search for new natural sources of antioxidants is of great interest, as several synthetic substances used in the industry have toxic characteristics (Lobo et al. 2010; Zhang et al. 2010).

4. Conclusion

The use of vinasse and NPK, as sources of carbon and nutrients to the cultivation *of C. sorokiniana*, respectively, had improved biomass yield and had enhanced the synthesis of secondary metabolites, in addition to being natural and low-cost

medium supplements. The concentrations of phenolic compounds and flavonoids, as well as the antioxidant activity found in the aqueous extract of *C. sorokiniana* produced in the enriched medium enriched show that enrichment of media with vinasse and NPK may be a viable alternative for a higher scale production; this may be used in the fields of cosmetics, pharmaceutics, 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 regarding this method of species production. For future work, it is also recommended to study the application of biomass of microalgae *C. sorokiniana* in value-added products.

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