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BIOTECHNOLOGICAL POTENTIAL OF *PSEUDOKIRCHNERIELLA SUBCAPITATA, SCENEDESMUS SPINOSUS,* AND *SCENEDESMUS ACUMINATUS*

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Microalgae are promising alternatives to sequestration of carbon and reduction of environmental problems, e.g. the greenhouse effect and industrial water pollution. Depending on the growth conditions, microalgae can differ in their metabolism products, leading them to grow at different rates. Intracellular reactions and nutritional requirements from cell metabolism, as well as biomass composition, may vary in function of the temperature. In this study, the biotechnological potential of three microalgae strains from the species was evaluated in terms of growth, biomass composition, fatty acid profile, and chlorophyll and carotenoids contents. Each of the three species demonstrated different potential depending on their metabolisms: *Scenedesmus spinosus* presented fastest growth and had the highest protein content (52.99%), *Pseudokirchneriella subcapitata* presented the highest content of lipid extracted (26.51%), and *Scenedesmus acuminatus* showed increased production of chlorophyll (5.25 mg l^{-1}) and carotenoid (1.02 mg l^{-1}) pigments.

Keywords: microalgae, kinetics, growth, composition, pigments, fatty acids

Microalgae are unicellular organisms of rapid growth. They are found in marine environment, freshwater, and soil. They have the potential to reduce emerging environmental problems, e.g. greenhouse effect and water pollution (Harun et al., 2010). The number of species of these organisms is not known exactly. However, the existence of 200 000 to several million of representatives of this group is estimated. This diversity is also reflected in their biochemical composition and their unlimited source of bioproducts (Coêlho et al., 2019).

Beyond the biofixation of CO_2 from the atmosphere, the biomass formed by microalgae can be used as sources of chlorophyll, fatty acids, tocopherols, sterols, proteins, carbohydrates, vitamins, minerals, antioxidants, and pigments (Kiss & Németh, 2019), for the production of biofuels, e.g. biodiesel, biogas, bioethanol and hydrogen, organic fertilizer, natural dyes, pharmaceutical compounds, and nutrients for animal feed or even human food (Ribeiro et al., 2019).

Some fatty acids synthesized by microalgae, e.g. ω-3 and ω-6, are important in food and pharmaceutical industries, as they are the main precursors of hormones, e.g. prostaglandins, prostacyclins, leukotrienes, and thromboxanes (Pereira et al., 2012). In addition, three main groups of pigments are found in their biomass: chlorophylls, carotenoids, and phycobilins. Carotenoids are the pigments of greater commercial interest. Some strains can accumulate high concentrations of β-carotene, astaxanthin, or canthaxanthin, which have a wide application, e.g. dyes and natural antioxidants (Kiss & Németh, 2019). Chlorophyll has antioxidant properties and high antimutagenic activity. Under ideal growth conditions,

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microalgae can accumulate about 4% of chlorophyll in dry weight. The species of green microalgae mostly have chlorophyll a and b (Harun et al., 2010).

The microalgae studied here have been extensively investigated for their role in production of biofuel (Nascimento et al., 2013, Dhup et al., 2016) and environmental protection (Minillo et al., 2013). However, few studies underline other biotechnological potentialities. Thus, the aim of this work was to evaluate the biotechnological potential of three microalgae strains from the species *Pseudokirchneriella subcapitata, Scenedesmus spinosus*, and *Scenedesmus acuminatus*, in terms of growth, biomass composition, fatty acid profile, and chlorophyll and carotenoids contents.

1. Materials and methods

1.1. Microorganisms, isolation, and preservation

Pseudokirchneriella subcapitata (Korshikov) Hindak (Chlorophyceae) was obtained from the Laboratory of Limnology, from the Federal University of São Carlos. It has been isolated from the Broa Dam (São Carlos, Brazil). *Scenedesmus spinosus* Chodat and *Scenedesmus acuminatus* (Lagerheim) Chodat were previously isolated by Minillo and co-workers (2013).

The cultures were maintained in synthetic medium (Chu-12) at 25 °C, 1 kLux light, 12 h photoperiod, and constant stirring in a BOD incubator (200 r.p.m.) (Minillo et al., 2013). This medium contained per litre of distilled water: $Ca(NO_3)_2$, 0.03 g; K_2HPO_4, 0.005 g; $MgSO_4.7H_2O$, 0.075 g; K_2SiO_3, 0.025 g; KCl, 0.005 g; Na_2CO_3, 0.02 g; $FeCl_3$, 0.0005 g; autoclaved (121 °C, 20 min) (Sipaúba-Tavares et al., 1999).

1.2. Cultivations

Main cultivations were carried out in static 5 l Erlenmeyer flasks containing 4.5 l synthetic medium (Chu-12). Agitation was supplied by aeration. Cultivations started by the addition of microalgae inoculum with optical density (OD) of 2 (λ = 670 nm) in the concentration of 1% (45 ml) of the useful volume of the flask (corresponding to an initial cell density of 0.06±0.03 g l^{-1}). Each treatment was represented by a single strain, which was cultivated in triplicate during 33 days in a BOD incubator at 25±1.0 °C, light intensity of 1.5 kLux, 12 h photoperiods, with filtered air provided at a flow rate of 0.45 l min^{-1} through aquarium pumps.

Samplings were carried out at intervals of 3 days until stationary growth phase. Algae growth and pH were monitored. The biomass formation was monitored by OD of the cultures at 670 nm in a UV-Vis spectrophotometer, which was correlated to dry weight via a standard curve previously constructed.

1.3. Biomass concentration

The biomass pellet was obtained after centrifugation (1100 r.p.m., 10 min.) and drying in oven (70 °C) until constant weight.

The dried cell mass (X, g l^{-1}) was determined by the quotient of the difference of weight by the volume of centrifuged medium. It was also indirectly determined via OD measurements performed with a spectrophotometer at 670 nm. For this purpose, the measured absorbance values were converted into mass values using a linear relationship determined for each experiment.

1.4. Biomass composition

Moisture, crude protein, and crude ash contents were determined in triplicate according to the methods described by AOAC (2000). Moisture was determined by the oven drying method at 105 °C until constant weight (method 950.46), protein by the Kjeldhal method (method 928.08) using a 6.25 factor to convert the nitrogen content into crude protein, and ash by using the muffle oven technique (method 920.153). Total lipid content was determined in triplicate according to Bligh and Dyer (1959). Carbohydrates were estimated by difference.

1.5. Determination of fatty acids composition

The fatty acid methyl esters (FAME) were identified in a gas chromatograph equipped with an HP-88 column (60 m × 0.250 mm × 0.20 μm). Analysis was conducted under the following conditions: carrier gas helium (1 ml min^{-1}) (split 1:50); injector and detector temperatures of 260 °C; detection system was flame ionization detector (FID), with the oven set at 140 °C for 5 min, with a temperature increase up to 240 °C at 4 °C min^{-1}, thereafter maintained at 240 °C for 5 min. FAMEs were identified by comparing the retention time of the constituents of the sample with a mixture of FAME standards and quantified by the area normalization method (AOCS, 2005).

1.6. Determination of chlorophyll

Chlorophyll was extracted by organic solvent until the biomass appeared colourless, and its concentration was determined by spectrophotometry (Lee & Shen, 2004). Equations and λ (649 and 665 nm) were modified due to changing the solvent for ethanol 95% (v v^{-1}) (Lichtenthaler & Wellburn, 1983).

1.7. Determination of kinetic parameters

The exponential growth phase (EGP) was identified as the linear region on an ln (OD) vs. time plot for batch cultivation data. The maximal specific growth rate (μ_{max}, day^{-1}) was determined as the slope of the linear region and the doubling time (DT, days) by the ratio of ln (2) and μ_{max}. The maximum biomass concentration (X_{max}, g l^{-1}) was indicated by the maximum dried cell mass concentration or OD observed in each experiment. The maximum cell productivity (P_{cell}, g l^{-1} day^{-1}) was obtained according to the equation $P_{cell}=(X_{max} - X_0)/(t–t_0)$, where X_0 is the biomass concentration (g l^{-1}) at initial time (t_0, day zero) and t the time (days) corresponding to X_{max} (Ribeiro et al. 2019). The same was carried out for the maximum carotenoids productivity (P_{Ct}, mg l^{-1} day^{-1}) and maximum ethereal extract productivity (P_{Ee}, g l^{-1} day^{-1}).

2. Results and discussion

Table 1 shows the cultivation parameters obtained in this study of microalgae cultivated in synthetic medium (Chu-12) under a photoperiod of 12 h light/dark. Growth and pH kinetics for *P. subcapitata, S. spinosus*, and *S. acuminatus* cultivations can be observed in Figs. 1, 2, and 3, respectively. For all studied strains pH remained within the range 7.8 to 8.3; increasing gradually during cell growth.

Table 1. Cultivation parameters of the microalgae *P. subcapitata*, *S. spinosus*, and *S. acuminatus* grown in the synthetic culture medium Chu-12

Parameters	Species		
	P. subcapitata	*S. spinosus*	*S. acuminatus*
Cultivation (days)	42	33	51
μ_{max} (day^{-1})	0.093±0.006	0.170±0.021	0.068±0.001
DT (days)	7.431±0.439	4.115±0.504	10.25±0.130
X_{max} ($g\ l^{-1}$)	0.754±0.024	1.657±0.085	1.190±0.084
P_{cell} ($g\ l^{-1}\ day^{-1}$)	0.018±0.001	0.052±0.003	0.024±0.001
P_{Ct} ($mg\ l^{-1}\ day^{-1}$)	0.006± 0.000	0.009±0.001	0.020±0.000
P_{Ee} ($g\ l^{-1}\ day^{-1}$)	0.005±0.000	0.178±0.009	0.226±0.009

DT: doubling time; μ_{max}: maximum specific growth rate; X_{max}: maximum biomass concentration; P_{cell}: maximum cell productivity; P_{Ct}: maximum carotenoids productivity; P_{Ee}: maximum ethereal extract productivity

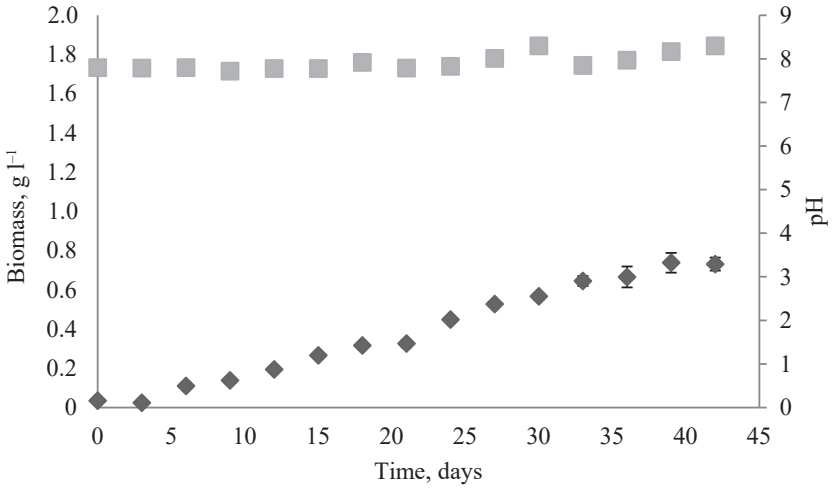

Fig. 1. Growth kinetics of *P. subcapitata* (ΔT = 26.0±3.1 °C; ◆: biomass; ■: pH)

In aqueous medium, the inorganic carbon may be in the form of CO_2, H_2CO_3, HCO_3^-, or CO_3^{2-}, and their proportions depend on pH, whereas, according to the pH increase, the proportions of bicarbonates and carbonates increase in the culture medium. Thus, it is possible to admit that the demand for CO_2 was similar in the experiments, as the pH variation (increase) was quite close between strains (Figs. 1, 2, and 3).

The lag phase indicates the period of adaptation for each species. *S. spinosus* presented shorter lag phase (Fig. 2) than the other species (Figs. 1 and 3). Though it took longer to get adapted, the species *S. spinosus* revealed the best growth parameters: higher μ_{max}, X_{max}, and P_{cell} under the studied conditions (Table 1). Because of this high P_{cell}, and its carbon sequestration capacity, cultivation of *S. spinosus* is proposed for the mitigation of CO_2 (Minillo et al., 2013).

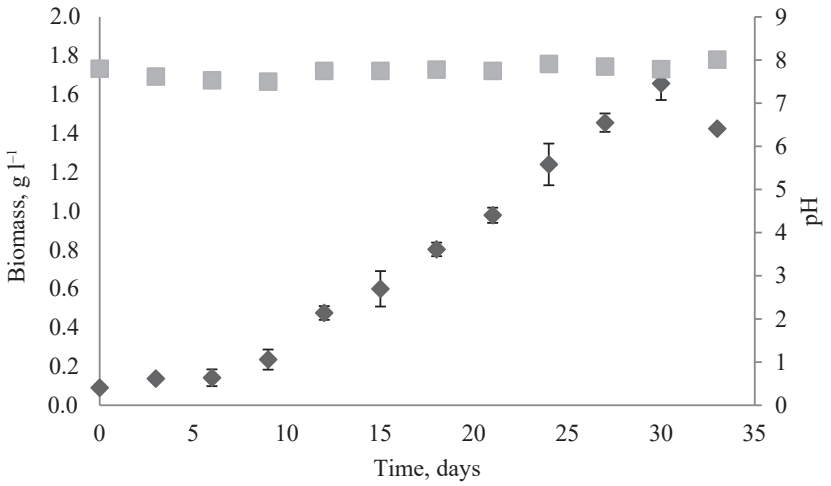

Fig. 2. Growth kinetics of *S. spinosus* (ΔT = 24.1±3.0 °C; ◆: biomass; ■: pH)

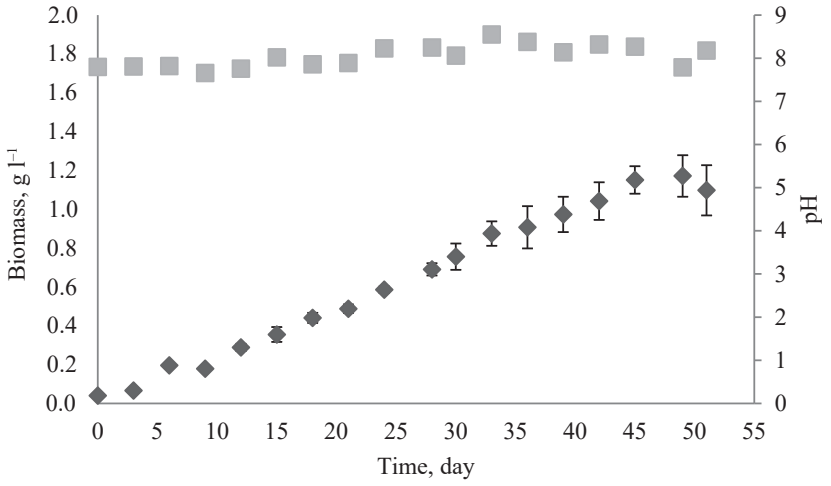

Fig. 3. Growth kinetics of *S. acuminatus* (ΔT = 26.6±1.5 °C; ◆: biomass; ■: pH)

The proximate composition of biomass showed highest protein content for *S. spinosus* (53%), revealing its potential in the enrichment of food products, for both animals and humans. A wide variety of algae have already been analysed for their biochemical composition and subjected to comprehensive nutritional and toxicological assessments demonstrating the suitability of biomass as a supplementary food or substitute for conventional animal feed sources (Harun et al., 2010).

Among the studied species, *P. subcapitata* presented the highest content of ethereal extract (26.51%; Table 2). The value was quite close to the 28.43±5.4% found in a previous study conducted with the same species (Nascimento et al., 2013). Due to its potential, this

species has been evaluated for biofuel production, despite the few results available (Adeyemi et al., 2011). *S. acuminatus* presented considerable fat content (19%; Table 2), which is comparable to the 17% obtained with another strain of this species, isolated in Pakistan, being considered a good oil producer (Musharraf et al., 2012). Despite the highest X_{max} observed here, the culture presented higher DT (10 days) compared to *P. subcapitata* (7 days) and *S. spinosus* (4 days) (Table 1).

The fatty acid profiles of the oils extracted from the final biomasses are shown in Table 3. The data show that the number of fatty acid compounds identified for each species is related to their lipid content, *i.e*. the higher the lipid accumulation is, the number of produced different fatty acids is also higher (Tables 2 and 3).

Table 2. Proximal composition of the dry microalgae biomasses obtained at the end of the cultivations of *P. subcapitata*, *S. spinosus*, and *S. acuminatus* grown in the synthetic culture medium Chu-12

Species	Protein (%)	Total lipids (%)	Ash (%)	Carbohydrates* (%)
P. subcapitata	41.60±0.90	26.51±1.67	6.61±0.04	25.28
S. spinosus	52.99±0.27	10.84±0.25	5.37±0.04	31.24
S. acuminatus	45.18±0.80	19.13±0.95	5.91±0.05	29.79

*By difference

Table 3. Fatty acid profile (%) of the oils extracted from the biomasses and contents of chlorophyll (a and b) and carotenoids (mg l^{-1}) from the microalgae biomasses obtained with the microalgae *P. subcapitata*, *S. spinosus*, and *S. acuminatus* grown in the synthetic culture medium Chu-12

Fatty acid		*P. subcapitata*	*S. spinosus*	*S. acuminatus*
C14:0	Myristic acid	3.36±0.01	n.d.	9.26±1.25
C15:1	*Cis*-10-pentadecanoic acid	5.73±0.10	n.d.	15.88±2.17
C16:0	Palmitic acid	21.79±0.94	45.70±3.21	23.25±2.05
C16:1	Palmitoleic acid	6.32±0.33	6.51±0.56	4.83±0.43
C18:1	Oleic acid	28.83±1.00	17.95±0.10	11.37±0.48
C18:1 t-11	Vaccenic acid	5.76±0.74	n.d.	4.37±0.48
C18:2 n-6c	Linoleic acid	13.83±0.60	10.48±0.40	8.98±1.02
C18:3	Alpha-linolenic acid	10.63±0.62	19.35±0.93	22.06±2.21
C20:5 n-3	Eicosapentaenoic acid	3.75±0.22	n.d.	n.d.
PUFA	Polyunsaturated fatty acids	28.21	29.83	31.04
MUFA	Monounsaturated fatty acids	46.64	24.46	36.45
SFA	Saturated fatty acids	25.15	45.70	32.25
Pigment		*P. subcapitata*	*S. spinosus*	*S. acuminatus*
Chl-a	Chlorophyll a	3.55±0.86	2.93±0.56	5.25±0.23
Chl-b	Chlorophyll b	1.35±0.35	1.15±0.16	0.81±0.18
Ct	Carotenoids	0.26±0.01	0.30±0.02	1.02±0.02

n.d.: not detected

In most organisms, fatty acids contain among 12 and 22 carbon atoms, with palmitic acid being the main compound, followed by oleic acid and then by other fatty acids typical of each species (Rezanka & Sigler, 2009). The same behaviour was identified in this study (Table 3). Nascimento and co-workers (2013) reported oleic acid as the main fatty acid in terms of concentration for *P. subcapitata* and palmitic acid for the genus *Scenedesmus* when evaluating 12 microalgae strains. Our results are in accordance with this study (Table 3).

All species studied here presented fatty acids of the ω-3 series, with the highest concentration obtained for *S. acuminatus* (22%, Table 3) in an ethereal extract of 19% (Table 2). Microalgae naturally contain ω-3 fatty acids, which can be purified to provide a high value food supplement. Due to the benefits to human health, studies have been carried out for obtaining and extracting this fatty acid from microalgae species (Harun et al., 2010).

The composition of fatty acids determines the quality of the biofuel (Nascimento et al., 2013). The most important characteristics affecting properties are the length of the carbon chain and the number of double bonds. The monounsaturated fatty acid (MUFA) and saturated fatty acid (SFA) fractions of the raw material are related to the properties of the biofuel, and an optimal raw material should contain higher concentrations of C16:1 and C18:1. Fuels rich in these FAMEs will have adequate cetane number, cold-flow parameters, and viscosities (Stansell et al., 2012). Regarding this analysis, the species *P. subcapitata* presented better results (Table 3), but its productivity in ethereal extract was the lowest compared to the other species studied (0.005 g l^{-1} day^{-1}) (Table 1).

Odd chain fatty acids are rare. The presence of C15:1 was detected in two species (*P. subcapitata*, 5.7%, *S. acuminatus*, 15.9%) (Table 3). High molecular weight lipids containing odd fatty acids have already been isolated from *Scenedesmus* spp. (Rezanka & Sigler, 2009). For *Scenedesmus abundans*, C15:0 and C17:0 were found (Rai & Gupta, 2017). In another study, four different odd fatty acids (C15:0, C15:1, C17:0, and C17:1) were obtained from *Scenedesmus* sp. cultivations (Dhup et al., 2016). However, in all these reports the concentration of these acids was always inferior in relation to the other fatty acids. Differently, C15:1 was the third main fatty acid identified for *S. acuminatus* (15.88%) (Table 3).

A study on the effect of nitrogen source on the accumulation of lipids by two species of microalgae (*Scenedesmus abundans* and *Chlorella ellipsoidea*) showed that by changing the nitrogen source only, the fatty acid profile could be altered. For both of these species, the alteration resulted in the production of C15:1 (González-Garcinuño et al., 2014). Thus, the nitrogen sources present in the Chu-12 medium may have stimulated the synthesis of C15:1 in *P. subcapitata* and *S. acuminatus*, too.

The profile of fatty acids produced by microalgae is dependent not only on the species, but also on extrinsic factors, e.g. nutrient concentration, pH, light intensity (Stansell et al., 2012). Thus, the conditions offered here may have stimulated this increased production of C15:1 by *S. acuminatus*. The odd-chain fatty acids (C15:0 and C17:0) have been shown to be associated with human diseases, e.g. multiple sclerosis and Alzheimer's disease (Jenkins et al., 2015). Thus, *S. acuminatus* could become a potential supplier of odd chain fatty acids to the human diet.

According to the data presented on chlorophyll and carotenoid contents, *S. acuminatus* species presented higher concentrations of chlorophyll-a (5.25 mg l^{-1}) and carotenoids (1.02 mg l^{-1}) (Table 3). It was observed that even species of the same genus, in this case of genus *Scenedesmus*, pigment contents were found very different. *S. acuminatus* presented higher concentration of pigments, which was expected as it also presented higher fat content, and the pigments are contained in the lipid fraction of the cells (Soto et al., 2011).

3. Conclusions

Each of the three microalgae species studied demonstrated different potential for the products of their metabolisms: *S. spinosus* presented faster growth and high protein content, *P. subcapitata* presented the highest content of lipids extracted, and *S. acuminatus* showed increased production of chlorophyll and carotenoid pigments. All produced fatty acids of the ω-3 series. These differences in terms of potential might be applied to obtain different products in optimized concentrations.

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