

Residual sugar from microalgae biomass harvested from phycoremediation of swine wastewater digestate

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ABSTRACT

The present study assessed the carbohydrate and sugar production from *Chlorella* spp. biomass harvested from a field scale reactor simulating phycoremediation of swine wastewater. The microalgae biomass was mainly composed by (%): carbohydrates (41 ± 0.4), proteins (50 ± 0.4), and lipids (1.3 ± 0.5). The residual sugar present in the biomass was extracted via acid hydrolysis. Among different concentrations of sulfuric acid tested (i.e., 47, 94, 188, 281 and 563 mM), significantly higher sugar content was obtained with 188 mM ($0.496 \text{ g-sugar g}^{-1}$ microalgae-DW). The concentration of sugar present in the microalgae did not differ significantly between the biomasses harvested by either centrifugation or coagulation-flocculation. Two commercially available strains of yeast (i.e., *Saccharomyces cerevisiae* and *S. cerevisiae chardonnay*) were tested for their capability to ferment sugar from lyophilized microalgae biomass. *S. cerevisiae chardonnay* showed a significantly faster consumption of sugar during the exponential growth phase. Both strains of yeast were capable of consuming most of the sugar added $\cong 8 \text{ g L}^{-1}$ within 24 h. Overall, the results suggest that carbohydrate-rich microalgae biomass obtained from the phycoremediation of swine wastewaters can play an important role in green design for industries seeking alternative sources of feedstock rich in sugar.

Key words | carbohydrate, *Chlorella* spp., phycoremediation, residual sugar, swine wastewater

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INTRODUCTION

Concerns about the uncertain availability of fossil fuels in the near future have motivated the scientific community to search for alternative sources of renewable energy. For instance, ethanol derived from crops, such as corn and sugar cane, has become a commodity with a multibillion-dollar industry that still threatens to push up the price of these plants for food (Ho *et al.* 2013a; Brasil *et al.* 2015). There are several environmental implications associated with current production of energy crops, such as atmospheric emissions associated with the use of fire in sugar-cane fields; the excessive use of water for irrigation; contamination of groundwater and soil by pesticides; territorial expansion; and soil erosion, among others (Abbasi & Abbasi 2010). In this regard, lignocellulosic materials have been considered, although production of sufficient biomass requires logging and deforestation (Cheng & Timilsina 2011). Production of ethanol from lignocellulosic biomass that does not compete

with the food industry is still struggling to take off due to high costs (Cheng & Timilsina 2011; Khoo 2015).

The use of microalgae for biofuel production has been discussed extensively. Compared to conventional crops, the growth rates and yield of microalgae are significantly superior. Other advantages include less demand for consumable resources (e.g. water and soil) (Mata *et al.* 2010; Khan *et al.* 2018) and the increased potential for CO₂ mitigation (Mu *et al.* 2014; Ullah *et al.* 2014). Microalgae biomass is composed of large amounts of carbohydrates (polysaccharides) in cell walls and across the intracellular matrix that can be converted into fermentable sugars (Harun *et al.* 2011). Residual sugar concentrations (wt wt⁻¹) of 80% (*Spirulina platensis* (Markou *et al.* 2013) and *Synechococcus* sp. (Möllers *et al.* 2014)), 45–70% (*Chlorella vulgaris* KMMCC-9; (Kim *et al.* 2014)), and 37.9–44.3% (*Scenedesmus* sp. CCNM 1077; (Pancho *et al.* 2016)) were reported.

It is worth noting, however, that the economic feasibility of microalgae production in an industry-relevant setting is largely influenced by the availability and costs of water and/or nutrients. In an attempt to reduce costs, the use of wastewater has been considered (Service 2011; Popp *et al.* 2014). Many industries are contemplating the use of algae-based phycoremediation treatment to remove nutrients from wastewater effluents while simultaneously producing valuable microalgae biomass (Brasil *et al.* 2015). The microalgae produced in this process can have different biochemical compositions depending on the nutrient concentration present in the wastewater used as growth medium (Lee *et al.* 2015a, 2015b). For instance, nutrient-rich wastewaters such as those generated from confined swine production may constitute an alternative growth medium to produce microalgal biomass rich in carbohydrates (Michelon *et al.* 2015; Özçimen & İnan 2015). Variations in carbohydrate content from microalgae can also occur depending on the harvesting method; that is, mechanical centrifugation or chemical coagulation/flocculation (Lee *et al.* 1998; Borges *et al.* 2011; Coward *et al.* 2014). This effect, however, is not always observed (Ndikubwimana *et al.* 2016), thus suggesting the need for assessment on a case-by-case basis.

Also, considering that microalgae biochemical composition can vary depending on the harvesting method used, ancillary investigation was performed to determine whether centrifugation or coagulation-flocculation (the two most conventional harvesting methods) could affect the total amount or residual sugar present in the biomass.

MATERIALS AND METHODS

Experimental set up

Microalgae inoculum was obtained from a field-scale lagoon used to remove nutrients from swine wastewater digestate originating from an anaerobic biodigester (Brazilian Agricultural Research Corporation, EMBRAPA, Concórdia, SC, Brazil). The inoculum was composed of a consortium dominated by *Chlorella* spp. as previously identified (Michelon *et al.* 2015). Experiments were performed at pilot scale using 500-L reactors (121.2 cm internal Ø; 58.4 cm height) placed inside a greenhouse, exposed to direct sunlight (photosynthetic photon flux density average and standard deviation of $321.5 \pm 411.4 \mu\text{mol m}^{-2} \text{s}^{-1}$) and under ambient average temperature of $31.7^\circ\text{C} \pm 16.3^\circ\text{C}$. These measurements ($n = 3$) were taken in the morning (8am),

mid-day (12pm) and afternoon (4pm). Reactors were inoculated with 30% of inoculum (volume-based) containing 70 mg dry weight microalgae L^{-1} . The growth medium was continuously mixed in the reactor using a submersible aquarium pump (flow rate of $1,200 \text{ L h}^{-1}$). The growth medium consisted of 6% v/v of raw digestate effluent diluted in the reactor's total volume of water. Dilution of digestate was necessary to enhance light penetration and microalgae growth. The chemical composition of the growth medium used in the reactor at the beginning of the experiments (i.e. at time zero) was (average $\text{mg L}^{-1} \pm$ standard deviation): total organic carbon (100 ± 5.2), biological oxygen demand (BOD_5 90.8 ± 0.9), alkalinity as CaCO_3 (190 ± 10), total nitrogen (50.3 ± 0.9), ammonia-N (40.1 ± 0.7) and phosphate-P (10.5 ± 4.6). pH was 7.9 ± 0.6 .

In this work, we focused on the microalgae only. The efficiency of phycoremediation as a treatment approach to remove nutrients from swine wastewater digestate was discussed elsewhere (Mezzari *et al.* 2013; Michelon *et al.* 2015; Prandini *et al.* 2016).

Harvesting

After 5 days of cultivation, the biomass in the reactor reached $0.3\text{--}0.4 \text{ g dry weight microalgae L}^{-1}$. At this point, biomass was harvested either by centrifugation ($3,000 \times g$, at 25°C for 30 min; EVODOS, T10, The Netherlands) or chemical coagulation-flocculation. A tannin-based cationic polyphenolic organic polymer produced through ammonium chloride and formaldehyde reaction was used as coagulant. The use of tannin was chosen due to its biodegradability (Beuckels *et al.* 2013; Vandamme *et al.* 2013) as opposed to other types of coagulants (e.g. aluminum) that may jeopardize water quality (Rosseland *et al.* 1990). The tannin used was extracted from Acacia tree (*A. mearnsii*) bark and is available commercially in liquid form with 30% w/v of tannic acid solution (flavan- 3,4-diol) and weight distribution of 830–1940Da (CAS # 85029-52-3; Veta OrganicTM, Brazilian Wattle Extracts, Canoas, Brazil). This tannin was chemically modified through the Mannich reaction to improve cationic strength properties by adding an ammonium quaternary functional group. Coagulation was performed directly in the reservoirs by adding $0.01\% \text{ v v}^{-1}$ of tannin. The coagulation/flocculation method proved to recover $>95\%$ of microalgae biomass at neutral pH (Mezzari *et al.* 2014).

The concentration of sugars present in microalgae can decrease significantly within days at room temperature or when stored in the refrigerator (4°C) (Adamson 2015). For

this reason, the harvested microalgae biomass was immediately frozen (-40°C) and lyophilized (Model 030-JJ LJI Scientific) on the same day.

Determination of carbohydrate, lipid, protein and ash content

The cellular lipid content was determined by ether extraction (Ankom XT15) (AOCS 2013). Protein content was measured by the combustion method (Leco FP-528) (AOAC 1990). Ash content was determined according to the Brazilian Compendium of Animal Nutrition, method 36 (BCAA 2009). Carbohydrate was determined by subtracting total cell dry weight from the measured lipid, protein and ash concentrations (Bi & He 2013).

Recovery of residual sugar

Acid hydrolysis was used to recover sugar from microalgae biomass. A fixed amount of collected biomass (15 g L^{-1} re-suspended in distilled H_2O) was used as substrate for reaction assays. Different concentrations of sulfuric acid were tested (i.e. 47, 94, 188, 281 and 563 mM) to determine the most effective concentration. Hydrolysis assays were conducted in Erlenmeyer flasks. Reaction took place at 100°C for 30 min (Waiser Lab. Products NC EST - 011). Samples were cooled at room temperature and then centrifuged at $3,200\times g$ at 20°C for 8 min (Excelsa[®] II model 206 BL). The supernatant containing the residual sugars was collected and the pH adjusted to 5.5 using 1 M NaOH. The residual sugar concentration was analyzed using the DNS (dinitrosalicylic acid) method with glucose as standard for calibration curves (Miller 1959). After mixing 0.75 mL of glucose with 0.5 mL of DNS reagent, samples were heated at 100°C for 5 min. Samples were cooled at room temperature and then 3 mL of water was added. Sugar concentrations were determined spectrophotometrically (Varian, Inc. Cary[®] 50 UV-Vis) at 540 nm.

Fermentation assays

Prior to fermentation tests, the supernatant containing the residual sugar was sterilized in an autoclave at 121°C for 15 min (Phoenix[®] Av-75/2). Fermentation assays were performed in triplicates using two different strains of yeasts: *Saccharomyces cerevisiae* (AEB Fermol[®]) and *Saccharomyces cerevisiae chardonnay* (Proenol[®]). Pre-inoculum was prepared in sterile Erlenmeyer flasks by adding 20 g L^{-1} of yeast into sterile deionized water containing 0.2 g L^{-1}

nutrient medium YPD broth medium (Himedia[®]). After approximately 1 h of incubation, yeast suspensions were washed three times in phosphate buffer and transferred to 500 mL ($3\% \text{ w v}^{-1}$) Erlenmeyer flasks containing 200 mL of the sterile hydrolyzed sugar solution. Incubation took place at 30°C for 48 h. Samples were taken over time for determination of sugar concentration as described above.

Statistical analysis

Experiments were performed in triplicate ($n = 3$). The results were presented as mean \pm standard error. Data were tested for normality and homoskedasticity and the statistical differences between group means were determined by one-way ANOVA. Tukey's honestly significant difference (HSD) post hoc test was conducted after the determination of the homogeneity of variances ($p \geq 0.05$). Statistical analyses were performed using SASTM (2012). The level of significance considered for all the analyses was 5% ($p \leq 0.05$).

RESULTS AND DISCUSSION

Biochemical composition of microalgae

Microalgae can accumulate considerable amounts of lipids and carbohydrates under different nutrient-deficient conditions, making them one of the most versatile and sustainable sources for biofuel production (Fan et al. 2014). Most microalgae have carbohydrate contents ranging between 5–25% of the total cell biomass, depending on the species (Bruton et al. 2009; Biller & Ross 2011; Prajapati et al. 2014). The amount of carbohydrate content in microalgae biomass can be increased once cells are deprived of nutrients and/or exposed to additional sources of atmospheric CO_2 (Chen et al. 2013; Ho et al. 2013b). Our previous studies corroborate these findings (Michelon et al. 2015). Table 1 compares the carbohydrate content from different species of microalgae grown under controlled conditions using synthetic growth medium amended or not with CO_2 . Most of the experimental conditions tested may not realistically represent the environmental dynamics (e.g. variations in light and temperature) expected at field scale. In this work, the microalgae produced from a pilot-scale experiment exposed to field conditions were rich in proteins (50.3%) and carbohydrates (41%) but low in lipids (1.3%). Thus, the cultivation of microalgae biomass as proposed here may not be attractive for industries seeking oil and its derivatives such as Omega-3; Omega-6, etc.

Table 1 | Biochemical composition of biomass changes according to species and growth conditions

Microalgae species	Growth medium	Growth conditions	Dry cell weight (g L ⁻¹)	Content (%)			Yield of hydrolysis (%)	Reference
				Carbohydrates	Proteins	Lipids		
<i>Chlorella variabilis</i>	Synthetic	2% CO ₂ (CO ₂ – air)	0.43	37.8	19.8	24.7	–	Cheng <i>et al.</i> (2013)
<i>Chlorella vulgaris</i> FSP-E	Synthetic	2% CO ₂ (CO ₂ – air)	7	13.3–54.4 ^a	20.1–58.8	11–15	90.4	Ho <i>et al.</i> (2013a)
<i>Chlorella vulgaris</i> KMMCC – 9	Synthetic	Bubbling air	–	22.4	–	–	45–70	Kim <i>et al.</i> (2014)
<i>Scenedesmus</i> sp. CCNM 1077	Synthetic	–	–	45.2	31.2	–	37.9–43.4	Pancha <i>et al.</i> (2016)
<i>Scenedesmus dimorphus</i>	Synthetic	2% CO ₂	4–5	45–50	10–32.5	7.5–35	–	Wang <i>et al.</i> (2013)
<i>Spirulina platensis</i>	Synthetic	Bubbling air	2–2.2	58 ^b	24	4	80	Markou <i>et al.</i> (2013)
<i>Synechococcus</i> sp.	Synthetic	1% CO ₂ (CO ₂ – air)	0.9–3.7	40–59	–	–	80	Möllers <i>et al.</i> (2014)
<i>Chlorella</i> spp.	Non-sterile swine digestate	Open to atmosphere	0.3–0.4	41	50.3	1.3	49.6	This study

^aCultivated under nitrogen-limited conditions.

^bCultivated under phosphorus-limited conditions.

Acid hydrolysis

Acid pre-treatment is a method used regularly to disrupt microalgae cell walls before proceeding to enzymatic hydrolysis. The disruption facilitates the release of entrapped carbohydrates present in the cell wall. To determine the most adequate concentration of acid for hydrolysis pretreatment, different concentrations of sulfuric acid ranging from 47 to 563 mM were tested (Figure 1). More diluted acid concentrations are always preferred for hydrolysis because the process becomes less harsh and costly. The use of low concentrations of acids for hydrolysis can still be more effective than other hydrolysis methods (e.g. enzymatic hydrolysis) (Ho *et al.* 2013c). The optimum concentration of sulfuric acid, which led to a significant ($p < 0.01$) increased sugar recovery (0.496 g-sugar g⁻¹ microalgae-DW; Table 2), was 188 mM (Figure 1). The lowest and highest concentrations of acid tested; that is, 47 and 563 mM, were not as effective in recovering sugar, with only 0.13 and 0.22 g-sugar g⁻¹ microalgae-DW, respectively. The sugar yield obtained here was comparable to other specific microalgae strains grown under controlled laboratory conditions using synthetic medium amended with CO₂ (29.5 to 98.2%) (Xu *et al.* 2013; Coward *et al.* 2014; Möllers *et al.* 2014; Lee *et al.* 2015a, 2015b; Wan *et al.* 2015) (Table 1).

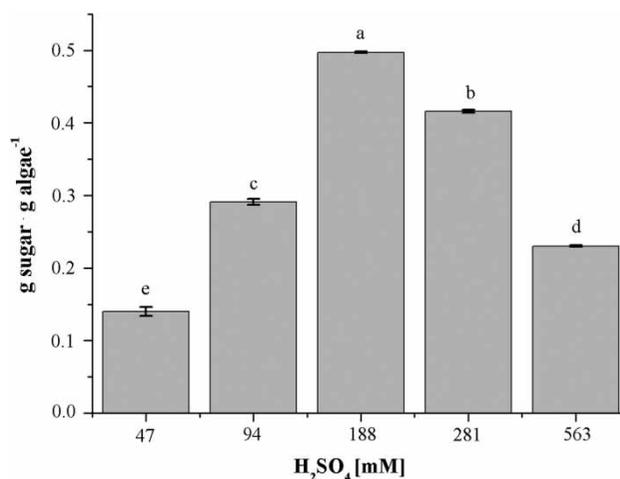


Figure 1 | Effects of different concentrations of sulfuric acid on acid hydrolysis and sugar recovery from microalgae biomass. Bars depict standard error. Different letters denote significant differences ($p \leq 0.05$) according to Tukey HSD test.

Effect of coagulation-flocculation or centrifugation on sugar content

There are various mechanical and chemical methods available for harvesting microalgae, such as centrifugation, flocculation, filtration and screening, gravity sedimentation, and flotation (Coward *et al.* 2014). Among these approaches, coagulation and flocculation with organic polymers are the

Table 2 | Consumption of sugar over time by the two strains of yeast tested in this work

Incubation time	Sugar consumption (g L ⁻¹)		p value
	<i>S. cerevisiae</i> (n = 3)	<i>S. cerevisiae chardonnay</i> (n = 3)	
10 min	8.617 ± 0.080 ^a	8.614 ± 0.003 ^a	0.9720
15 min	8.680 ± 0.039 ^a	8.543 ± 0.038 ^a	0.0166
30 min	8.549 ± 0.041 ^a	8.556 ± 0.004 ^a	0.8643
50 min	8.549 ± 0.041 ^a	8.350 ± 0.008 ^b	<0.0001
4 h	6.887 ± 0.426 ^b	4.763 ± 0.012 ^c	<0.0001
6 h	4.997 ± 0.008 ^c	2.441 ± 0.008 ^d	<0.0001
20 h	3.020 ± 0.855 ^{cd}	0.691 ± 0.031 ^d	0.0104
24 h	2.299 ± 0.779 ^d	0.452 ± 0.008 ^d	0.0240
p value	<0.0001	<0.0001	

Data shown as means ± standard error.

Different letters denote significant differences ($p \leq 0.05$) according to Tukey HSD test.

most appropriate and cost-effective option for large-scale operations (Xu et al. 2013; Mezzari et al. 2014; Wan et al. 2015). There are conflicting lines of evidence showing that different harvesting processes result in changes to microalgae biochemical composition. (Borges et al. 2011; Coward et al. 2014; Michelon et al. 2015; Ndikubwimana et al. 2016). Thus, it is important to determine on a case-by-case basis if the harvesting method of choice can ultimately affect microalgae composition and residual sugar concentration. To address this question, the microalgae residual sugar yield harvested from centrifugation was compared to microalgae collected via coagulation-flocculation. No significant ($p \leq 0.05$) differences in sugar concentration were observed independently of the method of harvesting used (Figure 2).

Sugar consumption

One limitation of bioethanol production from microalgae carbohydrates is that not all residual sugars are suitable for yeast fermentation (Lee et al. 2015a, 2015b). In this regard, less complex sugars such as glucose or fructose are usually preferred (Markou et al. 2013). Two different commercially available strains of *Saccharomyces* were used in the fermentation assays to investigate which yeast could lead to higher sugar consumption. These yeast strains were used because of their broad metabolic capabilities and capacity to adapt in response to changes in the environmental conditions, ultimately increasing bioethanol yield (Sharma et al. 2016; Mohd Azhar et al. 2017). Suspended cells of *S. cerevisiae* (Fermol Aromatic Group – AEB[®]) and *S. cerevisiae* (Fermol Chardonnay Group – AEB[®])

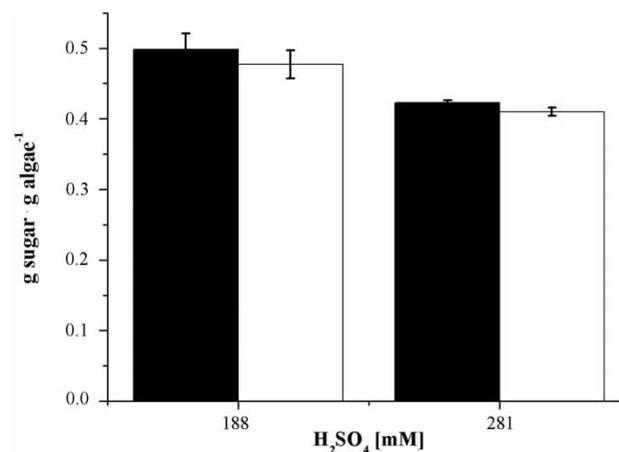


Figure 2 | The concentration of residual sugar recovered from biomass did not change significantly as a result of harvesting methods; that is, coagulation-flocculation (white) versus centrifugation (black). The p value obtained from one-way ANOVA was 0.89 and 0.26 for the 188 and 281 [mM] sulfuric acid, respectively. Bars depict standard error.

consumed 39.8% and 70.6%, respectively of the initial glucose concentration after 12 h of experiment. *S. cerevisiae chardonnay* was capable of removing 91.6% of the initial glucose present in the medium after 24 h of incubation (Table 2). The rate of sugar consumption was significantly higher ($p < 0.0001$) for *S. cerevisiae chardonnay* during the exponential growth phase (between 50 min and 6 h of incubation) (Table 2).

Table 3 shows the theoretical ethanol yield expected from microalgae biomass in comparison with other conventional feedstocks. Data from microalgae were estimated based on microalgae yield coefficient (0.3 g L⁻¹ obtained every 5 days of cultivation) and the concentration of sugar recovered from biomass. Microalgae biomass yield was eight-fold higher than corn and two-fold lower than sugarcane. However, the higher concentration of carbohydrate present in microalgae biomass outweighs its lower yield in comparison to sugar cane. Hence, the potential for sugar production from microalgae (43.4 ton ha⁻¹ yr⁻¹) is comparatively

Table 3 | Theoretical production of sugar from conventional agricultural feedstock sources

Feedstock	Yield (ton ha ⁻¹ yr ⁻¹)	Residual sugar (ton ha ⁻¹ yr ⁻¹)	Reference
Corn	10.73	–	USDA (2015)
Sugar-cane	150	18	FAO (2014)
<i>Chlorella</i> spp.	87.6 ^a	43.4 ^b	This study

^aEstimated using the equation = $[10^4 \text{ m}^2/\text{ha} \times 0.4 \text{ m (} h \text{; raceway depth)} \times \text{microalgae yield (0.3 g-algae/L/5d)} \times 10^3 \text{ L/m}^3 \times 1 \text{ ton/ } 10^6 \text{ g} \times 365 \text{ d/yr}]$.

^bEstimated using the equation = $(87.6 \text{ ton/ha/yr} \times 0.496 \text{ wt-reducing sugar/wt-biomass})$.

superior to sugarcane (18 ton ha⁻¹ yr⁻¹). In this regard, microalgae biomass can play an important role in the development of sustainable biorefineries that are less dependent on arable land and water for irrigation. The existing concerns about the use of arable land for production of biofuels instead of food are diminished because microalgae can be produced in areas unsuitable for agricultural practices (e.g. desert, sand, etc.) (Mussgnug *et al.* 2010). The water footprint to produce microalgae can range between 200 to 1,000 m³ ton⁻¹ (assuming typical yields of 1–5 g fresh weight L⁻¹) which is considerably higher than the estimated global average water footprint of sugar cane; that is, 209 m³ ton⁻¹ (Gerbens-Leenes & Hoekstra 2012). However, the water used for microalgae growth can be reused postharvest (Mezzari *et al.* 2014), thus significantly minimizing the amount of water needed. Another advantage of the use of microalgae is the short harvesting cycle (1–20 days) compared to sugarcane, which is harvested once or twice a year. The frequent harvesting provides uninterrupted supply of raw material to meet the constant demands imposed by industries. Once in the industry, the operational costs associated with biomass pretreatment are expected to be lower with the use of microalgae because cells lack hemicellulose and lignin, facilitating saccharification (Babadzhanov *et al.* 2004; Carrieri *et al.* 2010). Despite these advantages, however, the industry of microalgae for biofuels is still struggling to take off, mostly due to high operating costs with harvesting and dewatering, as well as low cell productivity (Khan *et al.* 2018). Consequently, the production costs of microalgae are still much higher (673 to 700 US\$ ton biomass⁻¹) (Kang *et al.* 2015; Hoffman 2016) than sugarcane (20–26 US\$ ton biomass⁻¹) (Cardoso *et al.* 2019). Yet, the high cost to produce microalgae can be offset by extracting and marketing residual byproducts of high added value for the nutraceutical and pharmaceutical industries. Although the production of microalgae biomass can unfold a promising feedstock alternative to ethanol production, it still needs to be further investigated by encompassing more comprehensive techno-economic analysis.

CONCLUSIONS

In this work, field scale experiments simulating phycoremediation of swine wastewater produced microalgae rich in proteins (50.3%) and carbohydrates (41.0%). Among the concentrations of sulfuric acid tested for the recovery of sugar from biomass, the concentration of 188 mM showed best results with 0.497 ± 0.001 g sugar g algae⁻¹. The use

of mechanical or chemical coagulation-flocculation for harvesting the microalgae biomass had insignificant effect on the biomass residual sugar. Compared to *S. cerevisiae*, *S. cerevisiae chardonnay* showed significantly faster consumption of sugar during the exponential growth phase, consuming 92% of the total sugar added (8 g L⁻¹) within 24 h. These results support the notion that phycoremediation used as tertiary treatment system for removal of nutrients from wastewaters could provide valuable microalgae feedstock rich in fermentable sugars.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors declare that they have no conflicts of interest.

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