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ORIGINAL ARTICLE

Amino acids, fatty acids, and peptides in microalgae biomass harvested from phycoremediation of swine wastewaters

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Abstract

Algae-based wastewater tertiary treatment systems have been drawing attention to eco-friendly companies due to high remediation effectiveness and production of valuable raw material. The amino acids, fatty acids, and peptides from microalgae harvested from a pilot-scale phycoremediation system treating swine wastewater were determined. The maximum microalgae concentration of 247 ± 3.4 mg L^{-1} was obtained after 11 days when phosphate and ammonium were completely removed. The AA content showed relatively high concentrations (as % of total protein) of essential amino acids such as leucine (4.1), lysine (2.5), phenylalanine (2.6), and threonine (2.4). The fatty acid profile was composed of 5.3% polyunsaturated (as C18:2 and C18:3) and ~ 10% of unsaturated (mainly C16:1 and C18:1). About 25 bioactive peptides related to antioxidative, anti-inflammatory, and anticarcinogenic properties were found. Therefore, microalgae biomass produced during phycoremediation of swine wastewaters seems promising as a source of alternative feedstock with high-added value molecules.

Keywords Amino acids · Biorefinery · Fatty acids · Peptides · Phycoremediation

1 Introduction

In recent years, the global demand for animal protein has increased significantly [1]. For instance, production of swine meat in Brazil (fourth larger exporter) increased from 3.2 in 2010 to 4 million tons, in 2019 [2]. Thus, the animal production system was enforced to increment productivity, which was mainly achieved by raising the number of confined animals per unit of area. However, one of the main drawbacks of such production systems is the large volumes of wastewater generated, containing high organic matter, nutrients, heavy metals and veterinary drug residues, in which if not properly treated prior to disposal, can harm the environment (e.g. eutrophication) [3].

Phycoremediation is an effective and low-cost wastewater tertiary treatment for the removal of N and P from wastewaters. The process aids CO_2 sequestration and the produced biomass utilized as feedstock or raw material to produce fertilizers, biofuels, food supplements, pharmaceutics and cosmetics [4]. For example, a wide range of high-value molecules synthesized by microalgae, such as essential FA, especially the long-chain polyunsaturated fatty acids (PUFAs), γ-linolenic acid (18:3 omega-6), arachidonic acid (20:4 omega-6), EPA (20:5 omega-3), and DHA (22:6 omega-3), are

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of special interest for nutraceutical, aquafeed, and animal industry [5, 6]. Moreover, the presence of several essential AA in microalgae biomass, such as threonine, methionine, lysine, cysteine and tryptophan, are also appealing as feed supplements [7]. Additionally, recent study showed that peptides extracted from microalgae have interesting nutraceutics properties including antioxidant, antihypertensive, immunomodulating, antithrombotic, and anticancer [8].

The composition of FA [9] and AA [10, 11] in microalgae biomass growing in piggery wastewaters was previously discussed. However, information on the characterization of peptide content in microalgae biomass grown in swine wastewater effluents remains unknown. Additionally, these studies focused on specific wastewater effluent physical-chemical characteristics but did not encompass comparative analysis of the microalgae biochemical compositions as result of changes in wastewater characteristics. Therefore, it is unknown if variations in swine wastewater effluents characteristics as result of different treatment systems used (e.g., anaerobic digestion or aerobic denitrification) can ultimately alter microalgae composition and if so to what extent.

Therefore, the aim of this study was to characterize the concentration profiles of AA, FA, and peptides in a consortium of indigenous microalgae biomass (mainly dominated by *Chlorella* spp.) cultivated in a pilot scale reactor, simulating phycoremediation of swine wastewater. Experiments were conducted using effluents from two commonly used approaches for treating swine wastewaters, i.e., anaerobic digestion and nitrification-denitrification (NR) to compare the effects of these effluents with distinctive physical-chemical characteristics on microalgae biochemical composition.

2 Material and methods

2.1 Microalgae inoculum

The microalgae consortium used as inoculum in this study was previously obtained from a field scale swine wastewater treatment system composed by an up flow anaerobic sludge blanket reactor (UASB) and a facultative pond as tertiary treatment (Brazilian Agricultural Research Corporation, EMBRAPA, Concordia, Brazil). *Chlorella* spp. were found to be dominant in the microalgae inoculum as previously characterized [12]. The collected microalgae inoculum was then acclimated in 12-L glass photobioreactors (PBRs; 20 cm Ø ID), filled with water containing 5% v v^{-1} of non-sterile digestate from the UASB. Dilution of digestate was needed to decrease effluent turbidity, enhancing light penetration required for microalgae growth. PBRs were kept at room temperature (23 °C) under mixotrophic conditions using 40-W fluorescent lamps (photosynthetic photon flux density (PPFD) of 44.8 μmol m^{-2} s^{-1}) and continuous agitation using recirculation mechanical pumps (Sarlobetter brand).

2.2 Pilot scale experiment

Experiments were conducted using three 500-L reactors, located in a greenhouse, exposed to natural sunlight (PPFD of 321.5 ± 411.4 μmol m^{-2} s^{-1}) and under temperature-controlled conditions (25 °C). Reactors were operated in fed-batch mode using effluents from either, a field scale UASB or an air-sparged nitrification-denitrification tank placed downgrading the UASB for the removal of nitrogen compounds. Effluent from the UASB was diluted by adding 30 L (6% v v^{-1}) into 320 L (64% v v^{-1}) chlorine-free tap water. Effluent from the nitrification-denitrification tank was also diluted by mixing 250 L (50% v v^{-1}) into 100 L (50% v v^{-1}) chlorine-free tap water. Reactors were inoculated with 75 ± 0.5 mg DW microalgae L^{-1} (30% v v^{-1}). The reactors were kept under continuous agitation using a mechanic pump (flow rate of 1200 L h^{-1}).

The chemical composition of the diluted UASB effluent, prior to inoculation (i.e., at time zero) was (mg L^{-1}): 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 (45.1 ± 0.7), and phosphate-P (10.5 ± 4.6). pH was 7.9 ± 0.6. The chemical composition of the nitrification-denitrification effluent prior to inoculation (i.e., at time zero) was (mg L^{-1}) the following: total organic carbon (210 ± 10), biological oxygen demand (BOD_5 100.8 ± 8.9), alkalinity as $CaCO_3$ (500 ± 15.1), total nitrogen (30 ± 0.7), ammonia-N (26.1 ± 0.2), and phosphate-P (11.5 ± 6.2). pH was 7 ± 0.5. After 12 days following reactors inoculation, N and P were completely removed. At this point in time the biomass in stationary growth phase was harvested by centrifugation at 3000×*g* (EVODOS, T10, Netherlands) and immediately frozen (−40 °C) and lyophilized (Model 030-JJ LJI Scientific) for further analyses. Fresh biomass average weight was 4 ± 1 g microalgae L^{-1}.

A third reactor was utilized to assess the effects of nutrients limitation on microalgae composition changes particularly on AA and FA. For this particular experiment, microalgae biomass previously grown in the UASB digestate [12] was harvested via centrifugation (3000×*g*; EVODOS, T10, Netherlands), and the cell pellet resuspended in the 500-L reactor containing fresh chlorine-free tap water. After 12 days following inoculation, the biomass was harvested via centrifugation (3000×*g*; EVODOS, T10, Netherlands) for further analyses. It is recognized that nutrients limitations (e.g., N and/ or P) can alter the composition of carbohydrates, proteins and lipids [13]. Therefore, studies were conducted to investigate the effects of N and/ or P starvation on microalgae AA and FA composition. Thus, one reactor after 12 days of phycoremediation, containing microalgae biomass was

harvested via centrifugation and cells were re-suspended in 500-L nutrient-free water and a second reactor containing N but not P. In the latter, to avoid N depletion during the tests, the concentration of nitrate was continuously monitored and added (50 mg $N–NO_3^-$ L^{-1}) when needed [12]. After 25 days, the cells were harvested by centrifugation (3000×*g*; EVODOS, T10, Netherlands) and the cell pellet stored for further analysis.

2.3 Analytical methods

Phosphate-P was quantified by the ascorbic acid colorimetric method (APHA, 2012). Ammonia ($N\text{-}NH_3$), nitrite ($N\text{-}NO_2^-$) and nitrate ($N\text{-}NO_3^-$) concentrations were determined by flow injection analysis (FIAlab – 2500). Total organic carbon (TOC) was measured using a TOC analyzer (Multi C/N 2100, Analytik Jena). Alkalinity (as mg $CaCO_3$) was determined by automatic titration (Metrohm 848 Titrino Plus). Light intensity was measured with a Luximeter (DX-100, Japan). pH was monitored using pH meter (pH–mV, Hanna Instruments, Inc.). A satisfactory correlation ($r^2 = 0.98$) between dry matter (DW) biomass content as measured by suspended solids (APHA, 2012) and optical density (OD_{570}) (mg DW $L^{-1} = 543.84 \times OD_{570}\text{nm} - 37.726$). Therefore, microalgae growth over time was assessed using a spectrophotometer (Varian, Inc. Cary® 50) analysis at 570 nm.

2.3.1 Quantification of AA and FA in microalgae

The concentration of AA was determined by the AOAC method 994.12 [14] using a Hitachi (L-8900) AA analyzer. The FA were first extracted with dichloromethane and methanol and then by volatilization using sodium hydroxide and methanol described in AOCS [15]. FA were dissolved in 1 mL hexane and solution was dried with anhydrous sodium sulfate. Two microliter was injected on a GC Varian CP-3800 (Walnut Creek, Palo Alto, CA, USA), equipped with a split/split less injector (1:100), a capillary column CP Sil 88 (50 m × 0.25 mm i.d. × 0.2 μm film thickness), a flame ionization detector (FID). Oven temperature was set to rise from 80 °C to 150 °C at 5 °C min^{-1}, then from 150 °C to 220 °C at 2 °C min^{-1} and held at 220 °C for 6 min. Nitrogen was used as carrier gas at 1 mL min^{-1}. FA were identified by comparison of the peak retention times between each sample and the authentic standards (Sigma-Aldrich). FA quantification in the sample solutions was done by external calibration using a methyl stearate curve ($r^2 = 0.992$). The AA and FA were reported as percentage of the total weight of protein and lipid, respectively.

2.3.2 Identification of hydrolyzed peptides from microalgal biomass

Digested peptides from microalgae biomass were obtained via protein digestion using trypsin enzyme (Sigma-Aldrich) solubilized in 400 μL of 50 mM ammonium bicarbonate (NH_4HCO_3), to a final concentration of 0.05 μg enzyme μL^{-1}. A 50 μL solution (1:50, enzyme: protein) was then prepared and incubated at 37 °C for 24 h. Then, 10 μL of 10% (v v^{-1}) trifluoroacetic acid was added into solution and kept for 90 min at 37 °C. Samples were centrifuged for 30 min at 17,400 *g*, kept at 6 °C for further analysis.

Digested peptides identification was performed by high performance liquid nano chromatography coupled to the mass spectrometer using a Nano LC-ESI-Q-TOF system (Thermo Scientific UltiMate 3000 nano LC and Bruker Daltonics ESI-Q-TOF Impact II model), containing nano electrospray ionization source and TOF quadrupole mass analyzer. The peptides were separated into PepMap nanocolumn (C18, 5 μm particles, pore size 300 Å, 15 cm long, 75 μm internal diameter; Thermo Scientific) using a gradient of 3 to 97% (v v^{-1}) of acetonitrile (Sigma-Aldrich) containing 0.1% (w w^{-1}) formic acid for 180 min at a flow rate of 0.3 μL min^{-1}. Positive mode ionization and the precursor ion (MS) spectra were acquired in the 50–3000 *m/z* range with a 2 Hz acquisition frequency, capillary voltage at 1.5 kV, source temperature at 150 °C, 3 L min^{-1} drying gas flow, and 0.2 bar nebulizer pressure. Precursor ion fragments (MS/MS) were acquired with an acquisition frequency of 4 to 16 Hz and collision energy between 23 and 65 eV.

Data files (.d) were imported into PEAKS Studio® 10 software (Bioinformatics Solution Inc., Waterloo, Canada) and MS/MS spectra were analyzed by searching the database using peaks DB, PTM and Spider [16]. The input parameters were configured: 20 ppm precursor mass tolerance, 0.025 Da fragment mass tolerance, trypsin as specific enzyme in use, maximum three cleavage failures, Cys carbamidomethylation (+ 57.02 Da) as fixed modification and Met oxidation (+ 15.99 Da) as variable modification. False discovery rates (FDRs) for digested peptides were set at a maximum of 1%.

The possible existing correlation between the measured peptides and bioactive functionalities was determined using the BIOPEP-UWM database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) [17].

2.4 Statistical analysis

Statistical differences between treatments data sets were determined using one-way analysis of variance (ANOVA) with Statistica® software. Turkey's significant difference (HSD) post hoc test was conducted after the determination of variances ($p \geq 0.05$).

3 Results and discussion

3.1 Phycoremediation efficiently removes nutrients from wastewater

Ammonia-N and phosphate-P were both completely removed (100%) from the effluents tested (UASB and NR) (Fig. 1a, b) after 11 days. It is worth mentioning that ammonia-N removal was unlikely attributed to microalgal assimilation only. In this case, bacterial-mediated nitrification and denitrification activities—as reported by Mezzari et al. [18]—very likely contributed to ammonia-N removal as suggested by the presence of nitrite as byproduct of nitrification in the samples collected 3 days after the beginning of the experiments (Fig. 1a). The maximum biomass concentration of 247 ± 3.4 and 188 ± 5.5 mg L^{-1} (DW) was obtained with the use of UASB and NR effluents, respectively (Fig. 1a, b). Overall, these data corroborate to the recent findings on phycoremediation as efficient approach for the removal of nutrients from piggery wastewaters [19, 20].

3.1.1 Microalgae grown in swine wastewaters contains high protein and carbohydrate contents

The protein, carbohydrate and lipid contents measured in the microalgae biomass harvested after complete removal of nutrients from UASB and NR effluent is shown in Fig. 1c. Variations in protein and carbohydrates contents were observed as a function of the effluents tested (UASB and NR). The biomass cultivated in the UASB showed protein and carbohydrate contents of 50.1% ± 0.7 and 34.4% ± 0.4, respectively. The microalgae biomass harvested from the reactor fed NR effluent had significantly lower ($p < 0.05$) protein (44% ± 0.9), and higher carbohydrate 41.9% ± 0.9 contents. Irrespective of the wastewater used, however, the lipid content measured in the biomass was relatively low ≤ 2%, suggesting that in the presence of sufficient amounts of N and P in the medium, cells preferentially store energy in the form of proteins and carbohydrates instead of lipids. Compared to UASB, the biomass grown on NR effluent had higher carbohydrate content (as short-term most favorable source of energy storage) due to lower N and P concentrations in this medium. The increase in protein content is likely attributable to culture medium composition that containing adequate concentrations of nitrogen compounds (e.g., ammonia-N) which is recognized to induce high-quality protein production in microalgae [21]. Chinnasamy et al. [22] observed similar trends in the biochemical composition of *Chlamydomonas globosa*, *Chlorella minutissima*, and *Scenedesmus bijuga* cultivated in industrial wastewater (also rich in N and P), i.e., protein (53.8%), carbohydrate (15.7%) and, lipid (5.3%). Contrariwise, some other specific microalgae can significantly accumulate more lipids in the cell. For instance, under controlled laboratory-scale conditions (temperature (25 ± 1 °C); injection air (5–6% CO_2) and continuous light irradiance (230 ± 20 μmol m^{-2} s^{-1})), *Chlorella zofingiensis* grown in piggery wastewater can accumulate lipid by as much 43% of total cell weight [23].

The effects of N and/or P limitations on lipid storage were observed (Fig. 1c). Lipid content increased to 16.1% ± 0.5 and 4.8% ± 1.5 when cultivated in the absence of both N and P or just P, respectively. The increase in lipid content associated with nutrients limitations was also reported previously in cells of *Nannochloropsis oculata* and *Chlorella* spp. [24]. These results suggest that the fraction of proteins, carbohydrates and/or lipids can be strategically manipulated by inducing nutrients-related stress conditions. Therefore, inducing the growth in N- or P-deficient conditions, the production of ROS in responses to intracellular stress can lead to incremental lipid production through autophagy [25].

3.2 Microalgae grown in swine wastewaters are rich in essential amino acids

Microalgae synthesize essential and non-essential amino acids [26] that can be further processed for human and animal nutrition [27]. The AA profiles found in the microalgae consortium cultivated with UASB or NR effluents as well as under the absence of nutrients (N and/or P) is shown at Table 1. Eighteen amino acids were identified, in which 11 are essentials i.e., histidine, arginine, threonine, proline, valine, methionine, isoleucine, leucine, phenylalanine, lysine and tryptophan. The concentration of the obtained AA (≈ 22% DW of total protein) was in agreement with previous studies using wastewater as growth medium for microalgae [11]. It is expected that the composition of AA is likely to change depending on the microalgae specie. For example, Canizares-Villanueva et al. [10] reported that the concentrations of AA found in *Spirulina* was comparatively higher than those found in *Phormidium* when both genus of microalgae were cultivated in the same diluted swine wastewater as growth medium.

After 2 days of cultivation, the wastewater containing only N (but not P) produced microalgae with considerably high concentrations of AA (59.6% DW) compared to the biomass harvested (after 12 days) from wastewater depleted of N (23.3% DW). A notable difference was found in methionine, cysteine and phenylalanine contents at these different growth stages, with an incremental production (6.8 ± 0.7%) within 8 days of cultivation (data not shown). Other AA remained at similar concentrations along the entire experimental time frame. Xupeng et al. [29] reported a 3-fold increase in phenylalanine during the early stages of microalgae growth when N was still bioavailable followed by a decrease in AA concentrations associated with the depletion of N towards the end of the experiments. Compared to previous studies, the concentration of AA measured in this study was comparatively much

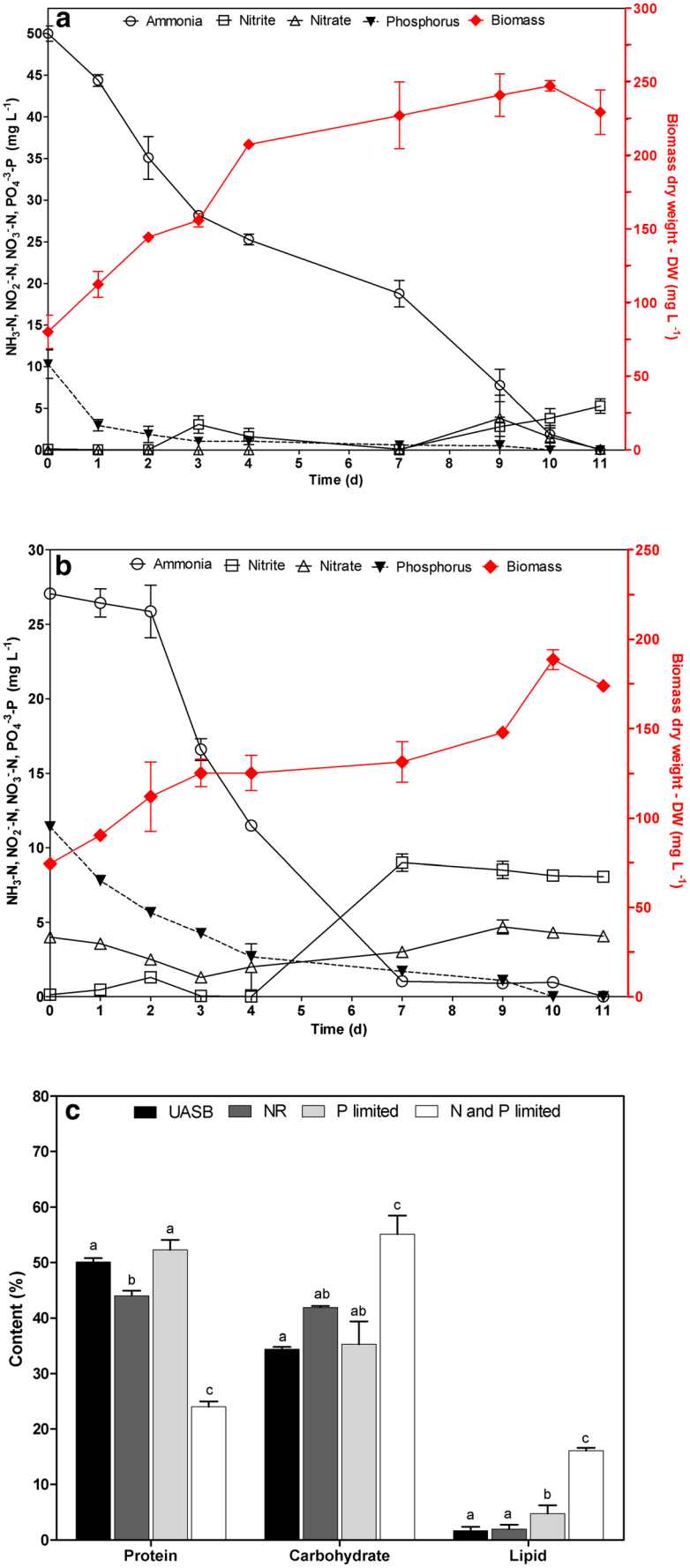

Fig. 1 Nutrient removal and biomass concentration during phycoremediation of UASB (A) and NR (B) effluents. Biochemical composition (%) of the microalgae consortium harvested in different stages of growth (C). Bars depict standard deviation of the mean and different letters denote significant differences ($p \leq 0.05$) according to Tukey test

Table 1 Amino acid composition (% of total protein dry weight) of the microalgae biomass grown in swine wastewater effluent and under N- and/or P-limiting conditions in comparison to typical amino acid (%) used to feed male pigs with high genetic potential

Amino acids (%)	*Chlorella* sp. and *Scenedesmus* sp.	*Spirulina maxima*	*Phormidium* sp.	Microalgae consortium					[Starting	Growing	Finishing]*
	Anaerobically digested piggery effluent	Aeration-stabilized swine waste		UASB	NR	P limited	N and P limited	*p* value			
Aspartic acid	2.4	7.3	4.7	**4.4 ± 0.4**^{a}	3.0 ± 0.1^{b}	4.1 ± 0.05^{a}	1.4 ± 0.12^{c}	< 0.001	-	-	-
Glutamic acid	3.6	12.3	5.01	**4.9 ± 0.04**^{a}	3.3 ± 0.2^{b}	4.9 ± 0.05^{a}	2.0 ± 0.03^{c}	< 0.001	-	-	-
Serine	1	3.9	2.1	1.8 ± 0.1^{a}	1.3 ± 0.06^{b}	**2.1 ± 0.04**^{c}	0.8 ± 0.02^{d}	< 0.001	-	-	-
Glycine	1.4	4.05	4.4	**2.8 ± 0.3**^{a}	1.9 ± 0.3^{b}	2.8 ± 0.01^{a}	1.1 ± 0.05^{c}	< 0.001	-	-	-
Histidine	0.2	2.3	0.85	**0.9 ± 1.2**^{a}	0.5 ± 0.2^{b}	0.6 ± 0.07^{b}	0.2 ± 0.04^{c}	< 0.001	0.4	0.3	0.3
Arginine	1.1	5.5	2.8	**3.9 ± 2.4**	1.9 ± 0.02	2.6 ± 0.03	0.9 ± 0.01	0.08	0.6	0.4	0.3
Threonine	1.1	4	2.6	1.9 ± 0.04^{a}	1.5 ± 0.04^{b}	**2.3 ± 0.04**^{c}	1.1 ± 0.03^{d}	< 0.001	0.9	0.8	0.6
Alanine	2.3	5.9	3.8	2.9 ± 0.6^{a}	2.3 ± 0.4^{ac}	**4.1 ± 0.01**^{b}	1.6 ± 0.1^{c}	< 0.001	-	-	-
Proline	1.3	3.9	1.1	1.9 ± 0.09^{a}	1.3 ± 0.05^{b}	**2.4 ± 0.04**^{c}	0.9 ± 0.02^{d}	< 0.001	-	-	-
Tyrosine	0.7	3.2	2.6	1.2 ± 0.4^{a}	0.8 ± 0.3^{ab}	**1.3 ± 0.02**^{a}	0.5 ± 0.01^{b}	0.01	-	-	-
Valine	1.4	4.1	2.1	**2.4 ± 0.3**^{a}	1.7 ± 0.2^{b}	2.8 ± 0.04^{a}	1.1 ± 0.01^{c}	< 0.001	1.0	0.8	0.6
Methionine	0.5	0.53	0.6	**2.8 ± 3.5**	1.02 ± 1	0.7 ± 0.01	0.3 ± 0.005	0.39	0.4	0.3	0.3
Cysteine	0.3	-	-	**2.9 ± 3.8**	1.9 ± 2.7	0.7 ± 0.06	0.2 ± 0.04	0.53	-	-	-
Isoleucine	0.8	3.3	2.3	1.5 ± 0.2^{a}	1.2 ± 0.2^{a}	**2.0 ± 0.02**^{b}	0.8 ± 0.05^{c}	< 0.001	0.8	0.6	0.5
Leucine	2	6.7	4.1	3.4 ± 0.1^{a}	2.5 ± 0.01^{b}	**4.1 ± 0.02**^{c}	1.7 ± 0.07^{d}	< 0.001	1.4	1.1	0.8
Phenylalanine	1.2	3.4	2.2	2.2 ± 0.2^{ab}	1.6 ± 0.3^{a}	**2.5 ± 0.1**^{b}	0.9 ± 0.02^{c}	< 0.001	0.7	0.6	0.4
Lysine	1.2	4.1	1.8	**2.5 ± 0.02**^{a}	1.2 ± 0.1^{b}	2.2 ± 0.1^{c}	0.9 ± 0.04^{d}	< 0.001	1.5	1.2	0.8
Tryptophan	0.3	-	-	**0.5 ± 0.01**^{a}	0.2 ± 0.01^{b}	0.3 ± 0.01^{c}	0.2 ± 0.005^{d}	< 0.001	0.2	0.2	0.2
Reference	Moheimani et al. [11]	Canizares-Villanueva et al. [10]		This study							

*Nutritional requirements of whole male pigs of high genetic potential [28]

Data shown as means ± standard deviation

Different letters denote significant differences ($p < 0.05$) according to Tukey HSD test

higher (up to 2-fold) than previous studies also using raw swine wastewater effluents from anaerobic digestion [11]. This could be attributable to variations in physical-chemical characteristics of the effluent as well as the type of microalgae used (i.e., *Chlorella* sp. and *Scenedesmus* sp.) in comparison to this study (*Chlorella* spp.).

Typical protein diets used in swine production requires supplementation with essential AA such as lysine, threonine, methionine, and tryptophan [30]. Deficiency and long-term effects of AA restriction may impair animal growth, immunity, increase the susceptibility to infectious diseases as well as encourage other digestive and reproductive problems [31]. The concentration of these same AA found in the microalgae biomass (ranging from 0.2–2.5%) exceeds the minimum AA requirements (Table 1). Thus, whereas microalgae produced during phycoremediation of wastewaters could be later processed as source of animal nutrition supplementation (circular economy) requires further investigations.

3.3 Microalgae grown in swine wastewater accumulates monounsaturated fatty acids

PUFAs in microalgae are known to promote health and prevent disease [32]. For instance, supplementation of PUFAs on swine diet is recognized for its beneficial effects on growth performance metabolism in the digestibility, and antimicrobial, anti-inflammatory, immunomodulatory activity, blood lipid profiles, and meat quality [33, 34]. To illustrate the presence of omega-3 fatty acids, eicosapentaenoic and docosahexaenoic acids that are considered the most important due to its important associated nutritional and economic value [35]. The effects of omega-3 polyunsaturated fatty acids obtained from microalgae were reported to decrease serum levels of triglycerides during swine gestation, improving swine birth weights [36]. Similarly, the supplementation of swine diets with omega-3 from microalgae *Schizochythrum* sp. increased the concentration of this fatty acid in swine, increasing overall meat value due to potential health benefits to consumers [37].

Table 2 Fatty acid content (% of total protein dry weight) of the microalgae biomass grown in swine wastewater effluent and under N- and/or P-limiting conditions

Fatty acids (%)	UASB	NR	P limited	N and P limited	*p* value
Myristic acid (C14:0)	0.01 ± 0.006^{a}	0.008 ± 0.002^{a}	0.02 ± 0.005^{a}	0.09 ± 0.005^{b}	< 0.001
Myristoyl acid (C14:1)	0.04 ± 0.006	0.02 ± 0.005	0.01	0.03 ± 0.04	0.394
Pentadecanoic acid (C15:0)	0.01 ± 0.006	0.009 ± 0.0005	0.009 ± 0.0005	0.01 ± 0.005	0.189
Palmitic acid (C16:0)	0.25 ± 0.04^{a}	0.35 ± 0.04^{a}	0.86 ± 0.03^{b}	5.48 ± 0.36^{c}	< 0.001
Palmitoleic acid (C16:1n7)	0.17 ± 0.23^{a}	0.04 ± 0.005^{b}	0.02 ± 0.01^{b}	0.04 ± 0.005^{b}	< 0.001
Margaric acid (C17:0)	0.02 ± 0.12^{a}	0.02 ± 0.01^{a}	0.03 ± 0.02^{ab}	0.05 ± 0.005^{b}	< 0.001
Estearic acid (C18:0)	0.04 ± 0.12^{a}	0.04 ± 0.005^{a}	0.03 ± 0.005^{a}	0.6 ± 0.3^{b}	< 0.001
Oleic acid (C18:1n9c)	0.08 ± 0.12^{a}	0.16 ± 0.03^{a}	0.2 ± 0.11^{a}	4.7 ± 0.2^{b}	< 0.001
Linoleic acid (C18:2n6c)	0.15 ± 0.04^{a}	0.2 ± 0.02^{a}	0.2 ± 0.08^{a}	1.5 ± 0.32^{b}	< 0.001
Linolenic acid (C18:3n6)	0.02 ± 0.012^{a}	0.03 ± 0.01^{ab}	0.02 ± 0.005^{ab}	0.04 ± 0.005^{b}	0.05
Linolenic acid (C18:3n3)	0.48 ± 0.017^{a}	0.5 ± 0.03^{a}	1.1 ± 0.04^{b}	3.9 ± 0.07^{c}	< 0.001
Arachic acid (C20:0)	-	-	-	0.02 ± 0.005	< 0.001
Eicosatrienoic acid (20:3)	-	-	0.009 ± 0.0005	0.02 ± 0.005	0.104
Behenic acid (C22:0)	-	0.009 ± 0.001^{a}	0.02 ± 0.01^{a}	0.04 ± 0.005^{b}	< 0.001
Erucic acid (C22:1n9)	-	-	-	0.01 ± 0.005	< 0.001
Eicosapentaenoic acid (C20:5n3)	-	-	-	0.02 ± 0.01	< 0.001
Lignoceric acid (C24:0)	-	-	0.01	0.01 ± 0.005	> 0.05
MUFAs	0.28	0.2	0.21	4.75	
PUFAs	0.61	0.71	1.22	5.27	
UFAs	0.92	0.91	1.45	10.03	
SFAs	0.27	0.39	0.90	5.83	
ω-3	0.48	0.49	1.04	3.9	
ω-6	0.15	0.22	0.18	1.37	
ω-9	0.09	0.14	0.21	4.7	

Monounsaturated (MUFAs), polyunsaturated (PUFAs), unsaturated (UFAs), and saturated (SFA) fatty acid

Data shown as means ± standard deviation

Different letters denote significant differences ($p < 0.05$) according to Tukey HSD test

As discussed earlier, the accumulation of lipids increased under nutrients deprived conditions. As expected, the increase in lipid content was accompanied by an increase in monounsaturated (MUFAs, from 0.2 to 4.7%), polyunsaturated (PUFAs; from 0.6 to 5.3%), unsaturated (UFAs; from 0.9 to 10%) and saturated (SFA, from 0.3 to 5.8%) FA (Table 2). Comparatively, other microalgae consortium (*Chlorella*, spp., *Nannochloropsis* sp., *Scenedesmus* spp., *Chlamydomonas* spp., *Oscillatoria* sp., *Kirchnella* sp., and *Microcoleus* sp.) found in the effluent of municipal wastewater treatment system, was composed mainly by saturated FA followed by MUFA and PUFA [5].

The concentration profile of FA measured in microalgae biomass during different states of cell growth was also evaluated (Table 3). Long-chain FA (from 13 to 21 C) were dominant. Very long FA (22 or more C), in particular 22:1 (ω11), 26:0 and 27:0 (Table 3). Essential FA commonly used as supplement in animal nutrition, i.e., α-linolenic acid (C18:3) and linoleic acid (C18:2) [38] were also present. Overall, the FA composition agrees with other reported values. For instance, Gan et al. [39] reported that *Chlorella vulgaris* produced high percentage of saturated FA, in particular palmitic acid (16:0) ≈ 38%. In addition, the authors described that *Chlorella vulgaris* can biosynthesize very long FA as 20:2, 20:3 and 20:4. Norashikin et al. [40] also observed high concentrations of C:18 (2) and C:18 (3) in *Chlorella vulgaris*. The relatively high concentrations of unsaturated FA observed in the indigenous microalgae consortium and growth conditions tested in this study may be of particular interest for industries seeking alternative sources of renewable and sustainable feedstock for product manufacture [41].

3.4 Potential bioactive peptides

Peptides consist of a diverse group of oligomeric structures usually composed of protein fragments or chains of different short amino acid sequences, usually 2–20 residues. These metabolites have been reported in the regulation of a number of cellular processes such as hormonal regulation, redox homeostasis, neuronal signal, cell signaling, transduction, growth and immune response [42]. In this study, twenty-five bioactive peptides were found in the microalgae consortium, with possible multiple bio functionalities including Angiotensin converting enzyme (ACE) Inhibitory, dipeptidyl peptidase-

Table 3 Fatty acid profile in microalgae harvested from phycoremediation of UASB digestate

Fatty acid	Days											
	1	2	3	4	5	6	7	8	9	10	11	12
C10:0	■											
C13:0			■									
C14:0												■
C14:1												■
C15:1												■
C16:0	■	■	■	■	■	■	■	■	■	■	■	■
* C16:1	■	■	■	■	■	■	■		■	■	■	■
C16:2 (7, 10)	■	■	■	■	■	■	■		■	■		■
C16:3 (7, 10, 13)	■				■	■		■	■			■
C18:0	■	■	■	■	■	■	■	■	■	■	■	■
† C18:1	■	■	■	■	■	■	■	■	■	■	■	■
C18:2 (9, 12)	■	■	■	■	■	■	■	■	■	■	■	■
C18:3 (9, 12, 15)		■	■	■	■	■				■	■	
C19:0		■	■									
C20:0							■			■	■	
C20:1 (ω11)		■				■						
C22:1 (ω-9)	■				■					■		
C26:0										■	■	
C27:0	■	■	■	■							■	

■ Presence of fatty acid

*Mainly C16:1 (9)

†Mainly C18:1 (9)

Table 4 Bioactive peptides identified in the microalgae consortium grown in UASB digestate using BIOPEP's "profiles of potential biological activities" tool

Bioactive peptides	Days							
	1	3	5	6	7	9	11	12
ACE Inhibitory	■	■	■	■	■	■	■	■
Dipeptidyl peptidase-IV	■	■	■	■	■	■	■	■
Dipeptidyl peptidase III inhibitor	■	■	■	■	■	■	■	■
Anti-amnestic	■	■	■	■	■	■	■	■
Antithrombotic	■	■	■	■	■	■	■	■
Immunomodulating	■	■	■	■	■	■	■	■
CaMPDE inhibitor	■	■	■	■	■	■	■	■
Renin inhibitor	■	■	■	■	■	■	■	■
Antioxidative	■	■	■	■	■	■	■	■
Activating ubiquitin-mediated proteolysis	■	■	■	■	■	■	■	■
Opioid	■	■	■	■	■	■	■	■
Regulating*	■	■	■	■	■	■	■	■
Stimulating**	■	■	■	■	■	■	■	■
Neuropeptide	■	■	■	■	■	■	■	■
Alpha-glucosidase inhibitor	■	■	■	■	■	■	■	■
Hypolipidemic	■	■	■	■	■	■	■	■
HMG-CoA reductase inhibitor	■	■	■	■	■			
Inhibitor of insulin secretion			■	■	■	■	■	■
Chemotactic			■	■	■	■	■	■
Anti-inflammatory			■	■	■	■	■	■
Bacterial permease ligand	■	■		■	■			
Anticancer	■	■	■					
Hypotensive	■	■	■					
Chymotrypsin inhibitor			■					
Dipeptidyl carboxypeptidase inhibitor								■

■ Presence peptides

*Peptide regulating ion flow or peptide regulating the stomach mucosal membrane activity

**Stimulating vasoactive substance release or glucose uptake stimulating peptide

IV, dipeptidyl peptidase III inhibitor, anti-amnestic, antithrombotic, immunomodulating, CaMPDE inhibitor, renin inhibitor, antioxidative, activating ubiquitin-mediated proteolysis, opioid, regulating, stimulating, neuropeptide, alpha-glucosidase inhibitor, hypolipidemic, HMG-CoA reductase inhibitor, inhibitor of insulin secretion, chemotactic, anti-inflammatory, bacterial permease ligand, anticancer, hypotensive, chymotrypsin inhibitor, and dipeptidyl carboxypeptidase inhibitor (Table 4). Most functional bioactive peptides are from the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) protein complex responsible for the conversion of atmospheric carbon dioxide into organic carbon through the Calvin cycle [43] and to a lower extent related to 50S Ribosomal Protein L7/L12, phosphoglycerate kinase, ATP Synthase Subunit Beta and Heat Shock Protein 70 [44]. It is worth noting that most of the peptides determined in microalgae biomass were dipeptides or tripeptides with diverse biological activities. To illustrate, a pepsin-hydrolyzed peptide with important antioxidant activities were obtained from *Chlorella vulgaris* and *Chlorella ellipsoidea* [45]. Similarly, bioactive peptides with antihypertensive, anticancer, and ACE inhibitory activities were isolated from *Chlorella* spp. and *Spirulina platensis* [46–49].

Overall, the microalgae biomass obtained from the phycoremediation of swine wastewaters seems to contain significant concentrations of important AA (e.g., arginine, lysine,

tryptophan, proline), FA (e.g., conjugated linoleic acids and EPA), and peptides with remarkable biological properties. It is estimated that phycoremediation could lead to microalgae biomass yield of ~ 40 ton ha^{-1} $year^{-1}$ (using the equation = 10^4 m^2 ha^{-1} × 0.4 m (depth of the cultivation system) × microalgae yield (0.25 g-algae L^{-1} 11 d^{-1}) × 10^3 L m^{-3} × 1 ton 10^{-6} g × 365 d $year^{-1}$). Comparatively, soybean yields can vary widely depending on water and fertilizer availability, and row spacing. Assuming rainfed conditions, good soybean yields vary between 1.5 and 3.4 ton ha^{-1} [50]. Despite of superior productivity, microalgae biomass also contains higher nutritional values (e.g., ~ 50% protein) than soybean (~ 37%). Nonetheless, the use of wastewaters to grow microalgae as source of animal nutrition still faces uncertainties raised by safety concerns. Some countries have more stringent regulations about the use of wastewater to grow microalgae than others [51, 52]. While further studies are certainly required to approve safety, recent studies [53, 54] reported that anaerobic digestion, pretreatment, and dilution of agro-industrial wastewaters can significantly decrease or even eliminate the risks associated with the presence of heavy metals, some antibiotics [55], as well as bacteria pathogens [56]. Integrating microalgae in wastewater treatment systems for nutrient recycle [57] and production of valuable biomass seems interesting for securing future food and feed production [58].

4 Conclusions

Phycoremediation efficiently removed ammonia-N and P (100%) from swine wastewaters effluents. Significant accumulation of lipids in microalgae biomass was observed in nutrients deprived growth conditions. The most abundant essential AA found were lysine, leucine, threonine, methionine and tryptophan. FA was mainly composed by linolenic acid (ω-3 and 6) and oleic acid (ω-9). It was identified, 25 peptides that are associated with relevant biological functions. The concept of biorefinery herein contributes to advancing our understanding of technological arrangements combining wastewater treatment with production of biomass rich in metabolites with a broad range of biotechnological applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Code availability Not applicable.

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