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Pig Slaughterhouse Wastewater: Medium Culture for Microalgae Biomass Generation as Raw Material in Biofuel Industries

Johanna Medrano-Barboza [1,2], Kevin Herrera-Rengifo [1], Alberto Aguirre-Bravo [1], José Rubén Ramírez-Iglesias [3], Rosalía Rodríguez [2] and Victoria Morales [4,*]

[1] Faculty of Engineering and Applied Sciences, Universidad Internacional SEK, Quito 170102, Ecuador
[2] Department of Chemical, Energy and Mechanical Technology, ESCET, Universidad Rey Juan Carlos, Móstoles, 28933 Madrid, Spain
[3] Faculty of Health Sciences, Universidad Internacional SEK, Quito 170102, Ecuador
[4] Department of Chemical and Environmental Technology, ESCET, Universidad Rey Juan Carlos, Móstoles, 28933 Madrid, Spain
* Correspondence: victoria.morales@urjc.es

Abstract: Microalgae are photosynthetic microorganisms with high lipid content, capable of degrading nutrients from wastewater. In this research, two strains of microalgae, *Scenedesmus* sp. and *Chlorella vulgaris* were cultivated in sterilized pig slaughterhouse wastewater using outdoor flat photobioreactors. Cell growth, total lipids, free fatty acids (FFA), fatty acid methyl esters (FAME) and physicochemical parameters of wastewater were measured. The results indicated that pig slaughterhouse wastewater is adequate to grow these species of microalgae, obtaining a higher biomass growth for *Scenedesmus* sp. compared to *Chlorella vulgaris* (0.41 g/L vs. 0.2 g/L); additionally, these species can be used in bioremediation processes due to the nutrient removal achieved in terms of Total Nitrogen (TN), Total Phosphorous (TP) and Total Organic Carbon (TOC). Methylcyclohexane, chloroform: methanol (1:2) and ethyl acetate had better yield of lipids and FFA. The percentages of FAMEs from FFA were in the range of 52.5–89.5 wt% for *Scenedesmus* sp. and for *Chlorella vulgaris* from 52–80.5 wt%. Although the values of lipids, FFA and FAME are below of the range reported by other authors, the use of this type of wastewater as culture medium for the two species cannot be ruled out for lipid extraction in biofuel production.

Keywords: microalgae; nutrient removal; pig slaughterhouse; lipids; free fatty acids

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1. Introduction

Currently, 80% of the energy production of the world is obtained from non-renewable sources such as oil. The combustion of these types of energy sources produces harmful gas emissions of carbon monoxide (CO), carbon dioxide (CO_2), sulfur and nitrogen oxide (SO_x and NO_x), among other gases, causing problems related to fossil fuel consumption such as environmental issues and affections to human health [1–3]. Facing this reality, new sources of energy have been studied such as biofuels. These are renewable energies obtained from a variety of biomass including animal or vegetable products and byproducts. Their combustion generates 50% less greenhouse gas emissions released into the atmosphere and can replace a portion of fossil fuel consumption for transport energy [1]. Biofuels are grouped according to the type of biomass they come from: the first generation of biomass comes from plants used for food with high contents of sugars and fats or oils. The second generation is composed of agricultural waste with high contents of cellulose and lignin and is used cooking oils [2]. The third generation is composed of organisms capable of feeding on light and CO_2, such as algae, microalgae,

fungi and yeasts, which have a high content of lipids, protein and carbohydrates. The fourth are genetically modified organisms capable of producing sugars and lipids, and possess greater capture of CO_2 [4].

Microalgae are photosynthetic unicellular organisms with autotrophic or heterotrophic growth capable of transforming CO_2 from the atmosphere, light and nutrients into oxygen and biomass [2]. As photosynthetic organisms, they use natural or artificial light and fix the CO_2, minimizing the pollution of the atmosphere, have easy adaptation to different ecosystems and have demonstrated a high capacity to reduce the organic and inorganic load of polluted waters [4,5]. These microorganisms produce 50 times more biomass compared to land plants [6,7], and produce ten times more liters of oil per hectare than soybeans and African oil palm [8]. The scientific community and industries have focused attention on microalgae as feedstock because of their high biomass production for energy and other bioproducts such as food pigments, proteins, fatty acids and lipids, among other valuable products [9]. Microalgae suitable for biodiesel production must meet certain conditions such as a high growth rate, the ability to adapt to variations in the culture medium and atmospheric conditions and a good capacity to form aggregates or flocs to simplify the harvesting process by sedimentation [10]. Oleaginous microalgae meet these conditions because they store energy in the form of lipids or triglycerides, for example, *Ankistrodesmus* sp., *Kirechneriella* sp., *Palmella* sp., *Eudorina elegans*, *Volvox* sp., *Chlorella* sp. and *Scenedesmus* sp. [11,12]. *Chlorella vulgaris* and *Scenedesmus* sp. are species with accelerated cellular growth, a high protein, vitamin and lipid production and are commonly used for bioremediation of wastewaters and biodiesel production [6,13–15].

Regarding lipid extraction from microalgae, it is important to consider that it varies according to the species, culture conditions and nutrient depletion. Additionally, the extracting solvent selection is fundamental to achieving high yields from microalgae, and normally organic solvents such as hexane, methanol, ethanol, chloroform: methanol (1:1; 1:2; 2:1 % v/v), isopropanol and some polar: non-polar mixtures of solvents are commonly used, coupled with other technologies for cell disruption to enhance lipid extraction [1,16]. Despite all the advantages of using microalgae as feedstock for biofuels and other high-added-value products, some constraints remain and need to be resolved for making large-scale production economically feasible. Large-scale microalgae cultures use large volumes of potable or natural waters, require a source of CO_2, nutrients and energy for aeration, light and instrumentation to control or measure certain parameters [2]. However, there are some limitations in these type of cultures due to the great amount of freshwater, engineering problems related to temperature, nutrient concentrations, aeration, antifoaming and pH control [17]. Another common limitation is the use of expensive fertilizers as a source of nutrients for microalgae.

Nowadays, the sustainable biorefinery concept is widely used in the microalgae field, and this concept is also linked to the circular economy principle to reduce the environmental impacts of this type of industry and the numerous bottlenecks and challenges associated with microalgal biorefineries [3,9]. Some less expensive and more efficient alternatives of medium cultures are being investigated to optimize the economic and environmental performance of a biorefinery. Among these alternatives, the use of urban, industrial or livestock wastewater to take advantage of their nutrient content is an option for pollutants treatment and biomass production [18–20].

Different research works support the use of wastewater for biomass and biofuel production from microalgae as a suitable and non-expensive method as compared to traditional cultivation methods for water reuse. Implementation of this type of water has been evaluated with different treatments such as non-treated or partially treated wastewater, sterilized and unsterilized wastewater, with nutrients addition or without them, among other types of treatment to improve the lipid content on blue and green microalgae, biomass production or bioremediation purposes [18,21]. Additionally, these studies showed the savings in nutrient addition, the reduction in the total costs of the

extracted bioproducts and the bioremediation aspects associated with the removal of nitrogen, carbon and phosphorus from wastewaters and their respective expenses. Among the diverse types of wastewater, domestic or urban, industrial, crop effluents, poultry, dairy and swine wastewaters have been used the most.

Some different microalgae strains of *Chlorella vulgaris* grown in urban, municipal, or domestic wastewaters have shown high efficiency for nitrogen and total organic carbon removal. It has the benefit of water reuse and algal biomass production with sufficient lipid content and fatty acids profile for potential use as feedstock in biodiesel generation [10,22,23]. *Chlorella vulgaris* grown in poultry manure anaerobic-digested effluents showed an efficient removal of ammonia (NH_4^+), total phosphorus (TP), Total organic (TOC), total carbon (TC), turbidity and bacteria, when combined with electrolysis for treatment of this type of wastewater [24]. Compared to blue-green medium BG11, raw dairy wastewater was demonstrated to be more suitable for biomass and lipid production for three microalgae strains (*Chlorella* sp., *Scenedesmus sp*. and *Chlorella zofingiensis)* [5]. Additionally, *Chlorella vulgaris* has shown high biomass and lipid productivity growing in dairy wastewater pretreated with sodium hypochlorite (NaClO) in the concentration of 30 ppm or 15 min of UV irradiation [25]. Pollutants in swine/piggery wastewater have been effectively removed by green microalgae and this biomass has shown a high potential of conversion for bioenergy products, despite the presence of toxic pollutants in the original wastewater [26,27]. Additionally, based on mass and energy balance analysis, swine wastewater has suitable characteristics for CO_2 mitigation and microalgal biodiesel production from *Chlorella vulgaris* [15,28].

Traditionally, pork is appreciated as a source of protein in the diet. Depending on the final purpose of a specific pig production system, it is possible to find businesses related to pig farming, breeding and/or slaughtering [29]; however, small or medium businesses are mainly focused on a single activity that allows retrieving wastewater with specific physiochemical conditions [30]. Pig slaughterhouse wastewater is generated during the sacrifice or butchering of pork and normally contains animal blood mixed with water and solid residues such as hair, skin, fat and meat residues, etc. [31,32]. Pig slaughterhouse wastewaters generated in small or medium butchers are a source of pollution for water bodies and soil when they are not treated in wastewater treatment plants; especially in developing countries where environmental legislation is deficient. Slaughterhouse wastewater contains a heavy load of organic and inorganic nutrients, mainly in the form of nitrogen, phosphorous and carbon that can be used for microalgae growth [31]. The use of this type of wastewater is preferred over conventional cultures with fresh water or natural sources of water since microalgae can reduce the pollutant load of nutrients in terms of Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), pathogens and heavy metals [33].

Some works support the use of swine/piggery wastewaters: different concentrations of pretreated swine slurry diluted with freshwater, non-treated or unsterilized swine wastewater and swine manure to take advantage of the residual nutrients from pig feed, pig farming or pig urine and manure [11,12,14,20–22,25,34]; however, there is scarce literature of the use of pig slaughterhouse wastewater for biomass cultivation or bioremediation purposes in American countries [31,32]. In this study, pig slaughterhouse wastewater was used as culture medium for *Scenedesmus* sp. and *Chlorella vulgaris* in outdoor photobioreactors exposed to atmospheric conditions in Quito, Ecuador, to test the feasibility of this specific type of wastewater for optimal growth of microalgae focused on lipids and potential production of biofuels.

2. Materials and Methods

2.1. Microalgal Strain and Culture Conditions

Chlorella vulgaris and *Scenedesmus* sp. strains were obtained from the Spanish Algae Bank and maintained in 1 L glass bottles filled with Bold Basal Medium (BBM) [35], with continuous aeration (4.2 L/min), artificial illumination with white fluorescent lamps at both sides of the bottles (light intensity of 60 ± 10 μmol photons/m^2 s) in 12:12 photoperiods and were kept at room temperature for seven days during laboratory-scale cultivation and before inoculation. The wastewater was collected twice (December 2019 and March 2020) from a pig slaughter farm. Large solid particles were removed by filtration with a filter cloth (2 μm pore size) [36] and then autoclaved (121.5 °C for 15 min). The nutrient composition of the wastewater was determined following the HACH DR 4000 Spectrophotometer Manual. Table 1 summarizes the physicochemical characteristics of the pig slaughterhouse wastewater used in this study. All data are expressed as means of triplicates with their corresponding standard deviation. Pretreated wastewater was transferred to 60 L glass flat panel outdoor photobioreactors (PBRs) (0.69 m long, 0.15 m wide, 0.58 m deep). The volume of the cultured media was 10 times the volume of the microalgae inoculum (10:1) for a final volume of 40 L. Temperature was controlled at 26 ± 2 °C with nickelines coupled to an electronic module for temperature control, and the culture was bubbled with compressed air (0.04% CO_2) without extra mechanical mixing. The initial concentration of microalgae was 1.88 × 10^5 cells/mL and 2.5 × 10^5 cells/mL for *Chlorella vulgaris* and *Scenedesmus* sp., respectively. Each microalgae strain was analyzed in separated PBRs exposed to the atmospheric conditions of Quito for 11 days, and no medium replenishment was made when the nutrients were depleted.

Table 1. Physicochemical characteristics of pig slaughterhouse wastewater used in this study.

Parameter	Initial Value for *Chlorella vulgaris*	Initial Value for *Scenedesmus* sp.
pH	6.5 ± 0.3	6.7 ± 0.5
SS (mg/L)	92.7 ± 5.6	96.5 ± 4.3
TN (mg/L N)	95 ± 0.9	75 ± 0.4
TP (mg/L PO_4^{3-})	60 ± 1.2	99.9 ± 0.6
TOC (mg/L C)	421 ± 0.8	438 ± 1.3

Abbreviations: SS, suspended solids.

2.2. Sampling, Nutrient Analysis, Cell Counting and Biomass Processing

Samples measuring 50 mL were collected daily for 11 days. Biomass growth was determined by direct cell counting in a Neubauer chamber under the microscope (Leica PLAN 40X) with Equation (1) [37]. All samples were centrifuged at 4000 rpm for 5 min and supernatants were collected and then filtered through a 0.2 μm membrane for determination of total organic carbon (TOC- mg/L C), total nitrogen (TN- mg/L N) and total phosphorus (TP mg/L PO_4^{3-}) using commercial HACH wastewater test kits and a UV spectrophotometer (DR2700; HACH, USA) and HACH method 10,072, 10,127 and 10,128 [38–40].

The level of suspended solids (SS) was determined by filtering the wastewater through glass-fiber filter paper (0.45 μm pore size) followed by drying at 105 °C for 2 h. Biomass was washed twice with deionized water; half of the harvested wet biomass was stored, and the other half was dried at 105 °C for 5 h for further analysis. Dry biomass weight (DBW) was determined with filtering paper at 60 °C and 24 h [41]. Biomass productivity was calculated by dividing the total collected biomass (wet and dry) by volume (L) and cultivation time (d) according to Equation (2). Specific growth rate was calculated as the slope of the straight line in the exponential growth phase when plotting

the natural logarithm of the biomass concentration versus culture time according to Equation (3) and efficiency of nutrient removal was calculated according to Equation (4).

$$Cell\ concentration = \frac{number\ of\ cells\ \times\ 10{,}000}{squares\ counted\ \times\ dilution\ factor}\left[\frac{cells}{mL}\right] \quad (1)$$

$$Biomass\ productivity = \frac{total\ biomass}{volume\ \times\ time}\left[\frac{g}{L\ d}\right] \quad (2)$$

$$Specific\ growth\ rate = \frac{Ln\ (cell\ concentration)}{\Delta t}\ [d^{-1}] \quad (3)$$

(4)

$$Nutrient\ removal\ efficiency\ (\%) = \frac{initial\ concentration - final\ concentration}{initial\ concentration}\ \times\ 100$$

2.3. Lipid Content and Fatty Acid Analysis

Total lipids in wet and dried biomass were extracted by physical and chemical methods following the Bligh and Dyer method using vortex, sonication and solvent extraction with six HPLC degree solvents: methanol, ethanol, hexane, methylcyclohexane, ethyl acetate and chloroform–methanol (1:2 v/v) [42]. Lipids results were determined according to Equation (5), where tube 1 is empty tube weight (g), tube 2 is the tube with dried lipid weight and biomass weight is the quantity of wet or dry biomass used for each extraction. Lipid productivity was calculated with Equation (6), by dividing the total lipid mass with volume (L) and time (d).

$$wt\%\ lipids = \frac{tube\ 2 - tube\ 1}{biomass\ weight} \times 100 \quad (5)$$

$$Total\ productivity = \frac{total\ lipid\ mass}{volume\ \times\ time}\left[\frac{g}{L\ d}\right] \quad (6)$$

Free fatty acid (FFA) extraction was performed in dry biomass with organic solvents and a basic catalyst (KOH) following the saponification process of FFA extraction [43]. FFA results were determined with Equation (7), where tube 1 is empty tube weight (g), tube 2 is the tube with dried FFA weight (g) and dry weight is the quantity of dry biomass used for each extraction (g).

$$wt\%\ FFA = \frac{tube\ 2 - tube\ 1}{dry\ weight} \times 100 \quad (7)$$

Fatty acid methyl esters (FAME) were extracted from dry biomass (direct extraction), total extracted lipids and FFA lipids (indirect extraction). In all cases, two types of acid catalysts were used, concentrated sulfuric acid (H_2SO_4) as homogenous catalyst and a commercial resin CT-269 as heterogeneous catalyst. This process was as follows: for the homogeneous catalyzed procedure, a relationship of (1.74:15:1) (catalyst:solvent:biomass) was used; on the other hand, for the heterogeneous procedure, a relationship of (1.1:70:1) (catalyst:solvent:biomass) was used. All substances were put in clean screw-top pressure glass tube reactors in a thermal bath at 90 °C for 4 h and 900 rpm. After the reaction, the organic phase was filtered in a Millipore filter and 2 mL of hexane: diethyl ether (80:20) solution was added for removing impurities from the catalyst. The mix was transferred to a separatory funnel to separate the organic phase, which was transferred to a previously weighted clean tube, washed with 5 mL of 0.1% NaCl solution and then dried at 60 °C for 24 h. After gravimetric measurement of FAMEs, results were obtained according to Equation (8), where tube 1 is empty tube weight (g), tube 2 is FAME content and tube

weight (g) and dry weight is the mass of the biomass used (dry microalgae biomass or extracted lipids) (g) [16]. Experiments were performed in triplicate, and data were expressed as mean with their corresponding standard deviation (±SD).

$$wt\%\ FAME = \frac{tube\ 2 - tube\ 1}{Dry\ weight} \times 100 \quad (8)$$

2.4. Statistical Analysis

Experiments were performed in triplicate, and data were expressed as means with their corresponding standard deviation (±SD). Mean comparison was made with the Kruskal Wallis test in Statgraphics software 18 version. Statistically significant differences between means were assumed when $p < 0.05$.

3. Results

3.1. Cell Growth

Cell growth of *Scenedesmus* sp. and *Chlorella vulgaris* in pig slaughterhouse wastewater for 11 days is shown in Figure 1 in terms of cell concentration (Figure 1a) and dry weight (Figure 1b). For *Scenedesmus* sp., initial cell concentration was $2.5 \times 10^5 \pm 3.31 \times 10^4$ cells/mL, and the stationary phase was reached after 9 days, where cell concentration was $1.98 \times 10^6 \pm 1.4 \times 10^5$ cells/mL. Dry weight varied from 0.22 g/L ± 0.01 to 0.41 ± 0.06 g/L and biomass productivity was in the range of 0.02 to 0.037 g/L/d. For *Chlorella vulgaris*, initial cell concentration was $1.88 \times 10^5 \pm 1.48 \times 10^5$ cells/mL, and the stationary phase was reached after 9 days with a cell concentration of $4.58 \times 10^6 \pm 3.8 \times 10^4$ cells/mL. Dry weight varied from 0.04 g/L to 0.2 ± 0.03 g/L at the end of the culture and biomass productivity was in the range of 0.004 to 0.018 g/L/d. The growth rate was 0.8309 d^{-1} and 1.3558 d^{-1} for *Scenedesmus* sp. and *Chlorella vulgaris*, respectively. In this study, dilution of the initial culture medium was not conducted to reduce the organic load and turbidity, thus reducing the consumption of fresh water. Furthermore, no nutrient replacement was carried out during cultivation time.

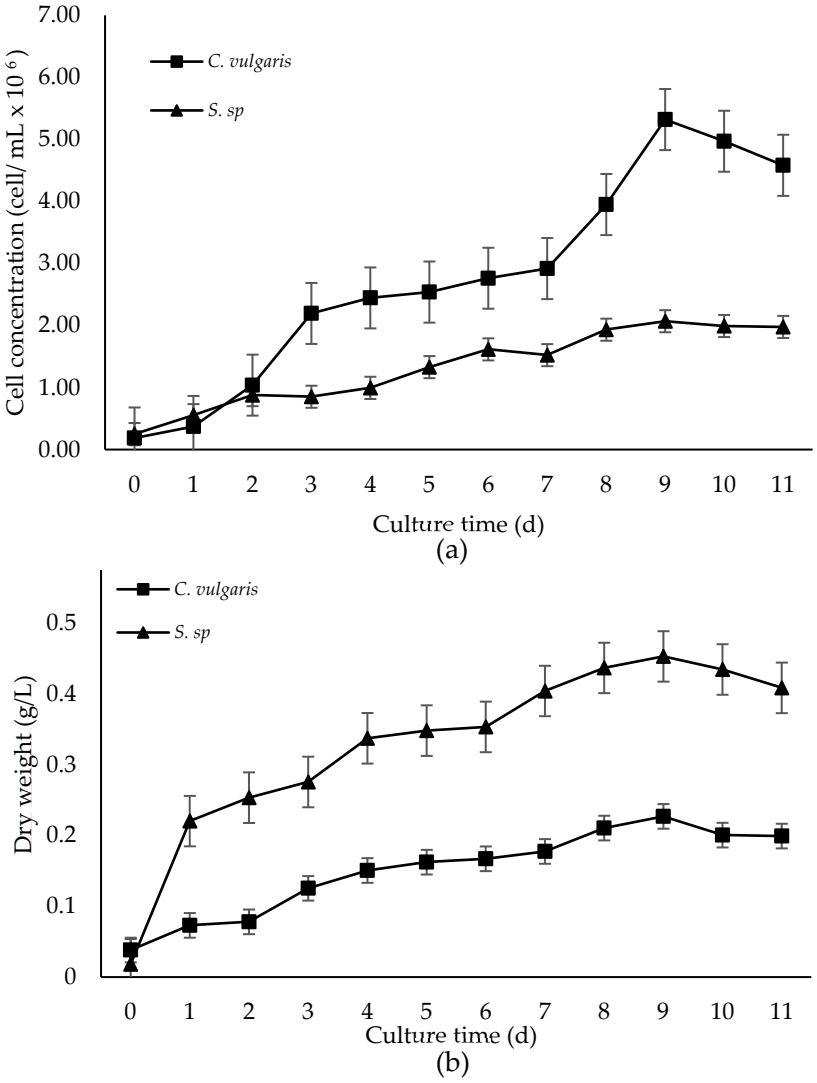


Figure 1. Cell growth of *Scenedesmus* sp. and *Chlorella vulgaris* in pig slaughterhouse wastewater for 11 days: (**a**) Cell concentration (cells/mL) vs. Time and (**b**) Dry weight (g/L) vs. Time. The values are presented as means of triplicate measures with their corresponding standard deviation (±SD).

3.2. Nutrient Removal

Figure 2 shows the behavior of the physicochemical parameters during cultivation time for *Scenedesmus* sp. and *Chlorella vulgaris*. It can be observed that for *Scenedesmus* sp., TN varied from 70.5 ± 7.12 g/L N on day 0 to 22 ± 3.41 g/L N on day 11, which represents 70.67% of TN removal (Figure 2a). TP varied from 98.45 ± 2.35 mg/L PO_4^{3-} to 39.05 ± 3.61 mg/L PO_4^{3-}, which represents 60.91% of TP removal (Figure 2b). In the case of TOC, it varied from 98.45 ± 1.84 mg/L C to 39.05 ± 4.41 mg/L C (Figure 2c), which represents 67.12% of removal. For *Chlorella vulgaris* cultivation, TN varied from 90.5 ± 6.12 g/L N at day 0 to 11.5 ± 3.53 g/L N; this represents 87.89% of TN removal (Figure 2a). For TP, the removal was 61.92% (from 49.55 ± 1.19 mg/L PO_4^{3-} to 22.85 ± 3.35 mg/L PO_4^{3-}) (Figure 2b). TOC varied from 371 ± 11.72 mg/L C at day 0 to 96.5 ± 6.12 mg/L C, which is 77.08% of TOC removal (Figure 2c). These figures show how microalgae are fixing nutrients into their metabolism to grow and maintain themselves during cultivation time.

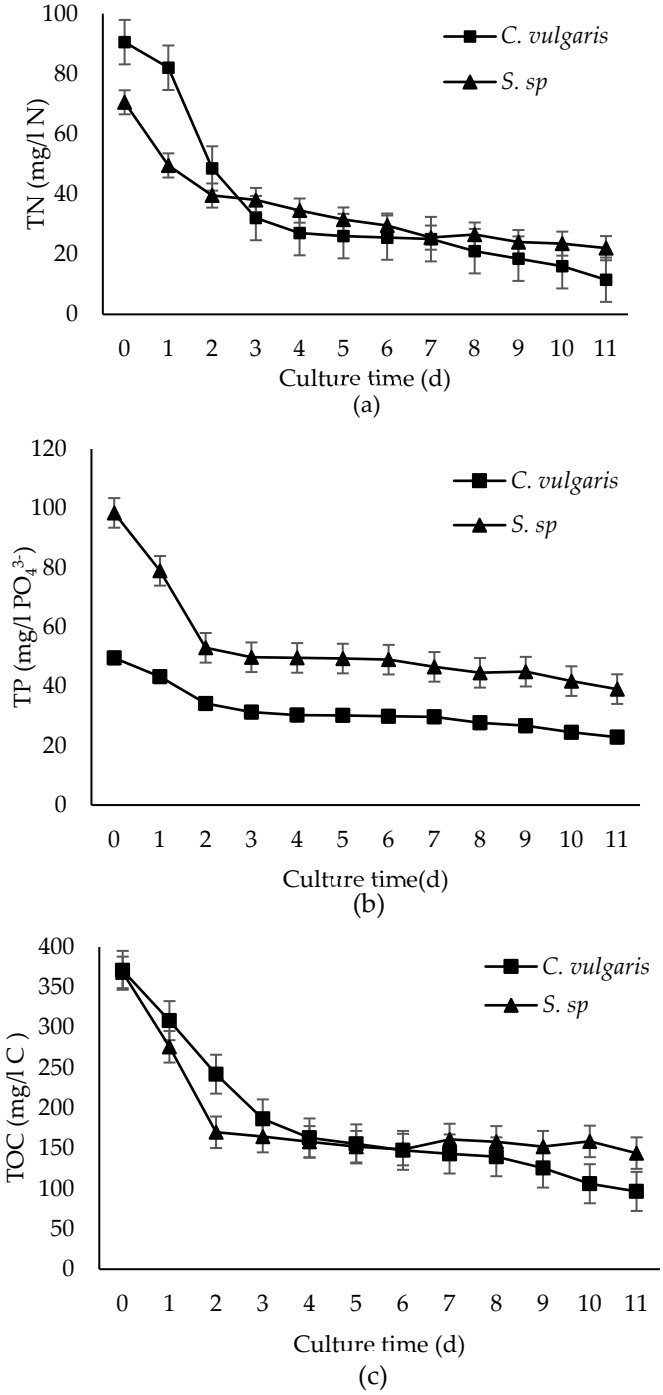


Figure 2. Nutrient consumption of *Scenedesmus* sp. and *Chlorella vulgaris* in pig slaughterhouse wastewater for 11 days (**a**) TN (mg/L N) vs. Time, (**b**) TP (mg/L PO_4^{3-}) vs. Time and (**c**) TOC (mg/L C) vs. Time. The values are presented as means of triplicate measures with their corresponding standard deviation (±SD).

3.3. Lipid Content and Fatty Acid Analysis

Figure 3 shows the lipid content of wet and dry biomass from *Scenedesmus* sp. with six organic solvents: hexane, methanol, ethanol, chloroform: methanol (1:2), ethyl acetate and methylcyclohexane. Among these solvents, methylcyclohexane, the mix of chloroform: methanol (1:2) and ethyl acetate showed the higher lipid extraction. For both wet and dry lipid extraction, methylcyclohexane was the best extracting solvent with 8.51 ± 0.35 %wt of lipids and productivity $2.89 \times 10^{-4} \pm 5.2 \times 10^{-5}$ g/L/d for wet biomass and

18.91 ± 1.15 %wt of lipids and productivity 3.18 × 10^{-4} ± 1.4 × 10^{-5} g/L/d for dry biomass. The second-best extracting solvent was the mix of chloroform: methanol (1:2) with 7.45 ± 0.91 %wt of lipids and productivity 2.55 × 10^{-4} ± 1.2 × 10^{-5} g/L/d for wet biomass and 16.25 ± 1.02 %wt of lipids and productivity 2.74 × 10^{-4} ± 0.94 × 10^{-5} g/L/d for dry biomass. Ethyl acetate was in third place with 6.31 %wt of lipids and productivity 2.18 × 10^{-4} g/L/d in wet biomass and 14.25 %wt of lipids and productivity 2.4 × 10^{-4} ± 0.49 × 10^{-5} g/L/d in dry biomass. The amount of lipids extracted by methanol, ethanol and hexane was lower than 5 %wt, which made them inefficient for lipid extraction.

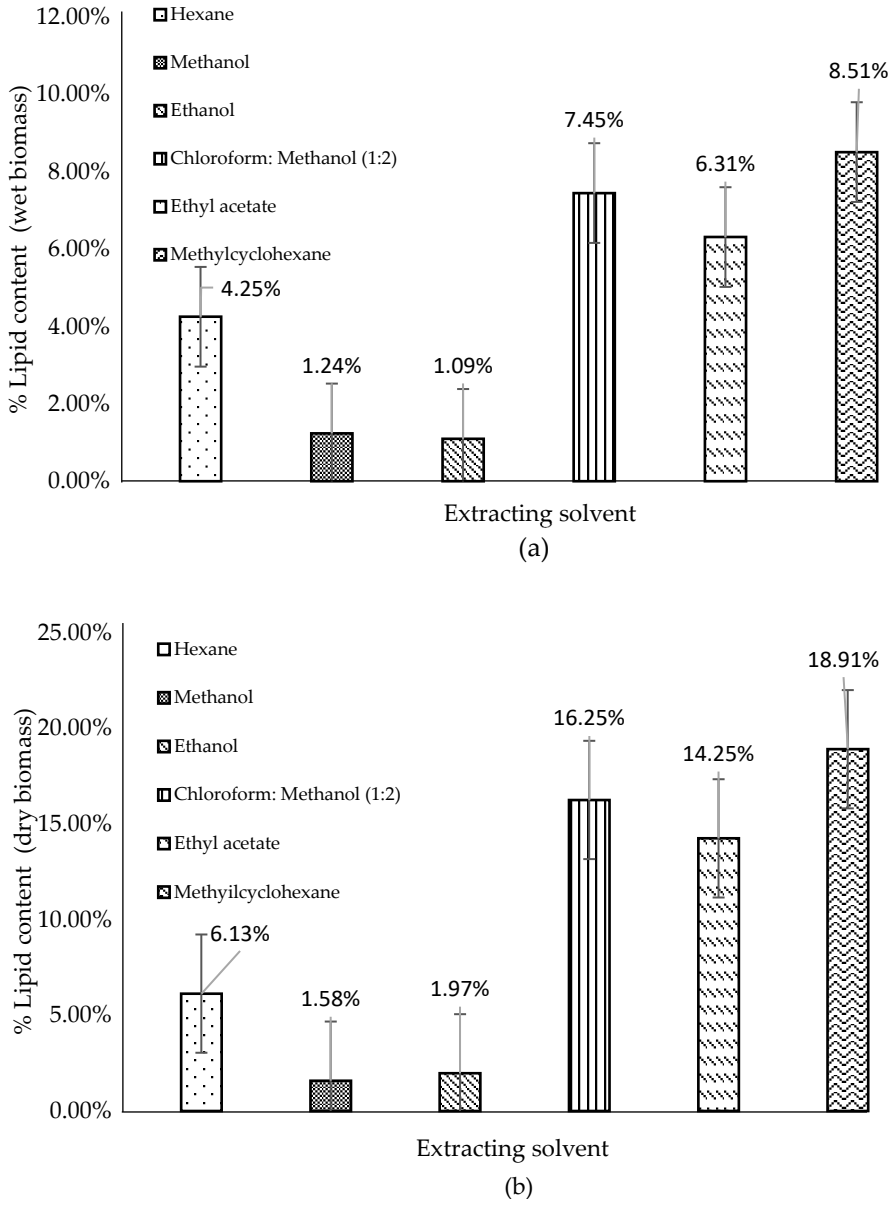


Figure 3. Lipid content of *Scenedesmus* sp. growth in pig slaughterhouse wastewater for 11 days in (**a**) wet biomass and (**b**) dry biomass. The values are presented as means of triplicate measures with their corresponding standard deviation (±SD).

Figure 4 shows lipid content from wet biomass (Figure 4a) and dry biomass (Figure 4b) of *Chlorella vulgaris* using hexane, methanol, ethanol, chloroform: methanol (1:2), ethyl acetate and methylcyclohexane; this last solvent showed the higher lipid extraction in wet basis (13.40 ± 0.15 %wt of lipids and 4.63 × 10^{-4} ± 1.1 × 10^{-5} g/L/d for productivity), while in dry basis the mix of chloroform: methanol (1:2) showed the higher lipid extraction

(21.49 ± 0.52 %wt of lipids and $3.62 \times 10^{-4} \pm 1.94 \times 10^{-5}$ g/L/d for productivity). The rest of the solvents recovered less than 20 %wt of lipids, which made them inefficient for lipid extraction of *Chlorella vulgaris* under culture conditions used in this work. Figure 5 shows the FFA percentages of dry biomass from *Scenedesmus* sp. and *Chlorella vulgaris* with the six different solvents used in this work. For *Scenedesmus* sp., methylcyclohexane, ethyl acetate and the mix of chloroform: methanol (1:2) were the best extracting solvents with 28.02 ± 1.15 %wt, 24.77 ± 1.03 %wt. and 19.40 ± 1.17 %wt, respectively. Figure 5 also shows the FFA weight percentages of dry biomass from *Chlorella vulgaris* with the group of solvents used in this work, being methylcyclohexane, ethyl acetate and the mix of chloroform: methanol (1:2) as the best extracting solvents, achieving 34.92 ± 2.05 %wt, 32.70 ± 1.82 %wt and 23.93 ± 1.33 %wt of FFA, respectively. The rest of the extracting solvents yielded less than 15 %wt of FFA.

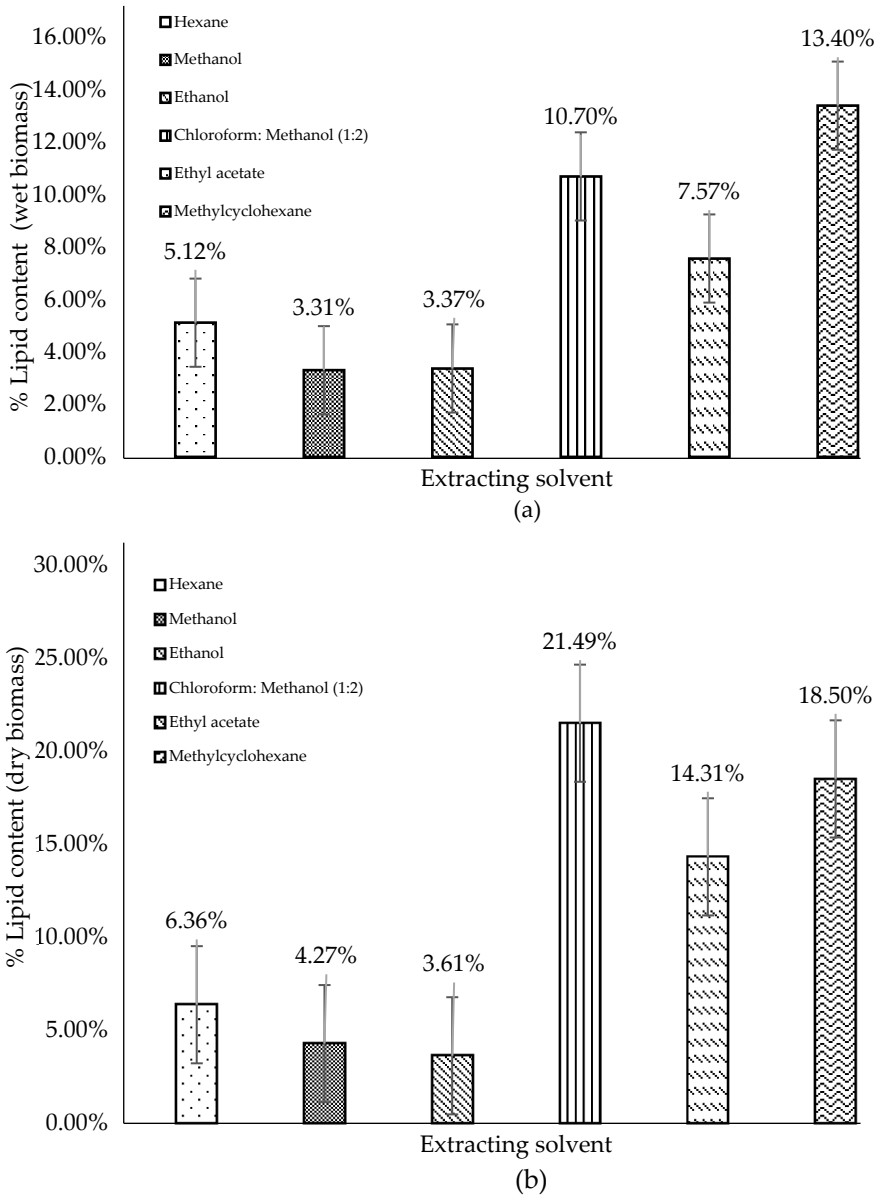


Figure 4. Lipid content of *Chlorella vulgaris* growth in pig slaughterhouse wastewater for 11 days in (**a**) wet biomass and (**b**) dry biomass. The values are presented as means of triplicate measures with their corresponding standard deviation (±SD).

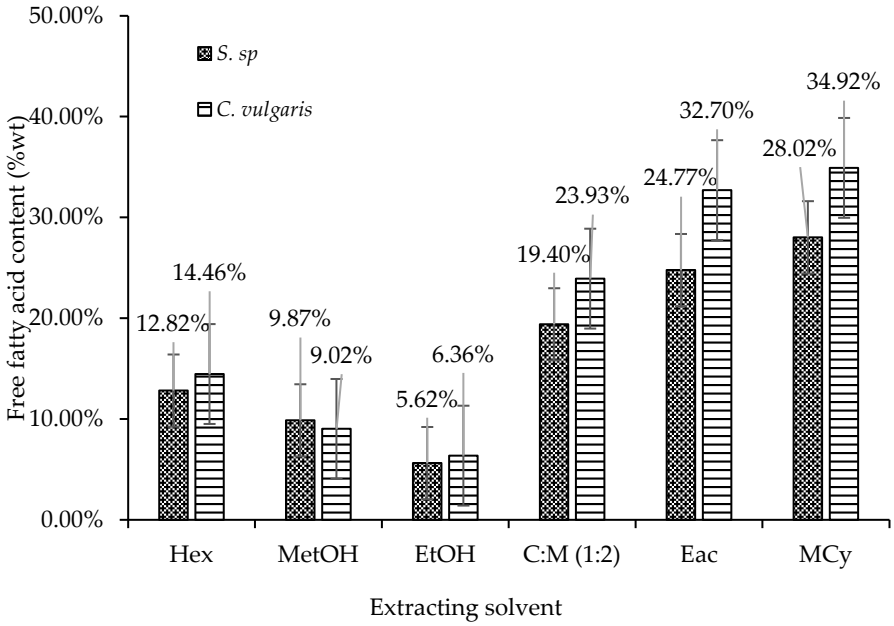
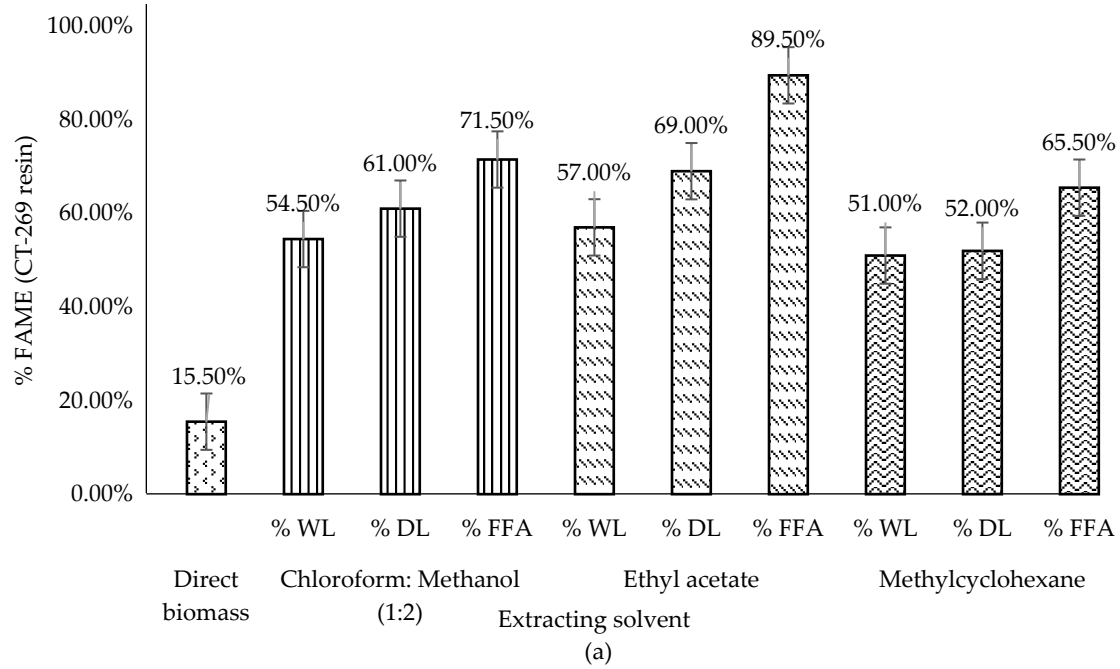


Figure 5. Free fatty acid weight content of *Scenedesmus* sp. and *Chlorella vulgaris* growth in pig slaughterhouse wastewater for 11 days and extracted using Hexane (Hex), Methanol (MeOH), Ethanol (EtOH), Chloroform: methanol (1:2) (C:M (1:2)), Ethyl acetate (EAc) and Methylcyclohexane (MCy). The values are presented as means of triplicate measures with their corresponding standard deviation (±SD).

Figure 6 shows the quantity of FAMEs obtained by the best three extractive solvents from the FFA procedure (methyl acetate, chloroform: methanol (1:2) and methylcyclohexane) for the species *Scenedesmus* sp. using three types of biomass: (i) direct dry biomass of *Scenedesmus* sp., (ii) lipids extracted from wet biomass (WL) and dry biomass (DL) and (iii) lipids from FFA procedure. Figure 6a shows the results of FAMEs using the commercial resin CT-269, which is an acid catalyst, obtaining a range from 65 to 90 %wt when using lipids from FFA procedure, a range from 52 to 69 %wt using lipids extracted from dry biomass and a range from 51 to 57 %wt using lipid from wet biomass. Meanwhile, for dry biomass, the percentage of FAMEs was only 15.50 %wt. Figure 6b shows the results of FAMEs using sulfuric acid as catalyst; the range varied from 52 to 84%wt using the lipids of FFA; from 47 to 59 %wt using lipids of the dry biomass; from 44 to 55 %wt using lipids from wet biomass and only 10.53 %wt of FAMEs using dry biomass directly.



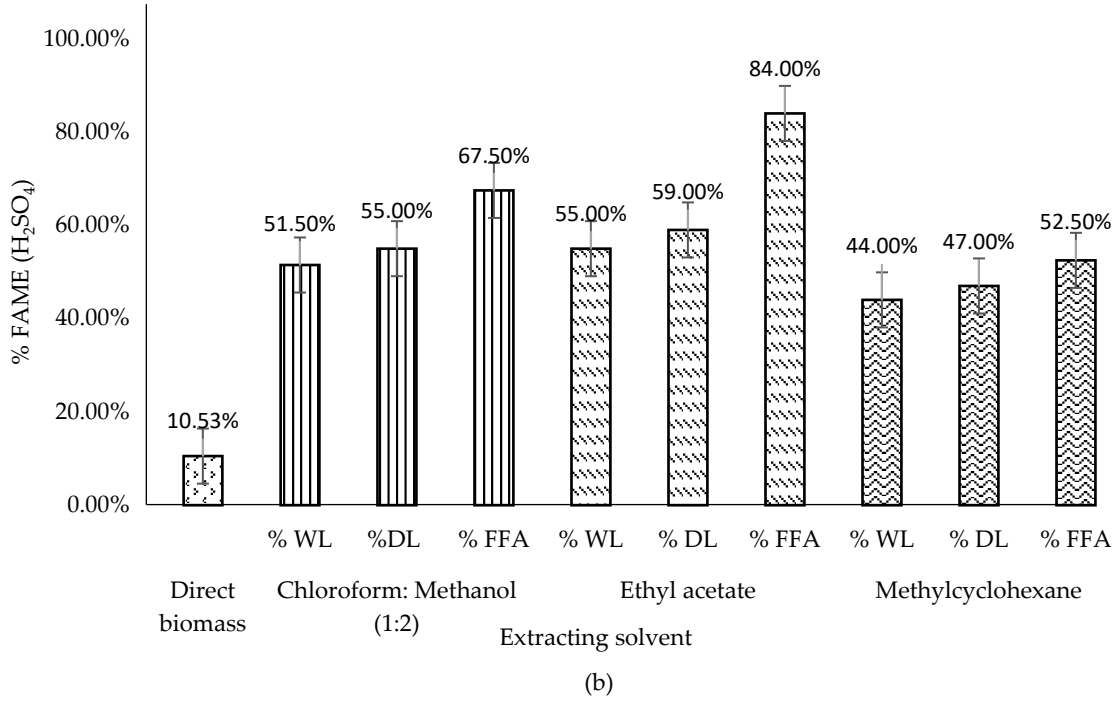
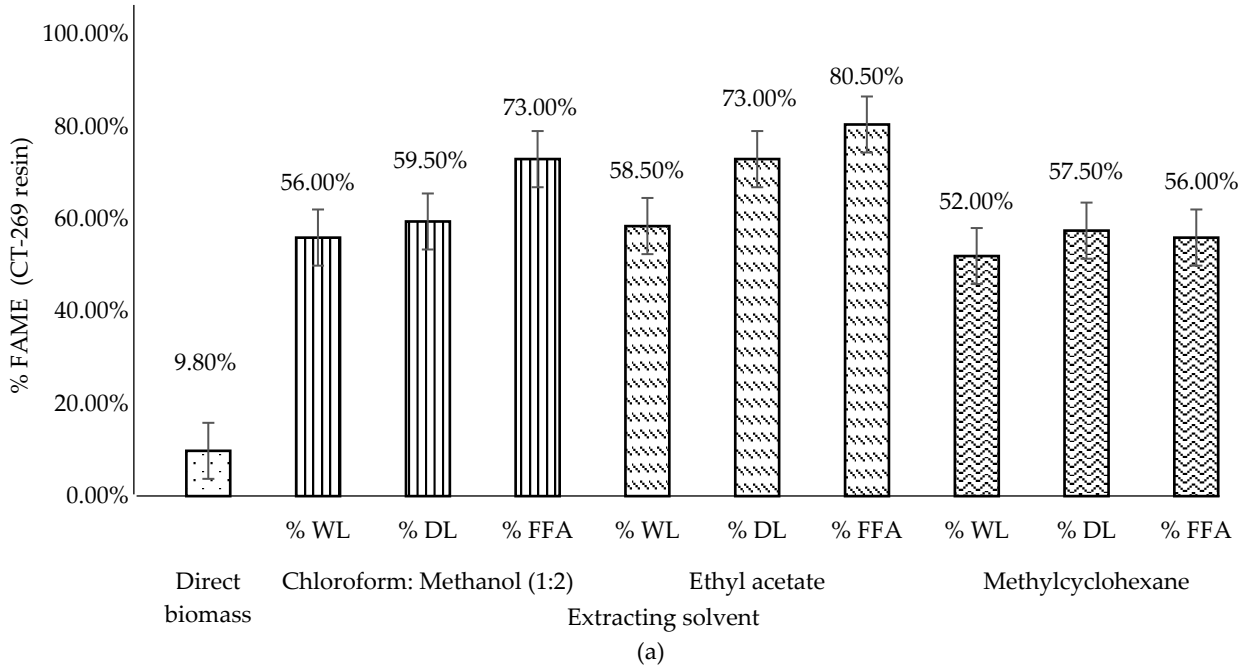


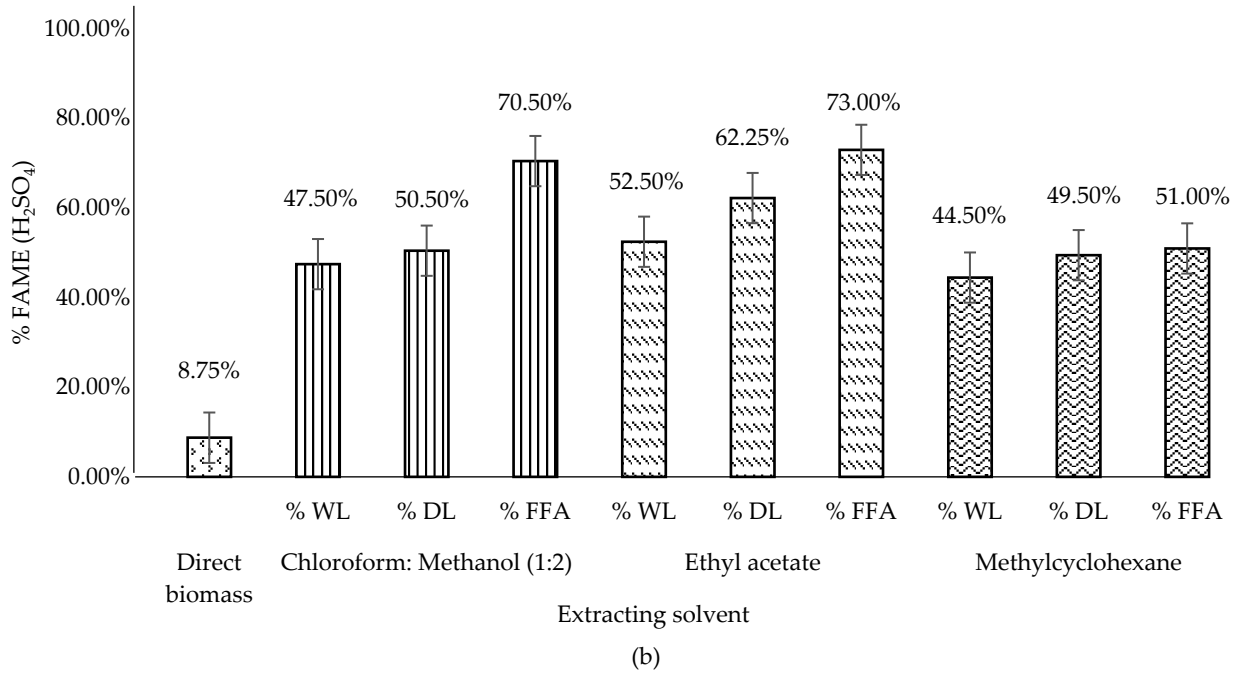
Figure 6. Fatty acid methyl esters weight content of *Scenedesmus* sp. with the three best extracting solvents using different direct biomass, lipids from wet biomass (WL), lipids from dry biomass (DL) and lipids from FFA (FFA) with two catalysts (**a**) CT-269 resin and (**b**) sulfuric acid (H_2SO_4). The values are presented as means of triplicate measures with their corresponding standard deviation (±SD).

Figure 7 shows the percentages of the FAMEs obtained from the three best extracting solvents using dry biomass of *Chlorella vulgaris*, as well as lipids extracted from dry and wet biomass and lipids from the FFA procedure. Figure 7a shows the results of FAME using the commercial resin CT-269, whose values varied from 56 to 80.50 %wt for lipids from the FFA procedure; from 57 to 73 %wt and 52 to 58.50 %wt for lipids from dry and wet biomass, respectively; and 9.80 %wt for direct biomass. On the other hand, Figure 7b shows the results using sulfuric acid as catalyst, with values that varied from 51 to 73 %wt

for lipids from the FFA procedure; from 49 to 62 %wt for lipids from dry biomass; 44 to 52 %wt for lipids from wet biomass and 8.75 %wt for direct biomass.



(a)



(b)

Figure 7. Fatty acid methyl esters weight content of *Chlorella vulgaris* with the three best extracting solvents using different direct biomass, lipids from wet biomass (WL), lipids from dry biomass (DL) and lipids from FFA (FFA) with two catalysts (**a**) CT-269 resin and (**b**) sulfuric acid (H_2SO_4). The values are presented as means of triplicate measures with their corresponding standard deviation (±SD).

4. Discussion

4.1. Cell Growth

Like other microorganisms, microalgae normally present four typical growth phases: lag, exponential, stationary and lysis. As seen in Figure 1a, no lag phase was observed for the studied species, which may indicate a prior adaptation of the microalgae to wastewater [44]. The stationary phase was more notorious for *Scenedesmus* sp., while *Chlorella vulgaris* had a well-marked lysis phase during cultivation time without nutrient reposition. Both species are well known for having high growth rates, high photosynthetic efficiency and adaptability to harsh environmental conditions [45]. Comparing the two species in this study, *Chlorella vulgaris* had higher cell concentration versus *Scenedesmus* sp. (Figure 1a), and the growth rate values obtained in this study confirm the robustness of this species and its adaptability to pig slaughterhouse wastewater. However, *Scenedesmus* sp. had higher biomass production in terms of dry weight (Figure 1b) and, therefore, higher biomass productivity compared to *Chlorella vulgaris.* Differences in biomass productivity are related to culture operation modes, nutrient availability, trace element concentration and organic material that increase cell growth, while inhibitory environmental agents and high pH reduce growth [10,46].

As mentioned before, there is scarce literature about microalgae growth in pig slaughterhouse wastewater for *Scenedesmus* sp. and *Chlorella vulgaris*. Table 2 indicates important parameters for microalgae cell growth, nutrient removal and lipid production with these species grown in swine/piggery wastewater. All these works used different experimental designs, cultivation methods and conditions that directly affect biomass growth. Biomass productivity for *Scenedesmus* sp. in this study was close to values reported for *Scenedesmus obliquus* grown in biological pretreated and filtered piggery wastewater (0.0265 ± 0.046 g/L/d) after 20 days of culture [35] and for *Scenedesmus* sp. grown in filtered swine wastewater for 10 days (0.0415 ±0.001 g/L/d) [27]. For *Chlorella vulgaris*, the achieved biomass productivity was close to the values for the same species grown in biological pretreated and filtered piggery wastewater (0.0245 ± 0.012 g/L/d) [35], but when grown in swine wastewater, biomass productivity reached higher values (0.0395 ± 0.003 g/L/d) [28].

When using swine wastewater, biomass productivity for microalgae can achieve higher values since N and P quantities are greater due to urine and manure in the culture medium, and nutrient availability is correlated to microalgae growth [26]. The final cell concentration of *Scenedesmus* sp. in the present study was lower compared to other research using fermented pig urine wastewater in a 60-day cultivation time where cell growth was ten times higher; this can be attributed to differences in culture conditions and time, also, the addition of nutrients at day 12 could be fundamental to the increase in biomass production [47]. Total biomass productivity for this species was lower than the range of productivity obtained by other researchers where this parameter varied from 0.084 to 0.095 g/L/d using filtered and anaerobically digested piggery wastewater diluted with Mayeux, Sandine and Elliker (MSE) medium at different concentrations after 14 days of cultivation [46]; this is expected considering the dilution of the original water. Cell growth of *Chlorella vulgaris* showed that the maximum growth was achieved after 9 days of cultivation, reaching the stationary phase. This growth cell is similar when using a combination of the corn cooking process and pig industries' wastewater with fresh water in different concentrations as a culture medium for *Arthrospira maxima* and *Chlorella vulgaris*, finding a growth cell of 1.481×10^6 cells/mL using 90% of piggery wastewater [48]. Total biomass productivity of *Chlorella vulgaris* in this work was higher compared with using synthetic sewage for the cultivation of *Chlorella* sp., *Chlorella ellipsoidea, Scenedesmus bijuga* and *Scenedesmus quadricauda* after 12 days of culturing (0.0286 g/L/d), directly related to the composition of the medium [49].

Swine wastewaters are colored and have high turbidity and high levels of ammonia and organic matter that inhibit microalgae growth; therefore, they are commonly diluted

with tap water. This practice is not recommended when applying circular economy principles. Other chemical options combined with adequate unitary operations used in the municipal wastewater treatment field can be applied to wastewater treatment based on microalgae, such as the photo-Fenton method to remove color and turbidity. Photo-Fenton slurry can be used as biofertilizer for animal feed crops and, thus, incorporate these new trends of sustainable biorefinery into microalgae cultures [50].

Table 2. Summary of literature using piggery/swine wastewater for microalgae biomass growth.

Wastewater Type	Wastewater Treatment	Microalgae Specie	Culture Time (d)	Biomass Production (g/L)	Biomass Productivity (g/L/d)	Removed Pollutant	Lipid Content (%)	Lipid Productivity (g/L/d)	FFA (%)	FAME (%)	Reference
Piggery/swine	Biological pretreated and filtered piggery wastewater	*Scenedesmus obliquus* *Chlorella vulgaris*	20	0.53 ± 0.30 0.49 ± 0.26	0.0265 ± 0.046 0.0245 ± 0.012	TIC, N-NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}	31 ± 0.8 29 ± 1.7	0.0124 ± 0.003 0.0105 ± 0.002	-	-	[35]
	Filtered and autoclaved piggery wastewater mixed with freshwater	*Chlorella zofingiensis*	10	2.646 ± 0.046	0.2678 ± 0.0455	COD, TN, TP	33.91	0.09081	-	89.17 Methanol containing 10% DMSO	[36]
	Non-Sterile urban: swine wastewater (1:2)	Microalgal consortium with a predominance of *Scenedesmus* sp.	21	1.1 ± 0.01 g/L	0.0524 ± 0.0215	NH_4^+, COD, NO_3^-, PO_4^{3-}, TOC, IC, BOD_5,	-	-	-	-	[44]
	Filtered swine wastewater	*Scenedesmus* sp.	10	0.4145 ± 0.023	0.0415 ± 0.001	COD, N-NH_4^+, TP	29.29	-	-	-	[27]
	Non-Sterile undiluted swine wastewater	*Chlorella vulgaris*	10	0.3945± 0.01	0.0395 ± 0.003	TN, TP, COD, N-NH_4^+	72.70	0.061 ± 0.0011	-	-	[28]
	Sterile undiluted swine wastewater	*Chlorella vulgaris*	13	0.2730 ± 0.001	0.021 ± 0.003	TN, TP, COD, N-NH_4^+	-	-	-	-	[51]

Abbreviations: Total nitrogen (TN), nitrites (NO_2^-), nitrates (NO_3^-), ammonia (N-NH_4^+), chemical oxygen demand (COD), total inorganic carbon (TIC), total organic carbon (TOC), phosphorus (SP), phosphate (PO_4^{3-}), Methylcyclohexane (MCy), Chloroform: methanol (1:2) (C:M (1:2)), Ethyl acetate (EAc), Dimethyl sulfoxide (DMSO), FFA (free fatty acids).

4.2. Nutrient Removal

Figure 2 shows the consumption of TN, TP, and TOC removal during growth of *Scenedesmus* sp. and *Chlorella vulgaris* for 11 days in separated photobioreactors, without nutrient or medium replenishment during cultivation time. In all cases, nutrient removal was greater than 60%, but *Chlorella vulgaris* removal percentages were higher than *Scenedesmus* sp. These values demonstrate that both species of microalgae are good options for bioremediation of pig slaughterhouse wastewater, especially for nitrogen and carbon removal. Mostly all research studies in microalgae-based processes for biomass

production report nutrient removal, but each author selects their own physicochemical parameters. Nitrogen in the form of total nitrogen (TN), nitrites (NO_2^-) and nitrates (NO_3^-); ammonia ($N\text{-}NH_4^+$); carbon in the form of chemical oxygen demand (COD), total inorganic carbon (TIC) and total organic carbon (TOC); and phosphorus in the form of soluble phosphorus (SP) and phosphate (PO_4^{3-}) are some of the evaluated parameters in available literature (Table 2).

No publications were found for TN, TP and TOC removal in pig slaughterhouse wastewater for the species in this study. However, since wastewater nutrient composition is key for microalgae growth and nutrient removal, and some authors reported that when applying dilution to wastewater, nutrient removal increases, it was observed that TN removal values for both microalgae in this study were slightly lower compared to the literature, where authors obtained 86.2% of ammonium nitrogen removal ($N\text{-}NH_4^+$) for *Scenedesmus* sp. cultivated in non-sterile urban:swine wastewater (1:2) during 20 days of cultivation [44]. TN for *Chlorella vulgaris* in this study was higher compared to TN removal for the same species grown in sterile undiluted swine wastewater for 13 days [51]. TP removal for *Scenedesmus* sp. and *Chlorella vulgaris* in this study are close to the values presented in some works achieving a TP removal range from 74.3 to 92.5% for *Chlorella vulgaris* cultivated in piggery wastewater [13] and a TP removal range from 53 to 88.7% for *Scenedesmus* sp. grown in filtered anaerobically digested piggery wastewater [46]. In the case of TOC, the removal achieved for *Scenedesmus* sp. is lower than the values obtained when using the same microalgae in non-sterile urban: swine wastewater (1:2), finding a removal of 82.42% [52]. These differences could be attributed to the fact that carbon utilization depends on available forms of carbon (organic and inorganic) and its assimilation by microalgae. On the other hand, *Chlorella vulgaris* TOC removal in this study was higher than the results when using piggery wastewater diluted with BG11 medium (58.03% of TOC removal) [15]. As mentioned before, when applying dilution to wastewater (mixing with freshwater or commercial medium), nutrient removal is affected.

In recent years, there has been a switch in wastewater treatment, considering water sanitation, nutrient removal, and recovery. This has included the development of more effective and energy-efficient technologies that considered microalgae as a biological primary treatment and microalgae-bacteria consortium [20]. Microalgae can improve bacterial activity by releasing certain extracellular compounds and bacterial growth can enhance microalgae metabolism by reducing O_2 in the medium and degrading compounds for microalgae [36]. An integrated system can contribute to circular economy applications by using available nutrients from animal manure in wastewater biologically pretreated with microalgae bacteria as feedstock for anaerobic digestion. Then, microalgae biomass can be used for animal feed (only if national legislation allows it) [20].

4.3. Lipid Content and Fatty Acid Analysis

In this study, six extracting solvents selected from the literature were used to check their ability to extract lipids from Scenedesmus sp. and Chlorella vulgaris, grown in undiluted autoclaved piggery slaughterhouse wastewater. Considering that drying processes to remove water content in microalgae biomass represent one of the most expensive stages in microalgal biorefineries, lipid extraction was made for wet and dry biomass (Figures 3 and 4), where it can be seen that *Chlorella vulgaris* had higher lipid content compared to *Scenedesmus* sp. Dry lipid extraction usually has better results compared to the wet basis extraction, but energy consumption related to the thermal removal of water is higher and more expensive [8,14]. Additionally, culture conditions and physicochemical composition of the culture medium affect lipid production. Therefore, some strategies to enhance lipid production must be applied to microalgae cultures.

Higher lipid content has been reported for *Scenedesmus* sp. cultivated in commercial mediums using other solvents, different concentrations of the extracting solvent and

extraction techniques with wet or dry biomass; for example, the mix of chloroform: methanol (2:1) extracted lipids from 12.7 to 28.3%wt from *Scenedesmus acutus* grown on municipal wastewater [10], and the mix of chloroform: methanol (1:2) extracted 29.90 %wt of lipids for *Scenedesmus* sp. cultivated in filtered swine wastewater [27]. These higher results can be related to using solvents with different polarities that enhance lipid extraction; although, lipid content decreases with the increasing dilution of wastewater [27]. Total productivity of lipids extracted from dry and wet biomass with the best solvents (methylcyclohexane, chloroform:methanol (1:2) and ethyl acetate) was similar to the literature, reporting values from 0.0198 to 0.027 g/L/d using the mix methanol-dimethyl sulfoxide, diethyl ether and hexane (1:1:1 v/v/v) [46] and values from 0.0637 to 0.125 g/L/d using the mix of chloroform: methanol (2:1) [37].

A group of researchers obtained 15.2 %wt of lipids from *Chlorella vulgaris* in a dry basis with the mix of chloroform: methanol (1:2) [13], a lower value than the one obtained in this work (21.49 %wt). This range of lipids is acceptable at an industrial scale for biofuel purposes [8]. Other works have used ethyl acetate, hexane and the mix of chloroform: methanol (1:2) with *Isochrysys galbana* cultivated in municipal wastewater, obtaining 17.9 %wt; 38 %wt and 7.5 %wt of lipids, respectively [16].

In terms of productivity, total lipid productivity in dry and wet basis is lower than the range published by Qin et al. [25] (from 0.027 to 0.051 g/L/d) using the mix hexane: ether (1:1), and it is also lower than the value of productivity obtained by Tejeda -Benítez et al. [53] using *Chlorella* sp. cultivated in modified Conwy medium and the mix of chloroform: methanol (1:2) as the extracting solvent (0.0396 g/L/d). Lipid content of microalgae depends on culture medium and external factors (if using outdoor photobioreactors), among other parameters [21]. Phosphorus concentration in the culture medium also affects lipid content, since microalgae convert phosphorus into inorganic orthophosphates such as nucleic acids, phospholipids and ATP, etc. [54].

Extracting techniques of FFA can enhance the content of FFA. The work of Zhang et al. [55] for *Scenedesmus* sp. cultivated in BG11 medium reported the use of commercial enzymes for cellular disruption before FFA extraction with chloroform: methanol (1:1 *v*/*v*), obtaining a range from 60.2 %wt to 68.9 %wt of unsaturated fatty acids. The work of Cho et al. [56] using lyophilized biomass of *Chlorella vulgaris* mixed with water to obtain wet biomass showed 8.76 %wt of FFA with the mix of chloroform: methanol (2:1) as the extracting solvent. This value is lower compared to the percentages of FFA in this work (Figure 5). The different polarities of the solvents used in the present work affected the solubility of compounds, such as lipids, pigments and sugars, and biodiesel production. Non-polar solvents such as methylcyclohexane, hexane and ethyl acetate are more efficient since they can dissolve non-hydro-soluble compounds (i.e., lipids and fatty acids); meanwhile, polar solvents such as methanol and ethanol can dissolve other compounds with no interest in biodiesel production [35]. The mix of chloroform: methanol (1:2), capable of solving polar and non-polar compounds, achieves satisfactory results in oil extraction from microalgae for biodiesel production [46].

Values of FAMEs between the studied species were close to those reported in the literature, which confirms the potential use of these microalgae for biofuel production purposes. Ethyl acetate was the best extracting solvent with the higher values of FAMEs for the treatments applied, (i) direct biomass, (ii) lipids from wet and dry biomass and (iii) lipids from FFA procedure. Differences between extracting solvents must be related to their polarity and types of lipids. In this work, FAMEs from lipids and FFA were higher compared to direct biomass, this could be related to the lack of a non-polar solvent such as ethyl acetate since, in the treatment with direct biomass, the solvent used was methanol. The use of ethyl acetate as an extracting solvent has been extended to large-scale production due to its lower toxicity compared to the traditional mix of chloroform: methanol [35].

Lai et al. [47] obtained a percentage of FAMEs that varied from 54 to 100%wt using *Scenedesmus* sp. in a direct extraction with a polymerized Spurr epoxy resin, isopropanol,

and surfactants (3_DAPS, MTAB, SDS). These values are higher than those obtained in the present work, thus, the surfactants cause ruptures in the cell walls that simplify lipid extraction from the microalgae. Han et al. [37] extracted FAMEs from direct biomass of *Chlorella vulgaris* cultivated in synthetic wastewater pretreated with autoclave sterilization and using sulfuric acid as a catalyst, obtaining 44.86 %wt of FAMEs, a higher value than the one obtained in this work with homogeneous and heterogeneous catalysts (sulfuric acid—9.8 %wt and CT-269 resin—8.75 %wt, respectively).

In the literature, there is no information available about FAME content from lipids or FFA extracted with methylcyclohexane for the species studied in this work. However, similar solvents and catalysts have been used; Sánchez- Bayo et al. [16] extracted FAMEs from lipids of *Isochrysis galbana* grown in municipal wastewater, obtaining values in the range from 52 to 53.3 %wt for the mix of chloroform: methanol (1:2) and from 74.1 to 75.9 %wt for ethyl acetate. These results are close to the results obtained in the present work, and the variations could be associated with characteristics of the species, culture conditions and nutrient availability.

FAME extraction requires several energy-demanding steps for lysis (vortex agitation, sonication, ultrasound, etc.) and to avoid these steps some researchers suggest the use of wet biomass directly since there is no need for the previous drying and, even though transesterification time is lower, recovery of the catalyst is not possible.

In general, if lipid extraction from microalgae is the main goal, cultures must be manipulated to improve lipid yield according to the final bioproduct (lipids for food, nutraceuticals, or biofuels). Some authors use environmental stress, manipulation of cultivation conditions, hybrid lipid extraction processes and metabolic or molecular approaches to enhance lipid production [9,26,45]. The results of the actual work confirm the application of pig slaughterhouse wastewater for microalgae growth and lipid extraction for biodiesel generation, although, including some of the above-mentioned strategies, these values could meet higher lipid content. A normal residue from microalgae experiments is residual biomass after lipid extraction, and recent literature focused on microalgae-based biorefineries pointed out that this type of residue can be used as source of valuable macromolecules for low-value products such as biofertilizers, biostimulants, or bio-oil or biogas production; all of this is possible by applying the principle of an integrated biorefinery, as well as other uses considering the wide range of valuable products that can be extracted in an algae biorefinery [16,19,57].

5. Conclusions

The use of pig slaughterhouse wastewater represents an efficient culture medium for growing biomass of *Scenedesmus sp*. and *Chlorella vulgaris* in outdoor photobioreactors. Biomass productivity was 0.02–0.037 g/L/d and 0.004–0.018 g/L/d for *Scenedesmus* sp. and *Chlorella vulgaris*, respectively. After 11 days of a batch culture without nutrient reposition, nutrient removal in terms of TN, TP and TOC was higher than 60 %wt, like the values reported by other authors for the two species, suggesting their use for bioremediation purposes. Lipid extraction from dry and wet biomass and the FFA procedure showed a high efficiency with methylcyclohexane, the mix of chloroform: methanol (1:2) and ethyl acetate, and FAME contents were higher using the lipids from the FFA procedure compared to the values when using direct biomass and lipids from dry and wet biomass. The results of this study represent a contribution to literature in terms of pig slaughterhouse wastewater treatment for microalgae biomass and lipid production, with a positive environmental impact. Authors recommend the inclusion of some adjustments to culture conditions such as feed batch operation mode in photobioreactors and nitrogen depletion to improve biomass and lipid yields; additionally, residual biomass could be used for biogas generation, thus, cost-effectively valuing all the biomass.

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