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# *Porphyridium cruentum* Grown in Ultra-Filtered Swine Wastewater and Its Effects on Microalgae Growth Productivity and Fatty Acid Composition

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**Abstract:** Microalgae have been extensively tested for their ability to create bio-based fuels. Microalgae have also been explored as an alternative wastewater treatment solution due to their significant uptake of nitrogen and phosphorus, as well as their ability to grow in different water types. Recently, there has been significant interest in combining these two characteristics to create economic and environmentally friendly biofuel using wastewater. This study examined the growth and lipid production of the microalgae *Porphyridium (P.) cruentum* grown in swine wastewater (ultra-filtered and raw) as compared with control media ($L^{-1}$, modified f/2) at two different salt concentrations (seawater and saltwater). The cultivation of *P. cruentum* in the treated swine wastewater media (seawater = 5.18 ± 2.3 $mgL^{-1}day^{-1}$, saltwater = 3.32 ± 1.93 $mgL^{-1}day^{-1}$) resulted in a statistically similar biomass productivity compared to the control medium (seawater = 2.61 ± 2.47 $mgL^{-1}day^{-1}$, saltwater = 6.53 ± 0.81 $mgL^{-1}day^{-1}$) at the corresponding salt concentration. Furthermore, no major differences between the fatty acid compositions of microalgae in the treated swine wastewater medium and the control medium were observed. For all conditions, saturated acids were present in the highest amounts (≥67%), followed by polyunsaturated (≤22%) and finally monounsaturated (≤12%). This is the first study to find that *P. cruentum* could be used to remediate wastewater and then be turned into fuel by using swine wastewater with a similar productivity to the microalgae grown in control media.

**Keywords:** microalgae; *Porphyridium cruentum*; wastewater treatment; ultrafiltration

## 1. Introduction

Microalgae have a significant potential to be used for the development of alternative bio-based fuels [1]. Under optimized conditions, microalgae have been reported to have a high productivity of lipids (for biodiesel) or carbohydrates (for bioethanol or biobutanol), depending on the type of microalgae and growth conditions. Additional advantages of microalgae include high lipid yield per unit area [2], short cultivation periods, better resistance to diverse environments like seawater or eutrophic waters [3], and the production of valuable co-products, such as proteins and residual biomass [4]. In addition, microalgae strains can thrive in saltwater, seawater, and wastewater [5–7]. Work by Solovchenko et al. (2015) showed that animal manure provides a very rich source of phosphorous required for microalgae growth [8]. Thus, the purpose of this paper is to show that swine

wastewater can be used to cultivate microalgae with little change, as compared with other nitrogen and phosphorous sources.

For growth and productivity, microalgae require significant amounts of nitrogen and phosphorous. One economical and environmentally friendly source for these nutrients is wastewater. Although nitrogen levels are often high in wastewater, phosphorus is often at lower levels than the desired ratio for algal growth. Animal (swine and dairy) wastewaters have been reported to contain a higher ratio of phosphorus to nitrogen than primary and secondary wastewaters (primary wastewater is a result of the capture of suspended solids and organics through sedimentation. The secondary wastewater is a removal of organic matter using microorganism) [9]. As nitrogen and phosphorous are expensive, and often come from a petroleum-derived source, microalgae growth from wastewater sources is attractive. Oswald et al. (1957) first proposed the use of microalgae to clean up wastewater [10]. There have been many reported cases of using microalgae to clean up wastewater, including *Chlorella vulgaris* for the clean-up of wastewater from ethanol and citric acid production [11], *Chlorella vulgaris* for the biodegradation of hydrocarbons [12], *Nannochloris* sp. for the treatment of trimethoprim, sulfamethoxazole, and triclosan [13]. While there have been numerous studies on the nitrogen and phosphorous removal abilities of microalgae on wastewater, and numerous studies on the growth of algal biomass on wastewater, there have been limited studies evaluating the fatty acid composition of the microalgae grown in swine wastewater. Since there are many different types of wastewater, here the focus will be on swine wastewater and the products produced, as compared with conventional growth media.

Several previous studies have explored the growth rates of different microalgae species on swine wastewater, such as *Scenedesmus intermedius* (0.014 mg chlorophyll $h^{-1}$), *Nannochloris* sp. (0.011 mg chlorophyll $h^{-1}$) [14], and *Chlorella vulgaris* (40 $mgL^{-1}day^{-1}$) [15]. Both of these studies found that swine wastewater was suitable for microalgae growth but did not look at the product breakdown of the fatty acids. However, a few studies have also evaluated the fatty acid content of microalgae grown in swine waste. Hu et al. (2012) compared the growth of *Chlorella* sp. in fresh and anaerobically digested swine wastewater [16]. They found a growth of 75.7 $mgL^{-1}day^{-1}$ in raw diluted swine wastewater and a growth of 164.3–224.7 $mgL^{-1}day^{-1}$ in diluted swine wastewater supplemented with volatile fatty acids (acetic, propionic, and butyric). Depending on the amount of volatile fatty acids added, the fatty acid composition was ~43–57% saturated, ~8–10% monosaturated, and 35–48% polyunsaturated fatty acids. However, the fatty acid composition was not determined for microalgae grown in the raw diluted swine wastewater, which was the medium for the study below. In a study by Mulbry et al. (2008), the microalgae were grown in raw swine wastewater at different effluent loadings and had a growth of 6.8–10.7 $gmL^{-2}$ $day^{-1}$ [17]. They found that the dominant algal species was *Rhizoclonium* sp. with a fatty acid composition of ~53–58% saturated, 16–20% monosaturated, and 22–26% polyunsaturated fatty acids, depending on the concentration of the swine waste. In this study, the polyunsaturated fatty acids were lower, but the microalgae culture was mixed. Another study by Wu et al. looked at the growth of *Nannochloropsis oculata* in anaerobically and aerobically treated diluted swine wastewater [18]. The growth rate was 0.59 $gL^{-1}day^{-1}$ (50% diluted) and 0.42 $gL^{-1}day^{-1}$ (25% diluted). The fatty acid composition was determined to be ~39% (25% diluted) and ~38% (50% diluted) saturated, ~19% (25% diluted) and ~17% (50% diluted) monosaturated, ~31% (25% diluted) and ~32% (50% diluted) polyunsaturated, and ~11% (25% diluted) and ~13% (50% diluted) undetermined fatty acids. Most of the studies above used digestion as a way of preparing the nutrients for microalgae growth, but none of these studies looked at ultrafiltration for swine wastewater purification. Further, *Porphyridium cruentum* was not used in any of these swine wastewater studies, and it is felt that this alga is important to characterize because of its ability to make pharmaceuticals, food products, and fuels.

The red, unicellular microalgae *Porphyridium (P.) cruentum*, also called *P. purpureum*, has often been studied for its ability to produce high-value products, including phycobiliproteins and omega fatty acids [19]. While there have been limited reports, it has also been explored for its biofuel potential [20,21].

*P. purpureum* had a lipid productivity and carbohydrate production similar to or higher than the green microalgae *Chlamydomonas reinhardtii* [20]. Sandefur et al. (2016) studied ultrafiltration for treating swine wastewater in the contaminant-free production of lipids using *Porphyridium cruentum* [22], and Kim et al. (2017) studied the same organism for use in bioethanol production [23].

To date, there have been no studies looking at *P. cruentum* grown in swine wastewater media for biofuel production and comparing this with culture media. It is desirable to establish that a biofuel-producing organism can be grown in swine wastewater with parity compared to culture media. The purpose of this study was to determine if the biofuel potential of *P. cruentum* would have significantly altered the growth or lipid composition when grown in swine wastewater. To our knowledge, this is the first study to report the fatty acid composition of *P. cruentum* grown in swine wastewater compared with standard culture media.

## 2. Materials and Methods

### 2.1. Strain and Culture Medium

The marine microalgae *P. cruentum* (CCMP1328) were obtained from the Provasoli-Guillard National Center for Marine microalgae and Microbiota (NCMA, East Boothbay, ME, USA). *P. cruentum* cells are red, spherical and 5–8 µm in length. In the experiments, six different media were used. Each of them differed by the type of water used, the addition of nutrients, the medium type, and the presence of raw swine wastewater. The medium type was either a control medium (CM) or a swine wastewater medium (SWM). L1-medium, which is a modified f/2 medium, was chosen as the control medium and for culture maintenance. It contained $NaNO_3$, $NaH_2PO_4{\cdot}H_2O$, $Na_2EDTA{\cdot}2H_2O$, $FeCl_3{\cdot}6H_2O$, $MnCl_2{\cdot}4H_2O$, $ZnSO_4{\cdot}7H_2O$, $CoCl_2{\cdot}6H_2O$, $CuSO_4{\cdot}5H_2O$, $Na_2MoO_4{\cdot}2H_2O$, $H_2SeO_3$, $NiSo_4{\cdot}6H_2O$, $Na_3VO_4$, $K_2CrO_4$, Thiamine HCl (Vitamin B1), cyanocobalamin (Vitamin B12), and seawater or saltwater. Filtered seawater was obtained from the National Center for Marine Algae and Microbiota (NCMA), W Eel Pond, Woods Hole, Massachusetts, USA. Sodium chloride and distillated water were used to make a stock solution of 2.5% NaCl in deionized water for the saltwater solutions. *P. cruentum* was pre-cultured at 22 °C with natural illumination in a 500 mL glass bottle containing 250 mL of the sterilized medium (autoclaved at 127 °C for 30 min).

### 2.2. Swine Wastewater Preparation and Ultrafiltration

Swine wastewater samples were obtained from a manure holding lagoon located at a grow-finish swine farm in Savoy, AR, USA, as previously described in Sandefur et al. [22]. An ultrafiltration system was used to remove biological contaminants and inorganic solids. Ultrafiltration (UF) has previously been used for wastewater treatment to achieve regulatory levels of total suspended solids, chemical oxygen demand, and coliform levels [24,25]. The UF system included 1-inch hollow fiber membrane cartridges (50,000 MWCO; Koch Romicon PM50, Wilmington, MA, USA) and was operated at a transmembrane pressure of 17.5 psi. The permeate samples were taken after two hours of ultrafiltration operation in the recycle mode. After processing, the permeate samples were cultured using the IDEXX Colilert method (IDEXX Laboratories, Westbrook, ME, USA) [26] to check for the presence of *E. coli* and coliforms. Additionally, the permeate samples were analyzed for total phosphorus (TP), total nitrogen (TN), total organic carbon (TOC), and ammonia-N using APHA (American Public Health Association) methods [26]. After ultrafiltration, the complete rejection of *E. coli* and coliforms was observed for the swine wastewater samples (<1.0 CFU $mL^{-1}$). The concentrations of TP, TN, ammonia-N, and TOC in the permeate samples were 69.1, 695.6, 422.8 and 598.0 $mgL^{-1}$, respectively. Additional characterization information for the ultra-filtered swine wastewater is available in Sandefur et al. [22].

### 2.3. Microalgae Growth Experiments

Algal cultivation was performed in 150 mL corning sterile bottles from VWR International, a global laboratory supplier (Radnor, PA, USA). The inoculum volume for each sample was 5 mL containing

5000 cells $mL^{-1}$, which was obtained from the pre-cultured microalgae in the early exponential growth phase. There were six different media used to investigate the effects of swine wastewater on microalgae growth rate. The total volume of the medium added to each microalgae sample was 95 mL. The control media (prepared as described in the culture medium section) were solutions based on either seawater or saltwater. The UF-treated swine wastewater media consisted of 65 mL of swine wastewater and 30 mL of appropriate control medium. The details of the microalgae growth media are given in Table 1.

**Table 1.** Microalgae growth media compositions.

| Sample Name | Medium | Water Type | Contents |
|---|---|---|---|
| **C-SEA** | Control | Seawater | 95 mL seawater medium, 5 mL algae |
| **SW-UF-SEA** | Swine Waste | Seawater | 30 mL seawater, 65 mL swine waste, 5 mL algae |
| **C-SALT** | Control | Salt Water | 95 mL saltwater medium, 5 mL algae |
| **SW-UF-SALT** | Swine Waste | Salt Water | 30 mL deionized water, 65 mL swine waste, 1.6 g NaCl, 5 mL algae |

During the experiment, the containers were maintained at ambient laboratory temperature (18–22 °C) and illuminated using four fluorescent lamps under a light-dark cycle of 13:11 h, respectively. The average light intensity was 140 (130–150) μE $m^{-2}s^{-1}$. This condition was selected according to previous studies on the optimum growth condition of *P. cruentum* [27]. The biomass was harvested after 24 days in the stationary phase, using centrifugation at 2800 rcf for 15 min in 50 mL falcon tubes. Microalgae was harvested in the stationary phase because, when making polyunsaturated fats, it is often required to do nutrient starvation to force the desired product breakdown. The harvested biomass pellets were washed with deionized water to remove mineral salt precipitates, and then were lyophilized for direct transesterification. The biomass productivity was calculated using the Equation below:

$$\text{Biomass productivity}\left(\text{mgL}^{-1}\text{day}^{-1}\right) = \frac{\text{dried microalgae biomass (mg)}}{\text{working volume (l)} \times \text{cultivation day}} \quad (1)$$

### 2.4. Direct Transesterification

Direct transesterification was used because it can be used on smaller sample sizes and has been shown to have consistent results with traditional transesterification. For direct transesterification, the samples were weighed and moved to 10 mL flasks [28]. Then, a solution of $H_2SO_4$/methanol with a final volume ratio of 5:100, respectively, was added into the flasks. The flasks were stirred at 70 °C for one hour since transesterification and extraction were being performed in the same step. After one hour, the flasks were cooled down to room temperature by running tap water over the outside of the flask. Next, 2 mL of hexane and 0.75 mL of distilled water were added to the flasks, and all of the flasks were vortexed for 30 s. After vortexing, the mixture had two phases: the upper hexane layer containing the fatty acid methyl esters (FAMEs) and the lower aqueous layer containing the residues. In the last step of direct transesterification, the upper hexane layer was transferred to gas chromatography (GC) vials.

Although lipids are traditionally extracted from microalgae before transesterification, in situ transesterification or direct transesterification can be performed by contacting biomass directly with the alcohol and catalyst required. This process reduces the number of unit operations to produce FAMEs from biomass [29]. This process was used to convert the biomass of *P. cruentum* into FAMEs. The major fatty acid composition of the tested microalgae was determined by using GC analysis. Mass fractions were normalized according to the total fatty acids found from the GC analysis.

### 2.5. Gas Chromatography Analysis

In order to analyze FAMEs, the gas chromatograph, GC-2014 (Shimadzu, Columbia, MD, USA) equipped with a flame ionization detector (FID) and an auto sampler was used. The GC column used to separate the FAMEs was a Zebron™ZB-FFAP polar capillary column (30 m × 0.32 mm × 0.25 μm)

film thickness; Phenomenex, Torrance, CA, USA). Helium was used as a carrier gas with a linear velocity of 35 cm/s. The column temperature was programmed from 150 (held for 3 min) to 240 °C at 1.5 °C/min. Sample volumes of 2 µL were injected with a split ratio of 10:1. The detector temperature was set at 250 °C. The peaks obtained from the GC were compared with Marine Oil Test Mix. and Fame #13 Mix (Restek Corp., Bellefonte, PA, USA) FAME standards.

### *2.6. Statistical Analysis*

Statistical differences in the data were determined using GraphPad (GraphPad Software Inc., San Diego, CA, USA). GraphPad QuickCalcs was used for the unpaired *t*-test and GraphPad Prism (version 8.3.1) was used for one-way, nonparametric ANOVA and Tukey analysis. While the *t*-test and Tukey compared the average of individual values to each other to determine statistical significance, ANOVA was used to compare multiple values to each other. Values were considered to have a statistically significant difference if the *p* value was less than 0.05.

## 3. Results and Discussion

### *3.1. Growth and Productivity*

The growth and fatty acid productivity of *P. cruentum* was evaluated in control and diluted ultra-filtered swine wastewater. Figure 1 shows the biomass productivity of each culture. For the samples grown in seawater, those containing treated swine wastewater (SW-UF-SEA) had almost double the average biomass productivity (5.18 $mgL^{-1}day^{-1}$) than those grown in the control medium (C-SEA, 2.61 $mgL^{-1}day^{-1}$). Alternatively, for the samples grown in saltwater, those containing swine wastewater (SW-UF-SALT) had about half the average biomass productivity (3.31 $mgL^{-1}day^{-1}$) of those grown in the control medium (C-SALT, 6.52 $mgL^{-1}day^{-1}$). However, it is important to note that there was wide variation among the samples and, therefore, the average biomass was statistically the same. A previous study by Lee and Bazin (1991) determined that the optimum growth for *P. cruentum* occurred at a similar concentration of NaCl to our saltwater samples (0.42 M ~ 24.5 ppt), and that the next highest growth occurred at a concentration of NaCl similar to our seawater concentration (0.59 M ~ 34.5 ppt) [30]. Overall, the average biomass productivity was statistically the same between the microalgae grown in the control media and the microalgae grown in the treated swine wastewater media, as indicated by $p \geq$, as shown in Table 2, for the different analysis methods. This indicates that ultra-filtered swine wastewater can be used to grow microalgae (specifically *P. cruentum*) without any significant loss to the biomass productivity.

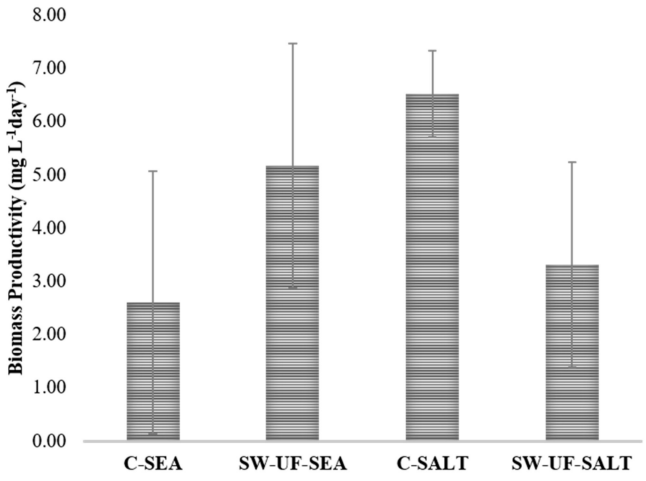


**Figure 1.** Total biomass productivity of each culture in $mgL^{-1}$ $day^{-1}$ repeat. C = control media, SW = swine wastewater media, UF = purified by ultrafiltration, RW = raw swine wastewater added after ultrafiltration, SEA = seawater, SALT = saltwater, $N = 3$.

**Table 2.** Statistical analysis of biomass productivity for different growth conditions.

| Growth Conditions | *t* test | ANOVA + Tukey | ANOVA |
|---|---|---|---|
| **C-SEA/SW-UF-SEA** | 0.26 | 0.32 | 0.14 |
| **C-SALT/SW-UF-SALT** | 0.06 | 0.08 | 0.08 |
| **C-SEA/C-SALT** | 0.06 | 0.13 | |
| **SW-UF-SEA/SW-UF-SALT** | 0.05 | 0.56 | |

Note: Values are considered statistically significant if $p < 0.05$. For the ANOVA from top to bottom the values compare all the seawater, all the saltwater, and all values to each other.

### 3.2. FAME Composition

There were interesting differences between the FAME compositions in all of the growth methods (Figure 2). *P. cruentum* observed for all of the growth conditions were C16:0 (palmitic acid; 42–51%), C18:0 (stearic acid; 19–30%), C20:5 (EPA; 6–10%), and C24:0 (lignoceric acid; 4–7%). Uncommon fatty acids (C14:0, C14:1, C22:0, C22:1, and C24:1) were either not observed or observed at very low values (<3%). These values agree with previous studies [31–33]. Several other fatty acids (C16:1, C18:2, C18:3, C20:0, C20:1, C20:3, and C20:4) were also either not observed or observed at low concentrations (≤5%). Although there was some variation in the exact composition of the fatty acids among the samples as shown in Figure 2, statistically they were the same. The values were statistically the same when comparing saltwater with seawater, as well as when comparing treated swine wastewater with control media. The similarity of the fatty acid compositions between the samples again indicates that swine wastewater media compares favorably with control media.

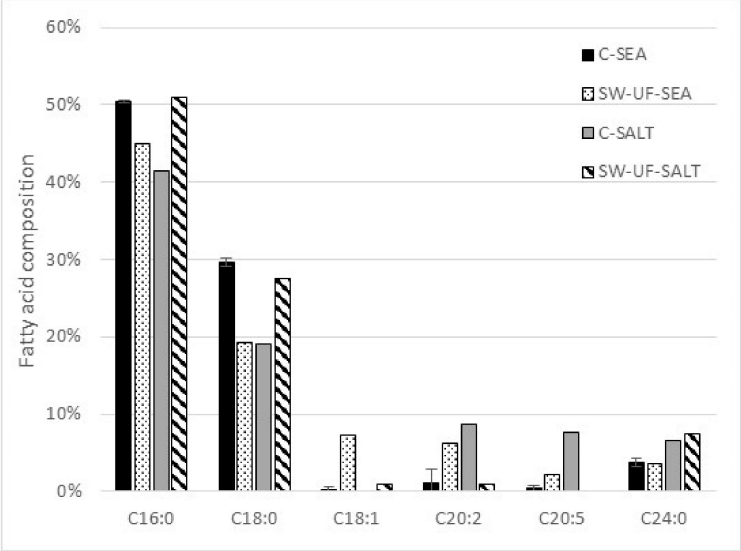


**Figure 2.** Fatty acid methyl esters (FAME) composition of *P. cruentum* grown in control and swine wastewater media. $N = 2$ for C-SEA and SW-UF-SEA, $N = 1$ for C-SALT and SW-UF-SALT.

As shown in Figure 3, *P. cruentum* has more saturated FAMEs (C-SEA: 91.3%, SW-UF-SEA: 74.09%, C-SALT: 77.40%, SW-UF-SALT: 93.47%) than the unsaturated (monounsaturated and polyunsaturated) FAMEs (C-SEA: 8.97%, SW-UF-SEA: 25.91%, C-SALT: 22.60%, SW-UF-SALT: 6.53%). The composition of saturated or unsaturated fatty acids affects the quality of the biofuel produced from the microalgae. High levels of saturated fatty acids provided better combustion but lead to high kinematic viscosity [34]. Biodiesel with high levels of unsaturated fatty acids has optimum chemical properties, but higher NOx emissions and a lower cetane number that lead to longer ignition delays [35]. The ideal biodiesel, as an alternative to fossil fuels, should contain both saturated and unsaturated fatty acids with a higher portion of saturated fatty acids for the efficiency of fuel [34].

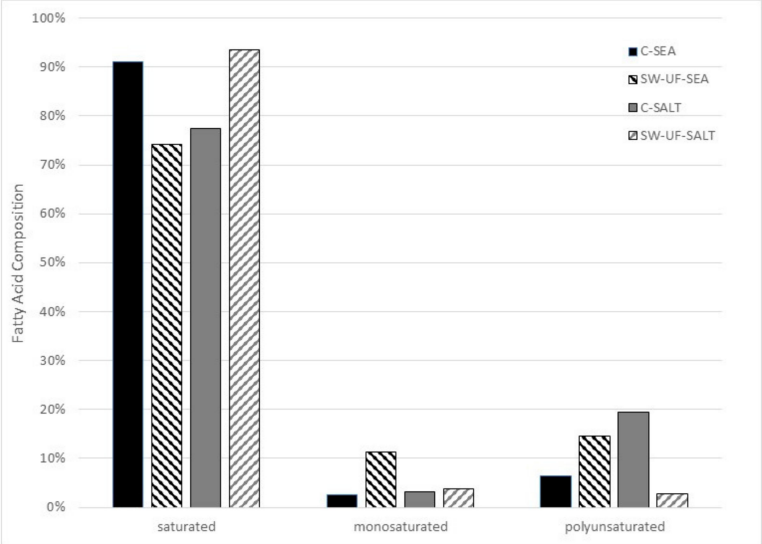


**Figure 3.** Saturated and unsaturated fatty acid compositions of *P. cruentum* grown in control and treated swine wastewater media. $N = 2$ for C-SEA and SW-UF-SEA, $N = 1$ for C-SALT and SW-UF-SALT.

## 4. Conclusions and Future Perspectives

In this study, the growth and productivity of *Porphyridium cruentum* were examined in swine wastewater versus control media with different salinities. While the biomass productivity of *P. cruentum* varied in the different media, there was no statistical difference between the swine wastewater and the control media. FAME analysis of *P. cruentum* grown in the control and swine wastewater media also showed no significant differences in composition. *P. cruentum* yielded a higher percentage of saturated fatty acids compared with unsaturated fatty acids, indicating that it has the potential to be used as a biofuel. Therefore, UF-treated swine wastewater has the potential to be used as an alternative growth medium for microalgae in biofuel production, which in turn will help with global issues of eutrophication.

The development of microalgae cultivation in swine wastewater has plenty of environmental benefits, due to the high growth rate of microalgae and environmental pollution control. However, these noteworthy results, achieved in swine wastewater-grown *P. cruentum*, promote the further investigation of this environmentally friendly method of microalgae cultivation, with the objective of improving their harvesting on a large scale. There are several studies that have focused on the strategies to achieve this target, such as the use of non-poisonous additives, bio-magnetic flocculant, and the genetic modification of microalgae [36–38]. These studies provided a good starting point for further research into overcoming the difficulties of the large-scale harvesting of *P. cruentum* and other microalgae grown in swine wastewater.

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