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Application of Two Indigenous Strains of Microalgal *Chlorella sorokiniana* in Cassava Biogas Effluent Focusing on Growth Rate, Removal Kinetics, and Harvestability

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Abstract: Microalgae cultivation in wastewater is an emerging approach to remove its contaminants and generate microalgal biomass. This study aimed to screen and isolate potential strains in a cassava biogas effluent wastewater (CBEW) treatment system and produce algal biomass. *Chlorella sorokiniana* strains P21 and WB1DG were isolated from CBEW and found to grow by utilizing various carbon sources. Experiments conducted in a batch reactor using an unsterilized substrate were done to evaluate the nutrient removal and growth of isolated strains from CBEW. The results showed that *C. sorokiniana* P21 and WB1DG could achieve biomass accumulation of more than 2564 and 1301 mg L^{-1}, respectively. The removal efficiencies of chemical oxygen demand (COD), total phosphorous (TP), and total inorganic nitrogen (TIN) were found up to be 63.42, 91.68, and 70.66%, respectively, in a WB1DG culture and 73.78, 92.11, and 67.33%, respectively, in a P21 culture. Harvestability of the P21 strain was examined using several coagulant–flocculants. $FeCl_3$ was found to remove more than 90% of the cells. Nutrient removal and growth rates resulting from these indigenous strains with application of untreated CBEW support the possibility of this strain being a promising candidate to couple a CBEW treatment and algal biomass generation with minimal process adjustment.

Keywords: microalgae; biogas wastewater; nitrogen; phosphorus; COD; harvestability

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1. Introduction

Cassava biogas effluent wastewater (CBEW) is the primary waste from the cassava starch industry apart from cassava pulp. Before biogas production was developed, cassava pulp was mainly used for generating methane through the anaerobic process of methanogenesis. The concentrations of carbon, nitrogen, and phosphorous in this wastewater are relatively low after anaerobic digestion for biogas production [1,2]. However, the effluent nutrient contents are still higher than the threshold for direct discharge to the environment. Most of the treatment methods nowadays rely on conditioning ponds employing the native organisms in the pond to treat the wastewater [3].

Cassava biogas effluent is in agro-industrial waste that contains relatively low levels of nitrogen (N) and phosphorus (P), which is different from biogas process effluents that use animal manure as a substrate. These contain high amounts of N and P [4]. Conversely, high chemical oxygen demand (COD) is usually found in this wastewater, since the C/N ratios in agricultural crop wastes are higher than those in manure [5]. Relatively low nitrogen removal has been previously reported in biogas generation systems [6]. COD in these effluents may come from extracellular polymeric substance and residual COD from the influent, which may be up to 92% removed [1] from materials with initial COD

concentrations of 4200–7000 mg L^{-1} [7]. Nutrients in these effluents remain because biogas generation does not effectively remove phosphorus [8]. Thus, these parameters may result in effluents that are unsuitable for direct discharge to the environment prior to further treatment. Several studies have reported levels of phosphate removal in terms of total phosphorous and orthophosphate in the wastewater [4].

Microalgae have been reported to be versatile and effective remediation agents in wastewater treatment [9,10]. Both engineered and indigenous strains of microalgae can be used. Each has its advantages and disadvantages. Engineered strains have been developed throughout long and rigorous processes. Thus, oil production and substrate intake can be remarkably high. A mixotrophic-engineered strain that overexpresses endogenous RuBisCO activase to produce a high amount of lipid by genetic engineering of microalgae for this particular objective has recently been achieved [11]. Nevertheless, the compatibility of the strains with use in wastewater is not high.

Conversely, isolated strains are well suited to the conditions of wastewater, which can change dramatically over a short period of time [12]. Several isolated strains from extreme environments have been reported with high lipid contents [13]. Similarly, it has been found that isolating strains from an environment where the substrate or similar environment occurs, e.g., wastewater, is a promising way to obtain highly tolerant and adaptable strains [14]. To achieve high removal efficiency of nutrients in wastewater, obtaining candidate algal strain from a similar environment is among the most promising methods [15,16]. Numerous strains have been isolated from various source of wastewater. Among the isolated, *Chlorella* genera stands as one of the naturally occurred strains in the wastewater [17,18]. These strains are widely known for their fast growth rate, wide range of substrate utilization, and high nutrient removals [19–21].

Indigenous strains isolated from cassava effluent wastewater ponds have never before been explored. Thus, the current study was aimed to screen and isolate potential strains for growth in CBEW and algal biomass production. To evaluate the nutrient removal and growth of isolated strains from CBEW, the performance of these strains was examined in an unsterilized reactor to study nutrient removal kinetics. Moreover, this study is the first of its kind to examine the removal of nutrients in moderate concentrations with concurrent biomass growth coupled with determination of nutrient removal kinetics of single indigenous cultures of microalgae. Furthermore, at the end of the cultivation period, several coagulant–flocculants for microalgae were tested with the optimal strain to examine the feasibility of using the selected strain for nutrient removal and biomass production. Results of this research were expected to obtain single versatile strains that can be applied as a potential phytoremediation agent using microalgal cultures.

2. Materials and Methods

2.1. Wastewater Source

Biogas effluent wastewater discharged from a biogas reactor at Korat Flour Industry Co., Ltd. Nakhon Ratchasima, Thailand (N 14°53′53″ E 102°04′00″) was collected. The total phosphorous (TP), soluble phosphate in the orthophosphate form (PO_4), nitrate (NO_3-N), nitrite (NO_2-N), ammonium (NH_4-N), total inorganic nitrogen (TIN), alkalinity, chemical oxygen demand (COD), and biological oxygen demand (BOD) were analyzed using standard methods [22]. Briefly, PO_4 and TP were measured using ascorbic acid method, COD and BOD were measured using closed reflux colorimetric and dissolved oxygen methods, NH_4-N was measured with titrimetric method, and NO_3-N and NO_2-N were measured using cadmium reduction and azo dye methods. pH, dissolved oxygen (DO), and conductivity of the samples were determined using a YSI 556 MPS Multiprobe System (Xylem, OH, USA). An additional probe was employed to detect CO_2 emissions from the system using a gas analyzer (Geotech GA 5000, QED Environmental Systems, Ltd., Coventry, UK).

2.2. Microalgal Isolation and Screening

The isolation process was based on previously reported methods [23]. Briefly, serial dilution was conducted before inoculating and spreading 100 µL of wastewater samples onto a sterile BG 11 agar medium. Individual microalgal colonies were observed under a light microscope and selected based on their morphological appearance.

Screening of mixotrophic microalgae was conducted using several sources of carbon. Glucose, sucrose, fructose, mannitol, and galactose were used as carbon sources. The strains were tested in BG 11 medium with the addition of 0.5% of each of the carbon sources. BG 11 medium with no additional organic carbon source was inoculated as a control. The algae were grown for 14 days in 250 mL of BG 11 until reaching their stationary growth phase. Then, the cultures were diluted to achieve an optical absorbance at a 680 nm wavelength (A_{680}) of 0.5 [24,25]. Next, 100 µL of each dilution was inoculated to individual wells of a sterile twenty-four-well microplate with 1 mL working volumes to culture the strains. After seven days of incubation, A_{680} of the culture was measured. These results were used to determine the most suitable microalgae for further experiments.

2.3. Morphological and Molecular Identification

Morphological characteristics of the microalgae were observed under light microscopy (Primo Star, Zeiss) and scanning electron microscopy (SEM) (Auriga, Zeiss) and identified using identification keys [26–29]. Molecular identification was conducted as well based on previous studies [30,31]. The 18s rDNA region of each test strain was amplified using the primers, NS1: 5′-GTAGTCATATGCTTGTCTC-3′ and NS8: 5′-TCCGCAGGTTCACCTACG GA-3′ [32]. After a PCR reaction was performed, the sequence of the gene was analyzed using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 12 July 2021). A phylogenetic tree was constructed using MEGA version X (MEGA, Philadelphia, PA, USA) after multiple alignments of data using the Muscle Tool. Evolutionary distances and clustering were constructed using a neighbor-joining method and evaluated using bootstrap values based on 1000 replications.

2.4. Cultivation Conditions and Biomass Generation

Mixotrophic strains, thus, were cultivated in a photobioreactor cylinder made from 0.5 cm thick acrylamide (Figure 1). The total cylinder volume was 12 L with a working volume of 10 L (diameter of 20 cm and height of 38 cm). The cylinder is also equipped with two paddles for agitation. Cultivation was conducted with no additional aeration. Agitation was done at 250 rpm with a 12:12 h dark/light cycle under illumination by a white bubble lamp (5000 lux) for 20 days. Selected microalgae were cultured in BG 11 medium until the biomass reached 70 mg L^{-1}, signifying the beginning of the exponential phase. The reactor was filled with 9.75 L of unsterilized wastewater from which the coarse particles were removed using screens. All the water quality parameters were analyzed daily based on a preliminary analysis of wastewater characteristics. The microalgae biomass and bacterial contamination during the production process were measured separately.

Measurements of microalgal biomass were conducted using a spectrophotometer at absorbance values of 630, 645, 665, and 750 nm to determine the chlorophyll A content [33] as a representation of microalgal density. The concentration of chlorophyll was used to estimate the total algal biomass in the culture. Based on a correlation between biomass and chlorophyll, standard curves were constructed for each selected isolate (Figure S1). Total biomass from the system was also measured using a gravimetric method [34]. The contaminant microbial biomass was estimated using Equation (1):

$$B_C = B_T - B_A, \quad (1)$$

where B_C is the contaminant biomass, B_T is the total biomass, and B_A is the algal biomass.

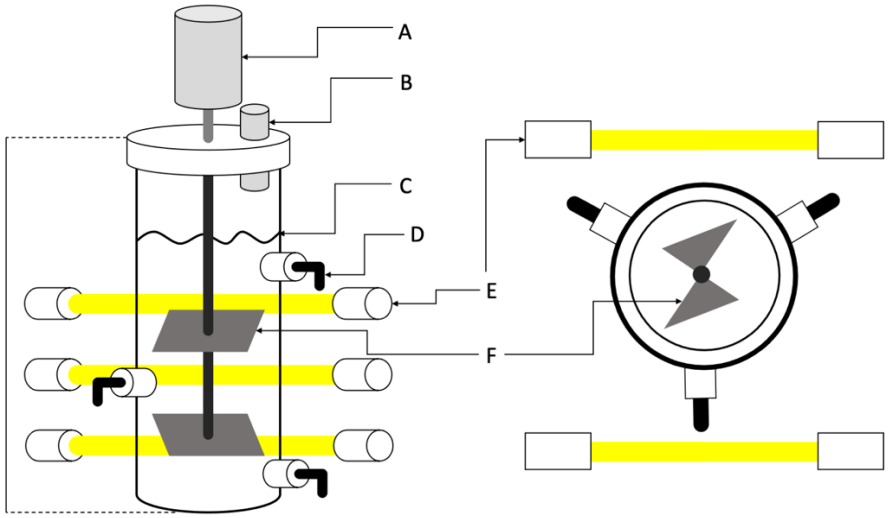

Figure 1. Reactor configuration. (**A**) Motor, (**B**) gas outlet sampling, (**C**) surface of culture, (**D**) sampling ports, (**E**) LED lamp, and (**F**) agitation paddle.

2.5. Kinetics Analysis

TP, PO_4, NO_3, NH_4, TIN, and COD are the main focus of the current study. From cultivation, the removal efficiencies of each of the nutrients were estimated using Equation (2):

$$E = ((S_0 - S_{na})/S_0) \times 100, \quad (2)$$

where E is the efficiency of nutrient removal, S_0 is the initial concentration of each particular nutrient, and S_{na} is the concentration of that particular unassimilated nutrient.

Currently, there are two commonly used models for determining the removal and growth rates of microalgae cultures, the conventional growth rate (Monod equation) [35,36] and the population model (Verhulst model) [37,38]. The current study used the Verhulst kinetic model. In this model, the maximum concentration of a microalgal biomass in wastewater was taken as the limiting factor in the growth of microalgae in Equation (3):

$$\frac{dX}{dt} = \mu X_0 (1 - \frac{X_0}{X_{max}}), \quad (3)$$

where X is the biomass at a given time, t is the time, X_0 is the initial biomass concentration, and X_{max} is the maximum biomass. The Verhulst model in Equation (4) was used to determine the biomass (X) value at a specific time (t):

$$X = \frac{X_0 X_{max} e^{-\mu t}}{X_{max} - X_0 + X_0 e^{-\mu t}}, \quad (4)$$

Productivity is a primary parameter in reactor operation, where biomass production is described over time. Verhulst model productivity (P), Equation (5), was obtained for the system as:

$$P = \frac{\mu(0.9X_{max} - 1.1X_{0)}}{\ln\left(\frac{9(X_{max} - 1.1X_0}{1.1X_0}\right)}, \quad (5)$$

Additionally, the consumption rate of nutrients as substrates for microalgal growth is also essential when coupling the reactor for two purposes: nutrient removal and wastewater treatment. The nutrient consumption rate was calculated using Equation (6):

$$CR_s = \left(\frac{S_t - S_{t+\Delta t}}{\Delta t}\right), \quad (6)$$

where CR_s is the consumption rate, S_t is the substrate concentration at a given time (t), and $S_{t+\Delta t}$ is the substrate concentration after a period of time. The Verhulst model also describes substrate removal in Equation (7):

$$S = \frac{\left(\frac{X_0}{Y_0} + S_0\right)(S_0 - S_{na}) - S_{na}(S_0 - \left(\frac{X_0}{Y_0} + S_0\right))e^{\mu t}}{(S_0 - S_{na}) - (S_0 - \left(\frac{X_0}{Y_0} + S_0\right))e^{\mu t}}, \quad (7)$$

where $1/Y_0$ is the nutrient content of the biomass, and other parameters are as described above. Several adjustments were made to the process. The nutrient content $1/Y_0$ was limited to values between 0.01 to 0.1 for nitrogen, 0.001–0.01 for phosphorus [37], and 0.1–0.6 for COD [36].

Biomass growth and nutrient removal results were adjusted using the Microsoft Excel Solver tool with minimal deviation from the model as the objective. The GRG non-linear solving method was used to fit the corresponding results with predicted values. Convergence of 10^{-4} and the forward derivative mode were constraints on variable states in the system.

2.6. Harvestability Tests

After 20 days of cultivation, treated wastewater was separated from the biomass suspension using coagulation–flocculation harvesting methods. Calcium chloride ($CaCl_2$), starch (S), iron (II) sulfate ($FeSO_4$), and ferric chloride ($FeCl_3$) at various level were used in this study to determine the harvestability of the cultures in a wastewater-based medium.

The experiments were conducted in capped 50 mL conical tubes. Briefly, 40 mL of the culture samples were added into the tubes, followed by the coagulants and flocculant stocks. The tubes were shaken at maximum speed for 1.5 min to ensure that the coagulant or flocculant was completely dissolved. After that, all the tubes were shaken at 50 rpm for 20 min to allow flocculation. Another 20 min of sedimentation time was given for the flocs to settle as sediment before measuring their optical density at the wavelength 680 nm (OD_{680}) values. Sampling of the clarified water was done at three water levels. These levels were the 10, 19, and 38 mL depths of the liquid level, to obtain homogeneity of the water samples. The biomass removal efficiency was calculated using Equation (8):

$$Removal\ Efficiency\ (\%) = \left(1 - \frac{A_{680\ in\ final\ condition}}{A_{680\ in\ initial\ condition}}\right) \times 100\%, \quad (8)$$

2.7. Statistical Analyses

All the experiments were conducted in triplicate. The obtained data were fit into the previously described models. A non-linear programming package in the Microsoft Excel Solver Tool was used for this purpose. Deviation of the actual and predicted model values was minimized using a GRG non-linear method. The Data Analysis tool pack was used to perform regression analysis and estimate the precision of the models.

3. Results and Discussion

3.1. Isolation and Screening of Microalgae

It is well established that indigenous strains of wastewater have shown remarkable removal activity in vast niches compensating for the environmental conditions of the wastewater. Thus, proper application of these indigenous strains can hasten their application with little adjustment for commercial applications. Alternatively, the introduced strain will first need to have the required properties such as fast growth, efficient removal of wastewater nutrients, adaptation to significant changes in environmental conditions, and capability to grow simultaneously with indigenous bacteria [39]. Here, isolation of indigenous microalgae was demonstrated. Twenty-four different strains were isolated based on their morphology from six different sites around wastewater effluent treatment systems. The early separation and labeling of microalgae were based on their presence at

several sampling sites and morphological characteristics. After obtaining pure cultures, all of the strains were tested for growth under mixotrophic conditions. From all the isolated strains, only ten could successfully grow under mixotrophic conditions. They grew optimally using the organic carbon sources present and in the absence of light (Table S1). Among the strains, P21 and WB1DG were found to grow using all of the carbon sources tested in this study.

Generally, phylogenetic tree construction was used to confirm the BLAST strain classification to conserve the genome and draw relationships among the isolated strains or represented traits [17]. In this study, 18s rRNA was selected for study of the phylogenetic traits of selected strains based on their ability to utilize carbon sources. This approach is widely used for identification of green microalgae, and it has been employed for identification to the species level of microalgae [40,41]. Nucleotide sequences from various reference *Chlorella* spp., *Miractinium* spp., *Dictyosphaerium* spp., and *Chlorellaceae* spp. were used to construct a phylogenetic tree to determine the relationships of microalgae strains among the members of the Chlorellaceae. Several strains of *Scenedesmus* spp. and *Symbiochloris pauciautosporica* were chosen as an outgroup of the root of the phylogenetic tree (Figure 2). Based on the 18s rRNA gene sequence similarity, it has been found that the chosen strains have the capability to use organic carbon. This is distributed among several genera of the Chlorellaceae family. The high carbon utilizing strains, WB1DG (MZ35987) and P21 (MZ359868), are *Chlorella sorokiniana* species. Furthermore, organisms of moderate organic carbon source utilization include RS 3 (MZ359869), which is of the Chlorellaceae family, while BP 3 (MZ359867) is *C. sorokiniana*. Last, SP22 (MZ359870) as the representative strain with no organic carbon utilization is a *Dictyosphaerium* sp. Nevertheless, there is no strong evidence to support the hypothesis that carbon utilization capability is based on evolutionary traits, as SP 22 (no carbon utilization strain) was closer to the two representatives of high carbon utilizing strains (WB1DG and P21), while C. sorokiniana is among the moderate- and high-carbon utilization algae.

Based on the carbon utilization results, the experiments focused on the P21 and WB1DG strains. Morphological observation revealed that the P21 and WB1DG strains have round and unicellular shapes (Figure 3), where WB1DG was found to be larger (4–6 μm) than P21 strain (3–5 μm). Isolation of microalgae in wastewater for bioremediation has been done for years. However, not all of the strains are suitable for the mixotrophic conditions in which removal for certain nutrients can be expected to be optimal. However, similar isolations from different kinds of wastewater have resulted in the isolation of various strains of this species. *Chlorella* has been widely recognized as one of the most common microalgae used to treat wastewater [15]. *C. sorokiniana* has been successfully isolated from many sources of wastewater, such as in palm oil mill wastewater [32], secondary effluent of municipal wastewater [21], dairy wastewater [42], chicken farm flushing wastewater [15], and urban wastewater [43].

C. sorokiniana has been reported to have the ability to grow using various carbon sources in mixotrophic culture [44,45]. The ability to grow under mixotrophic conditions is essential to achieve higher yields of biomass than possible under autotrophic conditions. However, various structures of organic carbon can affect the structure and composition of *C. sorokiniana* [46]. Glucose, sucrose, fructose, mannitol, and galactose are among the small and easily degraded carbon molecules for microbial growth under mixotrophic and heterotrophic conditions. The ability of the microalgae to grow and utilize various carbon sources is expected to be a function of the carbon sources utilized by microalgae cells [47].

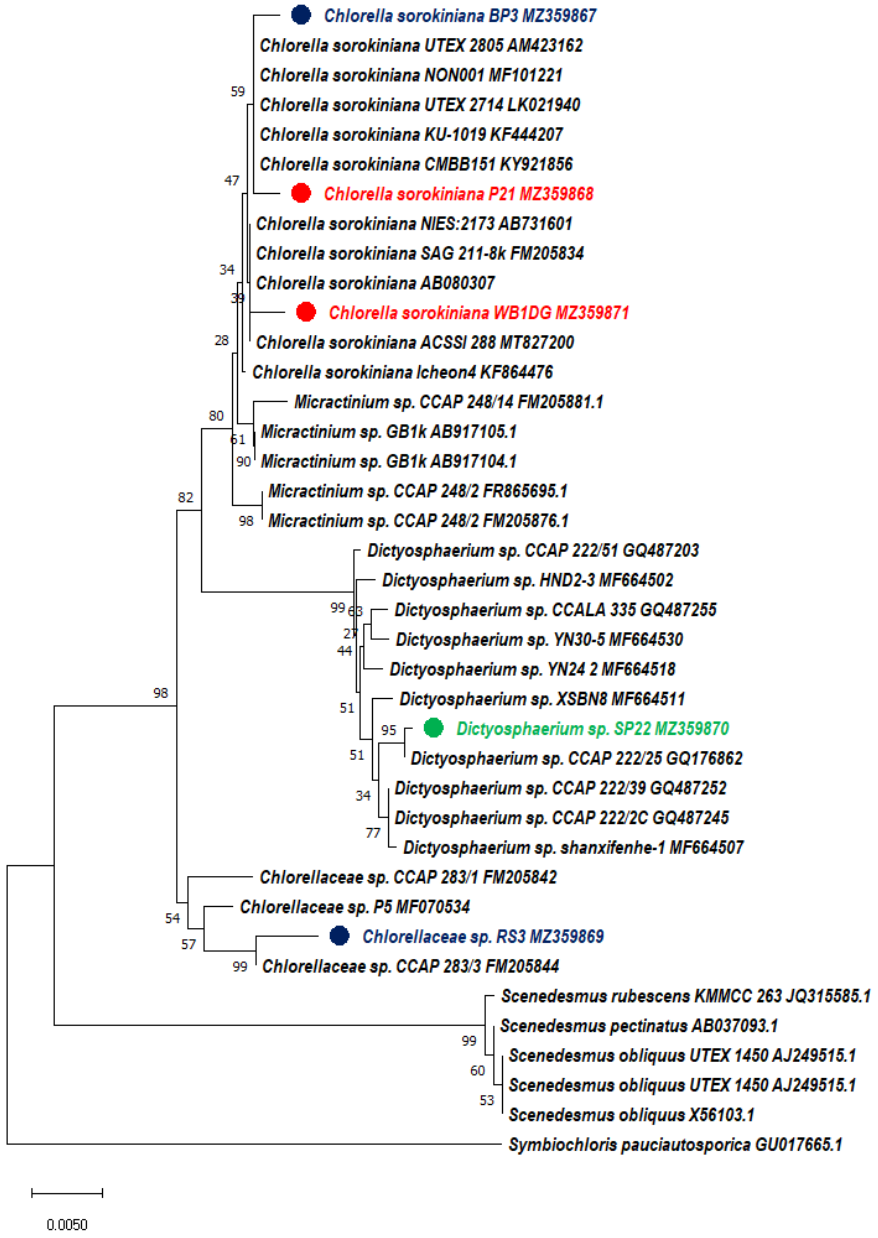

Figure 2. Phylogenetic tree of represented strains with various levels organic carbon utilization. The colors of the dots indicate high (●), moderate (●), and no carbon utilization (●). This tree was made using the neighbor-joining method after 1000 rounds of bootstrap resampling.

3.2. Culture Conditions during Cultivation

Growth of the P21 and WB1DG strains was examined in CBEW wastewater after particulate removal. There was no adjustment in the nutrient content from the actual conditions (Table 1). After inoculation in the CBEW, the lag phase of the culture occurred during the first three days of the culture for both strains. After that, the logarithmic phase of growth occurred until day six, as the biomass dramatically increased. After day 6, the growth of P21 tended to be stable in the stationary phase, while the growth of WB1DG increased slowly (Figure 4a). The lag phase of a microalgal culture is usually related to the acclimatization mechanisms of the organisms. The current study found that the lag phase occurred due to the culture's need to acclimate to a variety of substrates, as the algae were previously cultured in BG 11 medium. Initially, the carbon source was only in an inorganic form. In line with this result, this phase was also reported in *C. sorokiniana* cultured in a tannery wastewater, where the lag phase occurred for up to 7 days [48]. When *C. vulgaris*

was cultivated in aerobic and anaerobic cultures, the lag phase occurred for 120 h due to acclimatization [49].

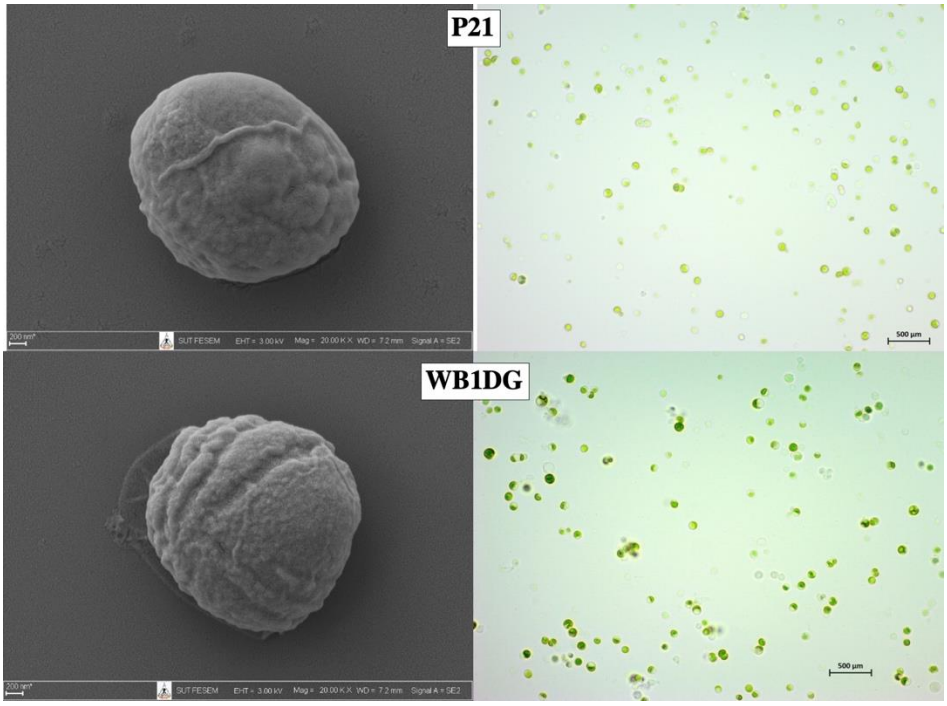

Figure 3. *Chlorella sorokiniana* strains P21 and WB1DG. The images were observed using SEM (**left**) and compound (**right**) microscopy.

Table 1. Wastewater characteristics.

Parameters	Value	Units
COD	205 ± 12.3	mg L^{-1}
BOD	75 ± 9.52	mg L^{-1}
TP	37.26 ± 2.05	mg L^{-1}
PO_4-P	23.53 ± 1.70	mg L^{-1}
TKN	54.1 ± 3.21	mg L^{-1}
NO_2-N	0.08 ± 0.02	mg L^{-1}
NO_3-N	16.43 ± 0.69	mg L^{-1}
NH_4-N	31.24 ± 1.67	mg L^{-1}
pH	7.6 ± 0.03	-
DO	3.21 ± 2.4	mg L^{-1}
Conductivity	2699 ± 43.60	mS cm^{-1}
Alkalinity	700 ± 32.57	mg L^{-1} as
Salinity	2.2 ± 1.3	parts per thousand (PPT)

After six days, a stationary phase occurred where the growth of biomass was nearly stagnant. However, the biomass still increased from 1036 mg L^{-1} to become 1301 mg L^{-1} on day 20 of the WB1DG culture, while P21 had a relatively stable biomass concentration from days 6 to 20 (2584 to 2640 mg L^{-1}). Interestingly, when the stationary phase began on the sixth day, noticeable growth of native microbial biomass was seen in both cultures (Figure 4b). This may have resulted from extracellular polymeric substances excreted from the microalgal biomass grown earlier that settled in the system. In this way, microalgae provided nutrients suitable for other microorganisms to grow. The growth of native microorganisms here did not affect microalgal growth, since neither detrimental effects nor competition were present. One reason to avoid native microorganisms is that they can compete for carbon and other nutrients in the culture [47].

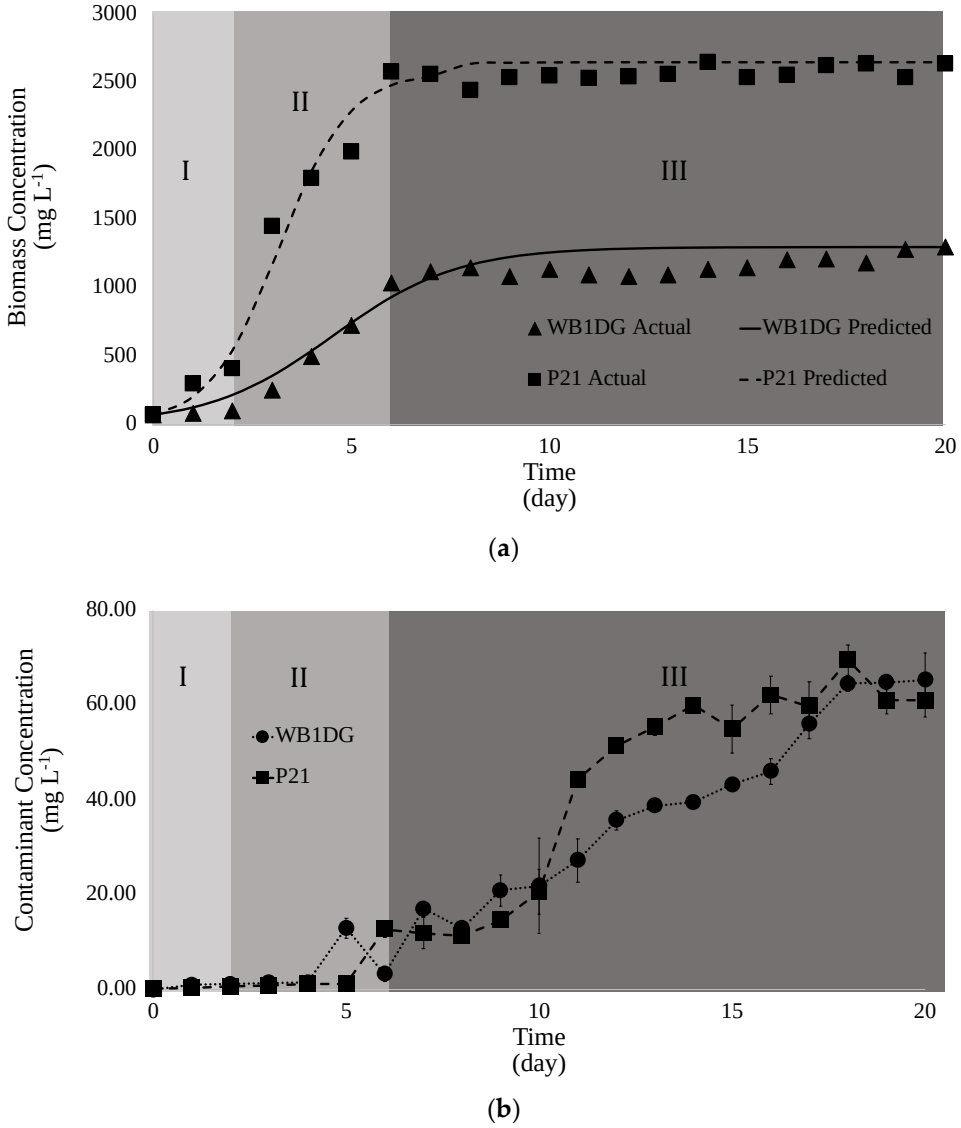

Figure 4. Biomass concentration of the culture of microalgae *C. sorokiniana* WB1DG and P21 over 20 days of cultivation in the different phase of growth; (I) lag, (II) exponential, and (III) stationary phases of the culture. (**a**) Algal biomass and (**b**) contaminant biomass. All the data were based on the mean values of triplicate experiments.

Microalgal *C. sorokiniana* P21 and WB1DG constituted a high percentage of biomass in the system. A noticeable production of biomass was first observed on day 5 of the WB1DG and day 6 of the P21 cultures. However, the end of the exponential growth stage occurred on day 6. Based on these observations, it could be concluded that the native microorganisms did not affect algal biomass generation, as microalgae were still the predominant organism in the system [50]. Moreover, microalgal P21 and WB1DG were the predominant strains after cultivation, as seen in the microscopic observations where the P21 strain was the dominant microalgae observed after the end of cultivation (Figure S2). Although the increase in microalgal biomass was still considerably high from days 6 to 20, where maximal biomass generation was achieved, an additional 14 days for this increased performance was not commercially suitable for mass culture. Thus, the performance of the first six days of the culture process was the focus of the current study.

Generally, the dissolved oxygen profiles showed a dramatic increase during the exponential phase in the early stages of the cultivation of both cultures (Figure 5). After a four-day cultivation period, a slight decrease in DO was observed. The lag and exponential

phases of algal biomass generation occurred during this cultivation period. However, CO_2 concentration was below the detection limit of the probe during the cultivation period. Removal of COD (Figure 6a) and the absence of CO_2 in this period indicate the system was in mixotrophic cultivation where organic and inorganic carbon were simultaneously degraded and fixed, respectively. Organic carbon may enable a heterotrophic mode, where CO_2 is generated.

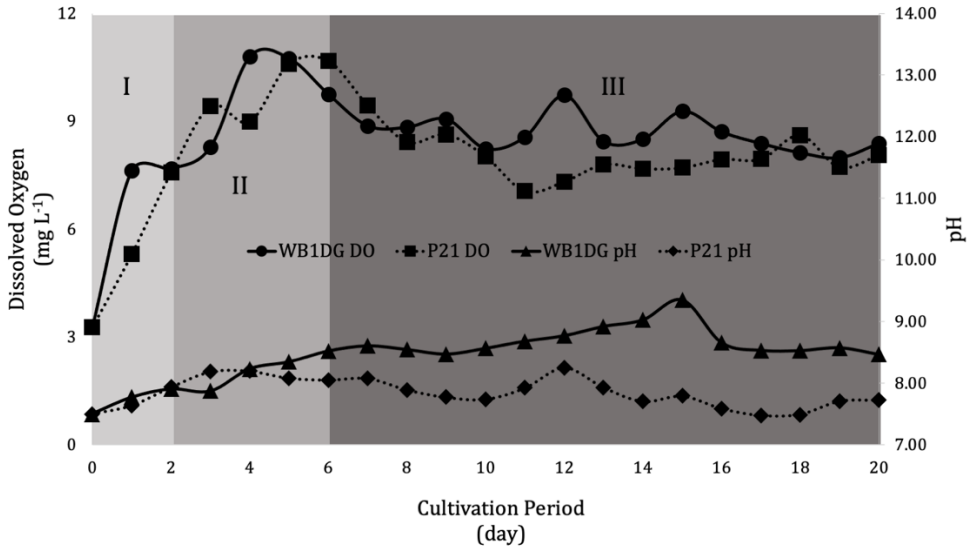

Figure 5. pH and dissolved oxygen in cultures of *C. sorokiniana* WB1DG and P21 during 20 days of cultivation. (I) lag, (II) exponential, and (III) stationary phases of the culture. All the data are based on the mean values of triplicate experiments.

In contrast, the autotroph mode of microalgae can be enhanced by the presence of higher levels CO_2 from heterotrophic activity [51]. As a consequence, dissolved oxygen was abundant. A slight increase in pH was also observed during the first four days of the cultivation of WB1DG, while a steady increase was found in the P21 culture until day 15, before it decreased. Interestingly, the reactor was not equipped with an aeration system, which usually causes a decreased pH due to dissolved CO_2. This indicates that there was active transport of carbon through the cell membranes of the microbial cells involving free H^+ ions and, thus, decreased the available free H^+ ions in the wastewater [36].

The results of biomass growth were consistent with the removal of nutrients from the wastewater. Their concentrations decreased slowly on the first day and exponentially from the second day onwards (Figure 6a). This demonstrates that the *C. sorokiniana* P21 and WB1DG strains utilize soluble organic carbon in the wastewater for generation of biomass. Removal efficiencies of COD by P21 and WB1DG were 73.78 and 63.42%, respectively. COD represents the oxygen needed for carbon oxidization in the wastewater. It can be reflected as the carbon source for completing the reactions needed for nutrient removal [52]. Previous studies reported various removal efficiencies of *C. sorokiniana*. Up to 80% removal was achieved by this alga in raw sewage with an initial COD of 3633 mg/L [53], 95.6% from chicken farm flushing wastewater with 525.7 mg L^{-1} of initial COD [15], 79.8% removal from municipal wastewater with an initial COD concentration of 44 mg L^{-1}, and 75% removal from swine manure wastewater with an initial COD of 2000 mg L^{-1} [54]. It can be seen that the lower removal efficiency of *C. sorokiniana* often occurred in wastewater with a low initial COD concentration. However, moderate COD removal from wastewater is related to the carbon being unbiodegradable by microalgae. As CBEW comes from a long hydrolysis process, methanogenesis. Most of its easily degradable carbon is utilized and the biodegradable carbon in the form of BOD was only 37.5% (Table 1). This assumption also explains why that in most studies of microalgae used for wastewater treatment, the removal efficiencies of phosphorus and nitrogen were higher than for COD removal [48,53].

Another scheme that might be possible is that degradation of nitrate and other nitrogen forms in wastewater that require organic matter are released in the system to incorporate nitrogen [55]. Thus, organic matter as COD could not be completely removed from the system.

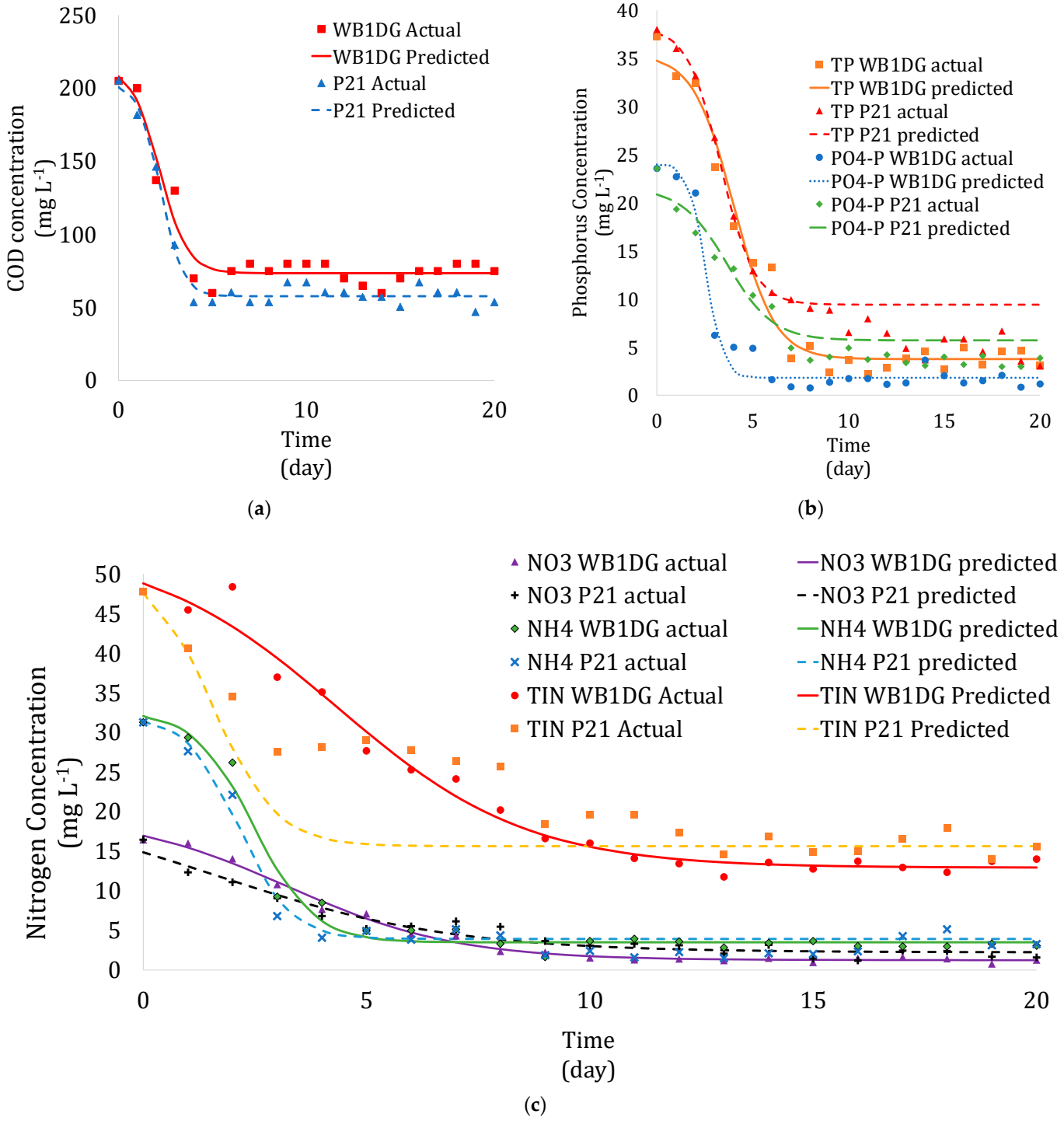

Figure 6. Evolution of nutrients concentration in CBEW during the cultivation of *C. sorokinaina* P21 and WB1DG. (**a**) COD, (**b**) phosphorus, and (**c**) nitrogen. All the data were based on the mean values of triplicate experiments.

Dissolved nitrogen plays an essential role in biomass generation. A lack of available nitrogen for assimilation can lead to a lower generation of biomass. In the current experiments, immediately after inoculation, NO_3 dramatically decreased until day ten, and the NH_4 concentration in both cultures plunged within four days (Figure 6c). NO_3 has been reported to be a nitrogen source that microalgae can quickly assimilate [56]. Similarly, ammonium is also useful for assimilation, since it requires less energy [57], even though ammonium was also reported to be toxic to microalgae at higher levels [58]. Thus, it was expected that the P21 and WB1DG strains could assimilate both forms of nitrogen. Total dissolved inorganic nitrogen in the CBEW decreased gradually until day 10 for WB1DG and day 13 for P21, indicating that another form of inorganic nitrogen (NO_2) was still assimilated as seen through a decrease in total inorganic nitrogen (Figure S3). A dramatic decrease in NH_4 can indicate that a nitrification process took place quickly and efficiently [39]. This result is supported by the control condition, where the NO_2 removal did not occur, while NH_4 was still reduced (Figure S4).

Nitrification was also found in the study of [59], where the decreased ammonium in the wastewater was in line with the sudden appearance of nitrite. Nevertheless, in both the P21 and WB1DG strains, nitrification was not the only cause of NH_4 removal, as the total NH_4-N removal was higher than just for NO_2. A high amount of dissolved oxygen in the system (Figure 5) emphasizes that denitrification was not the primary removal mechanism in the present study [60]. This phenomenon supports the concept that algae consume ammonium simultaneously during nitrification, rather than a single nitrification process of NO_2-N into NO_3-N [56]. Similarly, several strains of *Chlorella* were reported to survive and take up ammonium in a pond-scale reactor [61]. Nitrogen in the form of ammonium is preferred for assimilation by microalgae, because it is a more economical for nitrogen reduction into organic nitrogen. Ammonium can also be directly converted into amino acids by glutamine synthetase (GS)–glutamate synthase (GOGAT) enzymes [58].

The removal efficiencies of *C. sorokiniana* strains WB1DG and P21 were, respectively, 92.42 and 90.4% for nitrate, 90.20 and 89.64% for ammonium, and 70.66 and 67.34% for total inorganic nitrogen. This result is relatively lower than reported in several previous studies. [37] demonstrated an ultimate removal of total N by *C. sorokiniana* SAG 211–8k in urban wastewater with an initial N concentration of 54.58 mg L^{-1}. More than 99% removal of nitrate was also reportedly removed by *Chlorella* sp. in artificial wastewater [39]. However, the amount of nitrogen remaining in the wastewater was still lower than the maximum threshold for wastewater discharge. Since one of the objectives of a coupled method of treatment by microalgae is to obtain a dischargeable wastewater condition to the environment, this particular result still needs further development [62].

Total phosphorous (TP) is a parameter indicating the amount of P in the solution, and it depicts how microalgae consume the overall phosphorous in the system. TP removal efficiency was measured and found to be relatively high (91.68% for the WB1DG culture and 92.11% for the P21 culture). Phosphate removal from the CBEW treated with the P21 algal strain was only 83.74% and 83.74% for the WB1DG strain. PO_4 depicts the amount of soluble and efficiently utilized phosphorous, but the particulate phosphorous (difference between TP and PO_4) was still considerably high. However, the culture managed to significantly reduce the amount of this phosphorous form as the removal of TP gradually reached the rate of PO_4 removal (Figure 6b). The pH was below 9.5 during most of the culture period (Figure 5), revealing that the removal via precipitation of insoluble phosphorous was low [63]. In the control, phosphorous removal was negligible (Figure S5). Thus, a possible mechanism, apart from the precipitation of this insoluble removal, is by converting the most labile portion of particulate phosphorous into PO_4 for further utilization by algal cells [64].

Interestingly, there was a difference between the starting time of the decrease in COD, N, and P with increased biomass. This result might have been caused by the uptake of wastewater constituents that were not directly converted into biomass. This reduction in wastewater constituents seemed to initiate the exponential phase of microalgae growth. This finding is similar to that of [52], where decreased levels of wastewater constituents occurred before the exponential increase in microalgae biomass. Later, this might have been due to a phenomenon called luxury uptake of polyphosphate. This is supported, since the PO_4 level was almost 50% of the total phosphorous in the wastewater. Moreover, removal of PO_4 in the first three days of cultivation was higher than the TP removal, strongly suggesting that this mechanism was taking place [65], since orthophosphate represents a soluble and easily assimilated form of phosphorous.

3.3. Kinetics of Microalgae Growth in Wastewater

Kinetic parameters play an important role in successfully applying and optimizing microalgae cultivation on larger scales [66]. Parameters such as growth rate and productivity are among those used to examine microalgae strains under various substrate conditions [35,37]. These kinetic parameters have been used to compare several single microalgae strains (de Mattos and Bastos, 2016) as well as mixed cultures (Mennaa et al., 2015). Simple parameters such as productivity are also used as comparisons [67]. However, various methods were used for determining even simple parameters such as growth rate and productivity in many previous studies. Generally, there are four widely used models to calculate growth rate, the Verhulst, Monod, Droop, and Haldane models [68]. Since CBEW contains relatively low substrate N and P concentrations, lower than the inhibitory concentrations for this microalga to grow (Figure S6), the Verhulst kinetic model was used to examine the isolated strains.

This study was focused on the kinetic parameters of *C. sorokiniana* P21 and WB1DG as robust candidate strains for cultivation in wastewater effluent systems. Thus, the comparison takes place using a similar strain isolated from wastewater to remove contaminants and generate biomass. As shown in Table 2, microalgae growth of both fit the Verhulst model of consumption ($R^2 > 0.95$). It was evident that there was a significant difference in all parameters of P21 and WB1DG. The maximal concentration of biomass in the P21 culture was twice that of the WB1DG culture, indicating different growth capabilities in CBEW conditions. Another distinct parameter was the growth rate. The P21 strain was found to have a higher growth rate than WB1DG. P21 had more than three times higher volumetric productivity than that of WB1DG. This noticeable gap might be caused by the strains' disparate capabilities in acclimating to the culture medium and reactor conditions [37]. As discussed above in the screening process section, the strains were chosen based on carbon utilization. Different metabolism traits were found in the P21 and WB1DG strains regarding carbon utilization (Table S1). Metabolic differences can become distinctive from one strain to another. It has been shown that different strains from similar isolation sites had disparate growth rates, implying varying levels of maximal biomass generated from the same substrate [17].

Table 2. Growth parameters of microalgae P21 and WB1DG.

Kinetic Parameters	P21	WB1DG
X_0 (mg L^{-1})	70	70
X_m (mg L^{-1})	2652.99	1301.85
μ (day^{-1})	1.11	0.61
R^2	0.99	0.96
P (mg L^{-1} day^{-1})	179.42	49.09

Table 3 shows the results of the Verhulst model applied in the removal of wastewater constituents over a 20-day period. All the parameters in the Verhulst kinetic model were suitable for modeling the growth and removal rates in the system. Interestingly, the removal rates of substrates (μs) of all parameters except NO_3-N and TIN-N were higher than the growth rate of the microalgae ($\mu_{biomass}$). This result indicates that the removal processes did not all result from algal biological process. Some of the algal biological processes might operate in the precipitation of phosphorous and nitrogen stripping of ammonium to form ammonia gas [37]. A reduction in NH_4 and NO_3 in the control experiment was observed (Figure S3). The resulting kinetic parameters are in line with depletion of NH_4-N as discussed above. Although significant removal of ammonium was assumed from the biological process, the results also suggest there must be another way of utilizing the source of NH_4-N, since the rate of removal was higher than in the biomass growth, such as precipitation [59]. Similarly, PO_4-P and orthophosphate were removed under the process called luxury uptake. However, precipitation might have contributed to removal, since the rate of P removal was higher than the biomass growth rate. It is interesting to note that COD removal by the P21 and WB1DG cultures—73.78 and 63.42%, respectively—can explain the PO_4-P and TP removal rate. Constant COD remaining in the wastewater suggests the presence of extracellular polymeric substances (EPS), mainly from photosynthesis. Removal of PO_4-P and TP might have been partially a result of precipitation mediated by algal photosynthetic activity [69].

Table 3. Kinetic parameters of nitrogen and phosphorus removal obtained from a Verhulst kinetics model.

Nutrient	Strain	Kinetic Parameters					
		S_0 (mg L^{-1})	S_{na} (mg L^{-1})	μ (Day^{-1})	R^2	$1/Y_0$ (% S)	CR_s (mg L^{-1} Day^{-1})
COD	P21	200.72	57.65	1.69	0.98	4.6	53.57
	WB1DG	207.92	73.36	1.35	0.94	10	62.85
NO_3-N	P21	14.88	2.21	0.36	0.95	10	4.10
	WB1DG	16.96	1.24	0.53	0.99	4	3.25
NH_4-N	P21	31.35	3.03	1.70	0.98	1	15.29
	WB1DG	32.07	3.50	1.50	0.98	1	16.92
TIN-N	P21	41.61	15.62	0.35	0.91	10	7.13
	WB1DG	48.83	12.91	0.47	0.98	6	11.41
PO_4-P	P21	19.85	4.37	1.26	0.94	1	4.33
	WB1DG	23.97	1.79	2.24	0.98	1	14.81
TP	P21	37.05	6.08	1.01	0.98	1	8.17
	WB1DG	34.75	3.71	0.94	0.98	1	9.47

Growth rate is the most used parameter to compare and study the application of biological agents in wastewater treatment systems. It depicts the process in which biomass grows from its initial concentration. Numerous researchers have used this particular parameter to determine whether studied strains were potentially suitable for development on different scales. Here, we obtained useful growth rates of *C. sorokiniana* strains P21 and WB1DG. It was found that the growth rates of these indigenous strains were higher than most of the previously studied strains [17,37,38,70]. However, there are still many aspects of strain development that need to be addressed for operations at a larger scale, even though the current study uses a relatively large closed photobioreactor (10 L).

3.4. Harvestability of Microalgae

Harvestability of microalgae is one of the main bottlenecks to industrialized algal-based biofuel. There are many harvesting methods available nowadays. However, coagulation–flocculation is one of the preferable harvesting methods in many recent studies [37,71]. Several coagulants and a flocculant were used to obtain an optimal removal, ferrous sulfate ($FeSO_4$), ferric chloride ($FeCl_3$), calcium chloride ($CaCl_2$), and starch (S).

Smaller-scale coagulation and flocculation tests are feasible with limited amounts of culture and more replications in harvesting experiments [37]. Here, a similar approach was employed to examine these four coagulants.

A 20-day culture of microalgal P21 self-sediments by up to 58%. Additional coagulants and flocculant have various impacts upon sedimentation. Among all the coagulants flocculant added, only $FeCl_3$ could remove up to 90%. In contrast, other flocculants showed removal effectiveness of 82% for $CaCl_2$ and 74% for $FeSO_4$. However, starch showed removal that was even worse than with no flocculant (52%) (Figure 7).

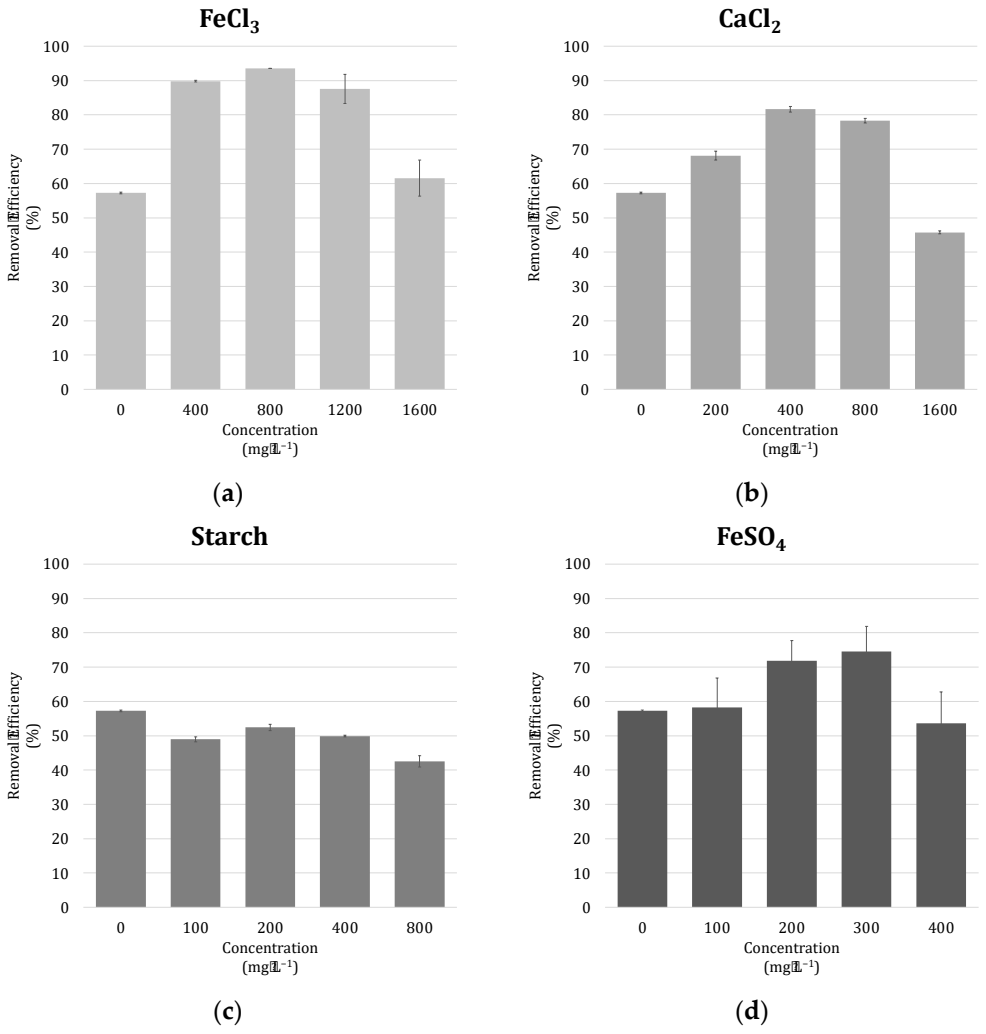

Figure 7. Flocculation efficiencies of several coagulant flocculants at various doses. (**a**) $FeCl_3$, (**b**) $CaCl_2$, (**c**) starch, and (**d**) $FeSO_4$.

$CaCl_2$ is widely used in bacterial harvesting processes, since it contains a sufficient amount of positively charge groups in its calcium ions. However, several studies have reported the feasibility of using this coagulant for algal harvesting. For this particular coagulant, removal of algal biomass in unsterilized wastewater was conducted, since the amount of biomass from native organisms at the end of cultivation was considerable (up to 60 mg L^{-1}) (Figure 4b). The optimal concentration of $CaCl_2$ in an *Arthrospira maxima* culture of 2 g L^{-1} was reported as 200–300 mg L^{-1} at a high pH [72]. $CaCl_2$ was also used as a flocculant aid for enhanced harvesting by bioflocculation of bacteria. [73] reported that 70 mg L^{-1} was optimal for enhancing the removal of algal from a suspension using *Streptomyces* sp. The mechanism of calcium binding in algal and other microorganisms in culture might result in removal of 74% of the biomass. Here, the removal of $CaCl_2$ through a

calcium-binding mechanism was assumed to take place (Branyikova, Prochazkova, Potocar, Jezkova, and Branyik, 2018). Microbial contamination can reach 60 mg L^{-1} during the coagulation–flocculation.

Modified starch was reported to require 20–40 mg L^{-1} doses for >80% removal [74]. Similarly, 20–30 mg L^{-1} doses of maize starch reduced turbidity in an algal suspension from >100 NTU to <20 NTU [75]. However, incomplete removal of starch has also been reported [76]. This result may be related to insufficient cationic groups in the modified starch. Higher doses of modified starch can increase the turbidity of water. It can be seen in Figure 8 that the formed flocs were not as compact as when other flocculants were used. Moreover, the P21 strain was found to settle more quickly in the absence of S as a flocculant. This result is similar to the use of *Scenedesmus rubescens*, which exhibits considerable self-sedimentation [77].

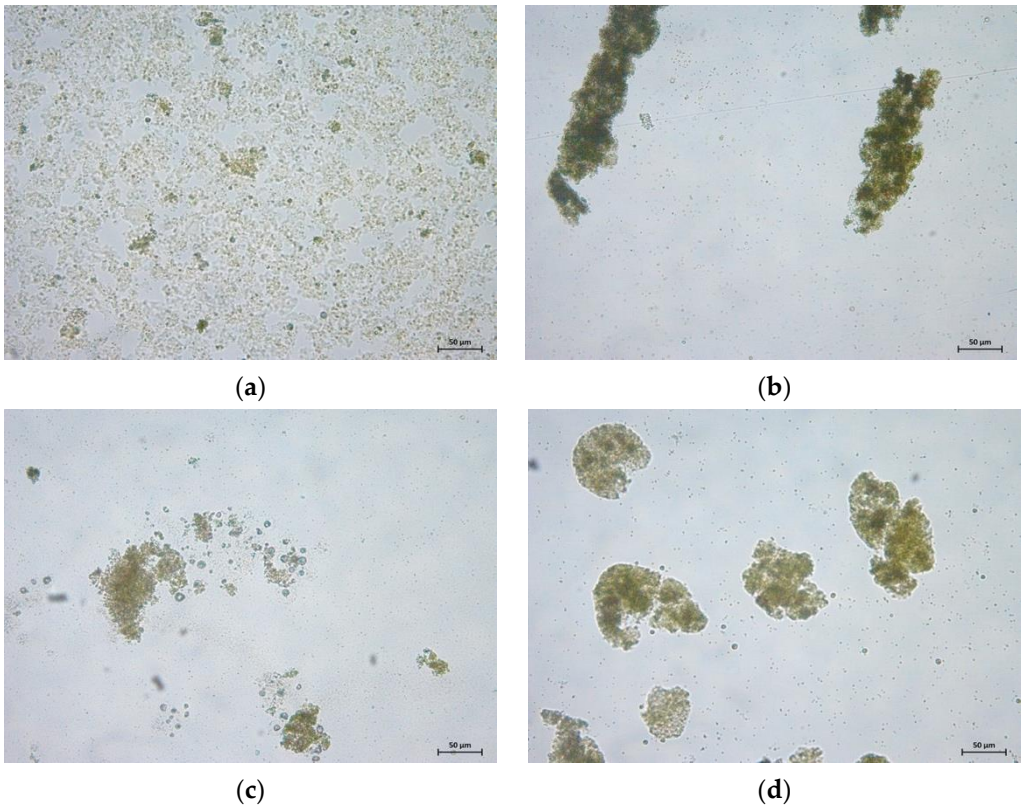

Figure 8. Various structures and sizes of algae flocs of four different coagulant–flocculants applied in sufficient concentrations for harvesting. (**a**) $FeCl_3$, (**b**) $CaCl_2$, (**c**) starch, and (**d**) $FeSO_4$.

Utilization of $FeCl_3$ for examining the harvestability of microalgae has been demonstrated in previous studies. $FeCl_3$ was reported to remove as much as 15 mg L^{-1} in a 55 mg L^{-1} algal suspension [78]. Mennaa, Arbib, and Perales [37] also reported obtaining high removal efficiency (>90%) in various cultures of microalgae, including a blooming algal seed at a concentration of 60 mg L^{-1}. $FeCl_3$ is a well-known coagulant for wastewater treatment systems and works suitably with the extracellular components of algae [79]. Another reason for the high removal activity displayed by $FeCl_3$ is the ability of Fe^{2+} ions to capture extracellular polymeric substances [80] that contribute to the turbidity of the culture where colloids are found in the suspension (Figure 8).

Conversely, $FeSO_4$ is an economical metal coagulant for wastewater. It was reported to obtain removal efficiencies of 57–86% in *C. vulgaris* culture with an initial concentration of 1.12 g L^{-1} [81]. With a 2.5 mg L^{-1} algal biomass in a urine supplemented culture, 300 mg L^{-1} of $FeSO_4$ was reported to remove 65% of the biomass present [82]. Although $FeCl_3$ and $FeSO_4$ rely on Fe ions to neutralize charges [83], the lower removal of $FeSO_4$

than $FeCl_3$ is related to the sensitivity of $FeSO_4$ flocculation to high pH values (>9) [84]. Moreover, a high concentration of $FeSO_4$ can generate a yellow-brown color caused by corrosion [82]. However, the optimal concentration of $FeSO_4$ was found to be relatively lower than that of $FeCl_3$, since the sulfate salt of ferrous sulfate can act as a coagulant via a charge neutralization mechanism.

Numerous studies have been conducted to remove algal suspensions from artificial media [77,81]. However, fewer studies focused on direct application in wastewater where algae were cultivated [76]. Various conditions can create different removal results in each medium [83]. Thus, it is important to examine the flocculation ability of several flocculants under actual conditions [85]. Here, several flocculants were examined to obtain a suitable flocculant for the selected strain. Among the flocculants, only $FeCl_3$ could achieve more than 90% removal. Thus, this flocculant is recommended for use in terms of removal efficiency for this particular strain. However, it is important to note that the improper dose of this coagulant can affect the quality of the recovered water. Application of $FeCl_3$ to harvest biomass can also limit the utilization of harvested biomass. Fe^+ ions can be toxic for feeding purpose [86]. Occurrence of this ions is also potential to inhibit the utilization of algal biomass for biogas generation, as the ions can potentially disturb the digestate in biogas reactor [87]. Nonetheless, utilization for biofuel purpose is still promising, since the Fe^+ occurrence does not affect the process of fuel extraction nor quality of extracted fuel.

4. Conclusions

Microalga *C. sorokiniana* strains P21 and WB1DG were isolated from biogas effluent wastewater and tested for mixotrophic growth in wastewater for nutrient removal and biomass generation. Microalgal biomass generation in an unsterilized culture was found in high concentrations. The WB1DG and P21 strains had maximal biomass concentrations of 1301.76 and 2652.99 mg L^{-1}, respectively. Both algal strains showed high removal efficiencies that could be advantageous in field and industrial scale-up. COD, N, and P removal were found up to 63.42, 91.68, and 70.66% in the WB1DG culture and 73.78, 92.11, and 67.33% in the P21 culture. Notably, a high growth rate was achieved, as much as 1.11 day^{-1} for P21 and 0.61 day^{-1} for WB1DG.

Consequently, microalgal culture using wastewater with no CO_2 injection has been shown to produce microalgal biomass. Considerable removal was also achieved. Harvestability of the P21 strain was examined using several coagulant–flocculants. $FeCL_3$ was found to achieve removal of more than 90% of the biomass. The promising results from these strains could be a benchmark for simultaneous coupling of algal biomass generation and removal of wastewater constituents. Nevertheless, further analysis of microbial contamination and oil content is important. Moreover, the dynamics of microalgae in a CO_2 supplemented system shall be further examined to address the possibility of higher results at reasonable costs.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13172314/s1, Figure S1. Standard curve for microalgae biomass determination based on chlorophyll A content. (**a**) P21 and (**b**) WB1DG, Table S1. Isolated strains of microalgae from the area around a CBEW pond and its growth on several carbon sources, Figure S2. Morphology of microalgae in the reactor after 20-days of cultivation. (**a**) P21, and (**b**) WB1DG, Figure S3. Nitrite levels during cultivation of Chlorella sorokiniana P21 and WB1DG, Figure S4. Nitrogen profile in the control reactor, Figure S5. Phosphorous profile in the control reactor, Figure S6. Density of two strains of Microalga *C. sorokiniana* P21 (Black) and WB1DG (white) in BG 11 medium with different concentration of nutrients after 7-day cultivation. (**a**) Nitrogen and (**b**) Phosphorus.

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