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Pretreatment of poultry waste anaerobic digested effluents by chitosan flocculation for *Chlorella pyrenoidosa* growth and pollutants removal

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ABSTRACT

Poultry waste anaerobic digested effluents are biorefractory for microalgal cultivation. Hence, proper pretreatment is required to improve the conditions of digested effluent and enable the microalgal cultivation. This study evaluated the efficiency of nutrient removal in the digested effluents by pretreatment of chitosan flocculation and determined the potential of pretreated effluents to cultivate *Chlorella pyrenoidosa*. Approximately 82.1% of chemical oxygen demand, 91.6% of turbidity, and 99.3% of color in the digested effluents (without pH adjustment) were removed by chitosan flocculation (dissolved in hydrochloric acid) at an optimum chitosan concentration of 1.65 g/L. The maximum biomass production of 0.719 g/L and chlorophyll-a content of 13.71 mg/L were obtained from *C. pyrenoidosa* Y3 cultured for 20 d in the flocculated effluent at the optimum chitosan concentration. NH_4-N in the pretreated effluent decreased significantly by 77.2%. However, total phosphorus was reduced by algae to a certain extent (19.5%). Overall, the results indicate that chitosan flocculation is a feasible pretreatment to improve decontamination and algal biomass yield.

Keywords: Microalgae; Poultry anaerobic digested effluents; Nutrient removal; Chitosan; Flocculation

1. Introduction

The effluent from anaerobic digestion of poultry wastes usually contains large amounts of nutrients and organic compounds, which can be recycled back to culture microalgae [1–3]. Microalgae, particularly green algae (*Chlorophyta*), exhibit the prominent characteristic of nutrient removal and the ability to generate significant quantities of biomass suitable for conversion to biodiesel or bio-crude oil [4]. Several species of microalgae have been well documented, including *Spirulina platensis*, *Chlorella minutissima*, *Chlorella sorokiniana*, and *Scenedesmus bijuga*, which were used for post-treatment of anaerobic digested (AD) effluents from poultry wastes [3].

AD effluents from poultry wastes are primarily biorefractory for microalgal treatment because of their opaqueness, dark color, and high concentration of organic matter (COD). The dark color and high turbidity, which are attributed to humic substances (the major components of colored dissolved organic matter) [5] and suspended particulates, can block light penetration for microalgal growth and hence reduce the effectiveness of nutrient recovery from effluents. Therefore, a desirable pretreatment is required to improve the conditions of effluents prior to nutrient recovery by microalgae [6]. Some physical and chemical pretreatments, such as sedimentation, centrifugation, filtration, coagulation–flocculation, and precipitation, have been

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recommended for solid–liquid separation of livestock slurry [7]. Dilution is another pretreatment method commonly used to avoid light limitation and reduce the nutrient concentration of digested effluents.

Flocculation involving coagulation and coacervation is a transport step in which finely divided particles and destabilized particles are induced to form large aggregates or flocs as a result of destabilization [8,9]. The chemicals or substances added to promote flocculation, which are known as flocculants, are classified into chemical coagulants/flocculants, natural bioflocculants, and grafted flocculants [8,10]. Chitosan, a linear biopolymer of acetylamino-D-glucose derived from the deacetylation of chitin, is a promising bioflocculant widely applied in water and wastewater treatments [11,12]. When compared with conventional chemical flocculants, chitosan presents many remarkable properties, including non-toxicity, biocompatibility, biodegradability, renewability, and ecologically acceptable and outstanding pollutant-binding capacities [12,13]. Additionally, chitosan exhibits excellent adsorption capacity for contaminants dilute manure [14], dairy [15], piggery [6], olive mill [16], and cardboard-mill [17] wastewaters because of the presence of amino and hydroxyl functional groups [18]. However, little information describing the feasibility of integrating chitosan flocculation (CF) with microalgal biological systems for pollutants, or regarding turbidity and color removal from digested effluents with concomitant enhancement of biomass production, is available. Nevertheless, Depraetere et al. [6] reported that chitosan treatment with piggery wastewater resulted in color removal and a 50% increase in final biomass yield of Arthrospira.

The current study evaluated the efficiency of nutrient removal from poultry waste anaerobic digested effluents (PWADEs) by CF and determined the potential for use of flocculated effluents to cultivate *Chlorella pyrenoidosa*.

2. Materials and methods

2.1. Poultry waste anaerobic digested effluent

AD effluent was collected from a large-scale biogas plant located in a poultry farm in Beijing, China. The effluent was transported to the laboratory and stored at 4°C until use. The physical and chemical properties of PWADE are listed in Table 1. The raw effluent contained high concentrations of ammonia nitrogen (NH_4-N), total phosphorus (TP), total organic carbon (TOC), and COD_{cr}.

2.2. Preparation of chitosan solutions

Commercial chitosan (95% deacetylated; molecular weight of 50,000–80,000 g/mol), a fine white powder with <80 mesh size, was purchased from Jinan Haidebei Marine Bioengineering Co., Ltd (Shandong, China). Two stock solutions of 15 g/L each were prepared by dissolving chitosan powder in acetic acid and hydrochloric acid separately (Table 2). To prepare the first stock solution, 1.5 g of chitosan powder was hydrated in 95 mL of deionized water and stirred overnight at room temperature (20°C). Afterward, 5 mL of acetic acid (CH_3COOH) solution was added. The solution was stirred for another 3–4 h. The second stock solution was prepared by dissolving chitosan powder (1.5 g) in 100 mL of 0.5% hydrochloric acid (HCl) solution by stirring for 3–4 h at 300 rpm.

Table 1
Physicochemical properties of the poultry waste anaerobic digested effluents (PWADE)

Parameters	Values
NH_4-N (mg/L)	3,456
TP (mg/L)	457
TOC (mg/L)	6,375
COD (mg/L)	18,227
K (mg/L)	1,876
Na (mg/L)	446
Ca (mg/L)	152
Mg (mg/L)	59.3
Turbidity (NTU)	5,868
pH value	8.4

Table 2
The addition volumes of chitosan stock solutions (dissolved in CH_3COOH or HCl) and corresponding initial concentrations

Chitosan dissolved in CH_3COOH		Chitosan dissolved in HCl	
Addition volume (mL)	Initial concentration (mg/L)	Addition volume (mL)	Initial concentration (mg/L)
10	150	10	150
15	225	20	300
20	300	30	450
25	375	50	750
30	450	60	900
–	–	80	1,200
–	–	100	1,500
–	–	110	1,650

2.3. Experimental procedure

Raw PWADE was diluted tenfold prior to the flocculation process. The jar-test method was adopted to study the flocculation. Several beakers that each contained 1 L of 10% (v/v) PWADE were amended with different volumes of the two stock solutions (10–30 mL/L for chitosan in CH_3COOH and 10–110 mL/L for chitosan in HCl). The addition volumes and corresponding concentrations are listed in Table 2. Each beaker was stirred at 300 rpm for 2 min and then settled down for 2 h. The pH of the digested effluents was adjusted to 4.5, 6.0, 7.0, or 10.0 using 0.1 M HCl or 0.1 M NaOH, after which chitosan was added. The 10% (v/v) PWADE at the "natural" pH of 8.5 was simultaneously investigated. Supernatant from the top 2 cm of the suspension was collected to assess the pollutants and cultivate the microalgae.

2.4. Microalgal strain and cultivation in pretreated PWADE

The green microalgae *Chlorella pyrenoidosa* Y3 were obtained from the College of Biological Sciences of China Agricultural University. The *C. pyrenoidosa* Y3 samples were incubated at 20% (v/v) in 500 mL Erlenmeyer flasks containing

200 mL of pretreated effluents under static conditions. Three pretreated effluents (PE1, PE2, and PE3) flocculated by chitosan dissolved in 1.2, 1.5, and 1.65 g/L HCl, respectively, were evaluated at "natural" pH of 8.5. The flasks were placed in an illuminating growth chamber (GXZ, Dongqi, China) at 28°C ± 2°C under a light intensity of 250 μmol/m^2/s (Li-250A, Li-COR Lightmeter) with a 15:9 h light:dark cycle. The initial microalgal cell density in each experiment was approximately 0.1 g/L. All experiments were conducted for 20 d, and the pH value was not adjusted during the whole cultivation. The untreated 10% (v/v) PWADE after centrifugation was used as a control.

2.5. Microalgal biomass assay

Dry weight and chlorophyll-a content were used to evaluate the biomass yield of *C. pyrenoidosa* Y3. The linear relationship between dry weight (*DW*, g/L) and optical density $(OD)_{680}$ was previously determined for this strain as follows:

$$DW\ (g/L) = 0.24 \times OD_{680} - 0.074,\ R^2 = 0.999 \quad (1)$$

The chlorophyll-a content was measured using the method described by Oncel and Sukan [19].

2.6. Measurements

The NH_4-N, TP, COD, and turbidity of the effluents were analyzed using an ultraviolet spectrophotometer (TU-1810, PGENERAL, Beijing, China). The pH values were determined using a pH meter (PB-10, Sartorius, Germany). The absorbance at 475 nm, which was characterized by a brown color, was used to measure the true color [20]. The samples were centrifuged at 8,000 rpm for 10 min before the color measurement.

Color removal (*CR*, %) in the experiments was calculated as follows:

$$CR = (OD_i - OD_f)/OD_i \times 100 \quad (2)$$

where OD_i and OD_f are the initial and final optical densities (before and after the treatment), respectively [6]. The removal rates of NH_4-N, TP, COD, and turbidity were calculated using the same method described above.

2.7. Statistical analysis

All experiments were performed in triplicate for each treatment and measurement. Values were reported as the means of triplicate measurements with standard deviation. Data were statistically analyzed using statistical product and service solutions statistics software (version 19.0, IBM, Armonk, NY, USA). Statistical analysis was performed using one-way ANOVA with significant differences set at $P < 0.05$.

3. Results and discussion

3.1. Effect of chitosan dosage on pollutant removal efficiency for PWADEs

Fig. 1 shows the effects of chitosan dosage on flocculation performance in PWADE. To determine the preferred solvent for improving the flocculation capacity of chitosan, two solvents (acetic acid and hydrochloric acid) were evaluated. Chitosan dissolved in acetic acid demonstrated its flocculation ability in a relatively narrow range of 150–450 mg/L. The maximum COD and turbidity removal efficiency were 36.6% and 98.3%, respectively, at the optimum chitosan concentration of 225 mg/L, while the color removal was 86.1%. The COD and turbidity removal efficiency decreased to 33.0% and 98.1%, correspondingly, as the chitosan dosage was reduced beyond the limit concentration of 75 mg/L; however, their values were not significantly lower than the maximum removal efficiency ($P > 0.05$). As the chitosan dosage increased to 375 mg/L, the COD and turbidity removal efficiency declined to 13.6% and 97.5%, respectively. Finally, the COD of the treated effluent conversely increased by 23.8% when the chitosan dosage reached 450 mg/L.

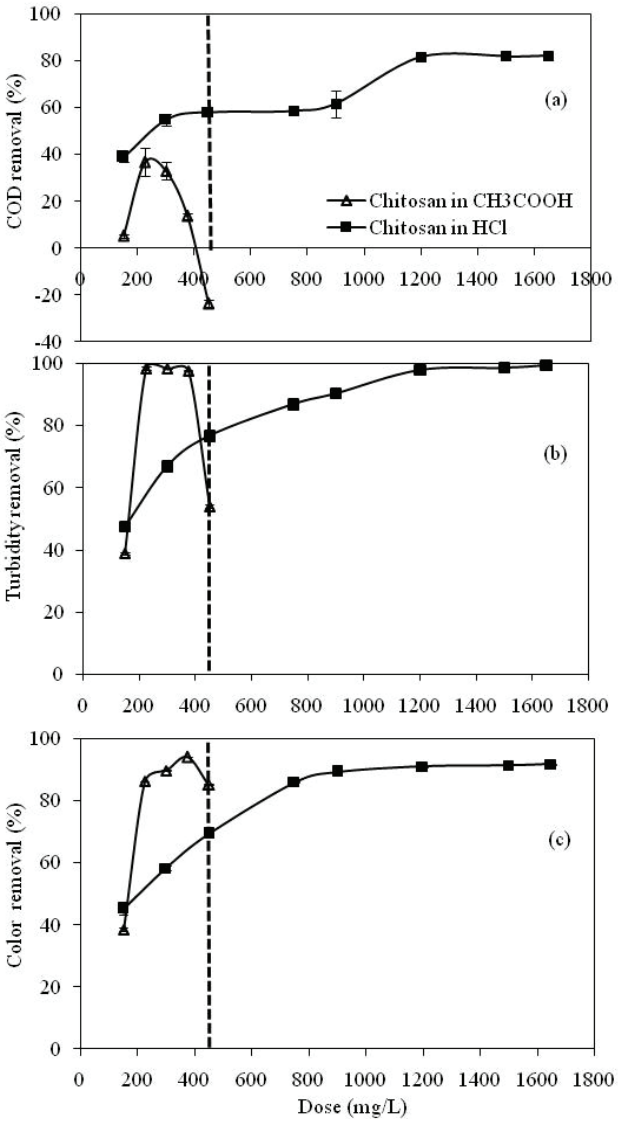

Fig. 1. Effect of chitosan dosage (dissolved in CH_3COOH and HCl) on the removal efficiency of COD (a), turbidity (b), and color (c) from pretreated effluents at the "natural" pH of 8.5. The dotted line represents the chitosan dose of 450 mg/L.

The optimum chitosan concentration was similar to the concentration selected to treat swine waste anaerobic digester effluent [14]. Altaher [18] obtained a similar result, indicating that the coagulation efficiency began to decrease when the chitosan dosage increased from 180 to 360 mg/L, and the final turbidity value reached 12.9 NTU at a dosage of 360 mg/L. These findings indicate that excessive addition of chitosan negatively affects the flocculation performance because overdosing can cause reversal of surface charge, as well as restabilization of coagulated particles [12,21,23]. Moreover, the efficiency of color removal increased from 38.5% to 93.9% as the chitosan dosage increased from 150 to 375 mg/L, but it declined to 85.1% at 450 mg/L (Fig. 1(c)). The flocculation process involves binding of insoluble particles (suspended solids or colloids) and/or dissolved organic matter (humic substances) into large agglomerates [17]. Humic substances such as humic acid and fulvic acid are primary components of the colored substances [5]. Therefore, chitosan may still reduce the true color of the effluent to a certain extent because excessive chitosan deprotonated in 10% (v/v) PWADE (pH 8.5) can promote flocculation via sweep-out mechanism [22,23].

Chitosan dissolved in hydrochloric acid showed an outstanding flocculation capacity at dosages ranging from 150 to 1,650 mg/L (Fig. 1), indicating that 1,650 mg/L chitosan did not reach the upper limit, and overdose phenomenon did not occur. The COD, turbidity, and color in PWADE decreased by 38.9%–82.1%, 47.4%–99.3%, and 45.2%–91.6%, correspondingly, with chitosan dosages varying from 150 to 1,650 mg/L. As shown in Fig. 1(a), the COD removal efficiency was not significantly affected by chitosan dosages ranging from 300 to 900 mg/L ($P > 0.05$). The COD removal efficiency peaked at 82.1% at a chitosan dosage of 1,650 mg/L. The COD and color removal efficiency remained constant when the chitosan dosage increased from 1,200 to 1,650 mg/L. These findings indicate that more chitosan can provide more adsorption sites and improve the opportunities for interaction of contaminants in wastewater with chitosan [24]. Furthermore, the adsorption sites eventually reached saturation because of the decreased adsorptive capacity of the adsorbent utilization [25].

The dotted line in Fig. 1 represents the chitosan dosage of 450 mg/L. The flocculation efficiency below this concentration was compared between chitosan dissolved in hydrochloric acid and chitosan dissolved in acetic acid. The former exerted better flocculation capacity resulting in COD removal of 58.0% at a dosage of 450 mg/L, whereas the latter removed COD by a maximum efficiency of 36.6% at the optimal dosage of 225 mg/L. Nevertheless, chitosan dissolved in acetic acid was superior to chitosan dissolved in hydrochloric acid, with respect to the turbidity and color removal below 450 mg/L. However, the final COD concentration (1,156.3 mg/L) of the treated effluent (flocculated by chitosan dissolved in acetic acid at the optimal dosage) was still higher and not suitable for subsequent microalgal cultivation. This finding may be ascribed to the presence of acetic acid, which not only increased the organic content of suspensions coagulated by chitosan but also increased the carbon content of the chitosan solution [18,26]. Accordingly, hydrochloric acid was selected as the optimal solvent for further studies, and the optimum chitosan concentration was 1,650 mg/L.

3.2. Effect of the initial pH of effluents on the pollutant removal efficiency for PWADE

Chitosan exerts double effects (coagulating and flocculating effects) on the coagulation–flocculation process in terms of its cationic behavior, molecular weight, and precipitation properties [22,27]. Different mechanisms involved in alleviating the pollutant concentrations depend on the pH of the effluent. The influence of the initial pH of the effluents (4.5, 6.0, 7.0, and 10.0) on the COD, turbidity, and color removal efficiency by chitosan dissolved in HCl at a concentration of 1.2 g/L is shown in Fig. 2. The maximum removal efficiencies of 88.5% and 99.7% for the COD and turbidity, respectively, were detected in the effluents at pH 4.5. Correspondingly, these efficiencies decreased to 85.6% and 96.8% at an initial pH of 6.0. The efficiencies for the removal of COD and

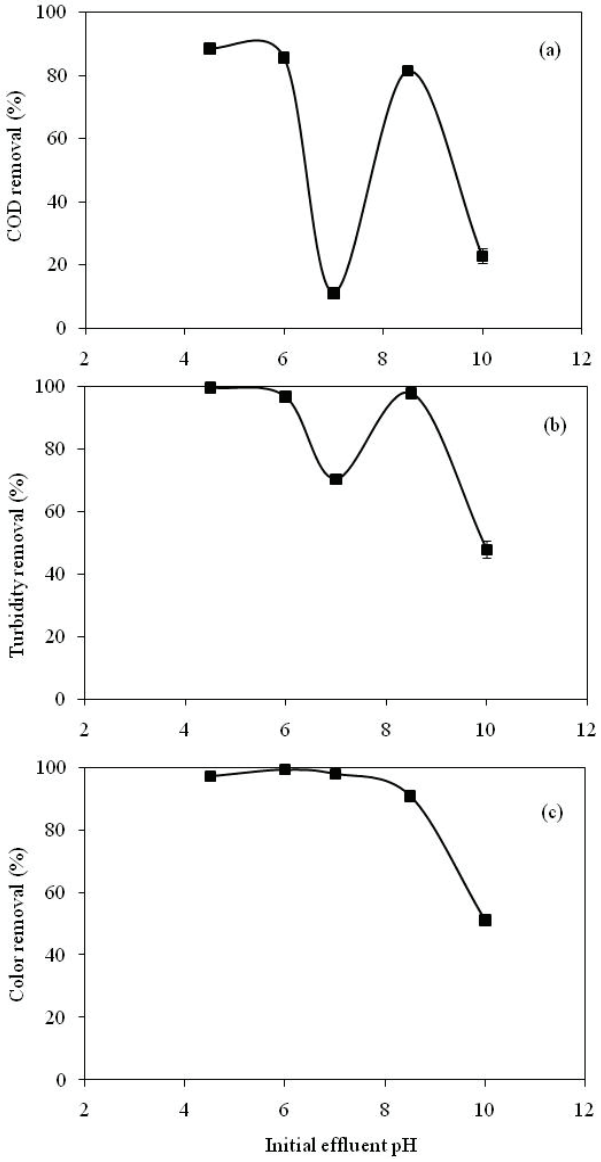

Fig. 2. Effect of the initial effluent pH on the removal efficiency of COD (a), turbidity (b), and color (c) by chitosan dissolved in HCl at 1.2 g/L.

turbidity decreased sharply at an initial effluent pH of 7.0 and recovered to 81.4% and 97.9% at the "natural" pH of 8.5. The COD and turbidity removal efficiencies then decreased again at pH 10.0. Chitosan demonstrated remarkable COD and turbidity removal efficiencies under acidic or slightly alkaline conditions. The turbidity removal observed in the present study was similar to that reported by Altaher [18]. The highest color removal efficiency of 99.4% was achieved at pH 6.0, which was in agreement with the findings reported by Bratskaya et al. [28]. The effectiveness of color removal (i.e., removal of humic substances) was mainly governed by the initial pH, mass ratio of chitosan/humic substances, and settling time [28,29]. In the present study, the optimum pH for color removal was determined by the three factors above.

Chitosan, as a cationic flocculant, can reduce negatively charged contaminants, such as dissolved contaminants (humic substances) and organic suspensions, through charge neutralization (coagulation mechanism) and particle entrapment (flocculation mechanism) either simultaneously or independently [22]. Huang and Chen [21] noticed that chitosan maintained a positive range while pH was below 8.0 and carried higher positive charges at lower pH values. The amino functional groups in chitosan were protonated, partially protonated, and fully deprotonated at acidic (4.5 and 6.0), neutral (7.0), and alkaline (8.5 and 10.0) pH, respectively [27]. At initial pH values of 4.5 and 6.0, charge neutralization was the dominant mechanism. At neutral pH, both coagulation and flocculation mechanisms were involved [22]. At high pH (8.5 and 10.0), chitosan became insoluble and exerted a flocculating effect. Polymer reprecipitation can physically entrap organic particles in its network through a bridging mechanism [27]. Additionally, greater dosages of chitosan were needed for higher pH values to reach the target COD or turbidity [22,27]. Our results indicated that 1.2 g/L chitosan at pH 4.5 did not lead to overdosing. The COD and turbidity removal efficiencies continuously decreased until the pH reached 8.5 because of insufficient amounts of chitosan at pH 6.0 and 7.0. At alkaline pH, the predominant mechanism was interparticle bridging, which was sensitive to the chain length and molecular weight of chitosan [27] instead of the dosing requirement. However, the precipitation of chitosan was limited in its ability to compensate for the loss of charge neutralization effect at pH 10.0. Finally, the removal efficiency declined again [29].

In the current study, the optimum pH value was determined not only by the flocculation performance, but also with consideration of subsequent microalgal cultivation. The final pH values of the flocculated effluents were 2.9, 5.7, 6.6, 7.3, and 9.7 (data not shown). For *Chlorella* growth, the optimum pH was approximately 6.3–7.5 [30,31]. Moreover, the AD effluents were highly buffered [32]. As a result, increased amounts of acid or base were needed for pH adjustment, which seemed uneconomical. Therefore, pH 8.5 (the "natural" pH of the AD effluent) was selected for cultivation of *C. pyrenoidosa* Y3.

3.3. Effect of chitosan flocculation pretreatment for PWADE on biomass production of C. pyrenoidosa Y3

Effect of CF on pollutant removal in 10% (v/v) PWADE is shown in Table 3. CF1, CF2, and CF3 represent the flocculation by chitosan dissolved in HCl at 1.2, 1.5, and 1.65 g/L, respectively, at a "natural" pH of 8.5. The untreated 10% (v/v) PWADE after centrifugation was used as a control. The COD, color, turbidity, and pH values in the PWADE were reduced significantly by CF treatment ($P < 0.05$). In contrast, NH_4^+ and TP were not decreased. The minimum COD, color, and turbidity values of 326.5 mg/L, 0.073 AU, and 4.3 NTU were obtained in the effluent flocculated by chitosan at 1.65 g/L. These values were significantly lower than those of the effluents flocculated by chitosan at 1.2 and 1.5 g/L ($P < 0.05$). The final pH of the flocculated effluents was approximately 7.0–7.3, which was suitable for microalgal cultivation. Up to 24.3% NH_4^+ and 17.2% TP were removed by centrifugation, and their removal efficiencies were approximately 20.6%–24.5% and 17.1%–26.1% in the three flocculated effluents, respectively. These findings implied that the inorganic N and P fractions, which existed in the form of soluble NH_4^+ and TP, were barely affected by the flocculation treatment [6,33]. Depraetere et al. [6] mentioned that the methods applied to remove color and organic pollutants should avoid reducing the nitrogen and phosphate concentrations from wastewater so that they can be reused.

In the current study, ammonia nitrogen was the primary form of nitrogen in the PWADE and could inhibit excessive algal growth. *Chlorella* can tolerate 18–400 mg/L NH_4-N [34]. In the current study, a NH_4^+ level ranging from 261.5 to 274.5 mg/L in the control and flocculated effluents did not inhibit the growth of *C. pyrenoidosa* Y3. As shown in Fig. 3, microalgae could survive in the three flocculated effluents and then accumulate biomass. While microalgae were

Table 3
Effect of the chitosan flocculation (CF) on the pollutant removal in the 10% (v/v) PWADE

Chitosan flocculation	NH_4-N (mg/L)	TP (mg/L)	COD (mg/L)	Color[1] (AU)	Turbidity (NTU)	Final pH
CF1	274.5 ± 4.0[a]	37.8 ± 0.4[a]	339.3 ± 1.7[b]	0.079 ± 0.003[b]	12.2 ± 0.9[b]	7.3 ± 0.2[b]
CF2	265.9 ± 2.0[b]	37.5 ± 0.4[a]	332.2 ± 0.4[c]	0.077 ± 0.002[b]	8.6 ± 0.0[c]	7.1 ± 0.0[b]
CF3	261.0 ± 1.4[c]	33.7 ± 0.2[b]	326.5 ± 1.9[d]	0.073 ± 0.001[c]	4.3 ± 0.3[d]	7.0 ± 0.1[b]
Control	261.5 ± 5.1[c]	37.8 ± 1.0[a]	1,099.5 ± 8.7[a]	0.867 ± 0.01[a]	31.1 ± 0.8[a]	8.5 ± 0.1[a]

Note: Values were the means of triplicate measurements ± standard deviation.
Control, CF1, CF2, and CF3 represent 10% (v/v) centrifuged PWADE, the flocculation by chitosan dissolved in HCl at 1.2, 1.5, and 1.65 g/L at "natural" pH, respectively.
Significant differences at $P < 0.05$ are indicated with different letters in the same column(a–d).
[1]AU: absorbance units measured at 475 nm.

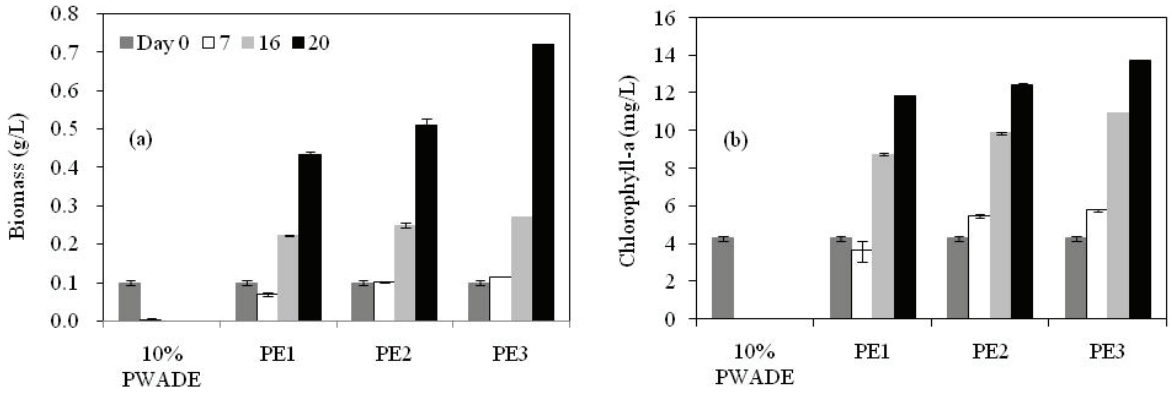

Fig. 3. Growth performance of *C. pyrenoidosa* Y3 in 10% (v/v) PWADE (control) and three pretreated effluents (PE1, PE2, and PE3 represent the effluents flocculated by chitosan dissolved in HCl at 1.2, 1.5, and 1.65 g/L at "natural" pH, respectively).

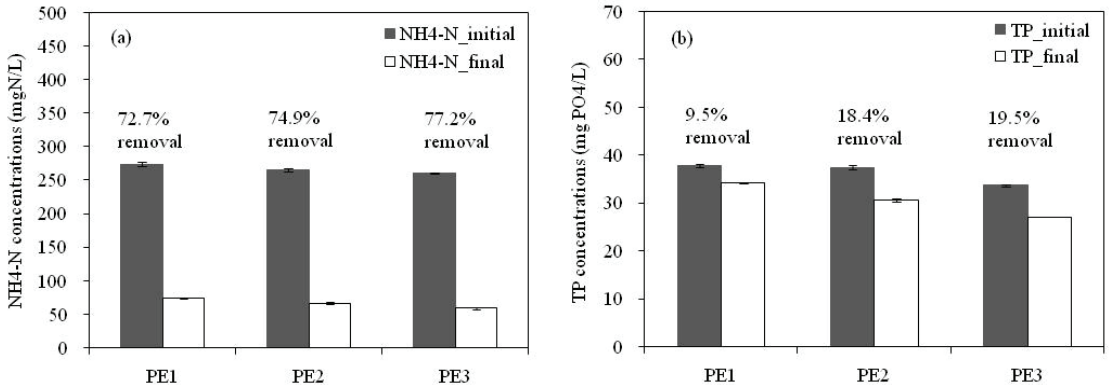

Fig. 4. (a) Initial and final NH_4-N concentrations and removal rates after 20 d cultivation, and (b) initial and final TP concentrations and removal rates after 20 d cultivation (PE1, PE2, and PE3 represent the effluents flocculated by chitosan dissolved in HCl at 1.2, 1.5, and 1.65 g/L at "natural" pH, respectively).

cultured in the control, microalgal growth was completely inhibited as previously reported [35], and microalgal death ultimately occurred on the 7th day after inoculation probably because of the limitation of other environmental factors such as high COD, dark color, high turbidity, and improper pH. In addition, a 7-d lag phase for microalgae grown in flocculated effluents was attributed to the high level of nutrients and low inoculation quantity. From day 8, microalgae began to grow rapidly. Eventually, the maximum biomass production of 0.719 g/L was yielded by *C. pyrenoidosa* Y3 cultured for 20 d in the effluent pretreated by CF at 1.65 g/L. Furthermore, the chlorophyll-a content reached 13.71 mg/L. Algal biomass accumulation in the two other flocculated effluents was significantly lower than the maximum ($P < 0.05$). At the end of the cultivation period, *C. pyrenoidosa* Y3 were still growing exponentially and had not entered into the stationary phase.

3.4. Nutrient removal in pretreated PWADE by C. pyrenoidosa Y3 cultivation

The initial and final concentrations of NH_4-N and TP with the removal efficiencies by *C. pyrenoidosa* Y3 during 20 d of growth are shown in Fig. 4. NH_4-N in the pretreated PWADE decreased significantly by 72.7%–77.2%. However, TP was reduced by algae to a certain extent (9.5%–19.5%). For microalgae, the efficiency of nutrient removal from wastewater depends on the Redfield ratio [7], and PWADE is characterized by nitrogen richness and carbon deficiency. Consequently, carbon became the limiting factor for nutrient removal during the whole cultivation in this study.

4. Conclusions

In PWADE, CF can efficiently remove COD, turbidity, and color but not NH_4-N and TP. Approximately 82.1% of COD, 91.6% of turbidity, and 99.3% of color in the digested effluents (without pH adjustment) were removed by CF (dissolved in hydrochloric acid) at an optimum chitosan concentration of 1.65 g/L. Flocculated effluents can be used as substrates to cultivate microalgae and accumulate algal biomass. The maximum biomass production of 0.719 g/L and chlorophyll-a content of 13.71 mg/L were obtained from *C. pyrenoidosa* Y3 cultured for 20 d in the flocculated effluent at the optimum chitosan concentration. The findings of this study indicate that CF is a potential pretreatment for AD effluents and algal biomass yield.

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