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Enhancing Algal Yield and Nutrient Removal from Anaerobic Digestion Piggery Effluent by an Integrated Process-Optimization Strategy of Fungal Decolorization and Microalgae Cultivation

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Abstract: The dark brown anaerobic digestion piggery effluent (ADPE) with a large amount of ammonium generally needs high dilution before microalgae cultivation due to its inhibiting effects on algal growth. Due to the strong decolorization of fungi by degrading organic compounds in wastewater, the process-optimization integrated strategy of fungal decolorization of ADPE and subsequent microalgae cultivation with ammonium-tolerant strain may be a more reliable procedure to reduce the dilution ratio and enhance algal biomass production, and nutrient removal from ADPE. This study determined a suitable fungal strain for ADPE decolorization, which was isolated and screened from a local biogas plant, and identified using 26s rRNA gene sequence analysis. Subsequently, ADPE was pretreated by fungal decolorization to make low-diluted ADPE suitable for the algal growth, and conditions of microalgae cultivation were optimized to achieve maximum algal yield and nutrient removal from the pretreated ADPE. The results showed one promising locally isolated fungal strain, Nanchang University-27, which was selected out of three candidates and identified as *Lichtheimia ornata*, presenting a high decolorization to ADPE through fungal pretreatment. Five-fold low-diluted ADPE pretreated by *L. ornata* was the most suitable medium for the algal growth at an initial concentration of ammonium nitrogen of 380 mg L^{-1} in all dilution treatments. Initial optical density of 0.3 and pH of 9.0 were optimal culture conditions for the algal strain to provide the maximum algal yield (optical density = 2.1) and nutrient removal (88%, 58%, 65%, and 77% for the removal rates of ammonium nitrogen, total nitrogen, total phosphorus, and chemical oxygen demand, respectively) from the pretreated ADPE. This study demonstrated that fungal decolorization and subsequent microalgae cultivation could be a promising approach to algal biomass production and nutrient removal from ADPE.

Keywords: bio-digester effluent; fungal decolorization; process optimization; algal growth; nutrient removal

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1. Introduction

Pork is the most prevalent consumed meat globally, according to the report named "Global Pork Meat Market 2017–2021" [1]. With a surging pork demand, quantities of pig farms were pursuing intensive and large-scale development, producing a large amount of piggery wastewater mainly derived from the manure, urine, and the water used for washing off piggery shed [2,3]. Anaerobic digestion (AD) is commonly applied in the large-scale treatment of piggery wastewater [4] because it can convert part of the organic matter into methane-rich biogas as an energy source.

AD effluent (ADE) from piggery (ADPE), the byproduct of anaerobic microorganism degradation of organics from piggery wastewater, is enriched in high levels of organic and inorganic compounds, heavy metals, antibiotics, and hormones [5]. Microalgae is a group of unicellular organisms with excellent performance in nutrient assimilation and value-added biomass accumulation during growth, widely applied for the treatment of municipal, agricultural, and industrial wastewaters [6–10], including ADPE [11]. For example, Debowski et al. [9] reported that a mixed-culture microalgae showed effective biomass production (3 g L^{-1}) and high nutrient removal rates in ADE from dairy wastewater, reaching above 90% of organic compound and nutrient removal. The harvested algal biomass with abundant carbohydrates, lipids, and proteins can be developed for multi-purpose valuable products such as biogas, biofuel, animal feed, and chemicals (e.g., carotenoids, antioxidants, and polyunsaturated fatty acids) [10]. In addition, microalgae were reported to significantly contribute to atmospheric carbon balance by CO_2 fixation and O_2 emission [12]. In recent decades, microalgae-based wastewater remediation has emerged into the limelight in the field of wastewater treatment owing to the above advantages.

Some recent studies explored the feasibility of using microalgae for ADPE remediation [13,14]. However, current barriers to adopting algal cultivation for ADPE treatment are the quite high turbidity (dark brown color) and ammonium (NH_4^+) concentration, inhibiting algal growth [13]. The dark brown ADPE prevents the passage of sunlight through water bodies and thus greatly inhibits photosynthesis for algal growth [15]. High NH_4^+ content easily shifts the chemical equilibrium from NH_4^+ to free ammonia (NH_3) at high pH during algal photosynthesis [13]. Free NH_3 can easily diffuse through cell membrane and accumulate in cytoplasm of algal cells, being toxic for most strains of microalgae due to its uncoupling effects on photosynthesis in chloroplasts [16,17]. Thus, ADPE pretreatment are required to alleviate the color inhibition and ammonia toxicity for subsequent microalgae cultivation. Li et al. [18] reported that ammonia stripping as an energy-intensive pretreatment could only maintain the level of NH_4^+ from ADPE below the microalgae tolerance, but the dark color still inhibited algal growth. Physical and chemical methods such as electro-fenton coagulation and inorganic coagulants are already known for ADE decolorization [19,20], but these methods are energy intensive and high cost, which do not comply with economic viability. Dilution method may be an economic solution overcoming the dark color of ADPE by dilution fold. However, in most previous studies, ADPE and other anaerobically digested manure needed high dilution to reduce color inhibition and NH_4^+ toxicity before microalgae cultivation [11,21]. Kwon et al. [11] investigated that the algal yield of *Chlorella vulgaris* peaked at an initial concentration of ammonium nitrogen (NH_4^+−N) of 100 mg L^{-1} in 50-fold diluted ADPE but was inhibited at higher NH_4^+−N concentrations in 10- and 20-fold diluted ones. Wang et al. [21] showed that higher algal yields were obtained at initial NH_4^+-N concentration of 110 mg L^{-1} in 20-fold diluted ADE from dairy manure rather than 10-, 15-, and 25-fold diluted ones during *Chlorella* sp. culture. These results might be because NH^+-N at a concentration of 110 mg L^{-1} was critical for NH_4^+ tolerance in green algae, including *Chlorella* spp. [22]. Indeed, high dilution pretreatment can alleviate the color inhibition and ammonia toxicity in ADPE but increase water consumption and reduce the nutrient concentrations, limiting algal growth. To reduce the high dilution of ADPE, a decolorization pretreatment of ADPE using NH_4^+-tolerant algal species may be required.

In recent years, fungi have become promising microorganisms in wastewater decolorization due to their low energy consumption and cost [15,23,24], compared to electrocoagulation and chemical reagents. Fungi have a strong ability to degrade complex organic compounds during decolorization by producing extracellular enzymes, such as laccase, manganese peroxidase and lignin peroxidase [25]. For example, Manimozhi and Kaviyarasan [24] isolated a fungal strain, *Achromobacter xylosoxidans* GRIRKNM11, from a textile dye effluent site, which could decolorize turquoise blue dye within 48 h. Currently, fungal decolorization is mostly used in the treatments of the paper mill and textile dyes' effluent [25]. For microalgae cultivation in wastewater through fungal decolorization, only Liu

et al. reported that *Phanerochaete chrysosporium* could achieve decolorization of 80% from ADPE at a pH of 6.3 and 25 °C [15], and *Chlorella vulgaris* could survive in the decolorized ADPE but with low removal rates of <12% for NH_4^+-N, TP, and COD [26]. The authors only focused on the feasibility of algal survival in the decolorized ADPE and its potential value of biofuel, but did not enhance the algal yield and nutrient removal from the ADPE through process optimization of microalgae cultivation [26]. Therefore, the integrated process-optimization strategy of fungal pretreatment and subsequent microalgae cultivation may be a more reliable procedure for algal biomass production and nutrient removal from ADPE. This novel concept of fungal pretreatment with high decolorization provides a promising way for microalgae-based wastewater remediation. The concept is needed to further optimize process to enhance algal yield and nutrient removal in microalgae-based wastewater remediation, including ADPE.

This study was designed to solve the technical problems encountered in ADPE remediation using microalgae. ADPE was pretreated by fungal decolorization to make low-diluted ADPE suitable for algal growth, and the conditions of microalgae cultivation were optimized to achieve maximum algal yield and nutrient removal from the pretreated ADPE. With the implementation of the novel concept of fungal decolorization pretreatment, microalgae-based ADPE remediation is expected to be a more practical process in terms of algal biomass production and nutrient removal.

2. Materials and Methods

2.1. ADPE Preparation

The ADPE used for all experiments was collected from the anaerobic digester in the Zhenghe Biogas Plant in Xinyu city, Jiangxi Province, China. The digester collected and digested piggery manure within 30 km around to obtain biogas by continuous stirred tank reactor (CSTR), simultaneously producing approximately 1000 m^3 of ADPE per day. All the collected ADPE were filtered through a solid-liquid centrifugal separator (LLW800, KAIDI, Suzhou, China) to remove large, suspended solids and stored at 4 °C until use.

2.2. Selection and Growth of Fungal Strains and ADPE Pretreatment

Three fungal strains were selected for ADPE pretreatment and isolated from soil samples near Zhenghe Biogas Plant using the dilution plating procedure. The isolated strains were named after Nanchang University (NU) and labeled as NU-3, NU-8, and NU-27, respectively. Afterward, they were cultured in Rose Bengal Agar slants (Table S1) at 30 ± 1 °C for 48 h and then stored at 4 °C until use.

To harvest fungal spores for mycelium pellet production, the agar slant was filled with 5 mL of distilled water and agitated by a vortex mixer for 1 min to make spores suspend in distilled water. This 5 mL spore suspension was inoculated into the PDB medium (Table S2) with a 500 mL working volume at an initial spore density of about 2×10^5 ind mL^{-1} in a 1 L Erlenmeyer flask. The spore suspension for each fungal strain was cultured at 30 ± 1 °C on an orbital shaker (TS-2102GZ, Tensuc, Shanghai, China) (140 rpm, 2 d) to form enough mycelium pellets.

These mycelium pellets were harvested using the double-layer gauze with a pore size of 0.425 mm and cultured with the diluted ADPE at initial pH 4 with a working volume of 10 L in a 15 L homemade airlift bioreactor (Figure 1a). In addition, the diluted ADPE in the bioreactor was added an extra 2 g L^{-1} glucose. This was because glucose supplementation was accessible for fungal consumption and could improve the stability and decolorization performance of the fungal pellets [27]. The temperature in the bioreactor was controlled at 30 ± 1 °C using an aquarium heater (YGR-002, Yee, Shanghai, China). Aeration inside the bioreactor was filtered through a syringe filter with a pore size of 0.45 μm (PES, Jingteng, Tianjin, China) and controlled at 1 L min^{-1} using an air pump (OKEY, Taizhou, China), which allowed the mycelium pellets to be mixed. All of the pretreated ADPEs were sterilized in the autoclave (BXM-30R, BOXUN, Shanghai, China) (121 °C, 40 min) to remove fungus and bacteria before microalgae cultivation.

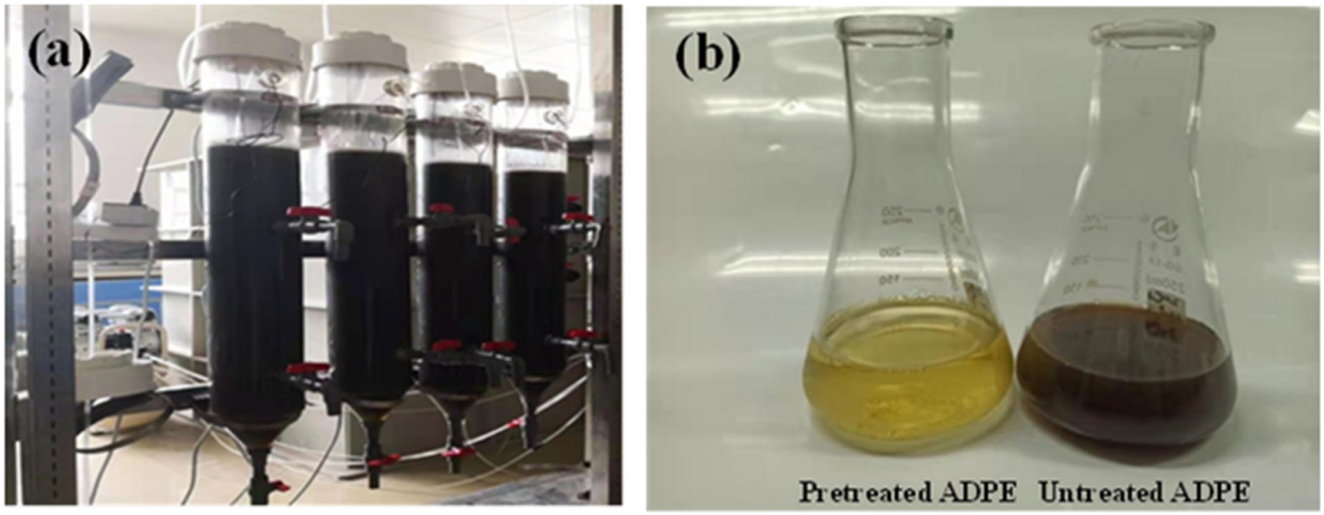

Figure 1. Homemade airlift bioreactor for the decolorization of anaerobic digestion piggery effluent (ADPE) through fungal pretreatment (**a**) and 10-fold (10×) diluted ADPE with and without fungal pretreatment (**b**).

In order to determine decolorization percentage, 2 mL of ADPE was collected to measure the maximum absorbance wavelength of 310 nm using a spectrophotometer (DR 6000, Hach, Loveland, CO, USA). The decolorization percentage (D, %) was calculated according to Equation (1).

$$D = (A_0 - A_t)/A_0 \quad (1)$$

where A_0 and A_t denote the absorbances of ADPE on day 0 and day t during the experiment of fungal pretreatment, respectively.

2.3. Algal Strain and Pre-Culture Conditions

One algal strain used for ADPE remediation was isolated from the waterbody in a peripheral ditch surrounding Maiyuan Landfill Plant, Nanchang City, Jiangxi Province, China, which was named Nanchang University (NCU)-39, and identified using our previous methods [28]. The genomic DNA of microalgae was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was conducted to amplify the extracted 18s rRNA gene. The amplified 18s RNA gene sequences were aligned and compared with the identified microorganisms in the GenBank database (National Center for Biotechnology Information). Based on sequence similarity (Table S3) and phylogenetic (Figure S1) analysis, NCU-39 was identified as a strain of *Chlorella pyrenoidosa*, which has been widely applied for the remediation of wastewater (including ADPE), because it can grow in a mixotrophic mode to achieve high biomass production and nutrient removal [29]. As the growth rate and plateau level of NCU-39 in Tap medium (Table S4) containing the abundance of organic acetic acid were quite higher than those in BG11 medium (Table S5) containing inorganic Na_2CO_3 (Figure S2), NCU-39 as a mixotrophic strain can be used for ADPE remediation.

NCU-39 was maintained in Tap medium (Table S4) and pre-cultured with 200 mL working volume in a 500 mL flask. The algae were incubated at 28 ± 1 °C with a continuous light intensity of 5000 lux in a growth cabinet (GTOP, TP, Hangzhou, China).

2.4. Chemical Analysis

To determine the chemical characteristics of ADPE and culture media, the collected samples were filtered through a 0.45 μm pore-size syringe filter (Jinteng, Tianjin, China) to remove suspended materials. The concentrations of NH_4^+-N, total nitrogen (TN), total phosphorus (TP) and chemical oxygen demand (COD) in the filtrates were measured using a Hach kit (DR/890 Colorimeter, Hach, Loveland, CO, USA).

2.5. Microalgae Cultivation

NCU-39 cells were centrifuged at 8000 rpm for 5 min. The centrifuged pellets were rinsed twice and re-suspended in distilled water to avoid the influence of medium nutrients. Each algal suspension was inoculated into 200 mL of culture medium (diluted ADPE, appropriately pretreated ADPE or Tap medium) at an initial optical density (OD) of 0.2–0.6 in a 500 mL flask. These flasks were incubated under the same condition of temperature and light as that of pre-cultivation. They were set on an orbital shaker (TS-2102GZ, Tensuc, Shanghai, China) at 120 rpm to avoid sticking and sedimentation of algal cells. OD of the algal suspension in the flask was measured daily or every two days at 680 nm with a spectrophotometer for monitoring algal growth.

To determine nutrient (NH_4^+-N, TN, TP, and COD) removal rates throughout the experiment (including fungal decolorization and microalgae cultivation), the algal suspension was filtered through the 0.45 μm pore-size syringe filter to remove suspended materials. Then, the nutrient concentrations in the filtrate were measured daily during the experiment using the Hach kit as described above. The removal rate (R, %) of nutrients was calculated using the following Equation (2):

$$R = (C_i - C_f)/C_i \times 100 \quad (2)$$

where C_i and C_f were the concentrations (mg L^{-1}) of nutrients (NH_4^+-N, TN, TP, and COD) on the initial day before fungal decolorization and the final day after microalgae cultivation, respectively.

2.6. Experimental Design

2.6.1. Microalgae Cultivation in Diluted ADPEs

The dark brown ADPE with a high NH_4^+ concentration inhibits algal growth, as described above. In order to avoid the inhibitions, NCU-39 was cultured in 10-fold (10×), 15×, and 20× diluted ADPEs with distilled water to determine dilution effects on algal growth in the ADPEs.

2.6.2. Screening of Fungal Strains

The 10× diluted ADPE was decolorized by each of three fungal strains (NU-3, NU-8, and NU-27) for five days in the homemade airlift bioreactor, followed by the fungal pretreatment as described above (see Section 2.2). Then, NCU-39 was incubated in the diluted ADPEs with and without fungal pretreatment, and the algal growth was monitored to select the most suitable fungal strain for ADPE pretreatment.

2.6.3. Identification of Fungal Strain

The genomic DNA of the selected fungus was extracted using the Fungi Genomic DNA Extraction Kit (Cat#D2300, Solarbio, Beijing, China). The selected fungus was identified by sequencing the 26s rRNA D1/D2 gene regions. The following primer sequences were employed: NL1: GCATATCAATAAGCGGAGGAAAAG and NL4: GGTCCGTGTTTCAAGACGG. PCR was carried out with a temperature program: initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, polymerization at 72 °C for 30 s, and final elongation at 72 °C for 10 min. The PCR products were separated by electrophoresis on 1% agarose gels and purified using the gel purification kit (D2500-02, OMEGA Bio Inc., Norcross, GA, USA) as instructed by the manufacturer. The DNA samples were sequenced using an ABI 3730XL sequencer (SG, Shanghai, China). The obtained sequences were trimmed and analyzed using BLAST analysis.

2.6.4. Process Optimization

As the maximum decolorization percentage was obtained in 3-day NU-27 pretreatment (see Results), NCU-39 was cultured in each of 3×, 5×, and 10× diluted ADPEs pretreated by NU-27 at initial pH 7.0 for three days, to determine the optimal dilution rate for algal growth.

Then, NCU-39 was cultured in the pretreated ADPEs at initial pH 7 but four different initial ODs of 0.2, 0.3, 0.4, and 0.6 to determine suitable inoculum density for algal growth. Finally, it was incubated in the pretreated ADPEs at different initial pHs (7.0, 8.0, and 9.0) to select the most suitable initial pH for reaching the maximum algal yield and nutrient removal. In addition, NCU-39 was cultured in the Tap medium (Table S4) as a control. Initial ODs of 0.2, 0.3, 0.4, and 0.6 in the pretreated ADPE were determined as initial inoculum densities of ca. 2.8, 5.6, 11.2, and 22.4 $\times$ 10^6 cells mL^{-1}, respectively. All initial pH levels in the pretreated ADPE were adjusted using hydrochloric acid (HCl) and sodium hydroxide (NaOH).

2.7. Statistical Analysis

All experiments in this study were conducted in triplicate (n = 3). The results were expressed as arithmetic means $\pm$ standard deviations. Differences in the concentrations of nutrients (NH_4^+-N, TN, TP, and COD) among the initial pH treatments at the end of the experiments were tested with analysis of variance (ANOVA). Further, upon a significant difference being observed in ANOVA, the Tukey–Kramer post hoc tests were conducted. All statistical analyses were performed using Origin software (OriginLab, Hampton, MA, USA), with a significance level set at $p < 0.05$.

3. Results and Discussion

3.1. Chemical Composition of ADPE

The concentrations of NH_4^+-N, TN, TP, and COD were examined to be 2098, 2306, 110, and 11,257 mg L^{-1}, respectively, in raw ADPE (Table 1), among which those of NH_4^+-N and TP values were substantially higher than those of Tap medium (Table S4). These major nutrients in ADPE, when compared with Tap medium, presented sufficient concentrations for algal growth.

Table 1. Chemical composition of raw anaerobic digestion piggery effluents (ADPEs). The results are presented as arithmetic means $\pm$ standard deviations (n = 3).

Nutrients and Metals (mg L^{-1})	Raw ADPE
NH_4^+-N	2098.4 $\pm$ 4.5
TN	2305.6 $\pm$ 6.1
TP	110.1 $\pm$ 1.5
COD	11,256.7 $\pm$ 20.7
Zn	64.2 $\pm$ 0.6
Fe	65.6 $\pm$ 0.8
Cr	0.3 $\pm$ 0.02
Cu	0.01 $\pm$ 0.001
Cd	0.01 $\pm$ 0.002
Pb	0.002 $\pm$ 0.001
As	0.05 $\pm$ 0.002
Hg	0.02 $\pm$ 0.001
pH	8.3

3.2. Dilution Effect on Algal Growth in ADPE

As the dark brown color and high NH_4^+ concentration of raw ADPE hindered algal growth as described above, 10$\times$, 15$\times$, and 20$\times$ diluted ADPEs were used for microalgae cultivation to determine the dilution effect on algal growth in ADPE. The findings showed that algal growth of NCU-39 was completely inhibited in 10$\times$ and 15$\times$ diluted ADPEs at initial NH_4^+-N concentrations of 210 and 140 mg L^{-1}, respectively; in contrast, algal growth was observed in 20$\times$ diluted ADPE at initial NH_4^+-N concentration of 105 mg L^{-1} (Figure 2 and Table 1). Generally speaking, NH_4^+-N concentration of 110 mg L^{-1} was critical for NH_4^+ tolerance in green algae, including *Chlorella* spp. [22]. Nevertheless, NCU-39 isolated from NH_4^+-rich waterbody in a ditch surrounding a landfill plant might present a great NH_4^+ tolerance above 110 mg L^{-1}. The complete growth inhibition might be attributed

to the dark brown color of the diluted ADPEs. To confirm this, we cultured NCU-39 in 10× diluted ADPE pretreated by fungal decolorization for the following experiment.

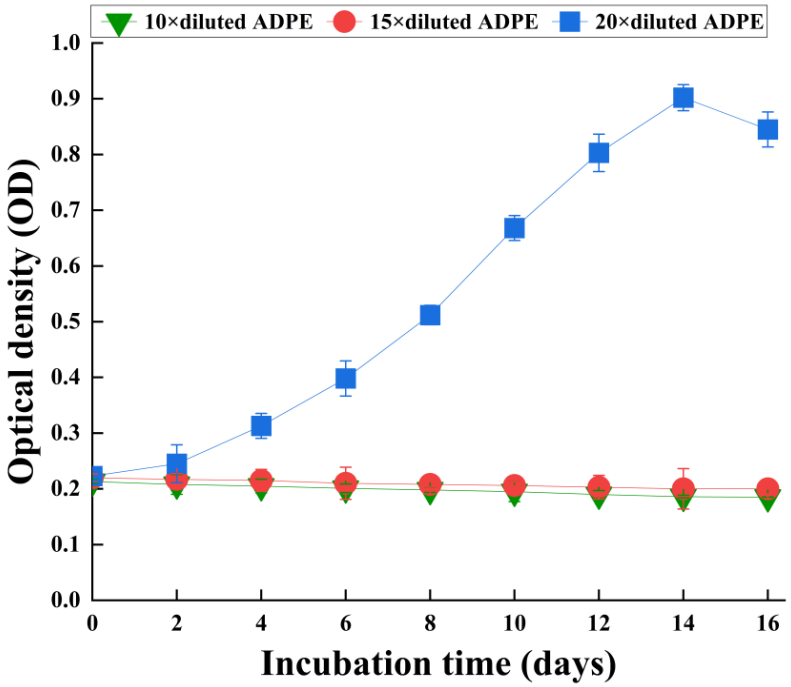

Figure 2. Growth curves of NCU-39 in different diluted anaerobic digestion piggery effluents (ADPEs) (10-fold (10×), 15×, and 20× dilution). Error bars represent standard deviations (n = 3).

3.3. Selection of Fungal Strain

The highest decolorization percentages were approximately 50% in 10× diluted ADPE for NU-3 and NU-27 on day 5 at the end of the experiment, followed by <10% for NU-8 (Figure 3a). Algal growths of NCU-39 were not completely inhibited in the ADPEs pretreated by NU-3 and NU-27, with the growth curves almost consistent with trajectories in the pretreated ADPEs (Figure 3b). However, algal growth was completely inhibited in the untreated and NU-8-pretreated ADPEs, corresponding to the decolorization percentage of <10% (Figure 3). These results indicated that the inhibition of algal growth may be related to low decolorization efficiency. To determine the effect of the brown color (turbidity) on algal inhibition, it may be needed to run an independent experiment with an inert source of turbidity in further study.

The highest decolorization percentages were achieved in the NU-27-pretreated ADPE by day 3 and NU-3-pretreated ADPE by day 4 in the airlift bioreactor (Figure 3a). Appropriate decolorization time may depend on various culture conditions such as fungal species and wastewater type [25]. Rodríguez Couto et al. [30] reported that the white-rot fungi *Trametes hirsuta* could decolorize textile dye indigo carmine almost 100% within three days. Liu et al. showed that *Phanerochaete chrysosporium* could achieve decolorization of 80% from ADPE by day five [15]. In this study, the isolated strain NU-27 with three-day decolorization was considered a potential candidate for ADPE pretreatment and subsequent experiments.

3.4. Identification of Fungal Strain

The partial 26s rRNA gene of NU-27 consisting of 695 bases was sequenced and submitted to the GenBank (Table S6). Based on the results of sequence similarity and phylogenetic analysis, NU-27 showed higher proximity (>99%) with *L. ornata* than with *Lichtheimia corymbifera*, *Lichtheimia ramose*, and *Lichtheimia hongkongensis* (approximately 94–98%) (Figure 4 and Table S6). Combined with morphological observation, NU-27 was identified as a strain of *L. ornata*, which was rarely reported for decolorization in wastewater treatment. Only in the report of Abd El-Rahim et al., *Lichtheimia* sp. presented the highest

decolorization percentages in mineral salts medium (MSM) supplemented with each of four azo dyes (i.e., Evans blue, direct blue, naphthol blue and direct violet) among the tested seventeen fungal strains [23].

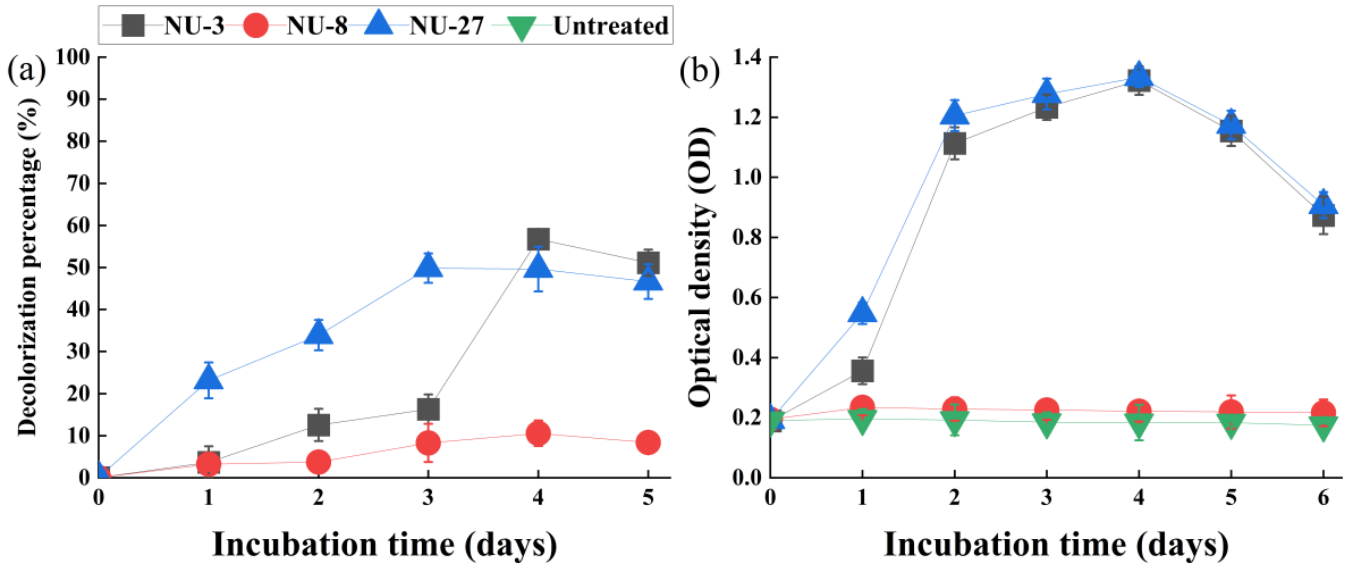

Figure 3. Decolorization percentage in 10-fold diluted anaerobic digestion piggery effluent (ADPE) pretreated by three isolated fungi (NU-3, NU-8, and NU-27) (**a**), and growth curves of NCU-39 in the ADPEs with and without fungal pretreatments (**b**). Error bars represent standard deviations (n = 3).

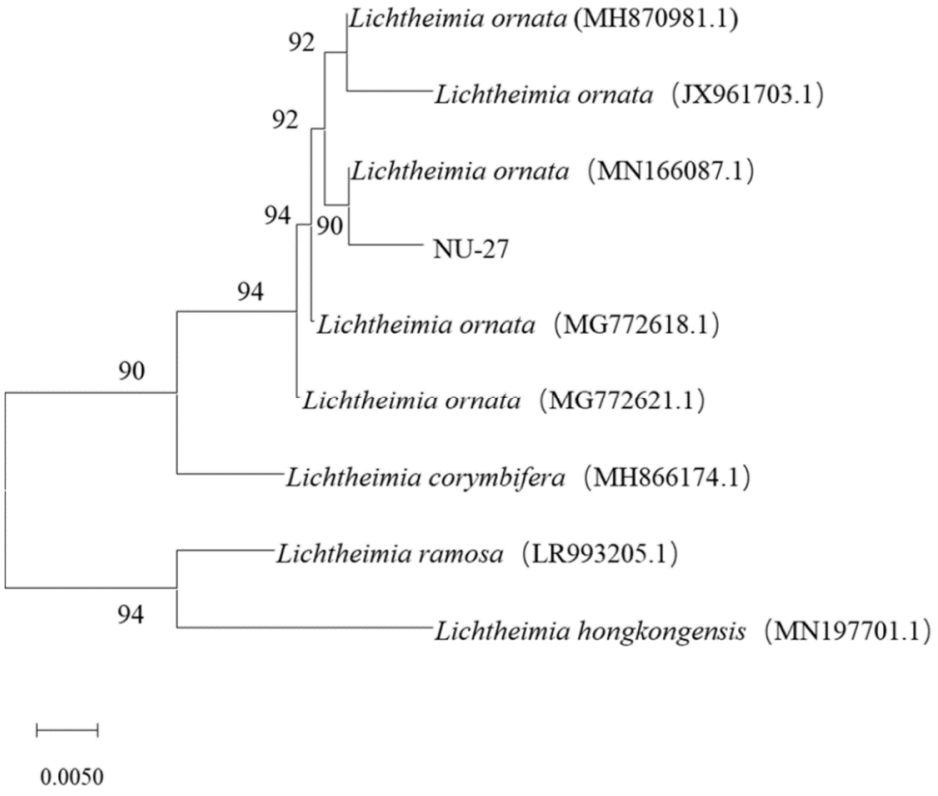

Figure 4. Phylogenetic tree of NCU-27 and *Lichtheimia* genus based on 26s rRNA gene sequences. GenBank accession numbers of each species are shown in parentheses. Numbers at the nodes indicate bootstrap values (expressed as %) with 1000 replicates, and the scale bar measures the distance between species.

3.5. Algal Growth in Different Diluted ADPEs Pretreated by Fungus

To further reduce the dilution ratio of ADPE through fungal pretreatment, NCU-39 was cultured in each of 3×, 5×, 10× diluted ADPEs pretreated by three-day NU-27 decolorization. Due to the addition of extra glucose for fungal decolorization as described above, initial COD concentrations in the diluted ADPEs (Table 2) were quite higher than those of the corresponding diluted one without glucose addition (Table 1). After fungal decolorization, the concentrations of NH_4^+-N, TN, and COD decreased by 10–29%, 7–19%, and 18–32% in all diluted ADPEs, respectively (Table 2). NH_4^+-N is one of the nitrogen sources for some fungal growths [27], including *Lichtheimia* spp. [23]. Moreover, fungi can also degrade nitrogenous compounds and other organic matters during wastewater remediation [27]. The above reasons decreased concentrations of NH_4^+-N, TN, and COD during fungal decolorization. In contrast, the TP concentration was improved by 43–45% after decolorization, maybe due to declining pH to 3.0 (Table 2). With declining pH in ADPE, phosphorus can be released from phosphate precipitates, such as struvite ($MgNH_4PO_4$ $6H_2O$) [31].

Table 2. Chemical composition of 3-fold (3×), 5×, and 10× diluted anaerobic digestion piggery effluents (ADPEs) before and after three-day fungal decolorization. The results are presented as arithmetic means ± standard deviations (n = 3).

Nutrient (mg L^{-1})	Time (Days)	3×	5×	10×
NH_4^+-N	0	653.7 ± 7.7	434.3 ± 3.9	216.6 ± 14.8
	3	586.3 ± 7.6	380.6 ± 12.9	153.4 ± 7.1
TN	0	656.8 ± 1.0	456.6 ± 1.9	235.5 ± 1.2
	3	611.0 ± 4.3	422.6 ± 1.8	191.8 ± 2.5
TP	0	23.1 ± 0.5	20.7 ± 1.3	9.4 ± 0.5
	3	33.0 ± 2.1	27.2 ± 1.5	13.6 ± 0.4
COD	0	4986.3 ± 66.7	4066.7 ± 70.7	2976.7 ± 45.3
	3	3410.2 ± 54.3	3083.3 ± 23.6	2443.3 ± 30.2
pH	0	4.0 ± 0.1	4.1 ± 0.1	4.2 ± 0.2
	3	3.1 ± 0.1	3.1 ± 0.1	3.2 ± 0.1

After fungal decolorization, algal growth was not completely inhibited in all diluted ADPE (Figure 5). Specifically, algal growth in 5× diluted ADPE was faster than that in 3× and 10× diluted ones in the first two days, which might be attributed to the inhibition effect of high NH_4^+ concentration (586 mg L^{-1}, Table 2) in 3× diluted ADPE, and limitation effect of low NH_4^+ concentration (153 mg L^{-1}) in 10× diluted ADPE, suggesting a high NH_4^+ tolerance of NCU-39 in 5× diluted ADPE at initial NH_4^+-N concentration of 380 mg L^{-1}. When NCU-39 was isolated from an NH_4^+-rich waterbody, it might already adapt to high NH_4^+ concentration at 380 mg L^{-1}. Vadiveloo et al. [32] showed that *Chlorella* spp. isolated from high NH_4^+ condition can be cultured in ADE from an abattoir at NH_4^+-N concentration of 220 mg L^{-1}. Ayre et al. [13] reported that *Chlorella* spp. isolated from high NH_4^+ conditions can even grow in ADPE with an NH_4^+-N concentration of 800–1600 mg L^{-1}. Using isolated algal species/strains capable of survival under high NH_4^+ conditions is highly feasible because of their high tolerance to NH_4^+, high algal yield and nutrient removal in NH_4^+-rich wastewater [13,32,33]. Although 5× low-diluted ADPE pretreated by NU-27 was more suitable for algal growth, it had quite lower algal yields than Tap medium (Figure 5). Therefore, subsequent process optimization of microalgae cultivation is needed for maximum algal yield in the pretreated ADPE.

3.6. Algal Growth at Different Initial Optical Densities

Algal growth curves of NCU-39 showed similar trends in the pretreated 5× diluted ADPE in all initial OD treatments (Figure 6). ODs rapidly reached about 2.1 until day 2 in the pretreated ADPEs at initial ODs of 0.3, 0.4, and 0.6, almost identical to those in Tap medium, while those increased to 1.6 at initial OD as 0.2 (Figure 6). Tap medium contains

inorganic nutrients and amounts of available organic carbon, i.e., acetic acid (Table S4), which can enhance the growth rate and achieve satisfactory maximum *Chlorella* sp. yield by short-time mixotrophy [34,35]. In all initial OD treatments, algal yields showed high growth rates in the pretreated ADPEs during the first two days (Figure 6), which might be attributed to amounts of residual added glucose from the pretreated ADPEs during fungal cultivation (see Section 2). Glucose has been demonstrated as an available organic carbon source for achieving a maximum algal yield of *Chlorella* sp. under mixotrophy [36,37].

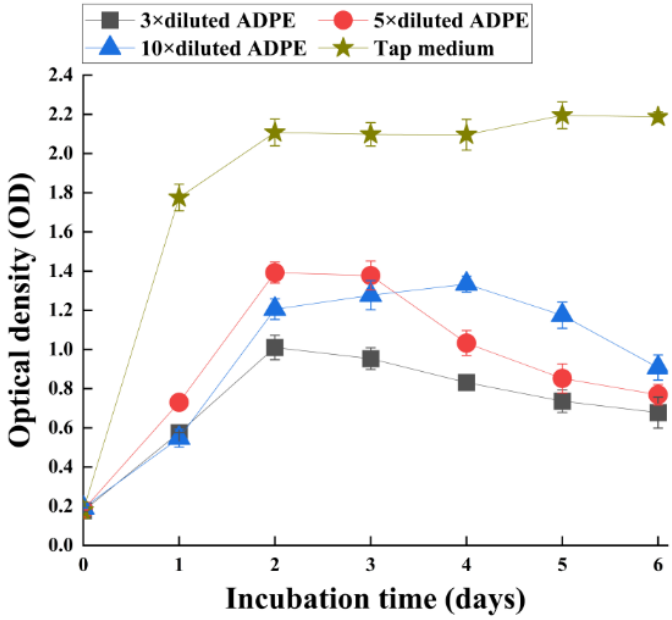

Figure 5. Growth curve of NCU-39 in different diluted anaerobic digestion piggery effluents (ADPEs) (3-fold (3×), 5×, and 10× dilution) pretreated by 3-day fungal decolorization. Tap medium was used as a control. Error bars represent standard deviations (n = 3).

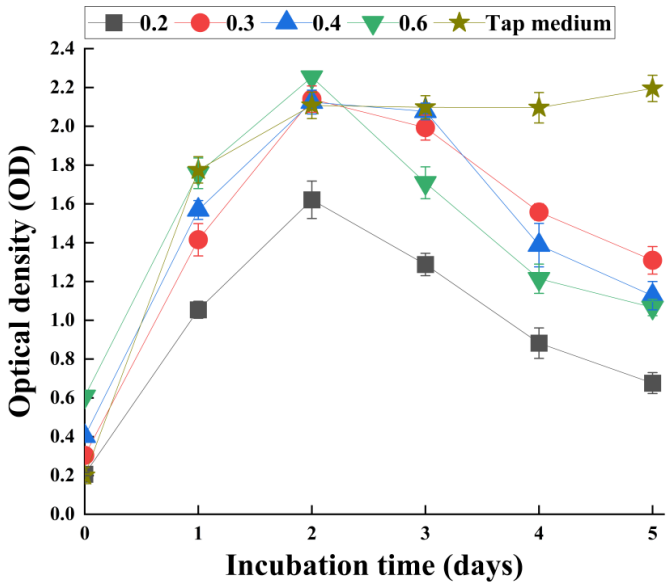

Figure 6. Growth curves of NCU-39 at different initial optical densities (ODs) (OD values = 0.2, 0.3, 0.4, and 0.6) in 5-fold diluted anaerobic digestion piggery effluents (ADPEs) pretreated by 3-day fungal decolorization. Tap medium was used as a control. Error bars represent standard deviations (n = 3).

Due to the nearly consistent maximum algal yields obtained at initial ODs of 0.3, 0.4, and 0.6, initial OD of 0.3 sufficed to achieve maximum algal yield in the pretreated ADPE. Generally, a higher initial cell density brings about a better algal yield. However, the obtained maximum algal yield would accumulate auto-inhibitors and reduce photosynthetic efficiency in culture medium, leading to a self-shading effect of limiting algal growth [38]. Thus, the almost same maximum yield obtained at higher initial ODs of 0.4 and 0.6 might be hindered by the self-shading effect. In order to reduce initial cell density as much as possible for high cost-efficiency, initial OD of 0.3 was therefore selected as inoculum density for subsequent experiments.

After day 2, ODs slumped in all pretreated ADPEs until the end of the experiment, while those remained constant in the Tap medium (Figure 6). The trend of algal yields might be related to pH variation. Throughout the experiment, pH rapidly declined from 7.0 to about 3.5 in the pretreated ADPEs but increased to 8.1 in the Tap medium (Figure S3). In theory, consumption of glucose and NH_4^+ during the mixotrophic growth results in pH decrease [34,39], while acetate uptake and photosynthesis contribute to the pH increase [40,41]. In the Tap medium, NH_4^+-N and acetate were respectively used as nitrogen and carbon sources, and the tris was used for pH buffer (Table S4). The slight increase of pH in the Tap medium under mixotrophic cultivation might be mediated by the pH buffer tris base, and the Tap medium kept constant algal yield under alkaline conditions. However, in the pretreated ADPE without such a pH buffer, much consumption of the residual glucose and NH_4^+ might lead to a pH decrease under mixotrophic cultivation. Levels of pH below 5.0 in the culture medium have been shown to induce cell damage in *Chlorella* spp. [42]. After day 2, pH levels below 5.0 might explain the rapid decrease of algal yields. In addition, levels of pH below 7.0 inhibit algal growth by suppressing intracellular enzyme activity [43,44] and enhancing metabolic costs [45]. In order to avoid pH declining below 7.0 during microalgae cultivation, elevating the initial pH in the pretreated ADPE might be needed before microalgae cultivation. Therefore, the following experiment of initial pH adjustment in the pretreated ADPE was conducted for subsequent microalgae cultivation.

3.7. Algal Growth in Different Initial pH Treatments

ODs rapidly increased to about 2.1 for maximum algal yield until day 2 in all initial pH treatments, almost the same as those in the Tap medium (Figure 7a), due to amounts of residual glucose from the pretreated ADPEs. After that point, ODs in pH 9.0 treatment showed almost identical plateau value to those in Tap medium, increasing until the end of the experiment, but ODs in pH 7.0 and 8.0 treatments declined to about 1.3 (Figure 7a). In the pretreated ADPE, pH values gradually decreased to 7.0 in pH 9.0 treatments but sharply declined to about 3.4 in pH 7.0 and 8.0 treatments (Figure 7b). The sharp drops of pH in both pH 7.0 and 8.0 treatments were attributed to consumption of residue glucose and NH_4^+ as described above, and resulted in decrease in algal yields due to cell damage induced by pH level below 5.0. However, these constant algal yields obtained in the pH 9.0 treatment might be because the level of pH above 7.0 (Figure 7b) failed to inhibit algal growth. As the hydroxide ion concentration at initial pH 9.0 was ten and hundred times quite higher than initial pH 8.0 and 7.0, respectively, the higher hydroxide ion concentration at initial pH 9.0, as well as that generated from algal photosynthesis, might partially neutralize hydrogen ion produced by consumption of residue glucose and NH_4^+ in the pretreated ADPE, resulting in the pH levels above 7.0 during algal cultivation. These results suggested that the initial pH of 9.0 in the pretreated ADPE was the optimal treatment for constant maximum algal yield during the experiment.

NH_4^+-N and TP concentrations were lessened during the first 2 or 3 days and then kept constant onward until the end of the experiments in all pH treatments (Figure 8a,c). NH_4^+-N concentrations decreased from 300 mg L^{-1} to 60 mg L^{-1} in pH 9.0 treatment, while those were lowered to 116 mg L^{-1} in both pH 7.0 and 8.0 treatments at the end of the experiment (Figure 8a), in response to final algal yields. In pH 9.0 treatment, partial removal of NH_4^+-N might include volatilization losses during the first two days due to pH

levels of more than 8.0 (Figure 7b). This was because high pH above 8.0 favored ammonia volatilization [46]. Likewise, TP concentrations decreased from 21 mg L^{-1} to 8 mg L^{-1} in pH 9.0 treatment and reduced to 14 mg L^{-1} in both pH 7.0 and 8.0 treatments through the study period (Figure 8c). pH levels of more than 7.0 helped to enhance phosphorus removal from wastewater through phosphate precipitation with metals, such as calcium phosphate and/or struvite [47,48]. Due to pH levels of over 7.0 in the pH 9.0 treatment (Figure 7b), the TP removal was associated with algal assimilation and phosphate precipitation. NH_4^+ volatilization and phosphate precipitation, as well as assimilation by algal growth, resulted in relatively high NH_4^+-N and TP removals in pH 9.0 treatment.

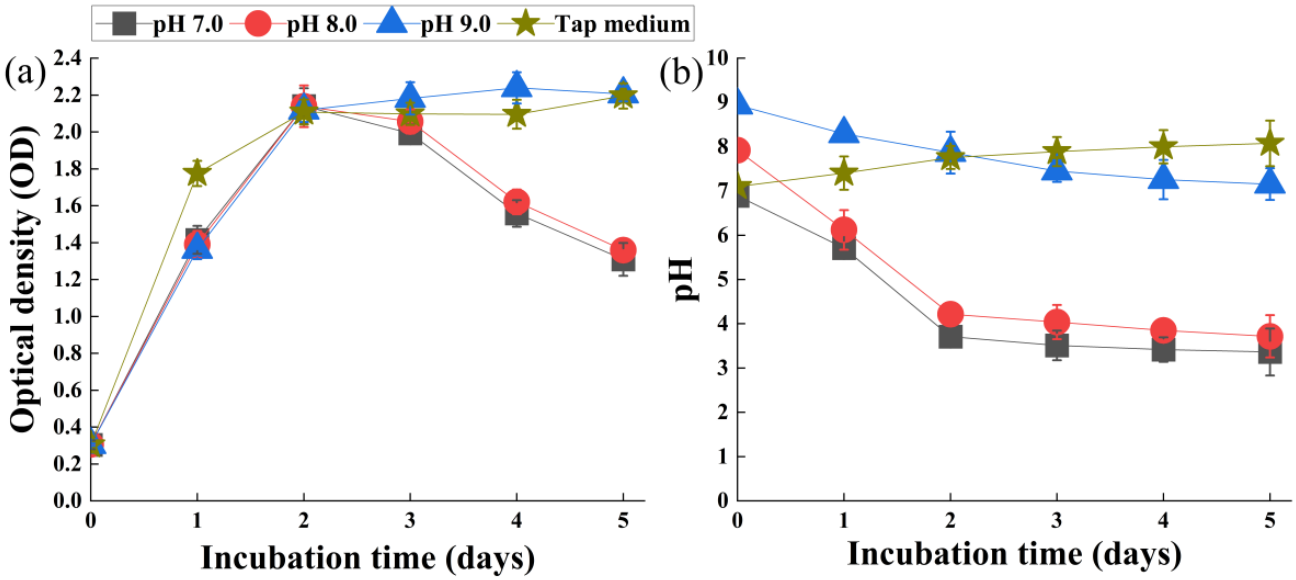

Figure 7. Growth curves of NCU-39 (**a**) and pHs (**b**) at different initial pHs in 5-fold diluted anaerobic digestion piggery effluents (ADPEs) pretreated by 3-day fungal decolorization. Tap medium was used as a control. Error bars represent standard deviations (n = 3).

TN concentrations decreased from 380 mg L^{-1} to 182 mg L^{-1} until day 2 in pH 9.0 treatment and then remained almost constant (Figure 8b). In both pH 7.0 and 8.0 treatments, TN concentrations declined to about 250 mg L^{-1} by day 3 but increased to 310–330 mg L^{-1} on the final day, 1.7–1.8 times higher than those in pH 9.0 treatment (Figure 8b). The concentrations of COD showed similar trajectories to those of TN, slumping from 3000 mg L^{-1} to about 617 mg L^{-1} until day 2 in pH 9.0 treatment and then being constant (Figure 8d). However, in pH 7.0 and 8.0 treatments, the concentrations declined to 700 and 600 mg L^{-1} by day 4 but increased to 900 and 785 mg L^{-1} at the end of the experiment, respectively. In all treatments, the results showed that a large amount of COD was consumed by microalgae under a mixotrophic mode, indicating that the large consumption was associated with consumption of residual glucose and some other organic matters from the pretreated ADPE. During pretreatment, fungi can significantly degrade larger complex organic matters to smaller simple ones with short chains in water [49,50], which can be easily utilized by microalgae for algal growth [51,52]. These simple organic compounds and glucose assimilated by microalgae resulted in a large amount of COD consumption in the pretreated ADPE. In pH 7.0 and 8.0 treatments, the increases of TN and COD concentrations on the final day may be relevant to pH values below 5.0 during the experiment (Figure 7b). Levels of pH below 5.0 have been shown to induce cell damage in *Chlorella* [42]. Dissolved organic matter (DOM) can be released from the dead and disrupted algal cells [53], among which those induced by decreasing algal yield may result in much DOM release and consequent increase of TN and COD concentrations. These results suggested that the initial pH of 9.0 was the optimum treatment for removing NH_4^+−N, TN, TP, and COD from the pretreated ADPE.

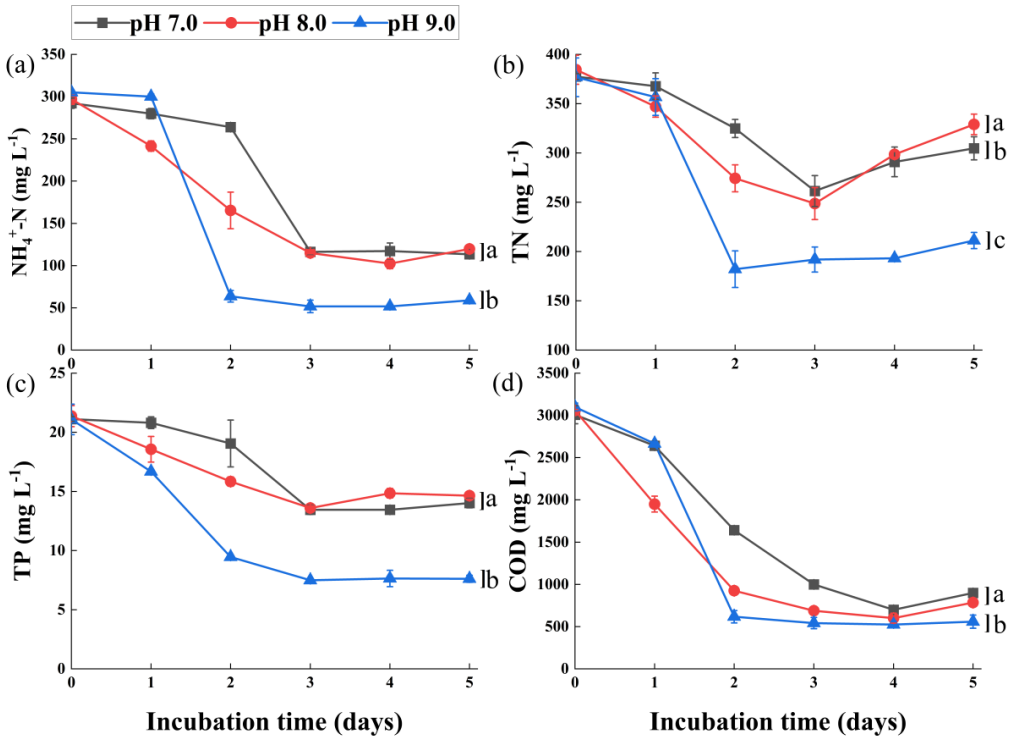

Figure 8. Concentrations of ammonium nitrogen (NH_4^+−N: (**a**)), total nitrogen (TN: (**b**)), total phosphorus (TP: (**c**)), and chemical oxygen demand (COD: (**d**)) for NCU-39 in different initial pH treatments in 5-fold diluted anaerobic digestion piggery effluents (ADPEs) pretreated by 3-day fungal decolorization. Tap medium was used as a control. Error bars represent standard deviations (n = 3). Different letters indicate significant differences with Tukey–Kramer post hoc tests ($p < 0.05$).

Based on nutrient concentrations on the final day (Figure 8) and those in 5× diluted raw ADPE (Table 1), the maximum removal rates of NH_4^+−N, TN, TP, and COD were calculated as 88%, 58%, 65%, and 77%, respectively, in the pretreated ADPE at initial pH of 9.0. This study achieved a significant process optimization in 5× low-diluted ADPE decolorized by NU-27 for three days and subsequent microalgae cultivation by NH_4^+-tolerant strain at initial OD as 0.3 and pH of 9.0 for ADPE remediation. The process optimization provided the optimum conditions for algal growth among the treatments, which allowed maximum algal yield and nutrient removal from the ADPE. However, *Lichtheimia* sp. as a pathogenic fungus implied a concrete risk for human health, and its application in microalgae-based wastewater treatment should receive careful evaluation [54]. Therefore, further study is required to select a non-toxic and eco-friendly fungus, such as white rot fungi [55], for ADPE decolorization. In addition, because fungi and microalgae are high-efficient organisms on heavy metal removal from diluted solutions [56,57], further exploration of their performance on heavy metal removal from ADPE is expected.

4. Conclusions

One promising local fungal strain, NU-27, was isolated and identified as *L. ornata*, with a high decolorization to ADPE through fungal pretreatment in an airlift bioreactor. Five-fold low-diluted ADPE pretreated by NU-27 for 3 days was the optimum medium for the growth of the NH_4^+-tolerant algal strain, compared with other dilution treatments. Subsequently, initial OD as 0.3 and pH of 9.0 were optimal conditions for the algal strain to provide the maximum algal yield (OD = 2.1) and nutrient removal (88%, 58%, 65%, and 77% for NH_4^+−N, TN, TP, and COD removal rates) from the pretreated ADPE. This study demonstrated that the integrated process-optimization strategy of fungal decolorization of ADPE and subsequent microalgae cultivation may could be a promising approach to algal biomass production and nutrient removal from ADPE.

Supplementary Materials: The information are available online at https://www.mdpi.com/article/10.3390/app12094741/s1.

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Abbreviations

ADE	anaerobic digestion effluent
ADPE	anaerobic digestion piggery effluent

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