Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2012.749377

51 (2013) 2768–2775
March

Taylor & Francis
Taylor & Francis Group

Identification of a newly isolated microalga from a local pond and evaluation of its growth and nutrients removal potential in swine breeding effluent

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Received 6 April 2012; Accepted 10 September 2012

ABSTRACT

In this study, an alga which grew robustly in a local pond was purified and identified, and its growth and nutrients removal potential in swine breeding effluent at concentration of 0, 20%, and 50% dilution were evaluated. The results showed that the isolated alga was identified as *Scenedesaceae* sp. with assistance of both DNA sequencing and morphological observation. Better growth curves were observed in 20% and 50% dilution with the growth rates being 0.275 and 0.279 d^{-1}, respectively, when compared with that in BG-11 medium. The maximum removal efficiencies of total phosphorus, COD, and ammonia nitrogen were 80.4, 37.1, and 98.2%, respectively, at the end of 12-day cultivation period. It is suggested that the isolated strain was highly promising for the ongoing efforts on mass cultivation of microalgae for treating swine breeding effluent coupled with biomass production.

Keywords: Identification; Growth; Nutrients removal; Swine breeding effluent

1. Introduction

Effluents from pig farming often contain high concentrations of nitrogen, phosphorus, and organic matter [1]. Serious environmental problems such as eutrophication of inland water body [2] or groundwater contamination [3] would be caused by direct discharge or improper treatment. There are some conventional biological disposal methods, including activated sludge process [4] and anaerobic treatments [5,6], could be adopted as the potential approaches, but the lower nutrients removal efficiency and higher energy inputs retarded widespread implementation in rural areas. In this context, it is crucial to develop and implement cost-effective technologies that can reduce discharge of nutrients into the watershed while increase farm profits for the establishment of sustainable farming [7].

Microalgae-based system grown in wastewater might be an alternative to solve the problem [8]. Microalgae could effectively assimilate the required organic matter and nutrients present in the wastewater for growth, and biomass production could serve as the replacement of the imported high-protein feed supplements [9]. Moreover, Mulbry et al. [10,11] pointed out that the annual energy input and cost of the projected system based on simple solar-powered

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photobioreactors would be minimized well below the costs cited for the existing wastewater treatment plants. With all the above-mentioned merits, more studies have been well conducted [12–17]. However, the success of such an algae-based treatment system for wastewater heavily depends on the performance of the used microalgae strains. Also the variations in the composition of wastewater would limit such a notion as only specific algae may perform to their potential, although a few strains' potential of efficient nutrients recovery have been proved. Therefore, it is particularly important and necessary to select the strains from local habitats [18].

Xiaoshan is a booming district of Hangzhou with a total area of 1420 square kilometers and a population of 1.2 million. Intensive animal breeding has dramatically developed since the regulation of animal feedlots in China, 2001. Swine farms are ubiquitous in the rural–urban continuum and rural areas. Large quantities of breeding effluents have come into being, and most of the effluents are always discharged into the nearby water bodies directly due to the lack of efficient disposal methods, performing the role of water quality degradation, and the eutrophication crisis. Besides odor nuisance, more serious disservices are imposed on local residents. This study is part of the ongoing efforts on mass cultivation of microalgae for products coupled with treating swine breeding effluent. The specific objectives of this study were: (1) to collect microalgae which grow robustly in local habitats; (2) to isolate, purify, and identify the dominated alga; and (3) to characterize the isolated strain for its biomass and nutrients removal when grew in swine breeding effluent.

2. Methods and materials

2.1. Isolation and purification of the used strain

The strain used in this study grew robustly in a local pond in Xinxin Swine Farm, Xiaoshan District, Hangzhou City, China. Water samples were taken from different locations around the pond, and then mixed and stored in sterile transparent plastic bottles, sent to the laboratory for algal isolation immediately. BG-11 medium recommended by Chinnasamy et al. [19] to enrich strains derived from natural water bodies was employed to enrich the candidate strain in this study and the ingredients of the medium were ($mg\,l^{-1}$) $NaNO_3$ 1500, $K_2HPO_4{\cdot}3H_2O$ 40, $MgSO_4{\cdot}7H_2O$ 75, $CaCl_2{\cdot}2H_2O$ 36, Na_2CO_3 20, $Fe(NH_4)_3C_{18}H_{10}O_{14}$ 6, Na_2-EDTA 1, H_3BO_3 2.86, $MnCl_2{\cdot}H_2O$ 1.81, $ZnSO_4{\cdot}7H_2O$ 0.222, $CuSO_4{\cdot}5H_2O$ 0.079, $Na_2MoO_4{\cdot}2H_2O$ 0.39, and $Co(NO_3)_2{\cdot}6H_2O$ 0.049. The protocols for isolating and purifying the dominated strain were as follows.

The water sample from above collection was centrifuged (5000 rpm, 4℃) to increase biomass concentration of the candidate algae. The concentrated biomass was diluted in sterile water, and then passed through 60 μm plankton net to remove zooplankton. After that, the sample was filtered through 0.45 μm pore-size membranes. The cells remained on the filter were rinsed several times with sterile salt water to remove as many bacteria as possible, then were inoculated onto a sterile 12-well tissue culture plate containing BG-11 medium for enrichment in a photo-incubator (JNG-1500E, China) under controlled conditions (25℃ with whole-day illumination at light intensity of 40 μmol photon $m^{-2}\,s^{-1}$). After enrichment for seven days, the candidate strains were transferred onto autoclaved 1.5% agar BG-11 medium plates for purification under the above-mentioned controlled conditions. Sequential subculturing was adopted until individual colony was obtained. Liquid cultures were grown by inoculating the individual colony from agar plates into autoclaved conical flasks containing BG-11 medium and placing on an orbital shaker at 150 rpm at 25 ± 2℃ with two-compacted fluorescent lights providing whole-day illumination at light intensity of 100 μmol photon $m^{-2}\,s^{-1}$.

2.2. Identification of the isolated alga

2.2.1. Morphological analysis

Morphological characters, including shape of cell and chloroplast, presence of pyrenoids/vacuoles/starch, were evaluated in four-week-old cultures using light microscopy (LEICA DFC300 FX, Germany) and TEM (JEM-1230, Japan). For TEM preparations, cells were collected by centrifuging at 3500 rpm for 15 min. The samples were prefixed for 2 h at 20℃ in 3% glutaraldehyde in 0.1 M cacodylate buffer and postfixed in 8% osmic acid for 2 h at 4℃, respectively. The cells were then dehydrated at room temperature in increasing concentrations alcohol (50, 70, 80, 90, and 100%, 15 min at each concentration). The dehydrated cells were kept in propylene oxide (two rinses of 20 min each). Embedding was carried out by using a mixture of 1:2 epon + propylene oxide, followed by 1:1, and finally 2:1 proportions of the same (1 h each step). Finally, the samples were left in pure epon overnight. They were stained at the same temperature with uranyl acetate for 2 h before rinsing in distilled water for 5 min. Ultrathin section of 60–80 nm were cut using an ultramicrotome and mounted on a carbon-coated copper TEM grid. Features were compared with the published descriptions [20] and incorporated with molecular analysis to aid the strain identification.

2.2.2. DNA extraction

DNA was extracted using Gem-CTAB method [21]. About 2 ml of the culture during exponential growth phase was centrifuged at 12,000 rpm for 5 min at 4℃. The supernatant was decanted and the pellet was re-suspended in 750 µl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH8.0). After centrifugation and careful decantation of the supernatant, 200 mg of fresh algal tissue was ground to fine powder in liquid nitrogen using prechilled mortar and pestle. The powder was transferred into 1.5 ml eppendorf tubes and 600 µl of extraction buffer was added. The contents were mixed three to four times by inverting the tubes gently and incubated at 65℃ for 1 h. The DNA extraction buffer of Gem-CTAB method had following composition: 100 mM Tris–HCl pH 8.0, 10 mM EDTA, 1.4 M NaCl, 2% CTAB, and 2% β-mercaptoethanol.

For removing protein, 0.8 volumes of phenol:chloroform:*iso*-amylalcohol (25:24:1) was added and mixed gently by inversion for 10 min to form an emulsion, and the residual phenol was removed by extraction with an equal volume of chloroform:*iso*-amylalcohol (24:1). The samples were centrifuged at 12,500 rpm for 10 min at 4℃. The top aqueous phase was transferred to a fresh tube. About 0.8 volume of cold isopropanol was added and mixed well, and then the mixture was incubated for 10 min at room temperature. Next, the mixture was centrifuged for 10 min at 12,500 rpm to collect the nucleic acid precipitate. The precipitated nucleic acid was eluted twice with 70% ethanol. The pellet was air-dried until all ethanol were removed and dissolved in 20 µl TE buffer, and stored at −20℃ prior to use. DNA was diluted fivefold and tenfold for use in PCR amplification.

2.2.3. PCR amplification and sequencing

For PCR amplification, the diluted portion of extracted DNA was used to amplify the 18S rDNA gene with two eukaryotic 18S ribosomal DNA-specific primers [22], 18S1N (forward, 5′-CAGGTCTGTGATGCCC-3′), and 18S2N (reverse, 5′-ACGGGCGGTG TGTAC-3′). Amplifications were performed in 20 µl reaction mixture containing 1 µl 50 ng of template DNA, 0.2 µl 1.6-unit of Taq polymerase (TaKaRa. Otsu, Japan), 2 µl 10× PCR buffer (TaKaRa. Otsu, Japan), 2 µl 2.5 mM dNTP, 0.5 µl 0.2 µM of 18S1N, and 18S2N primers, respectively. The amplification was carried out using BioMetra thermocycler. The PCR program was run for pre-denatured at 94℃ for 5 min, after that, 40 s at 94℃, 35 s at 58℃, 35 s at 72℃ for 36 cycles, and a 10-min final extension at 72℃. Amplified products were sent to BGI Company in Shanghai for purification and DNA sequencing. The result of 18S rDNA gene sequences was aligned and compared to the nucleotide sequences of some known species in GenBank database of the National Center for Biotechnology Information. For constructing the phylogenetic tree, the sequence alignment associated with other selected sequences was carried out using MEGA4.0 software according to the neighbor-jointing method with 100 bootstrap samplings.

2.3. Growth and nutrient removal potential of the alga grown in swine breeding effluent

An experiment aimed at providing baseline information on performance of the isolated alga alone in swine breeding effluent including growth and nutrient removal potential was conducted.

2.3.1. Characteristics of swine breeding effluent

Swine breeding effluent was collected from the same pond in the above-mentioned swine farm in May, 2011, which was mainly composed of urine and flushing water discharged from the breeding zones directly. Effluents from different locations around the pond were sampled, mixed, and stored in two 25 l buckets. After being sent to the laboratory, portion of the effluent required for the experiment was transferred into sterile transparent plastic bottles and stored in a refrigerator maintained at 4℃ to avoid the variation of wastewater composition with collection time. The characteristics of the effluent are shown in Table 1.

2.3.2. Experimental layout

In order to provide baseline information on performance of the isolated alga alone, the effluent was

Table 1
Characteristic of the collected effluent used in the present study

Parameter	Value	Parameter	Value
pH	7.85 ± 0.07	$NH_4^+ - N$ (mg l^{-1})	322.9 ± 0.67
Conductivity (mS)	3.13 ± 0.00	$PO_4^{3-} - P$ (mg l^{-1})	6.42 ± 0.22
COD (mg l^{-1})	1117.6 ± 43.7	$NO_3^{2-}N$ (mg l^{-1})	7.41 ± 0.13

sedimentated, filtered through 0.45 μm pore-size membranes, and then autoclaved to eliminate bacterial contamination and excess heterotrophic growth. The autoclaved effluent at 0, 20, and 50% dilutions was referred as T-100%, T-50%, and T-20% treatment, respectively. Algal culture ($OD_{690} = 1.24$) was inoculated at 20% (v/v) in 500 ml capacity Erlenmeyer flasks with 250 ml growth medium. The flasks were in stationary culture for 12 days in the laboratory at 25 ± 2°C with whole-day illumination at light intensity of 100 μmol m^{-2} s^{-1}. BG-11 medium was adopted as a positive control to judge the performance of autoclaved effluent as growth medium for algal cultivation, while no inoculation was served as the corresponding negative control to observe the variation of the composition of the autoclaved effluent with cultivation time elapse. Eighteen flasks were employed with each treatment in triplicate, and the average of the triplicate was reported in this study.

2.4. Growth and chemical analysis

2.4.1. Determination of algal growth

Optical density (OD) of the inoculated effluent at 690 nm was measured every two days as the algal density indicator using a spectrophotometer (Thermo Evolution 220, USA). A linear relationship between OD and dry weight (g l^{-1}) was determined for this strain:

$$\text{Dry weight } (\text{g l}^{-1}) = 0.9914\text{OD}_{690} - 0.0216, R^2 = 0.9455$$

The growth rate (GR, d^{-1}) obtained in the dilution was calculated by fitting the OD to an exponential function [23]:

$$\text{GR} = (\ln\text{OD}_t - \ln\text{OD}_0)/t$$

where OD_0 is the optical density at initial day, OD_t is the optical density for day t, and t is the time between the two measurements.

2.4.2. Nutrients analysis

Liquid sample for nutrient analysis was collected and filtered through a 0.45 μm pore-size membrane. The filtration was collected and properly diluted for analysis of ammonium ($NH_4^+ - N$), total phosphorus (TP), and chemical oxygen demand (COD). $NH_4^+ - N$, TP and COD were determined according to the standard methods [24]. The determination of COD was based on the dichromate method, $NH_4^+ - N$ was determined using the modified phenate method while total phosphorus was determined by ascorbic acid method. Removal efficiency for those nutrients was calculated by the difference in concentration between the initial day and final day.

3. Results and discussion

3.1. Morphological analysis

Morphological characters of the isolated algal were shown in Fig. 1. The alga was green and often performed the colony of multicells (or partially single). The single cell was oval with round deeply divided **chloroplast**, and the **chloroplast** segments were band shaped and assumed parietal positions, containing some droplets. The algal cells contained a distinct pyrenoid, nucleus.

3.2. Molecular analysis

The 18S rDNA gene of the candidate strain was amplified successfully from the whole genomic DNA

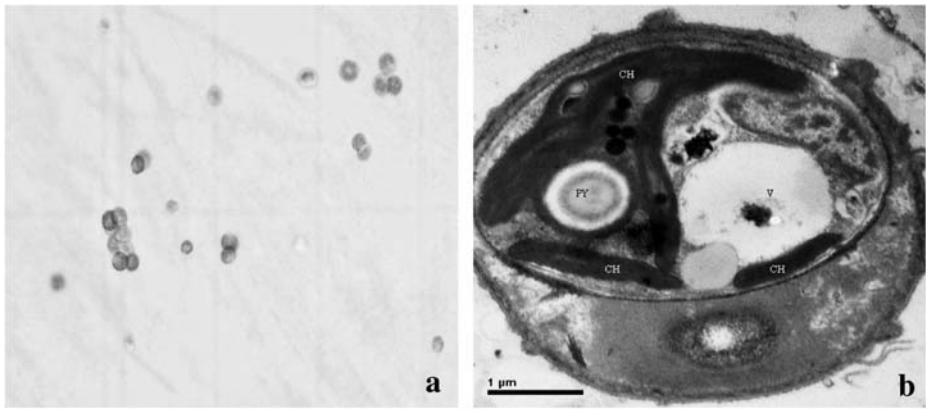

Fig. 1. Morphological characters of the isolated algal in light microscope (a) and TEM (b), CH, PY, and V as abbreviation for **chloroplast**, pyrenoid, and nucleus, respectively.

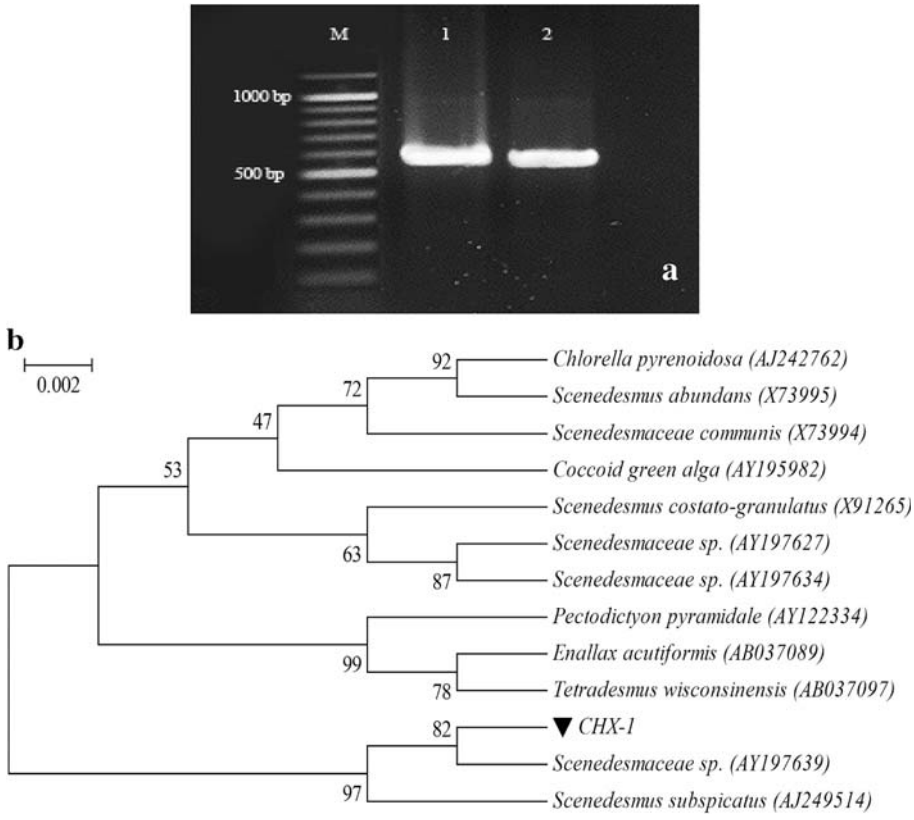

Fig. 2. PCR amplification of 18S rDNA gene with fivefold (lane 1) and tenfold-diluted DNA samples (lane 2) (a) and neighbor-joining phylogenetic tree by bootstrap values being calculated with 100 repeats (b).

using the following primer pairs: 18S1N and 18S2N. The band was single and an expected length was obtained with about 500bp as indicated in Fig. 2(a).

Based on the 18S rDNA gene library results, the phylogenetic tree was constructed (shown in Fig. 2(b)). The result showed that the isolated alga performed close relative relationship with *Scenedesmus*, and positioned the same branch with the known specie *Scenedesmaceae* sp. (Genbank accession number: AY197639) with 96% of sequence similarity. According to the blast result from algae gene library combined with morphological observation, the isolated strain was identified as *Scenedesmus* sp., although *Scenedesmus* always form groups of two or more cells in natural. Nevertheless, the cells can also exist separately in cultivation [25].

Previous researchers reported that *Scenedesmus* sp. which could be used for treatment of different wastewater streams, such as agricultural wastewater [26], animal manure wastewater [15], industrial wastewater [19,27], and domestic municipal wastewater [18,28,29], can perform high potential of growth and nutrients removal efficiency as well as lipid productivity.

3.3. Growth potential of the isolated alga grown in swine breeding effluent

Algal growth curves as indicated by optical density measured at 690 nm in T-100%, T-50%, T-20%, and BG-11 medium treatment are shown in Fig. 3. It should be worth noting that the observed values were the difference between the values of OD_{690} measured in inoculation treatment and those in the corresponding negative control. Jimenez-Perez et al. [30] pointed out that algae isolated from wastewater or real water bodies could usually adapt to culture conditions and grew better. In this study, the isolated alga could survive in all treatments, and no lag phase was observed in BG-11 medium and T-20% treatment until the end of cultivation, while a lag phase was visible in T-100% and T-50% treatment. The growth curve of T-50% treatment sharply leveled up after the fourth day, whereas, after the sixth day, the existing algal strain acclimatized itself to the raw effluent and picked up a slight growth. Compared with BG-11 medium, the strain grew faster in 20% and 50% dilution with the growth rates being 0.275 and 0.279 d^{-1}, respectively, which was higher than the result of 0.2 d^{-1} reported by Li

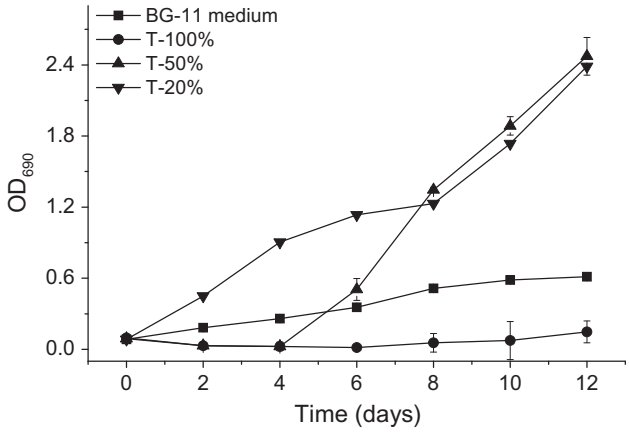

Fig. 3. Growth curve of the isolated strain grown in different growth media.

et al. [29] when a high-lipid producing strain *Scenedesmus* sp. grew in a secondary wastewater. Moreover, higher biomass of around 2.4 g l^{-1} was obtained in comparison with the common biomass level (0.3–0.5 g l^{-1}) expected in typical microalgae cultivation [31], suggesting that the isolated alga could be considered promising for biomass production on the swine breeding effluent with moderate dilution (see Fig. 3).

3.4. Nutrients removal potential of the isolated alga grown in swine breeding effluent

3.4.1. Total phosphorus removal

Total phosphorus was drastically reduced during the cultivation period of the inoculation treatments, whereas almost no variation was observed in the corresponding negative control (shown in Fig. 4(a)). During the 12-day experiment, total phosphorus was removed 80.4% from 5.67 mg l^{-1} to 1.11 mg l^{-1} in T-50% and 75.7% from 3.42 mg l^{-1} to 0.83 mg l^{-1} in T-20%, respectively. The phosphorus removal was greater than those reported in other studies

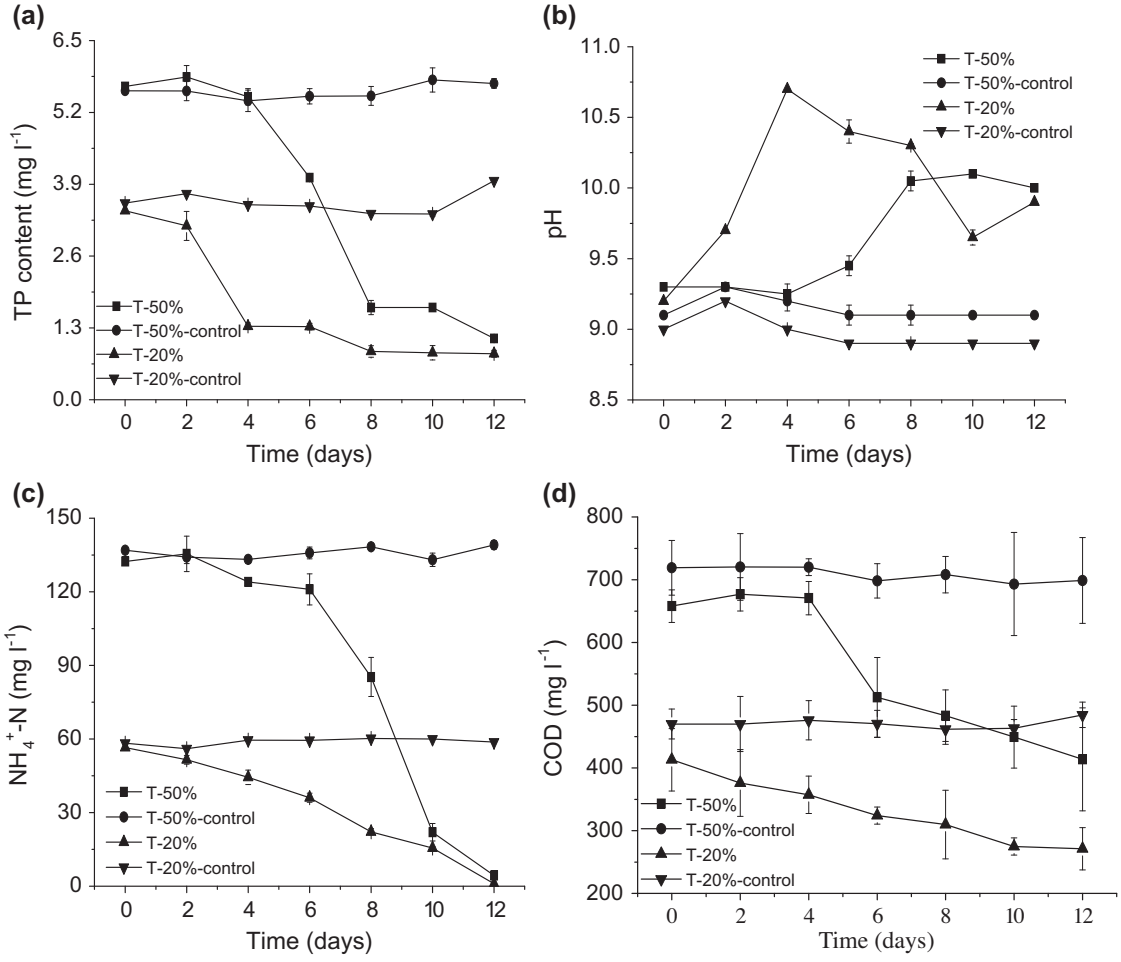

Fig. 4. Changes of TP (a), pH (b), $NH_4^+ - N$ (c), and COD (d) in growth media, respectively (TP: total phosphorus; $NH_4^+ - N$: ammonia nitrogen; COD: chemical oxygen demand).

using swine and dairy wastewater [11,14,16,32], suggesting that the isolated strain had greater potential of phosphorus removal grown in swine breeding effluent at 20% and 50% dilutions.

Previous researchers [28,33,34] reported that growth metabolism of algae could ascend the pH of the culture, and on condition that the pH value went above 8, algae system might cause coagulation and adsorption of inorganic phosphates. In this study, the medium pH was significantly increased in inoculation treatments, and exceeded 9.5 during the cultivation period as indicated in Fig. 4(b). It might be reasonable to conclude that the removal of phosphorus was due to both algal metabolic uptake and phosphate precipitation.

3.4.2. $NH_4^+ - N$ removal

$NH_4^+ - N$ was significantly removed in the inoculation treatment, but in the negative control, the concentration was slightly increased as indicated in Fig. 4(c). The concentration dropped from $132\,mg\,l^{-1}$ to $4.4\,mg\,l^{-1}$ in T-50% and from $56\,mg\,l^{-1}$ to $1.0\,mg\,l^{-1}$ in T-20%, respectively, and over 96% of $NH_4^+ - N$ was removed by the end of the cultivation. The removal efficiency was greater than those reported in other studies using swine wastewater [14,16], and the isolated strain performed high ammonium tolerance regardless of how high the initial concentration ($56–132\,mg\,l^{-1}$) was. The loss of $NH_4^+ - N$ might attribute to both utilization by the alga to synthesize new biomass under light condition [35] and NH_3 stripping under alkaline condition [36] due to the medium pH exceeded 9.5 during the cultivation period as indicated in Fig. 4(b).

3.4.3. COD removal

Fig. 4(d) showed that the concentrations of COD in the inoculation treatments were gradually reduced while no visible variation was observed in the control group. COD was removed 37.1% from $658\,mg\,l^{-1}$ to $414\,mg\,l^{-1}$ in T-50% treatment and 34.4% from $413\,mg\,l^{-1}$ to $271\,mg\,l^{-1}$ in T-20% treatment by the end of the cultivation, respectively. The removal efficiency was much greater than 23% reported by Cheunbarm and Peerapompisal [16], but significantly lower than the reported value of 76% by Godos et al. [14], who treated piggery wastewater associated with microbe in high rate algal ponds. The experiment was performed in axenic condition, and the reduction of COD could only be attributed to consumption by the isolated alga alone, which indicated that the alga could utilize different organic compounds as carbon sources, and was probably cultured under mixotrophic conditions to obtain more yields [37].

4. Conclusions

The isolated alga from a local pond was identified as *Scenedesaceae* sp. performing fast growth and high nutrients removal efficiency alone grown in diluted swine breeding effluent under controlled lab conditions. The candidate alga could be potentially used for mass cultivation and nutrients removal in swine breeding effluent at concentration of moderate dilution. However, there is a serious restriction for the dilution prior to biological decomposition to be used commercially. Therefore, more researches are required to screen otherwise pretreatment methods other than dilution and to find the optimum balance between nutrients removal and biomass production before further pilot-scale experiment.

Acknowledgments

The study is supported by the National Natural Science Foundation of China (40871101), the Natural Science Foundation of Zhejiang province, China (NO. Y12E080084) and China Postdoctoral Science Foundation (20110491792). The authors are grateful to Zeng Zhang-hui and Li Xiao-yan for providing guidance on molecular analysis, and also gratefully acknowledge all the reviewers for their great contribution to the improvement of this article.

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