Biotreatment Of Aquaculture Wastewater With Chlorella Sp. In Tubular Photo-Bioreactors

Le Hung Anh, Vo Thi Kim Khuyen

Abstract: Microalgae are microorganisms with high performance of wastewater treatment and potential for biofuel production thanks to the ability to convert contaminants in the wastewater into their biomass. Based on these advantages, strains of Chlorella sp. isolated from the shrimp wastewater were pre-cultured in F/2 medium and later applied to treat shrimp aquaculture wastewater using tubular photo-bioreactors (PBR) in both laboratory and outdoor conditions. The wastewater collected from shrimp ponds in Can Gio coastal district was found to be suitable for the growth of Chlorella sp. (salinity 8 ‰, pH 7.33, $N\text{-}NH_4^+$ 4.32 mg/L and $P\text{-}PO_4^{3-}$ 0.12 mg/L), and a new Chlorella strain isolated from the wastewater could remove nutrients in the wastewater with high speed and efficiency. Nutrient concentrations decreased after the first 4 days of culture, wastewater treatment yields in the laboratory and outdoor culture reached 86.34% and 77.54%, respectively for ammonium removal; 72.5% and 79.1% for phosphate treatment, respectively. Furthermore, the results showed the growth of Chlorella sp. was faster in outdoor culture, compared with laboratory condition. The success of isolating Chlorella sp. strains and using them for shrimp farming wastewater treatment in tubular photo-bioreactors is the basis for further applications on the pilot scale before being implemented in practice.

Index Terms: microalgae, Chlorella sp., shrimp farm wastewater, tubular photo-bioreactors (PBR), Can Gio district

1. INTRODUCTION

There has been an increase in national consumer demand as well as export demand for shrimp over the last decades. The majority (88%) of shrimp worldwide was supplied from Asia, including Vietnam [1] with 14% annual increase in shrimp production from 93,503 metric tons in 2000, to 628,231 metric tons in 2015 [2]. Since shrimp production brought large income, farming of whiteleg (Litopenaeus vannamei) and black tiger (Penaeus monodon) has been expanded throughout Vietnam, more importantly in the Mekong Delta region since 2005. Can Gio – a coastal district of Ho Chi Minh city is also a developing brackish-water shrimp farming area in coastal wetland connected to the mangrove system. However, the rapid expansion of intensive shrimp culture has discharged directly untreated wastewater from shrimp ponds to the nearby river, causing destruction of mangrove wetlands and algal bloom in open shrimp ponds. The shrimp production wastewater contains mainly remaining shrimp food, dead shrimp and shrimp faeces, particular amount of antibiotics, pharmaceuticals and hormones from shrimp food. The non-scientific feeding regime with industrial food containing high amount of nitrogen and phosphorus has led to food discharge to the water. Other organic substances available in river have created the eutrophication and ideal environment for the development of microalgae and zooplankton in shrimp ponds. The current trend in wastewater treatment field focused on biological treatment, and microalgae species N. oculata, T. chuii Scenedesmus, Spirulina, Nannochloris, Botryococcus brauinii and cyanobacter Phormidium have been applied in treatment of domestic wastewater [3], [4], livestock wastewater [5], and aquaculture wastewater [6], [7], [8], [9]. After wastewater treatment, biomass can be separated by microfiltration [10], coagulation, flocculation [11], then served back for aquaculture as a source of lipids, proteins (>55% dry weight) [12], vitamins and pigments for shrimp food, such as Dunaliella sp., Chlorella sp., Spirulina sp. [8], [13], [14], [15]. The algal biomass has been used as commercial fertilizers due to its high N and P content and very low cellulose [16], [17]. Lipid-rich biomass of green algae Chlorococcum sp., Neochlorosis oleabundans, Chlorella sp. has been also used as an alternative to fossil fuels for biofuel production in laboratories and small industries at this time, which contributes to reduce air pollution [18], [19].

Therefore, microalgae are commercialized for aquaculture wastewater in fixed biofilm or suspended growth operations [20], [21]. In fact, high-rate algae ponds (HRAP) [7] and corrugated raceways [5] are considered more simple but highly efficient and energy-saving compared with traditional biological treatments such as aerobic, anoxic, and anaerobic methods because algae can use sunlight as energy source, atmospheric CO_2 as carbon source to assimilate pollutants into organic compounds essential for their growth and creation of biomass [22], [23], [24]. Although open systems are easier and cheaper to build and operate, microalgae have low light use and more easily to be contaminated with bacteria, fungi, protozoa and other unknown microalgae available in the wastewater. To overcome these limitations, achieve maximum productivity and highest amount of biomass, closed systems using photobioractor models made from transparent materials have been developed [25]. Photobioreactors can be classified into categories: photobioreactors planes, plastic bag photobioreactors, cylindrical photobioreactors such as a triangular photobioreactors [6], and tubular photobioreactors [26], [27], [28], and a special type of reactor in the absence of light:fermenters [19]. In this study, an idea of culturing Chlorella isolated directly from aquaculture wastewater in acrylic tubular photo-bioreactors for shrimp wastewater treatment was developed in the laboratory and outdoor systems.

2 METHODOLOGY

2.1. Materials

Chlorella sp. was isolated from the shrimp production wastewater, which was taken in discharge pines of shrimp ponds. The isolated strains were cultured until getting the purified colonies. According to Safi et al., 2014, Chlorella is a

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unicellular green algae with a spherical cell of 2–10 µm diameter [12]. Further biological tests were performed to confirm the isolated strain was belonged to Chlorella species. The purified strain, named TH31 was stored at 5-6^{o}C at Renewable-project laboratory of Institute of Environmental Science, Engineering and Management, Industrial University of Ho-Chi-Minh City, Ho-Chi-Minh City, VietnamThe isolation and purification medium was modified F/2 medium [29], which contains 75 mg $NaNO_3$, 5.0 mg $NaH_2PO_4.H_2O$, 30.0 mg $Na_2SiO_3.9H_2O$, 4.36 mg $Na_2C_{10}H_{14}O_8N_2.H_2O$, 0.01 mg $CoCl_2.6H_2O$, 0.01 mg $CuSO_4.5H_2O$, 3.15 mg $FeCl_3.6H_2O$, 0.18 mg $MnCl_2.4H_2O$, 0.005 mg $Na_2MoO_4.2H_2O$, 0.022 mg $ZnSO_4.7H_2O$, 0.1 mg Thiamin HCl, 0,0005 mg Biotin and 0,0005 mg B12. The salinity of medium was adjusted to around 4‰, similar to the salinity of shrimp production wastewater.

2.2. Algal culture

Purified Chlorella sp. was pre-cultured in 10 ml of F/2 medium in 15-ml falcon tubes, then transferred to 250-mL Erlenmeyer flasks containing 100 mL of F/2 medium (Fig. 1A). The culture system was controlled at room temperature of 25-28°C, continuous light by using fluorescent light bulb 40W on the mechanical shaker to prevent algae from sticking to the bottom. To obtain high amount of the microalgae for wastewater treatment experiments, a particular volume of Chlorella sp. solution was transferred to 1.5-liter plastic bottles (Fig. 1B), then 5-liter bottles containing 3 liters of F/2 medium (Fig. 1C). Each bottle was continuously aerated by aeration pump under the continuous light (24/24h).

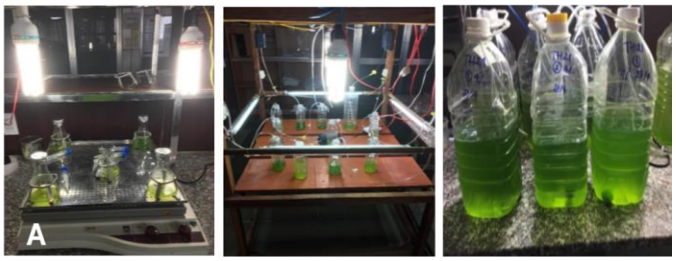
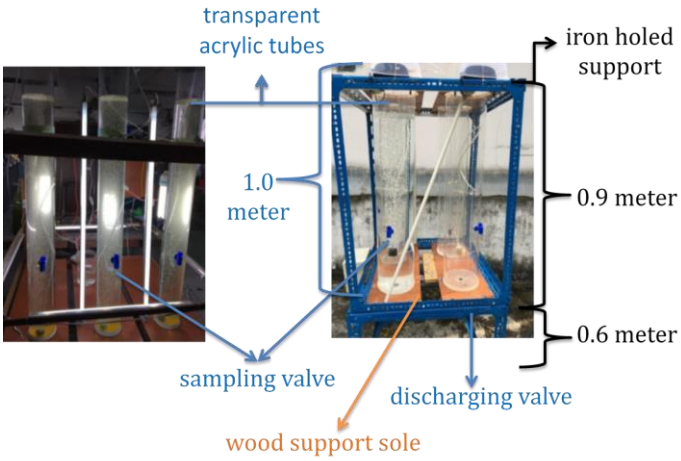


***Fig. 1**. Algal culture systems in the laboratory*
(A) First pre-culture of isolated Chlorella sp. in F/2 medium
(B) and (C) sub-culture of isolated Chlorella sp. in F/2 medium

2.3. Tubular photobioreactor models

When the algal concentration reach a particular amount as required, Chlorella sp. was transferred to tubular photo-bioreactor models. The laboratory models (Fig. 2A) include three transparent acrylic tubes. Each tube had the diameter (Ø) of 100 mm, working height of 1 meter, and working volume of 6 liters. Each tube had one sampling valve located at 1/3 from the bottom of the working height. The outdoor models (Fig. 2B) include 4 transparent acrylic tubes in pair, 1 meter in high, with the illumination degree of 93%. Each tube had the diameter (Ø) of 180 mm, working height of 0.8 meter, equivalent to 20 liter of water. There were one discharging valve at the bottom and one sampling valve on each tube. The support sole was made of good insulating wood. The holed support frame was made of iron, totally 1.5 meter in high, including 0.6-meter height from the land to the support sole and 0.9-meter height from the support sole to the top of tubes.

***Fig. 2.** Tubular photo-bioreactor systems for culturing isolated Chlorella sp. in the wastewater*
(A) Algal culture model in the laboratory
(B) Outdoor algal culture model



The culture conditions were controlled: 3000-4000 lux, 27^{o}C for laboratory model. And for outdoor model, the sunlight intensity was measured in the range of 50000- 10000 lux from 8h to 12h, 5000-1000 lux from 13h to 16h, the temperature varied from 29^{o}C to 31^{o}C.

2.4. Wastewater treatment in tubular photo-bioreactors

Two duplicate wastewater treatment experiments with Chlorella sp. (TH31) were carried out in the laboratory, and triplicate similar experiments were conducted in outdoor models. There was one control containing only wastewater for each model. The initial algal concentration was roughly 2 ×10^{6} cells/ml. The algal cell density was recorded based on the photon absorption of algal cells at the optimal wavelength. Thus, absorbance of samples was read by spectrophotometer at 420 nm and a regression curve was drawn to calculate the algal cell number in unknown samples. Most important parameters, such as pH, ammonium ($N\text{-}NH_4^+$) based on Vietnam standards [30], phosphate ($P\text{-}PO_4^{3-}$) [30] of the input wastewater were measured by spectrometric methods to ensure the tolerance of microalgae to the concentration of nutrients in the wastewater. Every day, these parameters were measured to investigate the algal growth in wastewater as well as the wastewater treatment ability of TH31, and the quality of output water.

3 RESULTS AND DISCUSSIONS

3.1. Algal growth cycle in F/2 medium

The correlation analysis showed a strong linear regression between the absorbance (A) and known algal cell density (C), producing a standard equation: A = 6.10^{6}C – 102641 with a high coefficient (R squared = 0.9932) at λ = 420 nm. Thus, this reliable method was used to calculate the algal concentrations in all samples.

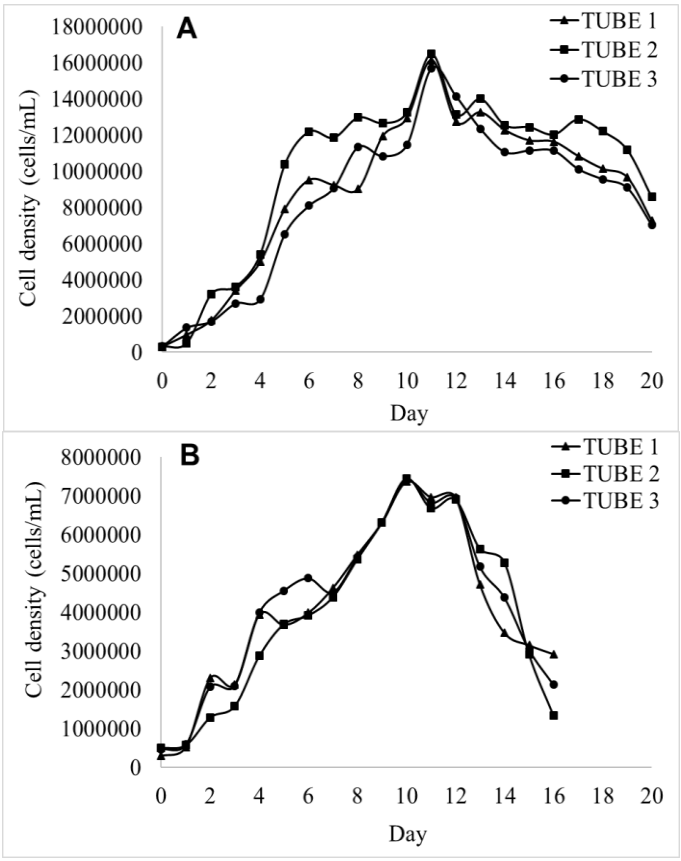


Fig. 3*. The growth curve of Chlorella sp. in F/2 medium: (A) laboratory condition, (B) outdoor condition*

The growth curve of laboratory culture (Fig. 3A) showed a very fast growth from the first day to the fifth day, called exponential (log) phase, from 316 000 cells/ml (the initial density, marked as "0" day) to 7×10^6 cells/ml, higher 23.3 times than the initial density. The algal number increased slightly on the sixth day, and later stayed stable in the range from 9×10^6 to 13×10^6 cells/ml until the sixteenth day. In this range, there was a maximum point where cell density reached the highest quantity 16.3×10^6 cells/ml, higher 50 times than the initial density on the eleventh culture day. The decay phase started on the seventeenth day when the cell number gradually decreased to 7.5×10^6 cells/ml on the twentieth. As a result, no lag phase prior to log phase was observed, which can be explained that TH31 strain experienced a long time to adapt gradually to F/2 medium in the pre and sub-culture period before being cultured in tubular photo-bioreactors. After 4-day exponential phase, the longest phase was stable phase in 10 days prior to short decay phase (3 days). Figure 3B illustrated the proliferation of TH31 in outdoor tubular photo-bioreactors. Similarly, there was no lag phase in the beginning of the growth cycle. In the log phase, the cell density raised significantly from 320 000 to 3.65×10^6 cells/ml, higher 12.2 times than the initial density just after 4 culture days. The increase continued marginally to about 5.38×10^6 cells/ml until the algal cell number reached the highest value, 7.5×10^6 cells/ml, higher 18.7 times than the initial number on the tenth culture day. The stable phase with the stable algal amount in the range of 5 – 7 million cells/ml occurred within 7 days. The 3-day decay phase started on the thirteenth day and lasted until the sixteenth day when the cell amount was only 2.14×10^6 cells/ml. In comparison with the 20-day culture cycle in laboratory condition, the growth cycle was shorter in outdoor condition, only 16 days. In contrast, the cell density reached the maximum number faster. This may be because Chlorella sp. cells received high intensity of natural sunlight, which is very suitable for photoautotrophic organisms including microalgae. Thus, the metabolism was better and proliferation was faster, compared to controlled conditions in the laboratory. The cell density and the maximum density in outdoor culture were obviously always lower about 3 times, 5.5×10^6 cells/ml in average, compared with 16×10^6 cells/ml in average in the laboratory. At the end of the decay phase, the cell number in both systems was still higher than the initial number; however, culturing TH31 in the laboratory could keep the cell concentration at higher value, 7.5×10^6 cells/ml in longer time, compared to outdoor tubes, only 1.2×10^6 cells/ml. These phenomena could lead to a conclusion that tubular photobioreactors with Ø100 mm (in the outdoor system) gave higher efficiency in biomass production than Ø180 mm tubes.

3.2. Wastewater treatment performance of Chlorella sp. in tubular photobioreactors

Before culturing TH31, wastewater was tested and results were interpreted: salinity 8 ‰, pH 7.33, $N\text{-}NH_4^+$ 4.32 mg/L and $P\text{-}PO_4^{3-}$ 0.12 mg/L. The results showed that nutrients of input wastewater were not high. The brackish water with neutral pH range was also suitable for algal growth and development.

Algal growth in the wastewater

The growth of TH31 cultured in laboratory tubes was described in Figure 4A. Its growth had a similar trend in two tubes. TH31 could adapt relatively fast to the wastewater in a short lag phase, with around 300 000 cells/ml, then developed quickly to 700 000 cells/ml on the second day. The cell number obtained the maximum value at 1.1×10^6 cells/ml on the fourth day. However, there was a difference from the culture in F/2 medium that the stable phase was almost not observed in wastewater environment. Instead, the cell density dropped immediately and strongly to a value lower than the initial concentration, nearly to the background value. In the control tube, although Chlorella sp. (TH31) was not added, there were also absorbance signals. They could be signals of some kinds of substances, or signals of micro-algal species available in wastewater. Nevertheless, the important remark is that these signals did not fluctuate significantly over days during the culture. Figure 4B illustrated the algal growth in outdoor systems. In the first and second tubular photo-bioreactors, the trend of cell density change was similar. Beginning with around 300 000 cells/ml, the cell numbers increased to the maximum peak at 850 000 – 900 000 cells/ml on the second culture day, despite of a small drop before in the second tube. After that the algal amount continued to decrease to nearly the value of baseline. The algal growth in the third tubes was quite different, in which the exponential phase lasted longer until the third day. While they entered the decay phase in the other tubes, the algae in the third tube just reached the peak of over 500 000 cells/ml.

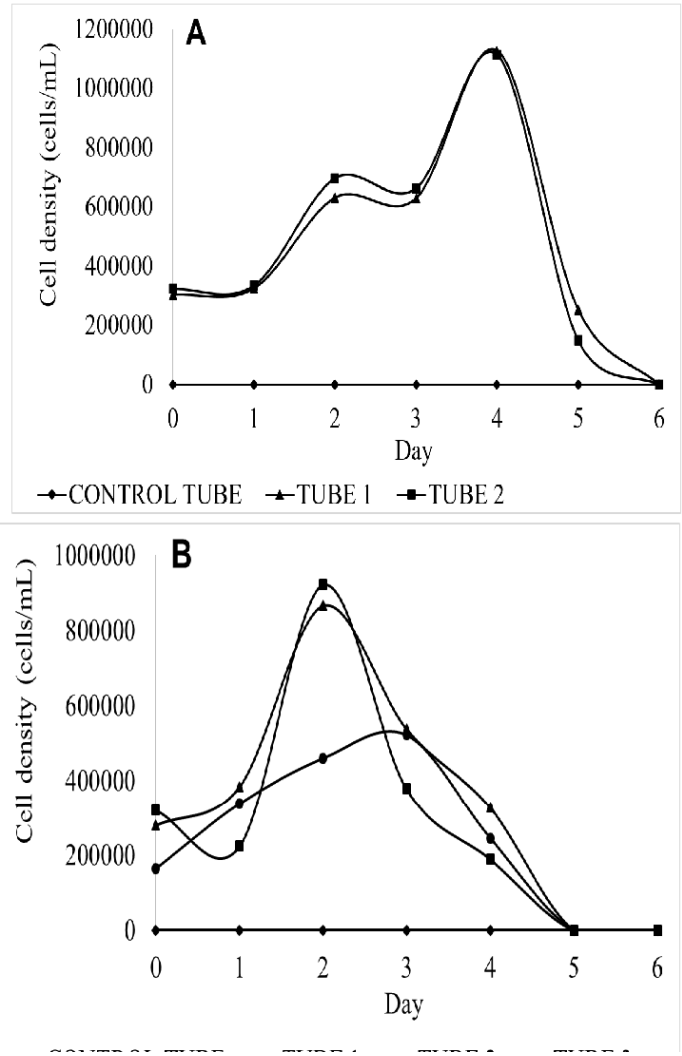


Fig. *4. The growth curve of Chlorella sp. in wastewater: (A) laboratory condition, (B) outdoor condition*

Different from the culture in F/2 medium, when culturing in wastewater, in either laboratory or outdoor condition, Chlorella sp. (TH31) could grow faster in aquaculture wastewater, possibly owing to the suitable amount of composition in the wastewater [32]. Nonetheless, Chlorella sp. needed more time for the multiplication, leading the delay of two days to obtain the maximum cell number in the laboratory models. These outcomes support a hypothesis, that outdoor culture using natural conditions could give higher algae growth, and thereby give higher wastewater treatment efficiency. The growth of microalgae occurred at the same time with the changes of phosphate and ammonium concentrations, which will be discussed in the next section. The pH of water also changed in the neutral to slight basic ranges over time. The pH values of the outdoor bioreactors were always higher than those of the laboratory tubes. Similar to Chlorella sp. growth curve, pH increased dramatically on the second culture day, from 7.3 to the maximum value of 8.1 (7.9 in the control tube) in the laboratory tubes, and to maximum of 8.7 (the first tube), or 8.2 (the control tube) in the outdoor systems. After that, the pH decreased gradually until the value of 7.7 on the last day in the laboratory bioreactors. In contrast, the pH value could remain within 2 days before decreasing gradually until the pH value of around 8.2.

Ammonium removal in the wastewater treatment

The general trend towards the decrease in ammonium concentration during the algal growth, described in Figure 5A. In the laboratory condition, ammonium amount decreased significantly just after one day, from the initial concentration (4.23 mg/L) to only 1.76 mg/L, obtaining 60 % of ammonium removal yield. The NH_4^+ amount continued to decrease until the fourth culture day when the treatment yield reached the maximum, 86.34 % equivalent to very small amount of NH_4^+ (0.59 mg/L). The maximum lasted in short time, just after one day, the ammonium concentration began to increase again. The increase was a signal of decay phase in algal growth when algal population increased with the reduction of nutrients. As a result, the increasing number of dead algal released a particular amount of ammonium in the environment. The similar trends occurred in the control bioreactor. There was also a short period that the ammonium suddenly rose to the peak of around 3.6 mg/L on the second day. After that, NH_4^+ concentration continuously decreased. The lowest NH_4^+ concentration was obtained on the fifth day, 1.08 mg/L converted to 75 % of treatment yield. There was stronger reduction of ammonium concentration in the outdoor culture system. The initial amount of nutrients in wastewater must be within the tolerance of Chlorella sp., they thereby did not need lag phase to adapt. Indeed, if the nitrogen doubled, for example, in case of wastewater effluent from treatment plants, there would be no dramatic decrease in ammonium matched with no growth of microalgae in the lag phase [33]. As can be seen from Figure 5b, from the initial concentration of 4.32 mg/L, the ammonium considerably decreased to 1.86 mg/L (57 %) after two treatment days, then reached the lowest value, up to 0.95 mg/L (77.54 %) on the fourth day. While the culture entered the decay phase on the sixth day in two tubes, the treatment process seemed to continue until the next day in the other tube (tube No. 3). In the control tube, the lowest NH_4^+ amount was 1.12 mg/L, corresponding to 73.52 %.

All in all, the change of ammonium was not highly different in both laboratory and outdoor systems. The both systems had four days to obtain the maximum treatment efficiency. Although NH_4^+ concentrations tended to increase at the end of the cycle, they were still very low, compared to the initial concentration. In comparison to the outdoor system (maximum 77.54 %), the treatment yields of the laboratory system were higher (86.34 %). Without addition of TH31, NH_4^+ concentration rose dramatically at the end of the culture in outdoor system.

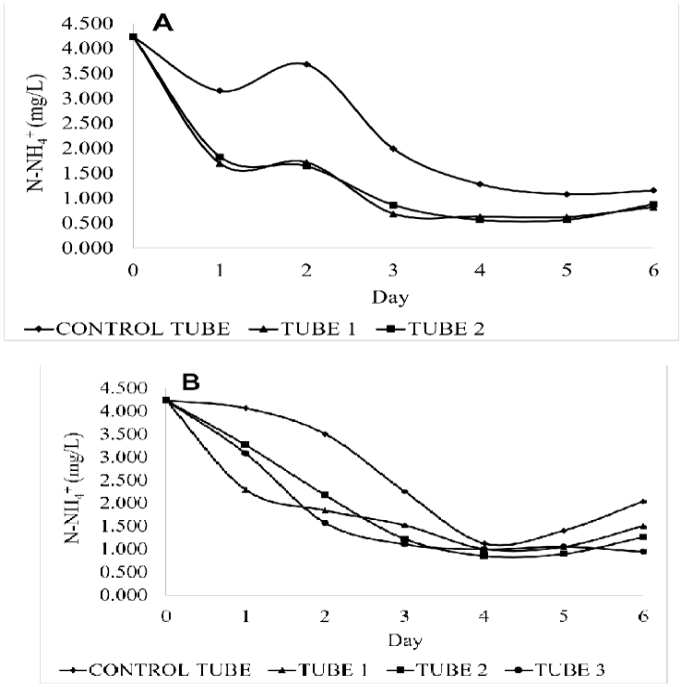


Fig 5. *The ammonium removal of Chlorella sp.: (A) laboratory condition, (B) outdoor condition*

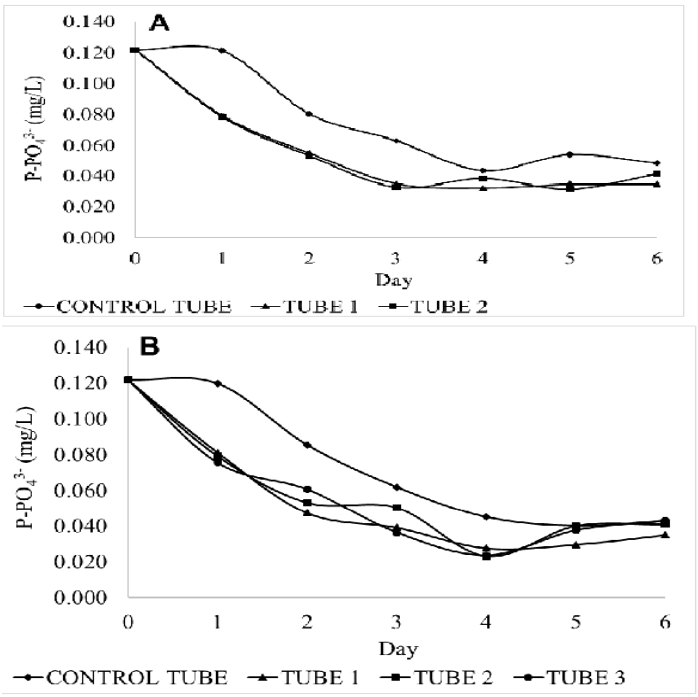


Fig. 6*. The phosphate removal of Chlorella sp.: (A) laboratory condition, (B) outdoor condition*

Phosphate removal in the wastewater treatment

The change of phosphate concentration during the algal culture in shrimp wastewater in the laboratory was described in Figure 6A. Phosphate amount decreased gradually during three days, from the initial concentration (0.12 mg/L) to around 0.3 mg/L, and held this threshold in some days. The lowest PO_4^{3-} measured was only 0.033 mg/L with the maximum yield of 72.5 % on the fourth day. The phosphate concentration then rose slightly on last days of the culture cycle owing to increasing presence of dead algae. Similarly, PO_4^{3-} concentrations decreased noticeably to 0.043 mg/L, corresponding to the maximum removal yield of 64.47 %, also on the fourth day, and seemed to remain or changed slightly on the final days. As showed in Figure 6B, phosphate also reduced noticeably in outdoor tubular photo-bioreactors during 4 days, and reached the lowest value at 0.032 mg/L. At this peak, the maximum treatment efficiencies varied around 79.17 % and tended to decrease at the end. There was a similar change of PO_4^{3-} amount in the control bioreactor. Although purified Chlorella sp. (TH31) was not added to the wastewater, the phosphate also reduced during the experiment time. The lowest concentration obtained was roughly 0.032 mg/L (64.47 %) on the fourth day, and then increased slightly until the end of the culture.

On the whole, the change of phosphate was similar in both laboratory and outdoor systems. The culture experienced four days to receive the maximum treatment yield. Although PO_4^{3-} concentrations tended to increase at the end of the culture, they were still very low, compared with those before treatment. In contrast to ammonium treatment, outdoor photo-bioreactors gave higher phosphate removal yields, up to 80 % maximum, compared with 73 % in the laboratory system. On the other hand, there was a similar trend in control bioreactors, which indicates the presence of unknown microalgae species with the same ability as Chlorella sp. Although the nitrogen and phosphorus removal yields increased over time before the decay phase, the yields of TH31-culture bioreactors were always higher than those in control bioreactors. This difference highlights the important function of purified Chlorella sp. (TH31) when being added to wastewater. In addition to this study, there have been some studies of using Chlorella sp. in outdoor ponds for aquaculture wastewater treatment in Vietnam. For example, according to a research of Tran et al (2015) in which Chlorella sp. was cultured in open ponds (500 liters) to treat wastewater of catfish farming. Surprisingly, the treatment yields were very higher, up to 88.36% and 87.98% of ammonium and phosphate treatment yields, respectively just within 2 days of waiting. The difference mentioned between the two studies is about treatment effectiveness and the treatment time to obtain the highest treatment efficiency. They could be dependent upon the volume of culturing model (tubular bioreactors with open ponds), and the designing ponds. Indeed, open ponds created more opportunities for microalgae to access to the environment and proliferate quickly. The amount of nutrients in the input wastewater is also a reason. In the study of Tran et al (2015), main nutrient sources for algae were NH_4^+ 5.3 mg/L, and PO_4^{3-} 4.1 mg/L [9], which were very higher, especially phosphate amount, 40

times higher than initial concentrations of shrimp production wastewater collected in Can Gio district.

4 CONCLUSION

The outdoor microalgae culture could give faster proliferation in the improved tubular photobioreactor (20 Liter) model with a cooling mist system to maintain a stable temperature of 29-31^{o}C, light intensity of 1000-50000 lux controlled by a roof system. Moreover, tubular PBR model (6 Liter) with smaller pipe size (Ø100 mm) could produce 2.5 times higher cell density than larger size tubes (Ø180 mm). New isolated Chlorella sp. strains (TH31) was able to grow rapidly during the wastewater treatment in the stable natural conditions. The algal population reached its maximum growth on the second day of culture cycle and then gradually decreased on the sixth day. The growth of microalgae was simultaneous with a gradual decrease in the concentration of ammonium and phosphate, obtaining the highest treatment efficiencies after only 4 days, 77.54 % of ammonium and 79.17 % of phosphate removal yield in photobioreactors. Indeed, tubular photobioreactor has been proved to give continuous production with easy control of temperature and contaminants, effective distribution of fresh air and CO_2 and better CO_2 transfer through interface of air and culture-lipid medium (Briassoulis et al., 2010). In other word, it is difficult to compare treatment effectiveness of nitrogen and phosphorus. In the laboratory, Chlorella sp. could remove nitrogen more effectively, up to 86.34 %, which is similar to result of Aslan & Kapdan, 2006 [3]. In contrast, phosphorus removal yield was slightly higher in outdoor bio-reactors (79.17 %). In conclusion, Chlorella sp. strains isolated from shrimp production wastewater were successfully cultured and used for removal of nutrients in shrimp production wastewater in tubular photo-bioreactors in the outdoor conditions. The treated wastewater met well Vietnamese standard of QCVN 14:2008/BTNMT column A for domestic wastewater discharge. These results indicate that isolation of microalgae from wastewater and their 'reuse' ability in wastewater treatment could be a potential technology for advanced wastewater treatment in practice. Nonetheless, it is necessary to look for a feasible way for microalgae harvesting after water treatment in terms of technology and cost.

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