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Batch Phytoremediation of Aquaculture Wastewater of Silver Barramundi (*Lates calcarifer*) Utilizing Green Microalgae; *Chlorella* sp.

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ABSTRACT

Microalgae could be utilized as an alternative to remove inorganic nutrients from wastewater and makes them a useful phytoremediation tool in wastewater treatment process. Phytoremediation is a process that utilized plant such as microalgae to treat the nutrient pollution in the aquaculture wastewater. *Chlorella* sp.'s morphology as unicellular microorganism could allow it to absorb nutrient more efficiently as compared to terrestrial plant. Thus, this study was performed to evaluate the performance of *Chlorella* sp. at different inoculation concentrations to determine its kinetic growth and at the same time their nutrient removal efficiency specifically Total Ammonia Nitrogen (TAN) and Total Phosphorus (TP). Silver Barramundi, *Lates calcarifer* wastewater was inoculated with microalgae culture with different concentrations of 10,15, 20, 25, 35 and 40% (v/v) in 14-days batch treatment period at room temperature (25±2°C). Determination of *Chlorella* sp. growth performance, TAN and TP removal was performed daily based on the APHA's standard method of examination of water and wastewater. In this study, results indicated that the best nutrient removal was in the range of 15-20% (v/v) *Chlorella* sp. inoculation concentrations which yield over 90% nutrient removal with final effluent of 0.08 mg L^{-1} TAN and 0.01 mg L^{-1} TP. The *Chlorella* sp. growth kinetics fitted the Monod model and the nutrient removal fitted with the first order kinetic. It could be concluded that *Chlorella* sp. is a potential candidate to treat aquaculture wastewater and served as green technology to mitigate the greenhouse-related problems.

Key words: *Chlorella* sp., *Lates calcarifer*, batch phytoremediation, total ammonia nitrogen, total phosphorus, monod model, first order kinetics

INTRODUCTION

Phytoremediation is a biological treatment utilizing any type of plant either terrestrial or marine plant. This type of biological treatment has gained its popularity due to its role in remediating pollution such as heavy metals, high nutrient concentration and also contributed positively to the environment. Microalgae is recognized as the most promising candidate for bioprocess due to a multiplicity of reactions (Rawat *et al*., 2011). The use of microalgae is desirable since, they are able to serve a multiple role such as bioremediation as well as generating biomass for biofuel production with concomitant carbon sequestration (Olguiin, 2003; Mulbry *et al*., 2008). In addition, wastewater remediation by microalgae is also an eco-friendly process since it does not release any secondary pollution as long as the biomass produced is continuously reused and efficient nutrient recycling is maintained (Munoz and Guieysse, 2006; Pizarro *et al.*, 2006; Mulbry *et al.*, 2008).

The release of untreated agricultural, industrial and municipal wastewater poses serious environmental challenges to the receiving water bodies (Arora and Saxena, 2005; De Bashan and Bashan, 2010). The major effect of releasing wastewater which is rich in organic compounds and inorganic chemicals such as phosphates and nitrates is mainly eutrophication (Olguiin, 2003; Pizarro *et al.*, 2006; Mulbry *et al.*, 2008; Godos *et al.*, 2009; De-Bashan *et al.*, 2002). According to Rawat *et al.* (2011), this global problem could be solved by utilizing microalgae whereby the wastewater is utilized as feed for the maintenance of microalgae growth. Throughout, the treatment period, there will be concomitant accumulation of biomass for downstream processing enabling the production of biodiesel feedstock (Munoz and Guieysse, 2006; Pizarro *et al.*, 2006; Pittman *et al.*, 2011). Cellular nitrogen is mainly used to build proteins, amino acids and nucleic acids while phosphorus is mostly constituent of nucleic acids and phospholipids (Geider and Roche, 2002). These findings has proven that microalgae are able to remove nutrients from wastewater to meet the stringent requirements according to international standards (Rawat *et al.*, 2011).

This research is motivated by the increasing interest in application of biotechnology and the implementation of environmentally friendly tools in treating wastewater (Yoshimoto *et al.*, 2005; Concas *et al.*, 2010; Sato *et al.*, 2010). *Chlorella* sp. was classified as a biological tool in wastewater treatment to reduce the present of nutrient content (Sydney *et al.*, 2010). At the same time, biomass of *Chlorella* sp. which is the by-product of the treatment process could be marketed as high-value products (Brennan and Owende, 2010). In addition, treatment of phytoremediation could also help in the uptake of various heavy metals and greenhouse gasses present in the wastewater (Davis *et al.*, 2003). This would contribute to overcome environmentally related problem of domestic wastewater reported around the world (De Godos *et al.*, 2010; Listowski *et al.*, 2011; Rawat *et al.*, 2011).

The aim of this study was to determine the possibilities of microalgae *Chlorella* sp. in treating aquaculture wastewater. This was implemented through monitoring of TP and TAN removal performance in aquaculture wastewater by phytoremediation using *Chlorella* sp. The most suitable inoculation concentration in batch mode and the growth kinetics of *Chlorella* sp. throughout the treatment period were investigated.

MATERIALS AND METHODS

Microalgae: The pure strain of green algae *Chlorella* sp. was obtained from the Institute of Tropical Aquaculture (AKUATROP) of University Malaysia Terengganu on October 2011. It was cultivated for 30 days to produce secondary culture for up-scaling. The medium used for culture was Conway media with autoclaved and filtered seawater. Normal air filtered with 40 μm air filter was provided as sterile aeration to prevent any bacterial contamination. Microalgae cultures were maintained at room temperature of about 25±2°C under a light intensity of 4100 lux from white fluorescent light for 24 h photoperiod. Maintenance of secondary culture was done for six months throughout the experimental period.

Fish culture: The aquaculture wastewater was supplied from the culture of *Lates calcarifer* also known as Barramundi or Silver Sea Bass as livestock. *Lates calcarifer* was chosen because of their ability to survive in water at various salinity levels, ranging from 0 ppt. up to 30 ppt. In this study, 30 fishes were reared in a tank under controlled temperature of 27±2°C with continuous aeration. The same type of feed was given based on 3% of body weight to control phosphorus and nitrogen content in the effluent. Nutrients content in the effluent were considered maximum on the fourth day of rearing period. At this time, the wastewater produced was channeled for phytoremediation by microalgae.

Microalgae phytoremediation: The treatment of wastewater was conducted in batch mode using 5000 mL flasks. This treatment commenced with the inoculation of *Chlorella* sp. culture into the wastewater. Six samples with different concentration of *Chlorella* sp. cultures i.e., 10, 15, 20, 25, 35 and 40% (v/v), were examined in this study. Illumination was provided continuously from the top of the flask. The experiments were conducted at controlled room temperature of 25±2°C for 14 days.

Analytical methods: Three parameters of Total Phosphate (TP), Total Ammonia Nitrogen (TAN) and biomass of *Chlorella* sp. concentrations were analyzed. The changes of concentrations in the nutrient content and *Chlorella* sp. biomass were monitored daily for a period of 14 days. Nutrient concentrations (phosphate and nitrogen) were the main concerned for evaluating removal efficiency in accordance to the growth of *Chlorella* sp. biomass as kinetic study.

***Chlorella* sp. biomass concentration analysis:** Analysis of *Chlorella* sp. biomass concentration was performed simultaneously with nutrient concentrations. Daily samples withdrawn from flasks were centrifuged at 4°C to separate algae biomass from water. Two-hundred-milliliters sample from each flask were poured into the four 50 mL centrifuge tubes and then they were centrifuged at 5000-6000 rpm for about 30 min to separate the *Chlorella* sp. cells from water. Then, 10 mL sample of algal suspension was again centrifuged at 3000 rpm and the supernatant was discarded. The algae were suspended in 3 mL methanol and heated for about 5 min in a water bath. The samples were cooled to room temperature and then the volume was made up to 5 mL by adding methanol. The Chlorophyll *a* concentration in the extract was determined using the Eq. 1 with the reading of absorption (A) of the pigment extract in a spectrophotometer at the given wavelength, λ (650 nm and 665 nm) against a solvent blank (Becker, 2008):

$$\text{Chlorophyll } a \ (\text{mg L}^{-1}) = (16.5\times A_{665})-(8.3\times A_{650}) \qquad (1)$$

TP and TAN concentration analysis: Analysis of TP and TAN concentrations were carried out by using HACH DR2400 kit. A 200 mL samples were daily withdrawn from the flasks. Then, the samples were centrifuged at 5000-6000 rpm to separate algae in order to obtain a clear supernatant. Measurements of TP and TAN were executed in clear supernatant by colorimetric methods i.e., Ascorbic Acid Method and Salicylic Acid Method, respectively. These methods were adapted from Standard Method for Examination of Water and Wastewater (APHA, 2012). They were equivalent to USEPA Method 365.2 and Standard Method 4500-PE for wastewater.

Statistical analysis: Microalgae cell growth, TP and TAN concentration were recorded in Microsoft Office Excel throughout the experimental period. Graphical analysis was performed using Originlab OriginPro 8.6 whereas statistical analysis involving ANOVA and Tukey's HSD Test was implemented using Minitab 16. Confidence interval of 95% was selected in order to strictly determine the significance of different *Chlorella* sp. inoculation concentrations on nutrient reduction rate, maximum biomass growth rate, remaining nutrient concentration and treatment period for maximum nutrient reduction.

RESULTS

The comparative studies of the growth performance of *Chlorella* sp., TAN and TP concentrations between different inoculations were determined as shown in Fig. 1. The growth kinetics of *Chlorella* sp. suited the Monod model in line with the growth phases such as lag, log, stationary and declining phases. Treatment with 15% (v/v) *Chlorella* sp. had maximum cell concentration of 49.99, 51.55 mg L^{-1} for 20% (v/v) and 48.36 mg L^{-1} for 40% (v/v) inoculation concentration. The TP and TAN reductions followed the First Order Kinetics where removal percentage continued to increase exponentially until reaching an asymptote where the removal percentage was higher than 99% with final nutrient concentration of 0.08 mg L^{-1} TAN and 0.01 mg L^{-1} TP.

In order to further investigate the effect of different *Chlorella* sp. inoculation concentrations on TP and TAN removal, correlation and regression analysis were performed specifically on Day 7 of the treatment period since the most obvious reduction occurred at this point. Figure 2 shows the remaining TP and TAN concentrations logarithmically decreased with the increased of *Chlorella* sp. inoculation concentration. At Day 7 which was the mid-point of treatment period, 0.23 mg L^{-1} TP and 0.08 mg L^{-1} TAN still remain for 10% (v/v) inoculation as compared to 0.10 mg L^{-1} TP and 0.05 mg L^{-1} TAN for 40% (v/v) inoculation concentration. Therefore, a higher inoculation concentration of *Chlorella* sp. would reduce the remaining nutrient concentrations.

Figure 3 shows the correlation between treatment periods required to achieve the maximum reduction and different inoculation concentrations of *Chlorella* sp. The increase in *Chlorella* sp. inoculation concentration contributed to a shorter treatment period required to reach the maximum TP and TAN removal. A very strong negatively linear relationship with slope of -0.1335 and adjusted R^2 of 0.9057 was established for TP, whereas a similar pattern of strong

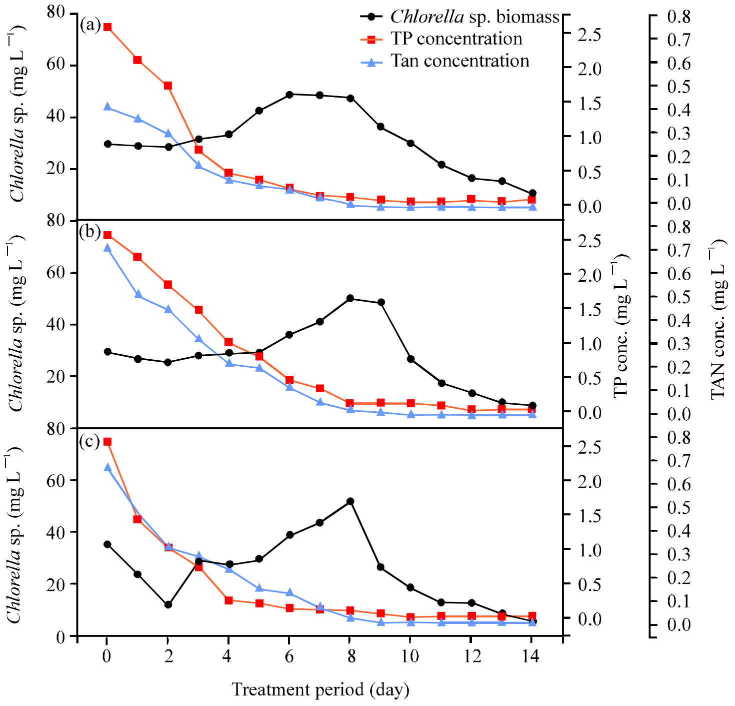

Fig. 1(a-c): Growth performance of *Chlorella* sp., TP and TAN reduction for 14 days treatment period for (a) 15 (b) 20 and (c) 40% (v/v) biomass concentration

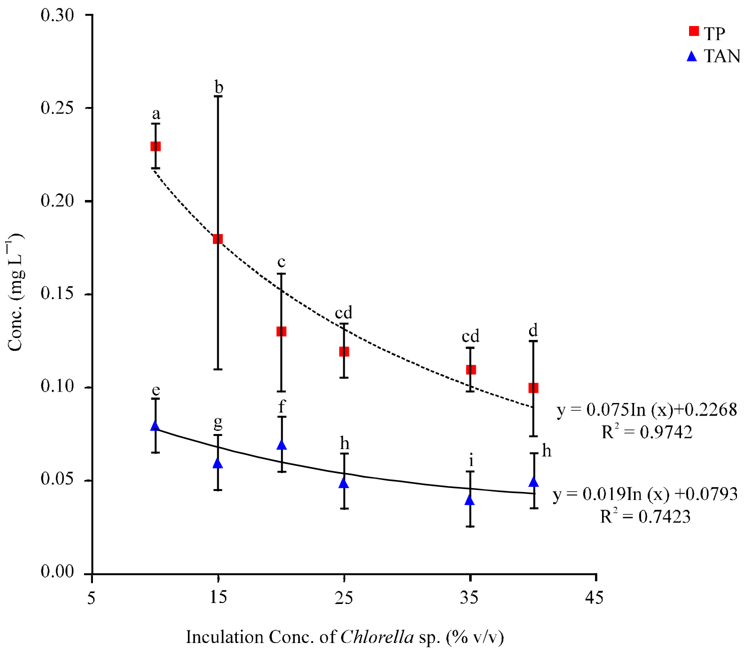

Fig. 2: TAN and TP remaining concentrations after Day 7 of the treatment period at different *Chlorella* sp. inoculation concentrations, Letters above error bar represent significantly different group based on 95% confidence interval

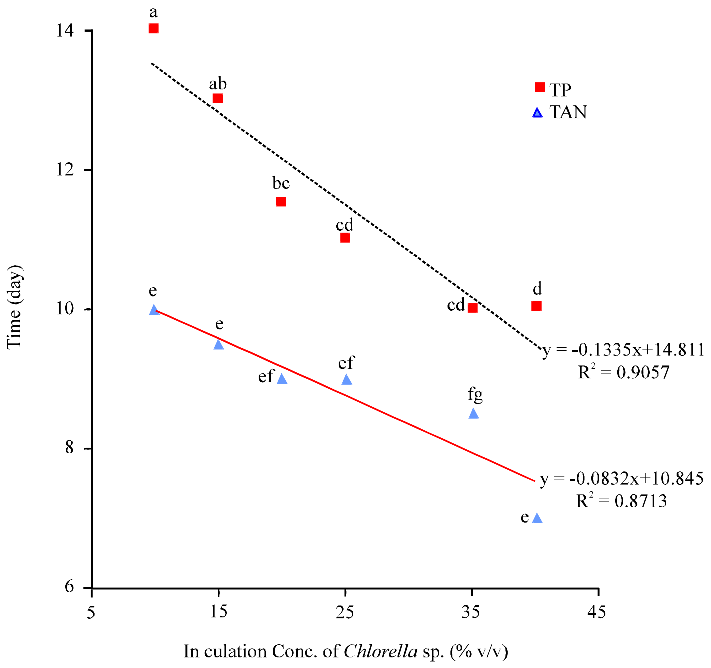

Fig. 3: Treatment periods required to reach maximum TAN and TP reduction at different *Chlorella* sp. inoculation concentration. Letters above data points represent significantly different group based on 95% confidence interval

Table 1: Kinetics coefficient of *Chlorella* sp. growth using Monod model for TP and TAN removal at different inoculation size

	Kinetics coefficient (TP)		Kinetics coefficient (TAN)	
Chlorella sp. (% v/v)	Max. growth rate constant, μ_m (1 day^{-1})	Half saturation constant, K_s (mg L^{-1})	Max. growth rate constant, μ_m (1 day^{-1})	Half saturation constant, K_s (mg L^{-1})
10	$0.521^{a,b}$	0.354^{a}	$0.5048^{a,b}$	$1.23\times10^{-6\,c}$
15	$0.525^{a,b}$	0.252^{b}	$0.4789^{a,b}$	0.0751^{b}
20	0.560^{a}	0.227^{b}	$0.4814^{a,b}$	0.703^{a}
25	0.481^{b}	0.191^{c}	0.4774^{a}	0.0653^{b}
35	0.480^{b}	0.166^{c}	0.5147^{a}	0.0617^{b}
40	0.471^{b}	0.164^{c}	0.4164^{b}	0.0622^{b}

Superscripted letters represent significantly different group based on 95% confidence interval

Table 2: TP removal over 14 days of treatment

	Orthophosphate reduction in effluent (%)													
	Treatment period (day)													
Chlorella sp. inoculation (v/v %)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
10	53.0	55.7	64.8	71.5	84.2	88.5	90.9	91.7	90.5	95.3	96.4	98.8	98.8	99.2
15	12.2	28.1	42.7	60.7	68.8	82.6	87.4	96.0	95.3	95.7	97.2	99.6	99.2	99.2
20	43.5	59.7	70.8	90.1	91.7	94.5	94.9	96.0	97.2	99.6	98.8	98.8	99.2	98.8
25	26.5	39.5	55.7	64.0	71.5	83.0	87.7	91.3	96.4	98.4	99.6	98.0	98.8	98.8
35	20.9	31.6	72.7	78.3	82.2	91.3	95.7	96.4	99.6	99.6	99.2	98.8	99.6	98.0
40	18.2	33.2	69.6	83.0	87.0	91.7	96.0	96.8	98.4	99.6	98.8	98.0	99.2	97.6

relationship with slope of -0.0832 and adjusted R^2 of 0.8713 for TAN removal with the increase of *Chlorella* sp. inoculation concentration. The higher inoculation concentration contributed to a higher readily available biomass which produces higher initial nutrient reduction. After that, *Chlorella* sp. would undergo subsequent mitotic cell division of growth to increase its biomass concentration by utilizing the remaining nutrients exist in the wastewater (De Bashan and Bashan, 2010).

Microalgae growth: Batch phytoremediation on aquaculture wastewater utilizing *Chlorella* sp. was successfully performed. During the study, the concentrations of *Chlorella* sp. used for phytoremediation was varied at 10, 15, 20, 25, 35 and 40% (v/v). All samples were treated for a period of 14 days after *Chlorella* sp. inoculation. Different concentrations of *Chlorella* sp. have shown a quite similar growth pattern and suited the Monod model. As shown in Table 1, the maximum growth rate constant and half saturation constant of TP show reduction with the increase of inoculation concentrations. For 10% (v/v), the half saturation constant, K_s was 0.354 mg L^{-1} and decreasing to 0.164 mg L^{-1} at 40% (v/v) *Chlorella* sp. However, there was no clear trend between kinetics coefficients of TAN and different *Chlorella* sp. inoculation concentrations.

Total phosphorus and total ammonia nitrogen removal: The initial concentrations of TP for all treatments were maintained at 2.50±0.05 mg L^{-1} orthophosphate, PO_4^{3-}-P in order to determine the percentage of TP removal for a period of 14 days. The reading for TP concentration was taken daily to observe its reduction in the effluent. From Table 2, TP concentration in each treatment decreased sharply at early period of *Chlorella* sp. growth due to the rapid increase of *Chlorella* sp.

Table 3: The percentages of TAN removal over 14 days of treatment

	Ammonia nitrogen reduction in effluent (%)												
	Treatment period (day)												
Chlorella sp. inoculation (v/v %)	1	2	3	4	5	6	7	8	9	10	11	12	13
10	33.3	48.6	55.6	61.1	73.6	80.6	88.9	94.4	95.8	98.6	93.1	90.3	83.3
15	27.5	36.2	53.6	68.1	71.0	82.6	91.3	95.7	97.1	98.6	92.8	88.4	84.1
20	28.1	50.0	56.3	64.1	76.6	79.7	89.1	95.3	98.5	95.3	92.1	89.1	87.5
25	11.1	25.9	50.0	64.8	72.2	83.3	90.7	96.3	96.3	98.1	88.9	83.3	74.1
35	10.0	24.0	54.0	70.0	80.0	86.0	92.0	96.0	100.0	86.0	78.0	70.0	60.0
40	11.9	56.2	57.1	71.4	76.2	81.0	95.2	85.7	76.2	66.7	61.9	45.2	45.2

biomass as indicated in log phase. All samples achieved the high percentage of TP removal, 99.6% which indicate that the effluent contain 0.01 mg L^{-1} PO_4^{3-} except for sample of 10% (v/v) *Chlorella* sp. The concentration of 0.01 mg L^{-1} PO_4^{3-} in effluent is being considered as a safe level for effluent to be released to water body and being used for water recycled in aquaculture. The earliest sample that achieved the maximum removal percentage of more than 99% of TP is the sample with 35% (v/v) *Chlorella* sp., followed by the samples with 20 and 40% (v/v) *Chlorella* sp. Next is the sample with 25% (v/v) *Chlorella* sp. and the last one is the sample with 15% (v/v) of *Chlorella* sp. As shown in Table 3, the initial TAN concentrations for all six aquaculture wastewater samples were approximately 0.80±0.05 mg L^{-1} before being inoculated with *Chlorella* sp. Once the treatment of phytoremediation completed, the final TAN concentration with 10% (v/v) *Chlorella* sp. was reduced to 0.12 mg L^{-1}. Therefore, the optimum percentage of microalgae for phytoremediation at specified treatment period was found to be in the range of 15-25% (v/v*)* *Chlorella* sp.

Figure 4 shows the maximum growth rate constant of *Chlorella* sp. analyzed using Monod model and reduction rate analyzed using First Order Kinetics with regard to TAN and TP. The characteristics of log phase were accurately quantified in accordance to the First Order Kinetics. The maximum growth rate constant was at 20% (v/v) inoculation concentration for TP whereas for TAN at 35% (v/v). However, the highest cumulative nutrient reduction rate was at 15% (v/v) slightly decreased as inoculation concentration increased to 25% (v/v). Reduction rate of TP was recorded as the highest within the range of 15-35% (v/v) whereas, for TAN at the range of 10-20% (v/v) inoculation concentrations. Thus, the most suitable concentration of *Chlorella* sp. inoculation that produced the optimum reduction for both nutrients was determined in the range of 15-20% (v/v).

DISCUSSION

The use of several species of microalgae as a tertiary wastewater treatment was proposed over a decade ago and various potential treatments continue to be evaluated today (De Bashan and Bashan, 2010). The underlying assumption is that the microalgae will transform some of the contaminants to non-hazardous materials and then the treated water can be reused or safely discharged (Borowitzka and Borowitzka, 1988). In this study, once the TP and TAN concentrations mostly depleted, *Chlorella* sp. growth began to decline. This was also supported by Droop-based model suggested by Bougaran *et al.* (2010). The interaction of TP and TAN concentrations on the growth of microalgae should be considered at the acquisition level rather than at the assembly level. Nutrient present in the wastewater (extracellular region) affect the *Chlorella* sp. growth more than those in the intracellular region. Thus, this phenomenon indicated

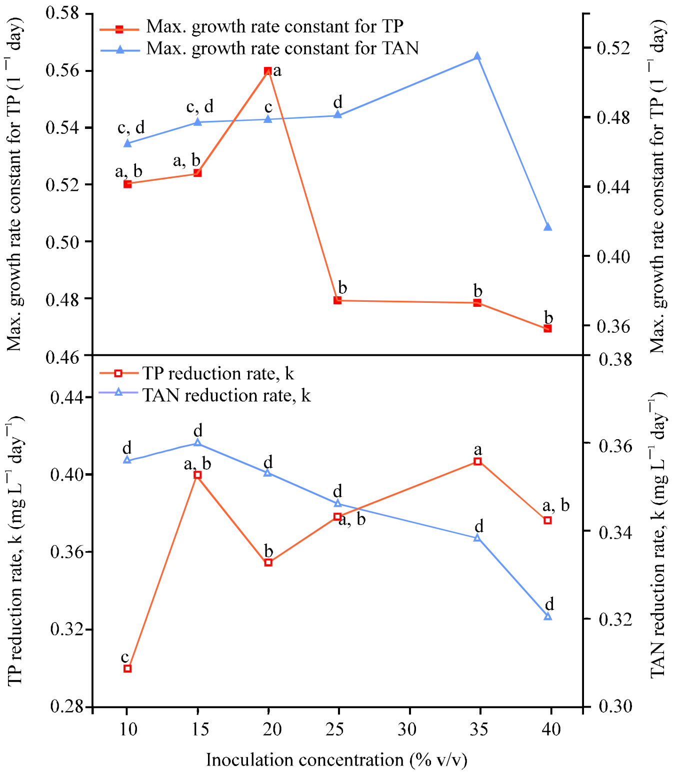

Fig. 4: Maximum growth rate constant of Chlorella sp. with regard to concentration of TP and TAN analyzed using Monod Model (top), reduction rate of TP and TAN analyzed using First Order Kinetics Model. Letters above data points represent significantly different group based on 95% confidence interval

that the present of these nutrients in wastewater contributed significantly on the growth of *Chlorella* sp.

Based on the previous study, microalgae can be effectively used to remove high amount of nutrient because they require large quantity of nitrogen and phosphorus for protein (45-60% of microalgae dry weight), nucleic acid and phospholipids synthesis (Rawat *et al*., 2011). In this study, the concentrations of *Chlorella* sp. used for phytoremediation was varied at 10, 15, 20, 25, 35 and 40% (v/v). Different concentrations of *Chlorella* sp. have shown a quite similar growth pattern and suited the Monod model which consists of lag phase, log phase, stationary phase and death phase. After the inoculation, reduction of microalgae growth occurred within a short period of about 2 days known as lag phase. In this phase, the reduction of growth occurred because microalgae were still adapting with the new environment (El-Sheekh and Fathy, 2009). When microalgae culture inoculated into the aquaculture wastewater, other pollutants present may also contribute to the reduction in its growth. The microalgae growth rate rebounded back once they were successfully adapted to the new environment. This stage was known as log phase. This is indicated by the rapid increase in the biomass as depicted with exponential growth of microalgae. Thus, microalgae utilized the TP and TAN that present in aquaculture wastewater as source of food contributing to the reduction of nutrient.

From nutrients removals aspect, TP in the form of orthophosphate was absorbed by *Chlorella* sp. and being stored as polyphosphates within the cells (Rawat *et al*., 2011). Under subsequent aerobic condition, the orthophosphate is oxidized to produce energy and re-accumulation of phosphate into polyphosphate. The energy generated in this transformation process was used by *Chlorella* sp. for its growth. However, phosphorus concentration above 6 mg L^{-1} will lead to explosive growth of algae. It has become a global problem where ponds and lakes gradually turn into marshes. Algae are considered to be a biological means of phosphorus removal which helps in detecting a potential eutrophication. Since phosphorus is ecologically significant in algal productivity, its removal from aquatic bodies is essential to prevent the occurrence of eutrophication problems.

During the experiment, it was found that microalgae started to degrade themselves after all substrate had been used. When degradation of microalgae occurred, the microalgae would release the nutrients that it had absorbed. Due to this phenomenon, the removal percentage of TAN had decreased after achieving its maximum value. The determination of proper inoculation concentration of *Chlorella* sp. is crucially important for the implementation on the real scale aquaculture wastewater treatment.

The final TAN concentration has been reduced to 0.08 mg L^{-1} after undergoing the full treatment period. In the real application of phytoremediation, this value would represent the actual concentration of TAN before it is disposed into the water body such as river or sea. As recommended by the USEPA Standard, effluent TAN concentration released must be less than 0.2 mg L^{-1} to be considered as environmentally safe since the effluent discharged did not exert significant impact on the flora and fauna. Thus, this indicated that the phytoremediation of aquaculture wastewater was an excellent method in removing nutrient especially TAN and TP.

CONCLUSION

It was shown that the used of microalgae *Chlorella* sp. in reducing harmful nutrients in the aquaculture wastewater was suitable and effective. Based on the kinetic coefficients using Monod model and First Order Kinetics on the removal of both TAN and TP, the optimum concentration of *Chlorella* sp. biomass occurred in the range of 15-20% (v/v) that contributed over 99% nutrient removal with final effluent of 0.08 and 0.01 mg L^{-1}, respectively. Microalgae *Chlorella* sp. did exhibit a normal growth patterns with clear distinction of growth phases. Aquaculture wastewater treatment using microalgae *Chlorella* sp. also known as phytoremediation could be considered as a novel innovation of wastewater treatment technology.

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