STUDY ON THE EFFECT OF MICROALGAE – BACTERIA GROWTH CONDITIONS IN SYNTHETIC WASTEWATER TREATMENT

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ABSTRACT: Microalgae has been widely used for wastewater treatment as wastewater contains nutrients that are essential for microalgal growth. In addition, the co-culture system of microalgae-bacteria was reported to be able to improve the efficiency of wastewater treatment compared to microalgae alone. Bacteria help microalgae break down the complex nutrients present in wastewater into simpler compounds, which will be easily assimilated by microalgae. However, to achieve high nutrients (ammonium and phosphorus) and COD removals in wastewater treatment, the best growth conditions of both microbes need to be investigated. The objective of this study is to investigate how the selected factors affect the removal of nutrients and COD at particular ranges through One-Factor-at-a-Time (OFAT) method. We successfully evaluated the effects of three significant growth conditions, namely the initial concentration of microalgae, initial concentration of bacteria, and inoculation time of bacteria on the removal of nutrients and COD in synthetic wastewater. The highest nutrients and COD removal were achieved when the initial concentration of microalgae and bacteria was at 0.77 g/L and 10^4 CFU/mL, respectively, and the best time to inoculate bacteria was at day 1.52. Moreover, models for all three factors were validated and verified using ANOVA, with percentage error of predicted values and experimental values was less than 10%. Hence, these conditions can be used as the basis for further optimization process in order to improve the wastewater treatment by microalgae-bacteria co-culture system.

KEY WORDS: *Co-culture, microalgae-bacteria, wastewater treatment, nutrients removal.*

1. INTRODUCTION

Conventional treatment methods, such as anaerobic and aerobic digestion process, have been used to reduce nutrients content, such as ammonium and phosphorus, from wastewater before it can be released for irrigation and other purposes. However, the conventional treatment plants are expensive due to the high operational energy cost, whereas some of the treatments are not efficient for nutrients removal [1]. Wastewater treatment plants using aerobic microbes generally require 1 kWh of electricity to supply oxygen for every 1 kg of biochemical oxygen demand (BOD) biodegradation [2]. This leads to high energy consumption for the aeration system, which contributes about 40 – 50% of the total energy consumption in wastewater treatment plants, thus increasing the operating cost [3].

On the other hand, microalgae treatment has gained massive attention as an alternative for biological wastewater treatment. Microalgae can fix carbon dioxide by photosynthesis and effectively remove excess nutrients at considerably low cost [4]. Wastewater could act as the growth media for microalgae since they require nitrate and phosphate present in wastewater to grow [5]. Besides providing major nutrients, wastewater also can be the source of organic carbon from various organic pollutants. Therefore, the use of microalgae in wastewater treatment would simultaneously reduce environmental pollution, as well as reduce the cost for microalgae cultivation [6]. For instance, research has proven that microalgae *Spirulina* was able to reduce COD, nitrogen and phosphorus up to 50.8%, 96.5% and 85.9% respectively when it was cultivated in palm oil mill effluent (POME) for 15 days [7]. The initial concentration of COD, nitrogen and phosphorus in the POME were 700 to 1200 mg/L, 200 to 300 mg/L and 25 ± 5 mg/L respectively. Besides that, *C. vulgaris* co-cultured with *P. putida* in synthetic wastewater for four days of cultivation was able to remove 80 %, 80 % and 60 % from the initial concentration of 490 mg/L of COD, 50 mg/L of nitrogen and 10 mg/L of phosphorus, respectively [8].

Furthermore, the co-culture of microalgae and bacteria was reported to be advantageous in the wastewater treatment system [9, 10]. Microalgae as a photo-synthetic microorganism releases O_2 to be used by the heterotrophic bacteria that could in turn help biodegrade organic pollutants in wastewater, while the bacteria provide the carbon dioxide for microalgae to grow in the presence of light. However, microalgae may exhibit several types of metabolism and they are capable of shifting metabolism in response to changes in environmental conditions, for example from autotrophic to heterotrophic metabolic when there is organic carbon source available in the system. Thus, microalgae may compete with the heterotrophic bacteria under this condition. The efficiency of nutrient uptake by the microalgae-bacteria is not only affected by the availability of nutrients but also depends on the physico-chemical factors, such as temperature, light intensity, pH and biological factors [10].

Although the microalgal – bacterial symbiotic relationship has previously been studied [8, 10, 11, 12], there is no specific literature on how growth conditions of these two microorganisms affect the wastewater treatment was reported. In order to achieve high removal of COD, ammonium and phosphorus in wastewater by microalgae-bacteria co-culture system, significant growth factors for both microbes need to be identified and optimized. Thus, the main focus of this study is to find and optimize the significant growth factors that influence COD and nutrients removals in synthetic wastewater before it can be applied in the real wastewater treatment. The experiment was designed based on One-Factor-at-a-Time (OFAT) design of the Design Expert software. The significance and the best values of the parameters were evaluated using analysis of variance (ANOVA) and least square regression methods and validated by laboratory experiments.

2. Materials and Methods

2.1. Microorganism and Inoculum Preparation

A species of unicellular microalgae, *Chlorella vulgaris* was purchased from Algae Research Laboratory, Institute of Ocean and Earth Sciences, University Malaya, Malaysia. The microalgal was cultured in Tris-Acetate-Phosphate (TAP) medium at 25 ^{o}C and 150 rpm in a rotary shaker. *Azospirillum brasilense* was purchased from Leibniz-Institut DSMZ, Germany and grown in nutrient medium at 30 ^{o}C and 120 rpm in a rotary shaker.

2.2. Synthetic Waste Preparation

Synthetic wastewater for the experiment was prepared using the following components (mg/L): glucose (512.82), NH_4Cl (588.83) and KH_2PO_4 (87.6) to obtain 600 mg/L of

Chemical Oxygen Demand (COD), 200 mg/L of ammonium and 20 mg/L of phosphorus [8, 13]. The pH of the synthetic wastewater was adjusted to 7±0.2 with KOH.

2.3. Experimental Design

The prepared inoculum of microalgae and bacteria were separated from their medium by centrifuging them at 3000 rpm for 5 minutes [8]. The supernatant was discarded, and the pellets were transferred into a 250 ml conical flask containing 150 mL of synthetic wastewater. The experiment was carried out for six days and the growth conditions were at temperature of 25 ºC, shaking speed of 150 rpm and under illumination of white fluorescent light (alternate light/dark periods of 12 h/12 h) of 45 – 50 μmol photon m^{-2} s^{-1} [14]. Samples were collected after six days and analyzed for COD, ammonium and phosphorus removals. Each experiment was performed in triplicate.

The design of experiment was based on One-Factor-at-a-Time (OFAT) design and three factors were tested for three responses (i.e. COD, ammonium and phosphorus removal), which were microalgae initial concentration, bacteria initial concentration and bacteria inoculation time. The experiment for each factor was conducted individually by varying one factor and fixed the other two factors. The conditions of the experiments are presented in Table 1.

The *p*-value and *R*-squared were evaluated using ANOVA and least square regression method, with $p < 0.05$ indicating that the model terms are significant. The design of experiment for the three factors based on OFAT is shown in Table 2.

Table 1: Factors and parameters for OFAT experiments

Factor	**Range Tested**	**Fixed Parameters**
Microalgae Initial Concentration	0.02 mg/L – 2.0 mg/L	10^5 CFU/mL bacteria inoculum concentration, bacteria inoculation at day 0
Bacteria Initial Concentration	10^2 CFU/mL – 10^6 CFU/ml	0.3 mg/L algae inoculum concentration, bacteria Inoculation at Day 0
Bacteria Inoculation Time	Day 0 – Day 4	0.32 mg/L algae inoculum concentration, 10^2 CFU/mL bacteria inoculum concentration,

Table 2: Design of experiment based on OFAT.

Run	Microalgae Initial Concentration (g/L)	Bacteria Initial Concentration (CFU/mL)	Bacteria Inoculation Time (day)
1	1.50	6.00	4.00
2	2.00	5.00	3.00
3	1.01	2.00	2.00
4	0.02	6.00	0.00
5	0.02	3.00	0.00
6	2.00	2.00	1.00
7	0.52	4.00	4.00

2.4. Microalgae and Bacterial Growth

The cell number of microalgae, C was counted for every two days using the Neubauer haemocytometer under a microscope and the specific growth rate was calculated using Eq. (1)[16].

$$\mu\,(day^{-1}) = \frac{\ln^{C_2}/_{C_1}}{t_2 - t_1} \tag{1}$$

where, C_1 and C_2 denoted the cell number of microalgae (cells/mL) at time t_1 and t_2 (d), respectively. Cell number of bacteria was counted using colony forming unit (CFU) method.

2.5. Analytical Methods

The performance of wastewater treatment by microalgae – bacteria co-culture system was indicated by the percentage of COD and nutrients removals. The percentage of removal was calculated using Eq. (2)[16].

$$\%\ \textbf{of removal} = \frac{\text{Initial Concentration}\left(\frac{mg}{L}\right) - \text{Final Concentration}\left(\frac{mg}{L}\right)}{\text{Initial Concentration}\left(\frac{mg}{L}\right)} \times \mathbf{100} \tag{2}$$

Standard methods for the examination of water and wastewater were used to analyze the COD, ammonium and phosphorus concentrations. COD, ammonium and phosphorus were respectively analyzed by closed reflux of colorimetric method, phenate method and ascorbic acid method [15].

3. Results and discussion

3.1. Effect of Microalgae Initial Concentration on Nutrients and COD Removals

The effect of microalgae initial concentrations on nutrients and COD removal was studied by varying microalgae initial concentrations from 0.02 g/L to 2 g/L, while the other two parameters were fixed at 10^5 CFU/mL of bacteria inoculum concentration and bacteria was inoculated during day 0.

The effect of the initial concentrations of the *C. vulgaris* in synthetic wastewater on nutrients and COD removals after six days of treatment is illustrated in Fig. 1, while the growth profiles of microalgae in the system during six days of treatment, is shown in Fig. 2.

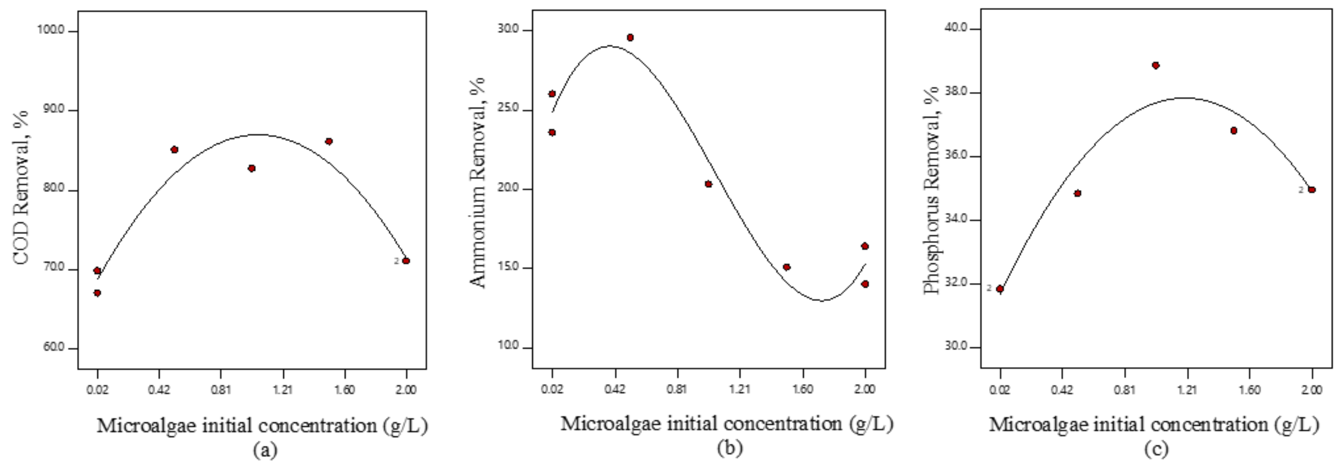


Fig. 1. The percentages of removal from OFAT at different initial concentration of *C. vulgaris* in culture medium varied from 0.02 g/L to 2.0 g/L, bacteria concentration fixed at

10^5 CFU/mL and bacteria inoculation time fixed at day 0. (a) % COD removal (b) % ammonium removal (c) % phosphorus removal.

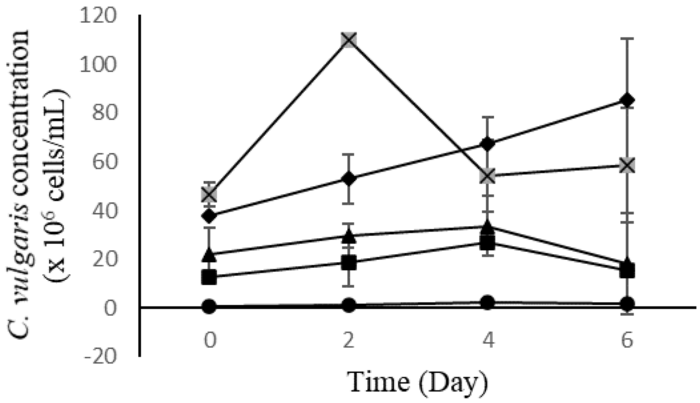


Fig. 2. Growth profiles of *C. vulgaris* for six days treatment in synthetic wastewater with different microalgae initial concentration (● 0.02 g/L, ■ 0.52 g/L, ▲ 1.01 g/L, ◆ 1.5 g/L and ☒ 2.0 g/L). Error bars represent standard deviation about the mean (n = 3).

From Fig. 1, the highest COD, ammonium and phosphorus removals were 86.1%, 29.6% and 38.9%, respectively. These were achieved at microalgae initial concentration of 1.5 g/L, 0.52 g/L and 1.01 g/L, respectively. The percentages of COD and nutrients removals were low at minimum (0.02 g/L) and maximum (2.0 g/L) of microalgae initial concentration. For lowest initial microalgae concentration, the removals of COD and nutrients were low due to the low population of microalgae, approximately 10^6 cells/mL compared to the other concentrations, which was more than 10^7 cells/mL.

However, too concentrated microalgae inoculum would inhibit the microalgae growth due to self-shading effect, an accumulation of auto-inhibitors and a reduction in photosynthetic efficiency in microalgae culture [16]. Thus, high concentration of microalgae after optimum concentration would not increase the growth of microalgae as well as the COD and nutrients removals. In addition to that, high specific growth rate of microalgae was recorded (0.429 d^{-1}) in the first two days of treatment. However, the population of microalgae drastically decreased afterwards, which results in low COD and nutrients removals after six days of treatment.

Based on the three responses obtained, the optimum value for microalgae initial concentration generated by ANOVA is 0.77 g/L. The model for this factor was significant for the three responses since p-value were less than 0.05 with adjusted and predicted R^2 is within the range difference of 0.2 as shown in Table 3. The equation for each removal is shown in Eqs. (3), (4) and (5), where A represents the microalgae initial concentration.

Table 3: ANOVA results for microalgae initial concentration factor.

Responses	p-value	Adjusted R^2	Predicted R^2
% COD Removal	0.0095	0.8537	0.7038
% Ammonium Removal	0.0169	0.9064	0.9532
% Phosphorus Removal	0.0049	0.8955	0.7936

$$\% \text{ COD Removal} = 86.99 + 1.28\,A - 16.92\,A^2\,0.9025 \quad (3)$$

$$\% \text{ Ammonium Removal} = 21.76 - 17.70A - 1.65A^2 + 12.90A^3 \quad (4)$$

$$\% \text{ Phosphorus Removal} = 37.71 + 1.60\,A - 4.40A^2 \quad (5)$$

3.2. Effect of Bacteria Initial Concentration on Nutrients and COD Removals

The effect of bacteria initial concentrations on nutrients and COD removal was studied by varying bacteria initial concentrations from 10^2 CFU/mL to 10^6 CFU/mL, while the other two parameters were fixed at 0.32 mg/L of microalgae inoculum concentration and bacteria was inoculated during day 0.

The individual effect of the second factor to nutrients and COD removals by *C. vulgaris* after six days of treatment is demonstrated in Fig. 3.

It was reported that the addition of phytohormones produced by *A. brasilense* could increase the microalgal population [9]. In fact, *C .vulgaris* can grow well under unfavorable aquatics conditions (high pH and presence of toxic substances) when they were co-cultured with *A. brasilense* [17]. However, as shown in Fig. 4, *C. vulgaris* cells population decreased or had a lag phase for the first two days in the system. A recent research proved the temporary decrease in microalgal population in the co-culture systems is due to the adaptation period for the cells in co-culture environment [8].

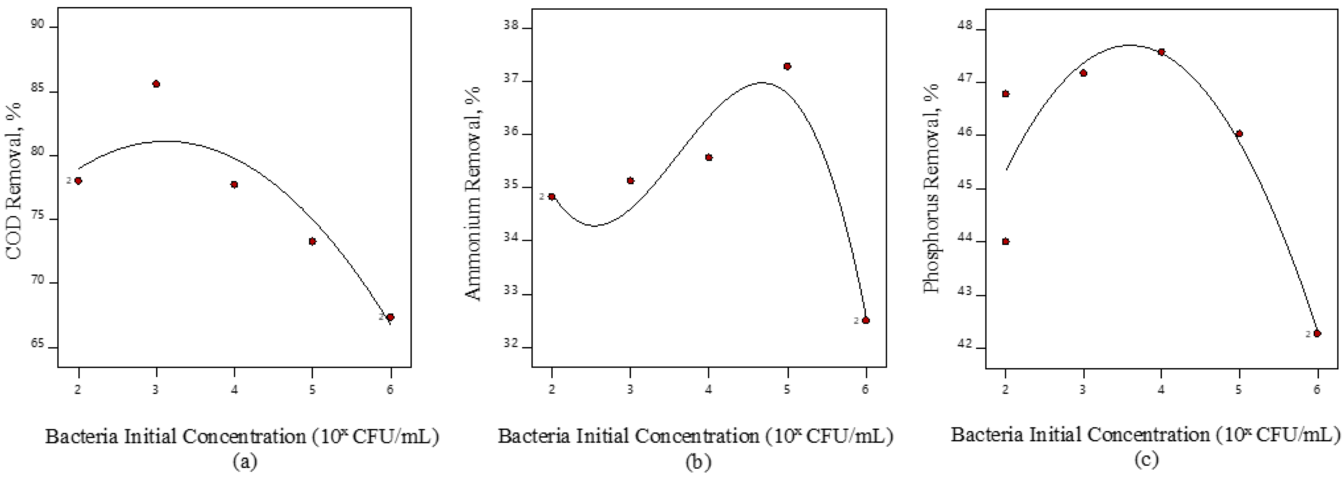


Fig. 3. The percentages of removal from OFAT when the bacteria initial concentration varied from 10^2 cfu/ml to 10^6 cfu/ml, microalgae initial concentration fixed at 0.32 g/L and bacteria inoculation time fixed at day 0. (a) % COD removal (b) % ammonium removal (c) % phosphorus removal.

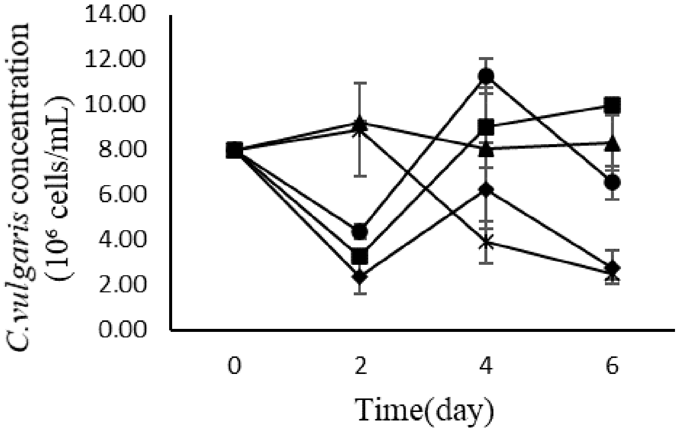


Fig. 4. The growth profile of *C. vulgaris* in the synthetic wastewater treatment with different bacteria initial concentrations (● 10^2 CFU/mL, ◆10^3 CFU/mL ▲10^4 CFU/mL, ■ 10^5 CFU/mL and ×10^6 CFU/mL). Error bars represent one standard deviation about the mean (n = 3).

Based on Fig. 3, the highest COD, ammonium and phosphorus removal were 85.6%, 37.3% and 47.6% at 10^3, 10^5 and 10^4 CFU/mL of bacteria initial concentration, respectively. On the other hand, COD, ammonium and phosphorus removal were low at the lowest and highest bacteria initial concentration. At low bacteria concentration (10^2 CFU/mL), the effect of bacteria in the co-culture system was not significant on the removal of the nutrients. On the other hand, low nutrients removal was recorded at maximum bacterial population due to the competition of the nutrients between the bacteria and microalgae. A similar study compared the nutrients removal by axenic and co-culture system [18] was obtained. The initial concentration of bacteria used was 1x10^6 cfu/ml. It shows that, co-culture system has improved only about 10% in the synthetic wastewater treatment. At this condition, the bacteria growth rate is higher than microalgae growth rate, hence the utilization of nutrients was dominated by the bacteria cells.

Based on the three responses obtained, the optimum value for bacteria initial concentration generated by ANOVA was 10^4 CFU/mL. The model for this factor was significant for the three responses since p-value was less than 0.05 with adjusted and predicted R^2 is within the range difference of 0.2 as shown in Table 4. The equation for each removal is shown in Eqs. (6), (7) and (8), where B represents the bacteria initial concentration

Table 4: ANOVA results for bacteria initial concentration factor.

Responses	p-value	Adjusted R^2	Predicted R^2
% COD Removal	0.0134	0.8264	0.7091
% Ammonium Removal	0.0286	0.8668	0.9334
% Phosphorus Removal	0.0159	0.8109	0.8739

$$\% \text{ COD Removal} = 79.76 - 6.11B - 6.92\,B^2\ 0.8842 \quad (6)$$

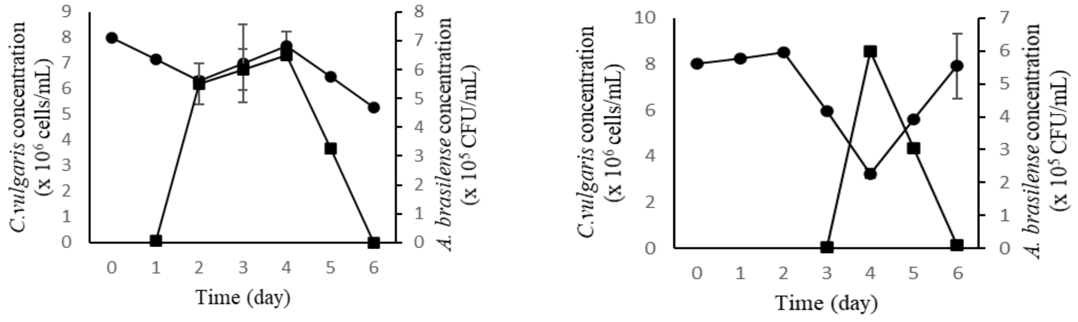
$$\% \text{ Ammonium Removal} = 36.35 + 3.27B - 2.62B^2 - 4.43B^3 \quad (7)$$

$$\% \text{ Phosphorus Removal} = 47.55 - 1.51B - 3.72B^2\ 0.8739 \quad (8)$$

3.3. Effect of Bacteria Inoculation Time on Nutrients and COD Removals

The effect of bacteria inoculation time on nutrients and COD removal was studied by varying time to inoculate bacteria in the system from day 0 to day 4, while the other two parameters were constant at 0.32 mg/L of microalgae inoculum concentration and 10^2 CFU/mL of bacteria initial concentrations.

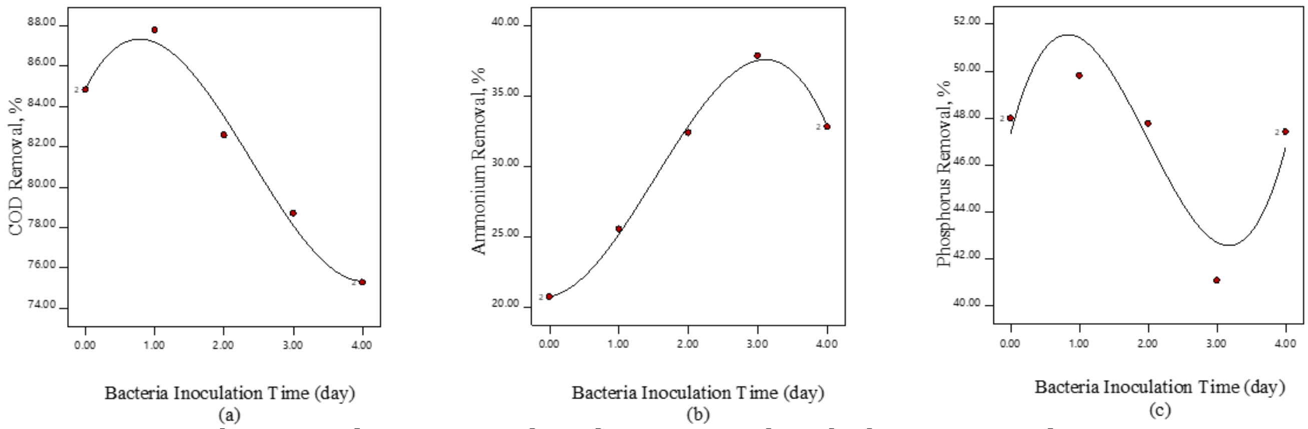
The growth profiles of *C. vulgaris* and *A. brasilense* in each experiment of different bacteria inoculation time with fixed microalgae and bacteria initial concentration are shown in Fig. 5.



(a) (b)

Fig. 5. Growth profiles of microalgae (● *C. vulgaris*) and bacteria (■ *A. brasilense*) in 150 ml of synthetic wastewater for different bacteria inoculation time (a) Day 1 and (b) Day 3. Error bars represent one standard deviation about the mean (n = 3).

The individual effect of the third factor to nutrients (ammonium and phosphorus) and COD removal by *C. vulgaris* after six days of treatment is demonstrated in Fig. 6.



(a) (b) (c)

Fig. 6. The removal percentages based on OFAT when the bacteria inoculation time varied from day 0 to day 4, microalgae and bacteria initial concentration were fixed at 0.32 g/L and 10^3 CFU/mL respectively. (a) % COD removal (b) % ammonium removal (c) % phosphorus removal.

The co-culture system was proved to have positive effect on the COD and nutrients removals in synthetic wastewater compared to the axenic system [18]. However, to optimize the effect of this co-culture system and ensure maximum removals of contaminants in wastewater treatment, the time to introduce bacteria in the system has to be investigated further. As presented in Figs. 5 (a) and (b), a few days after the adaptation period, the *C. vulgaris* growth increased when *A. brasilense* was introduced in the synthetic wastewater. It shows that, the presence of bacteria in the system could enhance the growth of microalgae. Bacteria help degrade the intractable compounds such as nitrogen, phosphate and ammonium, so that they can be easily taken up by microalgae [19], hence increasing the population of microalgae.

Figures 6 (a) and (c) show that, the highest COD and phosphorus removals were achieved when bacteria were inoculated at day 1, which were 87.8% and 47.8%, respectively. COD and phosphorus removals were higher when the bacteria were introduced at day 1 compared to day 3 because the population of bacteria was higher. Early introduction of bacteria into the system would help microalgae degrade and remove more COD and phosphorus. The mechanism of COD removal by the bacteria is by oxidative degradation through heterotrophic metabolism for carbon and energy usage [8].

On the other hand, ammonium removal was higher when the bacteria were introduced at day 3 compared to day 1 when the specific growth rate of microalgae was higher on day 1, which was $0.45d^{-1}$ compared to $0.10d^{-1}$ on day 3. High microalgae population would promote higher removal of ammonium which are used for the cell's growth.

Based on the three responses obtained, the optimum value for bacteria inoculation time generated by ANOVA was at day 1.52. The model for this factor was significant for the three responses since p-value was less than 0.05 with adjusted and predicted R^2 is within the range difference of 0.2 as shown in Table 5. The equation for each removal is shown in Eqs. (9), (10) and (11), where *C* represents the bacteria inoculation time.

Table 5: ANOVA results for bacteria inoculation time factor.

Responses	*p*-value	Adjusted R^2	Predicted R^2
% COD Removal	0.0018	0.9788	0.8877
% Ammonium Removal	0.0001	0.9966	0.9818
% Phosphorus Removal	0.0260	0.7583	0.8389

$$\% \text{ COD Removal} = 83.47 - 10.50C - 3.35C^{2} + 5.73C^{3} \quad (9)$$

$$\% \text{ Ammonium Removal} = 32.88 + 14.39C - 6.06C^2 - 8.36C^3 \quad (10)$$

$$\% \text{ Phosphorus Removal} = 47.07 - 11.52C + 11.24C^{3}0.8389 \quad (11)$$

According to the ANOVA and polynomial model regression analysis in the OFAT design, the models for all factors are significant since all the *p*-values are less than 0.05 with adjusted and predicted R^2 is within the range difference of 0.2. Thus, it shows that these three factors, namely microalgae initial concentration, bacteria initial concentration and bacteria inoculation time, were significant on the removal of COD and nutrients in the synthetic wastewater. The models for percentage removal of COD, ammonium and phosphorus were validated based on the solutions suggested in OFAT design values as shown in Table 6. The responses values were based on triplicate experiments. As presented in Table 6, the percentage error predicted values with experimental values were less than 10%. This suggested that the model is valid and thus verified.

Table 6: Result of validation model by comparing the predicted and experimental response (COD, ammonium and phosphorus removal).

A (g/L)	B (CFU/ mL)	C (days)	% COD Removal			% Ammonium Removal			% Phosphorus Removal		
			Pre	Exp	% Error	Pre	Exp	% Error	Pre	Exp	% Error
0.77	10^5	0	85.7	80.0	7.5	25.8	28.5	8.8	37.1	35.2	5.1
0.32	10^4	0	79.7	72.9	9.8	36.4	39.4	8.6	47.5	44.0	8.0
0.32	10^2	1.52	85.7	79.0	8.9	29.2	29.1	0.4	49.7	45.5	9.2

A: Microalgae initial concentration, B: Bacteria initial concentration, C: Bacteria inoculation time, Pre: Predicted, Exp: Experimental

4. CONCLUSION

In this study, we successfully evaluated three factors that significantly influenced the removal of nutrients and COD in synthetic wastewater treatment by microalgae-bacteria co-culture system by using OFAT method. Based on the generated equation models, the highest removal for COD, ammonium and phosphorus are 86%, 29% and 50%, respectively. These values were obtained at 0.77 g/L microalgae initial concentration, 10^4 CFU/mL bacteria initial concentration and bacteria inoculation time of 1.52 day. Subsequently, the values of these three factors could be further investigated using Face Centered Central Composite Design (FCCCD) coupled with Response Surface Methodology (RSM) of Design Expert Software to study their interactions.

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