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Wastewater treatment by microalgae-bacteria co-culture system

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ABSTRACT

Aim: Microalgae is one of the bioremediation agents in wastewater treatment due to its ability to degrade nutrients and organic compounds. Several studies reported that co-cultivation of microalgae and bacteria, i.e. Microalgae Growth Promoting Bacteria (MGPB) could improve the nutrients removal process. This MGPB helps to degrade complex nutrient compounds into smaller components before being taken up by microalgae. The objective of this study is to investigate the effectiveness of co-cultured (microalgae and bacteria) system compared to axenic microalgae system in the removal of major nutrients (ammonium and phosphorus) and chemical oxygen demand (COD) in synthetic wastewater.
Methodology and result: In this study, two different strains of microalgae (*Chlorella vulgaris* and *Scenedesmus quadricauda*) were each co-cultured with a MGPB (*Azospirillum brasilense*) and their effectiveness in the removal of major nutrients and COD were compared. The results show that, the nutrients and COD removal were higher in co-cultured system compared to the axenic microalgae under similar cultivation conditions for both microalgae strains. Higher percentage removal was obtained from co-cultured *C. vulgaris* compared to that from co-cultured *S. quadricauda* which were 86% and 48%, 44% and 31%, 62% and 35% for ammonium, phosphorus and COD removal respectively.
Conclusion, significance and impact of study: The findings of this study demonstrate the potential of the co-culture of *C. vulgaris* and *A. brasilense* to be applied in wastewater treatment, specifically replacing the aerobic digestion process in secondary stage of conventional wastewater treatment. This study provides an important insight into developing an efficient and environmental friendly method to treat wastewater by incorporating the green technology in the treatment system.

Keywords: Bioremediation, co-culture, microalgae, synthetic wastewater

INTRODUCTION

Eutrophication in water bodies such as municipal wastewater occurs when inorganic nutrients like nitrogen and phosphorus are present in excess. These nutrients need to be removed before the wastewater can be discharged into river to prevent the water pollution, which would affect the aquatic life. As regulated by the Department of Environment Malaysia, the Chemical Oxygen Demand (COD), Ammoniacal Nitrogen and Phosphorus concentrations in water for irrigation purpose should be less than 100 mg/L, 2.9 mg/L and 0 mg/L, respectively. The conventional nutrient removal process is costly and complex, which requires a few stages of process with different conditions. Therefore, developing an efficient and cost effective technology for the wastewater treatment is crucial (Su *et al*., 2011).

Recently, microalgae have received massive attention as an alternative system for biological wastewater treatment. Microalgae have high potential to be used for wastewater treatment because they are photosynthetic microorganisms that can grow in harsh conditions due to their simple multicellular structure (Martins *et al*., 2010). Microalgae have the ability to take up the nutrients in wastewater and convert them into biomass that can be used to produce different products such as biodiesel, fertilizer, and aquaculture feed.

It was previously reported that, the removal of nutrients in wastewater can be improved with co-culture of microalgae and heterotrophic bacteria (De-Bashan *et al*., 2004; Mutjaba *et al*., 2015; Delgadillo-mirquez *et al*., 2016). This bacteria is known as Microalgae Growth Promoting Bacteria (MGPB) (Hernandez *et al*., 2006), which helps to degrade organic pollutants from wastewater. Besides that, one of the direct mechanisms of microalgae and bacteria is O_2 and CO_2 exchange. Microalgae undergo photosynthesis process and produce O_2, which will be consumed by heterotrophic bacteria, while, bacteria in return released CO_2 that can be used by microalgae for growth. This shows the symbiosis of these microorganisms where the O_2 and CO_2 were freely exchange for their growth and metabolism. However, microalgae can exhibit many types of metabolism (e.g. heterotrophic, autothrophic and mixothrophic) and are capable of shifting the metabolism when the environmental conditions change (Martins *et al*., 2010). In such a case, they may compete with the heterotrophic bacteria under certain conditions.

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Selection of the microalgae species for wastewater treatment is important in order to achieve high removal of nutrients. Green microalgae are usually the species that can tolerate to many wastewater conditions (high amount of nutrients and COD). Some species that have relatively high efficiency to remove more nutrients, as well as to produce more lipids are *Chlorella* and *Scenedesmus* species. They are the predominant microalgal communities in waste stabilization ponds and high rate algal pond (Wu *et al*., 2014). There was study that compared these two microalgae species in nutrients removal from wastewater but they were not co-cultured with the MGPB (Kamyab *et al*., 2015).Thus, the aim of this study is to evaluate the efficiency of these two microalgae species co-cultured with MGPB for nutrients and COD removal in synthetic wastewater before it can be applied to real wastewater treatment.

MATERIALS AND METHODS

Microorganism and inoculum preparation

Two species of unicellular microalgae*, Chlorella vulgaris* and *Scenedesmus quadricauda* were used in this study. These two microalgae were purchased from University of Malaya, Malaysia. Both microalgae were maintained in Tris-Acetate-Phosphate (TAP) medium at 25 °C and 150 rpm in a rotary shaker and MGPB *Azospirillum brasilense* was grown in M9 nitrogen-free medium (De-Bashan *et al*., 2004).

Synthetic wastewater preparation

Synthetic wastewater for the experiment was prepared using the following components (mg/L): Glucose (256.41), NH_4Cl (35.33) and KH_2PO_4 (43.8) to obtain 300 mg/L of Chemical Oxygen Demand (COD), 12 mg/L of ammonium and 10 mg/L of phosphorus (Hernandez *et al*., 2006; Mujtaba *et al*., 2015). The pH of the synthetic wastewater was adjusted to 7±0.2 with KOH.

Experimental set-up

The inoculum for both cells (microalgae or bacteria) were separated from their medium by centrifuging them at 3000 rpm for 5 minutes (Mujtaba *et al*., 2015). The supernatant was discarded and the pellets were transferred into a 250-mL conical flask containing 150 mL of synthetic wastewater as shown in Figure 1. The initial concentration of microalgae was approximately 3×10^6 cells/mL and $3\text{x}10^5$ cells/mL for *C. vulgaris* and *S. quadricauda* respectively. The bacteria initial concentration was $1\pm0.8\times10^6$ cells/mL. The experiment was carried out for 8 days and the growth conditions were 25 °C, 150 rpm and under illumination of white fluorescent light (alternate light/dark periods of 12 h/12 h). Samples were taken every two days to record the growth of microalgae and bacteria as well as analyze the COD, ammonium and phosphorus removal in the system. Each experiment was performed in triplicate.

Analytical methods

Microalgae cell numbers were determined by Neubauer haemocytometer counting and bacterial concentrations were measured as colony forming unit (CFU) on M9 medium agar plates. While, COD, ammonium and phosphorus removal were measured using Standard Methods for the Examination of Water and Wastewater (APHA, 1999). COD was analysed by the closed reflux, colorimetric method while ammonium by the phenate method and phosphorus (ortophosphate) by the ascorbic acid method.

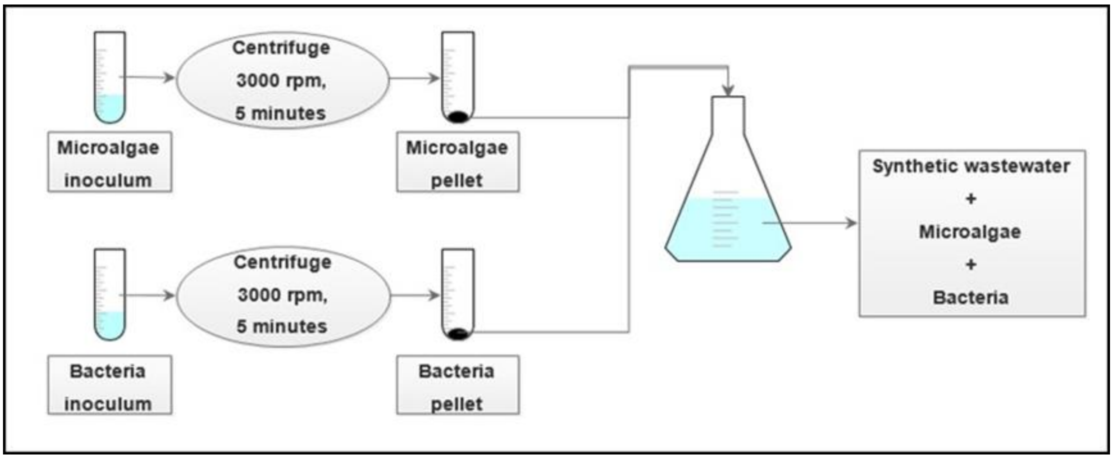

Figure 1: Experimental set-up.

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RESULTS AND DISCUSSION

Microalgae population

Growth profile of both microalgae species (*C. vulgaris* and *S. quadricauda)* for axenic and co-cultured systems are shown in Figure 2.

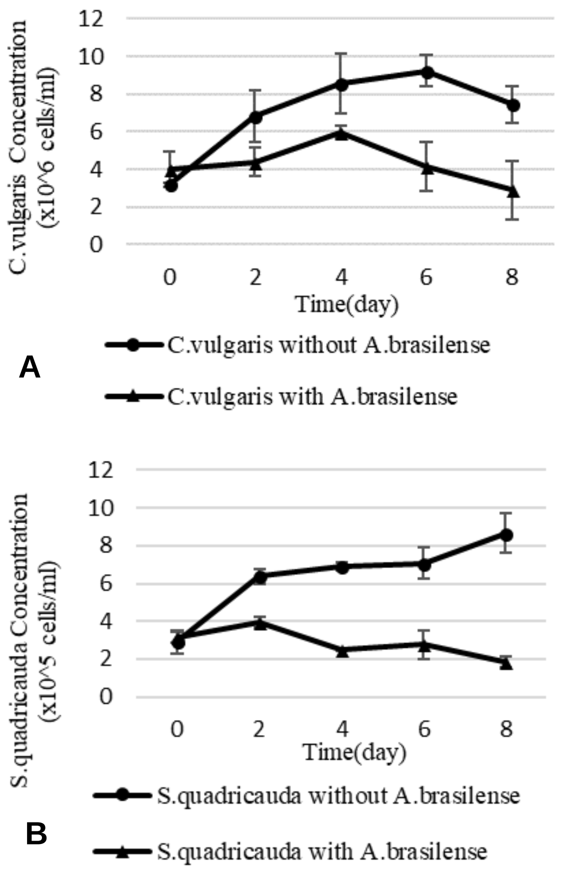

Figure 2: Growth profile of axenic and co-cultured microalgae in synthetic wastewater (A) *C. vulgaris* ($\mu_{C.vulgaris}$ = 0.275/day, $\mu_{C.vulgaris\ with\ A.brasilense}$ = 0.203/day) (B) *S. quadricauda* ($\mu_{S.\ quadricauda}$ = 0.216/day, $\mu_{S.\ quadricauda\ with\ A.brasilense}$ = 0.118/day). Cultivation was done in 250 mL conical flask containing 150 mL synthetic wastewater, pH 7±0.2 at 25 °C, 150 rpm, under illumination of white fluorescent light of (alternate light/dark periods of 12 h/12 h), initial concentration of *A. brasilense* was 1±0.8×10^6 cells/mL and error bars represent one standard deviation about the mean (n = 3).

Figures 2A and 2B illustrate that, both microalgae strains (*C. vulgaris and S. quadricauda*) have no lag phase in axenic culture and short lag phase in co-culture system. Besides that, these two figures show that the growth of both microalgal strains were affected when they were co-cultured with the MGPB. The specific growth rate of co-culture *C. vulgaris* was slightly lower (0.203/day) compared to that of axenic (0.275/day). The same pattern was also observed to *S. quadricauda,* where the co-culture system had lower specific growth rate (0.118/day) compared to the axenic system (0.216/day). This occurrence could be due to the inappropriate initial concentrations of MGPB introduced in the co-culture system. As previously reported, the increase in population of MGPB did not contribute towards the increase in the population of microalgae (Luz, 2005). Bacteria could help microalgae in nutrients and COD removal, but at the same time they would also compete with microalgae to get the nutrients for their growth. Thus, it reduced the growth rate of the microalgae in co-culture system.

Nutrients removal

The removal of ammonium and phosphorus in synthetic wastewater were analyzed for both axenic and co-cultured systems. The percentages of ammonium and phosphorus removal are presented in Figures 3 and 4, respectively.

Synthetic wastewater was used in this study to compare the efficiency of nutrients and COD removal by two different systems (axenic microalgae and microalgae-bacteria co-culture). Figure 3 shows the removal of ammonium by both microalgae species (*C. vulgaris* and *S. quadricauda*) which were slightly higher in co-culture system compared to axenic system. In this study, with initial concentration of 12 mg/L, 85% and 86% of ammonium were removed by axenic *C. vulgaris* and co-culture system respectively, after four days of cultivation. Similar trend of result was reported by De-bashan *et al*. (2002), whereby 86% (axenic) and 99% (co-culture) system of ammonium were removed after six days of cultivation with lower initial ammonium concentration, 3 mg/L. In other study, higher percentage removal of ammonium was obtained by *C. vulgaris* on municipal wastewater with native bacteria after four days with initial concentration of 30 mg/L (Delgadillo-mirquez *et al*., 2016). This indicates that the removal efficiency of the nutrients was improved by the presence of the MGPB compared to the axenic culture, however the effect was not very significant in this study.

Besides that, he ammonium removal by co-culture system for both microalgae strains remained constant after four days of co-cultivation. This could be due to the decrease in microalgae cell number (Figure 2), when they were co-cultured in synthetic wastewater. In addition, *S. quadricauda* recorded higher rate of ammonium removal per cell in both systems, 10^{-9} $mgNH_4$/cells·day compared to that of *C. vulgaris* of 10^{-10} $mgNH_4$/cells·day. Similar pattern was obtained in other study (Zhan, 2016). Strong aeration and high pH in a system may also strip out ammonium in solution (Mun and Guieysse, 2006). However, in this system the aeration involved was only flask-shaking and the pH was low to promote the stripping of ammonium. Therefore, the ammonium removal in this system was only due to the microalgae uptake.

Figure 4 shows the comparison of phosphorus removal between two culture systems for *C. vulgaris* and *S. quadricauda* species. Co-culture systems of both

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microalgae strains were able to remove slightly higher amount of phosphorus, 44% compared to 37% of axenic microalgae within four days of cultivation. This result was in agreement with the report which recorded 36% and 19% removal for co-culture and axenic culture, respectively from 9 mg/L of initial phosphorus concentration (De-Bashan *et al*., 2004).

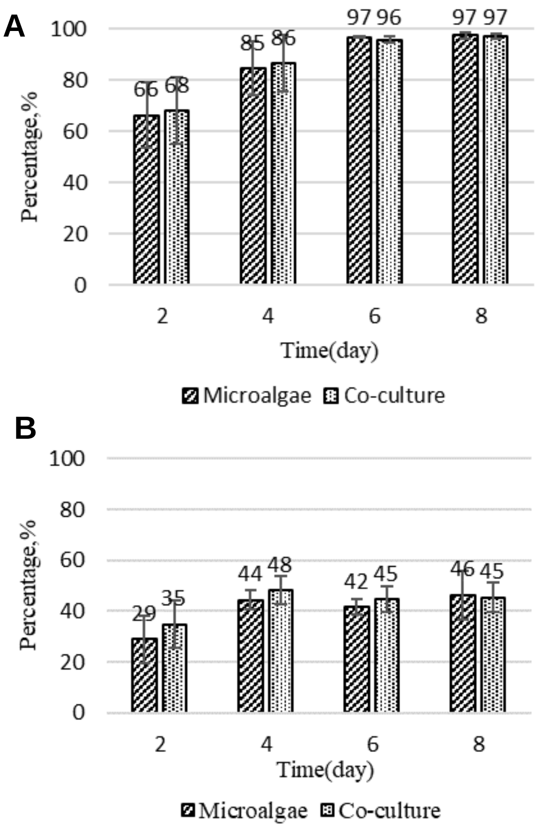

Figure 3: Percentage of ammonium (NH_4^+) removal in synthetic wastewater (A) *C. vulgaris* (initial concentration= 3±1×10^6 cells/mL (B) *S. quadricauda* (initial concentration = 3±1×10^5 cells/mL. Cultivation was done in 250 mL conical flask containing 150 mL synthetic wastewater, pH 7±0.2 at 25 °C, 150 rpm, under illumination of white fluorescent light of (alternate light/dark periods of 12 h/12 h), initial concentration of ammonium was 12 mg/L and error bars represent one standard deviation about the mean (n = 3).

During eight days of cultivation in both systems, the rate of phosphorus removal by *S. quadricauda* is higher than *C. vulgaris* with approximately 10^{-9} mgPO_4/cells·day and 10^{-10} PO_4/cells·day, respectively. Comparable result was obtained by Mujtaba and Lee, whereby higher rate of phosphorus removal (4.68×10^{-11} mgPO_4/cells·day) by *S. quadricauda* was recorded compared to *C. vulgaris* at 2.875x10^{-12} mgPO_4/cells·day. Phosphorus is one of the most important nutrients to microalgae as constituents of phospholipids as well as provide energy to the cells (Mujtaba and Lee, 2016). Since, the size of *Scenedesmus* species is bigger (11-18 µm) compared to *Chlorella* species (2 to 10 µm), *Scenedesmus* absorbs more phosphorus to be used for their growth which leads to the higher rate of phosphorus removal despite of the lower cells number compared to *Chlorella* species as observed in this study. The phosphorus can also be removed in the form of orthophosphate precipitation at high pH, 9 ~ 1 (Nurdogan and Oswald, 1995). As mentioned earlier, the pH in our system was sufficiently low. Hence, this precipitation effect was negligible and it can be concluded that, the main mechanism of phosphorus removal in this experiment was due to the uptake by microalgae.

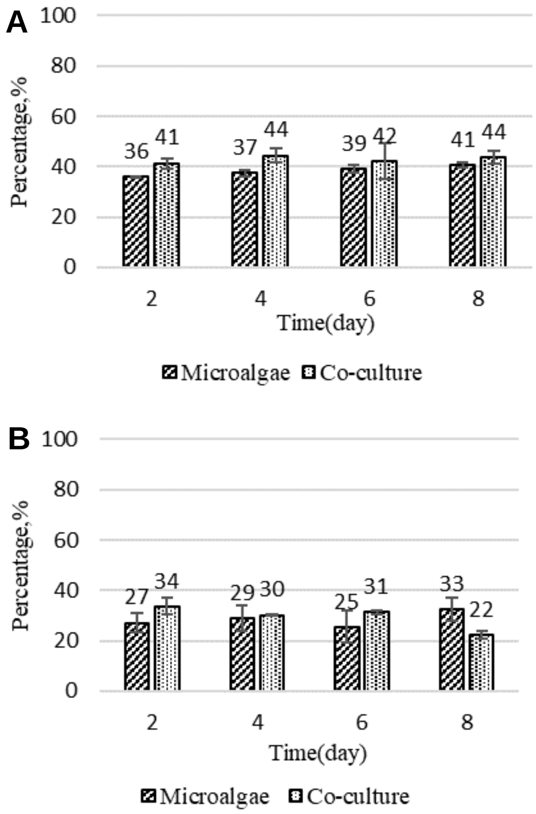

Figure 4 Percentage of phosphorus (PO_4^{-3}) removal in synthetic wastewater (A) *C. vulgaris* (initial concentration = 3±1×10^6 cells/mL) (B) *S. quadricauda* (initial concentration = 3±1×10^5 cells/mL). Cultivation was done in 250 mL conical flask containing 150 mL synthetic wastewater, pH 7±0.2 at 25 °C, 150 rpm, under illumination of white fluorescent light of (alternate light/dark periods of 12 h/12 h), initial concentration of phosphorus was 10 mg/L and error bars represent one standard deviation about the mean (n = 3).

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COD removal

Besides ammonium and phosphorus, COD is also one of the parameters that was analyzed to examine the characteristic of the wastewater. Figure 4 shows the percentage of COD removal for both microalgae species. Chemical oxygen demand (COD) is also an important parameter to be analyzed in wastewater. The removal of COD by both systems were presented in Figures 5A and 5B for *C. vulgaris* and *S. quadricauda* strains respectively. COD removal percentage of *C.vulgaris* was higher in co-culture system, which recorded 62% removal in four days. While co-cultured *S. quadricauda*, displayed 52% removal after 8 days of cultivation. Even though, in this study the COD removal is higher in co-culture system, this removal percentage was slightly lower compared to a previous study which achieved 80% of COD removal in 3 days (Mujtaba *et al*., 2015).

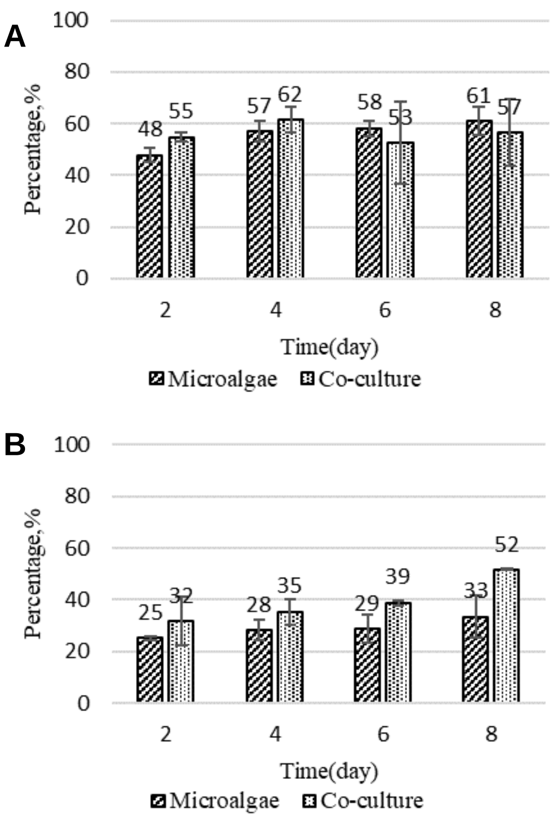

Figure 5: Percentage of COD removal in synthetic wastewater (A) *C. vulgaris* (initial concentration = $3\pm1\times10^6$ cells/mL) (B) *S. quadricauda* (initial concentration = $3\pm1\times10^5$ cells/mL). Cultivation was done in 250 mL conical flask containing 150 mL synthetic wastewater, pH 7±0.2 at 25 °C, 150 rpm, under illumination of white fluorescent light of (alternate light/dark periods of 12 h/12 h), initial concentration of COD was 300 mg/L and error bars represent one standard deviation about the mean (n = 3).

Normally, in the presence of light and inorganic carbon such as CO_2, microalgae follow photoautotrophic metabolism to acquire energy and carbon. However, some microalgae species like *C. vulgaris* was able to switch their metabolism from autotrophic to heterotrophic or mixotrophic, based upon the availability of carbon source as well as the environmental conditions for growth (Perez-garcia *et al*., 2010). Heterotrophic metabolism is activated when there is organic carbons and no light presence. On the other hand, mixotrophic turns on when there is light and in the presence of both organic and inorganic carbon (Mayo, 1994; Nurdogan and Oswald, 1995; Perez-garcia *et al*., 2010). In this experiment, both microalgae undergo mixotrophic since both carbon sources and light were presence in this system. Besides microalgae uptake, the heterotrophic bacteria also contributed in COD removal through oxidative degradation mechanism (metabolism for energy and carbon usage). Hence, COD removal is higher in co-cultured system compared to axenic microalgae system.

CONCLUSION

In conclusion, the specific growth rate of both microalgae strains decreased when they were co-cultured with the bacteria. The growth of both microalgae in this system were not enhanced in the presence of bacteria due to the improper of growth conditions such as pH and ratio of initial concentration of microalgae and bacteria in co-culture system. Therefore, there were only slight differences on the removal of the nutrients between axenic and co-culture system. However, this study still proved that, co-culture system for both microalgae species improved the nutrients and COD removal in synthetic wastewater. *C. vulgaris* recorded 85% and 86% of ammonium removal, 37% and 44% of phosphorus removal, 57% and 62% of COD removal for axenic and co-cultured system respectively. While, *S. quadricauda* recorded 44% and 48% of ammonium removal, 29% and 31% of phosphorus removal, 28% and 35% of COD removal for axenic and co-cultured system respectively. Therefore, it shows that *C. vulgaris* and *A. brasilense* co-culture system has higher potential to be applied for real wastewater as their percentage of removal and the growth rate was higher compared to the other species. Finally, further studies on the effect of the other growth conditions such as initial microalgae/bacteria ratio concentration and pH can be conducted to improve the percentage removal of the nutrients and COD.

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