SHRIMP WASTEWATER BIOREMEDIATION

ARTICLES FOR FACULTY MEMBERS

Title/Author	Bioremediation potential of macroalgae Gracilaria edulis and Gracilaria changii co-cultured with shrimp wastewater in an outdoor water recirculation system / Mawi, S., Krishnan, S., Din, M. F. M., Arumugam, N., & Chelliapan, S.
Source	*Environmental Technology & Innovation* Volume 17 (2020) 100571 Pages 1-8 https://doi.org/10.1016/J.ETI.2019.100571 (Database: ScienceDirect)

Title/Author	Dechlorination of wastewater from shell-based glucosamine processing by mangrove wetland-derived fungi / Han, Z., Moh, E. S. X., Santos, A. L. S., Barcellos, I. C., Peng, Y., Huang, W., & Ye, J.
Source	*Frontiers in Microbiology* Volume 14 (2023) Pages 1-11 https://doi.org/10.3389/fmicb.2023.1271286 (Database: Frontiers)

Title/Author	Highly effective reduction of phosphate and harmful bacterial community in shrimp wastewater using short-term biological treatment with immobilized engineering microalgae / Krasaesueb, N., Boonnorat, J., Maneeruttanarungroj, C., & Khetkorn, W.
Source	*Journal of Environmental Management* Volume 325 (2023) Part A 116452 Pages 1-9 https://doi.org/10.1016/J.JENVMAN.2022.116452 (Database: ScienceDirect)

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Title/Author	Immobilized Tetraselmis sp. for reducing nitrogenous and phosphorous compounds from aquaculture wastewater / Khatoon, H., Penz Penz, K., Banerjee, S., Redwanur Rahman, M., Mahmud Minhaz, T., Islam, Z., Ara Mukta, F., Nayma, Z., Sultana, R., & Islam Amira, K.
Source	*Bioresource Technology* Volume 338 (2021) 125529 Pages 1-9 https://doi.org/10.1016/J.BIORTECH.2021.125529 (Database: ScienceDirect)

Title/Author	Improving microbial bioremediation efficiency of intensive aquacultural wastewater based on bacterial pollutant metabolism kinetics analysis / Dong, D., Sun, H., Qi, Z., & Liu, X.
Source	*Chemosphere* Volume 265 (2021) 129151 Pages 1-12 https://doi.org/10.1016/J.CHEMOSPHERE.2020.129151 (Database: ScienceDirect)

Title/Author	Increasing CO2 concentration impact upon nutrient absorption and removal efficiency of supra intensive shrimp pond wastewater by marine microalgae Tetraselmis chui / Tahir, A., Rukminasari, N., Yaqin, K., & Lukman, M.
Source	*International Journal of Phytoremediation* Volume 23, 2021 Issue 1 (2021) Pages 64–71 https://doi.org/10.1080/15226514.2020.1791051 (Database: Taylor & Francis Group)

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Source	*Algal Research* Volume 72 (2023) 103110 Pages 1-10 https://doi.org/10.1016/J.ALGAL.2023.103110 (Database: ScienceDirect)

Title/Author	Trends in shrimp processing waste utilization: An industrial prospective / Nirmal, N. P., Santivarangkna, C., Rajput, M. S., & Benjakul, S.
Source	*Trends in Food Science & Technology* Volume 103 (2020) Pages 20–35 https://doi.org/10.1016/J.TIFS.2020.07.001 (Database: ScienceDirect)

Title/Author	Utilization of microalgae, Chlorella sp. UMT LF2 for bioremediation of Litopenaeus vannamei culture system and harvesting using bio-flocculant, Aspergillus niger / Nasir, N. M., Jusoh, A., Manan, H., Kasan, N. A., Kamaruzzan, A. S., Wan Abdul Karim Ghani, W. A., Kurniawan, S. B., & Lananan, F.
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Bioremediation potential of macroalgae *Gracilaria edulis* and *Gracilaria changii* co-cultured with shrimp wastewater in an outdoor water recirculation system

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ABSTRACT

Effluent from the aquaculture industry discharged into water bodies and it impacts the environment severely. The shrimp industry is one of the developing aquacultures that releases a high amount of organic matters in the form of wastewater. As an effort to reduce the environmental impact, an integrated system with shrimp and macroalgae researched abundantly as the macroalgae are naturally capable of removing nutrient from wastewater. As a bioremediation potential, this study investigates the nutrient uptake and macroalgal growth performance in short term (21 days) using an outdoor recirculating water system stocked with two local macroalgae species *Gracilaria edulis* and *Gracilaria changii* as biofilter. The stocking density of 3 kg/m^{2}with the flow rate of the water system set to 200 L/hr during the operation. The temperature, pH, dissolved oxygen (DO) and salinity was measured daily throughout the experimental period. Water temperature in all tanks were almost constant and ranged between 28.5 °C to 29.1 °C. The higher mean of pH of around 8.26 ± 0.15 and 8.28 ± 0.05 was observed in tanks with *G. edulis* and *G. changii* respectively. In the control tanks, mean pH was 7.87 ± 0.09. The mean concentrations of dissolved oxygen in *G. edulis*, *G. changii* and control tanks were 6.89 ± 0.05 mg/L, 6.84 ± 0.06 mg/L, and 6.10 ± 0.03 mg/L respectively. The mean growth rates of *Gracilaria edulis* and *Gracilaria changii* were found to be 4.3% day^{-1}, 4.1% day^{-1} with carbon to nitrogen (C:N) ratio of 8.3 to 8.5 respectively. The removal rate of ammonium and nitrate by the two species were found to be 72.5%, 71.0%, and 58.8%, 56.8% respectively. The macroalgal biofilter is found to be an ecologically sustainable that has improved the shrimp water quality to an acceptable level that in turn ultimately enhanced shrimp and macroalgae productivity.

1. Introduction

The rapid expansion of the aquaculture industry has contributed to the degradation of the coastal environment, due to the discharge of aquaculture wastewater directly into the sea or water bodies before treatment (Anh et al., 2010). There are

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alarming concerns to reduce the adverse environmental impact pertained to aquaculture. Shrimp wastewater contains a high concentration of organic matter in the form of excretory wastes and excess leftover of feeding materials (Krasaesueb et al., 2019). Consequently, the high amount of nutrient and organic load causes eutrophication and the occurrence of the red tide which affects the marine organisms and degrades the sustainability of the coastal environment. Granada et al. (2018) had revealed on the nutrient requirement in a closed shrimp pond system where the shrimp could assimilate total inputs of nitrogen and phosphate of 23%–31% and 10%–13%, respectively. A large amount of nitrogen discharges into rivers are notable in Cambodia, Malaysia, Thailand, and Vietnam with a value of 350,000 tonnes of nitrogen per year from various sources (Phillips et al., 2018). Cost-effective techniques with minimal environmental impact for high shrimp yield are pivotal for the sustainable growth of the shrimp industry (Kang et al., 2011).

Therefore, incorporation of macroalgae (weeds type) cultivation in aquaculture wastewater was researched and considered as an ecological solution due to the ability of macroalga as a potential nutrient remover to assimilate the excess nutrient from wastewater (Abreu et al., 2011). The suitable macroalga to integrate into an aquaculture operation is characterized by its rapid growth and accumulation of high levels of nitrogen (N) and phosphate (P) in its cells or tissue. The most common genera of macroalgae in aquaculture biofiltration are *Ulva* and *Gracilaria* (Msuya et al., 2006; Marinho-Soriano et al., 2011; Rabiei et al., 2014; Samocha et al., 2015). Conventional biofiltration studies depict that *Ulva* species were commonly preferred for pollutant removal, owing to a high biomass production and biofiltering efficiency (Masri et al., 2018). Recently, the genus *Gracilaria* (Rhodophyta sp) is found to be more suitable for bioremediation potential in intensive aquaculture as its ability to absorb more nutrients very rapidly as an efficient nutrient pump (Poblete et al., 2018). Besides offering commercial value as agar-agar, food for human consumption and also as fodder for other high valued aquaculture organisms such as abalone (Chew et al., 2018), Gracilarioid species such as *Gracilaria* and *Gracilariopsis* is the proven low-cost technology that can contribute to the efficient removal of dissolved N and P wastes in contaminated water streams along with the economic activity output (Sudhakar et al., 2018).

As *Gracilaria* species face difficulties in land-based aquaculture due to its rapid growth rate and fragmentation, integrated aquaculture of shrimp and macroalga is a better option for practical production strategies (Sarkar et al., 2019). As an effort to develop an eco-friendly integrated aquaculture technology, the potential of two locally available macroalgae species *Gracilaria edulis* (*G.edulis*) and *Gracilaria changii (G.changii)* for bioremediation of shrimp effluents was evaluated. Therefore, the aim of this study is to test and develop integrated aquaculture innovations relevant to local conditions prior to increasing the scale using the above mentioned two different macroalgae. The specific growth rate, ammonium, nitrate, and phosphate removal efficiency of the *Gracilaria edulis* (G.edulis) and *Gracilaria changii* (G.changii) was evaluated in the outdoor water recirculation system (OWRS). Overall this study is designed to mitigate the environmental impact of shrimp farms by converting shrimp wastewater into a resource for macroalgae growth and filter feeders organisms. In addition, this study provides the solid base for commercial-scale design of OWRS farms and thus increasing overall process efficiency and sustainability.

2. Materials and methods

2.1. Macroalgae sampling

The macroalgae chosen for this study were collected from Pulau Merabong, Gelang Patah, Malaysia (1.315851°N 103.610160°E). The collected macroalgae were then cleaned from epiphytes, mud, sand, and debris (Lavania-Baloo et al., 2014). and then held for 3 days in seawater to keep both alive and refreshing.

2.2. Outdoor Water Recirculation System (OWRS)

The design of OWRS is as shown in Fig. 1. Its an indigenous system developed by the Brackish Water Aquaculture Research Division, Fisheries Research Institute, Gelang Patah, Johor, Malaysia. Each experimental unit consists of a reservoir tank; a macroalgae tank and a biosand filter (BSF). Each reservoir tank and macroalgae tank has a volume of 150 L (100 cm x 47 cm x 42 cm) and an area of 0.47 m^2. The diameter of BSF is 60 cm and height is 80 cm. The shrimp wastewater flow by gravity to the macroalgae tank and then recycled back to wastewater tank via BSF at a controlled flow rate by a submersible pump. The experiments were conducted outdoor so that light and the temperature at natural and ambient conditions would be similar to that in the field and shaded with a transparent roof to avoid rainfall effect on the experiments.

2.3. Experimental set-up

Two macroalgae species *G. edulis* and *G. changii* were selected to test their biofiltration capacity and growth. The experiments were conducted using OWRS at a flow rate of 200 L/h. To assess the biofiltration capacity of *Gracilaria* species, 1.41 kg of *G. edulis* and 1.41 kg of *G. changii* were placed in separate tanks containing shrimp pond effluent. This stocking density was based on the experiment conducted by Abreu et al. (2011), who reported that 3 kg/m^2 is the best stocking density to attain a high production level of *Gracilaria* species. The experiments were conducted outdoor for 3 weeks at 28 °C in triplicates with one experimental unit as a controlled study (without *Gracilaria* species). The outdoor experiments mimic the light and temperature conditions similar to that in the field with a transparent roof shading to avoid the rainfall effect.

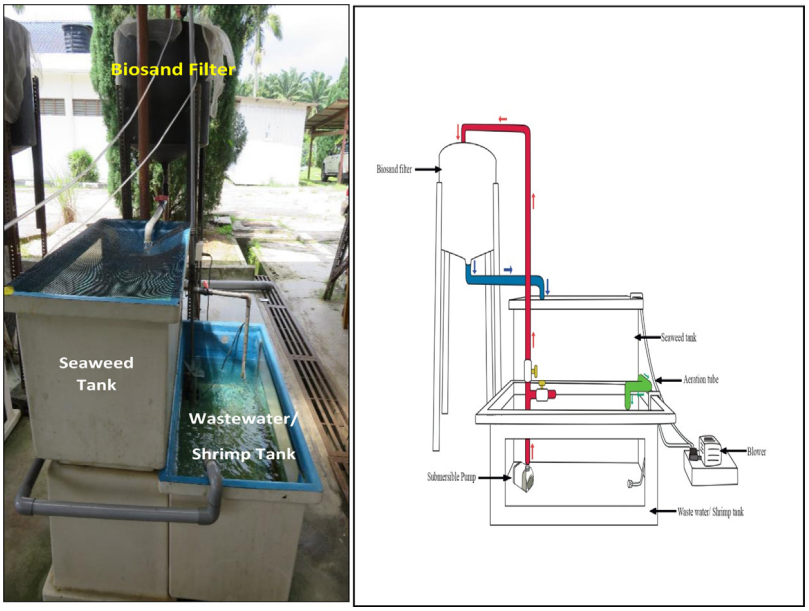

Fig. 1. Picture of Outdoor Water Recirculation System (OWRS).

2.4. Macroalgae growth

Initial and final fresh weight was measured to determine the specific growth rate (SGR). Fresh weight of macroalga was recorded fortnight by first blotted using a paper towel to remove excess water and then, weighed before restoring into macroalgae tanks. Specific growth rates (SGR, % d^{-1}) were determined as follows in Eq. (1):

$$SGR = 100x\,(\ln W_t - \ln W_0)/t \tag{1}$$

where W_o and W_t are initial and final weight of macroalga in grams, and t is the time in days (Yousef et al., 2012).

2.5. Nutrient removal rate

Nutrient removal (NR %) in the system was estimated using formula as Eq. (2):

$$NR = 100 * (C_{initial} - C_{final})/C_{initial} \tag{2}$$

where, $C_{initial}$ and C_{final}, are the initial and final nutrient concentrations respectively (Lavania-Baloo et al., 2014).

2.6. Carbon (C) and nitrogen (N) composition in tissue

Macroalga tissues were grounded finely into powder for the composition of C and N analysis in the tissues. Fresh tissues (50 g) of both macroalgae species were collected and then dried at 65 °C for 24 h followed by storage in a dry space until C and N analysis (Yousef et al., 2012). Carbon, Hydrogen, and Nitrogen (CHN) Analyzer (Perkin Elmer Model PE 2400) was used for analysis.

2.7. Measurement of physico-chemical parameters

Salinity, pH, temperature, and Dissolved Oxygen (DO) are the variables of water that were monitored daily using a multi-parameter probe (YSI Professional Plus). Water samples were collected at early morning weekly to analyse the dissolved nutrients' level such as ammonium (NH_4^+), nitrate (NO_3^-) and phosphate (PO_4^{3-}). All of these measures were conducted triplicates and analysed calorimetrically with a Hach spectrophotometer (DR5000: Hach Dusoldof, Germany).

2.8. Statistical analysis

The replication was expressed in terms of mean ± standard deviation. The software SPSS 16.0 version (from SPSS Inc, USA) was used to perform data analysis. Test conducted to determine the significant difference in mean between two groups. In addition, one-way analysis of variance (ANOVA) was used to determine the significant nutrient removal rate and SGR of macroalgae. In all instances, a significance level of 95% ($P < 0.05$) was set (Rabiei et al., 2014).

3. Results and discussions

3.1. Specific growth rate of macroalgae

The mean specific growth rate (SGR) of *G. edulis* and *G. changii* in this experiment are 3.9 ± 1.0% day^{-1} and 3.7 ± 0.9% day^{-1} respectively. In this study, *G. edulis* has presented a larger growth rate compared to *G. changii*. *G. edulis* has displayed a decreased growth rate which corresponds to the duration of cultivation, with the highest SGR recorded on the first week (4.5% day^{-1}) and decreased in the subsequent weeks to 3.43% day^{-1} on the third week. Meanwhile, as for *G. changii*, the SGR elevated considerably and a peak during the second week with SGR of 4.2% day^{-1} was recorded. Subsequently, the growth rate dropped with almost similar value as that of the first week (3.5% day^{-1}).

The cultivation types and species specificity determines the growth performance of the macroalgae because different macroalga has a diverse capability in the growth. In Brazil, the study on the growth rate and biofiltration capacity of the macroalga *G. birdiae* in the tank showed a SGR of 3.6% day^{-1}. The specific growth rate of *G. caudata* cultivated in cages in shrimp pond was 3.3% day^{-1} (Marinho-Soriano et al., 2009). Yang et al. (2006) obtained a mean growth rate of 3.95% day^{-1} for *G. lemaneiformis* in shellfish farming. Hoang et al. (2019) reported SGR of 2.27–2.54% day^{-1} for *G. birdae* in integrated fish cultivation while, Masuya and Neori (2018) reported a growth rate of *G. crassa* of 1.5% day^{-1} cultivated in the fishpond outflow channels. Azman et al. (2014) reported that a mean growth rate of 4.0% day^{-1} for *G. edulis* cultivated in an outdoor tank shrimp wastewater recirculation system. The SGR obtained for *G. edulis* and *G. changii* in this study was comparable with those recorded findings.

The macroalga growth is affected by the nutrient availability as nitrogen and phosphate source are known to be as the major nutrient source and limiting factors for algal growth (Borba Gurpilhares et al., 2018). Besides, the periodic harvesting of the macroalga from the treatment system has improved the biofiltration efficiency, because the stocking density influences the growth performance. Once the maximum carrying capacity of the system has been achieved, appropriate biomass harvesting increases the production and nutrient removal percentage in the treatment system (Balina et al., 2017).

3.2. Nutrient removal rate

In this experiment, ammonium (NH_4^+), nitrate (NO_3^-) and phosphate (PO_4^{3-}) removals from water have been observed in a recirculation system for three weeks as for two different treatment tanks each stocked with *G. edulis and G. changii* and one control tank (without macroalga). Fig. 2 shows the ammonium removal efficiency in each tank. Initial NH_4^+ concentration was in the range between 1.3 to 1.5 mg/L. At the end of the third week, a significant reduction in NH_4^+ concentrations was observed in treatment for both *G. edulis* and *G. changii* with both exhibited 72.5% and 71.0%, ammonium removal efficiency respectively. The control tank has shown ammonium removal of 2%. The ammonium reduction in control tank could be due to the initial microbial and phytoplankton activity that assimilates the ammonium for their cell metabolism and growth, and volatilization to the external environment (Copertino et al., 2009).

Fig. 3 shows that nitrate removal in the three tanks with the initial concentration was detected at 3.3 ± 0.1 mg/L. Moderately, high removal achieved by *G. edulis*, 58.8% and G. changii had 56.8% removal in the continuous recirculation system. While in the control tank (T3-C), the final concentration of nitrate was higher than the initial concentration indicate the ammonification occurs naturally. Fig. 4 shows the phosphate removal efficiency of *G. edulis* and *G. changii*. Initial phosphate concentration was reported in the range of 2.4 ± 0.2 mg/L. *G edulis* and *G. changii* recorded phosphate removal efficiency of 45.9% and 43.5% respectively. However, the control tank has shown an increase in phosphate concentration due to the absence of macroalga in this tank to serve as a biofilter.

G. edulis and *G. changii* exhibited varying biofiltration capacity in this experiment. Out of the two nitrogen sources, ammonium absorption occurred more rapidly. This was due to the immediate incorporation of ammonium into the amino acid pool and its supply was ample for the growth of macroalgae. The preference of macroalgae for ammonium intake rather than nitrate was sue to the availability of ammonium ions in the reduced form (Guttman et al., 2018). However, the significant reduction in the nitrate concentration indicated that they are assimilated by the macrolagae simultaneously along with ammonium, neither by creating a competitive environment for both nitrogen sources nor producing an inhibitory effect.

It was explicitly shown that both *G. edulis* and *G. changii* has high preferences to ammonium and nitrate compared to phosphate. There are studies of different co-cultured aquaculture system with *Gracilaria* species support this phenomenon. The removal of ammonium by G. lemaneiformis co-cultured in an aquarium with fish presented a higher removal of 85.53% ammonium compared with 65.97% phosphate after 23 days of treatment (Yang et al., 2006). Yousef et al. (2012) reported removal of 80.15% of total ammonia and 41.06% of phosphate by *G. arcuata* in an integrated aquaculture system with marine fish for 30 days at a flow rate of 225 L/h. However, Leila et al. (2018), is reported the red macroalga *G. verrucosa* removed more than 80% of ammonium in co-culture with mussels *Mytilus galloprovincialis* after three weeks. The ammonium concentration decreased by 85.5% and phosphate decreased by 65.97% in a laboratory experiment (fish and macroalga culture) carried out by Yu-Feng et al. (2006) after 23 days. An average of 76.7% total ammonia nitrogen removal was obtained with 5 kg m^{-2} stocking density of *Gracilaria bursa pastoris* at a water flow rate of 140 L/h. Hernández et al. (2002) reported that a minimum biofiltering efficiency of 61% was observed in unstarved cultures of *G. gracilis* with

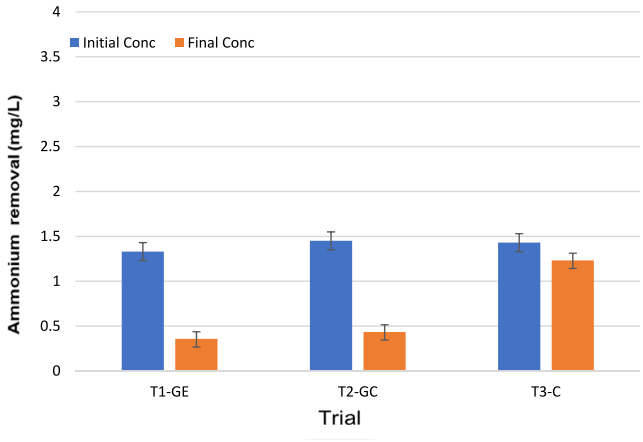

Fig. 2. Ammonium removal efficiency by *G. edulis* and *G. changii* in OWRS (T1-GE: Tank with *G. edulis*; T2-GC: tank with *G.changii* ; T3-C: tank without macroalgae).

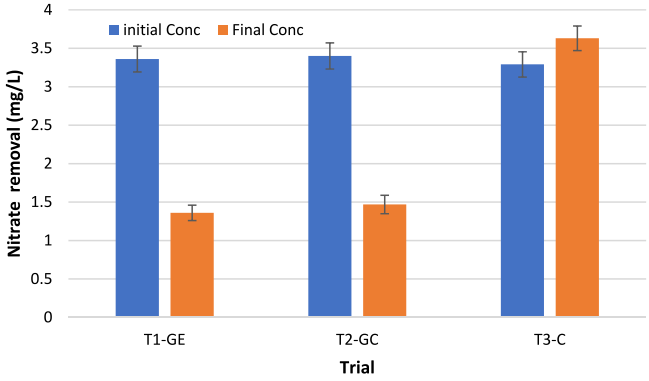

Fig. 3. Nitrate removal efficiency by *G. edulis* and *G. changii* in OWRS (T1-GE: Tank with *G. edulis*; T2-GC: tank with *G.changii* ; T3-C: tank without macroalgae).

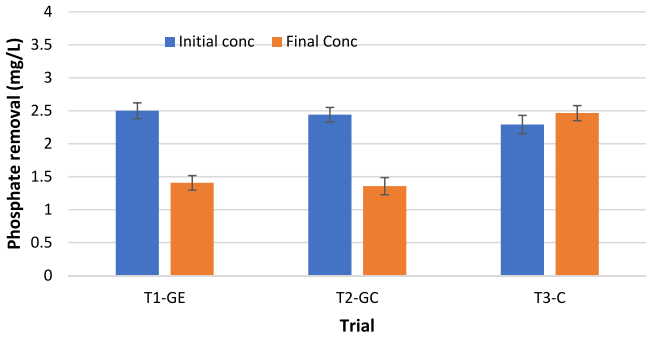

Fig. 4. Phosphate removal efficiency by *G. edulis* and *G. changii* in OWRS (T1-GE: Tank with *G. edulis*; T2-GC: tank with *G.changii* ; T3-C: tank without macroalgae).

seabass wastewater at a flow rate of 2 volumes/day for 7 days. Azman et al. (2014) reported a 70% removal of ammonium by *G. edulis* in an outdoor tank shrimp wastewater recirculation system for a period of 2 weeks. Data from the present study on the biofiltration capacity of *G. edulis* and *G. changii* are well fitted within the range of those recorded from other species of this genus. An increase in water flow means increases the nutrient flux to the surface of algal thalli (types of ropes) and thus increase in nutrient uptake rate. The results showed that the uptake rates of the macroalgae were much higher than those in the static condition. However, it should be noted that the nutrient removal efficiency will decrease if the water flow rate is too high. This is due to the low water retention time near the surface of macroalgae's

Table 1
Carbon, Nitrogen contents and ratios of C:N in *G. edulis* and *G. changii*.

Species	Tissue C content (%)		Tissue N content (%)		C:N ratio	
	Initial	Final	Initial	Final	Initial	Final
G. edulis	21.6	23.2	1.5	2.8	14.4	8.3
G. changii	20.4	22.0	1.4	2.6	14.6	8.5

thalli (Skriptsova and Miroshnikova, 2011). In order to achieve both high nutrient removal efficiency and uptake rate, the water flow rates should be carefully regulated and monitored to enhance the absorbing capability of the seaweeds.

3.3. Carbon (C) and nitrogen (N) composition

Total carbon and nitrogen and the ratio of carbon to nitrogen (C: N) were determined at both initial and end of the experimental period and the values are as presented in Table 1. The carbon content of *G. edulis* and *G. changii* tissues did not vary significantly over time or between species, respectively ($P > 0.05$). In the case of N content, there was no significant difference between species ($P > 0.05$), but there was a significant change in time ($P < 0.05$). The C/N ratio of *G. edulis* and *G. changii* showed significantly higher values ($P < 0.01$) at the beginning of the experiment and reached average values of 8.3 and 8.5 respectively towards the end. No significant differences ($P > 0.05$) were found for C/N ration between the two species.

The nutrient uptake capacity of a macroalga is indicated through C/N and the results show that both species were N-limited because of its high initial C/N values (nearly 15). The C/N ratio inversely correlates with N-enriched shrimp effluents that allowed reducing the C/N values down to nearly 8 and C/N values below 10 will not influence the N uptake rate (Abreu et al., 2011). The fact that both studied macroalgae species showed a linear increment in N concentration (especially in the tissues of *G. edulis* and *G. changii*) when cultivated using shrimp effluents appears to be an indication of improved growth conditions, including high water movement and a continuous supply of ammonia without temperature and light limitation (Harrison and Hurd, 2001; Abreu et al., 2011).

Nutrient removal by macroalgae for cell growth is influenced by various environmental factors, the algal structural physiology and also the nutrient past history of the macroalgae. The internal concentration of nutrients in a macroalga is the best indicator of the nutrient status of their tissues. This study found that the N content in the macroalga tissues increased with the presence of dissolved nutrients in the water. Torres et al. (2019) reported that the N storage capacity of *G. cornea* allows it to grow over a 7 days period with low N (or critical N) concentration. Critical N concentration of a macroalga also influences their N uptake rate. Critical N concentration is defined as the minimum N concentration required for a maximum growth rate at any point of time. Therefore, a low value of the initial N content in both macroalgae in this current study indicates the low level of N presence in their tissue. The final N content in this study is supported well with *G. caudata* that grown in cages and tubular net in a static condition of shrimp farm with $2.61 \pm 0.26\%$ N content (Marinho-Soriano et al., 2009).

A lower initial value observed in *G. edulis and G. changii* portrays its character in assimilating the stored N in the tissues and relatively small quantities of N are fixed into the cellular tissue. The high SGR in *G. edulis and G. changii* might be associated with the increase in N removal rate and intracellular N content in the tissue. A positive correlation between external N availability and internal tissue N that contributes to the growth was observed. Other factors that influence the uptake rate for nutrients by macroalgae are known as environmental conditions, such as light, temperature, water motion, and susceptibility to epiphytes. A stable DO and pH condition in this study also provides a suitable growth condition for the macroalgae. However, at the natural environment, both macroalgae species definitely will not achieve the maximum rates as a result of limited availability of external inorganic nutrients, which only favours macroalgae's growth than stored in the tissue.

3.4. Physico-chemical parameters

Water quality parameters for the treatment system is presented in Table 2. Temperature, pH, dissolved oxygen (DO) and salinity were measured daily throughout the experimental period. Water temperature in all tanks was almost constant throughout the course of study and ranged between 28.5 °C to 29.1°C. However, the pH and dissolved oxygen in *G. edulis* and *G. changii* had demonstrated significantly higher value compared to control tanks. In the control tanks, the mean pH was 7.87 ± 0.09. The higher mean of pH 8.26 ± 0.15 and 8.28 ± 0.05 was observed in respective tanks with *G. edulis* and *G. changii*. The mean concentrations of DO in *G. edulis* and *G. changii* were 6.89 ± 0.05 mg/L and $6.8\ 4 \pm 0.06$ mg/L respectively and significantly varied ($P < 0.01$) from control tanks, 6.10 ± 0.03 mg/L. High DO was observed due to continuous movement of shrimp wastewater. No significant variation ($P > 0.05$) was observed for salinity in all tanks.

The previous study reported that both macroalgae species successfully are grown in a wide range of salinity and temperature (Baloo, 2015). Therefore, the constant temperature and no significant variation in salinity did not affect the studied system. The DO and pH in the control experiments (without macroalga), were found slightly lower than the system integrated with macroalgae. These environmental parameters varied significantly in treatment and control

Table 2
Water physio-chemical parameters the outdoor water recirculation system.

Tank	Temp (°C)	Dissolved oxygen (mg/L)	pH	Salinity (ppt)
G. edulis	28.9 ± 0.2[a]	6.89 ± 0.05[a]	8.26 ± 0.15[a]	25.1 ± 0.6[a]
G. changii	28.7 ± 0.2[a]	6.84 ± 0.06[a]	8.28 ± 0.05[a]	24.9 ± 0.8[a]
Control	28.8 ± 0.1[a]	6.10 ± 0.03[b]	7.87 ± 0.09[b]	25.1 ± 0.09[a]

Note: Data are mean ± S.D. (n = 3) and different superscript indicate statistical different at P < 0.01.

tanks ($P < 0.01$). Stable DO concentration was recorded in the presence of macroalgae and stable culture condition exists within the treatment system resulted in the optimum macroalgae yield of 3 kg/m^2. Physio-chemical parameters such as temperature, light, water motion, salinity, and nutrient availability were found as one of the major factors affecting the macroalgae growth rate during the experimental period.

4. Conclusion

The potential of both *G.edulis* and *G.changii* species have studied and both showed a remarkable perspective as biofilters in shrimp wastewater. Both macroalgae have proved a significant positive response to the increasing amount of ammonium, phosphate, and nitrate in the wastewater medium. Both macroalgae have demonstrated a relationship between the specific growth rates, nutrient removal rate and internal nitrogen content. The mean growth rates of *G.edulis* and *G.changii* were found to be 4.3% day$^{-1,}$ 4.1% day^{-1} with C:N ratio of 8.3 and 8.5 respectively. The ammonium removal was recorded as 72.5% and 71.0% and nitrate removal of 58.8% and 56.8% respectively The C:N ratio below 10 was also a strong indicator of the biofiltration capacity. Both macroalgae have reduced these nutrients efficiently from the medium and incorporated into their biomass. The biomass is an economically valuable source to generate income to the country with versatile by-products derived from macroalgae upon harvesting. The data provide a solid base for commercial-scale design of OWRS farms and thus increasing overall process efficiency and sustainability.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Saberi Mawi: Data curation, Investigation, Writing-original draft, Methodology. **Santhana Krishnan:** Formal Analysis, Conceptualization, Investigation, Writing - review & editing. **Mohd Fadhil MD Din:** Supervision. **Nithiya Arumugam:** Software. **Shreeshivadasan Chelliapan:** Software.

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Dechlorination of wastewater from shell-based glucosamine processing by mangrove wetland-derived fungi

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Wastewater from processing crustacean shell features ultrahigh chloride content. Bioremediation of the wastewater is challenging due to the high chloride ion content, making it inhospitable for most microorganisms to survive and growth. In this study, mangrove wetland-derived fungi were first tested for their salt tolerance, and the highly tolerant isolates were cultured in shrimp processing wastewater and the chloride concentration was monitored. Notably, the filamentous fungal species *Aspergillus piperis* could remove over 70% of the chloride in the wastewater within 3 days, with the fastest biomass increase (2.01 times heavier) and chloride removal occurring between day one and two. The chloride ions were sequestered into the fungal cells. The genome of this fungal species contained Cl^- conversion enzymes, which may have contributed to the ion removal. The fungal strain was found to be of low virulence in larval models and could serve as a starting point for further considerations in bioremediation of shell processing wastewater, promoting the development of green technology in the shell processing industry.

KEYWORDS

fungi, industry wastewater, inorganic chloride removal, bioremediation, environmental safety

1. Introduction

Inorganic chloride (Cl^-) pollution is one of the problems faced by industries producing chitin, chitosan, and glucosamine from the cuticles of crustaceans such as shrimp, lobsters, and crabs, as the cuticles are only soluble in highly concentrated hydrochloric acid (Bastiaens et al., 2019). It has been reported that around 8.5 tons of 30% (v/v) hydrochloric acid has to be used for processing 1 ton of shrimp cuticles, resulting in a high concentration of Cl^- in the effluents (around 70 g/L; Bertuzzi et al., 2018). Currently, four strategies are primarily used in industry for removal of Cl^- from shrimp processing wastewater, including electrodialysis, precipitation, adsorption, and microporous separation (Association A.W.W, 2006; Sathasivan et al., 2017; Li

et al., 2022). These strategies concentrate Cl^- as a result, and may cause salinization if improperly disposed (Li et al., 2022).

New technologies are emerging in two directions to prevent Cl^- pollution caused by shell processing. One is to develop a green method for chitin extraction, which has drawn lots of attention and can be basically divided into chemical and biological methods (Kozma et al., 2022). In the chemical approaches, alternative solvents, such as citric, acetic, and lactic acids as well as ammonium-based ionic liquids were used to extract chitin from shrimp shells (Cira et al., 2002; Tolesa et al., 2019; Kozma et al., 2022). Regarding biological approaches, enzymatic or microorganism fermentation methods have been employed to break down proteins and calcium carbonate in shells (Arbia et al., 2013; Hossin et al., 2021; Kou et al., 2021). The other direction is to develop alternative Cl^- removing methods from waters.

Bioremediation is environmentally friendly but not widely used in the Cl^- removal industries, since most bacteria commonly used in wastewater treatment are not resistant to >3 g/L Cl^- under normoxia (Liu et al., 2020; Li et al., 2022). On the other hand, biological removal of organic chlorine pollutants has been used and studied extensively with two common groups of prokaryotes, namely sulphate-reducing and denitrifying bacteria. Basically, these bacteria perform different adaptations to degrade chlorine pollutants, like aerobic fermentation on the surfaces of water or soil, denitrification response to changes in the oxygen concentration, and oxidation–reduction reactions in anoxic environments (Xu et al., 2019; Xing et al., 2020; Sobiecka, 2022). The marine bacterial species *Staphylococcus xylosus* has been reported to be able to convert Cl^- into organic cellular compounds and eventually lower the Cl^- concentration in wastewater (Abou-Elela et al., 2010). As an alternative to bacteria, one can also consider fungi as a potential candidate in Cl^- removal industries, as they can also be halophilic.

Microorganisms naturally growing in polluted environments are supposed to be capable of biodegrading toxic compounds (Sobiecka, 2022). Mangrove forest land may be an ideal location for isolating fungi capable in Cl^- removal from shrimp processing wastewater, though there has yet to be a report on this issue to our knowledge. Mangroves are a type of salt-tolerant submerged sclerophyllous plant species growing in coastlines worldwide at low tide areas in the tropics and subtropics. Mangrove forests form an interface between terrestrial and marine habitats and provide excellent support for migrating waterbirds, offshore fish, and other water fauna (Thatoi et al., 2013). The wetlands in mangrove forests feature not only high salt concentration (0.5–80 g/L) but also abundant macronutrients from decomposition, providing a unique biosphere for colonization of halophilic microorganisms (Schmitz et al., 2009; Hamzah et al., 2018; Jia et al., 2020).

Till now, plenty of filamentous fungi have been isolated from mangrove forest wetlands, including several species belonging to the following genera: *Penicillium, Aspergillus, Trichoderma, Stemphylium, Talaromyces, Setophoma, Mucor, Lasiodiplodia*, *Annulohypoxylon*, *Rhytidhysteron*, *Phomopsis*, *Diaporthe*, *Neosartorya*, *Beauveria*, *Eupenicillium*, and *Dipodascus* (Bonugli-Santos et al., 2015; Ancheeva et al., 2018; Jia et al., 2020). Similarly, yeast have been recovered from this habitat, particularly species belonging to the *Candida, Kluyveromyces, Pichia*, *Kodamaea*, *Debaryomyces*, and *Williopsis* genera (Chi et al., 2012; Bonugli-Santos et al., 2015). Among these fungi, some of them have been studied on their industrial application. For example, *Aspergillus sclerotiorum* (strain CBMAI 849) and *Mucor racemosus* (strain CBMAI 847) are able to degrade polycyclic aromatic hydrocarbons (Passarini et al., 2011; Bonugli-Santos et al., 2015). *Aureobasidium* sp. (strain P6), *Penicillium janthinellum* (strain P1) and *Tinctoporellus* sp. (strain CBMAI 1061) are able to decolor various synthetic dyes such as bromothymol blue, eriochrome black T, crystal violet, malachite green, and methyl orange (Chen et al., 2014; Rodriguez et al., 2015; Lu et al., 2018; Aung et al., 2019).

In this study, fungi growing near a sewage drain outlet in mangrove wetlands located in an industrial park in Zhanjiang, the southernmost city on the coast of mainland China, were isolated and studied in terms of fungal species, salt tolerance, chloride removal capacity and virulence. The results have the potential to offer valuable insights into the application of fungal strains in the remediation of Cl^- in the processing industry.

2. Materials and methods

2.1. Sampling campaigns

Sampling was conducted on three occasions in total over five afternoons before the tide rising, at three sites (seaward zone, mid zone and landward zone) in the mangrove forest (Leizhou, China, 21°68'N, 110°33'E), where soil samples were collected from 0 to 10 cm below the surface sediments. The samples were collected using a sterilized hand shovel (around 4 cm × 6 cm, with 18 cm handle) and large debris was removed by hand. All the samples from all sites were combined and mixed thoroughly before transferring into sterile 500 mL canning jars and transported to laboratory in an icebox.

2.2. Fungal isolation assay

Fungal isolation was carried out immediately after arrival, around 30 min after the collection, following Ahumada-Rudolph's method with some modifications (Ahumada-Rudolph et al., 2016). Briefly, the sediment was suspended in 4 L sterile seawater, homogenized, and left to settle. The supernatant was collected by centrifugation at 100 g for 15 min and stirred well afterwards. Supernatant (2 mL) was diluted in series with sterilized seawater (10–100 folds in 10-fold intervals) with two replicates. An aliquot of 100 µL was transferred to yeast extract peptone dextrose agar (YPD) prepared using sterilized seawater supplemented with ampicillin, kanamycin and streptomycin (final concentration of 50 µg/mL, 50 µg/mL and 25 µg/mL respectively). The aliquot was spread onto the agar using a sterile glass coating rod and the incubation was conducted at 28°C.

Subcultures were performed till individual colonies were obtained. Morphology of individual colonies was verified under the microscope. Spores of isolated fungi were collected using sterilized water with 0.05% Tween 80 and stored at 4°C for up to 1 week. The agar with fungal growth was sliced off from the dish using sterilized scalpel (about 4 mm × 4 mm each piece), submerged in 80% glycerol solution (v/v) and stored at −80°C for 4 months.

2.3. Molecular methods for fungal identification

Fungal genomic DNA was extracted using the Qiagen DNEasy Plant Extraction kit (Qiagen Inc., Valencia, CA, United States).

Primers (ITS1: 5'-TCCGTAGGTGAACCTGCGG-3′ and ITS4: 5'-TCCTCCGCTTATTGATATGC-3′) were used to amplify by polymerase chain reaction (PCR) the whole region of ribosomal internal transcribed spacer (*ITS1-5.8 s-ITS2*; Korabecna, 2007). The PCR reaction mixture included 10 μL of PCR master mix (Promega, 2X), 1 μL of the forward primer ITS1, 1 μL of the reverse primer ITS4, 1 μL of DNA extraction, and 7 μL of ddH_2O. The thermal cycler was programmed with the following conditions: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 1 min, concluding with a final extension at 72°C for 10 min. PCR products were purified using the QIA quick PCR purification kit and sent to Sangon Biotech (Shanghai, China) for bidirectional sequencing. The assembled sequences were analyzed using BLAST[1] against the standard database on NCBI.

2.4. Salt tolerance assay

Salt tolerance assay was performed on YPD agar supplemented with sodium chloride (NaCl, w/v) at different concentrations (50, 100, 150, 200 and 250 g/L). The spores of isolated fungi were inoculated on the salt-contained agar, cultured at 28°C for up to 10 days, and the fungal growth was monitored.

2.5. Cultivation of salt tolerance strains in the wastewater

Real wastewater originating from processes utilized for glucosamine production was used in this study. The wastewater featured with a low pH (0.39 ± 0.05), dark color, fishy smell, and a high Cl^- concentration (84.39 ± 1.21 g/L). Other physical properties and chemical composition were not characterized in this study.

Fungi tolerant to salt concentrations equal to or above 100 g/L were adapted gradually into 20, 40, 60 and 80% glucosamine processing wastewater. Peptone, yeast extract and glucose were added into the diluted wastewater to a 20% concentration of the regular YPB (yeast extract peptone dextrose broth) medium. Liquid cultures were performed in 250 mL conical flasks containing 50 mL of growth medium inoculated with 5×10^5 conidia and incubated at 28°C on an orbital shaker at 180 rpm with three individual flasks dedicated for each time point. Conidia collected from low wastewater-containing medium were used for the subsequent culture. Culture supernatants were collected every day from flasks dedicated to each time point by centrifugation at 4,500 g for 30 min. The biomass was weighed after drying and the supernatant was filtered through a 0.22-μm membrane (Millipore, China) at 4°C. The cleared supernatants were then aliquoted in 1.5 mL Eppendorf tubes and stored at −80°C.

2.6. Acidity-tolerance assay

Acidity-tolerance assay was performed in liquid culture with 80% wastewater as a medium with different pH values (3.5, 4.0, 4.5, 5.0 and 5.6). The cultivation was performed at 28°C at 180 rpm for 7 days, and the fungal biomass, pH values as well as the Cl^- concentration in the wastewater were measured daily.

2.7. Measurement of chloride removal from the wastewater

The fungal strains capable of growing in 80% wastewater (Cl^- concentration of 67.512 g/L) were further studied and their Cl^- removal capacity was detected by ion chromatography at room temperature using a Metrohm Model 761 Compact Ion Chromatograph (Metrohm, Herisau, Switzerland) with suppressor module, equipped with an ICSep AN2™ analytical column (250 mm × 4.6 mm). The injection volume was 20 μL. The eluent used was a 1.8 mM Na_2CO_3 + 1.7 mM $NaHCO_3$ mixture and the suppressor regenerating solution was 0.1 M H_2SO_4. The eluent was freshly prepared and filtered through a 20-μm filter before usage. Data acquisition and processing were performed automatically using the integration software MagIC Net™ software 1.1. Standard solutions (GBW (E)082048, China) of known concentrations of Cl^- were analyzed in order to create a calibration curve (Supplementary Figure S2).

2.8. Microscopic examination and Cl^- tracing

Fungal cells were stained using a chloride sensitive fluorescent probe MQAE (*N*-[ethoxycarbonylmethyl]-6-methoxy-quinolinium bromide, Beyotime, China) and then observed with a fluorescence microscope equipped with an imaging system at an excitation wavelength of 360 nm (D-35578 Wetzlar, Leica, Germany).

To observe the distribution of Cl^- in fungal cells, MQAE and Congo Red were used to conduct double-staining for the fungal cells. MQAE was loaded first following the manufacturer's manual. Briefly, the mycelia were washed three times with Krebs-HEPES buffer (0.02 M, pH 7.4), placed onto glass slides immersed in a drop of MQAE (5 mM), incubated for 30 min at 37°C in the dark, and finally washed again using the buffer. The resulting specimens were then stained using 0.01% Congo Red for 10 min, washed with ddH_2O to remove the excess stain, and then covered with 4% paraformaldehyde fix solution and a coverslip. A Leica TCS SP5 confocal laser microscope (Leica Microsystems, Germany) equipped with epifluorescence microscopy (Leica DMI 6000B microscope) was used to observe the specimens at excitation of 360 nm (emission at 460 nm) and 497 nm (emission at 614 nm) separately.

2.9. *In vivo* infection assays using *Tenebrio molitor* and *Galleria mellonella* models

For these experiments, *A. piperis* was grown on Petri dishes containing yeast peptone dextrose agar (YPD) at 28°C. After a

1 www.ncbi.nlm.nih.gov/BLAST

7-day-culture period, conidia were obtained by washing the plate surface with phosphate-buffered saline (PBS; 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) and filtering them through a 40-μm nylon cell strainer (BD Falcon, Franklin Lakes, NJ, United States) in order to remove the hyphal fragments. The conidial cells were counted in a Neubauer chamber. *Tenebrio molitor* larvae exhibiting clear and uniform color and weighing between 70 and 100 mg were selected for the survival studies (de Souza et al., 2015). *Galleria mellonella* larvae were maintained and fed as previously described until reaching 200–300 mg in weight (Silva et al., 2018). The survival curves (virulence assay) were performed through injection of different fungal inocula (10^2, 10^3, 10^4, 10^5 and 10^6 conidia/larva). Larvae (10 per each assayed group) were inoculated with fungal conidia using an insulin syringe (10 μL/larva) and incubated at both 28 and 37°C in Petri dishes containing rearing diet. The inoculation was performed by the injection of fungal suspensions into the *T. molitor* larvae hemocoel in the ventral portion at the second visible sternite above the legs or in the last right proleg of the *G. mellonella* larvae (de Souza et al., 2015). Larvae inoculated with sterile PBS were used as control groups. Larvae were assessed daily, up to 7 days, to check their survival, being scored as dead when they displayed no movement in response to touch. Survival analyses were determined using the log-rank test and the Kaplan–Meier survival curves (GraphPad Prism 6). The experiment was conducted in two independent experimental sets.

3. Results

3.1. Morphology of the mangrove wetland-derived fungi

A total of 34 fungal strains were isolated from the wetlands of a mangrove forest, named H1 to H34 (Figure 1), displayed on a dark background showing the diverse morphology of the fungal population isolated from the mangrove. Detailed taxonomy information for these strains can be found in Supplementary Table S1. The fungal strains' DNA was successfully amplified using the universal primers ITS1 and ITS4. BLAST searches revealed their identities as members of 3 different phyla (Supplementary Table S1), namely Ascomycota, Basidiomycota and Mucoromycota, in which the Ascomycota accounted for the majority (44.1%). The dominating fungal genera identified in this study were *Aspergillus* (35.3%) and *Penicillium* (20.6%). Representatives of *Trichoderma*, *Furarium, Mucor, Candida, Amanita* and *Talaromyces* genera were additionally identified. Despite *Amanita loosii* is generally recognized as an edible mushroom, the strain identified in this study is not recommended for consumption, considering its origin in an industrial waste environment.

3.2. Salt tolerance and chloride removal assay

An initial screening of these fungal strains for salt tolerance was carried out. The results (Figure 2) showed that 4 strains (H6, H10, H15 and H16) were tolerant to a salinity of 200 g/L, while 8 strains (H1, H4, H8, H12, H13, H14, H22 and H25) tolerant to 150 g/L, 7 strains (H7, H9, H11, H19, H20, H29 and H32) tolerant to 100 g/L, and 10 strains (H2, H3, H8, H23, H24, H26, H28, H30, H31 and H34) tolerant to 50 g/L. Strains H17, H21, H27, and H33 showed tolerance to lower salt levels. However, they were not subjected to further testing because low tolerance was deemed unfeasible for processing shrimp shell effluents, which typically contain salt levels exceeding 50 g/L. The mangrove wetland-derived fungal strains with a salt tolerance level greater than 100 g/L were subsequently gradually adapted into the 80% wastewater and Cl^- concentration in the water was measured using ion chromatography. Most of the fungal strains, including those tolerant at 200 g/L NaCl on YPD medium, were not able to grow in the wastewater with pH adjusted to 5.6. Only 7 strains (Figure 2, yellow bars, H4, H6, H7, H12, H16, H18 and H32) adapted to the highest wastewater concentration (80% wastewater). Among these strains, H16 (*Aspergillus piperis*) removed the highest amount of Cl^- (approx. 30%) from the wastewater.

3.3. pH tolerance and Cl^- removal capacity assay

The original pH value of the wastewater was highly acidic (pH 0.39 ± 0.05), in which none of the tested fungal strains survived. A great amount of base (NaOH) was required to neutralize the pH to a level able to support the fungal growth, which would be problematic at an industrial scale. To determine the lowest possible pH for fungal growth, the pH of the wastewater was adjusted to 2.0–5.0, 0.5 intervals and 5.6 to assess viability for strain H16 that had the highest chloride removal. It turned out that H16 could survive down to pH 3.5, with a slow growth rate. Wastewater with pH 5.0 supported the best fungal growth (Figure 3A) and the fungal strain was able to neutralize the acidity of the medium as biomass increased (Figure 3B). Interestingly, while H16 neutralized the acidity of the medium at similar rates at pH 5.0 and pH 5.6, chloride removal was more rapid at pH 5.0 than it was at pH 5.6 (Figure 3C), and the biomass peaked earlier (Figure 3A).

The removal of Cl^- was observed to occur in conjunction with fungal exponential phase (Figures 3A,B). When normalized to biomass, chloride reduction varied by days among the tested pH range (Supplementary Figure S1). The fungus apparently tended to reduce more Cl^- in a strong acidic environment, as seen from the reduction rates at pH 3.5 and 4.0, which were greater than those at higher pHs. However, the values kept decreasing day by day due to slow growth. pH 4.5, 5.0 and 5.6 brought out smaller Cl^- reduction rates at the beginning of the cultivation, but the reductions were enhanced gradually with the highest chloride removal per unit biomass by day 7 at pH 4.5. Exploring the interplays between fungal growth, chloride uptake and medium pH will be an interesting avenue for future research.

The wastewater had a dark color (Figure 4), low pH (1.7 in this study) and a high Cl^- concentration (Figure 3C). After adjusting to pH 5.0 using NaOH, strain H16 was able to clarify the broth in pace with the fungal growth, along with the decrease in the Cl^- concentration (Figure 4). A visible lightening in the color of the wastewater was also observed, suggesting that other components in the wastewater were remediated by the fungi, but the identification of which was not within the scope of this study. At day 3 post-inoculation, the Cl^- concentration in the broth dropped to 17.478 g/L from the original level of 67.512 g/L, corresponding to a 74.11% Cl^- removal. Further investigations are needed to lower the Cl^- concentration to less than 5 g/L, creating conditions conducive for the growth of most microorganisms. This, in turn, will enable subsequent bio-treatment

FIGURE 1
Diversity of fungal colonies isolated from a mangrove wetland located near the industrial park where the high salt wastewater was obtained from. All strains cultivated on YPD medium for 5 days at 28°C.

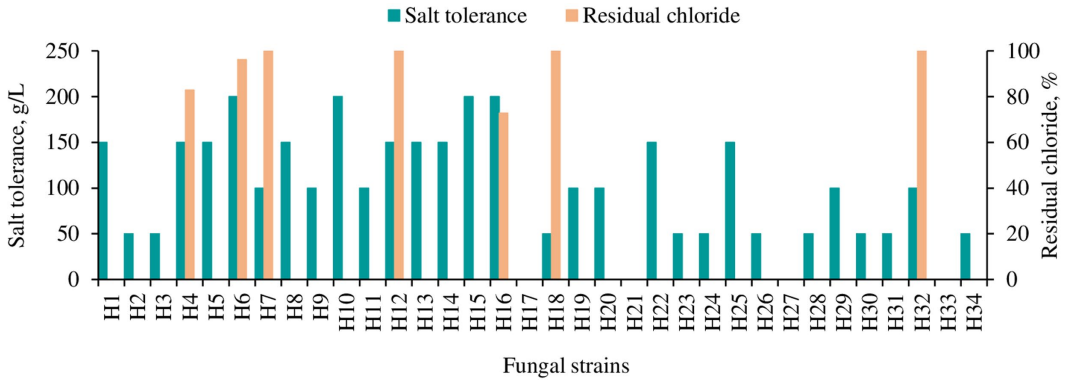

FIGURE 2
Salt tolerance and chloride removal capacity of different fungal strains isolated from the mangrove forest. Salt tolerance was tested on YPD agar plate. The yellow bar indicates the strains which could grow in 80% wastewater, and residual chloride from the media was measured in liquid culture 2 days post-inoculation with 80% wastewater as a medium (pH 5.6) with Cl^- concentration of 67.512 g/L.

processes aimed at reducing the chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in the wastewater (Liu et al., 2020).

3.4. Distribution of Cl^- in fungal cells

The Cl^- and fungal cells were stained with MQAE (blue) (Figure 5) and/or Congo red (red) (Figure 6) fluorescent stains, and observed using a fluorescent microscope and a confocal laser microscope separately. MQAE is a sensitive chloride ion indicator, and the fluorescence was observed throughout the fungi, including both the hyphae and spores, distributed evenly in the cells, suggesting a strong uptake from the wastewater by strain H16, consistent with the reduction in media Cl^- content (Figures 3, 4). Compared to strain H4, which had a reduced chloride clearance ability than H16 (Figure 2), the fluorescence intensity observed was lower (Figure 5). Confocal imaging with Congo Red, which bound to the glucans in the fungal

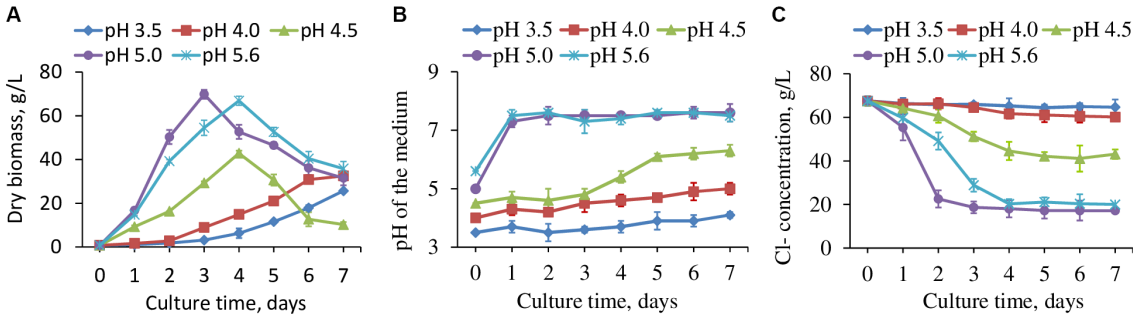

FIGURE 3
The fungal strain H16 grew in wastewater with different starting pH, the production of biomass **(A)**, pH changes in the water **(B)** and concentration of residual Cl^- **(C)**. Data represent mean ± SD ($n = 3$) of three biological replicates. Data for pH 2, 2.5 and 3 were not included as fungi did not grow.

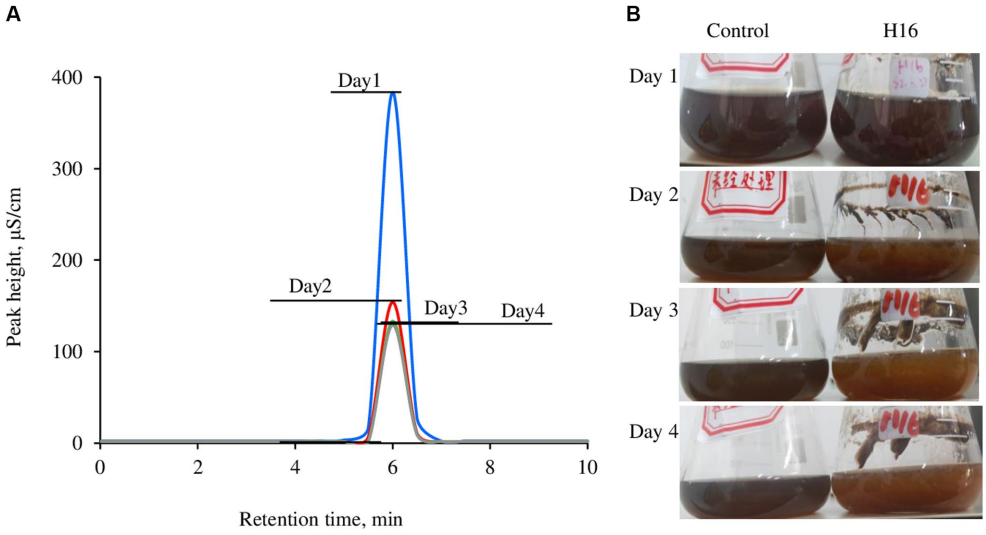

FIGURE 4
Overlay of Cl^- concentration in the wastewater brought by the growth of *Aspergillus piperis*. Cl^- was detected using a Compact ion chromatograph **(A)** and the changes in media color over the culturing time **(B)**. All the samples were diluted 500 times before measurement. The wastewater was used as a control. The individual ion chromatographs were available in the Supplementary Figure S3.

cell wall, showed markedly different outcomes (Figure 6); chloride ions entered H16 cells, while being bound to the exterior of H4 cells. Further studies will be necessary to understand the mechanism behind the uptake of Cl^- into the fungal cells.

3.5. Virulence capability of *Aspergillus piperis*

The pathogenicity of the isolated *A. piperis* was investigated using *T. molitor* and *G. mellonella* virulence models at both 28 and 37°C. The results of the present assay showed that mortality in both insect models was typically dose-dependent, with doses lower than 1×10^5 conidia having little to no effect on larval mortality within 7 days after fungal inoculation (Figure 7). The fungal infection capability was also found to be modulated by temperature, as incubation at 37°C (host temperature) led to more significant larval killing induced by *A. piperis* in both insect models compared to 28°C (an environmental temperature; Figure 7). It is worth noting that *G. mellonella* larvae were found to be less sensitive to *A. piperis* conidial infection than *T. molitor* larvae under both tested temperatures (Figure 7).

4. Discussion

The removal of Cl^- from seafood shell processing wastewater using a biological method is highly sought after due to the high global consumption of seafood, such as crabs, lobsters and oysters. By discovering new biological solutions to remediate the high chloride-containing wastewater, recycling efforts for shell wastes can be improved to achieve better sustainability outcomes. In this work, the fungal strain H16, of *A. piperis* genus, isolated from a mangrove swamp near the high-salt wastewater-producing industry, demonstrated the capability to bioremediate the high chloride content of the wastewater. Many fungi have been reported to be halophilic (Musa et al., 2018). Among them, the most salt-tolerant is the

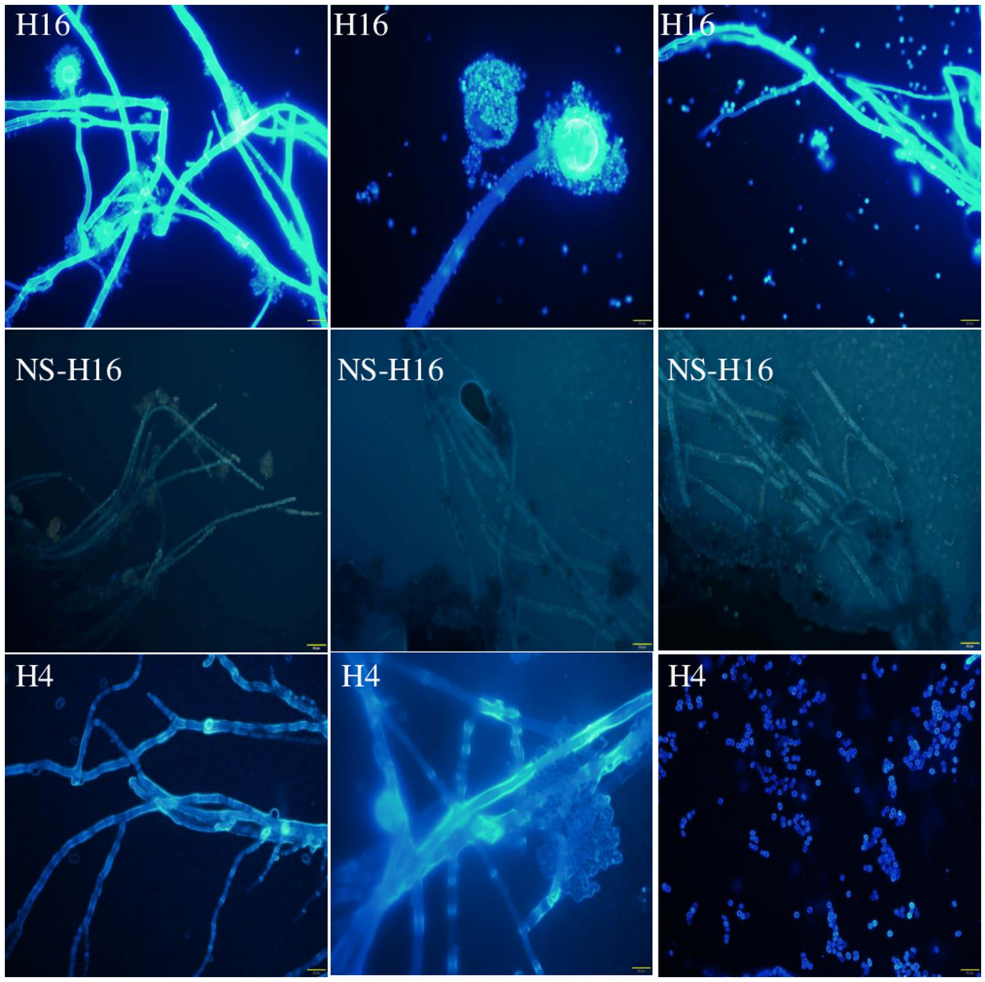

FIGURE 5
Observation of *A. piperis* stained with Cl^- fluorescent probe MQAE (H16). The unstained fungus was used as a control to show the fluorescence of Cl^- (NS-H16). H4, growing in wastewater and taking up less Cl^- was used as a control as well (H4). The brightness of the NS-H16 images was enhanced 40% to make visible. Magnification: ×400. Scale bars = 20 μm.

basidiomycete fungus *Wallemia ichthyophaga,* which can tolerate as much as 320 g/L of NaCl and grows fast in a high-salt environment (Jančič et al., 2016; Musa et al., 2018). However, there are no reports regarding their capabilities of growing in shell processing wastewater. Furthermore, the wastewater from shell processing not only features a high Cl^- concentration but also contains heavy oligosaccharides, COD, BOD, oxidized proteins, and pigments with an extremely dark color and low pH (Gincy et al., 2021). Strain H16 not only demonstrated the ability to survive in the 80% wastewater but also effectively reduced the chloride content and diminished the pigmentation of the wastewater, suggesting multiple modes of bioremediation. Some microorganisms are capable of maintaining different pH levels across the plasma membrane, demonstrating a pH optimum for growth and another pH optimum for tolerance in extreme environment (Baker-Austin and Dopson, 2007). This homeostasis is supported by mechanisms such as organic acid, DNA and protein repair systems, and complex cell wall structures, including reversed transmembrane potentials, highly impermeable cell membranes, and secondary active transport systems (Baker-Austin and Dopson, 2007). Further study showed that the tolerance of acidophilic bacteria to Cl^- was highly greater at pH 3.0 than at pH 2.0, although the maximum Cl^- resistance level varied greatly between species (Carmen and Barrie, 2018). In our study, *A. piperis* strain H16 demonstrated more efficient Cl^- removal from wastewater at pH 5.0 than at pH 3.0 (Figure 3C). Further investigation into nitrogen and carbon conversion will provide insight into the underlying mechanisms. *A. piperis* strain H16 was also able to neutralize the low pH environment of the medium, reducing the amount of base required to transform the highly acidic wastewater environment into a viable one (Figure 3B). The phenomenon has been observed in some fungal species such as *A. nidulans* (Vylkova, 2017), *Saccharomyces cerevisiae* (Palková et al., 2002), and *Candida albicans* (Davis et al., 2000). The increase in pH is believed to be linked to ammonia production, as suggested by existing literature (Vylkova et al., 2011; Vylkova, 2017). Ammonia can be produced either through amino acid catabolism under carbon deprivation conditions or *via* the reduction of nitrate/nitrite under nitrogen catabolite repression mode (Palková et al., 1997; Alkan et al., 2008). Further investigations will be necessary to understand whether strain H16 of *A. piperis* employs a similar mechanism to neutralize the low pH environment.

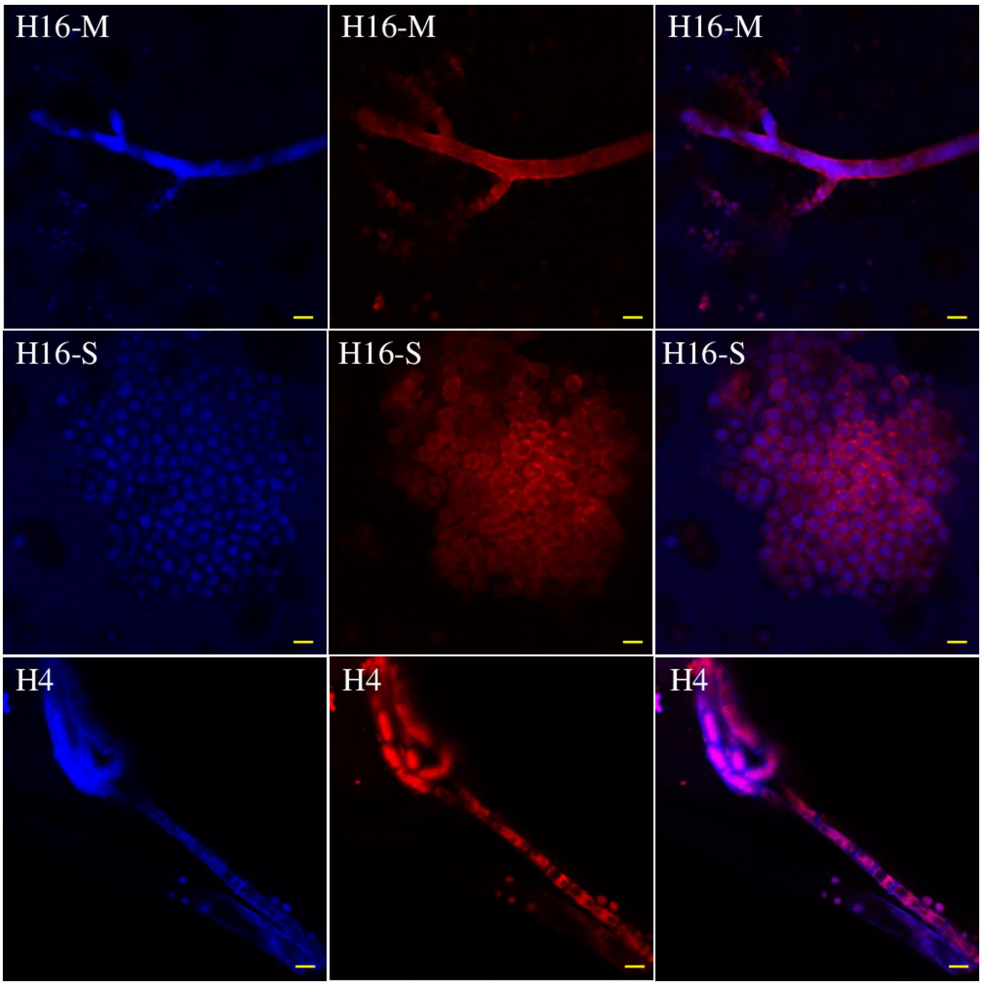

FIGURE 6
Distribution of Cl^- in the mycelia (H16-M) and spores (H16-S) of *A. piperis* 3 days post-inoculation observed using a confocal laser microscope. H4, growing in wastewater and taking up less Cl^- was used as a control to verify the staining protocol (H4). Magnification: ×400. Bars = 20 μm.

Chloride ions were sequestered into *A. piperis* (strain H16) rather than being biosorbed, as observed using a confocal laser microscope (Figure 6). Microorganisms exhibit varying responses in salty conditions. Some may succumb to osmotic pressure (Mille et al., 2005), while others some maintain low intracellular salt content relative to the extracellular medium and regulate their metabolism and membrane fluidity accordingly (Plemenitaš et al., 2008). Some microorganisms adsorb and immobilize ions at the cell surface or within biofilms, akin to the phenomenon observed in fluoride ion removal (Yao et al., 2009; Silva et al., 2019). Additionally, some microorganisms sequester and convert the chloride ions into organic chlorine compounds, such as polychlorinated halogenated alkanes, chloroacetic acid and halogenated aromatic compounds (Geng et al., 2009). Further investigation is warranted to elucidate the mechanism by which strain H16 of *A. piperis* adapts to high intracellular chloride concentrations and the specific compounds into which it channels chloride. Previous research has highlighted the involvement of certain enzymes in the biological conversion of Cl^- to organic chloride, including S-adenosyl-L-methionine (SAM) chlorinase, SAM methyltransferase, SAM halogenase (Atashgahi et al., 2018), flavin-dependent halogenase (Hopwood, 2012), and chloroperoxidase (Bengtson et al., 2013). A search of the NCBI database for *A. piperis* proteins reveals the presence of four SAM methyltransferase enzymes (GI: 1407039482, GI: 1419162826, GI: 1407048220, and GI: 1407048162). This finding suggests that *A. piperis* might employ SAM methyltransferases to process the excess chloride ions, indicating a potential pathway for chloride ion utilization. However, the overall pathway in fungi remains largely unknown and warrants further studies.

To the best of our knowledge, there are few reports regarding the pathogenesis of *A. piperis*. In this study, larvae of *G. mellonella* and *T. molitor* were employed to investigate this concern. These larvae are highly convenient in *in vivo* models for a variety of research purposes as these invertebrates possess a humoral immune response that is highly compatible with that of mammals. Examples of studies using these insect models include assessing the activity and toxicity of antimicrobial agents, evaluating the virulence capability of microbial agents, and studying the immune response to pathogens (Piatek et al., 2021). In addition, insect larvae are also inexpensive to purchase and house, easy to inoculate, and their use is not subjected to legal or ethical restrictions. Due to these numerous advantages, insect models have become widely adopted in both academic and industrial research

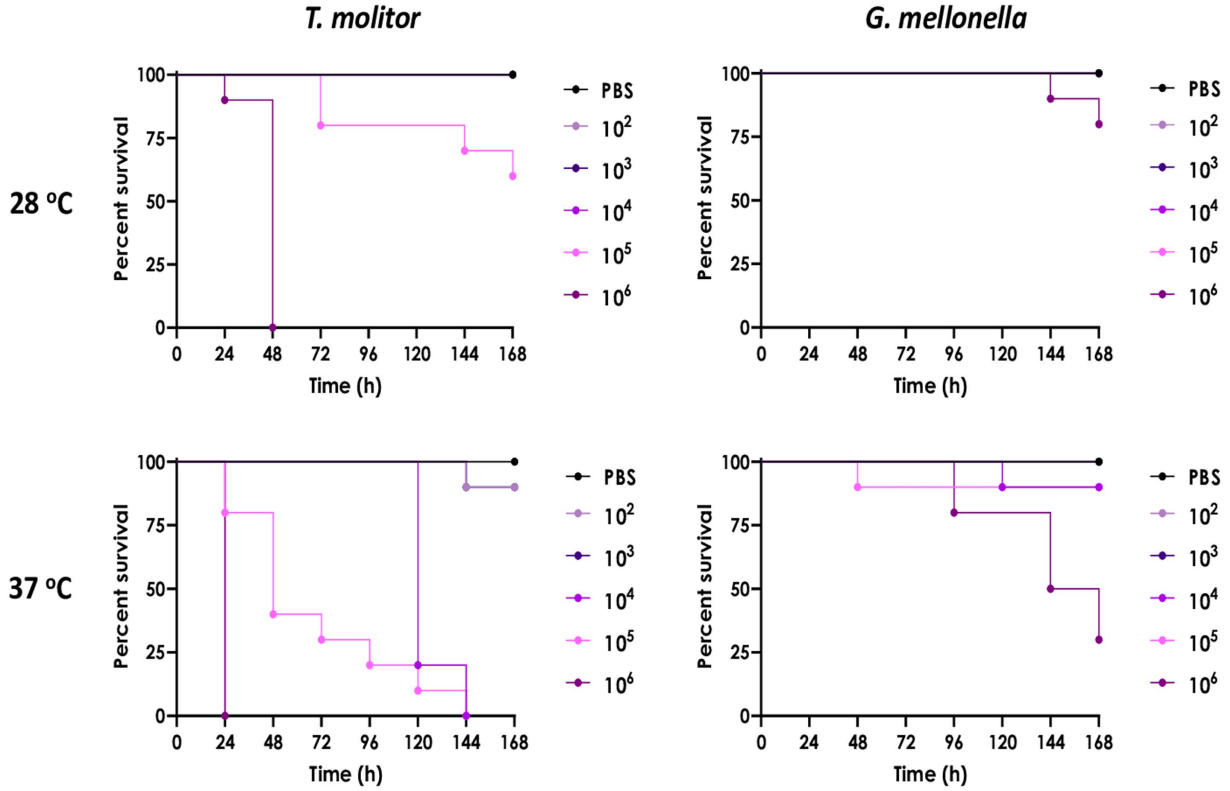

FIGURE 7
Comparison of *A. piperis* virulence in *Tenebrio molitor* and *Galleria mellonella* larvae infected with different fungal inocula (10^2, 10^3, 10^4, 10^5 or 10^6 conidia/larva, $n = 10$ per condition) and incubated at both 28 and 37°C up to 7 days. PBS is used as a control for physical injury caused by inoculation, demonstrating the lack of larval mortality. The results are expressed as percent survival in comparison to uninfected larvae.

settings (Piatek et al., 2021). The fungus isolated in the present study (*A. piperis*) was observed to exhibit low virulent, which was dependent on cell density and temperature (Figure 7). However, it is important to note that every microorganism has the potential to become a pathogen under specific host and environmental conditions. For example, *Saccharomyces cerevisiae*, a yeast species commonly used in the production of bread, pizza and wine, was described as an opportunistic pathogen recently and might cause sepsis in immunosuppressed individuals (Ramos et al., 2023). In contrast, *A. piperis* demonstrates significant potential in bioremediation of pollutants, not only for Cl^- as shown in this study but also for heavy metal. Other studies have demonstrated that this fungus is capable of removing heavy metals such as Se (IV), Pb (II), and Zn (II) from water, with a maximum Pb (II) adsorption capacity predicted by isotherm models to be 275.82 mg/g (De Wet et al., 2020; de Wet and Brink, 2021). Further analysis showed that heavy metals were adsorbed on the surface of fungal cells and displaced sodium (Na) and potassium (K) (de Wet and Brink, 2021), which is a different remediation pathway from this study.

Using microorganisms to reduce Cl^- concentration in wastewater has been studied in some fungal species. *A. terries* has been found to be able to reduce 43% of Cl^- in wastewater samples with a Cl^- concentration of 0.478 g/L collected from an urban wastewater treatment plant, while *A. niger* and *Penecillium digitatum* could reduce 22 and 34%, respectively (Kadhim et al., 2021). The authors discovered that the plasma membrane played a role in regulating the elimination of Cl^-, yet the specific mechanism responsible for this regulation remains unsolved. Sobiecka (2022) used a mixture of both sulphate-reducing and denitrifying bacteria isolated from a petrochemical wastewater sedimentation tank to remove Cl^- in a synthetic chloride-rich medium in anaerobic conditions, and achieved a highest removal rate of 15.85% on the third week post-inoculation (Sobiecka, 2022).

5. Conclusion

Cl^- pollution originates from multiple sources and is considered hazardous at high concentrations. Bioremediation is one of the sustainable approaches to address this issue. Most studies showed that microorganisms conduct Cl^- bioremediation through passive processes like adsorption or biosorption. In this study, a mangrove wetland-derived *A. piperis* was found to be able to actively transport chloride ions into their cells, resulting in the removal of 74.11% of Cl^- from glucosamine processing wastewater with a salt concentration exceeding 60 g/L in just 3 days. The metabolic processes of Cl^- inner the fungal cells deserve further investigation to elucidate the underlying mechanisms and optimize fungal bioremediation techniques. This strain may become an industrially viable chloride bioremediation organism, contributing towards a global push for sustainability.

Data availability statement

The data presented in the study are deposited in the GenBank repository, accession number OR393858.

Author contributions

ZH: Data curation, Investigation, Writing – original draft. EM: Methodology, Writing – review & editing. AS: Writing – review & editing, Formal analysis, Supervision. IB: Formal analysis, Investigation, Validation, Writing – original draft. YP: Conceptualization, Methodology, Writing – review & editing. WH: Data curation, Investigation, Writing – original draft. JY: Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1271286/full#supplementary-material

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SHRIMP WASTEWATER BIOREMEDIATION

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Research article

Highly effective reduction of phosphate and harmful bacterial community in shrimp wastewater using short-term biological treatment with immobilized engineering microalgae

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ABSTRACT

Shrimp farming wastewater includes high amounts of phosphate and microbiological contaminants, necessitating further treatment before release into receiving water bodies. After 24 h of shrimp wastewater treatment, alginate beads containing the blue-green algal *Synechocystis* strain lacking the phosphate regulator gene (mutant strain ΔSphU) at 150 mg L^{-1} reduced phosphate content from 17.5 mg L^{-1} to 5.0 mg L^{-1}, representing 71.5% removal efficiency, with phosphate removal rate reaching 6.9 mg gDW^{-1} h^{-1} during photobioreactor operation. For short-term treatment, removal rates of nitrate, ammonium and nitrite were 42.7, 48.5 and 92.9%, respectively. Microalgal encapsulated beads also impacted the bacterial community composition dynamics in shrimp wastewater. Next-generation sequencing targeting the V3–V4 region of the 16S rDNA gene showed significant differences in bacterial community composition after 24 h of treatment. Proteobacteria are the most abundant phylum in shrimp wastewater. After 24 h of bioremediation, reductions of harmful bacteria in the Cellvibrionaceae and Pseudomonadaceae families were recorded at 5.85 and 3.18%, respectively. Engineered microalgal immobilization under optimal conditions can be applied as an alternative short-term bioremediation strategy to remove phosphate and other harmful microbial contamination from shrimp farming wastewater.

1. Introduction

The aquaculture market is one of the fastest growing and developing industries for food production, especially shrimp culture. Thailand is a major global exporter of shrimp, with production of inland and marine farming over 0.3 million tons from 2017 to 2021 (FAO, 2022). However, controlling environmental contamination from aquaculture wastewater discharge is a significant issue. Primary pollutants in shrimp wastewater discharge are organic matter and dissolved nutrients containing nitrogen and phosphorus (Thomas et al., 2010). In shrimp aquaculture, discharge wastewater is generated during the harvesting process, with shrimp feed, excreta, and dead shrimp all contributing to the microbial pollutant load (Ge et al., 2019). Untreated shrimp effluent causes eutrophication which is harmful to the natural ecosystem (Nasir et al., 2015). The variety of species grown and production techniques also affect the nutritional load of shrimp aquaculture effluent. Accumulation of phosphorus (P) from feed and urine excretion in a closed aquaculture system can reach 20 mg P L^{-1}, either insoluble or soluble, with inorganic phosphate being the most frequent component (Trépanier et al., 2002). Phosphorus has lower toxicity than ammonia or nitrite but the indirect consequences of eutrophication are hazardous to aquatic life (Epifanio and Srna, 1975).

Currently, nitrification and denitrification procedures are used to remove inorganic nitrogen from aquaculture effluent, while inorganic phosphorus removal requires additional sophisticated processes (Sesuk et al., 2009; Martins et al., 2010). Chemical precipitation or biological processes have typically been used to remediate inorganic phosphate in wastewater. Phosphate accumulating organisms (PAOs) have been

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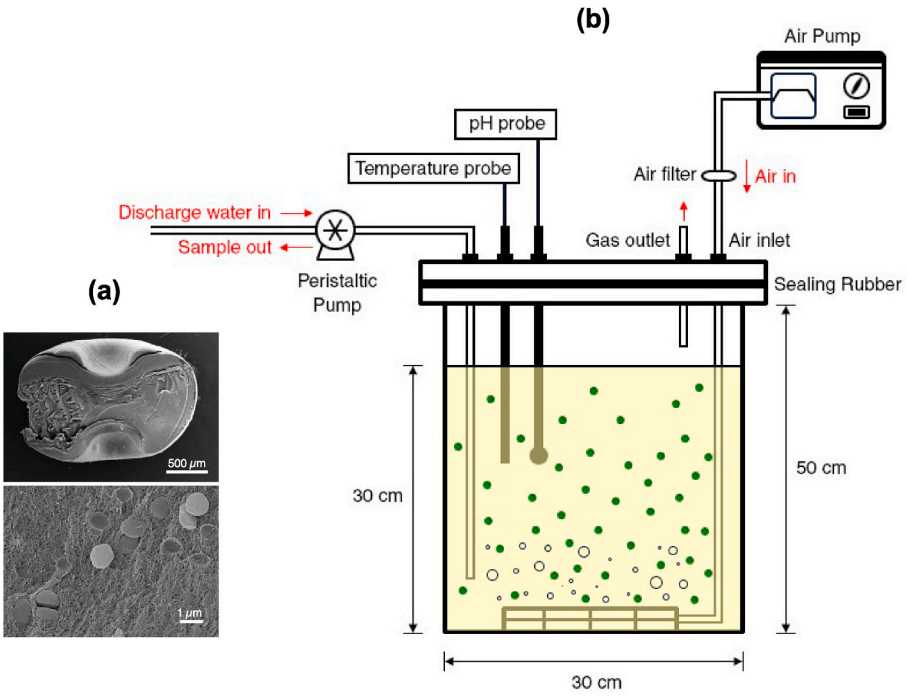

Fig. 1. (a) SEM images of the cross-sections of alginate bead for immobilized *Synechocystis* sp. PCC 6803 mutant strain ΔSphU. (b) Schematic diagram of the flat-plate photobioreactor for batch cultivation of immobilized engineered microalgae in shrimp wastewater.

extensively explored to optimize enhanced biological phosphorus removal (EBPR) but a sophisticated reactor is required for transitioning between anaerobic and aerobic conditions together with well-controlled organic carbon addition (Toerien et al., 1990; Barak et al., 2003; Hu et al., 2003). A photosynthetic phosphate removal process using phototrophic organisms such as green algae and cyanobacteria (blue-green algae) presents a more attractive and less complicated alternative to PAOs because these organisms can assimilate nitrogen and phosphorus molecules into their biomass and release oxygen to the water body through the photosynthesis pathway without the requirement of an organic carbon source (Zhang et al., 2008; Delgadillo-Mirquez et al., 2016). Blue-green algae can store phosphate intracellularly as polyphosphate granules (Burut-Archanai et al., 2011). Under high level phosphate conditions, algal cells can regulate phosphate uptake through their phosphate sensing system (SphU) (Juntarajumnong et al., 2007). Burut-Archanai et al. (2013) found that the blue-green algal *Synnechocystis* sp. PCC 6803 strain lacking SphU function (mutant strain ΔSphU) showed significant phosphate removal efficiency in a closed recirculating aquaculture system, taking 24 h for complete phosphate removal from wastewater containing 5 mg L^{-1} of inorganic phosphate. Inactivation of a negative SphU regulator leads to the up-regulation of genes involved in phosphate transport and utilization of mutant strain ΔSphU (Juntarajumnong et al., 2007). The mutant strain ΔSphU has also been used for phosphate removal in shrimp wastewater and reduced phosphate concentration of 8.22 mg L^{-1} at a rate of 20.16 mg g^{-1} d^{-1} (Krasaesueb et al., 2019). Thus, engineered microalgal immobilization is an interesting alternative for wastewater treatment in shrimp aquaculture with unrestricted capacity for phosphate removal.

The separation of cell biomass following wastewater treatment adds to the cost of microalgae-based processes (Mennaa et al., 2015). Therefore, the application of engineered microalgal immobilization in aquaculture wastewater is an attractive choice with many benefits such as simple cultivation, increased nutrient removal, easier biomass collection and improved cell endurance to hostile conditions including salinity and metal toxicity (De-Bashan and Bashan, 2010; Eroglu et al., 2015). This study determined the effect of immobilizing the blue-green algal *Synechocystis* strain ΔSphU for nutrient removal from shrimp wastewater under short-term biological treatment in a photobioreactor. Release of aquaculture wastewater into the environment has dangerous biological consequences and microbial contaminants require adequate treatment before discharge. Therefore, after passing through the photobioreactor, wastewater samples were collected for analysis of microbial diversity and bacterial community composition using high-throughput DNA sequencing technology. The findings of this study assist in wastewater treatment management of aquaculture systems using an alternative biological treatment technique with engineered microalgal immobilization technology.

2. Materials and methods

2.1. Shrimp wastewater collection and analysis

The wastewater sample was collected from a shrimp farm in Samut Songkhram Province, Thailand, transferred into 20 L plastic containers and kept at 4 °C to avoid chemical and biological changes before experimentation. Physiochemical water qualities including pH, temperature, salinity, nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) were analyzed according to the standard method for the Examination of Water and Wastewater (Rice et al., 2017).

2.2. Stock culture and cell immobilization in calcium alginate matrix

The *Synechocystis* mutant strain ΔSphU was grown in BG11 medium at room temperature (27 ± 2 °C) using a 125 mL flask with continuous shaking at 160 rpm under 40 μmol photons m^{-2} s^{-1} cool white fluorescence illumination. Then, cells at log phase (OD_{730} ~ 0.4) were harvested by centrifugation (2,790×*g*, 10 min) before cell immobilization, as described by Limrujiwat et al. (2022). Briefly, alginate solution (2.0% w/v) was prepared by dissolving sodium alginate in warm distilled water. Harvested cells at various concentrations were then combined with alginate solution, gently stirred for 5 min, and added dropwise using a peristaltic pump into $CaCl_2$ solution (2.8% w/v) for bead forming. The mutant strain ΔSphU ranging from 0 to 600 mg L^{-1} was selected for alginate encapsulation to investigate the effect of algal

Table 1
Physiochemical characterization of shrimp wastewater before and after treatment using immobilized engineering microalgae.

Water quality index[a]	Initial shrimp wastewater	Shrimp wastewater after 24 h of treatment
Salinity (ppt)	7.64 ± 0.05	8.46 ± 0.03
pH	8.41 ± 0.01	8.48 ± 0.02
Temperature (°C)	28.00 ± 0.01	28.20 ± 0.02
Phosphate; PO_4^{3-} (mg L^{-1})	17.53 ± 0.54	5.00 ± 0.25 (71.5% removal)
Nitrate; NO_3^- (mg L^{-1})	4.90 ± 0.12	2.81 ± 0.16 (42.7% removal)
Nitrite; NO_2^- (mg L^{-1})	0.42 ± 0.04	0.03 ± 0.08 (92.9% removal)
Ammonium; NH_4^+ (mg L^{-1})	4.02 ± 0.19	2.07 ± 0.08 (48.5% removal)

[a] Each value represents the mean ± S.D. ($n = 3$).

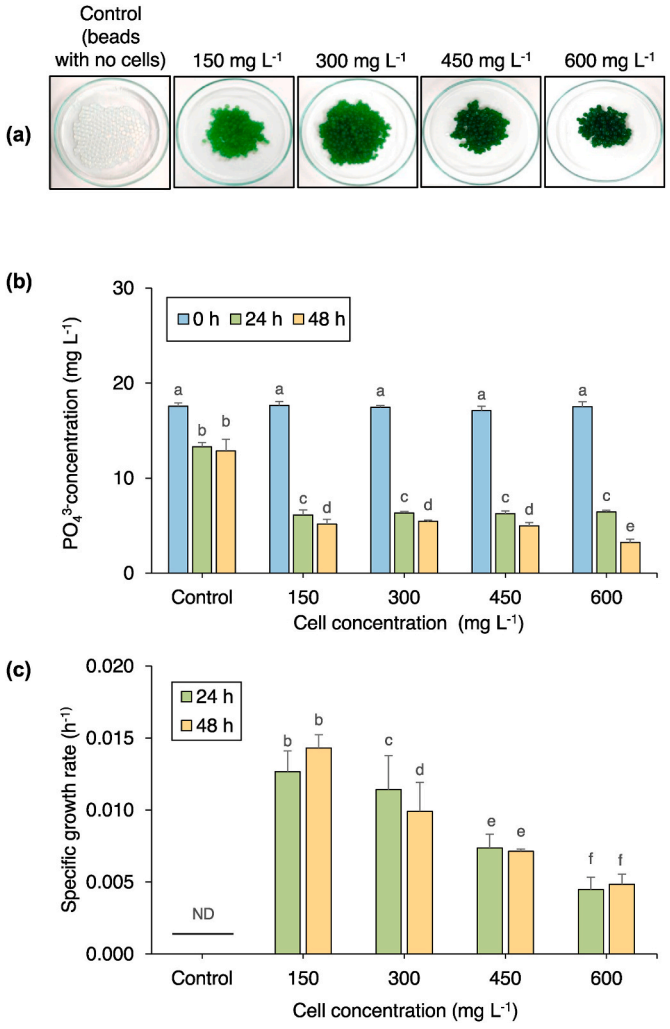

Fig. 2. Alginate beads containing mutant ΔSphU cells at various concentrations (a), and the effect of different concentrations of mutant cells in alginate beads on phosphate removal (b) and specific growth rate (c) when using shrimp wastewater as medium culture (means ± S.D., $n = 3$). The letters indicate the significant difference according to Duncan' s multiple range test at $p < 0.05$, ND means data not detected.

concentration inside the alginate bead on phosphate removal efficiency. Then, immobilized microalgae were transferred to a 125 mL flask using shrimp wastewater (50 mL) as a culture medium and incubated under growth conditions. The volume ratio of alginate-immobilized microalgae to shrimp wastewater was fixed at 1:5. Alginate beads were observed under a scanning electron microscope, as shown in Fig. 1a.

2.3. Shrimp wastewater treatment in a photobioreactor

A 10 L volume of shrimp wastewater was transferred to a flat-plate photobioreactor manufactured of acrylic plastic, as illustrated in Fig. 1b for biological wastewater treatment. The alginate-immobilized microalgae were inoculated into a photobioreactor at the same ratio used in the previous experiment. The reactor was continuously aerated from the bottom with ambient air filtered through a 0.22 μm cellulose acetate membrane, with airflow rate 1.0 L min^{-1}. The experiment was run for 24 h under light intensity of 40 μmol photons m^{-2} s^{-1} via a steel plate equipped with cool white fluorescent lamps at 2.5 cm from the wall of the photobioreactor. The wastewater was analyzed for physiological parameters every 6 h during the treatment period.

2.4. Analytical methods

The wastewater was filtrated through a 0.45 μm glass microfiber filter before physicochemical characterization using the standard method (Rice et al., 2017). The nutrient removal rate and efficiency were calculated following Krasaesueb et al. (2019) by determining residual nutrients remaining in the wastewater. Alginate-immobilized microalgae were collected to measure specific growth rate by determining chlorophyll *a* content, as reported by Rai et al. (2016). Photosynthesis efficiency was analyzed in terms of the capacity of microalgal cells to produce O_2 under saturating white light using a Clark-type O_2-electrode (Hansatech Instruments, UK) at 25 °C, as described by Krasaesueb et al. (2021). All experiments were performed as three biological replicates, with results presented as mean ± S.D. One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used to examine significant differences (p-value < 0.05) across the various treatments using SPSS software version 15.0.

2.5. Microbial community analysis

The wastewater was collected from the reactor and immediately stored at −20 °C before analysis of the microbial community. Total DNA was extracted using a Soil DNA Isolation Plus Kit (Norgen, Canada) according to the manufacturer's instructions. Agarose gel (1.0% w/v) electrophoresis was used to confirm the DNA quality before the extracted DNA was sent to Vishuo Biomedical in Singapore to conduct next-generation sequencing using the Illumina MiSeq platform. Briefly, a MetaVx™ Library Preparation Kit (GENEWIZ, Inc., South Plainfield, NJ, USA) was applied for library preparation. The V3–V4 region of 16S rDNA was selected to generate amplicons using the specific forward primer (CCTACGGRRBGCASCAGKVRVGAAT) and reverse primer (GGACTACNVGGGTWTC TAATCC). A Qubit 2.0 Fluorometer was used for nucleic acid quantitation. DNA libraries were multiplexed and loaded on an Illumina MiSeq Instrument (Illumina, San Diego, CA, USA) and DNA sequencing was performed using a 2x300 paired-end (PE) configuration.

High-throughput 16S rDNA data analysis was operated using the QIIME data analysis package mainly based on the protocol published by Li et al. (2017). Representative operational taxonomic units (OTUs) were annotated using the Ribosomal Database Project (RDP) classifier Bayesian algorithm, and the Silva database was used for taxonomic classification. The α-diversity was calculated in QIIME (1.9.1) from rarefied samples using the Shannon and Chao1 indices for diversity and richness analysis. The β-diversity was calculated using unweighted UniFrac analysis, and principal coordinate analysis (PCoA) was performed based on Bray-Curtis distance metrics. Analysis of similarities (ANOSIM) was performed to determine differences among the groups. The Spearman correlation coefficient between environmental factors and OTUs was used for the correlation analysis between community composition and environmental factors. A heatmap, relative abundance, PCoA and ANOSIM plots were generated using R (3.3.1) software and applied to the statistical analysis. All data were expressed as means ± S.

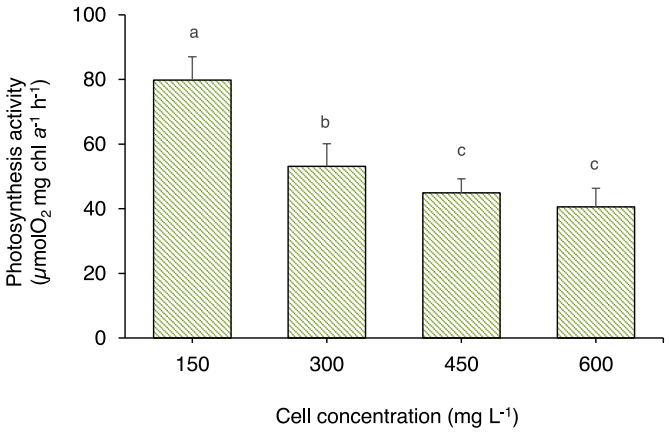

Fig. 3. Photosynthesis activity of mutant strain ΔSphU at different concentrations immobilized in alginate matrix (means ± S.D., $n = 3$). The different letters represent significant differences according to Duncan' s test ($p < 0.05$).

D.(n = 3).

3. Results and discussion

3.1. Shrimp wastewater characterization

Nitrogen and phosphorus-containing molecules in aquaculture wastewater are the most critical nutrients for microalgae development

Table 2
Sequencing data information performed on the Illumina MiSeq platform of each sample.

Samples	Raw reads	Filtered reads	Quality score (%)	Observed OTUs
0h-1	100,357	83,446	93.9	156
0h-2	90,081	77,665	94.3	152
0h-3	116,237	90,091	95.1	162
24h-1	81,048	71,627	92.6	156
24h-2	132,134	109,563	95.6	155
24h-3	138,281	110,069	95.3	161

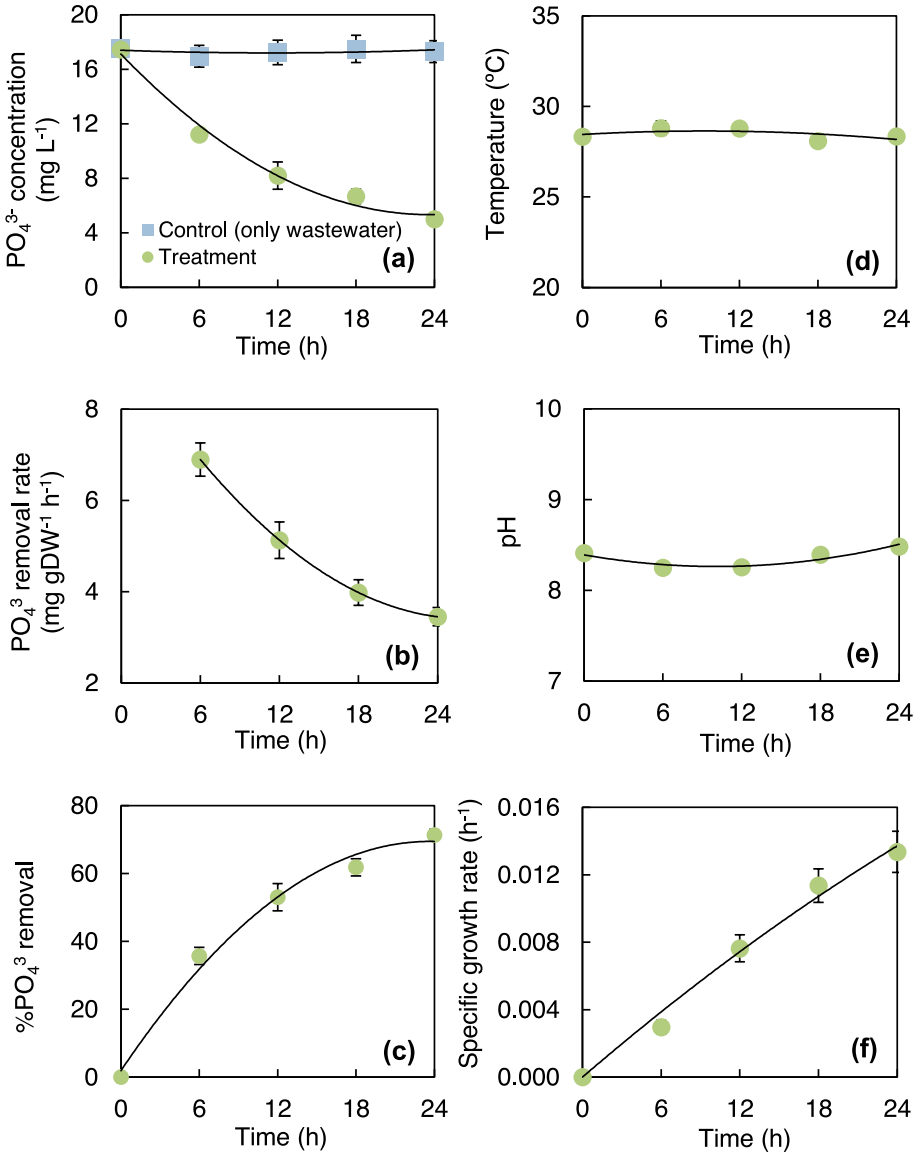

Fig. 4. The efficiency of phosphate removal from shrimp wastewater when treatment with immobilized mutant strain ΔSphU under bath photobioreactor for 24 h, phosphate concentration (a), phosphate removal rate (b), %phosphate removal (c), temperature (d) and pH (e) change in shrimp wastewater, and specific growth rate of ΔSphU cell (f). Data are the mean ± S.D. ($n = 3$).

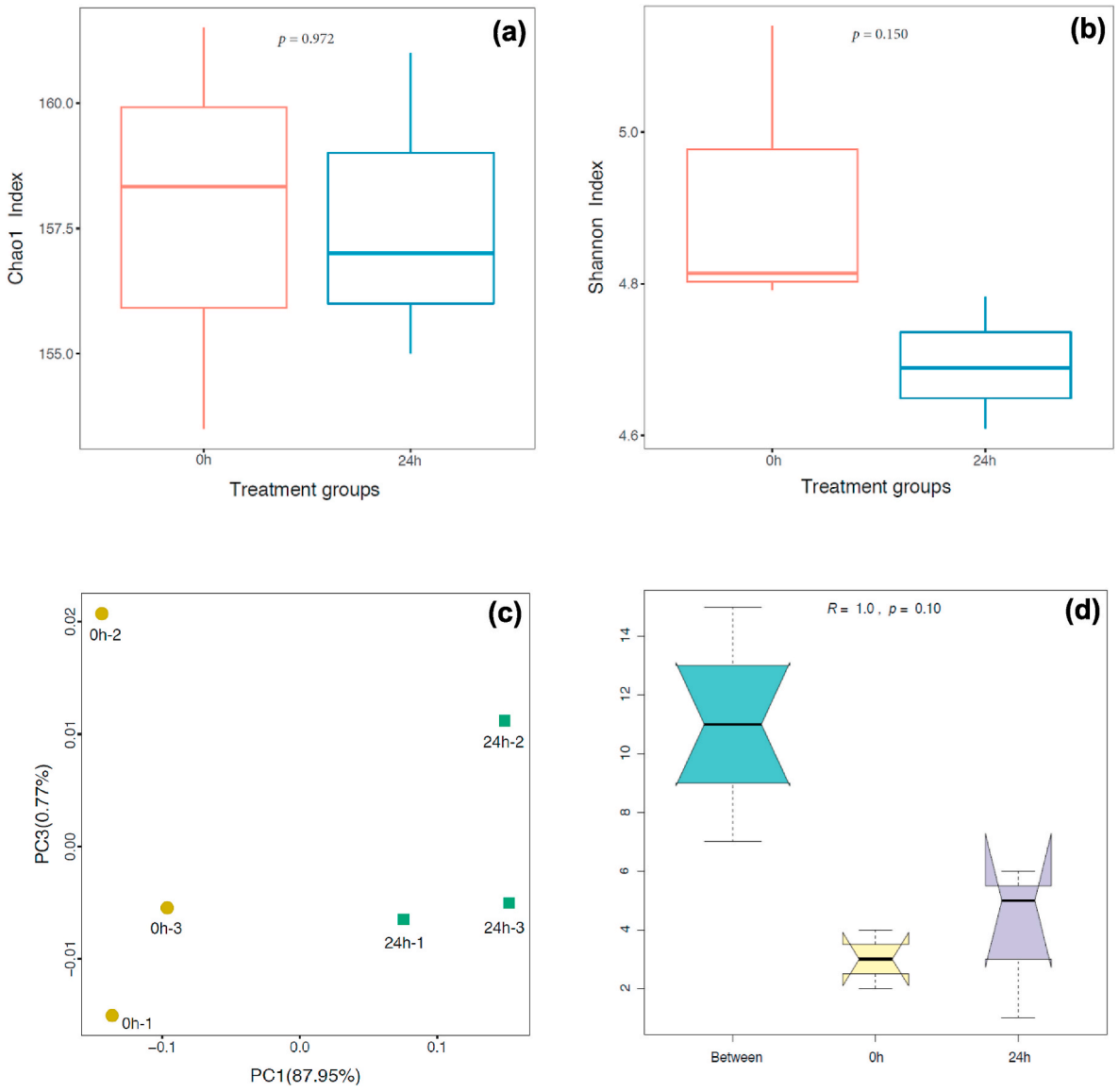

Fig. 5. Comparison of microbial diversity and community composition in shrimp wastewater when treatment with immobilized mutant strain ΔSphU under bath photobioreactor for 0 and 24 h. Between-group differential analysis using α-diversity indices (a, b), Principal coordinate analysis (PCoA) plot represents the β-diversity index (c), and group comparison by ANOSIM analysis (d).

and metabolism (Zhu et al., 2013). In this study, high levels of nutrients such as nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) were observed in shrimp wastewater. Table 1 shows the physicochemical water quality index of shrimp wastewater. Results revealed that nitrogen-containing molecules were found in several inorganic forms including nitrate (4.90 mg L^{-1}), nitrite (0.42 mg L^{-1}) and ammonium (4.02 mg L^{-1}). Surprisingly, the shrimp wastewater had high phosphorus levels in the form of inorganic phosphate (17.53 mg L^{-1}) that required treatment before reuse or release into the environment. The blue-green algal *Synechocystis* sp. PCC 6803 mutant strain ΔSphU has previously been found to thrive effectively in shrimp wastewater, with high pH and salinity having little effect on metabolism (Krasaesueb et al., 2019). As a result, high salinity (7.64 ppt) and alkaline pH (8.41) in the shrimp wastewater did not affect cell survival of the mutant strain ΔSphU (Fig. 2).

3.2. Optimal engineered microalgal concentration for cell immobilization on growth and phosphate removal

Separating the microalgae from treated wastewater is one of the primary issues when employing them for wastewater treatment (Mennaa et al., 2015). Immobilization technology that encases microalgal cells in an alginate matrix can solve these harvesting issues. Many studies determined that immobilized microalgae *Chlorella vulgaris* were more efficient in removing nutrients than freely suspended cells (Tam and Wong, 2000; Abdel Hameed, 2002). However, the stacking density of beads and microalgal content in beads are challenging factors for nutrient removal efficiency using microalgal immobilization. Fig. 2a shows the growth and phosphate removal of various concentrations of mutant ΔSphU cells (150–600 mg L^{-1}) used to optimize the microalgal content in alginate beads. Results indicated that after 24 h of treatment, encapsulated mutant ΔSphU cells removed phosphates compared to the control (beads without microalgae). Phosphate concentration of 17.5 mg L^{-1} in shrimp wastewater decreased rapidly to less than 10 mg L^{-1} in all treatments containing mutant ΔSphU cells (Fig. 2b). Surprisingly, increasing the amount of mutant ΔSphU cells in the beads to above 150 mg L^{-1} gave no significant improvement in phosphate removal. Hameed (2007) reported that ammonia and phosphate molecules in medium culture were adsorbed on bead surfaces before slowly soaking into the alginate and becoming accumulated by algal cells. Therefore, the

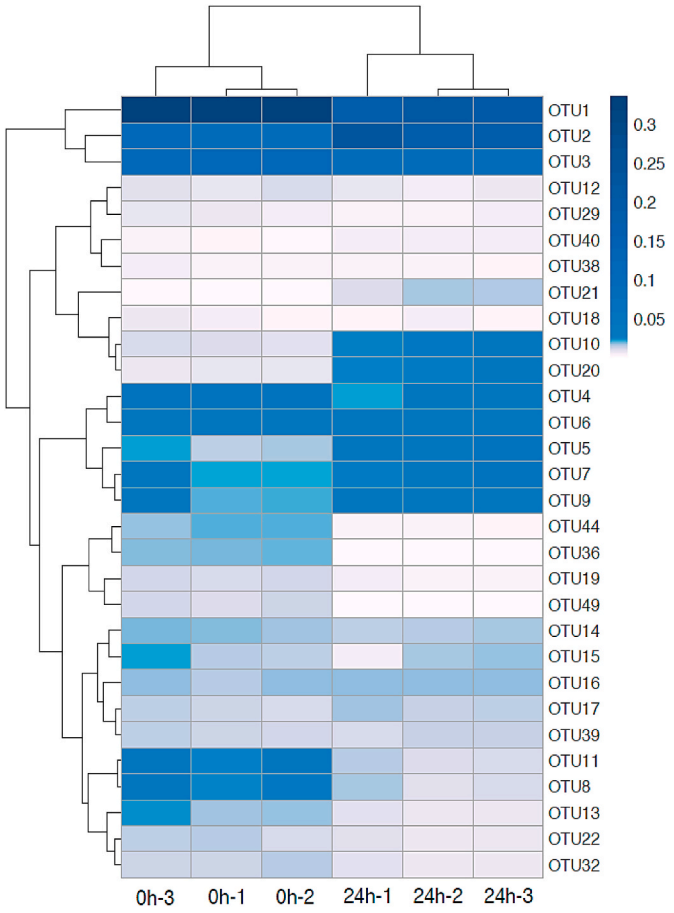

Fig. 6. Heatmap of the 30 most OTUs abundant clustering represents different microbiota between treatment groups. The column name is the sample information, and the row name is OTU ID. The sample clustering is represented by the dendrogram above the heatmap, while the dendrogram shows the OTU cluster to the left. The value of each colored box is the relative abundance of each OTU after normalization.

specific growth rate of ΔSphU cells was also studied, as shown in Fig. 2c. Results revealed that increased concentration of ΔSphU cells in alginate beads exceeding 150 mg L^{-1} limited their specific growth rate, possibly because higher cell concentration restricted nutrient diffusion through the alginate pores (Jimenez-Perez et al., 2004). A significant decrease in photosynthesis activity was also observed with increased microalgal concentrations (Fig. 3), resulting in reduced growth and nutrient utilization of mutant ΔSphU cells. *Synechocystis* mutant strain ΔSphU at a concentration of 150 mg L^{-1} proved optimal for bead encapsulation and was used for phosphate removal in shrimp wastewater.

3.3. Phosphate treatment using immobilized ΔSphU strain under a photobioreactor

The blue-green algal *Synechocystis* sp. PCC 6803 ΔSphU strain lacks a phosphate regulator gene (*sphU*), and can accumulate phosphate molecules inside the cell at higher amounts than the wild type (Burut-Archanai et al., 2011). Burut-Archanai et al. (2013) reported that this strain removed phosphate in a closed recirculation aquaculture system at 0.79 mg gDW $^{-1}$ h^{-1}. This study found that the immobilized mutant strain ΔSphU was highly effective for phosphate treatment in shrimp wastewater. Residual PO_4^{3-} concentrations in non-sterilized shrimp wastewater were drastically reduced from 17.5 to 5.0 mg L^{-1} in 24 h under a photobioreactor (Fig. 4a). Removal of PO_4^{3-} reached 6.9 mg gDW^{-1} h^{-1} during 6 h of treatment (Fig. 4b), faster and more effective than determined by Krasaesueb et al. (2019) utilizing free-living mutant

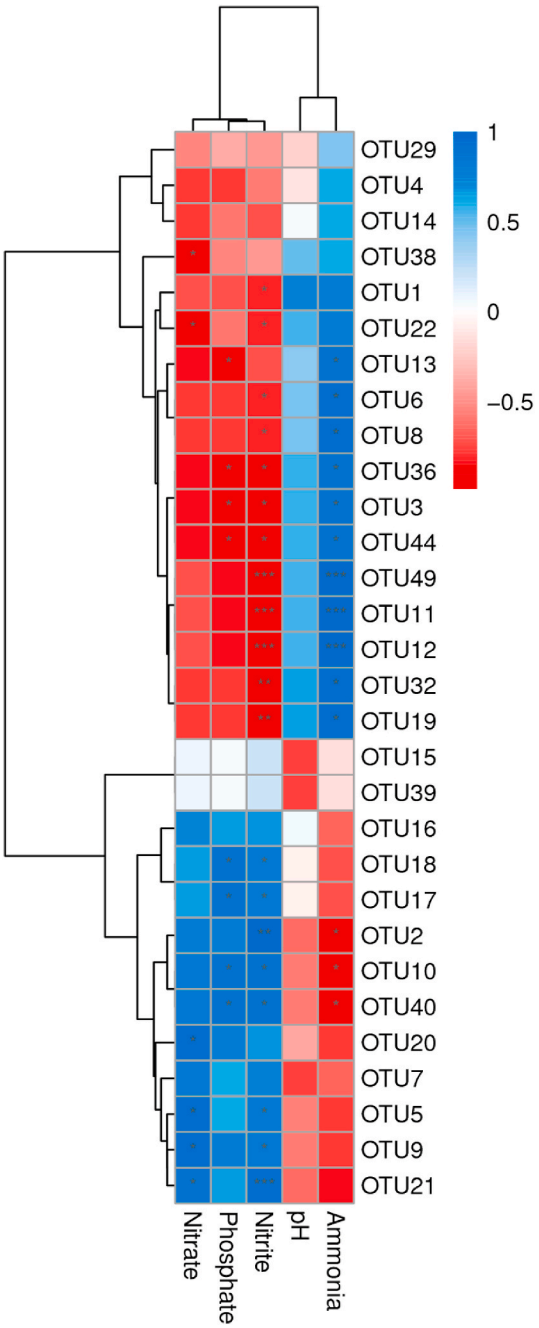

Fig. 7. Heatmap of Spearman correlation between OTUs and environmental factors. The colors in the heat map represent the Spearman correlation coefficient (r) ranges between −1 and 1, which indicates positive correlation ($r > 0$) and negative correlation ($r < 0$). Data are the mean ± S.D. ($n = 3$), significances are indicated by *($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cells. Results revealed that the immobilized mutant strain ΔSphU showed a higher rate of PO_4^{3-} removal than cyanobacterial biofilms dominated by *Phormidium* and *Oscillatoria* spp. monitored by Rai et al. (2016). Within 24 h of treatment, the control shrimp wastewater was evaluated to ensure that reduction of PO_4^{3-} was unaffected by precipitation of phosphate accumulating organisms (PAOs) in the wastewater (Fig. 4a). The immobilized mutant strain ΔSphU showed phosphate removal efficiency of 71.5% after 24 h of photobioreactor treatment (Fig. 4c). By contrast, the immobilized green algae *Chlorella vulgaris* required treatment time of longer than 48 h (Hameed, 2007).

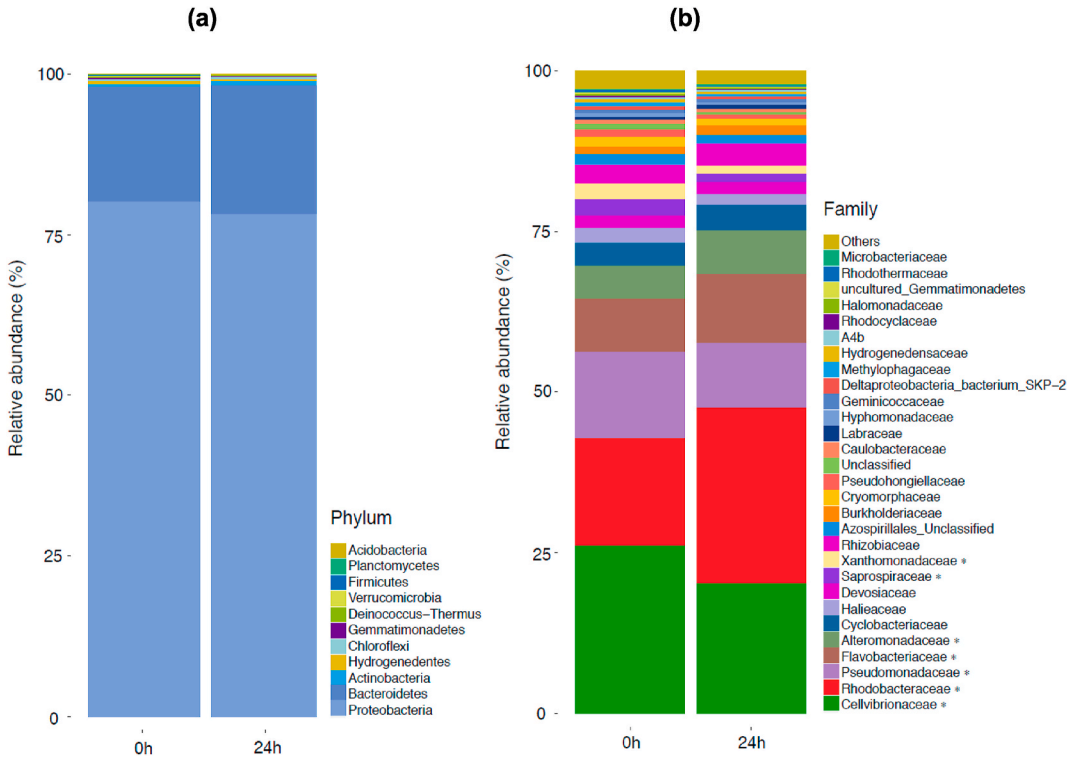

Fig. 8. The relative abundance of microbial communities at the phylum (a) and family (b) levels in shrimp wastewater samples between 0h and 24h of treatment. Data are the mean ± S.D. ($n = 3$). The asterisks indicate taxa with significant differences in relative abundance between treatment groups ($p < 0.05$).

Temperature and pH changes in shrimp wastewater were also monitored, with no notable alterations in both factors (Fig. 4d and e). Immobilized ΔSphU cells were grown in shrimp wastewater for 24 h and their specific growth rate gradually increased (Fig. 4f), demonstrating that alkaline pH and high salinity did not impact cell permeability and nutrient utilization. Employing the immobilized microalgal strain ΔSphU for shrimp wastewater treatment demonstrated high potential, with removal efficiencies of 71.5% for phosphate, 92.9% for nitrite, 42.7% for nitrate and 48.5% for ammonium within 24 h of treatment (Table 1).

3.4. Microbial diversity and community structure dynamics in shrimp wastewater treatment by engineered microalgal immobilization

Next-generation sequencing (NGS) approaches have enabled recovery of DNA sequence data from environmental samples such as freshwater, marine, soil, terrestrial and gut microbiota (Shokralla et al., 2012). These techniques have been applied to water quality assessment topics, such as developing bioindicators for water contamination and studying the relationship between waterborne microbial communities and water quality (Tan et al., 2015). Furthermore, it can also be applied to hazard assessment topics in the critically damaged river and terrestrial ecosystems caused by heavy metal contamination from industrial wastes or pollution from the mining process, as some recent studies (Koley, 2021; Jafarpour and Khatami, 2021). Studying changes in microbial communities as bioindicators using NGS technology may help in issue solving and lead to long-term environmental management strategies. Therefore, this study demonstrated the composition of microbial community dynamics in shrimp wastewater after treatment using engineered microalgal encapsulated beads by NGS technologies. Microbial community composition was evaluated using 16S rDNA metabarcoding at each time point during treatment (0 and 24 h). Fig. S1 displays the effective sequences used in data analysis. Numbers of observed OTUs from each sample achieved a plateau, as shown in Fig. S2, indicating that the sample was large enough to represent all OTUs present. Filtered sequence numbers obtained in all samples ranged from 77,665 to 110,069 reads, with up to 162 OTUs classified. (Table 2). The Chao1 and Shannon indices were used to determine the richness and α-diversity of microbiota in shrimp wastewater, as shown in Fig. 5. At 0 and 24 h of treatment, the Chao1 values of shrimp wastewater were 157.78 and 157.67, respectively ($p > 0.05$) (Fig. 5a), while the Shannon index values were 4.92 and 4.69, respectively ($p > 0.05$) (Fig. 5b). These results indicated no significant differences in microbial richness and α-diversity in shrimp wastewater treated with immobilized mutant ΔSphU cells for 24 h. However, the β-diversity characterizing data similarities and differences in each sample, as in the principal coordinate analysis (PCoA) plot (Fig. 5c), revealed that the microbiota was separated between treatment groups, with significant differences in microbial communities during 0 h and 24 h of treatment. Analysis of similarities (ANOSIM) (Fig. 5d) also demonstrated that inter-group differences in microbial composition were more significant than intra-group differences (R-value close to 1.0). Clustering of the 30 most abundant OTUs also confirmed different microbiota patterns between the 0 h and 24 h treatments (Fig. 6). Results in Fig. 7 demonstrated that several OTUs had a negative correlation with environmental factors, especially phosphate and nitrite. Thus, short-term biological treatment by engineered microalgal immobilization reduced microbial abundance and impacted community composition in shrimp wastewater.

Previous studies that investigated microbiota composition reported that Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria were the dominant phyla in various shrimp aquaculture systems but their relative abundances in wastewater and shrimp intestine were significantly different (Xiong et al., 2015; Huang et al., 2016; Zhang et al., 2016). Similarly, in this study, the top three phyla obtained in shrimp wastewater were Proteobacteria (78.28–80.20%), Bacteroidetes (17.81–20.01%) and Actinobacteria (0.42–0.61%). The most abundant phylum found in wastewater after treatment was Proteobacteria (Fig. 8a). Proteobacteria comprises a large diverse group of bacteria that are part of the normal human microbiota and many pathogens (Zhang et al., 2019). As shown in Fig. 8b, relative abundances between 0 h and

24 h of treatments showed significant differences ($p < 0.05$) at the family level. Interestingly, relative abundances of Cellvibrionaceae, Pseudomonadaceae, Xanthomonadaceae and Saprospiraceae in shrimp wastewater decreased by 5.85, 3.18, 1.28 and 1.20%, respectively after treatment with immobilized mutant ΔSphU cells for 24 h ($p < 0.05$). By contrast, relative abundances of Rhodobacteraceae, Flavobacteriaceae and Alteromonadaceae were higher in the wastewater sample at 24 h compared to 0 h ($p < 0.05$). Therefore, nutrient removal by engineered microalgal immobilization impacted microbial community structure dynamics in shrimp wastewater, with numbers of harmful bacteria in the Cellvibrionaceae and Pseudomonadaceae families greatly reduced within 24 h of treatment. A higher abundance of Flavobacteriaceae (most species are aerobic and composed of environmental bacteria) was discovered, suggesting that heterotrophic bacteria grew well and broke down organic materials in shrimp wastewater by utilizing oxygen produced from microalgal photosynthesis (Mathew et al., 2021). Therefore, integrated bacteria-microalgae approach might provide a potential method for the biological treatment of shrimp aquaculture systems. However, synthesis of nanocomposites with strong antibacterial activity requires further study to promote the reduction of hazardous bacterial communities in wastewater, as previously reported by Yousefi et al. (2021) and Mahdi et al. (2022).

4. Conclusions

The immobilized *Synechocystis* sp. PCC 6803 mutant strain ΔSphU, which lacks phosphate regulator activity, was used to improve water quality by reducing the concentrations of inorganic phosphate and harmful bacteria in shrimp wastewater. Engineered microalgal immobilization demonstrated high efficiency, with phosphate removal 6.9 mg gDW^{-1} h^{-1} after 24 h of treatment. The dynamics of microbial community structures were also altered following treatment with immobilized mutant cells, while microbiota of pathogenic bacteria in families Cellvibrionaceae and Pseudomonadaceae significantly decreased. Engineered microalgal immobilization can be applied to shrimp farming systems as a short-term bioremediation strategy. Further studies on the stability and number of microalgal bead reuse cycles are required, together with investigating optimal use of immobilized algal beads on a large scale, such as on a shrimp farm.

Credit author statement

Conception and design of study: W. Khetkorn; Acquisition of data: N. Krasaesueb, W. Khetkorn; Analysis and/or interpretation of data: N. Krasaesueb, W. Khetkorn; Drafting the manuscript: W. Khetkorn; Revising the manuscript critically for important content: W. Khetkorn; Approval of the version of the manuscript to be published: N. Krasaesueb, J. Boonnorat, C. Maneeruttanarungroj, W. Khetkorn.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2022.116452.

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ARTICLES FOR FACULTY MEMBERS

SHRIMP WASTEWATER BIOREMEDIATION

Title/Author	Immobilized Tetraselmis sp. for reducing nitrogenous and phosphorous compounds from aquaculture wastewater / Khatoon, H., Penz Penz, K., Banerjee, S., Redwanur Rahman, M., Mahmud Minhaz, T., Islam, Z., Ara Mukta, F., Nayma, Z., Sultana, R., & Islam Amira, K.
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Immobilized *Tetraselmis* sp. for reducing nitrogenous and phosphorous compounds from aquaculture wastewater

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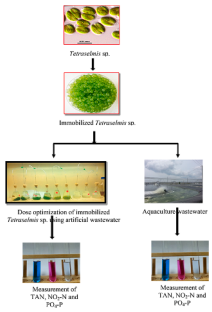
HIGHLIGHTS

- *Tetraselmis* sp. bead reduces TAN, NO2-N and PO4-P from artificial and aquaculture wastewater.
- Aquaculture wastewater can be treated with immobilized *Tetraselmis* sp.
- *Tetraselmis* sp. beads are cost-effective biological method to treat aquaculture wastewater.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Wastewater
Nitrogenous compounds
Phosphorous compounds
Reduction rate
Aquaculture

ABSTRACT

Removal of nitrogenous and phosphorus compounds from aquaculture wastewater by green microalgae (*Tetraselmis* sp.) was investigated using a novel method of algal cell immobilization.

Immobilized microalgae removed nitrogenous and phosphorous compounds efficiently from aquaculture wastewater. Results showed that *Tetraselmis* beads reduced significantly ($p < 0.05$) the total ammonia nitrogen, nitrite nitrogen and soluble reactive phosphorous concentration (0.08; 0.10 and 0.17 mg/L, respectively) from the initial concentration of 7.7, 3.1 and 2.0 mg/L respectively within 48 h compared to other treatments. Removal rate of total ammonia nitrogen, nitrite nitrogen and soluble reactive phosphorous were 99.2, 99.2 and 94.3% respectively, for the artificial wastewater within 24 h. For the shrimp pond wastewater, total ammonia nitrogen, nitrite nitrogen and soluble reactive phosphorous were reduced 98.9, 97.7 and 91.1% respectively within 48 h. It is concluded that *Tetraselmis* sp. beads is an effective means to reduce nitrogen and phosphorus levels in aquaculture wastewater.

1. Introduction

In this developing century, alongside with the growing human population, there has been an increase in contamination due to the release of effluents such as chemicals, and fertilizers in to the aquatic ecosystem which can eventually lead to eutrophication (Ngatia et al.,

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2019). One of the sectors that have been contributing to the nutrient contamination in the aquatic ecosystem is through the aquaculture industry. Wastewater discharged from aquaculture contains nitrogenous compounds (ammonia, nitrite and nitrate), phosphorus, total suspended solids, volatile suspended solids, biochemical oxygen demand, and chemical oxygen demand and dissolved organic carbon, which cause environmental deterioration at high concentrations (Lananan et al., 2014). In aquaculture, farmers feed their fish with food such as fish pallet, trash fish and etc. that contains high percentage of protein which are beneficial for the fish growth. However, uneaten food degrade very fast in the water and thus increase the nitrogenous compound particularly ammonia, nitrite and phosphorous (Dauda et al., 2019). When nitrogenous compound are released into the environment at a dangerous rate, they can create severe damage to the aquatic life and in a roundabout way changing the food chain. These nitrogenous waste can be toxic to the cultured animals by increase their vulnerability to diseases, especially the bottom organism like shrimp (Dvorak, 2004). In addition, the drained water may increase the occurrence of pathogenic microorganisms and introduce invading pathogen species (Cabral, 2010).

Ammonia is the main source of nitrogenous compounds in aquaculture wastewater. It is produced from the excretory product of the aquatic organisms in the form of ammonium and also from the uneaten feed which eventually decomposes and release nitrogenous compounds. These compounds are products from metabolic activity in high culture density, feeding rates, fertilizer and decaying matters in the culture system. Therefore, treatment of this water is necessary to prevent adverse environmental impacts. In a real-world application, the treatment of wastewater by aeration, filtration, and anaerobic-anoxic–oxic (A2O) system (Altmann et al., 2016) increases energy consumption and the total cost of aquaculture and financial burden of industry (Longo et al., 2016). Moreover, in traditional technologies, nutrients, including nitrogen, phosphorus, and carbon, in wastewater could not be completely utilized and recycled as resources (Lu et al., 2019). To overcome environmental and economic barriers in the aquaculture industry, a lot of efforts have been devoted into the application of microalgae for wastewater remediation, biomass production, and water quality control (Han et al., 2019). Microalgae assimilate nutrients in a eutrophic water body and have been proven to be a good way for wastewater remediation (Leng et al., 2018). Recently, the use of microalgae for wastewater management has become a common choice (Khatoon et al., 2016; Abdel-Hameed and Hammouda, 2007) for treatment and reuse of wastewater in intensive aquaculture systems. Microalgae has been proven in many previous research to be able to improve water quality as it utilizes the nitrogenous waste and convert it to energy for its own use (Khatoon et al., 2016; Renuka et al., 2015).

In the field of microalgae biotechnology there are four major research fields which consist of wastewater treatment, carbon dioxide sequestration, biofuel production and high value-added molecules production (Levasseur et al., 2020). Marine microalgal, *Tetraselmis* sp. is flagellated chlorophytes with rapid growth rate, and can stand a broad range of temperatures and pH values (Khatoon et al., 2014). *Tetraselmis* sp. is a widely used species in aquaculture as it contains adequate amounts of protein, lipid, carbohydrate and fatty acids which are essential for the cultured organisms (Khatoon et al., 2017). *Tetraselmis* sp. is found to be able to lower phosphates and nitrogenous waste level in wastewater efficiently. Using free suspended microalgae to manage the water quality in open water, also known as the green water system, there is difficulties during harvesting. In addition, free floating microalgae in filtration tanks have been reported not efficient in terms of harvesting, biomass obtained and cost (Gonzalez-Bashan et al., 2000). Therefore, immobilization technique is introduced to fix microalgae in alginate and improve harvesting (Huntley et al., 1989) and nutrient absorption rate (Kumar and Sarama, 2012). Immobilizing microalgae is a common approach in several bioremediation applications (de-Bashan and Bashan, 2010). In the making of immobilized microalgae beads, the most regular polymer used is alginate. Immobilization of microalgae in alginate beads has been commonly used and is applied in a range of biotechnological processes (Prasad and Kadokawa, 2009). Immobilization in alginate beads of the microalgae protects the microalgae when they perform wastewater treatment. The beads act as a physical barrier between the two environments. This maintains the treatment agents within the bead and keeps the native wastewater organisms out, thus allowing uninterrupted tertiary wastewater treatment (Covarrubias et al., 2012). Abdel-Hameed (2007) reported that the immobilized algal is more efficient in removing nutrients than the freely suspended cells of the same algal species. Nutrient removal efficiency can be affected by many factors, such as algal species, immobilization matrix, cell and bead concentration, aeration, and retention time. Therefore, the nutrients from the wastewater will become feed for the microalgae, which in turn become either a feed or a fuel source. The resulting wastewater will be of quality and suitable for many industrial applications (Khatoon et al., 2016). Hence, in this experiment, immobilized marine *Tetraselmis* sp. was used to remove nitrogenous and phosphorous compounds in aquaculture wastewater.

Table 1
Preparation of Conway Medium (Tompkins *et al.,* 1995).

(A) Main Mineral Solution	
Names of Chemicals	Quantity
$NaNO_3/KNO_3$	100.00 g/116.00 g
Disodium EDTA (C_{10} $H_{16}N_2O_8$)	45.00 g
H_3BO_3	33.60 g
$NaH_2PO_4{\cdot}4H_2O$	20.00 g
$FeCL_3{\cdot}6H_2O$	1.30 g
$MnCL_2{\cdot}4H_2O$	0.36 g
Trace metal solution	1.00 mL
Dissolving in deionized/distilled water and make the volume 1 L	
(B) Trace Metal Solution	
Names of Chemicals	Quantity
$ZnCl_2$	2.10 g
$CoCl_3{\cdot}6H_2O$	2.00 g
$(NH_4)_6MO_7O_2{\cdot}4H_2O$	0.90 g
$CuSO_4{\cdot}5H_2O$	2.00 g
Dissolving in deionized/distilled water and make the volume 1 L	
Vitamins	
Names of Chemicals	Quantity
Thiamine, B1	0.20 g
Cynocobalamin, B12	0.01 g
Dissolved in deionized / distilled water and make the volume 100 mL	

2. Materials and methods

2.1. Microalgae sample collection and culture

The pure culture of *Tetraselmis* sp. was obtained from School of Fisheries Sciences and Aquaculture, University Malaysia Terengganu. Preparation of Conway medium involves the preparation of stock solutions which consist of macronutrient, trace metal solution and vitamins (Table 1). All the macronutrient, trace metal and vitamins were prepared according to Tompkins et al. (1995). Stock suspension of *Tetraselmis* sp. was cultivated in Conway medium at pH 6.5–7.2 under aseptic conditions. Two subcultures of pure stocks were maintained at 100 mL in a conical flask without aeration with controlled conditions where pH, temperature and lighting were kept constant. After seven days of culture, 80 mL of culture was harvested and top up with 80 mL of Conway media. The harvested 80 mL of microalgae was centrifuge at 8000 rpm for 5 min and was stored properly. Sub-culturing was done every two weeks in order to maintain healthy and good stocks. Then the stock culture was used during the experiment.

2.2. Culture of Tetraselmis sp. for preparation of beads

Microalgae *Tetraselmis* sp. was cultured separately in a 300 mL conical flask with filtered aeration and proper lighting in Conway medium. At the exponential phase, microalgae were harvested. An aliquot amount of an exponentially growing *Tertaselmis* sp. culture was centrifuged at 8000 rpm for 5 min and was re-suspended in 1 mL of algal growth medium. This was the concentrated microalgae cells that were used for preparation of immobilized microalgae beads.

2.3. Preparation of immobilized microalgae beads

Alginate solution, 100 mL was prepared (Solution A + B) by first preparing Solution A: 3 g of alginate powder (Protanol) was dissolved in 70 mL of distilled water by slow stirring with a magnetic bar on a magnetic stirrer. Solution B: 3.5 g of sodium chloride (NaCl) was dissolved in 30 mL distilled water to obtain a saline solution. After alginate powder has completely dissolved, the two solutions (A and B) were thoroughly mixed using a magnetic stirrer bringing a total volume of 100 mL of sodium alginate solution. One mL of the concentrated microalgae cells was thoroughly mixed with the 100 mL sodium alginate solution by gentle stirring for a period from 45 min to get cell density concentration of 10^3 cells/mL. This was the 10^3 cells/ mL the algal alginate solution. For beads without algae that act as control.

The beads were formed by adding drop wise the mixed *Tetraselmis* sp. and microalgae alginate solution using a burette from a height of 15 cm at a rate of 30–40 drops/min forming 5 mm diameter/ beads in 4% strontium chloride solution. Magnetic stirrer was added into the beaker to prevent beads from sticking together. The beads were kept in the strontium chloride solution for a period of 45 min to 1 h to allow complete hardening. The beads were later washed several times with filtered and sterilized seawater to remove any traces of strontium chloride (Mallick 2002). The beads were stored in an algal Conway medium at 4 °C in the dark overnight (was sealed with aluminum foil) for further use. Blank beads were made without any algal cells as control.

Microalgae beads were tested in two phases of experiment. In the first phase, artificial wastewater was used and in the second phase, aquaculture wastewater was used. Artificial wastewater is prepared as follow.

2.4. Artificial wastewater

For this study three different nutrients such as total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N) and soluble reactive phosphorous (SRP) were used. Ammonium, nitrite and phosphate were added respectively at a concentration of 3, 2 and 2 mg/L in the sterilized seawater. Ammonium sulphate, sodium nitrite and potassium dihydrogen phosphate were used to increase total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N) and soluble reactive phosphorous (SRP) concentration in the seawater respectively.

2.5. Efficacy of immobilized microalgae beads in reducing nitrogenous and phosphorous compounds from artificial wastewater

Tetraselmis sp. immobilized microalgae beads at concentration of 10^3 cells/mL starting with 0, 0.5, 1, 1.5, 2, and 2.5 beads/mL was added into 300 mL of artificial wastewater each with triplicates in conical flasks. Five different dosages of beads, and 2 control (one control only the wastewater and another control BB – beads without microalgae cells) with triplicate each. All the experimental flasks were covered with cotton to minimize the evaporation. Experimental flasks were placed in a growth chamber with controlled conditions such as temperature (24 ± 1 °C), aeration and continuous illumination at a light intensity of 50 μmol/m^2/s. TAN, NO_2-N and SRP was measured every day according to the standard method by Parson et al. (1984). The optimum doses of beads were determined for the second phase experiment.

2.6. Efficacy of immobilized microalgae beads in reducing nitrogenous and phosphorous compounds from aquaculture wastewater

Aquaculture wastewater was collected from I-Sharp Blue Archipelago Shrimp Farm in Setiu, Kuala Terengganu, Malaysia. Collected wastewater was first filtered using a filter pump with the 541 Whatman quantitative filter paper (pore size: 22 μm). After that, the wastewater was filtered with GF/C Whatman glass microfiber filters (pore size: 1.2 μm). This filtered wastewater was used as experimental aquaculture wastewater. Using the optimal dosage of beads tested with artificial wastewater, which is 2 beads/mL was used in the second phase experiment. In this experiment there were three treatments such as i) 2.0 beads/mL (T2.0), ii) blank beads (BB – beads without microalgae cells) and iii) control (only the pond wastewater). The filtered wastewater 300 mL was poured into a 500 mL conical flask for each treatment with triplicate. All the experimental flasks were placed in a growth chamber with controlled conditions such as temperature (24 ± 1 °C), aeration and continuous illumination at a light intensity of 50 μmol/m^2/s. Concentration of TAN, NO_2-N and SRP was measured every day according to the standard method by Parsons et al. (1984).

2.7. Measuring total ammonia nitrogen (TAN), nitrate nitrogen (NO_2-N) and soluble reactive phosphorous (SRP)

Concentration of TAN, NO_2-N and SRP of the artificial wastewater were measured according to the following methods:

2.7.1. Determination of total ammonium nitrogen (TAN)

Total ammonia nitrogen was determined according to Parson et al. (1984). Standard stock solution was prepared by weighing 9.343 g of anhydrous grade (NH4)2 SO4 (dried at 110 °C for 1 h, cooled in dessicator before weighing) and dissolving in 1000 mL deionized water. From the stock solution (1000 mg/L of total ammonia–nitrogen), a series of standard solutions (0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5 and 1.0 mg/L) were prepared by mixing with appropriate ratio of deionized water.

Samples and standard solutions (10 mL) were placed in test tube and 0.4 mL of phenol solution (20 g of analytical grade phenol was dissolved in 200 mL of 95% v/v ethyl alcohol and 0.4 mL of sodium nitroprusside (1 g of Na2[Fe(CN)5 NO]2H2O, dissolved in 200 mL of DDH$_2$O water) was added in sequence. Finally, 1 mL of oxidizing solution was added and allows cooling at room temperature (20–27 °C) for 1 h. The test tubes were covered with parafilm (the color is stable for 24 h after the reaction period). The extinction was measured at 640 nm with Shimadzu spectrophotometer model UV-1601. Oxidizing solution was prepared by mixing 100 mL of alkaline reagent (dissolve 100 g of sodium citrate and 5 g of sodium hydroxide in 500 mL of DDH2O) and 25 mL of sodium hypochlorite solution [commercial hypochlorite (e.g. clorox) which should be about 1.5 N].

2.7.2. Determination of nitrite nitrogen (NO_2-N)

Nitrite was determined according to Parsons et al. (1984). Standard stock solution was prepared by weighing 4.9259 g anhydrous grade $NaNO_2$ (dried at 110 °C for 1 h, cooled in dessicator before weighing) and dissolving in 1000 mL deionized water. From the stock solution (1000 mg/L of NO_2-N), a series of standard solutions (0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5 and 1.0 mg/L) were prepared by mixing with deionized water.

Samples and standard solutions (10 mL) were placed in test tube. Then 0.2 mL of sulfanilamide solution (5 g of sulfanilamide was dissolved in a mixture of 50 mL of concentrated hydrochloric acid and dilute to 500 mL with DDH2O) was added. After>2 min but<10 min, 1 mL of NED reagent (0.5 g of the N- (1-napthyl)-ethylenediamine dihydrochloride was dissolved in 500 mL of distilled water) was added and mixed immediately. Between 10 min and 2 h afterwards, the extinction

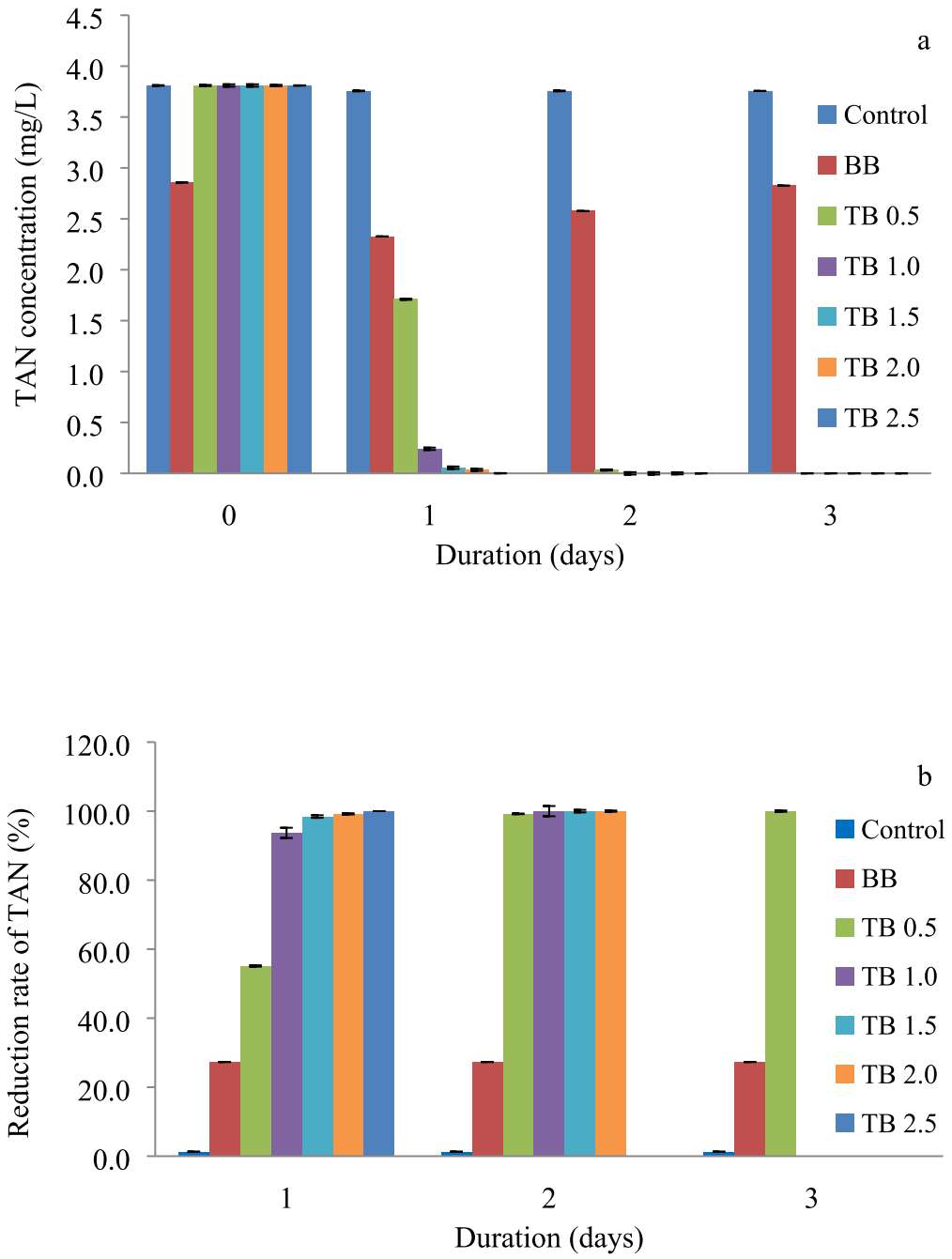

Fig. 1. (a) Concentration (mg/L) and (b) reduction rate (%) of total ammonia nitrogen (TAN) against day using different doses of *Tetraselmis* sp. beads (TB) and blank beads (BB) treated artificial wastewater.

was measured at a wavelength of 543 nm by using the Shimadzu spectrophotometer model UV-1601.

2.7.3. Determination of soluble reactive phosphorous (SRP)

Soluble reactive phosphorous (SRP) was determined according to Parsons et al. (1984). Standard stock solution was prepared by weighing 4.3937 g of anhydrous grade potassium dihydrogen phosphate, KH_2PO_4 (dried at 110 °C for 1 h, cooled in dessicator before weighing) and dissolving in 1000 mL deionized water. From the stock solution (1000 mgLof PO_4-P) a series of standard solutions (0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5 and 1.0 mg/L) was prepared by mixing with deionized water.

Ten milliliter of samples and standard solutions (10 mL) were placed in test tubes and 1 mL of mixed reagent was added. After 5 min and preferably within the first 2–3 h, the extinction was measured at 885 nm by using Shimadzu spectrophotometer model UV-1601. Mixed reagent was prepared by mixing 100 mL of ammonium molybdate (dissolve 15 g of analytical reagent grade ammonium paramolybdate $(NH_4)6Mo7O_{24}$ in 500 mL of distilled water), 250 mL sulfuric acid, 100 mL ascorbic acid (dissolve 27 g of ascorbic acid in 500 mL of distilled water) and 50 mL of potassium antimonyl-tartrate solution (dissolve 0.34 g of potassium antimonyl-tartrate (tartar emetic) in 250 mL of water.

2.8. Statistical analysis

The collected data were analyzed by using one-way analysis of variance (ANOVA). Significant differences among the different treatments were determined using Minitab software (Tukey's test at 0.05 level of probability).

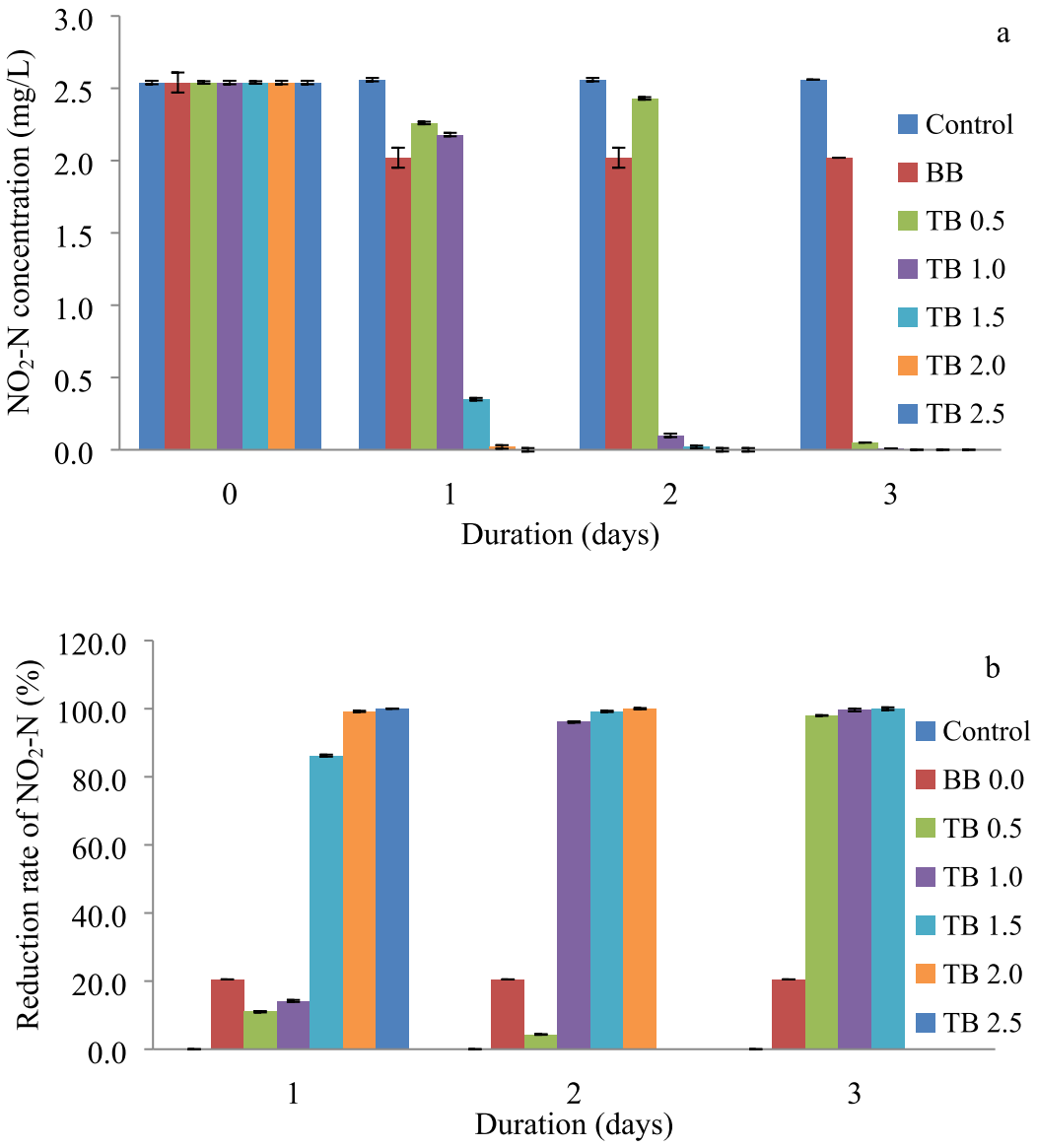

Fig. 2. (a) Concentration (mg/L) and (b) reduction rate (%) of nitrite nitrogen (NO_2-N) against day using different doses of *Tetraselmis* sp. beads (TB) and blank beads (BB) treated artificial wastewater.

3. Results and discussion

3.1. Efficacy of immobilized microalgae beads in reducing nitrogenous and phosphorous compounds from artificial wastewater

In this experiment, different removal efficiencies of nutrients were observed using different doses of immobilized *Tetraselmis* sp. and it showed that the nutrient removal rate is highly dependent on the doses of the microalgae beads. The results that were obtained might differ if it is applied in a different culture condition. This is because this experiment was conducted in a controlled condition where lighting was provided 100% artificially. However in the natural environment, light intensity is 12 h light and 12 h dark plus the intensity varies throughout the day. Besides that, there might also be other suspended solids that are in the water column which could reduce or block the light penetration for algae to grow.

For the phase one experiment different doses of immobilized *Tetraselmis* sp. beads were used to treat artificial wastewater with initial concentration of 3.81, 2.54 and 1.59 mg/L of TAN, NO_2-N and SRP respectively.

Results showed that after one day (24 h), TAN concentration significantly ($p < 0.01$) dropped from 3.81 mg/L to 1.71, 0.24, 0.06, 0.03, and 0.00 mg/L for artificial wastewater treated with 0.5 beads/ml followed by 1.0, 1.5, 2.0 and 2.5 beads/mL. In terms of percentage (%), reduction of TAN was 55.1, 93.7, 98.4, 99.2 and 100.0% for treatment 0.5, 1.0, 1.5, 2.0 and 2.5 beads/mL respectively. After two days (48 h) concentration of TAN was 0.03 mg/L for 0.5 bead/mL and 0.0 mg/L for 1.0, 1.5, 2.0 and 2.5 beads/mL. In terms of reduction, TAN was reduced by 99.2% for 0.5 bead/ml and 100.0% for 0.0, 1.5, 2.0 and 2.5 beads/mL. After three days (72 h) TAN concentration was 0.0 mg/L for all five different treatments. In terms of reduction rate, TAN was reduced by 100.0% for all five different treatments (Fig. 1a, b).

For the control (only wastewater without beads), TAN concentration dropped from 3.81 mg/L to 3.76 mg/L after one day (24 h) and remains the same on the second day and third day. At the same time, the percentage of reduction was similar for the first day, second day and third day which was 1.3%. In case of blank beads (Beads without microalgae cells), TAN concentration dropped from 3.81 ppm to 2.77 mg/L after one day (24 h) and remains the same on the second day and third day. At the same time, the percentage of reduction was similar for the first day,

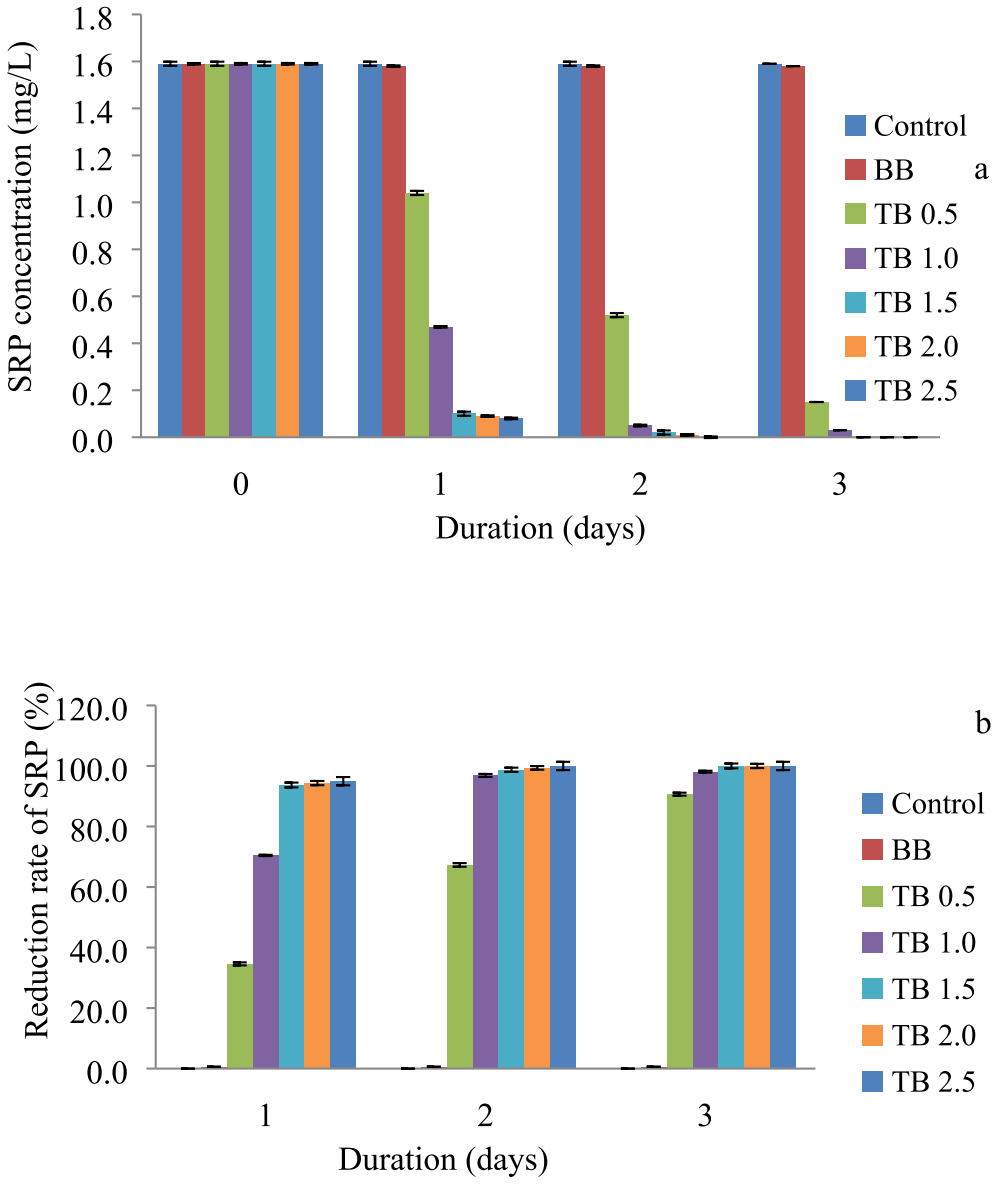

Fig. 3. (a) Concentration (mg/L) and (b) reduction rate (%) of soluble reactive phosphorous (SRP) against day using different doses of *Tetraselmis* sp. beads (TB) and blank beads (BB) treated artificial wastewater.

second day and third day which was 27.3% (Fig. 1a, b).

Based on Fig. 1a, b, immobilized *Tetraselmis* sp. beads showed excellent removal of TAN using 2.5, 2.0, and 1.5 beads/mL. Thus results showed significant reduction compared to the beads without microalgae. Treatment with the highest doses of beads which is 2.5 beads/mL achieved 100% TAN removal within 24 h followed by 2.0 and 1.5 beads/mL. This proves that the results obtained are better than the research conducted by Abdel-Hameed (2002) using immobilized *Chorella Vulgaris* where 10.66 beads/mL with initial cell concentration of 1.5×10^6 cells were used. For beads dosage of 1.5, 2.0 and 2.5 beads/mL, statistically they were grouped in the same category which means there were not much difference probably due to the limitation of nutrients and light intensity. This result proved that the uptake of ammonium and assimilation into algal cells are an essential process for the growth of microalgae cells. Nitrogen is an essential nutrient required for microalgae growth which subsequently contributes to the biomass produced (Farahin et al., 2021). The most predominant source of nitrogen is ammonium which is exists in urban, agricultural and aerobic digested effluents with a wide variation in its concentrations, ranging from low (10 mgL − 1 -N) to high concentrations (2,000 mgL − 1 -N) (Krishna Reddy et al., 2017). Therefore, in this experiment, *Tetraselmis* sp. beads used ammonia as their nutrient to grow.

In case of nitrite nitrogen results showed that concentration significantly ($p > 0.01$) dropped from 2.54 mg/L to 2.26, 2.18, 0.35, 0.02, and 0.00 mg/L for artificial wastewater treated with 0.5 beads/mL followed by 1.0, 1.5, 2.0 and 2.5 beads/mL after 24 h. In terms of reduction of NO_2-N was 11.0, 14.2, 86.2, 99.2 and 100.0% for treatment with 0.5, 1.0, 1.5, 2.0 and 2.5 beads/mL respectively. After two days (48 h) concentration of NO_2-N was 2.43 mg/L, 0.1 mg/L, for 0.5 and 1.0 bead/mL, and 0.0 mg/L for 1.5, 2.0 and 2.5 beads/mL. In terms of percentage NO_2-N was reduced by 20.5, 4.3, 96.1, 99.2 and 100.0% for 0.5, 1.0, 1.5, 2.0 and 2.5 beads/mL. After three days (72 h) NO_2-N concentration was 0.05 mg/L for 0.5 bead/mL and 0.0 mg/L for 1.0, 1.5, 2.0 and 2.5 beads/mL. In terms of percentage NO_2-N was reduced by 98.0 and 99.6% for 0.5 and 1.0 bead/ml and 100.0% for 1.5, 2.0 and 2.5 beads/mL (Fig. 2a, b). For the control beads, NO_2-N concentration did not change throughout the three days of treatment. And the percentage of reduction was similar for the first day, second day and third day which was 0.0%. In case of the blank beads, NO_2-N concentration dropped from 2.54 mg/L to 2.02 mg/L after one day (24 h) and remains the same on the second

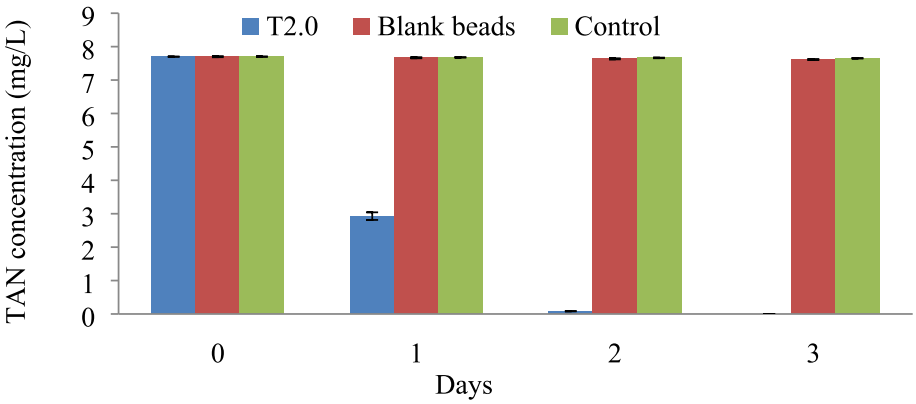

Fig. 4. Concentration (mg/L) of TAN against day using *Tetraselmis* sp. beads (T2.0 beads/mL) treated shrimp pond wastewater.

day and third day. At the same time, the percentage of reduction was similar for the first day, second day and third day which was 20.5% (Fig. 2a, b).

Based on the Fig. 2 (a) and 2 (b), immobilized Tetraselmis sp. beads were proven to be effective in removing nitrite nitrogen from the wastewater. 100% and 99.2% nitrite nitrogen removal was achieved for 2.5 and 2.0 beads/mL within 24 h. Results in this experiment thus showed 100% removal of both TAN and N02-N using 2.5 beads/mL and ± 99% removal using 2.0 beads/mL respectively. Many research found that the algae will take up ammonium and other reduced forms of nitrogen first before nitrite and nitrate (Lachmann et al., 2019; Ghaly and Ramakrishnan 2015) however in this experiment, both TAN and $N0_2$-N were removed at the same time most probably because the concentration of nutrients were much lower therefore the microalgae utilized the nutrients at the same time. It is also doubtful that the nitrogen was converted to N gas by de nitrification process because constant aeration was supplied to the flask (Tam and Wong, 2000). Otherwise it means that *Tetraselmis* sp. is more efficient in removing both TAN and $N0_2$-N compared to other species of microalgae.

Soluble reactive phosphorous concentration significantly ($p > 0.05$) dropped from 1.59 mg/L to 1.04, 0.47, 0.10, 0.09, and 0.08 mg/L for artificial wastewater treated with 0.5 beads/ml followed by 1.0, 1.5, 2.0 and 2.5 beads/ml after 24 h. In terms of percentage, reduction of SRP was 34.6, 70.4, 93.7, 94.3 and 95.0% for treatment with 0.5, 1.0, 1.5, 2.0 and 2.5 beads/mL respectively. After two days (48 h) concentration of SRP was 0.5, 0.1 mg/L for 0.5 and 1.0 bead/ml, and 0.0 mg/L for 1.5, 2.0 and 2.5 beads/mL. In terms of percentage SRP was reduced by 67.3, 96.9, 98.7, 99.4 and 100.0% for 0.5, 1.0, 1.5, 2.0 and 2.5 beads/mL. After three days (72 h) SRP concentration was 0.2 mg/L for 0.5 bead/mL and 0.0 mg/L for 1.0, 1.5, 2.0 and 2.5 beads/mL. In terms of percentage SRP was reduced by 90.70%, 98.1% for 0.5 and 1.0 bead/mL and 100.0% for 1.5, 2.0 and 2.5 beads/mL (Fig. 3a, b). For the control beads (only wastewater without beads), SRP concentration did not change throughout the three days of treatment. And the percentage of reduction was similar for the first day, second day and third day which was 0.0%. On the other hand, blank beads (Beads without microalgae cells), SRP concentration dropped from 1.59 mg/L to 1.58 mg/L after one day (24 h) and remain the same on the second day and third day. At the same time, the percentage of reduction was similar for the first day, second day and third day which was 0.62% (Fig. 3a, b).

The rate of phosphate removal was slow as compared to TAN and N02-N. Treatment with 2.5 and 2.0 beads/mL achieved 95% and 94.3% removal of soluble reactive phosphorous from the wastewater. Similarly, significant reduction of ammonia and phosphate in wastewater was achieved in reactors containing high density of algal beads using Chlorella vulgaris (Tam and Wong, 2000). Abdel-Hameed (2007) also reported that increasing cell stocking in beads did not cause any improvement in the efficiency of treatment, but caused some leakage problems. Also, increasing the beads concentrations in wastewater caused reductions in light penetration and enhanced self-shading effects and the beads settled at the bottom of the reactor. Since 2.5 and 2.0 beads/mL gave results that were insignificantly different, therefore, 2.0 beads/mL was chosen as the optimal dosage of beads that was used to test with shrimp pond wastewater because it is more cost effective. From a cost perspective, microalgal removal of phosphorus from wastewater could be a superior choice over chemical precipitation and engineered wetland based phosphorus removal (Christenson and Sims, 2011).

3.2. *Efficacy of immobilized microalgae beads in reducing nitrogenous and phosphorous compounds from aquaculture wastewater*

For phase two, immobilized *Tetraselmis* sp., 2.0 beads/mL was used to treat the wastewater collected from I-Sharp Setiu, Blue Archipelago Shrimp Farm. On day zero, the initial concentration was 7.7, 3.1 and 2.0 mg/L for TAN, NO_2-N and SRP respectively. TAN concentration *significantly* ($p > 0.01$) dropped from 7.7 mg/L to 2.9 mg/L, 0.1 mg/L and 0.0 mg/L on day one, two and three respectively. In terms of percentage, TAN reduction was 61.9, 98.9 and 100.0% on day one, day two and day three respectively. For the control (only wastewater without beads), TAN concentration was 7.7 mg/L for day one, two and three. Percentage of TAN reduction was 0.3, 0.5 and 0.6% for day one, two and three. In the blank beads (Beads without microalgae cells), TAN concentration was 7.7 mg/L for day one and 7.6 mg/L for day two and day three and the percentage of reduction was 0.4, 0.8 and 1.1% respectively (Fig. 4).

Based on the results obtained testing of immobilized microalgae beads in shrimp pond wastewater, immobilized *Tetraselmis* sp. beads were used to treat wastewater collected from I-sharp Blue Archipelago Shrimp farm in Setiu, Kuala Terengganu, Malaysia. Using 2 beads/mL, 7.7 mg/L TAN concentration which is double the dosage of the concentration in artificial wastewater was able to be reduced by 61.9% after 24 h of treatment and 98.9% after 48 h. In comparison with the experiment conducted using artificial wastewater, TAN was reduced by 99.2% using the same dosage of beads due to lower TAN concentration which was 3.8 mg/L. The results showed that relatively lower removal of TAN in terms of percentage because microalgae beads that were added into the wastewater require a certain period to undergo physiological adaptation to the new environment (Barsanti and Gualtieri, 2006) however, in comparison to the initial concentration of TAN, the microalgae showed better results which is>50% since the TAN of the shrimp pond wastewater was double the amount of the artificial wastewater. In recent years, due to microalgae's ability to use inorganic and organic nitrogen for their growth, their use in treating wastewaters, particularly those rich in ammonium, has been studied (Molazadeh et al., 2019)

Concentration of NO_2-N significantly ($p > 0.01$) dropped from 3.1 mg/L to 2.7 mg/L, 0.1 mg/L and 0.0 mg/L on day one, two and three

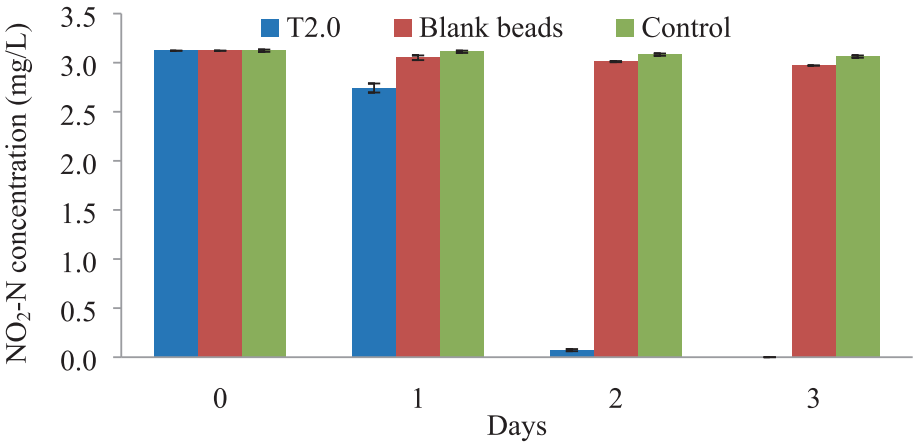

Fig. 5. Concentration (mg/L) of NO_2-N against day using *Tetraselmis* sp. beads (T2.0 beads/mL) treated shrimp pond wastewater.

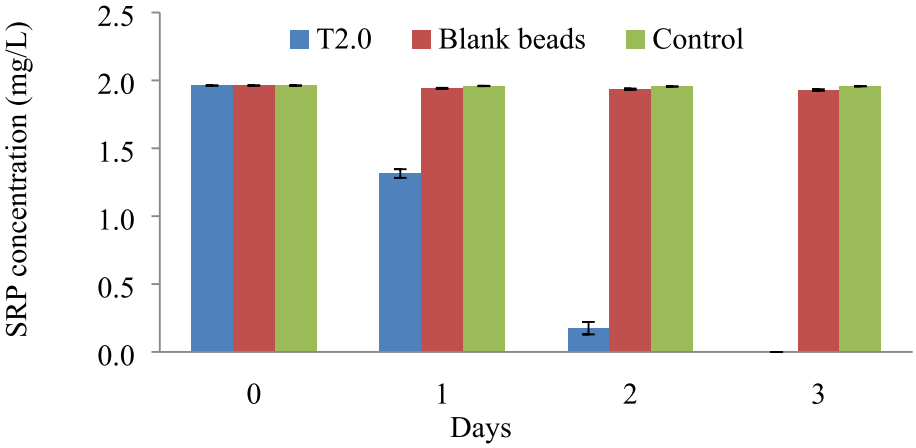

Fig. 6. Concentration (mg/L) of SRP against day using *Tetraselmis* sp. beads (T2.0 beads/mL) treated shrimp pond wastewater.

respectively. In terms of percentage NO_2-N reduction was 12.2, 97.7 and 100.0% for day one, two and three. For the control beads, NO_2-N concentration was 3.1 mg/L for day one, two and three. Percentage of NO_2-N reduction was 0.4%, 1.3% and 1.9% for day one, two and three. In the blank beads, NO_2-N concentration was 3.1 mg/L for day one and 3.0 mg/L for day two and three and the percentage of reduction was 2.3, 3.5 and 4.8% respectively (Fig. 5).

Reduction of NO_2-N was 12.2% which is comparatively low compared to the results obtained from the artificial wastewater which was 99.2% using the same dosage of beads. But reduction percentage increased up to 97.7% on day two. This is because at the same time, ammonia concentration was already reduced up to 98.9%. The nitrite reduction has a correlation with the ammonia reduction as their performance improved after the exhaust of most of the ammonia (Abdel-Hameed, 2007). It is also documented that certain level of ammonium nitrogen concentration is toxic and can inhibit microalgae productivity. Thus, further elucidation of microalgae ammonium nitrogen tolerance is needed, as some previous studies had pointed out that different strains of microalgae require different levels of nitrogen uptake (Feng et al., 2020).

Concentration of SRP significantly ($p > 0.01$) dropped from 2.0 mg/L to 1.3, 0.2 and 0.0 mg/L respectively. Similarly, SRP was reduced by 33.1, 91.1 and 100.0% for day one, two and three. For control beads, SRP concentration was 2.0 mg/L for day one, two and three. Percentage of SRP reduction was 0.2%, 0.4% and 0.3% for day one, two and three. For blank beads, SRP concentration was 1.9 mg/L for day one, two and three and the percentage of SRP reduction was 1.1, 1.4, and 1.7% respectively (Fig. 6).

Compared to the reduction of TAN and NO2-N, SRP reduction is the slowest. The *Tetraselmis* sp. beads took longer time in removal of phosphate-phosphorus from the wastewater. In the treatment with the highest dosage of beads which is 2.5beads/ mL, 100% removal of phosphate-phosphorous from the wastewater was achieved after 2 days. Different microalgae species have different ability on phosphate removal. Immobilized microalgae *Scenedesmus* was stated (Chevalier and de la Noüe, 1985) to be capable in removing 100% of phosphate within 2 h from a typical effluent. The uptake of phosphorous is related to the algal biomass (Tam and Wong, 2000). According to Jiménez-Pérez et al. (2004), the reaction of calcium ions that were presence in the alginate beads with the raised pH values from the wastewater causes the phosphate to be precipitate as calcium phosphate.

4. Conclusion

In this study, firstly improvement of water quality by reduction in nitrogenous waste from aquaculture wastewater using immobilize marine microalgae *Tetraselmis* sp. were monitored and secondly, the optimum dosage of beads required in reduction of nitrogenous waste from aquaculture wastewater was determined. It has demonstrated that immobilized *Tetraselmis* sp. in alginate beads were able to remove the TAN, NO_2-N and SRP from both artificial and aquaculture wastewater. Hence, further studies on the stability of the microalgal beads needs to be investigated and determined how long the beads can last. In addition, more study need to be done on how to apply the immobilized algae beads on big scale like in the hatchery of fish or shrimp farm.

CRediT authorship contribution statement

Helena Khatoon: Conceptualization, Project administration. **Kwan Penz Penz:** Methodology, Data curation. **Sanjoy Banerjee:** Methodology, Data curation. **Mohammad Redwanur Rahman:** Methodology, Data curation. **Tashrif Mahmud Minhaz:** Methodology, Data curation. **Zahidul Islam:** Methodology, Data curation. **Fardous Ara Mukta:** Data curation, Formal analysis. **Zannatul Nayma:** Data curation, Formal analysis. **Razia Sultana:** Data curation, Formal analysis. **Kafia Islam Amira:** Data curation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ARTICLES FOR FACULTY MEMBERS

SHRIMP WASTEWATER BIOREMEDIATION

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Improving microbial bioremediation efficiency of intensive aquacultural wastewater based on bacterial pollutant metabolism kinetics analysis

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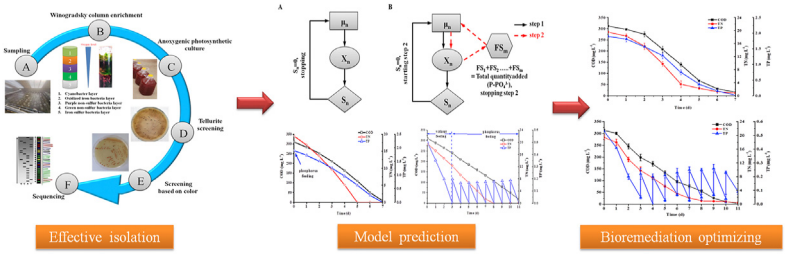
HIGHLIGHTS

- PNSB are effectively and conveniently isolated via the Winogradsky column method.
- Nutrients ratio of aquacultural wastewater determines bioremediation effect.
- Bioremediation is improved via pollutant metabolism kinetics analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

How to effectively bioremediate aquacultural wastewater using microbes is an urgent issue for the application of aquaculture beneficial microorganisms. Purple non-sulfur bacteria (PNSB) are beneficial in preventing related pollution in aquaculture applications. An autochthonous PNSB *Rhodobacter sphaeroides* was employed in this study to explore an effective bioremediation strategy of aquacultural wastewater. The test bacterium showed high performance in the removal of ammonium (97.50% ± 0.78% of 42 mg L^{-1} NH_4^+-N) and phosphate (93.24% ± 0.71% of 50 mg L^{-1} PO_4^{3-}-P) in the synthetic wastewater, which are the two crucial indicators of the aquacultural wastewater bioremediation. The study also unveiled that the imbalanced ratio of nutrients in water was the principal reason for limiting the efficient bioremediation of shrimp-culture wastewater. Therefore, an effective microbial bioremediation strategy was proposed by comprehensively considering bacterial pollutant metabolism kinetics constants such as specific consumption yields of chemical oxygen demand (COD)/phosphorous and nitrogen/phosphorous. Finally, COD, total nitrogen (TN), total phosphorus (TP), and ammonium (NH_4^+-N) in the wastewater were examined, and the results showed that they all decreased to the acceptable values. In conclusion, this study suggested a novel method for improved bioremediation efficiency of aquacultural wastewater, and the findings revealed that this strategy is promising due to its characteristics to be used in various aquaculture wastewater types.

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1. Introduction

Seafood is an essential source of healthy food for human beings (Troell et al., 2015). The availability of natural fishery resources is becoming harder due to the overexploitation of natural resources of oceans; therefore, intensive aquaculture with offshore water has acted as a guarantee for satisfying people with the yearly increased seafood demand (Cao et al., 2007; Farmaki et al., 2014). During the intensive aquaculture, farmers periodically exchange rich organic-load aquaculture wastewater with freshly obtained seawater and directly drained the contaminated wastewater into adjacent coastal waters (Gelfand et al., 2003; Farmaki et al., 2014). The action, therefore, quickly leads to eutrophication of coastal water with adverse consequences for marine ecology and aquaculture, as it carries many excessive nutrients coming from the aquaculture ponds (Farmaki et al., 2014; Edwards, 2015). The frequent occurrence of algal bloom affairs made people aware of reducing pollutant content of aquaculture wastewater to permitted standard was necessary before discharge (Cao et al., 2007; Edwards, 2015).

Compared to terrestrial sewage, the characteristics of pollutants found in aquaculture wastewater are simple, with low concentrations but high ratios of the organic matter (Rijn, 2013; Edwards, 2015). It means that the conventional techniques of sewage treatment do not meet the essential cleaning criteria for the aquaculture wastewater. In recent years, microbial bioremediation of intensive aquaculture wastewater has developed quickly (Ebeling et al., 2006; Prasenjit et al., 2017). Bioremediation is with aids of environmentally friendly microorganisms and has many advantages such as the low cost, in-situ restoration and no secondary pollution, which is considered as a foreground developing direction for aquaculture wastewater management (Prasenjit et al., 2017; Gao et al., 2018). Among the environmentally friendly microorganisms, purple non-sulfur bacteria (PNSB), which belong to the group of anoxygenic photosynthetic bacteria, show high research value and application significance for such approaches (Idi et al., 2015b; Alloul et al., 2019). PNSB have different metabolisms, such as anoxygenic photosynthetic, aerobic, and anaerobic growth, and widely distributed in the natural waters, such as rivers, lakes, and oceans (Idi et al., 2015b). PNSB can metabolize the excrement of aquaculture animals, residual feed, inorganic nitrogen, and hydrogen sulfide for quick reproducing; therefore, they can quickly form the dominant flora in aquaculture water (Seangtumnor et al., 2018). Among the PNSB, many strains of *Rhodobacter* genus have been widely used in the bioremediation field (Takeno et al., 1999; Idi et al., 2015a; Liu et al., 2015). For instance, *R. sphaeroides* has been applied to remove ammonium, phosphorus, heavy metal, nitrobenzene compounds and pesticides in the pollutant environments (Liu et al., 2015; Hay et al., 2016; Peng et al., 2018b). The bacteria from *Rhodobacter* genus are commonly considered as generally regards as safe organisms. Furthermore, some *Rhodobacter* strains show apparent antagonistic effects on aquatic pathogens (Seangtumnor et al., 2018). Hence, bioremediation of intensive aquaculture wastewater by *Rhodobacter* genus holds promise in avoiding the issue of aquaculture wastewater related pollution and aquatic animal diseases. Excavation of the resources of these organisms is highly valuable for the sustainable development of aquaculture. Water samples obtained from nature usually contain low numbers of these microbes. Although culture enrichment in liquid medium is available for the isolation of microorganism, slow-growing strains may be out-competed by their faster growing counterparts (Loss et al., 2013). Unfortunately, strains belong to the *Rhodobacter* genus generally show low growth rates than those shown by other bacteria. Therefore, the traditional enrichment-isolation method is often inefficient for the isolation of *Rhodobacter* bacteria. How to effectively and conveniently isolate this type of microorganisms is worth exploring.

Currently, the method of utilizing beneficial microorganisms for fishery wastewater bioremediation is quite coarse. The usual method is roughly added a certain ratio of a microbial agent into wastewater just based on the volume of water (Prasenjit et al., 2017; Gao et al., 2018), which often makes treating effect unsatisfactory. The component of nutrients in aquaculture wastewater is often not harmonious, which limits microbial metabolism activity that influences bioremediation efficiency (Wang et al., 2015). On the other hand, different sources of aquaculture wastewater often show evident variation in kinds and contents of nutrients (Cao et al., 2007). In this case, the current microbial bioremediation method for aquaculture wastewater is unreasonable. In this study, an autochthonous *R. sphaeroides* isolated from an intensive shrimp-culture factory was used for the wastewater treatment. The pollutant indicators in wastewater, namely COD, TN, TP, and ammonium nitrogen (NH_4^+-N), decreased to quite low concentrations with the treatment of the bacterium via a rational nutrient feeding strategy based on the comprehensive analysis of the features of bacterial pollutant metabolism kinetics. The study provided new guidance for improving microbial bioremediation efficiency of aquacultural wastewater.

2. Materials and methods

2.1. Media

Medium A was used as a seed medium for PNSB culture. The components of medium A were 3 g L^{-1} glucose, 2 g L^{-1} NaCl, 8 g L^{-1} yeast extract, 0.256 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1.3 g L^{-1} KH_2PO_4, 15 μg L^{-1} biotin, 1 mg L^{-1} thiamine hydrochloride, and 1 mg L^{-1} nicotinic acid. The medium used for PNSB isolation was a modified version of the medium A, in which sterilized K_2TeO_3 (150 mg L^{-1}) was added after sterilization at 121 °C for 20 min K_2TeO_3 was sterilized through a 0.22 μm filter. The modified synthetic wastewater medium (MSW) was used as an essential medium to detect bacterial ammonium and phosphorus removal ability. The MSW components were 5.34 g L^{-1} CH_3COONa, 0.912 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.36 g L^{-1} KCl, 1.88 g L^{-1} $(NH_4)_2SO_4$, 0.3 g L^{-1} KH_2PO_4, 15 μg L^{-1} biotin, 1 mg L^{-1} thiamine hydrochloride, 1 mg L^{-1} nicotinic acid, and 10 mL of the trace element solution. The trace element solution was composed of 99.922 g L^{-1} EDTA, 4.4 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 16.361 g L^{-1} $CaCl_2 \cdot 2H_2O$, 10.118 g L^{-1} $MnCl_2 \cdot 4H_2O$, 9.98 L^{-1} g $FeSO_4 \cdot 7H_2O$, 3.073 g L^{-1} $Na_2Mo_7O_{24} \cdot 2H_2O$, 3 g L^{-1} $CuSO_4 \cdot 5H_2O$ and 3.213 g L^{-1} $CoCl_2 \cdot 6H_2O$. The removal experiments for ammonium or phosphorus were carried out via adjusting the concentration of $(NH_4)_2SO_4$ or KH_2PO_4 in the MSW. Medium B (40 g L^{-1} glucose, 6.3 g L^{-1} $MgSO_4$, 4 g L^{-1} corn steep liquor, 3 g L^{-1} sodium glutamate, 3 g L^{-1} $(NH_4)_2SO_4$, 3 g L^{-1} KH_2PO_4, 2 g L^{-1} $CaCO_3$, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} thiamine hydrochloride, 15 μg L^{-1} biotin) was used for fermentation inorder to harvest a large amount of bacteria. The pH of all the above-mentioned media was adjusted to 7.2–7.4.

2.2. Bacteria isolation and identification

In the study, a Winogradsky column method as described by Babcsányi et al. (2017) was used to enrich purple non-sulfur bacteria (PNSB). The equipment was easily set up with a glass tube, which was approximately 30 cm tall and 5 cm in diameter. Mud from the bottom of aquaculture ponds was supplemented with cellulose, Na_2SO_4 and $CaCO_3$, and was then added to the lower one-third portion of the tube. The rest of the tube was filled with water from the ponds, and the tube was capped and placed in an

incubator with supplementary strip lights. In this study, the mud sediment was obtained from the culture ponds with a high yield of shrimp (Dongying Dazhen Biotechnology Co., Ltd., Shandong Province, China). Results of the Winogradsky column experiment showed that PNSB generally occupy the middle water layer of aquaculture ponds and display reddish color because of the synthesis of a large number of intracellular carotenoids (Whitton, 2012; Benoit, 2015). It meant that PNSB was primarily enriched when the middle layer of the column wall became dark red. When the whole column was covered by microbial membranes with different color, a loop of the middle layer of microbial membrane was inoculated into medium A and cultured at 30 °C under light without aeration till the broth color became red. Because most of the isolated *Rhodobacter* strains have a high resistance to tellurite (such as 150 mg L^{-1} of K_2TeO_3) and catalyze K_2TeO_3 to form a black compound (O'Gara et al., 1997); therefore, the black colonies on the solid media plate containing K_2TeO_3 had high probabilities of being the aimed bacterium. Subsequently, 0.1 mL of the red broth was serially diluted (up to 10^{-7}) and plated on the screening medium plate to obtain single colonies with black color. Then, a few big colonies were selected and draw on the medium A solid plate for the incubation. Finally, the isolated bacteria with a fast growth rate were picked out for identification. The protocol for isolating *Rhodobacter* strains was presented in Supplementary Material Fig. S1.

The bacterial genome was extracted using the Wizard genomic DNA extraction kit (Solarbio, Beijing, China). The 16 S rRNA genes were amplified using the 27-F and 1429-R primers. Previously described polymerase chain reaction (PCR) operations were used (Gao et al., 2018). Then, the PCR products were visualized on 0.8% (w/v) agarose gel electrophoresis using Goldview dye. The initial nearest neighbor sequences were made by the online BLAST program in the NCBI database. Sequences were aligned using the Clustal-X program, and the phylogenetic tree was constructed with the MEGA 6.0 program.

2.3. Evaluation of ammonium and phosphorus removal

Excessive ammonium (NH_4^+-N) (>0.2 mg L^{-1}) in a water body can easily result in disease and death of aquatic animals (Cao et al., 2007). Many strains of *Rhodobacter* genus have possessed a superior ability to assimilate NH_4^+-N and have been widely used in enhancing the removal of NH_4^+-N from sewage (Idi et al., 2015a, 2015b). In this regard, we evaluated whether *R. sphaeroides* S1 had a high ability to remove NH_4^+-N. For ammonium removal experiments, different quantities of NH_4Cl were added into MSW with different initial concentrations of NH_4^+-N (42, 137, 245, 465 and 666 mg L^{-1}). Subsequently, one loop of *R. sphaeroides* S1 stock was inoculated into an unbaffled Erlenmeyer flask containing 50 mL of medium A and incubated at 30 °C for 48 h under 150 rpm continuous shaking. When cells reached the exponential phase centrifugation was done at 6000 g for 10 min at room temperature. Pellets were resuspended in physiological saline solution (0.8%, w/v) to prepare inoculants. 1 mL of bacterial solution was added to 100 mL of the MSW, and the incubation was done at 28 °C and 150 rpm lasted for 5 d. During the cultivation period, the cells density (OD_{600}) and the residual NH_4^+-N were measured every day. As a negative control, a group having 137 mg L^{-1} initial NH_4^+-N with no bacterial inoculant was utilized.

Redundant phosphorus (>0.2 mg L^{-1}) in water bodies is another serious issue for aquaculture. A large amount of phosphorus can be assimilated by algae, which causes harmful algae fast proliferation that leads to a vast amount of phycotoxin release and hypoxia of the water body (Rocha et al., 2018). Therefore, the issue makes a serious threat to the survival of aquatic animals. In this study, the phosphorus removal capacity of *R. sphaeroides* S1 was measured. Similar experiments as NH_4^+-N removal ability evaluation were conducted for different concentrations of phosphorus as that of ammonium removal experiment. Phosphorus found in aquaculture water is commonly of two types, organic- and inorganic phosphorus. Compared to organic phosphorus, inorganic phosphorus has a direct and quick effect on the eutrophication, because organic phosphorus is firstly degraded into inorganic phosphate (PO_4^{3-}-P) before assimilated by the organisms. Therefore, phosphate was selected as the phosphorus source in this study. Phosphate was separately added into MSW to form different initial concentrations of PO_4^{3-}-P ranging from 50 to 172 mg L^{-1}. A group having 137 mg L^{-1} of initial PO_4^{3-}-P was set as the blank group. All experiments were done in triplicate unless stated otherwise.

2.4. Kinetics studies for phosphorus removal and bacterial growth

For the kinetics study of the removal of phosphorus, the reaction rate constant k and saturation constant K_m were derived based on the Michaelis–Menten kinetics as seen in Eq. (1).

$$R = \frac{R_{max} S}{K_m + S} \tag{1}$$

where R_{max} is the maximum substrate removal rate, and S is the substrate concentration. The initial substrate concentrations at time t and the corresponding substrate removal rates (R_t) were considered in the batch system. Therefore, Eq. (1) takes the following form:

$$R_t = \frac{R_{max} S_t}{K_m + S_t} \tag{2}$$

where $R_{max} = kX_t$ is the maximum substrate removal rate, and S_t is the corresponding substrate concentration. Eq. (2) can then be written as:

$$R_t = \frac{kX_t S_t}{K_m + S_t} \tag{3}$$

where k is the reaction rate constant, and X_t is the cells density at the time t. Dividing both terms by X_t gives the specific substrate removal rate (q_t)

$$q_t = \frac{R_t}{X_t} = \frac{kS_t}{K_m + S_t} \tag{4}$$

Moreover, Eq. (4) can be linearized to form Eq. (5):

$$\frac{1}{q_t} = \frac{K_m}{k} * \frac{1}{S_t} + \frac{1}{k} \tag{5}$$

Similarly, the kinetics model used for bacterial growth can be written as Eq. (6):

$$\frac{1}{\mu} = \frac{K_m}{\mu_{max}} * \frac{1}{S_t} + \frac{1}{\mu_{max}} \tag{6}$$

2.5. Bioremediation for intensive aquaculture wastewater

Eight samples from different shrimp ponds were treated with *R. sphaeroides* S1 to investigate the bioremediation effect. In such a bioremediation case, the removal rate of COD, TN, TP, NH_4^+-N and PO_4^{3-}-P is mainly considered by people. The experiments were performed in four parallel aquarium systems with 40 cm height, 60 cm length, and 37 cm width, where the total water holding

capacity and the working volume of each aquarium were 90 and 80 L, respectively. 1 mL of *R. sphaeroides* S1 bacterial solution was inoculated into 100 mL medium B and cultivated at 30 °C and 200 rpm for 60 h. When the OD_{600} was >15, 1 mL of the culture was inoculated into 66 L of wastewater samples (>10^5 CFU mL^{-1}) in three aquarium systems, and incubated at approximately 28 °C with aeration for 7 d. Moreover, the fourth aquarium system was set as the blank group (negative control) which filled with the same wastewater sample without bacterial inoculant and ran similarly. Every aquarium was equipped with additional airflow to maintain the dissolved oxygen (DO) levels at approximately 1–2 mg L^{-1}. During the treatment, COD, TN, TP, NH_4^+-N, and PO_4^{3-}-P measurements were done at every 24 h. 1 OD_{600} equaled to approximately 0.5 g of dry cell weight (DCW) per L.

2.6. Improving bioremediation effect of aquaculture wastewater

Phosphorus inadequacy in these wastewater samples was found as a key factor influence bioremediation efficiency. To solve the problem, we tried to add a certain amount of phosphorus into the samples. In this study, batch and fed-batch phosphorus feeding modes were investigated. First, the optimal quantities of phosphorus added into the samples were determined, because adding excessive phosphorus could also produce an imbalance of nutrients. Inorganic phosphorus was preferred as the source for *R. sphaeroides* S1; therefore, phosphate was utilized as the feeding phosphorus source. In this study, sample 1# was used to figure out the optimum feeding strategy of phosphorus. The initial COD of sample 1# was 3.11 ± 0.02 g L^{-1}. According to Eq. (7), the optimum quantity of TP required for removing the corresponded COD was approximately 1.9 mg L^{-1}. On the other hand, the original TN was 19.50 ± 0.97 mg L^{-1} in sample 1#. The optimum TP needed for complete removal of the TN was approximately 1.3 mg L^{-1} according to Eq. (8). *R. sphaeroides* strains were reported to utilize N_2 as the nitrogen source when NH_4^+-N was limited in the surroundings (Imam et al., 2015). In addition, *R. sphaeroides* S1 could grow in the medium without any nitrogen source added and the nitrogenase gene was overexpressed under the conditions (data omitted). The bacterium possibly activates nitrogen fixation to synthesize NH_4^+-N. In summary, the TP required for the removal of COD and TN of sample 1# should not be less than 1.9 mg L^{-1} (PO_4^{3-}-P). Similarly, it was understood that the optimum TP quantity needed for other samples should follow the same rule. Afterwards, the two feeding modes were compared to choose the best operation way.

Before conducting the above experiment, we simultaneously simulated the changes of COD, TN and TP in the two feeding modes. The predictive concentration curves of COD, TN and TP were obtained based on the following procedure. For the prediction procedure (see Supplementary Material Fig. S3), briefly, the value of specific growth rate was calculated according to the model listed in Table 2a; subsequently, the quantities of biomass growth and phosphorus consumption for each day were separately achieved based on the formulas listed in Tables 2b and 2c. Simultaneously, the amount of COD and TN consumed corresponding to that of TP removed could be obtained by Eq. (7) and Eq. (8).

2.7. Further analyses

The periodically obtained samples were used for the determination of cell density and chemical analyses. The cell density was measured by a UV–Vis spectrophotometer (Model no. UV2300II, Techcomp, China) at 600 nm (OD_{600}). The ammonium-nitrogen (NH_4^+-N) concentration was determined using the Nessler's method that works at 420 nm. Before each NH_4^+-N measurement, wastewater samples were pre-treated with the Rochelle salt. TN, PO_4^{3-}-P and TP were measured using the alkaline potassium persulfate digestion method, and the ammonium-molybdate method, respectively (Takeno et al., 1999). COD was determined by the potassium dichromate method (Gao et al., 2018). All of the samples were centrifuged at 10,000 g for 10 min before each chemical analysis, and then the absorbance reads were accomplished accordingly. The concentration of DO was measured by an automated water analyzer instrument (Orion Versa Star, Thermo Scientific, USA).

In this study, all the experiments were performed triplicate. Statistical significance was analyzed using the SAS statistical analysis program (Version 8.01; SAS Institute, Cary, NC, USA). The data

Table 2a
Theoretical specific growth rate versus phosphorus content during wastewater treatment.

Specific growth rate	Formula
μ_0	$\frac{\mu_{max}S_0}{K_m+S_0}$
μ_1	$\frac{\mu_{max}S_1}{K_m+S_1}$
μ_2	$\frac{\mu_{max}S_2}{K_m+S_2}$
μ_3	$\frac{\mu_{max}S_3}{K_m+S_3}$
……	…
μ_n	$\frac{\mu_{max}S_n}{K_m+S_n}$

Note: μ_n was the specific growth rate (d^{-1}) at time "*n*" (day), μ_{max} = 15.63 d^{-1}, K_m = 117.34 mg L^{-1}.

Table 1
Bioremediation result of intensive aquaculture wastewater samples by *R. sphaeroides* S1.

	1#		2#		3#		4#		5#		6#		7#		8#	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
NH_4^+-N (mg L^{-1})	1.90 ± 0.10 b	0.21 ± 0.03 g h	2.36 ± 0.19 a	0.27 ± 0.06 g	1.97 ± 0.12 b	0.11 ± 0.04 ghi	1.13 ± 0.08 e	ND i	1.36 ± 0.15 d	0.08 ± 0.03 hi	1.95 ± 0.12 b	0.15 ± 0.05 ghi	0.80 ± 0.12 f	ND i	1.59 ± 0.05 c	0.12 ± 0.04 ghi
TN (mg L^{-1})	19.50 ± 0.97 b	11.64 ± 0.74 e	12.83 ± 0.71 d	4.92 ± 0.30 hi	13.14 ± 1.37 d	4.07 ± 0.43 i	19.28 ± 0.55 b	1.54 ± 0.16 j	8.27 ± 0.08 g	2.07 ± 0.22 j	11.63 ± 0.47 e	5.85 ± 0.07 h	23.48 ± 0.52 a	16.81 ± 0.47 c	16.60 ± 0.63 c	10.21 ± 0.41 f
PO_4^{3-}-P (mg L^{-1})	0.15 ± 0.01 cde	ND h	0.11 ± 0.05 ef	ND h	0.26 ± 0.06 b	0.07 ± 0.03 f	0.54 ± 0.04 a	0.15 ± 0.02 cde	0.20 ± 0.06 c	ND h	0.14 ± 0.02 de	0.05 ± 0.03 fg	0.19 ± 0.04 cd	ND h	0.20 ± 0.02 cd	0.06 ± 0.03 fg
TP (mg L^{-1})	0.48 ± 0.04 e	ND g	0.58 ± 0.02 cd	0.07 ± 0.03 g	0.89 ± 0.07 b	0.23 ± 0.04 f	1.65 ± 0.07 a	0.45 ± 0.03 e	0.51 ± 0.04 de	0.04 ± 0.02 g	0.46 ± 0.03 e	0.07 ± 0.03 g	0.47 ± 0.04 e	ND g	0.61 ± 0.06 c	0.19 ± 0.04 f
COD (mg L^{-1})	311 ± 20 c	243 ± 24 g	330 ± 15 b	247 ± 20 fg	270 ± 14 d	165 ± 23 j	360 ± 20 a	165 ± 17 j	260 ± 13 e	190 ± 17 i	205 ± 21 h	143 ± 20 k	330 ± 13 b	252 ± 15 f	110 ± 14 l	38 ± 4 m

Note: ND meant nothing detection. Different letters within the same line indicated significant difference ($p < 0.05$).

Table 2b
Theory cells density change model during the wastewater bioremediation.

X	Formula
X_0	X_0
X_1	$X_0+\mu_0X_0$
X_2	$X_0+\mu_0X_0+\mu_1X_1$
X_3	$X_0+\mu_0X_0+\mu_1X_1+\mu_2X_2$
X_4	$X_0+\mu_0X_0+\mu_1X_1+\mu_2X_2+\mu_3X_3$
……	…
X_n	$X_0+\mu_0X_0+\mu_1X_1+\mu_2X_2+ \ldots +\mu_{n-1}X_{n-1}$

Note: X_n represented dry cell density (g DCW L^{-1}) at time "*n*" (day).

Table 2c
Theory phosphorus concentration change model for batch wastewater bioremediation.

Phosphorous concentration	Formula
S_0	S_0
S_1	S_0-kX_1t
S_2	S_0-kX_1t -kX_2t
S_3	S_0-kX_1t -kX_2t-kX_3t
……	…..
S_n	S_0-kX_1t -kX_2t-kX_3t- … …- kX_nt

Note: S_n was the residual phosphorous concentration (mg L^{-1}) at time "*n*" (day); k was the reaction rate constant, k = 1851.85 mg (PO_4^{3-}-P) g^{-1} (DCW) d^{-1} and t was treating time, t = 1 day.

shown in the tables and figures were the mean values of the experiments, and the error bars indicated the standard deviation. ANOVA was done to show significant ($p < 0.05$) results.

3. Results

3.1. Isolation and identification of Rhodobacter strains

For the aim bacteria isolation, single black colonies, which had a high resistance of tellurite, appeared on the screening medium plate after incubating for more than 4 d (Fig. 1A). A few black colonies were picked and could form a single red colony after drawing on the solid medium A plate (incubating for 4 d) (Fig. 1B). After that, several red colonies were selected to identify the bacteria based on their 16 S rRNA gene sequences. The result revealed that most of the microorganisms belonged to PNSB, which was consistent with the study reported (Whitton, 2012; Benoit, 2015). Among the bacteria identified, one strain with a fast growth speed (named as strain S1) was chosen for the following study. The phylogenetic tree revealed a very close phylogenic relationship between the strain S1 and *R. sphaeroides* 2.4.1 (Fig. 1C). Therefore, the strain S1 was finally named as *R. sphaeroides* S1, and its 16 S rRNA gene sequence was available in the GenBank (accession number MN640796).

3.2. Ammonium removal capacity of R. sphaeroides S1

When the initial concentration of NH_4^+-N was between 42 and 666 mg L^{-1}, all experimental groups decreased the NH_4^+-N concentration continuously compared to the control (Fig. 2A). It appeared that the bacterium removed NH_4^+-N with high speed over a wide range of NH_4^+-N concentration at the early phase, and then the removal rate decreased gradually till the end, which was similar to the performance of many other bacteria on removing NH_4^+-N such as *Bacillus subtilis* A1 (Yang et al., 2011). Moreover, the instant NH_4^+-N removing rate was calculated (Fig. 2B). The results showed that it had a similar change trend as that of the residual NH_4^+-N, which directly reflected the change of metabolic status of microbe. After 5 d, the removal percentage of NH_4^+-N vs the initial concentration from 42 to 666 mg L^{-1} was 97.50% ± 0.78%, 95.09% ± 1.39%, 67.73% ± 0.95%, 50.12% ± 1.61% and 37.09% ± 2.53%, respectively. Although the data was relatively low for the groups with a high concentration of initial NH_4^+-N, the removal performance was much more than the groups with low concentration. Simultaneously, we compared the NH_4^+-N removal ability with other bacteria. It was revealed that *R. sphaeroides* S1 displayed an apparent advantage in removing NH_4^+-N (see Supplementary Material Table S1). Since the content of NH_4^+-N in aquaculture water bodies is far less than the minimum value (42 mg L^{-1}) setting of this experiment, therefore, *R. sphaeroides* S1 can afford the NH_4^+-N removal task in aquaculture wastewater.

The growth curves of *R. sphaeroides* S1 was presented in Fig. 2C. On the 1st day of incubation, cells density (OD_{600}) of all experimental groups showed a fast increase compared to the control group. Afterwards, bacterial growth appeared in variation. In the group-1 having 42 mg L^{-1} of initial NH_4^+-N, and group-2 having 137 mg L^{-1} of initial NH_4^+-N, cell density continuously increased despite the increasing rate becaming slow. In the groups with 345 mg L^{-1} (group-3) and 666 mg L^{-1} (group-5) of initial NH_4^+-N, bacterial growth went into a stationary phase. In the group-4 having 465 mg L^{-1} of initial NH_4^+-N), cell density emerged reduction which meant the starting of bacterial aging. Since group-4 showed the fastest NH_4^+-N removing rate and consumed nutrients quickly, early bacterial aging emerged. Afterwards, cell density of all the groups decreased. The change of growth status was found similar to that of *R. sphaeroides* ADZ101 cultured in different concentrations of NH_4^+-N as well (Idi et al., 2015a). The phenomenon might be due to the inadequate nutrients in the surroundings in the late period, which made an apparent inhibition on bacterial growth. For the experimental groups, the maximum cell density was 3.16 ± 0.17 (OD_{600}) obtained in group-4. Although there was an apparent discrepancy in the initial NH_4^+-N, the maximum biomass showed relatively small differences in these groups (maximum OD_{600} ranged from 2.29 ± 0.28 to 3.16 ± 0.17). There might also be other nutrients inadequate in the medium that limited the microbial growth.

3.3. Phosphorus removal capacity of R. sphaeroides S1

The concentration of PO_4^{3-}-P was measured at every 24 h during the experiment and the results were presented in Fig. 3A. On the 1st day, of all experimental groups decreased the phosphate concentration compared to the blank. After that, PO_4^{3-}-P removal rate decreased in the 2nd day, but the content continuously reduced till the end. After 5 d, PO_4^{3-}-P removals of each group were determined as 93.24% ± 0.71%, 89.61% ± 0.74%, 84.62% ± 0.83%, 73.70% ± 0.46% and 68.97% ± 0.33%, respectively. The results suggested that *R. sphaeroides* S1 possessed a high ability to remove PO_4^{3-}-P like other *Rhodobacter* strains (Takeno et al., 1999). Furthermore, the kinetic coefficient of PO_4^{3-}-P removal for *R. sphaeroides* S1 was analyzed. According to Eq. (5), the kinetic coefficients were achieved after plotting 1/q against $1/S_t$ (Fig. 3B), which produced a straight line with slope K_m/k and Y-axis intercept of 1/k. S_t and q were obtained using the curves of PO_4^{3-}-P change (Fig. 3A). The slope was used to calculate the kinetic coefficients (Fig. 3B) as k = 1851.85 mg (PO_4^{3-}-P) g^{-1} (DCW) d^{-1} and K_m = 685.19 mg L^{-1} ($R^2 = 0.9818$), which suggests it had a fast removing speed for PO_4^{3-}-P.

In these experiments, bacterial growth was measured and the result was presented in Fig. 3C. On the 1st day, the cell density (OD_{600}) sharply increased in all the experimental groups compared to that in control. In group-1 (50 mg L^{-1} of initial PO_4^{3-}-P), cell density kept continuously increasing until the end of the experiment. In other groups, cell density showed an apparent reduction

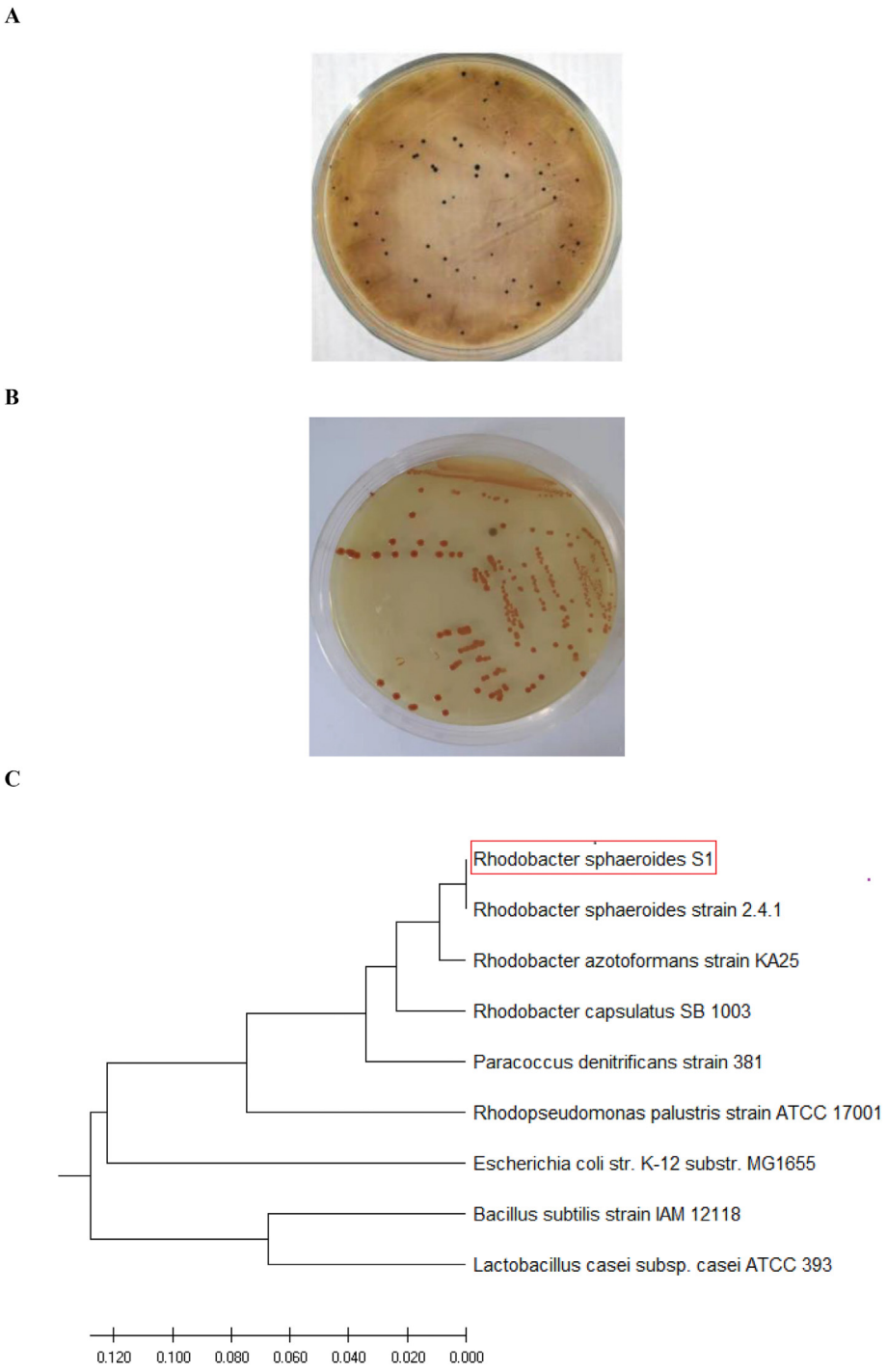

Fig. 1. Bacterial phenotype and homology identification. (A) The phenotype of PNSB grew on the K_2TeO_3 solid plate; (B) the phenotype of PNSB grew on the medium A solid plate; (C) phylogenic tree construction based on 16 S rRNA gene homology analysis between *R. sphaeroides* S1 and other bacteria.

after the 1st day, and the phenomena lasted almost to the end. We supposed that the depression of growth status might be due to bacterial aging after dramatically growing at the early period. Interestingly, we found the maximum biomass showed a big discrepancy among these groups that the maximum value of OD_{600} ranged from 2.73 ± 0.07 to 5.07 ± 0.15. Zhang et al. (2019) reported that phosphorus was a key nutrient deciding the growth of *R. sphaeroides*. The above results suggested that PO_4^{3-}-P in environments was also a key factor that significantly influences the growth of *R. sphaeroides* S1. Furthermore, the effect of organic phosphorus on the growth of *R. sphaeroides* S1 was also evaluated (see Supplementary Material Fig. S2). It was shown that organic phosphorus had a significant impact on the growth of bacteria. The difference was inorganic phosphorus made microbial decrepitude phase appeared much earlier than organic phosphorus. In this case, we can conclude that the abundance of phosphorus in surroundings significantly influences the growth of *R. sphaeroides* S1. To quantitatively describe the relationship between phosphorus

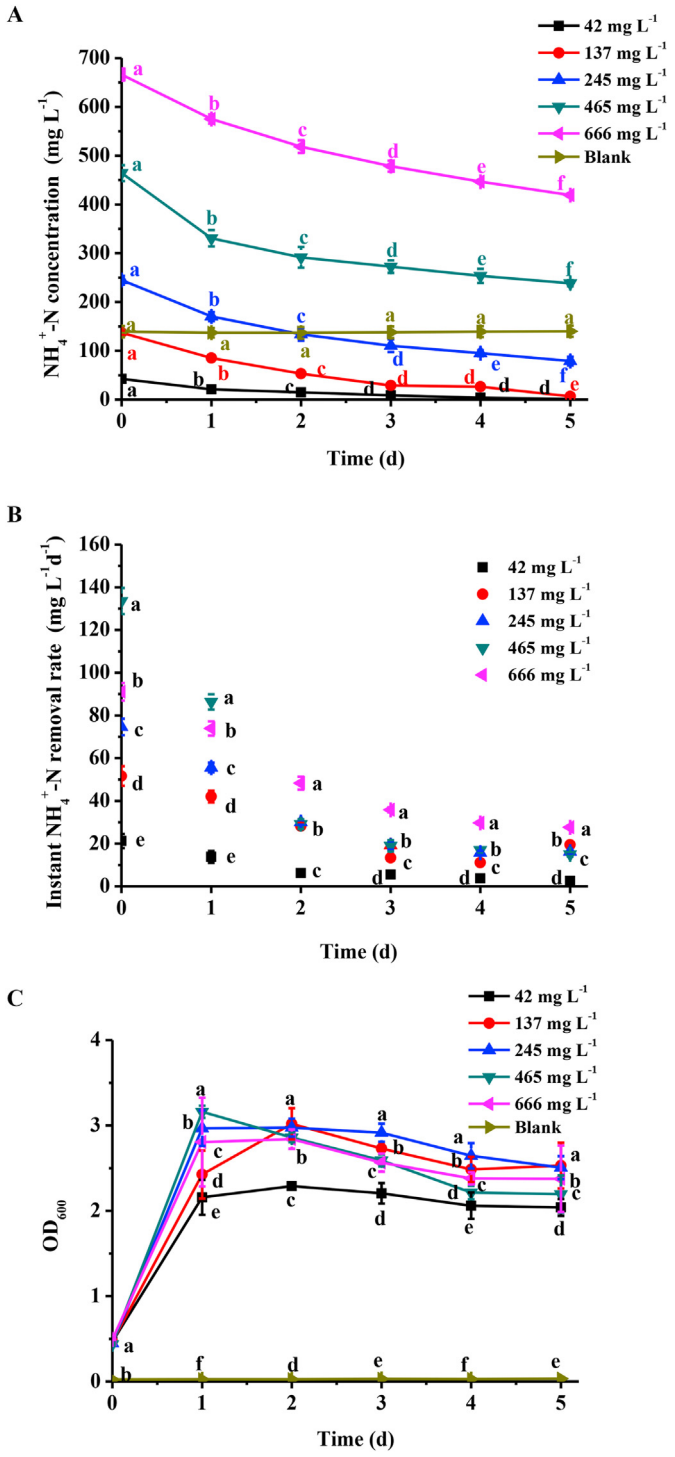

Fig. 2. Analysis of ammonium (NH_4^+-N) removal by *R. sphaeroides* S1. (A) The curves of NH_4^+-N concentration change during the experiment, and different letters within the same group indicated significant difference ($p < 0.05$); (B) the instant NH_4^+-N removing rate of *R. sphaeroides* S1 during the experiment, and different letters within the same day indicated significant difference ($p < 0.05$); (C) bacterial growth of *R. sphaeroides* S1 during the experiment and the meaning of different letters was same as that of (B).

concentration and bacterial growth, a kinetic model for specific growth rate (μ) versus phosphorus concentration was calculated based on the growth curves in Fig. 3D. The kinetic coefficients were also calculated after plotting $1/\mu$ against $1/S_t$ using Eq. (6). The kinetic coefficients as μ_{max} = 15.63 d^{-1}, K_m = 117.34 mg L^{-1} (R^2 = 0.9559).

3.4. Aquacultural wastewater bioremediation by R. sphaeroides S1

As presented in Table 1, the indicators were varied, and the ratio of C/P and N/P was quite high. After 7 d of treatment, the pollutants reduced compared to negative controls. The maximum removal of COD was 65.45% ± 0.07% measured in sample 8#. Nevertheless, the residual COD, as well as the TN, were high and did not meet the discharge standards. At the same time, the phosphorus content in all the samples regardless of TP or inorganic phosphorus declined and was undetectable in some samples. Wang et al. (2015) reported the imbalanced nutrients in aquaculture water, limiting the microbial bioremediation, and the zero-water exchange of aquaculture through optimizing the ratio of nutrients was observed. Considering the importance of phosphorus on the growth of *R. sphaeroides* S1, we surmised insufficient phosphorus in water body was a key factor limited the bioremediation efficiency. The equations of specific consumption yields of COD/phosphorous and TN/phosphorous were achieved to verify the hypothesis. Generally, both the inorganic- and organic phosphorus in aquaculture wastewater can be utilized by *R. sphaeroides* S1; thus, the TP was used in the equation to describe the consumption relationship between COD and phosphorus. Also, TP was used in the equation of specific consumption yield of TN/phosphorus. The linear fitting results were presented in Fig. 4 and the equations were described as follows:

$$Y = 164.01X - 1.74 \quad (7)$$

where X was the reduced quantity of TP (mg L^{-1}) and Y was the corresponding yield of COD (mg L^{-1}) consumed, R^2 = 0.99 (Fig. 4A),

and

$$Y = 14.65X + 0.17 \quad (8)$$

where X was the reduced quantity of TP (mg L^{-1}) and Y was the corresponding yield of TN (mg L^{-1}) consumed, R^2 = 0.98 (Fig. 4B).

According to Eq. (7) and Eq. (8), the contents of the original phosphorus were far less than that need for thoroughly removing COD and TN in the samples. For instance, the optimal quantity of TP needed for completely removing the COD was approximately 1.91 mg L^{-1} for sample 1# based on Eq. (7), while the actual content of TP was only 0.48 ± 0.04 mg L^{-1}. Conclusively, the imbalance of COD/TP and TN/TP in the samples directly influenced the efficiency of microbial bioremediation.

3.5. Improving the microbial bioremediation in aquacultural wastewater

For the batch mode, the concentration of initial phosphorus was adjusted to 1.9 mg L^{-1} before treatment. The predictive concentration curves of COD, TN and TP were obtained based on the formulas listed in Tables 2a–c and the curves presented in Fig. 5A. As seen in Fig. 5A, all the curves showed a similar trend for the removal of COD, TN and TP. Based on the prediction, TP and COD would be disappeared within 7 d, and TN would be exhausted at the end of the 5th day of incubation. In this sense, the time for bioremediation should be at least 7 d. Additionally, the actual change of COD, TN and TP were also shown in Fig. 5B, and the curves presented that COD, TN and TP showed a trend that basically fitted with the predicted results within the first 5 d. At the end of 5th day, the concentrations of COD, TN and TP fell to 0.53 ± 0.08 g L^{-1}, 2.32 ± 0.27 mg L^{-1} and 0.37 ± 0.12 mg L^{-1}, respectively. The removal ratio of COD, TN and TP was 82.96% ± 0.19%, 88.09% ± 0.24% and 80.53% ± 0.07%, respectively. As

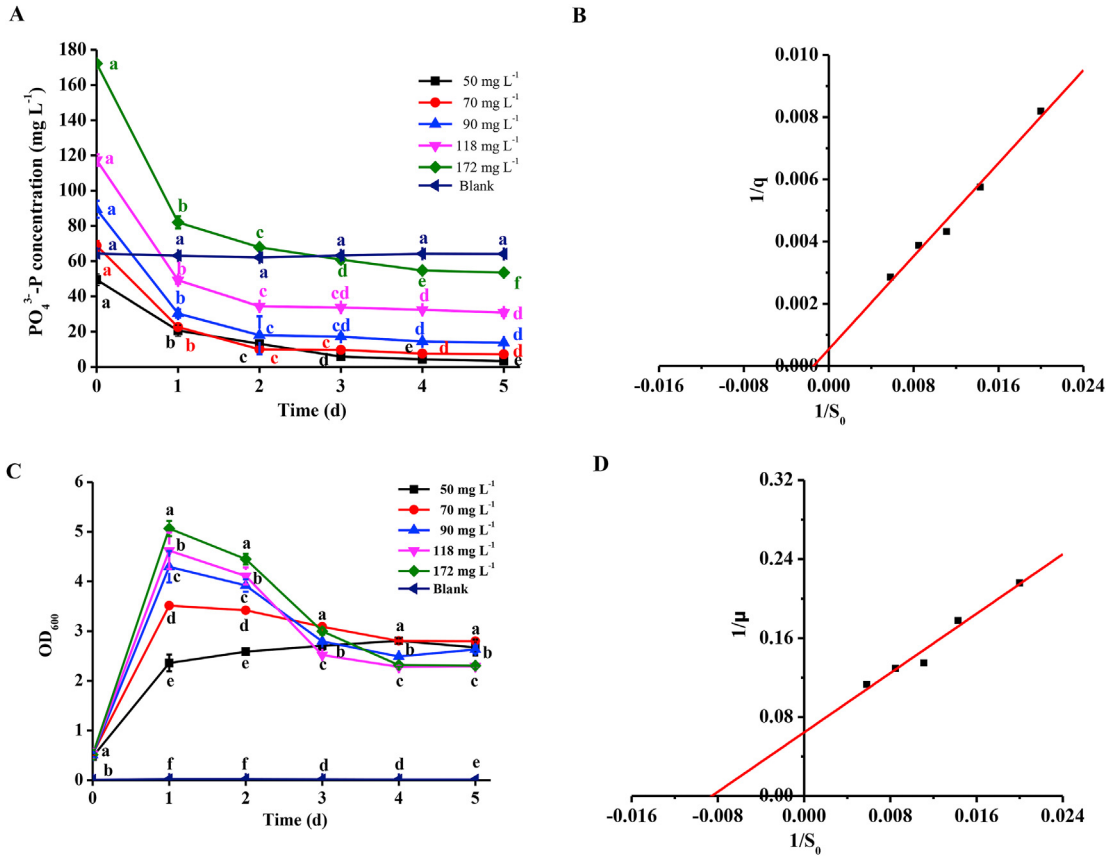

Fig. 3. Analysis of inorganic phosphate (PO_4^{3-}-P) removal by *R. sphaeroides* S1. (A) The change curves of PO_4^{3-}-P concentration during the experiment, and different letters within the same group indicated significant difference ($p < 0.05$); (B) PO_4^{3-}-P removing kinetic assay for *R. sphaeroides* S1, S_0 is the initial substrate concentration (mg L^{-1}) and q is the specific substrate removal rate versus the corresponding S_0 (mg d^{-1} g^{-1} DCW); (C) bacterial growth status of *R. sphaeroides* S1 during the experiment, and different letters within the same day indicated significant difference ($p < 0.05$); (D) growth kinetic assay for *R. sphaeroides* S1, the meaning of S_0 is the same as that of (B) and μ is the specific growth rate versus the corresponding S_0 (d^{-1}).

the treatment progressed, it was revealed that the content of COD, TN and TP continuously decreased although the removal rate became slow. It could be supposed that the phenomena were mainly due to poor nutrient conditions at the later phase. After 7 d treatment, the final concentrations of COD, TN and TP reduced to 0.23 ± 0.05 g L^{-1}, 1.85 ± 0.05 mg L^{-1} and 0.02 ± 0.01 mg L^{-1}, which were far less than the treatment without optimization. The removal percentage of COD, TN and TP was 92.50% ± 0.06%, 90.51% ± 0.17% and 98.97% ± 0.01%, respectively.

For the fed-batch procedure (see Supplementary Material Fig. S3), initially, the treatment without the addition of phosphorus was performed and the change of TP was presumed based on the formulas listed in Table 2d (without phosphorous feeding phase), which was same to the procedure of batch mode. It was predicted that the TP would be exhausted within 3 d. Hence, it was decided that additional phosphorus feeding should begin at the end of the 3rd day. Besides, theoretical cell density at the end of the 3rd day was determined using the formulas listed in Tables 2a and 2b The theoretical consumption yield of TP versus the biomass was also obtained according to the reaction rate constant k = 1851.85 mg (PO_4^{3-}-P) g^{-1} (DCW) d^{-1} (Table 2d, phosphorous feeding phase), which was also the feeding quantity at the end of the 3rd day. In summary, the quantity of phosphorous to be added for the following procedures was found through the same method. As a result, almost the same amount of phosphorus was periodically added to sample 1# during the bioremediation experiments. Subsequently, the residual COD and TN were figured according to Eq. (7) and Eq. (8). The predicted change of COD, TN and TP in the fed-batch mode was depicted in Fig. 5C. The content of COD and TN almost displayed a linear change. For TN and COD, 8 and 10 d, respectively, were needed for the complete bioremediation and therefore the results suggested the natural process should be at least 10 d. By following the predicted results, microbial bioremediation was carried out for 10 d, and the results were presented in Fig. 5D. The COD, TN and TP showed similar changes as predicted. After 10 d of incubation, the residual COD, TN and TP were 0.53 ± 0.02 g L^{-1}, 1.97 ± 0.19 mg L^{-1} and 0.12 ± 0.03 mg L^{-1}, respectively. The removal ratios of COD, TN and TP were 82.50% ± 0.26%, 89.72% ± 0.03% and 93.67% ± 0.07%, respectively.

After comparing the two incubation modes, it was concluded that the batch mode revealed better bioremediation efficiencies than the fed-batch mode within the same time. Furthermore, the batch operation was simple and easy. Hence, the batch mode was selected for the bioremediation of other samples. After effective treatment, COD, TN and TP in these samples significantly decreased. The removal ratio of COD in these samples was between 79% and 95%. For TN removal, the removal ratios were found to be between 86% and 92%. The detailed results were shown in Table S2. Simultaneously, the residual NH_4^+-N in all samples carried the amounts that fitted well with the discharge standards. It means that *R. sphaeroides* S1 showed promising applicability for the bioremediation in aquaculture wastewater and the bioremediation strategy was fitting for the wastewater treatment.

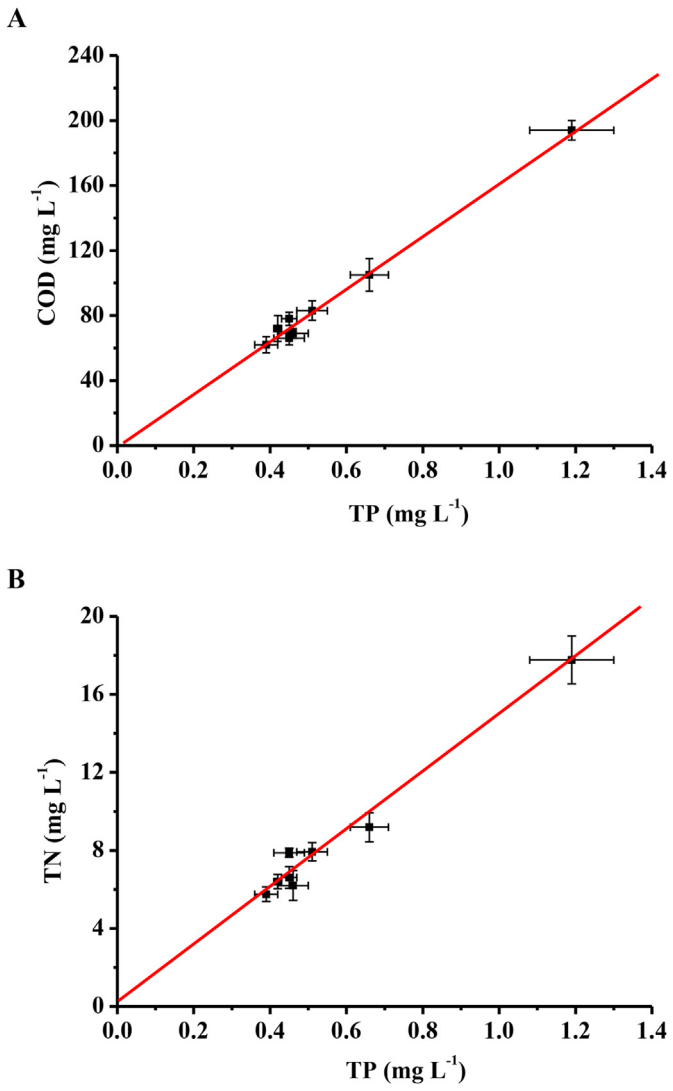

Fig. 4. The specific consumption relationship between phosphorus and other pollutants. (A) The specific consumption equation for COD verses TP ($R^2 = 0.99$) and (B) the specific consumption equation for TN versus TP ($R^2 = 0.98$).

4. Discussion

4.1. Effective isolation of Rhodobacter strains

As previously described, *Rhodobacter* strains are beneficial and can be used in the sustainable development of aquaculture. In this study, purple non-sulfur bacteria (PNSB) including *Rhodobacter* were easily enriched using a Winogradsky column. The device provided a suitable enrichment environment for the bacteria, which were inefficiently enriched via traditional methods (Loss et al., 2013; Benoit, 2015). After a few weeks of incubation, many stratified colored bands were observed along the decreasing O_2 gradients from the top to the bottom of the column. Cyanobacteria were in the top green aqueous layer followed by microaerophilic photoautotrophs with reddish-purple layer of PNSB and an olive-green colored layer of greenish non-sulfur bacteria, and in the bottom, there was a dark-purple and greenish-brown anoxygenic layer of iron sulfur bacteria respectively (Whitton, 2012). The principle of the Winogradsky column enrichment used is to simulate the niche diversity of different anoxygenic photosynthetic bacteria in nature. A study by Babcsányi et al. (2017) investigated the biogeochemical processes and microbial communities changed of heavy metal polluted wetland sediments with time and depth based on the Winogradsky column enrichment method. Furthermore, researchers have also studied the relationship between microbes and diseases with the help of this method. A study by Quinn et al. (2015) using a modified Winogradsky method revealed the physiology of polymicrobial communities in the lungs affected by cystic fibrosis, these microorganisms contributed to pulmonary exacerbations and lung-function decline. Overall, the Winogradsky column method is excellent for studying issues related to microbial niches, such as the enrichment of different niche microorganisms.

Rhodobacter like other PNSB prefer to inhabit the middle layer of aquaculture ponds or waste lagoons and undergo photosynthesis using organic compounds (Whitton, 2012). Researchers proposed that such characteristics were beneficial to avoid singlet oxygen damage to cells, although they grow faster under aerobic compared with the microaerobic conditions (Makhneva et al., 2020). According to the microbial niche feature of *Rhodobacter*, we chose the red pellicle in the middle of the device for bacterial separation. Considering the slow growth rate of PNSB, we used a high concentration of K_2TeO_3 to further distinguish the study bacteria from others. *Rhodobacter* strains were the most isolated bacteria. To sum up, the enrichment-isolation method used in this work showed a higher efficiency in *Rhodobacter* isolation.

4.2. Phosphorus significantly influenced the growth of R. sphaeroides S1

For the bioremediation aquaculture wastewater, microbial phosphorus removal capacity is an important factor of major concern. *R. sphaeroides* S1 like other *Rhodobacter* strains identified possessed a strong ability to assimilate phosphorus (Fig. 3A). Studies have verified that these *Rhodobacter* strains accumulate a large number of intracellular polyphosphates, thus showing their strong capacity to remove phosphorus from water bodies (Takeno et al., 1999). For instance, *R. sphaeroides* IL106 can remove more than 97% of phosphorus in the acidogenic fermentation of wastewater of oyster pond mud (Takeno et al., 1999). Interestingly, compared with the ammonium of nitrogen source, phosphate was a more significant promoter of the growth of *R. sphaeroides* S1 (Figs. 2C and 3C), though the bacterium also had a high capacity to assimilate ammonium (Fig. 2A). Phosphorus starvation leading to reduced growth of bacteria has been widely reported (Geske et al., 2013), but the above phenomenon is rarely reported. Phosphorus is an essential element for organisms and it influences cell physiology, nucleotide and phospholipid biosynthesis, and energy metabolism (Geske et al., 2013; Yadav et al., 2016). It was reported that under stress associated with phosphate limited conditions, *R. sphaeroides* altered its membrane composition such that membrane phospholipids were partially replaced by lipids, which lacked phosphorus (Zhang et al., 2019). Additionally, it was discovered that oxidative phosphorylation and cytochrome C biosynthesis were significantly affected by phosphate limitation (Zhang et al., 2019), these two proteins are involved in energy synthesis (Peng et al., 2018a). Therefore, an explanation for the significant promotion of bacterial growth by phosphorus compared with ammonium could involve the enhancement of energy production or increasing substrate acquisition or both through the phospholipids rich membrane from the medium under adequate phosphorus conditions. Moreover, phosphorus is not only necessary for their growth, but also important for survival when competing with other microorganisms in nature, such as fungus. A study by Nottingham et al. (2018) reported that the bacteria out-competed fungi for growth where phosphorus content was abundant. Considering that photosynthetic bacteria have a slow growth rate compared with

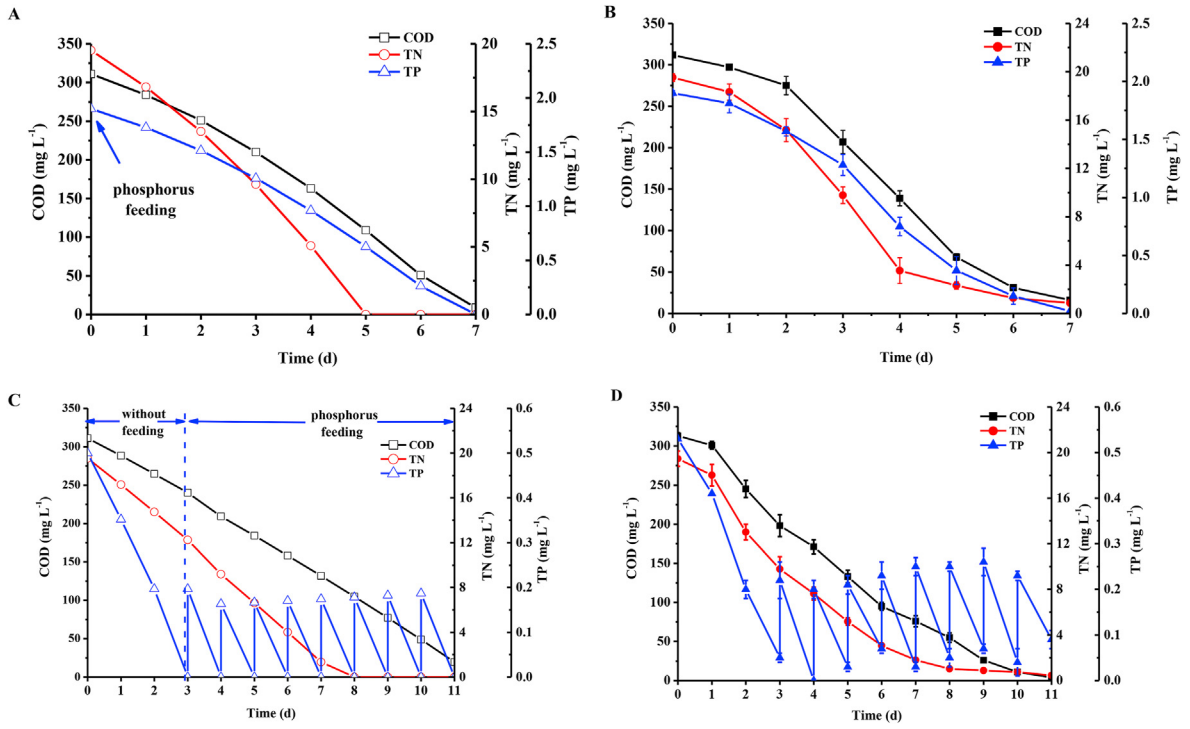

Fig. 5. Optimizing microbial bioremediation of aquaculture wastewater (sample 1#). (A) The predicted treating process based on batch phosphorus feeding strategy; (B) the actual bioremediation process based on the corresponded batch phosphorus supplying strategy; (C) the predicted treating process based on fed-batch phosphorus supplying strategy; (D) the factual bioremediation process with the corresponded fed-batch phosphorus feeding strategy.

Table 2d
Theory phosphorus concentration change model for fed-batch wastewater bioremediation.

Phosphorous concentration	Formula
Without phosphorous feeding	
S_0	S_0
S_1	S_0-kX_1t
S_2	S_0-kX_1t -kX_2t
S_3	S_0-kX_1t -kX_2t-kX_3t
……	…..
S_n	0
Phosphorous feeding	
FS_1	kX_nt
FS_2	$kX_{n+1}t$
……	…
FS_m	$kX_{n+m-1}t$

Note: FS_m was the phosphorous concentration (mg L^{-1}) at time "*m*" (day) during phosphorous feeding phase.

non-photosynthetic bacteria (Idi et al., 2015b), elucidating the mechanisms of phosphorus promoting bacterial growth is required especially for bioremediation.

4.3. Nutrients ratio of aquacultural wastewater determines bioremediation effect

In this study, the COD/phosphorus ratio of aquacultural wastewater samples was demonstrated to significantly influence bioremediation using *R. sphaeroides* S1. In sewage treatment plants, the ratio of COD/nitrogen is a key factor that influences the efficiency of treatment, and less attention is paid to the influence of phosphorus on sewage treatment (Yuan et al., 2017). From the experiences of sewage treatment plants, the ratios of COD/nitrogen in wastewater samples obtained from shrimp culture are appropriate, majorly between 15:1 and 20:1 (mg), but the practical efficiency of bioremediation is unideal. Through the analysis of pollutant metabolism of *R. sphaeroides* S1, combined with the ratios of major pollutant indicators in these wastewater samples, we proposed that inadequate phosphorus in water bodies may had inhibited its bioremediation efficiency. After adjusting the ratios of COD/phosphorus to a value of approximately 160:1 (mg) based on bacterial pollutant metabolism kinetics analysis, the bioremediation efficiencies were evidently improved (Fig. 5 and Table S2). This ratio was similar to the theoretically calculated optimal value (140:3) for bacterial cell growth, and is frequently recommended for biostimulation in soil bioremediation (Leys et al., 2005). In microbial ecology, the influence of phosphorus has been a major focus for researchers (Tang et al., 2016). Especially, for aquaculture ecosystems, the ratios of COD/nitrogen/phosphorus have immense importance in mineralization and aquatic productivity, which influences microbial activity of water, thereby, affecting the rate of nutrient cycle of from organic manures (Ghosh and Chattopadhyay, 2005). Therefore, the improvement of bioremediation efficiency obtained after phosphorus contents optimization may be related to the above reason, and the mechanisms need to be further elucidated in future studies.

Fed-batch operation strategy, commonly used in bacterial wastewater treatment, has high efficiency on sewage treatment (Beyde and Karapinar, 2018). To further optimize the bioremediation efficiency, a fed-batch operation (adding phosphate) was explored. The results revealed that the bioremediation efficiency of this method was lower than the batch method. The concentration of nutrients in sewage is much higher than that of aquacultural wastewater. In sewage treatment, fed-batch operation alleviates the inhibition of high-concentration nutrients for the growth of bacteria, and continuously provides new sewage to maintain the concentration of nutrients in an appropriate content, which is suitable for bacterial growth and metabolism (Beyde and Karapinar,

2018). However, the concentration of phosphorus nutrient was maintained at a low level required for the metabolic needs of bacterium during the fed-batch process. This may have caused the lower efficiency of the fed-batch method than the batch method.

5. Conclusion

Microbial bioremediation in intensive aquacultural wastewater by PNSB promises the avoidance of issue related to aquacultural pollution. A PNSB (*R. sphaeroides* S1) was effectively and conveniently isolated from a shrimp-culture pond using a modified Winogradsky column method. The autochthonous bacterium showed a high capacity to remove NH_4^+-N and PO_4^{3-}-P, that is 97.50% ± 0.78% of NH_4^+-N (42 mg L^{-1}) and 93.24% ± 0.71% of PO_4^{3-}-P (50 mg L^{-1}) in synthetic wastewater, which were consumed within 5 d. For shrimp-culture wastewater bioremediation, a quantitative phosphorous feeding strategy to adjust the ratio of COD/TN/TP to 160:15:1 (mg) was proposed after comprehensively considering the pollutant metabolism kinetics analysis of *R. sphaeroides* S1. Finally, using this strategy, the contents of COD, TN, TP and NH_4^+-N in the wastewater decreased to the emission standard. Conclusively, this study provides new guidance for aquacultural wastewater treatment with a more efficient microorganism *R. sphaeroides* S1. For bioremediation, we therefore plan that in our further study, bioremediation of aquacultural wastewater will be conducted at a pilot-scale.

Credit author statement

Zhengliang Qi designed the research; Haoyu Sun, Zhengliang Qi and Die Dong performed the experiments; Zhengliang Qi and Die Dong analyzed data; Zhengliang Qi, Die Dong and Xinli Liu wrote and revised the paper. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.129151.

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Increasing CO_2 concentration impact upon nutrient absorption and removal efficiency of supra intensive shrimp pond wastewater by marine microalgae *Tetraselmis chui*

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ABSTRACT

The objective of this study was to investigate the effect of increasing CO_2 concentration on the growth and the capability of *Tetraselmis chui*. in removal of nitrate, ammonium and phosphate from shrimp pond wastewater (SPWW). The factorial experimental design was used with the treatment of SPWW percentage in culture medium, namely: 100% SPWW, 75% SPWW + 25% Sea Water (SW) and 75% SW + 25% SPWW coupled with three CO_2 concentration treatments: 390 ppm, 550 ppm and 1000 ppm using CO_2 system. Growth of *T. chui*. for lengh of cultivation period tended to be higher at treatments of 390 ppm CO_2 and 100% SPWW, however there was a declining growth over period of cultivation for both treatments. The growth rate *of T. chui* was higher for all percentage of SPWW treatments in culture medium at 390 ppm CO_2 concentration compared to other percentage of SPWW treatments and CO_2 concentration treatments. There was a decreasing of growth rate with increasing CO_2 concentration at 100% SPWW and 75% SPWW + 25% SW in culture medium. Nitrogen removal efficiency and removal rate by *T. chui*. were strongly affected by CO_2 concentration. However, there was no significant effect of increasing CO_2 concentration to removal efficiency and rate of PO_4 by *T. chui*.

KEYWORDS

microalgae Tetraselmis chui and CO_2 concentration; nutrient removal; shrimp pond wastewater; supra intensive aquaculture technique

Introduction

Nowadays, there is a serious environmental problem faced by most countries around the globe in the form of increasing concentration of greenhouse gases, CO_2 in particular. Anthropogenic activities contribute more than 7% (v/v) of global CO_2 emissions from burning coal at power plants (Ramanathan 1988), and De Morais *et al.* (2007) found that around 10–15% (v/v) of exhaust gases from power plants is carbon dioxide. Photosynthetic microorganisms can convert CO_2 from sources into biomass (De Morais and Costa 2007a) and microorganisms are most effective in the absorption of CO_2 from the atmosphere. Comparing microalgae and higher plants, there are several advantages of microalgae in terms of a high level of photosynthesis efficiency, higher biomass production and faster growth (Tang *et al.* 2011). CO_2 fixation by algae through photosynthesis is estimated to be a flexible technology with energy storage and more environmentally friendly (Dote *et al.* 1994; Minowa *et al.* 1995; Miao and Wu 2006).

Some previous research results show several species of microalgae can reduce CO_2 concentrations, such as *Chlorococcum littorale* (Skjånes *et al.* 2007), *Chlorella kessleri, Scenedesmus obliquus* (Ota *et al.* 2009), *Chlorella vulgaris* (de Morais and Costa 2007a) *Dunaliella tertiolecta, Botryococcus braunii, Spirulina platensis* (De Morais *et al.* 2007), *Chlorella* sp. (Sydney *et al.* 2010), *Nannochloropsis oculate* (Chiu *et al.* 2008). Chiu *et al.* (2008, 2009) reported the growth of *Chlorella* sp. and *N. oculata* was inhibited when CO_2 concentrations are above 5%. De Morais and Costa (2007a, 2007b) and De Morais *et al.* (2007) also found that four microalgae *C. kessleri, S. obliquus, Spirulina* sp. and *C. vulgaris* at their best growth when CO_2 concentrations are below 6%, and Sydney *et al.* (2010) calculated carbon dioxide assimilation for four species of microalgae, *D. tertiolecta, B. braunii, S. platensis* and *C. vulgaris* at 5% CO_2.

Intensive aquaculture at the coastal area has a potential for increasing seafood biomass production, such as shrimp and fishes. However, this activity has led to an environmental problem due to increasing organic waste. Gondwe *et al* (2012) and Vezzulli *et al.* (2008) indicated that aquaculture is the major contributor to the increasing levels of organic waste and toxic compounds. Without proper treatment, aquaculture waste would potentially cause harmful algal bloom (Hegaret 2007). Wastewater effluent from aquaculture industry contains nitrogenous compounds (ammonia, nitrite and nitrate), phosphorus and dissolved organic carbon which may lead to environmental deterioration at high concentration (Ali *et al.* 2005). Ammonia (NH_3) is the product of fish respiration and decomposition of excess organic matter (Lananan *et al.* 2014). Lananan *et al* (2014) found nitrogenous compounds present in excess amount are responsible

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for generating eutrophication which disrupt the aquatic ecosystem balance and could leads to massive mortality of aquatic fauna.

Besides having the ability to absorb CO_2, microalgae also has the ability to remove nitrogen and phosphorus from wastewater efficiently, so that microalgae can also be used as organisms for the bioremediation of tertiary treatments of liquid waste. Biological treatment using algae has several advantages, such as high efficiency, minimum cost, easy and simple operation and only require small concentration (Sabeti *et al.* 2019). Aslan and Kapdan (Chiu *et al.* 2009) found that *C. vulgaris* very effective in removing nutrient concentrations as NH_4-N0 < 22 mg L^{-1} and PO_4-P0 < 7.7 mg L^{-1}. The use of a wide range of microalgae such as *Chlorella, Scenedesmus, Phormidium, Botryococcus, Chlamydomonas* and *Spirulina* for treating domestic wastewater has been reported that those microalgae showed an effective nutrient removal (Olguin 2003; Chinnasamy *et al.* 2010; Kong *et al.* 2010; Wang *et al.* 2010). Microalgae require high amounts of N and P for proteins (40–60% of dry weight) so they could potentially be a nutrient removal from organic waste water (Olguin 2003; Chinnasamy *et al.* 2010; Kong *et al.* 2010; Wang *et al.* 2010).

Several studies have been conducted on the use single species of microalgae (Silva-Benavides and Torzillo 2012) and cyanobacteria (Tam and Wong 1996; Voltolina *et al.* 2005; de-Bashan *et al.* 2008) for waste water applications, and focused more on the function of microalgae as CO_2 absorbents and nutrients removal separately. However, research on combining the function of microalgae as CO_2 absorbent and absorbent of organic waste are still scarce. The aim of this study was to investigate the effect of increasing CO_2 concentration on the growth and the capability of *Tetraselmis chui* in nitrate, ammonium and phosphate removal from shrimp pond wastewater. While the effect of increasing CO_2 concentration on algal growth and nutrient removal, the cultured media enrichment with CO_2 system was examined, and pH was controlled daily. Different percentages of shrimp pond waste waters were used as culture media to examine nutrient removal efficiency.

Material and methods

Microorganisms and culture medium

The microalgae *T. chui* were obtained from Culture Collection of Algae, Research Center for Coastal Aquaculture and Fisheries Extension, Maros, South Sulawesi, Indonesia. Stock solution of Conway for microalgae stock culture media was made with material composition (per liter): 15 mg $NaNO_3$, 12 mg $MgCl_2 \cdot 6H_2O$, 18 mg $CaCl_2 \cdot 2H_2O$, 15 mg $MgSO_4 \cdot 7H_2O$, 1.6 mg KH_2PO_4, 0.08 mg FeCl3 $\cdot 6H_2O$, 18 mg $CaCl_2 \cdot 2H_2O$, 15 mg $MgSO_4 \cdot 7H_2O$, 1.6 mg KH_2PO_4, 0.08 mg $FeCl_3 \cdot 6H_2O$, 0.1 mg $Na_2EDTA \cdot 2H_2O$, 0.185 mg H_3BO_3, 0.415 mg $MnCl_2 \cdot 4H_2O$, 3 µg $ZnCl_2$, 1.5 µg $CaCl_2 \cdot 6H_2O$, 0.01 µg $CuCl_2 \cdot 2H_2O$, 7 µg $Na_2MoO_4 \cdot 2H_2O$ and 50 mg NaCO. Microalgae stock was incubated at 2000-mL flasks at room temperature, under continuous fluorescence lighting. Stirring was done by an aerator. Initial concentration of *T. chui* inoculated into aquarium was 9976 ind/ml.

Characteristic of wastewater

Wastewaters were collected from supra intensive shrimp pond of Research Center for Brackishwater Aquaculture and Fisheries Extension, Maros, South Sulawesi. Waste water from the pond were then filtered with 200 µm mesh-size to remove large particles and indigenous bacterium. Ammonia nitrogen (NH_3–N), nitrate nitrogen (NO_3–N) and total phosphorus (TP) were determined for all samples using Spectrophotometer.

Experimental setup and cultivation condition

Experiments were performed in 4-L glass aquarium with working volumes of 3.6 L. CO_2 system were used to manipulate CO_2 concentration in the culture media. CO_2 concentration treatment consisted of three treatments, namely 390 ppm as a control treatment (concentration of CO_2 in the water), 550 ppm pCO_2 as a prediction CO_2 concentration at 2050 based and 1000 ppm pCO_2 as a prediction CO_2 concentration at 2100 (based on IPCC CO_2 prediction). The CO_2 system is a tool that was assembled from a number of components, including supporting equipment such as a CO_2 supply, an O_2 compressor and a mass flow controller (MFC). This system functioned as a regulator of carbon dioxide (CO_2) concentration in the water. Carbon dioxide from CO_2 gas cylinders and oxygen from the O_2 compressors both enter the mass flow controller. The MFC regulates the flow rate and has CO_2 meters with digital displays so that the rate of carbon dioxide concentration flowing into the aquaria can be measured and regulated. In this research, the mass flow controller was set for two concentrations: (i) 550 ppm pCO_2 (CO_2 gas flow rate range $7.95 - 8$ mL min^{-1} and O_2 range 2:49 to 2:55 L min^{-1}) and (ii) 1000 ppm pCO_2 (CO_2 gas flow rate range $9.95 - 10$ mL min^{-1} and O_2 range 1:10 L min^{-1}). Waste water of supra intensive shrimp pond were used as culture medium. Filtered seawater (SW) was used to dilute SPWW accordingly to achieve percentages of SPWW used in this work. Experimental design used is completely randomized design with three replicates for each treatment. There were two factors of treatment, namely: CO_2 concentrations (390 ppm, 550 ppm and 1000 ppm) and supra intensive pond waste water (SPWW) concentrations were 100% SPWW, 75% SPWW+ 25% SW and 25% SPWW + 75% SW). The experiment was conducted for 21 days. The schematic of experiment setup was presented in Figure 1.

Growth monitoring and kinetic growth parameters

Cell density of *T. chui* was monitored by calculating microalgae cell numbers every 3 days using a Sedgwick Rafter Counting Cell. The growth rate was calculated using the formula (1):

$$\mu = (\ln N_t - \ln N_o)/t \quad (1)$$

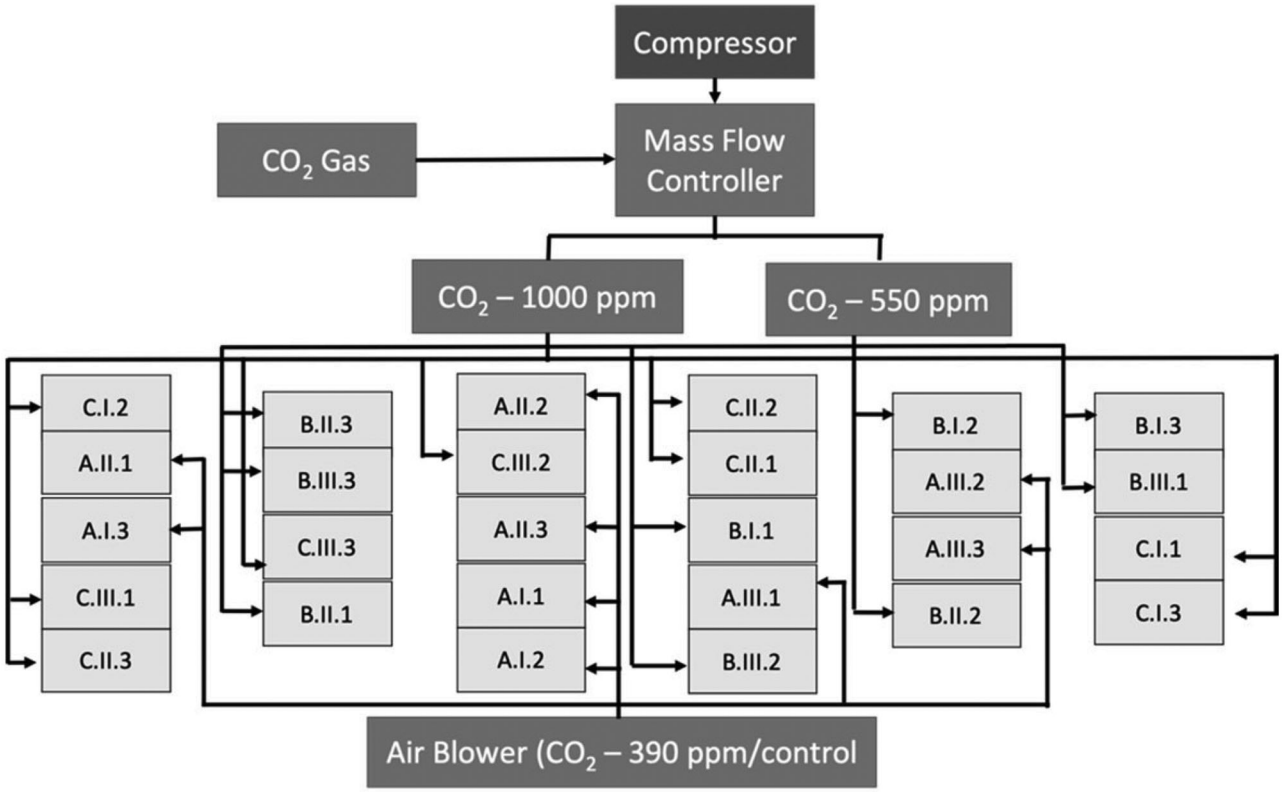

Figure 1. CO_2 system and schematic experimental design. Note: A: CO_2 concentration (390 ppm), B: CO_2 concentration (550 ppm), C: CO_2 concentration (1000 ppm); I : 100% shrimp pond wastewater (SPWW), II : 75% SPWW + 25% Seawater (SW), III: 25% SPWW + 75% SW; 1, 2 and 3 : replicate number.

where:

μ = growth rate (cell.day^{-1})
N_t = number of cells at time t (t)
N_o = number of cells at time 0 (to)
t = culture duration (hour.day^{-1})

Nutrient removal

Nutrients removal was determined by quantification of nitrate, ammonia and phosphate in the culture medium within the cultivation time. For nutrient analysis, 100-mL samples from each culture were weekly collected. Ammonium content was quantified by using Nessler's reagent with using colorimetric method, 0.3 ml of Nessler's reagent was added to 1 ml of wastewater and the resulting orange-red color was measured in the UV/Vis spectrophotometer at 420 nm. The standard curve was prepared from NH_4Cl to calculated ammonium content (Pouliot *et al.* 1989; Chevalier *et al.* 2000; Mallick 2002; Olguin 2003). Nitrate concentration was determined through UV spectroscopy at 220 nm using a T80 UV/VIS Spectrophotometer (PG Instruments, UK), according to Brucine Method. On the other hand, inorganic phosphate quantification was performed by measuring absorbance at 650 nm of an ammonium phosphomolybdate complex formed by reaction of inorganic phosphate with ammonium molybdate in spectrophotometer. Nutrient concentrations within the cultivation time were then used to determine nutrients removal efficiencies (R, in %).

Nutrients removal efficiencies were determined according to Eq. (2) (Nayak *et al.* 2016):

$$\%R = \frac{S_i - S_f}{S_i}.100 \quad (2)$$

where S_i and S_f correspond to nutrients concentration (in mg L^{-1}) in the beginning and at the end of cultivation time, respectively.

The rate of nutrient removal was calculated using the following equation (3) (Nayak *et al.* 2016):

$$\text{Removal rate } \left(\text{mg d}^{-1}\text{L}^{-1}\right) = (S_i - S_f)/Dt. \quad (3)$$

where, S_i and S_f are the mean values of nutrient concentration at the beginning (t_o) and at the end (t_i) time respectively. Δt is the cultivation time in days.

Statistical analysis

For each parameter, the average and the standard error values were calculated. The statistical significance of the results was evaluated using two ways ANOVA to investigate whether the differences between treatments could be considered significant and Tukey's multiple comparison test was performed to examine the difference between two treatments. This analysis was performed using the statistical software GrapPad Prism 7.05. Statistical tests were carried out at a significance level of 0.05.

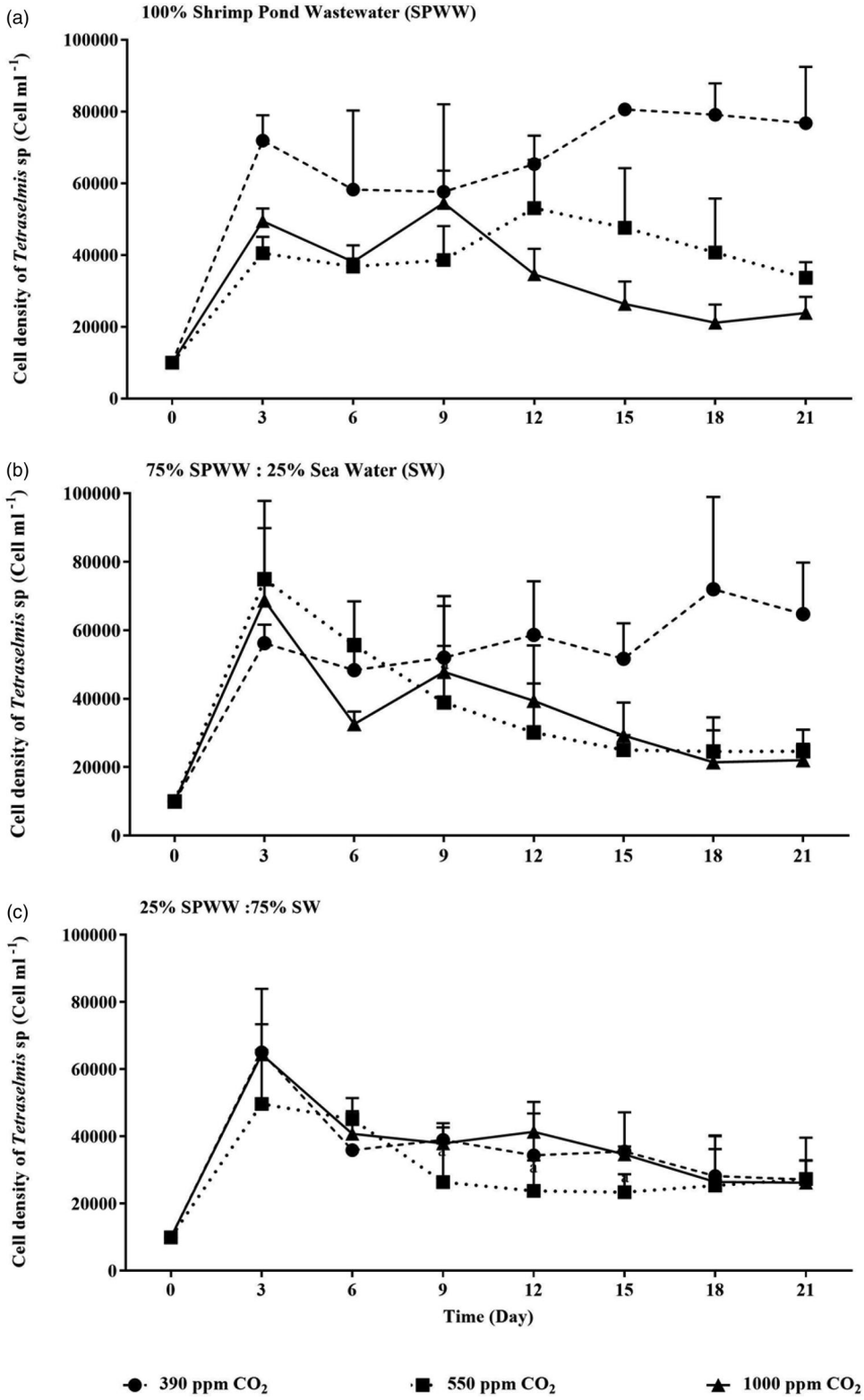

Figure 2. The growth curves obtained for *Tetraselmis chui*. (x ± SE, $n = 3$). (a) 100% SPWW, (b) 75% SPWW + 25% SW and (c) 25% SPWW + 75% SW.

Results and discussion

Microalgal density and growth rate

In this study, the selected microalgae, *T. chui* was cultivated in small aquarium using shrimp pond wastewater (SPWW) as a culture medium, supplemented with different concentration of CO_2; 390 ppm, 550 ppm and 100 ppm. The growth profile of *T. chui* in various CO_2 concentration with the incubation time of 21 days was assessed. *T. chui* density was examined every third day of culture for 21 days for determining growth rate and the pattern of *T. chui* and the study also examined the effect of increasing CO_2 concentrations and percentage of SPWW on the growth behavior of *T. chui*. (Figure 2). Figure 2 showed that the density of *T. chui* for all cultivation periods tended to higher

at treatments of 390 ppm CO_2 and 100% SPWW, however there was a declining density over period of cultivation for both treatments (CO_2 concentrations and percentage of SPWW in culture medium). The increasing CO_2 concentration at 100% SPWW in the culture medium of *T. chui* caused decreasing cell density over period of culture. This finding showed that *T. chui* was less tolerance to high concentration of CO_2, unlike other species of marine green algae, such as *Chlorella* sp. which showed high tolerance to CO_2 level *(Nayak et al.* 2016). The growth profile of *T. chui* at most of SPWW percentage in culture medium and CO_2 concentration treatment showed a dramatic increased at the first three days of culture and gradually decreased in the following day. This finding also indicated that there was an increasing growth pattern in different of SPWW percentage. This finding was in line with previous study by (Tripathi *et al.* 2019) who found that growth pattern of *Scendesmus* sp. was increasing when they growth in different percentage of wastewater ($25-100\%$). Statistical analysis showed that there was a significant difference of *T. chui* abundance between CO_2 concentration treatments and percentage of SPWW (100% SPWW and 75% SPWW + 25% SW) for every third days of observation. However, there was no significant difference of *T. chui* density between CO_2 concentration treatments at 25% SPWW + 75% SW for all days of observation. This finding showed that increasing CO_2 concentration and decreasing SPWW percentage in culture medium did not affect cell density of *T. chui* over period of culture. It assumed that *T. chui* could adapt well when there was low nutrient concentration event they were exposed by a high CO_2 concentration. There are two factors affecting the growth of microalgae when excess CO_2 is added to the mass culture, i.e., a) supply of carbon to the cell, and b) the pH (Raeesossadati *et al.* 2014), however species tolerant to a lower pH can grow at higher CO_2 concentrations (Moheimani 2013).

The growth rate of *T. chui* cultivated in SPWW under different CO_2 concentration were determined after 21 days cultivation and were used to describe the influence of different CO_2 concentration on this kinetic growth parameter (Figure 3). The result showed that the growth rate of *T. chui* was higher for all percentages of SPWW in culture medium at 390 ppm CO_2 compared to other percentages of SPWW treatment in the culture medium with other CO_2 concentration treatments. The highest growth rate was found at 100% SPWW in the culture medium at 390 ppm CO_2 concentration accounting for 0.096 cell day^{-1}. On the other hand, the lowest growth rate of *T. chui* was found at 75% SPWW + 25% SW treatment at 1000 ppm CO_2 concentration, accounting for 0.044 cell day^{-1}. The range of growth rate for different percentage of SPWW in the culture medium and CO_2 concentration treatments from 0.037 ± 0.0058 cell day^{-1} (1000 ppm CO_2 concentration at 75% SPWW + 25% SW in culture medium) and 0.096 ± 0.006 cell day^{-1} (390 ppm CO_2 concentration at 100% SPWW in culture medium). This finding showed a lower growth rate of *T. chui* compared to other marine green algae species *Spirulina* sp. (Keffer and Kleinheinz 2002). In general, there was a decreasing of growth rate with increasing of CO_2 concentration at 100% SPWW and 75% SPWW + 25% SW in culture medium. This finding was in line with previous study which found that the growth rate of *Scenedesmus* sp. was lower at 80% CO_2 than other percentage of CO_2 treatment (de Morais and Costa 2007b). The decreasing growth rate of *T. chui* was assumed due to decreasing pH with elevating concentration of CO_2 in culture media as a result of increasing acidification. Raeesossadati *et al.* (2014) found that microalgae could grow under 100% CO_2, however their growth was inhibited due to acidification. Microalgae consumed CO_2 in photosynthesis process resulted in increasing pH, this changes condition may affect growth rate of microalgae species (Pires *et al.* 2012)

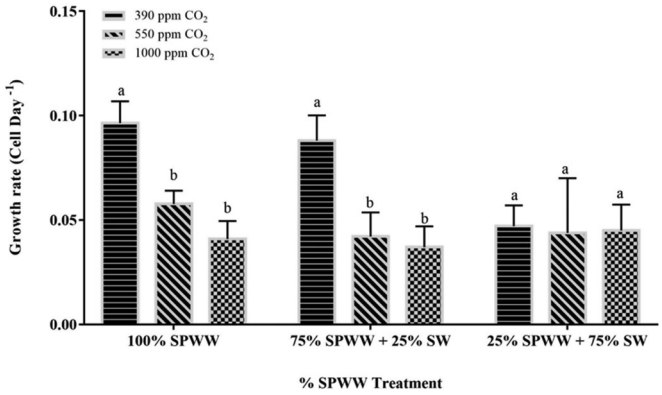

Figure 3. The growth rate of *Tetraselmis chui* at different CO_2 concentrations and treatment of SPWW percentage in culture medium ($x \pm SE$, $n = 3$). The same lowercase letter denotes a non- significant difference and different lowercase letter denotes a significant difference of growth rate between CO_2 concentration treatment ($p < 0.005$).

The effect of CO_2 concentration on nutrient removal and removal rate

The integration of wastewater with CO_2 sequestration is encouraging for higher growth rate of microalgae (Gonçalves *et al.* 2016). Moreover, the use of SPWW as a culture medium will minimize the requirement for nutrient. Figue 4 showed the removal efficiency of nitrate (NO_3), ammonium (NH_4) and phosphate (PO_4) by *T. chui* at different CO_2 concentration using different percentage of SPWW in culture medium. The concentration of CO_2 was varied (390 ppm, 550 ppm and 1000 ppm) and their effect on nutrient removal was assessed. The efficiency of bioremediation by *T. chui* was determined by measuring the overall nutrient removal percentage and nutrient removal rate as shown in Figure 4. Nutrient removal efficiency was higher for NH_4 than for NO_3 and PO_4 and the lowest nutrient removal was found for PO_4 at all CO_2 concentration and treatment of SPWW percentage. Nitrate removal efficiency was higher at treatment of SPWW percentage in culture medium than other SPWW treatments. NO_3 removal efficiency for this treatment was accounting for 99%, 98% and 89% for 390 ppm, 550 ppm and 1000 ppm CO_2 concentration treatment, respectively. This study found that NO_3 removal efficiency was slightly increasing with increasing CO_2 concentration for the treatment of SPWW percentage in culture medium, except the opposite trend was occurred for the treatment of 25% SPWW + 75% SW. This finding indicated that CO_2 concentration affected NO_3 removal efficiency by *T. chui.* It was increasing NO_3 removal efficiency with increasing CO_2 concentration. Our

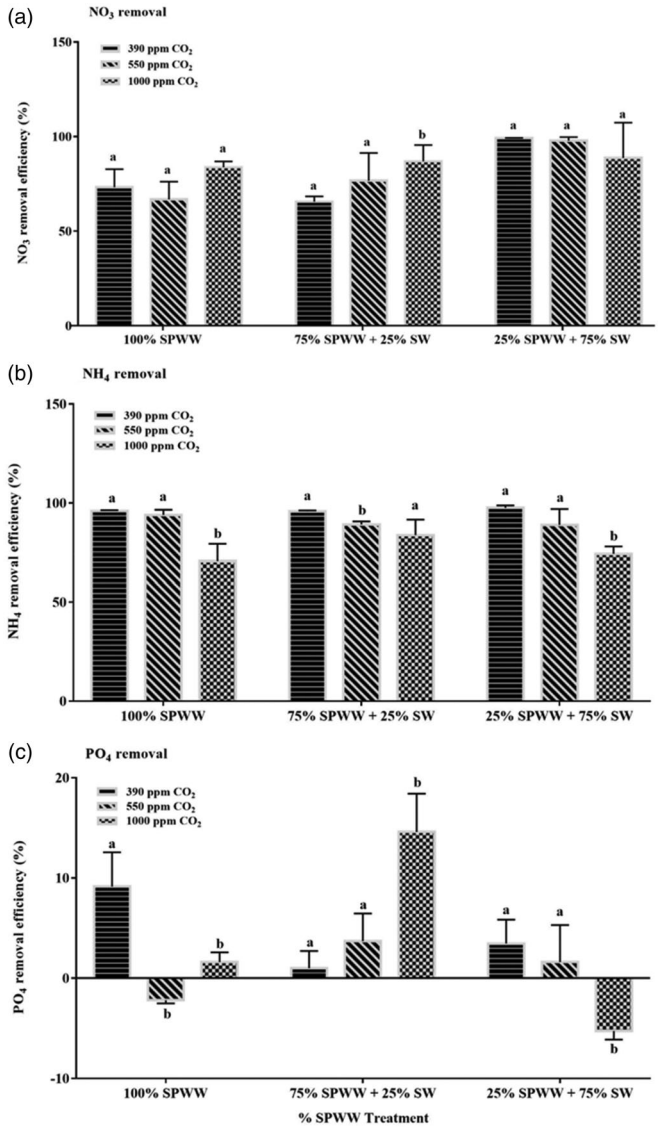

Figure 4. Nutrient removal efficiency at different CO_2 concentrations and treatment of SPWW percentage (x ± SE, $n = 3$). The same lowercase letter denotes a non- significant difference and different lowercase letter denotes a significant difference of nutrient removal efficiency between CO_2 concentration treatment ($p < 0.005$).

finding was in line with previous study by (Pires *et al.* 2012) and Nayak *et al.* (2016) who found that *Chlorella vulgaris* which was cultured with non-enriched-air stream had a lower nitrogen removal than those of cultured with CO_2 enriched-air and nutrient removal capacity improve with increasing CO_2 concentration. Our finding also indicated that the initial concentration of NO_3 will affect NO_3 removal efficiency. It showed at Figure 4a which was the treatment of 25% SPWW+ 75% SW in culture medium with average initial NO_3 concentration accounting for 0.092 mg L^{-1} resulting in 99.23% NO_3 removal efficiency. This finding was supported by Hariz *et al.* (2019) who found that there was increasing nutrient assimilation when CO_2 was adding into the microalgae culture media.

NH_4 removal efficiency tended to higher at 390 ppm CO_2 concentration for all treatment of SPWW in culture medium than those of other CO_2 concentration treatments. The highest NH_4 removal efficiency was found at the treatment of 25% SPWW + 75% SW at 390 ppm CO_2 concentration accounting for 97%. On the other hand, the lowest NH4 removal efficiency was 74% for the treatment of 75% SPWW + 25% SW in culture medium at 1000 ppm CO_2 concentration (Figure 4b). This finding indicated that NH_3 removal efficiency was decreasing with increasing CO_2 concentration. Our finding was also in agreement with previous study by Nayak *et al.* (2016) who found NH_4 removal efficiency by *Chlorella vulgaris* reached 98%. Moreover, Tam and Wong (1996) found that *Chlorella vulgaris* could remove NH_4 efficiently at 95% in batch culture.

PO_4 removal efficiency was the lowest than other nutrient removal efficiency for all the treatments of SPWW percentage in culture medium and CO_2 concentration treatments. The highest PO_4 removal efficiency was found at 1000 ppm CO_2 treatment for 75% SPWW + 25% SW in culture medium with mean value of 36.76 ± 11.99%. However, our PO_4 removal efficiency was lower compared to previous study by Gonçalves *et al.* (2016) who found that PO_4 removal efficiency at *Chlorella vulgaris* ranged between 49.0 ± 4.3% and 83.5 ± 0.3%. This finding indicated that there was a species-specific response on absorbing PO_4, depending on environmental condition and media composition. Aslan and Kapdan (2006) reported that media composition and environmental condition (such as the initial nutrient concentration, the light intensity, the nitrogen/phosphorus ratio, the light/dark cycle or algae species) were factors affecting nitrogen and phosphorus removal efficiency by microalgae. Interestingly, we found a negative value of removal efficiency at 100% SPWW and 25% SPWW + 75% SW in culture medium at high concentration of CO_2 (550 ppm and 1000 ppm), with mean values −2.19% and −5.77%, respectively. This finding indicated that initial PO_4 concentration in the culture medium affected the efficiency of PO_4 removal by *T. chui.* Furthermore, Amini *et al.* (2019) found that up-take capacity and removal efficiency of NO_3^- and PO_4^- by *Dunaniella salina* increases with an elevated initial NO_3^- and PO_4^{3-} concentrations. It showed that with elevated ions concentration in solutions, the removal efficiency by algae was increased (Amini *et al.* 2019). In this study, there was no significance different of nutrient removal between CO_2 concentration and the treatment of SPWW percentage except for 75% SPWW + 25% SW treatment, there was a significant difference of nutrient removal between CO_2 concentration treatment.

Nutrient removal rate was calculated for determining the capability of *T. chui.* in absorbing nutrient for their growth. Results of the present study showed that there was a different pattern of nutrient removal rate between the treatment of SPWW percentage in culture medium and CO_2 concentration (Figure 5). The maximum removal rate in this study was 0.09 mg L^{-1} of N. This finding indicated that increasing CO_2 concentration could escalate the uptake of NO_4 by *T. chui.* On the other hand, for NH_4 removal rate increasing CO_2 concentration could decline ammonia uptake. Statistically, there was a significance different of nutrient removal rate for NO_3 and NH_4 between the treatment of SPWW percentage and CO_2 concentration treatments ($p < 0.05$). This finding indicated that CO_2 concentration was strongly affecting an uptake of nitrogen by *T. chui.* This result was contradicting with previous study by Gonçalves et al (2016) who found that the uptake rate of nitrogen by *Chlorella vulgaris* was not strongly depended on CO_2

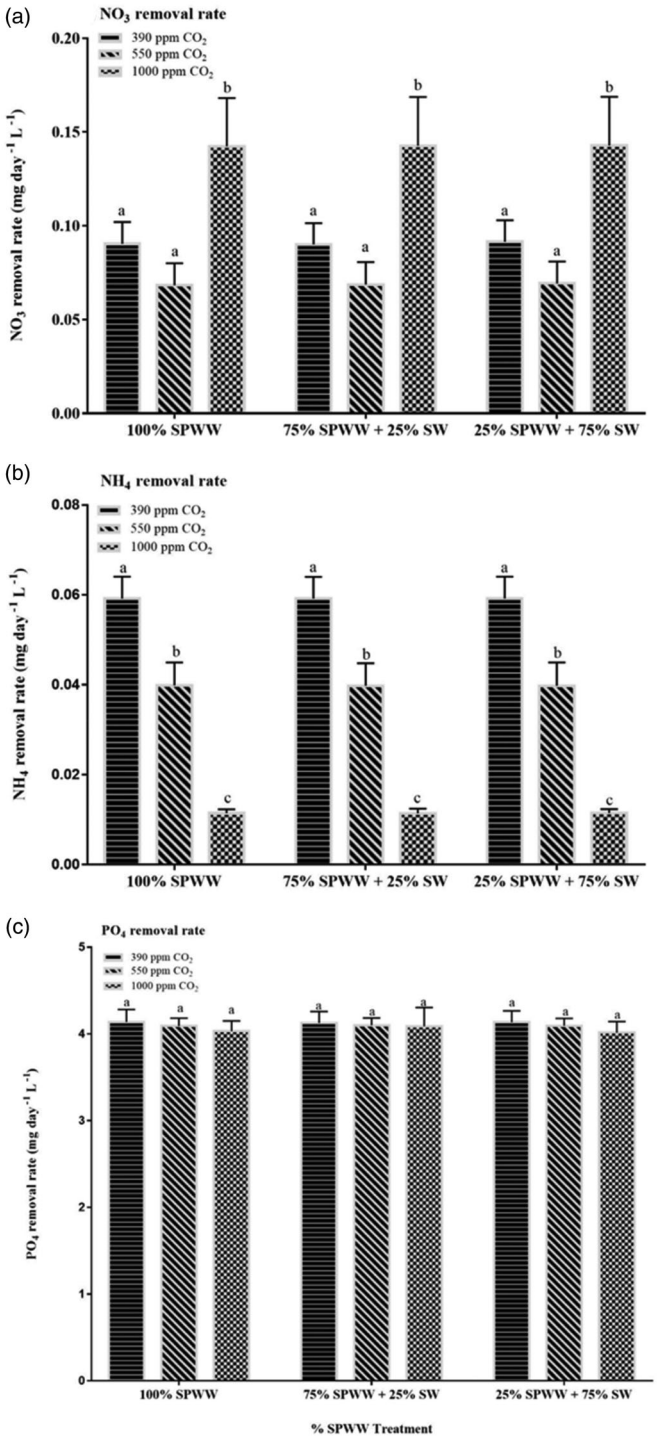

Figure 5. Nutrient removal rate at different CO_2 concentrations and the treatment of SPWW percentage ($x \pm SE$, $n = 3$). The same lowercase letter denotes a non- significant difference and different lowercase letter denotes a significant difference of nutrient removal efficiency between CO_2 concentration treatment ($p < 0.005$).

concentration. Contradictive result was presumably due to *T. chui*. was less tolerant to increasing CO_2 concentration, compared to *Chlorella vulgaris* which was more tolerant to increasing CO_2 concentration (Raeesossadati *et al.* 2014).

Nutrient removal rate for PO_4 tend to be similar in all treatments of SPWW percentage in culture medium and CO_2 concentrations. However, PO_4 removal rate was not significantly different between the SPWW percentage and CO_2 concentration treatments, indicating there was no significant effect of increasing CO_2 concentration to removal rate of PO_4. This finding was in line with previous study at *Chlorella vulgaris*, *Phormidium subcapitata* and *Spirulina salina* by (Raeesossadati *et al.* 2014) who found that phosphorus uptake rate was not strongly influenced by CO_2 concentration.

Conclusion

The results of this study demonstrated that *Tetraselmis chui*. was less tolerant to increased CO_2 concentration, mainly through decreasing growth rate in elevated CO_2 concentration. Although, *T. chui* could well adapt in a high CO_2 concentration when low nutrient concentration present. NO_3 removal rate by *T. chui* was increased with escalating CO_2 concentration, whilst NH_4 removal rate decreased with increasing CO_2 concentration. In conclusion, CO_2 concentration was significantly affecting nitrogen removal efficiency and rate by *T. chui*, but not significantly affecting removal efficiency and rate of PO_4 by *T. chui*.

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SHRIMP WASTEWATER BIOREMEDIATION

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Nutrient consumption of green microalgae, *Chlorella* sp. during the bioremediation of shrimp aquaculture wastewater

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ABSTRACT

Aquaculture products are among the biggest contributor to food supplies to meet the global food demands of the growing population over these past few years. For aquaculture to continue developing, an effective wastewater treatment is required to lessen the environmental effects. This study examined the potential of *Chlorella* sp. to reduce nutrients in shrimp aquaculture wastewater and correlate with the growth kinetics of the algae during the bioremediation process. Six different *Chlorella* sp. inoculation dosages ranging from 0 to 60 % (v/v) were used in this study. Marine water wastewater (MW) and Freshwater wastewater (FW) where the two types of shrimp wastewater were employed. Results indicated that the 30 % (v/v) and 40 % (v/v) were the optimum dosage for MW and FW. During the treatment, microalgae cell density increased more than tenfold compared to the initial value. Moreover, batch culture resulted in the specific growth rate concentration of 0.18 k day^{-1} and 0.15 k day^{-1}, respectively. Those dosage also resulting the highest removal efficiencies with removal of ammonia, nitrite and orthophosphate of 96.77 %, 82.07 %, 75.96 % and 90.10 %, 87.09 %, 95.60 %, respectively. The application of FTIR spectroscopy was employed in this study to analyze the functional group in the microalgae biomass. The results of the scanning electron microscopy (SEM) and Energy Dispersive Spectroscopy Analysis (EDS) also included to further illustrate how microalgae biomass was affected by the treatment in this study. Therefore, the research from this study could be used in design novel microalgae treatments that offer a thorough and environmentally beneficial method of treating shrimp aquaculture wastewater.

1. Introduction

The rapid growth of the human population has led to the fast expansion of aquaculture industries to support the global demand. Aquaculture effluent discharge has increased dramatically over the world. Approximately 82 m^3/kg production/year estimation of wastewater generated from aquaculture industries [1]. Wastewater from the aquaculture industry has a large amount of chemical, microbial pollutants, suspended solids and nitrogenous compounds [2]. With concern to the pollution generated by aquaculture, the pollutants discharged from aquaculture industries could destroy the receiving aquatic environment such as eutrophication and deterioration towards the natural ecosystem [3]. Many technologies have been created and applied to minimize the water pollution and one of those technologies that are being developed bioremediation.

Bioremediation uses naturally existing microorganisms and alternative aspects of the natural environment to treat discharged water of its nutrients. It has been demonstrated that bioremediation is more affordable than other technologies for the cleanup of hazardous waste [4]. Algae are used in phytoremediation, a sort of bioremediation, to enhance the water quality. Bioremediation has utilized plant-based remediation such as macro and microalgae. It has been found that microalgae effectively use the nitrogen and phosphorus in wastewater for cell development. Microalgae can take up these chemicals and transform them into biomass that can be used.

Microalgae biomass has become a very promising feedstock in recent

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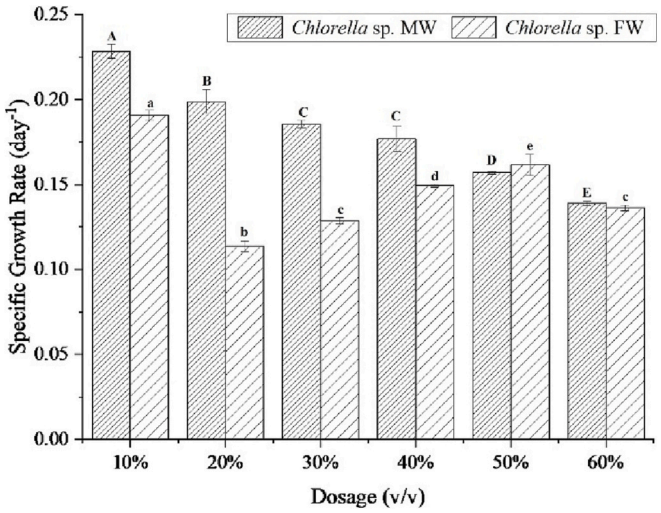

Fig. 1. Specific growth rate of *Chlorella* sp. at exponential phase during bioremediation.
Different capital letters (A-B-C-D-E) indicate significant difference of SGR among used dosage for MW while different lowercase (a-b-c-d-e) indicate significant difference of SGR among used dosage for FW.

years for sustainable biofuels such as biodiesel, bioethanol and biogas [5,6]. The increased cost required for microalgae cultivation is one of the difficulties. This is due to the continued usage of expensive chemicals like Conway or Walne fertilizers to replenish nutrients in growth media [6,7]. The production of microalgae biomass and its nutritional will be significantly influenced by the total nutrient composition and suitable nutrient concentration. Therefore, to meet the suit nutritional needs of microalgae during culture, it is necessary to replace the culture media with macronutrients and micronutrients. One viable substitute for culture media is wastewater.

Microalgae have been recognized as promising agents for improving wastewater quality while collecting nutrients from wastewater at low cost and in an environmentally friendly manner [8–10]. Additionally, heavy metal compounds and pesticides produced by industrial and agricultural wastewaters can be removed using microalgae [11]. Utilizing nutrient-rich of aquaculture wastewater as a growth medium for the development of microalgae could reduce the reliance on chemical pesticides. However, there is currently little research on the simultaneous production of microalgae and bioremediation of aquaculture wastewater. It is also yet to be determined how nutrient uptake and microalgal growth differ between fresh and marine aquaculture wastewater.

This study aims to determine the biomass yield and nutrient uptake by the *Chlorella* sp. microalgae species in aquaculture effluent during bioremediation. The ratio of microalgae and wastewater also considered as an important factor affecting the algae growth and the bioremediation performance. In addition, FTIR spectroscopy was used to examine the functional groups in the biomass of the microalgae. The organic chemical groups -OH, -COOH, NH_2, and C═O were detected in the microalgae biomass by FTIR analysis. SEM was used to characterize the shape of the microalgae cell and EDS was used to examine the chemical characterization of the nitrogen and phosphorus content in the microalgae biomass. The results of this research could enhance microalgae capacity to remove nutrients from different aquaculture effluent. Technologies based on microalgae offer a promising alternative for treating aquaculture wastewater. The success or failure of aquaculture output depends on how well water quality is maintained.

2. Materials and methods

2.1. Wastewater collection

Aquaculture wastewater was collected from the hatchery pond of shrimp, *Penaeus vannamei* for marinewater bioremediation (MW) and *Macrobrachium rosenbergii* for freshwater bioremediation (FW) at Universiti Malaysia Terengganu (UMT), Malaysia. Filtered sterile wastewater was prepared by autoclaved for 20 min at 120 °C. This method was used to ensure unnecessary species were killed. As a result, it remove other microorganisms from samples while preventing changes of the nutrient content in wastewater such as undergo the nitrification process before it was employed in the bioremediation process.

2.2. Microalgae cultivation

Pure cultivation of green microalgae genus *Chlorella* was obtained from Live feed Laboratory, Institute of Tropical Aquaculture Hatchery UMT. It was grown in Guillard's F/2 media for marine species, *Chlorella* sp. UMT LF2 and Bold's Basal Medium (BBM) for freshwater species, *Chlorella* sp. UMT LF1 under sterile conditions. Microalgae were cultures with an initial concentration of 1.0×10^5 cell·mL^{-1} algal cells. Cell density was calculated every two days using an improved Neubauer Haemocytometer. The specific growth rates (μ) of microalgae were determined during the exponential growth phase by the Eq. (1):

$$\mu = \left[\frac{ln N_2 - ln N_1}{t_2 - t_1}\right] \quad (1)$$

where μ is the specific growth rate, and N_1 and N_2 are the biomass at time 1 (t_1) and time 2 (t_2), respectively [12].

2.3. Bioremediation process

Green microalgae genus *Chlorella* was used for the bioremediation of shrimp aquaculture wastewater due to its simple cell cycle, high growth rate and having photosynthetic and metabolic pathways similar to higher plants [13]. *Chlorella* sp. were cultured until early exponential growth phase, Day 4 to Day 6 of the cultivation period. The batch bioremediation process was conducted using six different inoculation dosages: 0, 10, 20, 30, 40, 50, 60 % (v/v) with a total experiment volume of 1.5 L. The growth performance and nutrient analysis of microalgae (removal efficiency) were monitored every 2 days. The removal efficiency (%) of nutrients were determined by the Eq. (2):

$$R\ (\%) = \frac{C_o - C_t}{C_t} \times 100 \quad (2)$$

where R (%) is the removal efficiency of nutrients, C_0 (mg L^{-1}) is the initial concentration of the nutrient, and C_t (mg L^{-1}) is the final concentration of the nutrient at time t.

Aquaculture wastewater can be a major source of food requirements for microalgae cultivation and contributes to the reduction of nutrient [14]. Therefore, the uptake of nutrients, ammonia, nitrite and orthophosphate in batch bioremediation under sterile condition was studied. The initial NH_3^+, NO_2^- and PO_4^- concentrations for all seven different treatment were approximately 2.80 ± 0.05 mg L^{-1}, 1.5 ± 0.05 mg L^{-1} and 4.1 ± 0.05 mg L^{-1}, respectively for MW and 3.6 ± 0.05 mg L^{-1}, 1.75 ± 0.05 mg L^{-1} and 4.1 ± 0.05 mg L^{-1} respectively for FW before inoculated with *Chlorella* sp.

2.4. Water quality monitoring

The water quality analyses were carried out with the collection of 50 mL of the water sample from each treatment and control at 2-day interval until 14 days treatment period. The water samples were clarified by centrifugation (Hettich Zentrifugen Universal 1200, Germany) at

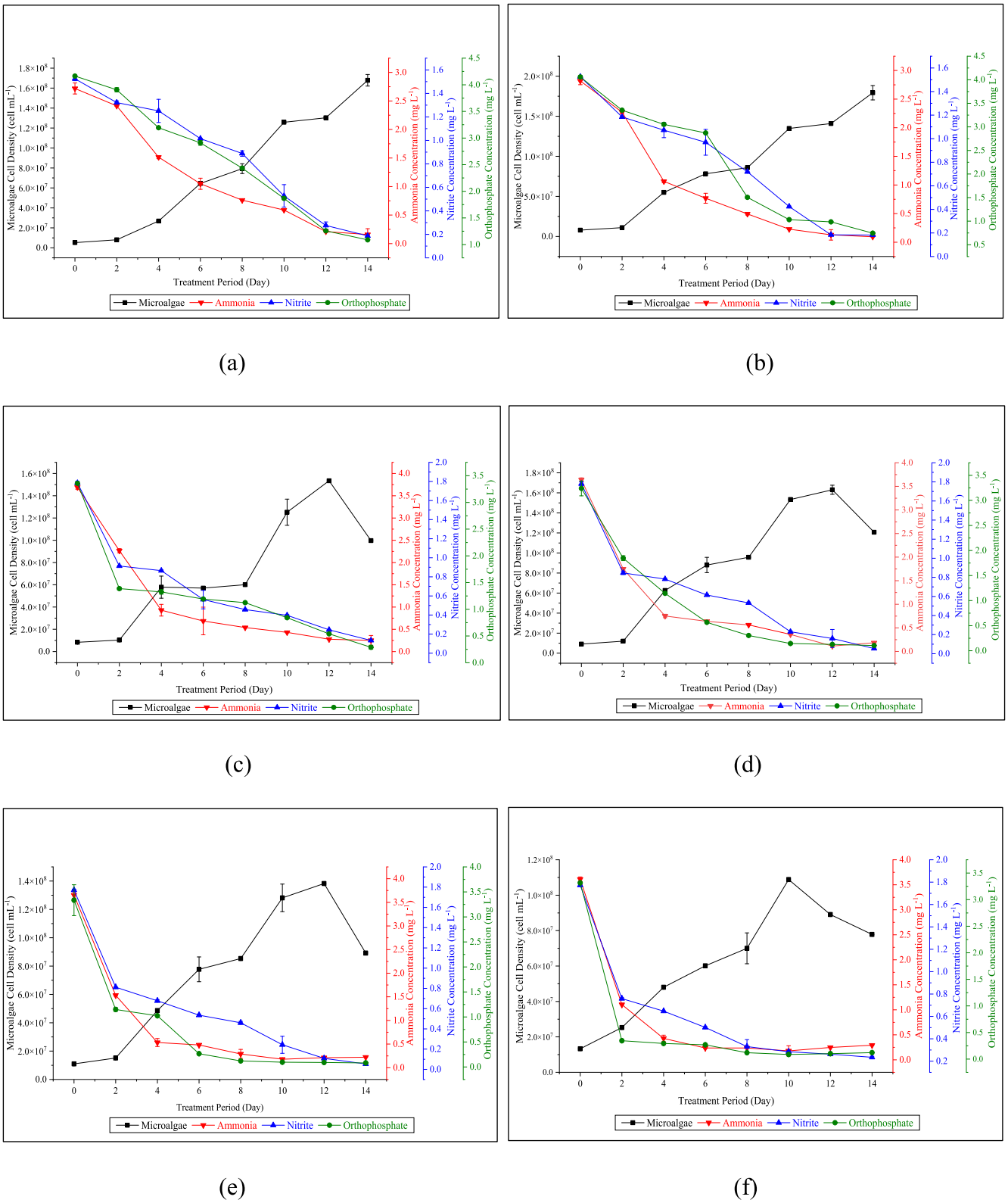

Fig. 2a. Bioremediation performance at (a) 10, (b) 20, (c) 30, (d) 40, (e) 50, and (f) 60 % (v/v) microalgae *Chlorella* sp. inoculation dosages throughout 14-days treatment period for Marine water Treatment (MW).

9000 rpm for 5 min to separate microalgae biomass for producing clear water to perform water quality analysis. The Ammonia (NH_3^+), Nitrite (NO_2^-) and Orthophosphate (PO_4^{3-}) determination were carried out using standard methods, Phenate Method (4500-NH_3.F), Colorimetric Method (4500-NO_2^-.B) and Ascorbic Acid Method (4500-P.E) adopted from APHA (2012). The Dual-Beam UV–Vis Spectrophotometer (Shimadzu UV-1800, Japan) was used to analyze nutrients concentration.

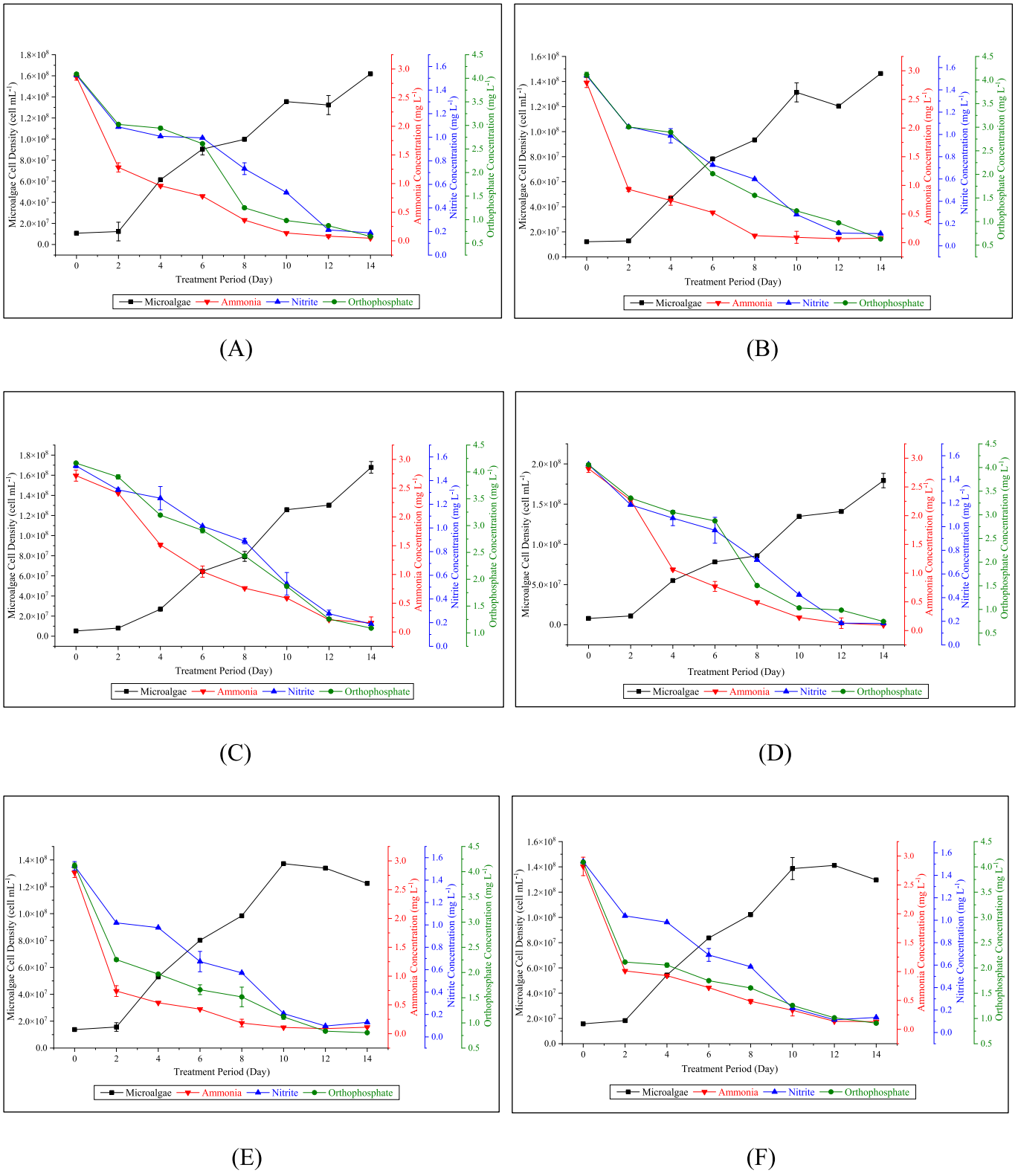

Fig. 2b. Bioremediation performance at (A) 10, (B) 20, (C) 30, (D) 40, (E) 50, and (F) 60 % (v/v) microalgae *Chlorella* sp. inoculation dosages throughout 14-days treatment period for Freshwater Treatment (FW).

2.5. Fourier Transform Infrared (FTIR) analysis

Microalgae cultures were cultured and harvested during late log phase for FTIR analysis. 100 mL of grown microalgae culture was centrifuged at 10,000 rpm for 10 min and the pellet was dried. Both the microalgae cultures, *Chlorella* sp. in media and treatment were processed. Microalgae biomass from marine cultures were rinsed with 0.5 M ammonium formate prior to centrifugation to remove salt from the biomass. The wet microalgae pellets were dried in freeze-dryer using Freezon 4.5 L −50 °C Benchtop Freeze Dryer (USA) for 24 h to form the

Table 1
The removal efficiency (%) of ammonia, nitrite, and orthophosphate for MW and FW at Day 10.

Dosage, % (v/v) / Nutrients	Ammonia (%)		Nitrite (%)		Orthophosphate (%)	
	MW	FW	MW	FW	MW	FW
0	0.77[a]	−3.05[a]	−0.11[a]	−0.17[a]	−0.01[a]	1.05[a]
10	78.36[b]	86.14[b]	65.41[b]	76.89[b]	55.19[b]	56.19[b]
20	91.94[c,d]	88.95[c]	65.23[b]	78.44[c]	74.59[c]	73.49[c]
30	96.77[c]	88.34[d]	82.07[b]	77.76[b]	75.96[c]	74.93[c]
40	95.65[c]	91.93[e]	81.63[c]	87.09[d]	70.19[d]	95.60[d]
50	96.14[c]	93.13[e]	86.42[c]	86.30[e]	72.89[e]	97.14[d]
60	88.32[d]	90.01[d]	85.72[c]	83.96[e]	69.31[d]	97.39[d]

Superscripts letter (a, b, c, d, e) refer to means for group in homogenous subset.

Table 2
The removal rate (k day^{-1}) of ammonia, nitrite, and orthophosphate for MW and FW at Day 10.

Dosage, % (v/v) / Nutrients	Ammonia (%)		Nitrite (%)		Orthophosphate (%)	
	MW	FW	MW	FW	MW	FW
0	0.0008	0.0013	0.0004	0.0013	0.0004	0.002
10	0.21	0.197	0.15	0.182	0.1	0.084
20	0.258	0.237	0.162	0.206	0.131	0.119
30	0.296	0.19	0.149	0.162	0.138	0.139
40	0.273	0.22	0.2	0.218	0.129	0.256
50	0.231	0.199	0.204	0.225	0.108	0.278
60	0.208	0.162	0.195	0.133	0.094	0.201

dried algae powders. The samples were analyzed using FTIR Spectrometer Thermofisher Scientific Nicolet™ iS™ 10 (USA). For this study, a view from the microscope was chosen from the transmission region between 4000 and 400 cm^{-1} wave number range, 4 cm^{-1} resolution and aperture of 20 × 20 μm square aperture, placed over a clear field (background) and 32 scans were taken as spectra.

2.6. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) analysis

The surface morphology of the microalgae was obtained using scanning electron microscopy analysis scan-brand Floor Top Scanning Electron Microscope (SEM) TESCAN/VEGA, CZECH REPUBLIC. The SEM was equipped with EDX BRUKER (Silicon Drift Energy Dispersive Spectrometer model Quantax Compact with XFlash 600Mini). Before the experiment, microalgae biomass was processed using the mentioned technique in FTIR analysis. To perform the analysis SEM, part of the microalgae biomass was bonded to stub with a tape of black carbon and coated with fine thin layer gold, Au to protect the sample and increase the conductivity.

2.7. Data analysis

All experiment data were analyzed in triplicate and graphical analyses were plotted using Origin 2022 software (Origin Lab Corp., USA) for the determination of interactions between factors. Statistical analyses were performed through IBM SPSS ver. 23.0. Normality and homogeneity of variances of the data were satisfied via Shapiro-Wilk test and Levene's test, respectively. Specific growth rate (SGR) of *Chlorella* sp. and removal efficiency of nutrient (%) in different concentrations (10 %, 20 %, 30 %, 40 %, 50 %, and 60 % (v/v)) inoculation in aquaculture wastewater was analyzed by One-Way Analysis of Variance (ANOVA), followed by Tukey HSD test. Results were considered as statistically significant at $p < 0.05$ in this experiment [15].

3. Results and discussion

3.1. Growth performance of microalgae

The performance of microalgae growth primarily governed by nutrients and yields of algae also can be boosted when the nutrients such as nitrogen and phosphorus are readily available in the growth medium [5]. Besides, the growth patterns of *Chlorella* sp. have depicted similar growth pattern at different dosages concentration. The growth kinetics of *Chlorella* sp. throughout bioremediation process suited with microbial growth kinetics by the growth phases of lag, exponential, stationary and declining phases [16]. Fig. 1 illustrated the growth performance of microalgae, *Chlorella* sp. by determining the specific growth rate (SGR) throughout the aquaculture wastewater bioremediation within 14 days treatment period.

The specific growth rate (SGR) of *Chlorella* sp. in this study was determined at exponential phase (Day 10). Fig. 2a and 2b shows that the specific growth rate for *Chlorella* sp. in both treatment, MW and FW. All the different treatment for MW consistently yielded the highest SGR than FW. For MW, the highest SGR (0.228 day^{-1}) was found at 10 % inoculation and lowest SGR (0.139 day^{-1}) was found at 60 % inoculation of microalgae. While for FW, the highest SGR (0.191 day^{-1}) was found at 10 % inoculation and lowest SGR (0.114 day^{-1}) was found at 20 % inoculation of microalgae. SGR for 60 % (v/v) was decreased due to the high competition between microalgae cell for limited available nutrient thus inhibiting effective absorption of nutrient into *Chlorella* sp. biomass [17].

SGR values at various inoculation concentrations (10 %, 20 %, 30 %, 40 %, 50 %, and 60 % (v/v)) were significantly different for FW with ($F = 175.029$, $p < 0.05$), while similar SGR was discovered between the inoculum concentrations of 30 % (v/v) and 40 % (v/v) via Post hoc Tukey's HSD test and Bonferroni test ($p > 0.05$). While for MW ($F = 131.133$, $p < 0.05$) and the SGR were found similar between inoculum concentration, 20 %, 30 % and 40 %. This implies that the microalgae cell growth rate was significantly affected by the amount of cell density that was inoculated [18].

3.2. Effect of microalgae concentration on nutrient consumption

The findings demostrate that microalgae can assimilate the nitrogen from a variety sources, including ammonium, nitrate, nitrite and urea [19]. Additionally, ammonia is the most energy-efficient nitrogen source since less energy is needed for its uptake. Table 1 tabulate the removal efficiency and nutrient availability for different dosages of microalgae for Day 10. According to the analysis, the ammonia concentration were significantly reduced for all different dosages both MW and FW except 0 % (v/v). However, the bioremediation performance of ammonia concentration in MV more effective than FW since the dosage of 20 % of MW already had produced 90 % removal as opposed to 40 % for FW. Generally, ammonium was the predominat nitrogen component in aquaculture, but this study also found that nitrite was present in significant amounts.

Within the first five days of the treatment period, *Chlorella* sp. bioremediation indicated low removal of nitrite and orthophosphate. However, the concentration of nitrite dramatically decreased throughout the treatment and efficiently removed >80 % when the dosages increased from 30 % (v/v) and 40 % (v/v) for MW and FW, respectively. It was noticed that the removal efficiencies were lower at lower dosages concentrations, below than 20 % (v/v). The concentration of nitrite was maintained in this study because the use of sterile microalgae culture and wastewater, without the effects of a complex microbiome that can convert the N and P concentrations. The process of nitrification was suggesting negligible throughout the treatment process. The nitrification process is the process involved the oxidation of ammonia to nitrite by ammonia-oxidizing bacteria, and nitrite to nitrate by nitrite-oxidizing bacteria.

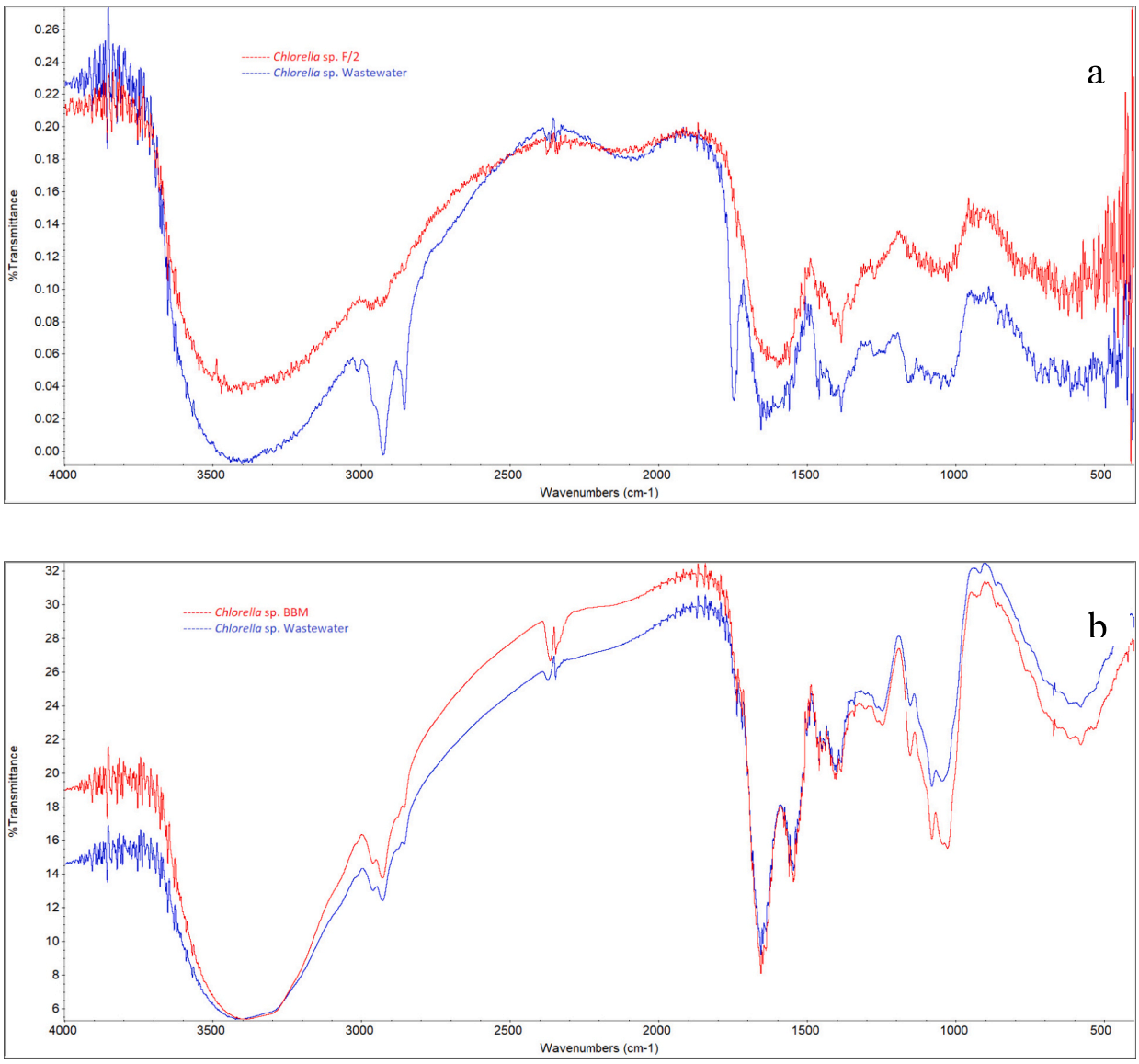

Fig. 3. FTIR Spectral image of *Chlorella* sp. culture in medium (red line) and aquaculture wastewater (blue line). a refer to marine water, Image b refer to fresh water. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3a
Functional group /Assignment of *Chlorella* sp. of Marine Water (MW). References adopted from [28].

Band	Main peak (cm^{-1})	Wave number range (cm^{-1})	Typical band assignment from literature
1.	3854.3	3900–3800	-NH_2 stretching vibration
2.	3401.50	3700–3100	Water v(O—H) stretching Protein v (N—H) stretching (amide A)
3.	2926.58/ 2853.91*	3000–2800	Lipid – carbohydrate Mainly vas(CH_2) and vs(CH_2) stretching
4.	1744.87*	1800–1700	Cellulose–Fatty Acids v(C=O) stretching of esters
5.	1637.09	1700–1600	Protein amide I band Mainly
6.	1458.25	1500–1400	Protein δas(CH_2) and δas(CH_3) bending of methyl, Lipid δas(CH_2) bending of methyl
7.	1155.91	1200–900	Carbohydrate v(C-O-C) of Polysaccharides

(*) Refer to peak present at Bioremediation Process only.

Table 3b
Functional group /Assignment of *Chlorella* sp. of Fresh Water (FW). References adopted from [28].

Band	Main peak (cm^{-1})	Wave number range (cm^{-1})	Typical band assignment from literature
1.	3854.31	3900–3800	-NH_2 stretching vibration
2.	3448.02	3700–3100	Water v(O—H) stretching Protein v (N—H) stretching (amide A)
3.	2927.34	3000–2800	Lipid – carbohydrate Mainly vas(CH_2) and vs(CH_2) stretching
4.	1654.28	1800–1600	Protein amide I band Mainly v(C=O) stretching
5.	1559.70	1600–1500	Protein amide II band mainly δ(N—H) bending and v(C—N) stretching
6.	1458.38	1500–1400	Protein δas(CH_2) and δas(CH3) bending of methyl, Lipid δas(CH2) bending of methyl
7.	1079.73	1200–900	Carbohydrate v(C-O-C) of polysaccharides Nucleic Acid (and other phosphate-containing compounds) vs (>P=O) stretching of phosphodiesters

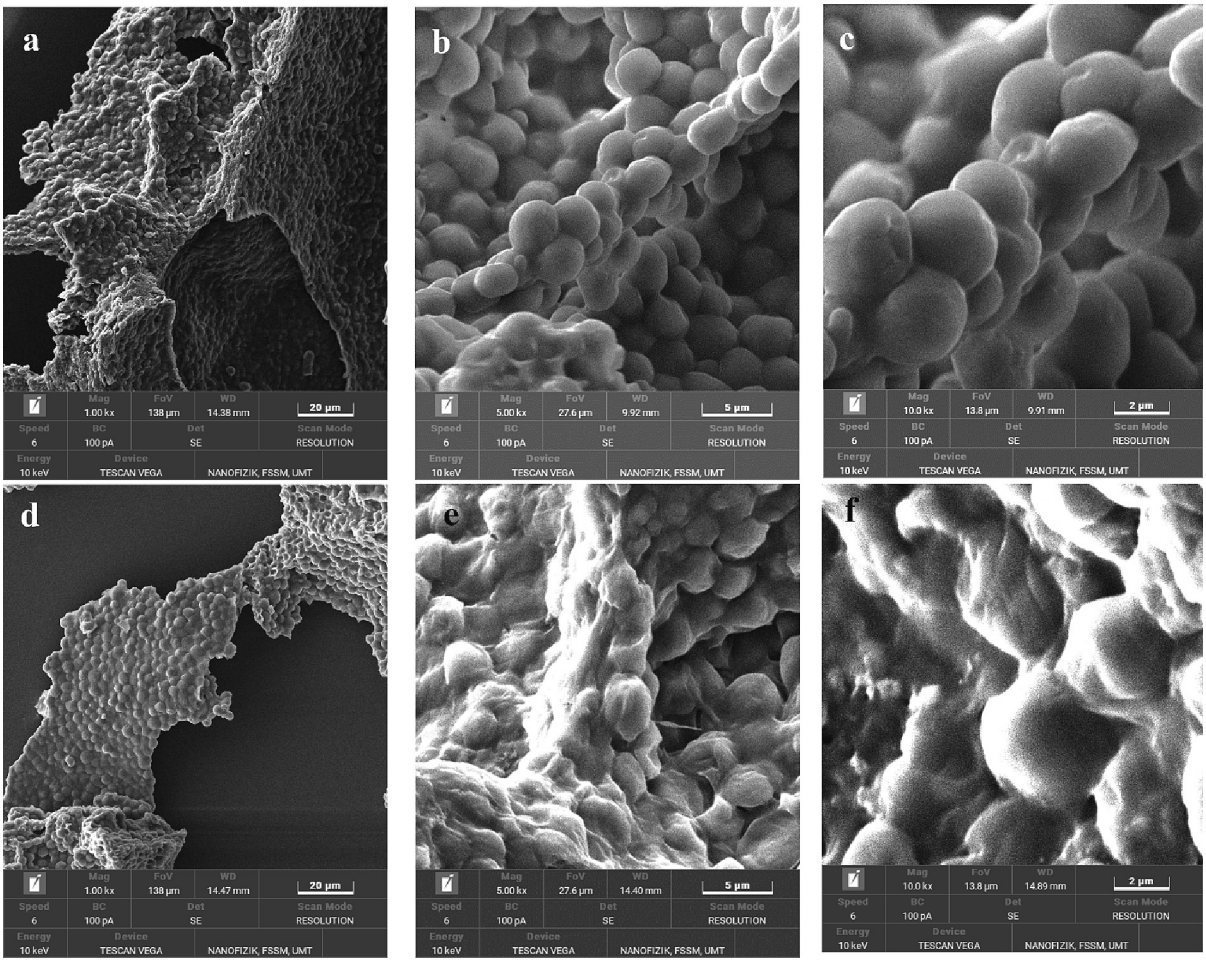

Fig. 4. SEM Image for microalgae magnification x1000, x5000, x10,000. Different letters refer to the genus *Chlorella* culture in different condition at different magnification, (a-b-c) refer to the genus *Chlorella* culture in Guillard's F/2 Media, (d-e-f) refer to the genus *Chlorella* culture in MW, (g-h-i) refer to the genus *Chlorella* culture in BBM, and (j-k-l) refer to the genus *Chlorella* culture in FW.

Phosphorus also crucial component for the growth of microalgae and frequently a major limiting factor for algal growth [20]. Typically, only orthophosphate that is assimilated by phytoplankton and can be utilize for cell development [21]. The absorbed phosphorus is usually retained as polyphosphate granules and will be useful to algae during their growth cycle. A study [22] mentioned that nutrient in the form of orthophosphate was reduced due to absorption by *Chlorella* sp. and stored as polyphosphates within the cells. Additionally, the overall findings show that phosphorus concentration in the form of orthophosphate, PO_4^{3-} for MW was eliminated with a lower removal efficiency <80 %, whereas in FW completely removed from the wastewater. This is postulated due to the green algae species like *Chlorella vulgaris* are capable of absorbing phosphorus only to a limited extent. Similarly, PO_4^{3-} removal in all treatments was higher was compared against the control.

Apart from that, the removal efficiency and removal rate of dosages 0 % (v/v) were the lowest, as could be seen in Fig. 1 and Table 1, and there were no significant differences among the other dosages for all nutrients because it was used as a control treatment and run without a microalgae inoculum.

The removal rate was positively affected by the dosage of microalgae (Table 2). The highest value of removal rate was observed different depending on the nutrients and dosages. The apparent removal rate (k. day^{-1}) at 30 % (v/v) was 0.296 k day^{-1} which is in accordance with the removal efficiency thats suggested as the maximum ammonia removal efficiency for MW. For FW, the removal rate for the 30 % (v/v) is 0.19 k day^{-1} and among the lowest compare to the other dosages. The dosage 20 % (v/v) in FW had achieved the faster rate of ammonia removal at Day 10, 0.237 k day^{-1} which remove about 88.95 % from the water sample.

As the 14 days treatment period, the dosages 30 % (v/v) was selected as the highest performance of nutrient consumption for marine wastewater treatment (MW) based on removal efficiency, with NH_3^+ and NO_2^- were 0.135 mg L^{-1} and 0.274 mg L^{-1} of nutrient availability and the removal efficiency were 96.77 % and 82.07 %, respectively. On the other hand, for the freshwater treatment (FW), the dosage 40 % was choosed as the best dosage, resulting the highest removal efficiency as compared to other dosages which were 90.10 %. 87.09 % and 95.6 % for ammonia, nitrite and orthophosphate, respectively.

For further investigation on the effect of *Chlorella* sp. inoculation concentrations on nutrients removal, the correlation analysis between growth and nutrient were performed individually on Day 10 treatment period for both MW and FW. The findings demonstrated that the positive correlation exists when nutrient concentrations decrease exponentially proportional throughout the treatment as the growth cell density rises and it's complied with the First Order Kinetic Model. This study was confirmed with the assumption make previously that the growth of microalgae was influenced by the reduction of nutrient content in wastewater.

Figs. 2a and 2b showed that the decreasing of nutrients (ammonia,

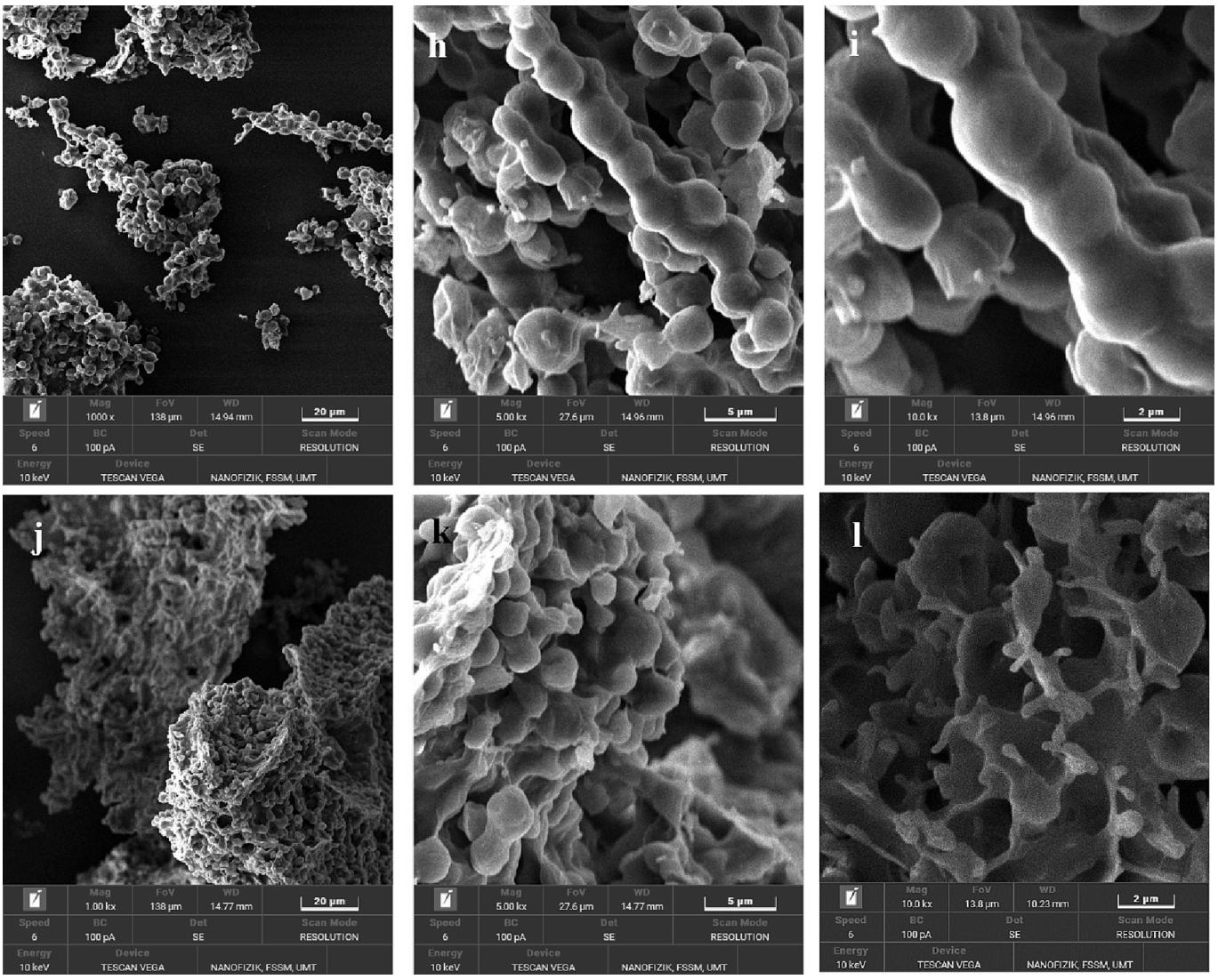

Fig. 4. (*continued*).

Table 4
Elemental identification by EDS.

Biomass sample	Contents of element by weight (%)			
	Chlorella sp. LF-2 Guillard's F/2 Media	*Chlorella* sp. LF-2 MW	*Chlorella* sp. LF-1 BBM	*Chlorella* sp. LF-1 FW
Carbon, C	73.56	58.26	53.27	57.75
Nitrogen, N	4.69	17.39	12.63	6.47
Oxygen, O	9.80	20.16	31.94	18.34
Phosphorus, P	5.87	2.94	0.97	12.18
Sulphur, S	6.08	1.24	1.2	5.17

nitrite, and ortophosphate) was in accordance with the microalgal biomass growth, suggesting the conversion of nutrient into biomass for both marine water (MW) and freshwater (FW) treatment. Similar growth pattern were depicted in the *Chlorella* sp. growth in different treatment with relatively short lag phase in the first two days and followed the exponential phase in the six to eight days. It was observed that the death phase began on Day 12 towards the end of the treatment period except 10 % and 20 % (v/v). As illustrated in Figs. 2a and 2b, the microalgae displayed a brief lag phase of one to two days when the cell concentration were increased about two-folds from the initial biomass density. Therefore, short lag phase revealed that the microalgae had excellent adaption characteristics to the aquaculture wastewater.

In the case of *Chlorella* sp., this species able to utilize both ammonium and nitrite for the syntesize of glutamine and glutamate with the involvement of glutamine synthetase (to gather energy from the breakdown of ammonium) and glutamate synthetase (to produce glutamate using nitrite) [23]. In addition to sufficient nitrogen, P also benefit the lipid content in *Chlorella* sp. followed by the increasing accumulation of poly-P inside cells [24]. The high uptake of ammonia, nitrite, and orthophosphate indicate a good utilization of nutrients by *Chlorella* sp. to support the cell growth [25]. According to the graph, the concentration of ammonia started to increase for all treatments between Days 12 and 14, especially for the 50 % and 60 % (v/v) treatments. Given that microalgae begin to enter the death phase on this day, it might be related to the microalgae's growth phase. As reported from my previous study, bioremediation using *Claries gariepinus* wastewater, this phonemonen happened due to release of absorbed nutrient from microalgae biomass as it experienced early death phase. During this growth phase, the *Chlorella* sp. biomass started to autolyze and degrade.

3.3. Characterization of microalgae morphology

3.3.1. FTIR analysis

FTIR spectroscopy played a crucial role for the characterization of the biochemical composition of phytoplankton [26]. In general, all chemical bonds have a number of bending and stretching vibrations with varying energies, which produce the various absorption bands. In addition, the composition and molecular functional groups can be determined by analysing the position, width, and intensity of infrared light absorption. The results of FTIR transmittance of microalgae biomass from wave number range of 4000–400 cm^{-1} indicates the presence of organic component groups of amine, alcohol, aromatic, alkyne, alkene, acid, ether and alkyl halide groups as well as organic contents such as carbohydrates, proteins, and lipids in *Chlorella* sp. The spectral absorption bands were identified in accordance with information that has been published.

Fig.3 shows the results of FTIR transmittance of four distinct microalgae biomass. Examinations of the infrared spectra of all biomass revealed the presence of the seven uniques bands at 3900–3800 cm^{-1}, 3700–3100 cm^{-1}, 3000–2800 cm^{-1}, 1800–1700 cm^{-1}, 1700–1600 cm^{-1} (MW only), 1600–1500 cm^{-1} (MW only), and 1200–900 cm^{-1}. This indicates that there are variances in the composition of the microlgae biomass despite the fact that they have comprable organic groups. The FT-IR spectrum of *Chlorella* sp. used in this study is similar reported by Ferreira et al. [27].

The typical band assignment from literature is summarized in Tables 3a and 3b. The band contributions were postulated from residual water (band 2), lipids (bands 3 and 6), cellulose (band 4), proteins (bands 5 and 6, 4, 5 and 6), and carbohydrate (band 7). The peaks located at 2853.91 and 1744.87 correspond to the lipid – carbohydrate and cellulose–fatty acids only obtained in bioremediation process of MW, suggesting the presence of additional constituents in nutrients from actual wastewater.

3.3.2. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy Analysis (EDS)

Advanced microscopy, such as scanning electron microscopy (SEM), is necessary to characterize microalgae [29]. After 14 days of treatment with aquaculture wastewater, the *Chlorella* sp. biomass cells were examined visually using light microscopy, SEM, as well as energy dispersive spectroscopy (EDS) to determine their elemental composition (EDS). In this investigation, SEM microscopy was employed to assess the surface features and morphological changes in the cell wall composition and shape of microalgae biomass after the bioremediation process.

Fig. 4 (a-b-c), scanning electron microscopy (SEM) visualization for all microalgae biomass revealed that cells were attached to each other. According to the findings, the sphericity and surface smoothness of microalgae particles were consistently observed throughout culture in widely used media, Guillard's F/2 Media. In contrasts, the irregular nonporous morphology with cavities on the surface of cells were discovered when subjected to bioremediation process. The *Chlorella* sp. LF1 might be associated with the component presenting in the aquaculture wastewater and created the cell-wall bound substance. This hypothesis was supported by the development of a new peak, which is demonstrable by previously findings, in FTIR analysis.

This result is further confirmed by [30] that the the surface of *Chlorella* sp. had irregular nonporous morphology with cavities on the surface after the treatment. By studying the structure of the particle, the results could serve as a foundation for understanding that the bioremediation using microalgae have affect's the cells of microalgae. The SEM analysis also revealed significant changes in the morphology of the investigated microalgae.

Characterization the chemical composition on cell surface microalgae was analysis using the combination of SEM accomplished with X-ray (EDX) (Table 4). EDS analysis is important to study since its enable to provide valuable information regarding the composition adsorbent surface for a sample. It should be highlighted that SEM provides only a qualitative evaluation of the surface structure and not able to specify the internal structure of cell [31]. When SEM is combined with EDX technique, it can provide valuable input in determining the distribution of various elements on the microalgae biomass. Tables 3a and 3b represented the result of elemental analysis of microalgae biomass. The data in terms of atomic percentages demonstrated the presence of C, N, O, P and S, which are the main components of cellular macromolecules [32].

After the bioremediation process, the percentages of N and O on the surface of the microalgae biomass showed a higher accumulation in MW, whereas C, P, and S were the lowest when compared to microalgae cultivated in Guillard's F/2 Media. In contrast to the microalgae biomass in FW, N and O were less abundant than C, P, and *S. Maximum* absorption peaks in the spectral region of lipids and carbohydrates were also produced by the greater oxygen accumulation in MW [33].

4. Conclusions

As conclusion, the results suggest that different wastewater types require different inoculation dosages for optimal bioremediation efficiency. For MW, the highest bioremediation efficiency was achieved at 30 % inoculation dosage and for FW, the highest bioremediation efficiency was achieved at 40 % inoculation dosage. This study also demonstrated that the varying concentrations of microalgae have significant impact on the growth performance of microalgae. Additionally, microalgae acknowledged able to transform nutrients; nitrogen and phosphorus from wastewater into biomass and bioproducts to boost the sustainability of wastewater treatment. Overall, the successful application of bioremediation approach was accomplished using microalgae-based by nutrient consumption from aquaculture wastewater and is relevant for future application in the aquaculture industry.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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CRediT authorship contribution statement

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Nurfarahana Mohd Nasir: performed the experiment and manuscript writing, Ahmad Jusoh: design, supervise the research project and comments on the critical part of manuscript, Nik Nor Liyana Nik Ibrahim: formatting, conduct on final revision of manuscript comments on the critical manuscript writing, Razif Harun: formatting, conduct on final revision of manuscript comments on the critical manuscript writing, Nazaitulshila Rasit: formatting, conduct on final revision of manuscript comments on the critical manuscript writing, Wan Azlina Wan Abdul Karim Ghani: supervise of the research project and comments on the critical manuscript writing. Setyo Budi Kurniawan: reviewed drafts of the paper.

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ARTICLES FOR FACULTY MEMBERS

SHRIMP WASTEWATER BIOREMEDIATION

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Trends in shrimp processing waste utilization: An industrial prospective

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ABSTRACT

Background: Shrimp farming and processing plants are the largest seafood industry around the world due to their high demand and market value. The shrimp processing industry produces 50–60% waste of the catch volume. These wastes contain a large quantity of bioactive compounds including chitin, protein, lipid, carotenoid and minerals. Bioactive compounds from shrimp processing waste exhibit various bioactivities, and can be used as food and feed as well as ingredient in functional food preparation. The recent trend of shrimp waste utilization focuses on the bioremediation and energy conversion sectors.

Scope and approach: In this review, shrimp processing and the main bioactive compounds from shrimp waste were outlined. The recent applications of bioactive compounds from shrimp waste briefly describe in terms of different bioactivities, food and feed applications, and other industrial approach. Hurdles and future prospectus of shrimp processing waste utilization have been addressed.

Key findings and conclusions: Shrimp processing industries generate tremendous amount of waste, which can be extracted to obtain active compounds including chitin, carotenoids and protein hydrolysates, etc. These active compounds act as antioxidant, antimicrobial, anti-hypertensive, anti-inflammatory and anti-proliferative agents. Moreover, due to their functional and nutritional properties, these compounds could be used as natural safe additives or functional food/feed ingredients. Active compounds in shrimp waste open the doors of energy, solid wastes, and wastewater bioremediation. Hence, the future trends of shrimp waste utilization are the movement towards eco-friendly energy conversion, bioremediation and bioplastic area.

1. Introduction

Crustacean aquaculture is the largest seafood production sector around the globe, providing protein-rich food supply. Shrimp and shrimp products are widely consumed all over the world and their demand is increasing yearly owing to the delicacy and nutritional value. The global shrimp production reach to 5.03 million tons in 2020 and is expected to grow up to 7.28 million tons by 2025 with compound annual growth rate (CAGR) of 6.1% from 2020 to 2025 (IMARC, 2020). Furthermore, the shrimp market turnover value is assumed to reach 67.6 billion US dollars by the end of 2025 (Marketstudyreport, 2019, p. 102). Asia is a major contributor to shrimp farming with more than eighty percent of global shrimp production (Mao, Guo, Sun, & Xue, 2017). In Asia, Thailand is the top leading exporter of farmed shrimp to the USA, Europe, Canada, Japan, and South Korea (Senphan, Benjakul, & Kishimura, 2014). Shrimp and shrimp products from Thailand are well appreciated around the globe for their quality, freshness, and taste. Pacific white shrimp (*Litopenaeus vannamei*) has become the major commercial farming species in Thailand and hold 90% shrimp production around the world (Nirmal & Benjakul, 2012).

Generally, shrimp are stored and exported in the frozen form with or without shell, depending on the market demand. Therefore, during the shrimp processing, approximately 50–60% of solid waste is generated as by-products containing the head, viscera, and shell, etc. (Senphan & Benjakul, 2012). Moreover, washing and cooking process also generate wastewater pollution, around 1 gallon wastewater per ton of cooked shrimp (Djellouli, López-Caballero, Arancibia, Karam, & Martínez-Alvarez, 2020). This by-product constitutes about 45–50% of the catch and causes environmental pollution and disposal problems due to unregulated discharge (Sila, Ghlissi, et al., 2015; Vázquez et al., 2017). Although, a small quantity of shrimp waste is utilized as animal feed and an ingredient in aquaculture feed formulation (Mahata, 2012). Large quantities of this byproduct are being wasted, resulting in the loss of valuable bioactive components and increase environmental pollution.

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The recovery of bioactive molecules from the waste would be beneficial for the economy of shrimp processors and the country. This would also help to reduce environmental pollution owing to shrimp waste dumping.

Shrimp waste contained valuable bioactive components such as protein/peptides (Cahú et al., 2012), chitin/chitosan (Paul et al., 2015), pigments (Sila, Ghlissi, et al., 2015), enzymes (Senphan et al., 2014), lipids (Senphan & Benjakul, 2012), minerals (Gómez-Estaca et al., 2019) and vitamins (Nair et al., 2017). The amounts of each constituent depend on the sources and processing conditions. Researches have been focused on the improvement of waste utilization to obtain a higher percentage of yields (Gómez-Guillén, Montero, López-Caballero, Baccan, & Gómez-Estaca, 2018; Sinthusamran, Benjakul, Kijroongrojana, Prodpran, & Kishimura, 2020). Galanakis (2012) reported the established and emerging five stages recovery of high-added value product which include i) Macroscopic pretreatment, ii) Macro-and micro--molecules separation, iii) Extraction process, iv) Isolation and purification, and v) Product formation. Value-added products based on bioactive components from shrimp waste have been developed and their applications in pharmaceutical, food, and feed, environmental industries have been intensively studied (Gulzar, Raju, Chandragiri Nagarajarao, & Benjakul, 2020; Mao et al., 2017; Prameela et al., 2017; Sinthusamran et al., 2020).

This review focuses on the shrimp processing and different bioactive components from shrimp waste. Applications of bioactive components from shrimp waste in medicine, food, environmental and energy conversion industries were briefly described. In addition, hurdles and future trends of shrimp processing waste utilization were outlined. The information could pave the new way for scientists and engineers to utilize shrimp waste for value-added products. Moreover, the information will be beneficial for the biotechnologist and industrial personnel to exploit the shrimp processing waste for the sustainable environmental development.

2. Shrimp processing

Shrimp and shrimp products have attracted the majority of the seafood market due to their significant taste and flavor. Shrimp is a high market value product among fish and shellfish and is generally sold fresh. However, shrimp is vulnerable to deterioration and quality changes occur promptly after harvest owing to the presence of non-

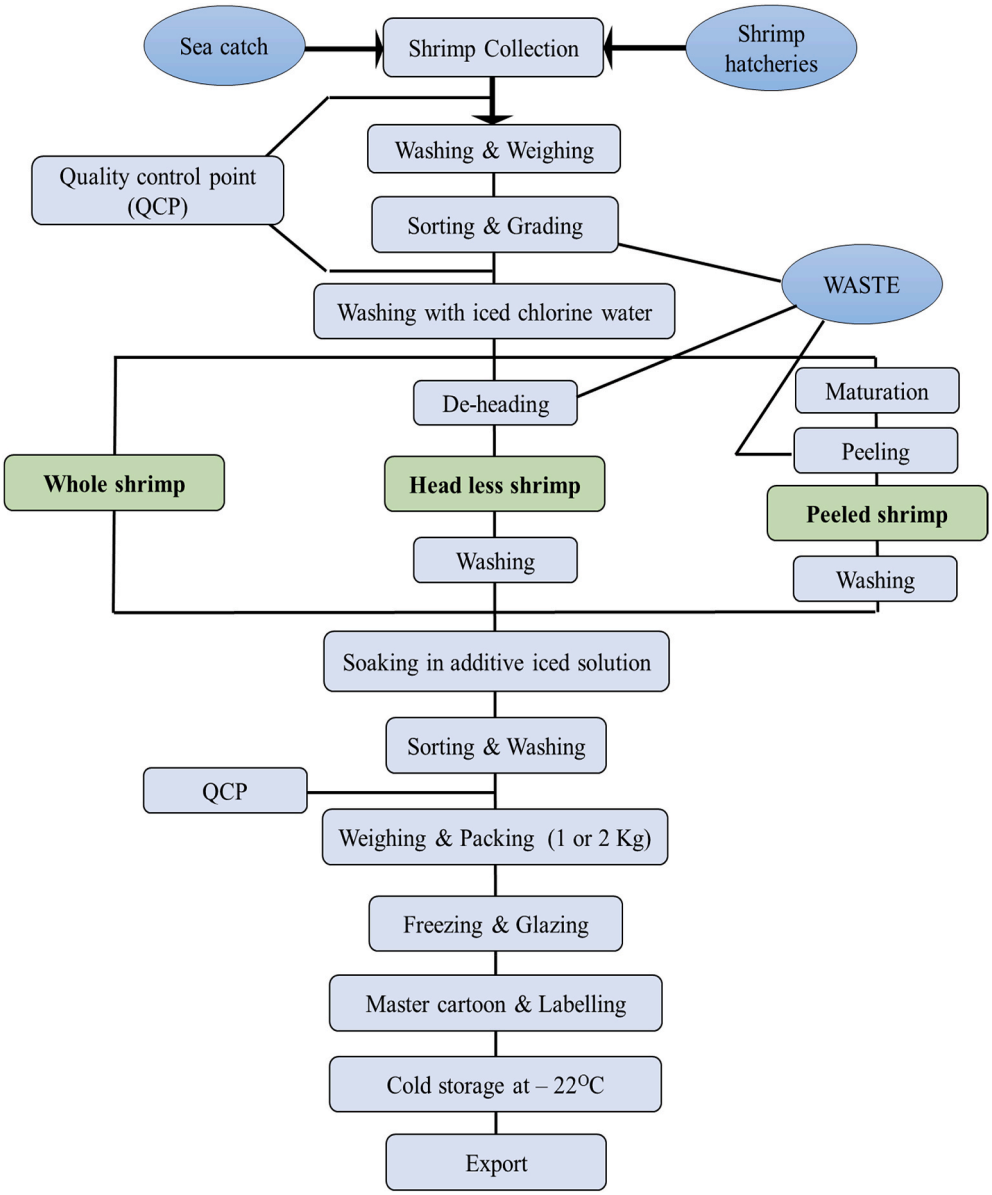

Fig. 1. Schematic presentation of shrimp processing.

protein nitrogenous compounds, high moisture content, neutral pH, and presence of digestive enzymes, as compared to fish (Nirmal & Benjakul, 2011b). This limits shrimp quality due to rapid microbial spoilage and black spot formation (Nirmal, Benjakul, Ahmad, Arfat, & Panichayupakaranant, 2015). Black spot formation (melanosis) in shrimp starts from head and spread up to the tail. Even though the blackening of shrimp does not harm consumer health, it impacts consumers' preference and value of the product (Nirmal & Benjakul, 2012). In this regard, shrimp processing starts onboard immediately after capture and stored in ice or frozen to avoid postmortem deterioration. Fig. 1 depicts the schematic presentation of fresh shrimp processing at the industry level. However, depending on the shrimp processing plant, more or less washing or additive can be implemented at different stages during processing.

Generally, shrimps are processed in three different categories such as i) whole shrimp, ii) de-headed shrimp, and iii) peeled shrimp (Anh, My Dieu, Mol, Kroeze, & Bush, 2011). As soon as shrimps arrive at the processing plants, shrimps can be processed on the same day or could be stored in a frozen room, if not process directly. Initially shrimps are washed by normal water to remove visible impurities. These shrimps get sorted and graded as per the requirement, where damage and unwanted shrimps are separated as a low-grade product or waste. Graded shrimp further undergo washing with iced chlorinated water for hygienic purpose (Anh et al., 2011). After the pre-wash treatment, shrimps are separated into three different sections as per the demand. The first section is whole shrimp, where whole shrimps are further processed with additive treatment to control the quality of the shrimps. Normally, 1.25% sodium metabisulfite is used as an additive in shrimp processing plants worldwide (Nirmal & Benjakul, 2011a). The second section consists of de-head shrimp, where shrimp are beheaded manually or using the machine. All de-headed shrimps are washed again to remove any unwanted debris and then soaked in an iced solution containing the formulated additives (Bini, Sudha, & Hatha, 2017). The third section is called as peeling section, in which shrimps are firstly soaked in iced water or brine water for maturation up to 48 h or more (Gringer et al., 2018). The maturation process loosens the muscle-shell attachment to facilitate the peeling process (Dang et al., 2018). After the peeling, shrimp meat is collected, washed, and treated with additive. All shrimps are washed again and the damaged shrimps are removed. At this point, a quick quality check is conducted and proceeds with weighing and packaging of 1 or 2 kg shrimps per package. Shrimps are frozen using the blast freezer or plate freezer. Frozen shrimps are again packaged in the master cartoon of various weight and size and labelled. Then those boxes are transferred to the frozen storage room at −20 °C until export.

Similarly, shrimps can be subjected to pre-cooking with the additional cooking step in the above process. Shrimps are pretreated with additives (sodium metabisulfite or phosphate) prior to the cooking process (Damasceno & Gonçalves, 2019). During sorting, beheading, peeling, washing, and cooking processes, nearly 50% of waste is generated as shrimp head, carapace, shell, cooking juice or washed etc (Gómez-Estaca, Montero, & Gómez-Guillén, 2018; Pérez-Santín, Calvo, López-Caballero, Montero, & Gómez-Guillén, 2013). Further, these shrimp processing waste were utilized as animal feed or fertilizer. Nowadays, the production of value-added compounds has gained increasing attention.

3. Bioactive compounds from shrimp waste

Biochemical content of shrimp waste revealed the presence of 10–40% protein, 15–46% chitin, 30–60% minerals, and 10–40% lipids (Bajaj, Winter, & Gallert, 2011; Takeungwongtrakul, Benjakul, & A, 2012; Tan, Lee, & Chen, 2020). Therefore, shrimp waste composition comprises nutritional and bioactive molecules. Nutritional components such as essential and nonessential amino acids, minerals (Ca, and P) and fat soluble vitamins (Vit. A, D, and E). Various bioactive compounds have been discovered such as chitin/chitosan, pigment (astaxanthin), protein hydrolysate (peptide), polyunsaturated fatty acids and α-tocopherol (Fig. 2).

3.1. Chitin and chitosan

Chitin is a polysaccharide accounted as the second most available biopolymer on the earth after cellulose and is widely present in the exoskeletons of shrimp or other crustaceans. Chitin is a biopolymer of β-1,4-N-acetyl glucosamine and chitosan is a deacetylated polymer form of chitin (Kandra, Challa, & Kalangi Padma Jyothi, 2012). The chitin content in shrimp waste varies from 14 to 30% of dry basis, depending on the extraction method (Tan et al., 2020). Extraction and isolation of chitin and chitosan have been major practice for researchers and industries, owing to their broad range of industrial applications (Hamed, Özogul, & Regenstein, 2016; Mao et al., 2017).

3.2. Pigment (carotenoid)

Pigments (carotenoid) play an important role in physiological function and also give characteristic pink-orange color to shrimp (Sila, Ghlissi, et al., 2015). Carotenoid is a fat-soluble pigment and can be extracted from head, hepatopancreas, and shell of shrimps (Gómez-Guillén et al., 2018; Nunez-Gastelum et al., 2016; Takeungwongtrakul et al., 2012). Astaxanthin is the major carotenoid 75–95% of total pigment) found in crustacean shells (Sila, Ghlissi, et al., 2015). The detailed chemistry, the role of astaxanthin in shrimp, extraction method, and application of astaxanthin have been reported by Prameela et al. (2017).

3.3. Protein hydrolysate

Proteins are the essential biomolecules for the physiological functions of living organisms. Shrimp waste discard contained high quality of protein, which is composed of 45% total proteins in shrimp (Kannan, Hettiarachchy, Marshall, Raghavan, & Kristinsson, 2011). When shrimp waste is hydrolyzed with proteolytic enzymes, more than 70% protein can be recovered as a hydrolysate (Gildberg, Arnesen, Sæther, Rauø, & Stenberg, 2011). Besides the protein, shrimp waste is a rich source of essential and non-essential amino acids (Latorres, Rios, Saggiomo, Wasielesky, & Prentice-Hernandez, 2018; Suparmi, Edison, Sari, Sumarto, & Susilo, 2020). Protein hydrolysate from shrimp waste has been reported to possess several functional and biological properties (Gildberg et al., 2011; Kannan et al., 2011; Latorres et al., 2018; Li-Chan, Cheung, & Byun, 2016).

3.4. Polyunstaurated fatty acids

Polyunstaurated fatty acids (PUFA) are well known as a good fatty acids with health promoting ability (Gómez-Guillén et al., 2018). Shrimp waste particularly hepatopancrease and cephalothorax are important source of highly unsaturated ω- 3 fatty acids such as eicosapentaenoic acid (EPA, 20:5n3) and docosahexanoenoic acid (DHA, 22:6n3) followed by oleic (C18:1n9c), palmitic (C16:0) and linoleic acid (C18:2n6c) (Gómez-Estaca, Calvo, Álvarez-Acero, Montero, & Gómez-Guillén, 2017; Senphan & Benjakul, 2012; Takeungwongtrakul et al., 2012). The lipid extracted from cephalothorax of Pacific white shrimp (*L. vannamei*) showed 37.5% of PUFA followed by 30.4% of saturated FA and 22.25% of monosaturated FA (Gulzar & Benjakul, 2018). Recently, extensive report on the extraction, composition, various bioactivities and industrial applications of oil from shrimp processing waste has been reviewed (Gulzar et al., 2020).

3.5. α –tocopherol (vitamin E)

α –Tocopherol (Vitamin E) is a fat soluble vitamin with anitoxidant properties and function in muscular and reproductive system (Afonso,

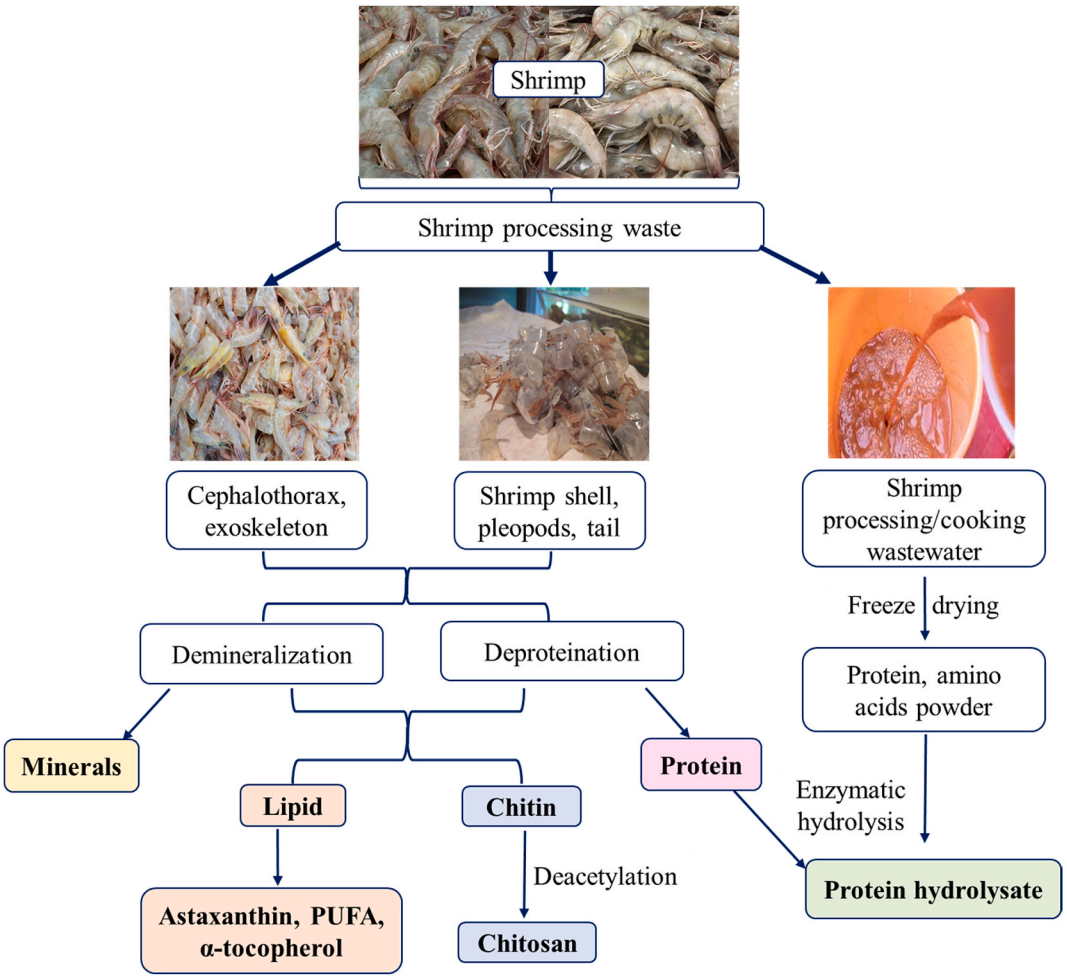

Fig. 2. Bioactive compounds recovery from shrimp processing waste.

Bandarra, Nunes, & Cardoso, 2016). Tocopherol content of the shrimp waste varied with different types or species, age, sex, shrimp waste part and the type of extraction process (Afonso et al., 2016; Gómez-Estaca et al., 2017; Gulzar & Benjakul, 2018). Tocopherol level found in brown shrimp meat (Merdzhanova, Dobreva, Stancheva, & Makedonski, 2014), and fermented shrimp waste (head and cephalothoraxes) (Sanches-Silva et al., 2011) was 7.73 and 50.5 mg/100 g, respectively. Higher tocopherol content (1.26 g/100 g) was reported in *L. vannamei* waste (cephalothorax, cuticles, tails and pleopods) extract (Gómez-Estaca et al., 2017). Tocopherol plays a vital role in the inhibition of lipid peroxidation in food system and inhibit oxidation of low density lipoprotein in living organism (Mathur, Ding, Saldeen, & Mehta, 2015).

4. Bioactivities of active compounds

Table 1 represents the active compounds extracted and isolated from various shrimp waste, extraction process, and their bioactivities. Bioactive compounds isolated from shrimp waste has been analyzed for various bioactivities such as antioxidant, antimicrobial, anti-inflammatory, ACE inhibitory, Anti-proliferative, wound healing, and antidiabetic activities, etc (Fig. 3).

4.1. Antioxidant activity

Oxidation is the normal physiological process occurring in a living organism. However, the excessive or unwanted process could cause the production of singlet oxygen or free radical by various routes (Sowmya & Sachindra, 2012). Bioactive compounds from shrimp waste including chitosan, protein hydrolysate, carotenoprotein, astaxanthin, and tocopherol show strong antioxidant effects by various mechanisms of actions (Ambigaipalan & Shahidi, 2017; Chintong, Phatvej, Rerk-Am, Waiprib, & Klaypradit, 2019). Chitin was enzymatically extracted from shrimp (*Metapenaeus Monoceros*) shell using *Bacillus mojavensis* A21 or *Balistes capriscus* proteases (Younes et al., 2014). The extracted chitin was further deacylated to chitosan and evaluated for antioxidant activity. Both chitosan showed reducing power, DPPH radical scavenging activity, and β-carotene bleaching inhibitory activity (Younes et al., 2014). Shrimp shell hydrolysates (SSH) and isolated shrimp shell protein hydrolysates (SPH) were prepared using various enzymatic treatments (Ambigaipalan & Shahidi, 2017). Both hydrolysates effectively scavenged free radicals and showed good reducing power and ferrous metal chelating activity. Moreover, SSH and SPH inhibited cupric ion-induced cholesterol peroxidation, β-carotene bleaching, and DNA damage induced by peroxyl and hydroxyl radical (Ambigaipalan & Shahidi, 2017). Shrimp (*Fenneropenaeus chinensis*) shell waste was hydrolyzed using neutrase to produce shrimp shell waste hydrolysate with DPPH radical scavenging ability, reducing power and lipid peroxidation inhibition capacity (Yuan, Li, Pan, Wang, & Chen, 2018). Protein hydrolysate prepared from shrimp shell waste by fed-batch biodegradation process using *Bacillus cereus* EW5 culture showed an improved antioxidant activity compared to batch biodegradation (Rashid, Jung, & Kim, 2018).

Astaxanthin was extracted and isolated from the head of *L. vannamei* using autolysis at 40 °C for 2 h, followed by ethanol fractionation (Santos et al., 2012). Isolated astaxanthin showed the inhibition against the oxidation induced by lipopolysaccharide in rat alveolar

Table 1
Active compound from shrimp waste and their bioactivities.

Active compound	Source	Extraction process	Bioactivities	References
Chitin/chitosan	Shrimp (*M. Monoceros*) shell	Enzymatic treatment	Antioxidant activity (DPPH radical scavenging activity, reducing power, and β-carotene bleaching activity) Antimicrobial activity Antitumor activity (Human bladder cell line, RT112)	Younes et al. (2014)
	Shrimp (*L. vannamei*) shell	Alkali and acid treatment	Antimicrobial activity (*Stenotrophomonas maltophilia, Enterobacter cloacae and bacillus subtilis*)	Vilar Junior et al. (2016)
	Shrimp (Nephropidae) shell	Chemical method	Application in dentistry, bone tissue engineering	Wedagama, Widjajanto, Parjianto, and Sumitro (2016)
	Shrimp head	Chemical method	Chitosan based film incorporated with gelatin, chondrioting-4-sulfate and zinc oxide for would healing properties	Cahu et al. (2017)
	Shrimp shell waste	Chemical method	Antibacterial activity against human pathogens and food contaminant (*E. coli, Shigella flexneri, B. subtilis, S. typhi, E. faecalis, and Proteus vulgaris*)	Jadhav and Diwan (2018)
	Shrimp (*P. mondon*) shell	Acid and Alkali treatment	Anticancer activity against ovarian cancer cell line (PA-1)	Srinivasan et al. (2018)
Astaxanthin (Carotenoid)	Pacific white shrimp (*Litopenaeus vannamei*) waste heads	Autolysis followed by solvent fraction	Antioxidant activity (Superoxide and nitric oxide scavenging activity). Anti-inflammatory activity (Inhibition of tumor necrosis factor-α in rat alveolar macrophages)	Santos et al. (2012)
	Shrimp waste (Head and carapace)	Fermentation followed by solvent fractionation	Hypoglycemic effect in alloxan induced diabetic mice	(J. J. Wang et al., 2012)
	Deep-water pink shrimp (*Parapeneus longirostris*) shells	Solvent fractionation	Antioxidant activity (DPPH radical scavenging activity, reducing power, β-carotene bleaching and DNA nicking assay) Antiproliferative activity against human laryngeal carcinoma (Hep 2) cells	Sila et al. (2013)
	Shrimp shell waste (head, thorax and appendix)	Solvent fractionation	Anti-diabetic activity (Astaxanthin lowered oxidative damages and pathological changes in diabetic mice)	Sila, Ghlissi, et al. (2015)
	Shrimp (*P. vannamei Boone*) waste	High pressure and solvent	Antioxidant activity (DPPH and superoxide anion radical scavenging activity)	Li et al. (2017)
	Shrimp (*L. vannamei*) shell	Solvent extraction	Antioxidant activity (DPPH and ABTS radical scavenging activity, singlet oxygen quenching activity and β-carotene bleaching assay) Tyrosinase inhibitory activity No cytotoxic effect on human dermal fibroblast cells	Chintong et al. (2019)
	Asian tiger shrimp (*Penaeus monodon*) shell	Methanol extraction	Antibacterial activity (*E. Coli, S. mutans, Pseudomanas auriginosa, S. typhi, S. aureus*) Anti-inflammatory activity	Sukmawati et al. (2019)
Caroteno-protein	Shrimp waste	Enzymatic hydrolysis using alkaline protease from the viscera of the *Serranus scriba*	Antioxidant activity (DPPH radical scavenging activity and β-carotene bleaching activity)	Nasri et al. (2015)
	Northern shrimp (*Pandalus borealis*)	Enzymatic hydrolysis followed by cation exchange chromatography	ACE inhibitory activity *In vivo* experiments with hypertensive rats exhibited anti-hypertensive activity. Two novel tri-peptide (Phe-Thr-Tyr and Phe-Ser-Tyr)	Gildberg et al. (2011)
Protein/protein hydrolysate/ peptide	Shrimp waste	Enzymatic hydrolysis using cryotin enzyme	Anti-proliferation activity against colon and liver cancer cells	(A. Kannan et al., 2011)
	Shrimp	Cooking juice	Antioxidant activity (ABTS radical scavenging activity, Ferric reducing antioxidant power, metal chelating activity, and photoluminescence assay) ACE-inhibitory activity	Pérez-Santín et al. (2013)
	Shrimp processing waste	Fermentation process using *Xerocomus badius*	ACE inhibitory activity	(Gao et al., 2014)
	Shrimp (*L. vannamei*) shell waste	Enzymatic hydrolysis using alcalase	ACE inhibitory activity	Feng et al. (2016)
	Shrimp (*P. vannamei*) cooked	Giant catfish viscera protease, Trypsin and Alcalase	Hypoglycemic and antidepressant (Dipeptidyl peptidase-IV (DPP-IV) and prolyloligopeptidase (PO) inhibitory activity)	Ketnawa et al. (2016)
	Shrimp (*Pandalopsis dispar)* waste	Enzymatic hydrolysis using portamex	β-secretase inhibitory activity Peptide identified as Asp-Val-Leu-Phe-His	Li-Chan et al. (2016)
	Shrimp shell	Enzymatic hydrolysis using alcalase	Antioxidant activity (DPPH, ABTS and hydroxyl radical scavenging activity, reducing power, ferrous chelating capacity, β-carotene bleaching activity, inhibition of peroxidation, Protective effect against peroxyl and hydroxyl radical induced DNA damage) ACE inhibitory activity	Ambigaipalan and Shahidi (2017)
	Pacific white shrimp (*L. vannamei*) shell	Biodegradation using *Bacillus cereus* EW5	Antioxidant activity (DPPH radical scavenging activity, reducing power, DNA protection activity)	Rashid et al. (2018)
	Oriental shrimp (*Fenneropenaeus chinensis*) shell	Enzymatic hydrolysis using neutrase	Antioxidant activity (DPPH radical scavenging activity, metal reducing ability and lipid peroxidation inhibition) α-amylase inhibitory activity	Yuan et al. (2018)
	Shrimp (*L. vannamei*) cooked juice and carapace	*Enterococcus faecalis* DM19 enzyme extract	Antioxidant activity (ABTS radical scavenging activity, FRAP, metal chelating ability) Antimicrobial activity (*Lactobacillus helveticus, L. innocua, S. aureus, Citrobacter freundii, E.coli,* and *P. fluorescens*)	Djellouli et al. (2020)

(*continued on next page*)

Table 1 (*continued*)

Active compound	Source	Extraction process	Bioactivities	References
Lipid (astaxanthin, α-tocopherol and PUFA)	Shrimp shell waste	Solvent extraction	Shrimp oil fed to obese rat showed improved glucose tolerance, insulin response, adiponectin, antioxidant capacity and lowered serum insulin, leptin, hemoglobin Alc, oxidative stress and inflammation, dose dependently.	Nair et al. (2017)
	Shrimp (*L. vannamei*) cephalothorax	Solvent fractionation	Antioxidant activity in lipophilic and hydrophilic medium. Anti-inflammatory activity.	Gómez-Guillén et al. (2018)

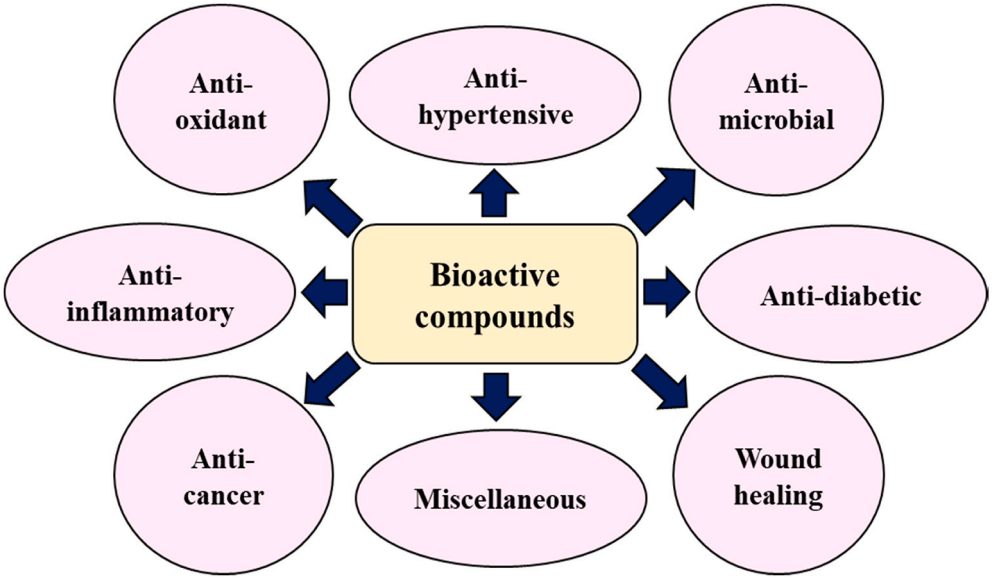

Fig. 3. Medicinal properties of bioactive compounds from shrimp processing waste.

macrophages. It was noted that astaxanthin successfully suppressed the synthesis of superoxide and nitric oxide free radicals in alveolar macrophage (Santos et al., 2012). In other study, Sila et al. (2013) reported that astaxanthin extracted from deep water pink shrimp shell waste showed higher antioxidant activity than commercial antioxidant BHA. Moreover they noted high lipophilic antioxidant activity in β-carotene bleaching assay and also confirmed DNA protection against hydroxyl radical (Sila et al., 2013). Carotenoproteins extracted from shrimp waste using alkaline protease from *S. scriba* viscera showed DPPH radical scavenging activity and inhibition of β-carotene bleaching (Nasri, Abed, Karra-châabouni, Nasri, & Bougatef, 2015). High pressure (HP) technique was also used for the extraction of astaxanthin from *P. vannamei boone* shrimp byproducts (Li et al., 2017). The extracted astaxanthin showed strong DPPH and superoxide anion radical scavenging capacity. DPPH radical scavenging ability of astaxanthin was 940 and 440 folds higher than those of the standard vitamin C and vitamin E solution, respectively. Similarly, superoxide anion radical scavenging activity was 710 and 350 folds higher than the standard vitamin C and vitamin E solution, respectively (Li et al., 2017). In their study, Gómez-Guillén et al. (2018) extracted lipid containing astaxanthin, α-tocopherol, and PUFA from the cephalothorax of *L. vannamei* and further encapsulated using spray-drying. Only the encapsulated lipid showed lipophilic and hydrophilic antioxidant activity, more likely related with stable astaxanthin, and α-tocopherol (Gómez-Guillén et al., 2018). Astaxanthin was extracted from shrimp (*L. vannamei*) shell using ethanol as a solvent (Chintong et al., 2019). Astaxanthin showed potent antioxidant activity for DPPH radical, ABTS radical, inhibition of β-carotene bleaching, and singlet oxygen quenching ability. In addition, astaxanthin did not have any toxic effect on human dermal fibroblast cells. The antioxidant effect of astaxanthin is related to its molecular structure, presence of hydroxyl (OH) group, keto (C═O) group, and the number of conjugated double bonds (Chintong et al., 2019).

4.2. Anti-microbial activity

Microorganisms are an integral part of the living system and play an important role in nature. However, some microorganisms are not friendly to a human being or food products, causing serious damage to life. Therefore, synthetic chemical drugs widely used by doctors, scientists, and microbiologists have been employed to control or eliminate the detrimental effect of microorganisms. In response to the continuous use of synthetic drugs, some of the microorganisms got resistance to the drug. Hence, a natural anti-microbial compound is inevitably required. Various plant, herbs, fruits, and their waste have been examined for the search of natural antimicrobial compounds. Similarly, seafood waste is one of the promising sources for the ample of bioactive compounds with various biological activities. Shrimp (*Metapenaeus Monoceros*) shell was enzymatically extracted using *Bacillus mojavensis* A21 and *Balistes capriscus* proteases to obtain chitin and further deacylated to produce chitosan (Younes et al., 2014). Chitosan produced by two different enzymes showed antimicrobial activities against Gram-positive (*S. aureus, M. luteus, B. cereus* and *E. faecalis*), Gram-negative (*E. coli, P. aeruginosa, K. pneumoniae,* and *S. typhi*) bacteria and fungi (*F. oxysporum, F. solani* and *F.* Sp) (Younes et al., 2014). Chitosan extracted from *L. vannamei* shell using chemical methods showed antibacterial activity against Gram-positive and Gram-negative bacteria (Vilar Junior, Ribeaux, Alves da Silva, & Campos-Takaki, 2016). The minimum inhibitory concentration of chitosan required to inhibit *S. maltophilia, B. subtilis,* and *E. cloacae* are 78, 625, and 156 μg/mL, respectively. Chitosan extracted and isolated from shrimp shell waste using chemical processes showed their antibacterial effect against human pathogenic bacteria and food contaminants (Jadhav & Diwan, 2018). Chitosan inhibited bacteria in a dose-dependent manner, in which the highest zone of inhibition was achieved for *E. coli* (12.70 mm), *P. vulgaris* (9.77 mm), *S.flexneri* (11.37 mm), *S. typhi* (8.90 mm) and *E. faecalis* (10.40 mm). The antibacterial

activity of chitosan was attributed to the free NH_2^+ groups (Jadhav & Diwan, 2018). In addition, chitosan could interact with the cell membrane and make lysis of cells. Chitosan could also form a polymeric layer on the surface of the cell and block the nutrient of the cell (Vilar Junior et al., 2016; Younes et al., 2014).

Protein hydrolysate prepared from shrimp cooking juice and carapace of *L. vannamei* shrimp were heated with glucosamine at 100 °C for the improvement of antimicrobial activity (Djellouli et al., 2020). Protein hydrolysate heated with glucosamine showed higher antimicrobial activity compared to hydrolysate alone. They concluded that glycation of protein hydrolysate enhanced the hydrolysate ability to inhibit the bacterial growth (Djellouli et al., 2020). Astaxanthin extracted from Asian tiger shrimp (*P. monodon*) shell using the solvent fractionation method (Sukmawati, Fawwaz, Pratama, & Hasrwati, 2019). Astaxanthin showed potent inhibitory effect against *E. coli, S. mutans, P. auriginosa, S. typhi* and *S. aureus*. The antibacterial effect of astaxanthin could be related to the lipophilic nature of the compound, which can interact and disrupt the cell membrane of bacteria (Sukmawati et al., 2019).

4.3. ACE inhibitory activity

Angiotensin I-converting enzyme (ACE) is the main enzyme in blood pressure regulation. The uncontrolled active form of ACE can cause high blood pressure and hypertension in individuals (Feng, Limwachiranon, Luo, Shi, & Ru, 2016). Protein hydrolysates or peptides from shrimp waste are proven to be safe and effective compounds to inhibit ACE (Ambigaipalan & Shahidi, 2017; Pérez-Santín et al., 2013). Northern shrimp (*P. borealis*) was enzymatically hydrolyzed and fractionated on cation exchange chromatography to obtain two new ACE inhibitory tri-peptide (Phe-Thr-Tyr and Phe-Ser-Tyr) (Gildberg et al., 2011). Spontaneously hypertensive rats fed with 60 mg hydrolysate per kg body weight per day showed anti-hypertensive effect, compared to the control. In another study, shrimp cooking juice was separated using centrifugal separator to obtain a protein and lipid rich concentrate (Pérez-Santín et al., 2013). The concentrate showed ACE inhibitory activity with IC_{50} value of 3.8 mg/mL and inhibitory activity was related with the presence of small peptide (502–1355 Da) (Pérez-Santín et al., 2013) Shrimp wastes were fermented with *Xerocomus badius* to obtain biodegraded functional broth with ACE inhibitory activity (Gao et al., 2014).

Shrimp (*L. vannamei*) shells hydrolyzed using neutral protease produced ACE inhibitory peptides (Feng et al., 2016). A peptide with less than 5 kDa showed 84.04% inhibitory activity against ACE. Similarly, shrimp processing discards were hydrolyzed using trypsin, chymotrypsin, pepsin, and alcalase to obtain shrimp shell protein hydrolysate (SPH) (Ambigaipalan & Shahidi, 2017). SPH showing the highest ACE inhibitory activity was fractionated using gel filtration and identified to be three potential bioactive peptides by MS. Peptide sequencing revealed the presence of hydrophobic, aromatic and branch chain amino acids (Val, Leu, Pro, Phe, and Glu) at higher proportions in all peptides (Ambigaipalan & Shahidi, 2017). Moreover, protein hydrolysate having ACE inhibitory activity contained proline or aromatic amino acid at the C-terminal end of peptide (Feng et al., 2016; Gildberg et al., 2011).

4.4. Anti-inflammatory activity

Inflammation is the body's protective physiological response against external harmful stimuli such as pathogens, free radicals, and dead cells. Anti-inflammatory compounds suppress the activity of pro-inflammatory cytokines such as NF-α, IL-1, and IL-6 (Santos et al., 2012). Astaxanthin extracted from the head of *L. vannamei* was examined for the inflammatory response in rat alveolar macrophages induced by phorbol myristate and lipopolysaccharide (LPS) (Santos et al., 2012). Astaxanthin at 43.5 μg/mL was not only non-cytotoxic but also increased the cell viability to 168%. Astaxanthin curtailed the formation of superoxide and nitric oxide free radicals by inhibiting TNF-α. (Santos et al., 2012). Lipid containing astaxanthin, α-tocopherol, and PUFA was extracted from the cephalothorax of *L. vannamei* by solvent extraction (Gómez-Guillén et al., 2018). Lipid treated with LPS induced RAW 264.7 cell line showed reduced nitric oxide production compared to the control. The anti-inflammatory effect of lipid was related to the antioxidant properties of lipids (Gómez-Guillén et al., 2018). Astaxanthin was obtained from the Asian tiger shrimp shell using the solvent extraction method and had the anti-inflammatory activity in the stability of the erythrocyte membrane (Sukmawati et al., 2019). Astaxanthin treated red blood cell showed dose-dependent stability on the erythrocyte membrane, and the concentration of 1000 ppm showed the highest anti-inflammatory activity (Sukmawati et al., 2019).

Anti-inflammatory properties of shrimp lipid containing astaxanthin, α-tocopherol, and PUFA were associated with their antioxidant capacity owing to its molecular structure, presence of hydroxyl, and ketone group (Gómez-Guillén et al., 2018; Santos et al., 2012).

4.5. Miscellaneous

Bioactive compounds from shrimp processing waste also have anti-diabetic, anti-proliferative, antitumor, wound healing, and enzyme inhibitory activities. Shrimp (*P. setiferus*) shell was hydrolyzed using food-grade cryotin enzyme to obtain gastrointestinal resistant peptide hydrolysate (Kannan et al., 2011). Furthermore, the hydrolysate was separated into three different fractions, depending on their molecular weight (<10, 10–30, and >30 kDa fractions). Fractionated samples were evaluated against colon and liver cancer cell growth and the result showed that fractions less than 10 and 10–30 kDa, significantly inhibited both cancer cell growth by 60%, thus proving the anti-proliferative activity of peptide (Kannan et al., 2011). Astaxanthin extracted from *P. longirostris* shell waste showed anti-proliferation activity (300 μg/mL) against human laryngeal carcinoma (Hep 2 cells) tumor cells (Sila et al., 2013). Chitosan recovered from *M. Monoceros* shell using bacterial proteases was evaluated for anti-tumor activity against human bladder cancer cell line (RT112) (Younes et al., 2014). It was noted that the cytotoxic effect of chitosan was dose and time-dependent as well as the lower molecular weight exhibited the highest antitumor activity. Chitin and chitosan obtained from *P. mondon* shell were examined for anti-cancer activity against human ovarian cancer cell line (PA-1) (Srinivasan, Velayutham, & Ravichandran, 2018). Results showed that chitin and chitosan potentially suppressed the 100% growth of PA-1 cell line at 50 and 10 μg/mL, respectively, indicating that chitosan was more effective than chitin. Furthermore, it was concluded that inhibitory effect of chitin and chitosan was related with their structure and the presence of positively charged groups, which probably bind with negative charge surface of cancer cell line and block their nutrient supply or cause cell lysis (Srinivasan et al., 2018; Younes et al., 2014).

Astaxanthin extracted from shrimp waste using solvent fractionation was assessed for the hypoglycemic effect in diabetic mice induced by alloxan (Wang, Chen, & Lu, 2012). Astaxanthin at 5 and 10 mg/kg fed to diabetic mice showed significantly lowered plasma glucose level compared to control, indicating the beneficial hypoglycemic effect of astaxanthin in Type-I diabetes mellitus. Hypoglycemic effect and antioxidant effect of astaxanthin prepared from shrimp processing waste were investigated on the kidney of diabetic rats (ADR) (Sila, Ghlissi, et al., 2015). ADR supplemented with astaxanthin showed significantly lowered glucose, creatinine, urea, and uric acid level, compared to ADR alone. Moreover, astaxanthin fed rat had reduced plasma and kidney malonadialdehyde and protein carbonyl levels, with increasing antioxidant enzyme activities. In addition, astaxanthin helps to improve glomerular and tubular appearance of the kidney, compared to diabetic rats (Sila, Ghlissi, et al., 2015). In another study, Sila, Kamoun, et al. (2015) mentioned the protective effect of astaxanthin extracted from *P. longirostris* against the liver oxidative stress in diabetic male rats. They found that astaxanthin treated ADR showed significantly lowered total lipid, lipid peroxidation, and glycemia in comparison with the control

group. Additionally, astaxanthin boosted the antioxidant enzyme system and lowered the aspartate and alanine transaminase, and alkaline phosphatase in plasma, thereby confirming the protective effect against the liver oxidative stress in diabetic rats (Sila, Kamoun, et al., 2015). In their study Nair et al. (2017) examined the effect of shrimp lipid (astaxanthin, α-tocopherol, and PUFA) feed on obese rats fed with high fat diet. Rats fed with shrimp oil effectively improved insulin response, glucose tolerance compared to normal diet fed rats. In addition shrimp oil, reduced serum insulin, leptin, free fatty acids, oxidative stress and chronic inflammation. Hence shrimp oil containing astaxanthin, PUFA and tocopherol significantly improved glycemic control in obese rats through multiple actions (Nair et al., 2017).

Amyloid beta (Aβ) production in excessive level in the brain is related to the Alzheimer's disease. β-secretase (an aspartic peptidase) plays a vital role in the synthesis of Aβ (Li-Chan et al., 2016). Shrimp (*P. dispar*) wastes were hydrolyzed using protamex and hydrolysate was fractionated using Sephadex G-25 column chromatography and β-secretase inhibitory activity was monitored (Li-Chan et al., 2016). The purified peptide was identified using Q-TOF MS/MS as Asp-Val-Leu-Phe-His with 629 Da molecular weight. Active peptide showed an IC_{50} of 92.70 μM against β-secretase with competitive inhibition kinetics (Li-Chan et al., 2016). One of the strategies to control type-II diabetes is to inhibit the α-amylase activity, which lowered serum glucose levels (Yuan et al., 2018). In this concern, shrimp shell waste hydrolysate was prepared using neutrase and examined for α-amylase inhibitory activity. Shrimp shell hydrolysate mainly contained smaller peptides (<4 kDa) and high essential amino acids (278 mg/g) with α-amylase inhibitory activity (43.4%) (Yuan et al., 2018). Results indicated that shrimp shell hydrolysate could be used as a functional food or nutraceutical for type-II diabetic patients. Ketnawa et al. (2016) produced protein hydrolysate from cooked shrimp (*P. vannamei*) using different enzymes (Giant catfish viscera protease, Tyrpsin and Alcalase) and studied their dipeptidyl peptidase-IV (DPP-IV) and prolyloligo peptidase (PO) inhibitory activity. They found that all hydrolysates at 1 mg/mL concentration effectively inhibited DDP-IV activity, whereas only hydrolysate prepared from trypsin and alcalase showed PO inhibitory activity. Therefore shrimp protein hydrolysate could be used as a potential hypoglycemic and antidepressant agent (Ketnawa et al., 2016).

Tyrosinase enzyme is important for the skin melanin synthesis and excessive uneven production of melanin can cause skin pigmentation (Chintong et al., 2019). Astaxanthin extracted from *L. vannamei* shell showed anti-tyrosinase activity with an IC_{50} value of 121.2 μg/mL. The presence of oxygenated groups in astaxanthin structure could involve in chelation of copper in the active site of tyrosinase, causing inactivation of enzyme (Chintong et al., 2019). Similarly, proteins have a tendency to form a complex with different enzymes and make them un-functional by blocking the active site pocket or change the active structure of the final complex (Li-Chan et al., 2016; Yuan et al., 2018).

Chitosan extracted from shrimp head by the chemical method and chitosan-based film incorporated with gelatin and chondriotin-4-sulfate with or without zinc oxide were prepared (Cahu et al., 2017). Furthermore, prepared films were evaluated for wound healing properties. Film-forming solutions showed no toxicity toward fibroblast or keratinocytes. In addition, chitosan was able to induce agglutination of red blood cells. The chitosan-based film significantly increased wound healing from 65 to 86% in full excision skin than control. Results suggested that chitosan based film could be able to produce biocompatible environment for fibroblast cell growth (Cahu et al., 2017).

5. Food and feed applications of active compounds

5.1. Food applications

Quality control of food during the process and storage is an important task for food industries. To avoid any quality deterioration of food, most food industries frequently use synthetic chemicals as additives. However, strict food additive regulation and consumer awareness for synthetic chemicals, open the way for natural food additives. The bioactive compounds from shrimp processing waste and their food and feed applications are presented in Table 2. Chitosan obtained from shrimp waste and nano form of chitosan was produced by chemical methods (Darwesh, Sultan, Seif, & Marrez, 2018). Chitosan and nano-chitosan showed non-toxicity using brine shrimp and rat bioassay. Furthermore, both chitosan showed antimicrobial activity against gram-positive and gram-negative bacteria, yeast, and fungi. Results showed that both chitosan can be used as a food ingredient due to their antimicrobial and non-toxicity (Darwesh et al., 2018). In their study, Abdelmalek et al. (2016) extracted the astaxanthin from shrimp byproduct and evaluated their effect on marinated chicken steak during storage at 4 °C for 7 days. Results showed that 10 mg astaxanthin/kg chicken steak effectively controlled lipid oxidation and microbial growth during storage. Moreover, quality control of chicken steak by astaxanthin was confirmed by sensory evaluation. The lipophilic antioxidant activity of astaxanthin played the major role in the quality control of chicken steak (Abdelmalek et al., 2016).

Protein hydrolysates produced from *P. monodon* and *P. indicus* waste using alcalase hydrolysis and their effect on the quality control of croaker fish fillet was studied during refrigerated storage for 10 days (Dey & Dora, 2014). Protein hydrolysate (5 mg/mL) retarded the lipid oxidation and maintained the fresh appearance of croaker fish fillet. Protein hydrolysate was reported with antioxidant activity in different assay systems (Dey & Dora, 2014). Ketnawa et al. (2016) reported that protein hydrolysate prepared from cooked shrimp (*P. vannamei*) showed good emulsifying properties, solubility (>97%) in broad pH range (3–10) and had oil binding capacity (0.86–1.83 g oil/g hydrolysate). Ghorbel-Bellaaj, Maalej, Nasri, and Jellouli (2018) fermented shrimp (*M. monoceros*) by-products using *Pseudomonas aeruginosa* A2 to obtain protein-rich liquid hydrolysate. The obtained hydrolysate was dried using oven drying at 70 °C and freeze-drying. Both powder hydrolysates showed good functional properties in terms of solubility, emulsifying properties, and oil retention ability. Furthermore, both powders exhibited good antioxidant activity in various assays with different capacities. Results showed that shrimp waste hydrolysate can be a promising food additive with functional and antioxidant properties (Ghorbel-Bellaaj et al., 2018). Protein hydrolysates were prepared from *L. vannamei* waste using alcalase and protamex at different hydrolysis rates (Latorres et al., 2018). Hydrolysates prepared from both enzymes showed good functional properties including solubility, emulsifying, and foaming properties. In addition, the antioxidant activity of hydrolysate varied with different degree of hydrolysis in a dose-dependent manner. The result indicated the potential of protein hydrolysate from *L.vannamei* as a natural antioxidant in the lipid food system (Latorres et al., 2018).

Functional food is a food with added nutrient or bioactive compounds from other sources (da Silva et al., 2017). Shrimp head fermented with *Bacillus licheniformis* OPL-007 to obtain bioactive supernatant (Mao et al., 2013). Bioactive molecules found in the supernatant were total phenols (888.8 mg/L), polysaccharides (402.7 mg/L), reducing sugars (85.9 mg/L), free amino acids (2061.8 mg/L) and organic acids (5426.7 mg/L). Moreover, the supernatant showed strong antioxidant activity in various assay systems. Therefore, supernatant produced from shrimp head fermentation by OPL-007 could be used as a nutritional ingredient for functional food preparation (Mao et al., 2013). Lipid extracted from *L. vannamei* waste contained abundant fatty acids (C16:0, C18:2n6C, C18:1n9c, C22:6n3, andC20:5n3), astaxanthin, and its ester form and α-tocopherol (Gómez-Estaca et al., 2017). The extracted lipid was stable until 120 days at room temperature owing to the presence of powerful antioxidants such as astaxanthin and α-tocopherol. It was concluded that lipid extract from shrimp waste could be used as a food ingredient due to its coloring capacity and antioxidant properties (Gómez-Estaca et al., 2017). In another study, shrimp (*L. vannamei*) cephalothorax was fermented with commercial

Table 2
Food and Feed applications of bioactive compounds from shrimp waste.

Active compound	Source	Application	Activity	References
Food industries				
Chitin and chitosan	Shrimp exoskeleton	Active food ingredient	Antimicrobial activity and non-toxicity up to 200 mg/kg body weight of rat	Darwesh et al. (2018)
Carotenoid/Lipid (astaxanthin, α-tocopherol and PUFA)	Pink deep-water shrimp (*P. longirostris*) head, cephalothorax and appendices	Natural antioxidant additive for quality control of marinated chicken steaks	Antioxidant activity	Abdelmalek et al. (2016)
	Shrimp (*L. vannamei*) shell waste	Functional ingredient and food coloring	Antioxidant activity Water solubility and gastrointestinal digestion capability	Montero et al. (2016)
	Shrimp (*Litopenaeus sp*) waste	Nutraceutical and coloring food ingredient	Antioxidant activity	Nunez-Gastelum et al. (2016)
	Shrimp (*L. vannamei*) waste	Functional food ingredient	Antioxidant activity, bioactive compound and coloring agent	Gómez-Estaca et al. (2017)
	Shrimp (*L. vannamei*) cephalothorax	Functional food ingredient	Antioxidant activity, functional properties and coloring agent	Gómez-Guillén et al. (2018)
Proteins/Protein hydrolysate	Shrimp (*P. mondon* and *P. indicus*) waste	Antioxidant for quality preservation of Croaker fish	Antioxidant activity Chemical and sensory quality control of Croaker fish during 10 days of refrigerated storage.	Dey and Dora (2014)
	Shrimp (*L. vannamei*) head	Nutritional supplement in diet	High protein content, higher protein digestibility, improve physiological parameters in Wistar male rats	da Silva et al. (2017)
	Shrimp (*M. Monoceros*) waste, cephalothorax, and appendix	Functional food additive	Solubility, emulsifying properties and oil retention capacity Antioxidant activity	Ghorbel-Bellaaj et al. (2018)
	White shrimp (*L. vannamei*) muscle	Functional and antioxidant properties in lipid food system	Solubility, foaming and emulsifying properties, Antioxidant activity	Latorres et al. (2018)
Polysaccharides, total phenols, sugars, organic acids, essentials, free, and non protein amino acids	Shrimp head waste	Functional food	Antioxidant activity	Mao et al. (2013)
Feed industries				
Shrimp waste meal	Shrimp waste	Feed source	Digestibility, and absorption of shrimp waste meal in juvenile cobia (*Rachycentrom canadum*), plus improved chitinase activity in cobia	Lu and Ku (2013)
Wet waste	Shrimp (*L. vannamei*) waste	Protein and mineral source	Dietary supplement for sea cucumber growth rate, and fecal production rate	Chen et al. (2014)
Chemical composition and total carotenoid	Shrimp waste (Heads and shells)	Aqua feed	*In-vitro* digestibility	Thongprajukeaw (2014)
Astaxanthin	Shrimp waste	Fish feed for goldfish	Antioxidant activity Antibacterial activity	Weeratunge and Perera (2016)
Protein hydrolysate	Shrimp waste	Dietary supplement and immunomodulatory effect in fish	Improved growth performance and immune system in European sea bass	Gisbert et al. (2018)
Carotenoprotein	Shrimp shell waste	Nutritive feed ingredient in animal diet	High protein, amino acid and carotenoid content. Antioxidant activity	Pattanaik et al. (2020)
Amino acids and proteins	Shrimp waste	Essential and non-essential amino acids content	None	Suparmi et al. (2020)
Active edible film/coating				
Chitosan, protein, and lipid	Shrimp (*L. vannamei*) waste	Active film formation for food applications	Structural properties, mechanical properties, antioxidant and antimicrobial properties	Arancibia, Alemán, et al. (2015)
	Shrimp (*L. vannamei*) waste	Active coating for shrimp	Antioxidant activity, antimicrobial activity. Improve organoleptic properties of shrimp during cold storage	Arancibia, López-Caballero, et al. (2015)
	Shrimp (*L. vannamei*) waste	Bioactive film formation	Antioxidant and functional properties	Gómez-Estaca et al. (2015)
	Shrimp waste (Head and skin)	Chitosan coating for shelf-life extension of banana (*Musa sapientum L.*)	Lowered respiration activity, antimicrobial activity, and antioxidant activity	Hossain and Iqbal (2016)
	Shrimp (*L. vannamei*) head and exoskeleton	Bio-coating for raw salmon for ready to eat purpose.	Nutritional content plus control fish spoilage microorganism.	Gómez-Estaca et al. (2019)

inoculum to obtain the lipid fraction (Nunez-Gastelum et al., 2016). The lipid fraction was characterized by the content of astaxanthin and its ester form using thin layer chromatography and HPLC. Lipid fraction contained free astaxanthin (44%), monoester (32%), and diester of astaxanthin (26%). All oils showed free radical scavenging activity against DPPH radical. Hence, oil extracted from shrimp waste could be a potential nutraceutical ingredient for human consumptions (Nunez-Gastelum et al., 2016).

Astaxanthin rich lipid was extracted from shrimp (*L. vannamei*) waste by solvent fractionation and encapsulated by a spray drying using maltodextrin and gum arabic (Montero, Calvo, Gómez-Guillén, & Gómez-Estaca, 2016). Encapsulated lipid showed stability for 110 days at 5 °C and high solubility, antioxidant activity as well as bio-accessibility were achieved. Therefore, the encapsulation process increases the stability and bio-accessibility of astaxanthin-rich oil, which could be the excellent functional ingredient and coloring agent for food (Montero et al., 2016). Shrimp waste protein hydrolysates (SPH) were extracted from the head of *L. vannamei* using enzymatic autolysis process

(da Silva et al., 2017). The obtained protein hydrolysate was fed to Wistar male rats for 20 days and their biochemical analyses were conducted to examine the effect of SPH on body composition, protein ratio, and digestion. Rats fed with SPH showed an increase in body composition and protein ratio and digestibility compared to rats fed with casein. The results indicated that SPH showed improved biochemical parameters of rats and could be used as a supplement in the human diet (da Silva et al., 2017).

5.2. Feed applications

Shrimp processing waste have been used in animal feed production owing to their nutritional and bioactive compounds such as protein, carotenoid, chitin, lipid, etc. (Table 2) (Pattanaik et al., 2020). Moreover, shrimp processing waste could improve the growth performance and immunity of farmed fish (Gisbert, Fournier, Solovyev, Skalli, & Andree, 2018; Lu & Ku, 2013). Shrimp waste meal (SWM) was prepared and incorporated in the diet of juvenile cobia farmed fish to evaluate the growth performance of fish for 6 weeks (Lu & Ku, 2013). Fish fed with different percentages of SWM (0–25%) showed increased weight gain and more than 87% of survival rate was attained. SWM helped to improve lean body mass plus chitinase activity but failed to protect cobia from *piscicida* infection induced by *Photobacterium danselae* ssp (Lu & Ku, 2013). In another study, wet waste (feces and residual feed) from *L. vannamei* (0–100%) was evaluated as feed ingredient for sea cucumber (Chen, Hu, & Ren, 2014). Results revealed that feed containing 50% shrimp wet waste and 50% sea mud showed higher growth performance and survival rate compared to other formulations. Thongprajukeaw (2014) investigated the different techniques (boiling, soaking, sundry, oven-dry and microwave irradiation) for the preparation of aqua feed from shrimp processing waste. Microwave irradiation retained high quality protein, chemical composition and total carotenoid of shrimp waste, thereby promoting feed digestion (Thongprajukeaw, 2014). Astaxanthin extracted from shrimp waste using solvent extraction was evaluated for the natural skin pigmentation in goldfish (Weeratunge & Perera, 2016). Extracted astaxanthin showed strong antioxidant and antibacterial activity. Goldfish fed with astaxanthin formulated feed (1% Asta +1% coconut oil + 1% gelatin +97% bread crumbs) showed faster skin color improvement compared to the group fed with shrimp waste (1% coconut oil + 99% shrimp waste). Hence the extracted astaxanthin from shrimp waste is an economic, safe, and effective source to improve the skin color of goldfish within 10 days (Weeratunge & Perera, 2016).

Protein hydrolysate produced from cephalothorax of *L. vannamei* was evaluated as a feed supplement for European sea bass and their impact on growth performance, and immune response was studied (Gisbert et al., 2018). The addition of protein hydrolysate (5%) in the fish meal sample did not affect the growth performance of the seabass, compared to control. However, the improved immune response was noticed by an increased survival rate (96.4%) of seabass affected by a *Vibrio pelagius* infection. The results indicated that shrimp waste protein hydrolysate could be used as a fish meal supplement to boost the immune response of fish without affecting their growth performance (Gisbert et al., 2018). Carotenoproteins (CP) were extracted from shells of different shrimp using papain and their antioxidant activity was determined by various methods (Pattanaik et al., 2020). CP extracted from *P. stylifera* showed higher protein content, essential amino acids, and carotenoid content, compared to that from other shrimp species. Similarly, the antioxidant activity of CP from *P. stylifera* shell was higher than others. Therefore, it was concluded that shrimp shell waste could be used as a nutritive feed supplement in animal feeds (Pattanaik et al., 2020). Similarly, shrimp flavor powder prepared from shrimp waste could be the best feed ingredient due to their thigh sensory properties and amino acid content (9 essential and 8 non-essential amino acids) (Suparmi, Sari, Sumarto, & Susilo, 2020).

5.3. Active edible film/Coating

Edible film or coating are made from the polysaccharides, proteins and other bioactive compounds extracted from food sources (Gómez-Estaca et al., 2019; Hossain & Iqbal, 2016). These edible coatings not only protect quality deterioration but also allow the incorporation of bioactive molecules (Gómez-Estaca, Calvo, Sánchez-Faure, Montero, & Gómez-Guillén, 2015). Recently, a lot of researches have been documented for edible active coating owing to their stability, bio-compability, ready-to-eat, and zero waste nature (Arancibia, López-Caballero, Gómez-Guillén, & Montero, 2015). In this interest, Arancibia, Alemán, López-Caballero, Gómez-Guillén, and Montero (2015) prepared active films using chitosan and protein concentrate from *L. vannamei* waste with lactic acid. They found that added protein concentrate improved light barrier properties, tensile strength, antioxidant and antimicrobial properties of the film. In another study, Arancibia, López-Caballero et al. (2015) developed an active coating solution using chitosan and protein-lipid concentrate obtained from *L. vannamei* processing waste. Additionally, the effect of active edible coating on the quality control of shrimp was studied during storage at 5 °C for 17 days. Active coating effectively delayed microorganism growth and the melanosis in the shrimp, thereby improving shrimp quality during storage. The quality improvement of shrimp was governed by the chitosan and the added lipid-protein concentrate (Arancibia, López-Caballero et al., 2015).

Gómez-Estaca et al. (2015) extracted protein from spoiled or damaged shrimp to prepare the active edible films incorporated with astaxanthin from shrimp cephalothorax or lycopene from tomato peel waste. The obtained edible films were easy to handle, transparent, and showed high water vapor permeability. However, after 30 days of storage at 22 °C, films become opaque, colored and less water soluble suggesting the reorganization of protein structure with active compounds. Antioxidant activity of active films remained unchanged during storage, indicating that other compounds could have exhibit antioxidant activity (Gómez-Estaca et al., 2015). Chitosan extracted from the shrimp shell waste was utilized as a coating solution (0.5, 0.75 and 1.0%) for shelf life extension of banana (*Musa sapientum* L.) fruits (Hossain & Iqbal, 2016). Results revealed that 1% chitosan coating effectively prolonged the total weight loss, color change, total soluble solids, and retarded the postharvest quality changes of the banana compared to other treatment. Moreover 1% chitosan extended the shelf-life of banana by 4 days during storage. The quality protection of banana was related with the antioxidant and antimicrobial properties of chitosan (Hossain & Iqbal, 2016). In another study, Gómez-Estaca et al. (2019) prepared shrimp shell extract (SSE) with a mild chemical treatment to obtain extract with all biochemical and minerals present in the shrimp shell including chitosan. The SSE film prepared and wrap to raw salmon pieces to produced ready to eat product. Quality changes of SSE wrap ready to eat salmon were studied during chilled storage of 19 days. Salmon coated with SSE film showed higher chemical and microbial quality compared to unwrap salmon. Additionally, shrimp extract coating provided the extra nutrients in the form of bio-calcium, MUFA, and PUFA (Gómez-Estaca et al., 2019).

6. Environmental and biotechnological applications of shrimp waste compounds

Shrimp processing waste is an abundant cheap source of carbon, nitrogen, oxygen, and active compounds such as chitin, protein, and lipids which could be utilized in various biotechnological applications either in crude form or isolated form. Fig. 4 represents the potential utilization of shrimp processing waste for other industries. Recently most of the studies documented the use of active compounds from shrimp processing waste in metal and dye removal from wastewater, bioplastic preparation, energy conservation, etc. (Table 3).

6.1. Metal and dye removal from wastewater

Wastewater from various industries such as mining, textile, leather, paper, and plastic, create tremendous water pollutants including metal, acid and dyes (He et al., 2020; Núñez-Gómez, Rodrigues, Lapolli, & Lobo-Recio, 2019). These heavy metals can be absorbed by living organisms and can be carried forward to humans through the food supply chain, thus causing health problems (Nunez-Gomez, Alves, Lapolli, & Lobo-Recio, 2017). Similarly, synthetic dyes are hazardous contaminants and difficult to remove from polluted water due to their stable and non-biodegradable nature (Druzian, Zanatta, Cortes, Streit, & Dotto, 2019), thereby causing a serious threat to a living organism and whole ecosystem (He et al., 2020).

Shrimp shell powder was considered as a biopolymer for the Fe, Al, Mn, Co, and Ni removal from mine-impacted water (Nunez-Gomez et al., 2017). Shrimp shell powder effectively removed heavy metals from mine impacted water due to its large content of chitin, and calcium carbonate (Nunez-Gomez et al., 2017). In their recent study, Núñez-Gómez et al. (2019) documented the sorption isotherm and continuous flow process of the metal ion and acid removal from the coal acid mine drainage system. They found out that metal removal followed the physisorption mechanism with 90% Fe and 88% Mn metal ions removal in the continuous flow process. Shrimp shell waste could be a low-cost economic adsorbent for heavy metal removal from wastewater. Chitosan was extracted from shrimp waste and chitosan nanoparticles were prepared using ionic gelation methods (Ali, Aboelfadl, Selim, Khalil, & Elkady, 2018). The prepared nano-chitosan particles were examined for the Fe (II) and Mn (II) ion removal from water. The result indicated that nano-chitosan effectively removed Fe (II) (99.8%) and Mn (II) (95.3%) ion with an adsorption capacity of 116.2 and 74.1 mg/g, respectively. Moreover, kinetic studies and adsorption isotherm analysis revealed the occurrence of pseudo-second-order and Langmuir sorption model (Ali et al., 2018).

Shrimp shells were sun-dried and ground to powder form and the powdered shrimp shell was examined for textile dye removal (Fabbricino & Pontoni, 2016). Powdered shrimp shell (2.1 mg/L) effectively removed 90% of the dye within 2 h. The mechanism of action revealed the monolayer adsorption process supported by the gradient diffusion and several anchorage points for the existing micro-porosity of every

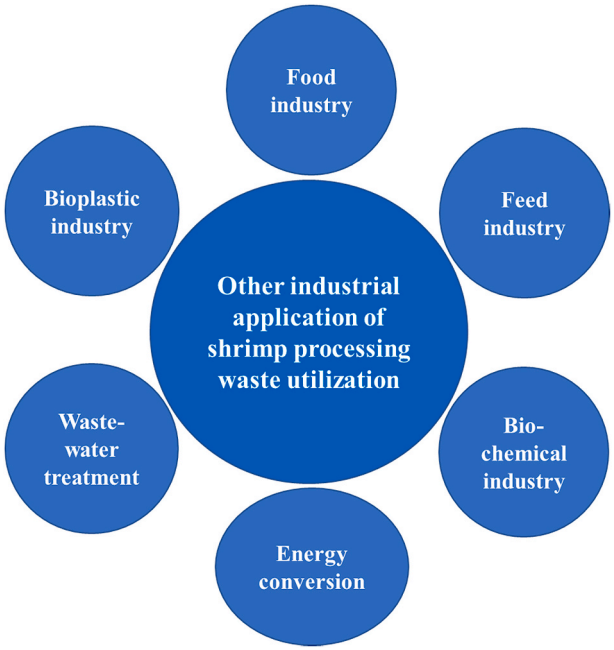

Fig. 4. Potential utilization of shrimp processing waste for other industries.

Table 3
Environmental and biotechnological applications of shrimp processing waste.

Active compound	Application	Activity/Function	References
Wastewater bioremediation			
Chitin, chitosan and chitosan nanofiber/	Textile dyes removal from wastewater	Adsorption capacity, micro-porosity and gradient diffusion ability	Fabbricino and Pontoni (2016)
Dried and ground shrimp shell	Water clarification	Chitosan biopolymer act as flocculation agent	Kadouche et al. (2017)
	Biomaterial for removal of heavy metal ions from mine impacted water	Metal-sorbent ability	Nunez-Gomez et al. (2017)
	Removal of iron and manganese from wastewater	Adsorption capacity and efficiency	Ali et al. (2018)
	Copper removal from wastewater	Adsorption capacity	Andreazza et al. (2019)
	Crystal violet dye removal from wastewater	Adsorption capacity and potential	Druzian et al. (2019)
	Biomaterial for removal of heavy metal ions and acids present in coal acid mine drainage	Physisorption mechanism	Núñez-Gómez et al. (2019)
	Metal removal from surface runoff of drainage	Bio-sorption activity	Rech et al. (2019)
	Uranium removal from aqueous medium	Adsorption capacity	Rostamian et al. (2019)
	Oil dispersion, Oil/water emulsion separation, Heavy metal ion removal from water phase.	Antifouling activity, emulsification activity	Yan et al. (2019)
	Anionic dye removal from aqueous medium	High adsorption capacity and regeneration performance	He et al. (2020)
Energy Conversion			
Carbon, hydrogen, nitrogen, and oxygen	Super capacitors	Phosphorous doped porous carbon with multiple pores and heteroatom functionalities	Qu et al. (2015)
	Energy, carbon sequestration and agriculture applications	Hydrochar formation with atomic carbon and calorific value	(S. Kannan et al., 2017)
	High performance lithium ion batteries and supercapacitors	Excellent electrochemical performance owing to porous structure, high specific surface area and nitrogen doping with superior cycling stability and specific capacitance	Mondal et al. (2017)
	Carbon and nitrogen source to prepare nitrogen, phosphorus co-doped catalyst (PA-SS 900)	Oxygen reduction reaction activity in microbial fuel cell	Zheng et al. (2020)
Biodegradable plastic			
Chitosan and Protein	Filler in wheat gluten-based bioplastics	Improved tensile strength, morphological and thermal properties	Thammahiwes et al. (2017)

(*continued on next page*)

Table 3 (*continued*)

Active compound	Application	Activity/Function	References
	Biodegradable plastic in conjunction with cassava peel powder	Physicochemical, mechanical and organoleptic properties	Dasumiati et al. (2019)
	Biodegradable plastic	Physical, mechanical, chemical and thermal properties	Elhussieny et al. (2020)
	Biopolymer for the preparation biodegradable plastic	Physicochemical properties, thermal stability, UV barrier properties, and antioxidant activity	Yuan et al. (2020)
Miscellaneous			
Chitin and Chitosan	Bio-based chemicals	Production of acetic acid and pyrrole from chitin and shrimp shell using catalytic process by metal oxide and oxygen gas	(Xiaoyum Gao et al., 2016)
	To determine multi-residue of veterinary drugs in the milk	Sorbent capacity	Arias et al. (2018)
Protein	Corrosion inhibitor for carbon steel	Adsorption on the carbon steel surface	Farag et al. (2018)
Shrimp head powder	Dry artificial fish bait for trap fishing in conjunction with tuna red meat	Stability and acceptability test	Karunanithi et al. (2018)
Shrimp waste hydrolysate	Oil dispersion	Critical micelle concentration and emulsification activity	Zhang et al. (2018)
Calcified shrimp waste	Benzene degradation	Supporter for Pd NPs catalytic activity	Odoom-Wubah et al. (2019)

single molecule (Fabbricino & Pontoni, 2016). Shrimp (*P. brasiliensis*) wastes were extracted by the chemical method to obtain chitin, and this chitin was further converted into chitin nanowhiskers (CNW) by acid hydrolysis (Druzian et al., 2019). Extracted chitin and CNW were studied as an adsorbent for crystal violet dye removal from water solution. The result showed that CNW (59.5 mg/g) effectively remove dye due to increase pore size and rod-like shape compared to chitin. Nevertheless, the adsorption was favorably endothermic, which showed the potential use of CNW in dye removal from wastewater (Druzian et al., 2019). Shrimp shell waste were utilized to prepare hydrochar (SHC) adsorbent using the deproteinization and deacetylation process followed by hydrothermal carbonization (He et al., 2020). Hydrochar is the carbon rich two phase mixture (solid and liquid) produced via hydrothermal carbonization. SHC had an excellent adsorption capacity (755.1 mg/g) for methyl orange dye at pH 4. The high efficacy of SCH was attributed to the higher specific area and presence of nitrogen-containing functional groups with long aliphatic chains. Kinetic study showed that SCH had electrostatic interactions and follows the monolayer adsorption patterns. Therefore, excellent adsorption ability and impressive regeneration capacity of SHC could be a potential adsorbent for anionic dye removal from wastewater (He et al., 2020).

6.2. Energy conversion

Porous carbon material provides a large surface area for electrochemical reaction, making it an attractive electrode material for energy applications (Kannan, Gariepy, & Raghavan, 2017; Mondal et al., 2017). Shrimp shell waste contained a high amount of nitrogen-based polysaccharides called chitin, which could be an abundant source of porous carbon (Qu et al., 2015). Furthermore, incorporation of heteroatom (like S and P) into such nitrogen-containing carbon source could improve their electrochemical activity, catalytic efficiency, and adsorption ability (Mondal et al., 2017; Zheng, Li, Ge, Xu, & Zhang, 2020).

Bohai shrimp shell treated with 10% HCl to remove $CaCO_3$ and shrimp shell was then activated with H_3PO_4 to obtain nitrogen, oxygen, and phosphorus-doped porous carbon electrode (PCE) material (Qu et al., 2015). PCE exhibits excellent specific capacitance of 206 F/g at 0.1 A/g in 6 m KOH solution due to the presence of mesopores, micropores, and high surface area. Additionally, the energy and power density of PCE can increase from 2.9 Wh/kg at 0.9 V to 5.2 Wh/kg at 1.1 V with the help of a doped PC. Therefore, PCE with a high number of micropore and heteroatom functionalities are excellent electrode materials for the production of high-performance supercapacitors (Qu et al., 2015). Shrimp waste was utilized to prepare hydrochar using hydrothermal carbonization (Kannan et al., 2017). The atomic carbon (39–40%), ash content (21–25%), and calorific value (18–23 MJ/kg) of hydrochar were determined. This carbon-rich hydrochar prepared from shrimp shell waste could be a potential raw material for energy conservation and carbon sequestration (Kannan et al., 2017). Shrimp shells were dried completely by the oven and vacuum drying and the dried shells were ground to powder and treated with 6 M KOH to remove $CaCO_3$ (Mondal et al., 2017). The dried powder subjected to carbonation at 900 °C for 5 h under argon atmosphere. The prepared PC with heteroatoms (N and O) (PCH) was used as an electrode for lithium-ion batteries and supercapacitors. When it was used in lithium-ion batteries as an anode material, it showed a higher capacity of 1507mAh/g at a current density of 0.1 A/g with better performance and excellent cycling stability. Similarly, PCH based supercapacitors showed specific capacitance of 239 F/g at a current density of 0.5 A/g in 6 M KOH solution. The prepared supercapacitors showed 99.4% capacitance retention after 5000 charge-discharge cycles, proving extreme stability. Hence, shrimp shell-based porous material with nitrogen doping could be the low-cost carbon source for both lithium batteries and supercapacitors long lasting electrochemical performance (Mondal et al., 2017).

Shrimp shell waste was also utilized as carbon and nitrogen sources to prepare phosphorous co-doped mesopores carbon material catalysts with a high specific area (Zheng et al., 2020). The prepared catalyst exhibited high oxidation-reduction reaction activity with half-wave potential (0.82 V) and current (4.47 mA/cm). When used in a microbial fuel cell, catalyst produced a power density of 802 mW/m^2 and open-circuit voltage of 653 mV, which was comparable with commercial catalyst. Moreover, the prepared catalyst from the shrimp shell showed excellent long-term stability than the commercial counterpart. Therefore, shrimp shell-based N, P doped catalysts could be efficient in air cathode microbial fuel cells for energy generation (Zheng et al., 2020).

6.3. Biodegradable plastic

Plastic bags fabricated from petroleum-based chemicals such as polyethylene, polypropylene, etc. are associated with long decomposition time and cause serious damage to the ecosystem (Elhussieny et al., 2020). Therefore, the development of biodegradable plastic from a natural biopolymer, particularly chitosan, which can be degraded by microorganisms is of interest (Wang, Qian, & Ding, 2018). In this instance, shrimp shell waste extracted for chitosan and starch obtained from cassava peel was used for the preparation of bioplastic using glycerol as a plasticizer (Dasumiati, Saridewi, & Malik, 2019). The prepared bioplastic showed good physical and mechanical properties with 7% chitosan (Dasumiati et al., 2019). In another study, Thammahiwes, Riyajan, and Kaewtatip (2017) used shrimp shell powder with or without calcified (2.5%) as filler for wheat gluten (WG) based bioplastic fabrication. The addition of shrimp shell powder either with or without calcination improved tensile strength of bioplastic compared to WG alone. However, calcified shrimp shell powder showed significant improvement of tensile, thermal and morphological properties owing to higher mineral content and altered layer structure of the bioplastic

(Thammahiwes et al., 2017).

Shrimp chitosan extracted from shrimp shell was used as a polymeric matrix and rice straw fiber was used as a reinforcement to prepare composite films using a casting technique (Elhussieny et al., 2020). The prepared composite films showed higher physical, chemical, mechanical and thermal properties, compared to the control. The reinforcement of rice straw and nano rice straw into chitosan improved the thermal degradation temperature of composite, compared to chitosan alone. Therefore, waste generated from shrimp shell and rice straw could be used as natural sources for the preparation of biodegradable bioplastic (Elhussieny et al., 2020). Recently, Yuan et al. (2020) prepared biodegradable composite films from the shrimp shell waste protein plus chitosan biopolymer and the active constituents from the extract of oolong tea, corn silk, and black soybean seed coat at different concentrations (1–5% w/w) (Yuan et al., 2020). The physic-chemical, mechanical and thermal properties of composite films were strongly influenced by the addition of the active constituents preferably at higher concentrations (5%). Moreover, at this concentration, films showed improved antioxidant properties and UV barrier capacity. The results showed that active film packaging could be prepared by the addition of active compounds into the bio-composite film (Yuan et al., 2020).

6.4. Miscellaneous

Shrimp shell waste and their active compounds also have diverse applications such as chitosan from shrimp waste used as a sorbent to detect the residue of veterinary drug, radioactive material removal, artificial fish bite, bio-based chemical formation, benzene destruction, oil spill dispersant and corrosion inhibitor for carbon steel (Arias et al., 2018; Farag, Ismail, & Migahed, 2018; Odoom-Wubah et al., 2019; Rostamian, Firouzzare, & Irandoust, 2019).

Shrimp shell waste was directly converted into the acetic acid by catalytic method using metal oxide and oxygen gas (Gao et al., 2016). The recovery of acetic acid showed 47.9% yield from direct shrimp shell catalysis. In addition to the acetic acid, nitrogen containing heterocyclic compound pyrrole was recovered as the major product (Gao et al., 2016). Chitosan obtained from shrimp waste was used as a sorbent in the cleanup step of the QuEChERs method to examine the multidrug residue in the milk (Arias et al., 2018). The chitosan in the cleanup step effectively reduced the extract turbidity and matrix effect. Moreover, it improved the correlation coefficient, the limit of quantification, and recoveries for all veterinary drugs in various milk samples (Arias et al., 2018). Shrimp shell waste proteins were extracted and evaluated as corrosion inhibitors for carbon steel in 1 M HCl (Farag et al., 2018). Shrimp shell protein effectively lowered the corrosion rate of carbon steel in a dose-dependent manner. The inhibition mechanism study revealed that protein adsorbs on the surface of carbon steel with chemisorption effect (Farag et al., 2018). Shrimp head powder (SP) incorporation with tuna red meat (TM) was utilized for the preparation of dry artificial fish bait for the fishing purpose (Karunanithi, Neethirajan, Padmanaban, & Robinson, 2018). The results showed that 61% TM and 15% SP with stick-shaped exhibited good stability in seawater and acceptance by tilapia. Further analysis revealed the protein bleaching rate and dry matter loss of selected bait were 24.82 and 36.6/mg/g/h, respectively. Moreover, the fish catch rate study proved that fish bait prepared from SP and TM showed significantly higher catch, compared to commercial bait. Nevertheless, shelf-life of SP + TM bait can be extended up to 6 months by the addition of 0.1% sodium benzoate (Karunanithi et al., 2018).

Northern pink shrimp (*P. borealis*) waste including head was hydrolyzed using alcalase to obtain hydrolysate or a green dispersant (GD) (Zhang et al., 2018). Green dispersants are those molecules which are prepared from natural sources and have oil and water binding capacity. The obtained GD was examined for the crude oil dispersion in seawater and the process was optimized to obtain high dispersant effectiveness of the GD. The optimized GD showed excellent critical micelle concentration, emulsification capacity, and stability. GD dispersion effectiveness was confirmed on three different crude oil spills including Alaska North Slope, Prudhoe Bay, and Arabian Light crude oil. The microtox acute toxicity test showed that GD is safe and non-toxic. Therefore, GD prepared from shrimp waste could be an efficient green solution to the oil spill problems (Zhang et al., 2018). Shrimp shell waste was calcified by normal calcination in the air to produce $CaCO_3$ and CaO active compounds (Odoom-Wubah et al., 2019). This calcified shrimp waste used as a support for Pd to obtain a catalyst in benzene oxidation process. The synthesized catalyst (0.5-Pd/SW@600) exhibited strong oxidation activity due to high Pd metal dispersion, a synergistic effect between metal and support base. Moreover, catalysts showed great stability, reusability, and high efficacy, which could be a promising green catalyst in benzene reduction (Odoom-Wubah et al., 2019). Chitosan was extracted from shrimp waste and converted into nano-fiber (NF) using the force spinning method (Rostamian et al., 2019). The obtained NF was examined for the adsorption capacity to remove uranium from the water solution. The results showed that NF effectively removes uranium while the efficacy depends on the pH, contact time and uranium concentration of the solution. Thus, chitosan nano-fiber from shrimp waste could be an inexpensive, eco-friendly, and promising approach to remove the radioactive compounds from aqueous solution (Rostamian et al., 2019).

7. Hurdles and future trends of shrimp waste utilization

Currently, the majority of shrimp waste has been utilized as an animal feed and some portion of the waste is converted into the value-added bioactive compounds such as chitin, chitosan, carotenoid, and protein. Although the extracted bioactive compounds are known to possess various biological activities, food, and pharmaceutical applications, their extraction process needs hazardous chemicals including strong acids and bases. This extraction process again leaves hazardous wastes and effluents which create a harmful burden on the ecosystem. Therefore, the future trends of shrimp processing waste lay in the complete utilization, without generating any new waste. Recently, shrimp shell waste showed promising applications in wastewater remediation, energy conversion, spill oil dispersant and base material for catalyst (He et al., 2020; Mondal et al., 2017; Núñez-Gómez et al., 2019; Zhang et al., 2018). In those applications, shrimp waste can be processed using very simple methods including drying and grinding, hydrothermal carbonization, and calcification in air. Hence the future prospectus of shrimp waste utilization will be diverted to bioremediation, and energy conversion, which are very important for a clean environment.

8. Conclusion

Shrimps are more demandable seafood in the market and are mainly processed for the meat, thus leaving more than 50% of the waste. The abundant amount of shrimp processing waste is generated every year, all over the world. Shrimp waste contains a bunch of bioactive components such as chitin/chitosan, protein, carotenoids, polyunsaturated fatty acid, α-tocopherol and minerals. These bioactive compounds have been reported to exhibit various bioactivities such as antioxidant, antimicrobial, antihypertensive, anti-inflammatory, anti-proliferative activities, etc. Nevertheless, these active compounds have been widely used in food and feed industries to improve the quality and functional properties of foods. Bioactive compounds like astaxanthin and protein hydrolysate can serve as functional foods, owing to their nutritional and nutraceutical properties. Recently, shrimp waste has been converted to porous carbon material, hydrochar, and nano-powder, which show the application in bioremediation, energy conversion, bioplastic, and other biochemical engineering applications. Hence the future shrimp waste utilization is more likely focused on environmentally sustainable energy and wastewater remediation.

Author contributions

NPN and CS conceive the work, search and collected literature, compiled data, and drafted the manuscript. SB interpreted the data and revised critically for important intellectual content. MSR analyzed data and drafting tables. All authors are agreed for their accountable contributions and approval of the manuscript.

Declaration of competing interest

None.

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SHRIMP WASTEWATER BIOREMEDIATION

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Utilization of microalgae, *Chlorella* sp. UMT LF2 for bioremediation of *Litopenaeus vannamei* culture system and harvesting using bio-flocculant, *Aspergillus niger*

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ABSTRACT

Microalgae are well known as autotrophic microorganisms in aquaculture wastewater treatment, however their role in the aquaculture system is rarely explored. The purpose of this study is to evaluate the influence of adding microalgae, *Chlorella* sp. UMT LF2, on the functionality for bioremediation and growth performance of Whiteleg shrimp, *Litopenaeus vannamei* in the nursery phase reared in a control culture system. Secondly, the response surface methodology (RSM) was used to investigate further on the optimal condition for the harvesting process of excess microalgae biomass using bio-flocculant from the filamentous fungus, *Aspergillus niger*. The experimental set-up with *Chlorella* sp. additions were compared to the control group without the microalgae addition based on the water quality, microalgae biomass and shrimp growth performance. Water samples were collected and underwent in-situ and ex-situ involving nine physicochemical parameters. The results showed that introducing microalgae with zero water exchange as treatment tanks reduced the NH_3, NO_2^- and PO_4^{3-} during the intermediate until the two days before the 30-day culture period's end. Microalgae also enhanced shrimp survival and growth performance significantly 89% as compared to the control, 68%. Furthermore, the culture with the addition of microalgae maintained pH stability and enhanced dissolved oxygen during daytime in the culture water. Therefore, this finding confirmed that *Chlorella* sp. UMT LF2 addition is promising in improving rearing environment and nursery performance for *L. vannamei* in culture system. However, further research is required to demonstrate the harvesting by-product from the culture system and the potential applications.

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1. Introduction

Aquaculture plays a vital role in Malaysia agriculture, particularly its growing contribution to economic growth. In addition, *L. vannamei* expanding quickly and is the largest contributor to Malaysian shrimp aquaculture (Thean et al., 2016). Malaysian Whiteleg shrimp aquaculture produces between 1.71 and 6.73 tonnes/hectare per crop or between 3.42 and 13.46 metric tonnes per hectare yearly. Although the increase in production is impressive, several issues and barriers still need to be overcome. Shrimp aquaculture has expanded quickly and the effluent contamination from the conventional shrimp farming model is raising growing concern due to high-density farming, excessive feed consumption, and intensive water exchange to maintain a desirable water supply for shrimp growth. A primary concern on the effluent discharged by shrimp aquaculture is that it can cause eutrophication towards the coastal water due to insufficient removal techniques (Aditya et al., 2022).

Despite its rapid growth, the aquaculture of shrimp also still confronts challenges because of viral diseases (El-Saadony et al., 2022; Seibert and Pinto, 2012). Instead of using chemical agents, many researchers have recommended that shrimp producers utilize microbial and microalgae in culture water to improve water quality and decrease disease levels. Microalgae can naturally remove nitrogen and phosphorus from culture water and create extracellular substances (such as tropodithietic acid) that prevent infections and other hazardous organisms (Yao et al., 2019).

Additionally, multiple advantages of producing microalgae alongside white-leg shrimp have been revealed in a new study published regarding *L. vannamei* (Huang et al., 2022). Microalgae are attractive alternatives for biomass production and pollutant removal due to their quick growth rates compared to terrestrial plants. Several researchers used the capability of microalgae to remove pollutants to conduct bioremediation to reclaim the contaminated environment (Kurniawan et al., 2022). Adding culture microorganism is a promising method for solving performance and efficiency issues throughout the wastewater treatment and bioremediation process in particular single microalgae species (Kassim et al., 2022). This method has been widely used to increase the degradation efficiency of recalcitrant toxic compounds from aquaculture wastewater (Ahmad et al., 2022; Maryjoseph and Ketheesan, 2020).

In bioremediation of aquaculture wastewater, microalgal species including *Chlorella, Phaeodactylum, Scenedesmus,* and *Nannochloropsis* are promising and frequently studied (Mohd Nasir et al., 2021; Nie et al., 2020). In other research, introducing microalgae to culture water boosted shrimp's survival and weight gain. For instance, a recent study by Huang et al. (2022) stated that, microalgae species *Nannochloropsis oculata* and *Thalassiosira pseudonana* benefited to water quality and shrimp development. Additionally, researchers found that throughout a farm cycle, adding *T. seudonana* to culture water significantly reduced levels of ammonia, suspended particles, orthophosphate, nitrite, and nitrate (Huang et al., 2022). Besides, microalgae boost dissolved oxygen levels in daylight and regulate pH levels.

Aquaculture for shrimp ponds typically uses large amounts of water. Using zero-exchange systems is practical substitute for conventional ponds methods of intense aquaculture production. The concept of zero-exchange systems has been implemented in indoor tank-based intensive production systems for *L. vannamei* (Wasielesky et al., 2006; Xu et al., 2022). However, the cultivation of microalgae is a more expensive process compared to conventional crops due to the high energy requirement (artificial light), supplementing the media (chemical usage), as well as aeration for the addition of oxygen (de Farias Silva et al., 2020). Therefore, the finding for minimizing consumption is vitally necessary to reduce the cost.

To further investigate the responses of microalgae under a zero-water exchange system, the current study was conducted to evaluate the bioremediation effects of additional microalgae, *Chlorella* sp. UMT LF2, on water quality parameters and growth performance status of *L. vannamei* raised in an indoor culture system. Furthermore, since the microalgae play a major role regulating inorganic nitrogen and phosphorus in the culture system, the harvesting process of microalgae is also discussed to guide the management process during shrimp culture to prevent algal blooms caused by an overabundance of microalgae. The ability of filamentous fungus, *Aspergillus niger* to harvest microalgae was also tested for harvesting protocol.

2. Material and methods

2.1. Isolation and identification of microalgae, Chlorella sp.

Locally isolated species can be chosen to ensure the originality of the species from the shrimp tank, even if many isolated microalgae species can be easily obtained from research facilities or hatcheries for aquaculture. The isolation is began with the water samples with a visible microalgal population collected from shrimp tank, AKUATROP, UMT. Fifty millilitres of water samples were collected and maintained refrigerated while transferred to the laboratory. Collections were made for the top and bottom of the tank. There are four primary isolation techniques for obtaining single cell species: streaking, spraying, serial dilution, and single-cell isolations (Bacha, 2013). The streaking and serial dilution were chosen for this study as isolation techniques. Initially, standard plating techniques were employed to separate algal populations from the water samples to isolate a single microalgal species. Then, the colonies were isolated using several medium recipes.

Microalgae isolation was conducted using serial dilution to obtain pure culture from water sample by employing Guillard's F/2 Media. Sterilized plastic petri dishes (90 mm × 15 mm) containing approximately 40 mL of agar medium were used to plate these diluted samples. A total of 1 mL of the diluted sample was transferred to a media plate and spread evenly across the surface. The inoculated plates were sealed up using parafilm to avoid contamination and were placed under a control temperature of 23 ± 2 °C and continuous illumination, approximately 27 μE/m/s where the algae were allowed to grow for about 14 days.

For molecular identification, the single cells were enriched with Guillard's F/2 media to promote the growth of microalgae stock. Microalgae cultures were harvested at the exponential growth phase by centrifuged using Eppendorf 5702 Variable Speed Multi-Purpose Centrifuge for 10 min at 6000 rpm to separate microalgae biomass and supernatant of culture medium. The biomass was set-

tled at the bottom, and the supernatant was discarded. The microalgae biomass was preserved using 95% ethanol for molecular identification purposes. The microalgae biomass was sent to GeneSeq Sdn. Bhd. for Polymerase Chain Reaction (PCR) amplified Deoxyribonucleic Acid (DNA) sequences.

The DNA sequence obtained from Geneseq was exported to FASTA format in notepad and analyzed using Bioedit (Dereeper et al., 2008). The nucleotides sequenced were assembled and searched against previously deposited sequences in BLASTn Program (NCBI BLAST, USA) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic analyses have been carried out using the software MEGA11. In this study, the phylogenetic analysis was conducted based on the 18S rDNA gene of microalgae. The phylogenetic tree was reconstructed using the maximum-likelihood method and bootstrap analysis (Hall, 2013).

2.2. Growth curve of microalgae

To determine the growth curve of microalgae, the microalgae were cultured in a 3000 mL conical flask in three replicates using 2000 mL of sterilized Guillard's F/2 media. Microalgae with 1×10^5 cells mL^{-1} density were inoculated into each flask. The culture flasks were placed under a control temperature 23 ± 2 °C and continuous illumination, approximately 27 μE m^{-1} s^{-1}, for 14 days. Aeration was supplied continuously to each flask using an air pump for providing oxygen and mixing to disperse oxygen throughout the culture flask (Ignatius, 2013).

Cell count, absorbance, and dry weight were analyzed to determine the microalgae cells growth. Microalgae cells were counted using a Neubauer hemocytometer for cell count analysis, and absorbance at an optical density of 680 nm using a UV/VIS spectrophotometer. GF/C filters were used to filter 10 mL of microalgae culture to determine the growth curve by dry weight analysis (Whatman, England). The filters were rinsed with 0.5 M ammonium formate, dried at 95 °C for the night, and reweighed. Using a lab analytical balance, the initial and final weight of filter paper were determined. Microalgae biomass was determined based on the dry weight of microalgae cells produced per litre (mg L^{-1}).

The growth curve with all parameters was plotted at the culture period's end. Based on the growth curve obtained, microalgae were cultivated with an initial concentration of 1×10^5 cell mL^{-1} until the early stationary phase for the experimental culture system and harvested to inoculate into the culture system tank.

2.3. Experimental set-up

The shrimp were acclimatized before being placed in the culture system. Shrimp first adapted to their new habitat during the acclimatization phase before being tested for the treatments culture system. As the L. *vannamei* being among the top aquatic species of commercial importance worldwide, the Specific Pathogen Resistant (SPR) *L. vannamei* from ISHARP Sdn. Bhd. was chosen (Wang et al., 2022). First, the post-larvae shrimp were acclimatized at an indoor hatchery for three days. Then, the acclimatization procedure was carried out in batch culture with 300 L working condition with continuous aeration, pH controlled at 7–8 and temperature maintained at 28 ± 2 °C. After three days of acclimatization in 30 ppt salinity marine water, *L. vannamei* in good condition was used for the experiment in the culture system.

There are three different types of experiments: i) cultures with water exchange (Positive Control) ii) cultures without water exchange (Negative Control) iii) cultures without water exchange with additional microalgae, *Chlorella* sp. UMT LF2 (*Chlorella* sp.). Each treatment was done in three replicates. This culture system was conducted in nine acrylic aquarium tank, 40 L that had been aerated overnight after being filled with 30 L of 30 ppt marine water at the hatchery. Post larvae L. *vannamei* weighing 0.05 g ± 0.02 were randomly dispersed into each aquarium at 100 shrimps per tank density and kept for 30 days. Shrimp were fed four times a day (8.00 a.m., 1.00 p.m., 6.00 p.m., 11.00 p.m.) with commercial shrimp feed, Star Feed 7701 and 7702 Blanca (STAR FEEDMILLS (M) Sdn. Bhd.). Each feeding amount was 2% of shrimp body weight based on feeding demand and feed was composed of 36% crude protein, 12% moisture, 5% fat and 4% fibre. Aerated marine water was added occasionally to replace water loss due to evaporation.

For the negative control tank, 20% of the water was exchanged on Day 14 in the middle of culture period, and zero water exchange for the positive control (Mohanty, 2001). Additionally, for the culture tank with the addition of microalgae, the *Chlorella* sp. UMT LF2 was introduced twice to the culture water, on Day 0 of the experiment and Day 14, nearly halfway through the culture process. The 3 L *Chlorella* sp. stock was harvested and inoculated to the tank.

2.4. Shrimp culture performance

According to Zhao et al. (2012), feed conversion rate (FCR), body weight gain (W, %) and survival rate (SR, %) were calculated to determine the culture performance. The FCR, W and SR were calculated using Eq. (1), Eq. (2) and Eq. (3). At the end of the culture period, all shrimp were collected from each tank and subsequently, these were weighed.

$$\mathrm{FCR} = \frac{\text{Feed Intake (g)}}{\text{Final Weight} - \text{Initial Weight (g)}} \tag{1}$$

$$\mathrm{W}\ (\%) = \frac{\text{Final Weight Gain (g)} - \text{Initial Weight (g)}}{\text{Initial Weight (g)}} \times 100 \tag{2}$$

$$\mathrm{SR}\ (\%) = \frac{\text{No. of Initial Shrimp} - \text{No. of Final Shrimp}}{\text{No. of Initial Shrimp}} \times 100 \tag{3}$$

2.5. Water quality analysis

A Multiparameter YSI Professional Plus (YSI Integrated, USA) instrument was used to measure the physicochemical water parameter: pH, dissolved oxygen (DO), temperature (°C), conductivity (μs cm^{-1}), total dissolved solids (TDS) and salinity (ppt). In addition, the selected water quality parameters such as ammonia (NH_3), nitrite-N (NO_2^-) and orthophosphate-P (PO_4^+) were performed every two days. Ammonia nitrogen content was determined using Phenate Method (4500-NH_3-F), nitrite was determined using Colorimetric Method (4500-NO_2^--B), and orthophosphate was determined using Ascorbic Acid (4500-P-E). Following the procedures provided by the Standard Method for the Examinations of Water and Wastewater (APHA 2012), the water samples were filtered using a 0.45 μm cellulose acetate microfilter before being analyzed.

2.6. Harvesting excess Chlorella sp. UMT LF2 biomass in culture system using bio-flocculant Aspergillus niger

The water sample containing *Chlorella* sp. biomass were collected from the culture system during the end of the culture period and harvested using bio-flocculant. For bio-flocculant formation, the filamentous fungus, *Aspergillus niger* was cultured in Richard Broth for three days with rpm 125 and pH 7 to achieve sufficient biomass concentration to agglomerate the suspended microalgae (Mohd Nasir et al., 2019). Regarding the previous result, the harvesting process was optimized by applying the response surface methodology (RSM). The term RSM refers to a group of statistical and mathematical methods used in experiment design, model construction, factor analysis, and the search for optimum condition. This study selected three factors with three level CCD experimental design combined with RSM to maximize the harvesting efficiency. Design Expert® Version 13 (Stat Ease, USA) was used to generate the experimental design, statistical analysis, regression model and graphical analysis of the data obtained. The optimization procedure was carried out using CCD depending on the three factors: dosage of bio-flocculant, pH and mixing rate. Based on the previous study by Mohd Nasir et al. (2019); Nasir et al. (2015) these parameters were chosen because they significantly differ in the microalgae biomass harvesting process. The harvesting process was carried out under controlled temperature of 27 °C. Table 1 listed the minimum and maximum ranges of variables investigated and the entire experimental strategy concerning their values in actual and coded form. All experiments in the model were carried out in triplicates.

2.7. Statistical analysis

All experiment data were expressed as the mean ± standard error (SE) from treatment replicates (n = 3). The graphs were plotted by Origin Pro Software (Origin Lab Corp., USA). Statistical analyses were conducted to verify the significant difference between control and treatment. The data for physical and chemical parameters throughout 30 days of the culture period were analyzed using One-way analysis of variance (ANOVA) by SPSS Statistics software version 20 (Analytical Software, USA). When significant differences were found, the Tukey HSD for multiple comparisons among means was applied to identify differences between culture tanks. The experiment's results were considered as statistically significant at ($p < 0.05$) in this experiment.

3. Results and discussion

3.1. Molecular identification of microalgae

Based on molecular identification using 18S rRNA gene sequences and Neighbor-Joining phylogenetic tree (Fig. 1), the isolated *Chlorella* sp. was found to be similar to *Chlorella* sp. TNBR1 (Accession number KR869729.1) and *Chlorella* sp. KAS012 (Accession number AB176666.1) with 99.94% of nucleotide similarity. It has been deposited as *Chlorella* sp. UMT LF2 with Accession number ON854139.1.

The Neighbor-Joining method inferred the evolutionary history (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein J., 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein J., 1985). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein J., 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. This analysis involved 10 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1157 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

3.2. Growth curve of Chlorella sp. UMT LF2

In this study, the single microalgae species *Chlorella* sp. UMT LF2 was cultured in batch mode for 14 days without any optimization factors that might influence the growth and biochemical composition. Growth media Guillard's F/2 are provides nutrients for the growth of marine algae and maintains algal strains. Fig. 2 shows the growth curve of microalgae *Chlorella* sp. UMT LF2 isolated from shrimp culture tank. The findings indicated that this species have typical microalgae growth curve with experienced lag, exponential,

Table 1
Coded values based on the factor at a time experiment for the 3 variables employed in the study.

Code	Parameter	−1	+1	0	-α	+α
A	Dosage (g L^{-1})	25	35	30	21.6	38.4
B	pH	6	8	7	5.3	8.7
C	Mixing Rate (rpm)	100	150	125	83	167

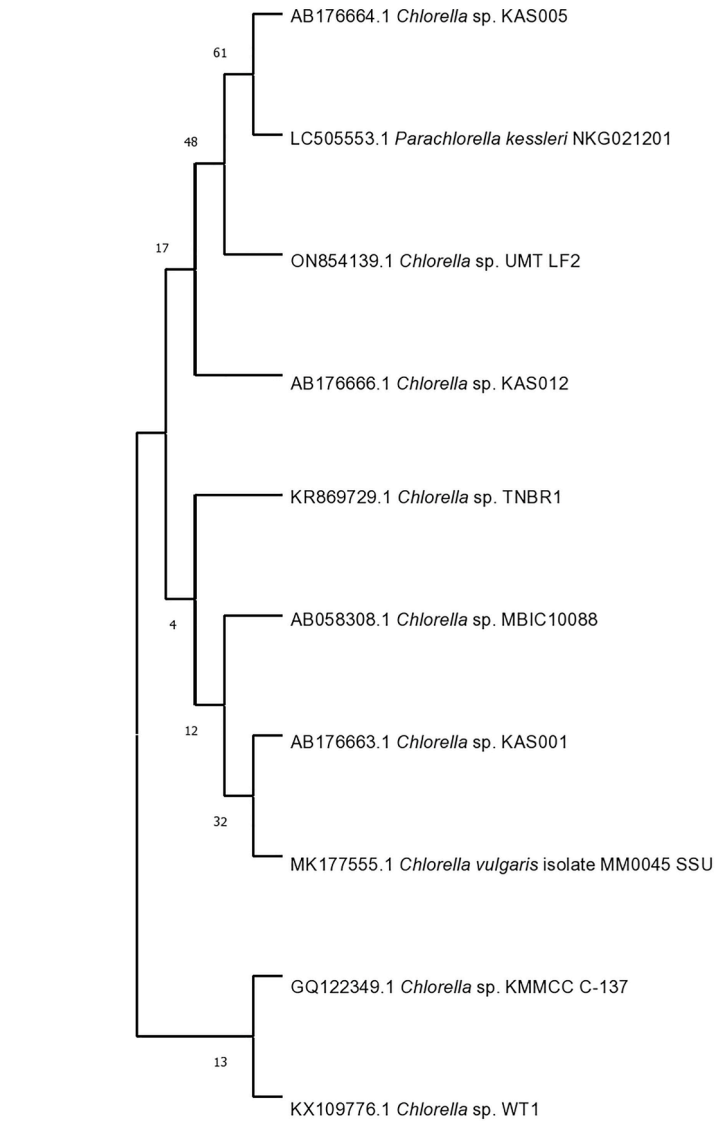

Fig. 1. Neighbor Joining Tree showing the phylogenetic relations among 18S rDNA sequences from microalgae used in this study and those obtained from NCBI GenBank Database.

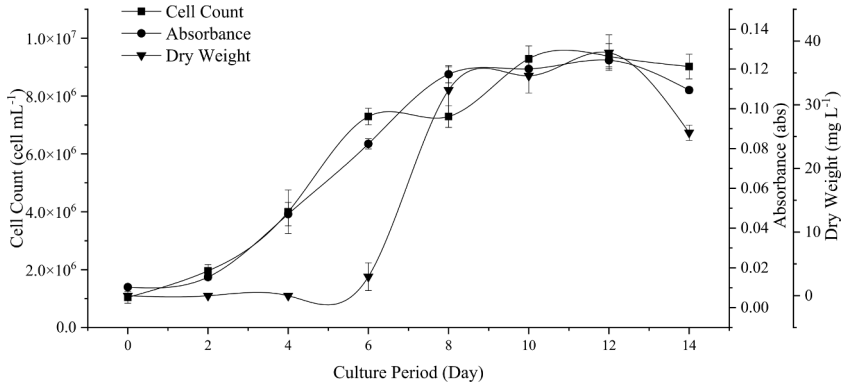

Fig. 2. The comparison growth curve of *Chlorella* sp. UMT LF2 based on cell count, absorbance, and dry weight.

stationary and decline phases. Microalgae have a short lag phase from Day 0 to Day 2, an exponential phase from Day 2 to Day 8, and start to enter the stationary phase (Day 8 to Day 12) for cell count and absorbance. By Day 14, the decline (death) phase had begun. Microalgae went through a lengthy lag phase and a short exponential phase for dry weight. Despite having high cell concentrations in counts and absorbance, *Chlorella* sp. had the smallest cells, which resulted in the lowest dry weight.

According to Anderson et al. (2020) some algal species grow by increasing the number of their cells (cell division), rather than their size. All eukaryotic, including aquatic plants and algae growth through mitosis where one cell divides into two cells (Yu et al., 2015). The *Chlorella* sp. used in this study in terms of dry weight suggest a prolonged lag phase before initiation of cell division and a brief exponential phase. The growth rate of cell division was also determined, and it varied according to the analysis. Cell regeneration, or the rate at which cells divide in a culture, is a specific growth rate. Since the methods of cell count and absorbance revealed a promising distinct growth curve, these method were selected to determine microalgae cell density in the 30-day culture system.

3.3. Microalgae cell density during shrimp culture system experiment

Fig. 3 illustrates the microalgae cell density throughout the culture period. The results indicated that white-leg shrimp culture water could increase the growth of *Chlorella* sp.. The microalgae biomass was drastically grown from Day 4 to Day 8 and increased from Day 14 to Day 26 due to the availability of sufficient nutrients in the culture tank. However, the microalgae biomass was reduced between Day 8 and Day 14, consistent with the previously observed growth phase, in which microalgae entered the stationary phase and decline phases. Reduced growth of microalgae in response to the increase of the nutrient concentration in the culture tank as reported in Section 3.5.

3.4. Physical – chemical water quality analysis in culture system experiment

Fig. 4 illustrates the dissolved oxygen concentration, pH, salinity and temperature throughout the 30-day culture period. According to the results, the dissolved oxygen concentration in the control tanks was steady for the first four days, then dropped precipitously on Day 8 and tended to fluctuate all the way up until the end of the culture period. A significant difference ($p < 0.05$) was found between culture tanks of control and *Chlorella* sp. regards to dissolved oxygen. The dissolved oxygen concentration in tank with *Chlorella* sp. was higher than that in control tanks because of microalgae photosynthetic activity predominated resulting oxygen releasing at daytime and heterotrophic carbon-oxidation and nitrification (Ge et al., 2016). Silva et al. (2022) recommended maintaining dissolved oxygen levels at or close to saturation and never below 5 mg L^{-1}.

The pH of the culture water was initially between 7 and 7.5. However as the culture developed, it was seen that this value increased up to pH 8.0 for the *Chlorella* sp. tank. According to Qiu et al. (2017), the microalgae addition cultures rises and fluctuate due to the uptake of inorganic carbon by microalgae photosynthetic activity. Therefore, rising pH levels are a favourable indicator that microalgae are truly growing in the growth medium. However, the pH tends to decrease from Day 8 because the conversion of NH_4^+ to NO_3^- involves in the transformation of an alkaline ion into an acid ion (Montalvo et al., 2014). The conversion formula is $NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+$. Romero et al. (2018) stated that in pond culture, photosynthesis by phytoplankton can increases the DO level, while respiration and microbial activities, such as nitrification and sulfur oxidation, may decrease the pH. Since rapid pH changes in shrimp ponds may affect the shrimp, especially their intestinal microbiome it is crucial to regulate the pH. In addition, shrimp also lead to stress and resulting lack of appetite and started illness (Furtado et al., 2015).

After 48 h, salinity slightly decreased to 29.14 ppt and 29.83 ppt in negative control and *Chlorella* sp. tanks, respectively. A higher salinity range was recorded for all tanks of 31.98 ppt–32.45 ppt on Day 18 and Day 26. The temperature range between culture tanks was narrow which from 26.6 to 28.1. Therefore, variation in salinity and temperature without significant differences between all cultures tank ($p > 0.05$). However, it is essential to control the temperature, due to temperature changes can affect the growth performance, survival rate and feed conversion ratio of cultured Pacific white shrimp, *L. vannamei* (Ponce-Palafox et al., 1997, 2019). Therefore, these factors need to be carefully monitored to prevent raising failure, such as by providing an adequate feed, maintaining the pH level and temperature, and routinely removing organic matter deposits (Romero et al., 2018). Furthermore, pH, temperature

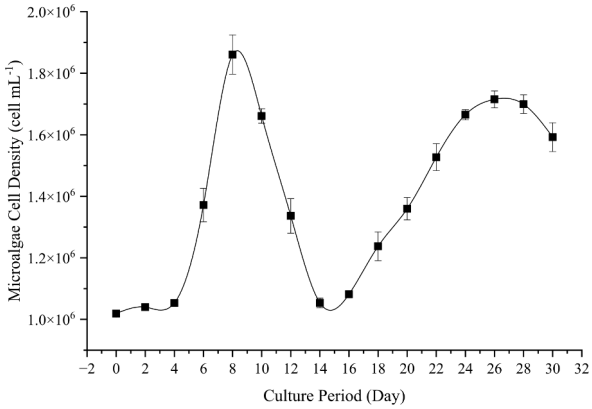

Fig. 3. Microalgae Cell Density throughout 30-day culture period.

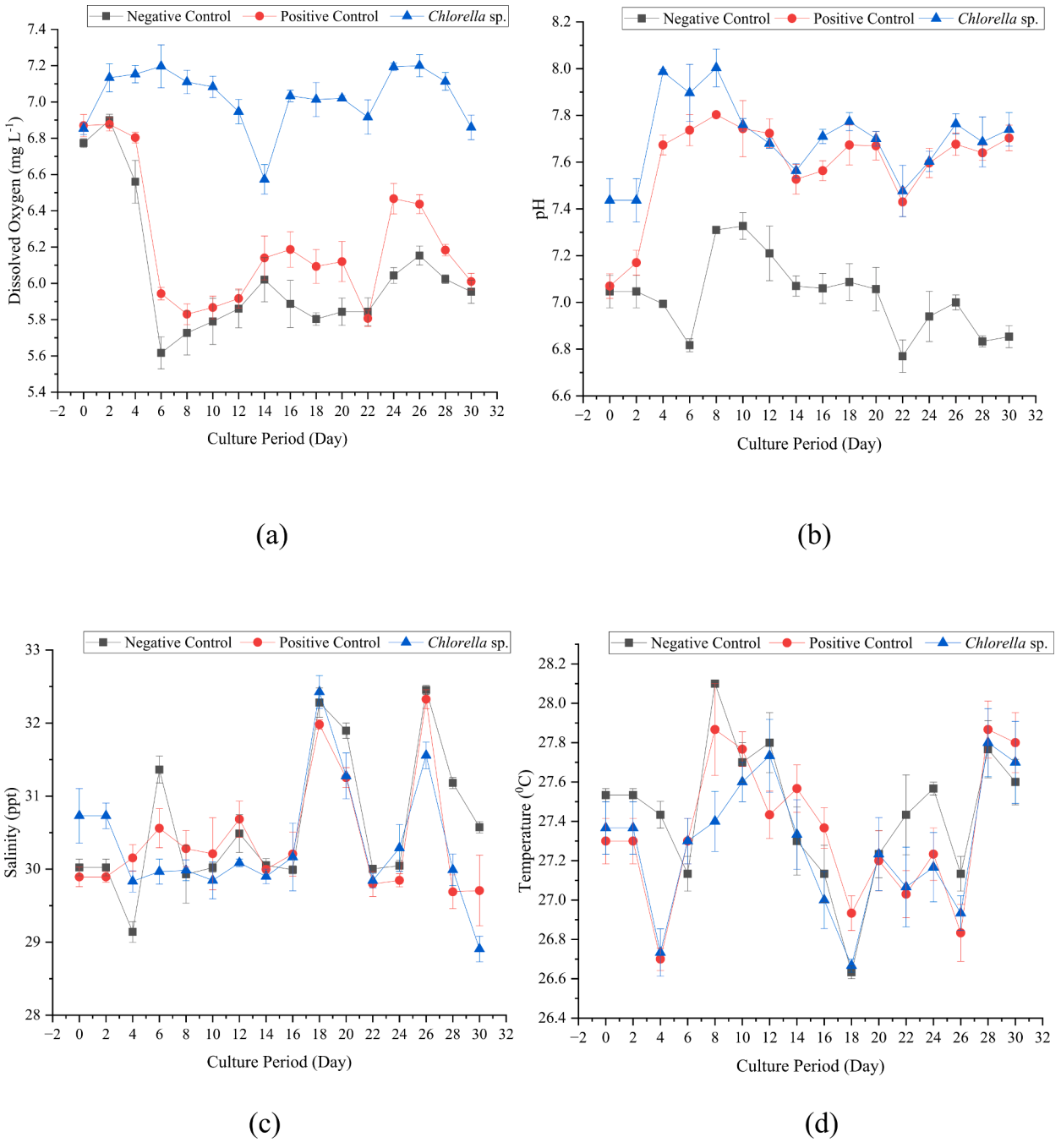

Fig. 4. The concentration of the dissolved oxygen (DO) (a), pH (b), temperature (c) and salinity (d) in the white-leg shrimp culture, *L. vannamei* with and without microalgae addition throughout 30 days of experimental period. Error bars represent standard errors of the means (n = 3).

and salinity of the environment main influenced the concentration of ammonia (NH_3) in water in the form of free ammonia or ionic ammonia (Mingming et al., 2020).

3.5. Nutrient analysis in culture system experiment

Generally, shrimp pond aquaculture involved with large volumes of water, which are regularly replenished by nutrients from left-over feeds, faeces, and other metabolite excretions of the cultured shrimps. Recently, microalgae are promising in maintaining water quality in system, particularly in removing nutrients; ammonia (NH_3), nitrite (NO_2^-) and phosphate (PO_4^{3-}) and producing oxygen through photosynthesis process. Therefore, main approach of *Chlorella* sp. addition in the present study minimizes waste production.

The findings indicated the concentration of nutrient with adding of *Chlorella* sp. UMT LF2 significantly affect ammonia, nitrite and orthophosphate concentrations ($p < 0.05$).

The findings illustrated in Fig. 5 show that the water quality parameters varied between the culture tanks. Ammonia concentration in Fig. 5(a) increased consistently in the control tank during the culture period, except for a drastic decrease from Day 16 in positive control tank due to 20% water changes. In aquaculture system, ammonia is nitrogenous waste that produced from many sources, including leftover feed, faeces (eliminated from the shrimp body) and microbial decomposition of organic matter in water bodies. As a result, the ammonia content in the culture tank was increased following the shrimp's development performance, and the waste was not siphoned during the culture period. The ammonia similarly rose dramatically increased in the *Chlorella* sp. tank but remained significantly lower than in the control tank until the end of the cultivation period. This is because microalgae will release the absorbed nutrient into the water bodies in the declination growth phase (Nasir et al., 2015).

Nitrite is also highly toxic to aquaculture. Fig. 5(b) shows that nitrite concentration increased in all treatments but decreased from Day 16 in the positive control tank and *Chlorella* sp. tank. On Day 26 of the culture period, the nitrite concentration was significantly higher than positive control tank and *Chlorella* sp. tank, which reached 3.73 mg L^{-1}. The concentration of nitrites in this study exhibited an increase in all culture tanks since Day 4 culture period. This suggest that the community of nitrifying bacteria requires time to grow for their establishment and the oxidation conversion of nitrogenous components (Howarth, 2022). In this study, ammonia, nitrite and orthophosphate concentrations were maintained within acceptable ranges for shrimp intensive culture which were <1.0 mg L^{-1}, <0.25 mg L^{-1} and 0.05–0.5 mg L^{-1}, respectively (Lucy Towers, 2015).

3.6. Shrimp growth performance

Table 2 tabulated the growth parameter performance of shrimp monitored through the 30-day culture period. At the end of the culture period (Day 30), the body weight of shrimp was significantly higher in the culture system addition of *Chlorella* sp. than in the control group without microalgae addition ($p < 0.05$). Meanwhile, the shrimp survival rate in positive control and *Chlorella* sp. was not significantly different ($p > 0.05$), which was more than 80%. The growth performance and survival rate of the shrimp in this study were comparable to that reported in previous studies that reported 83–88% for culture with addition of microalgae (Angela et al., 2021). This result demonstrated a comparable state for a modified extensive farming system with a 20% water exchange and a culture with zero water exchange with addition of microalgae.

The final weight (g) and survival rate (%) in *Chlorella* sp. tank were significantly higher than in the control group tanks. In contrast, the feed conversion rate (FCR) was considerably lower than that of the control group. These results indicate that adding mi-

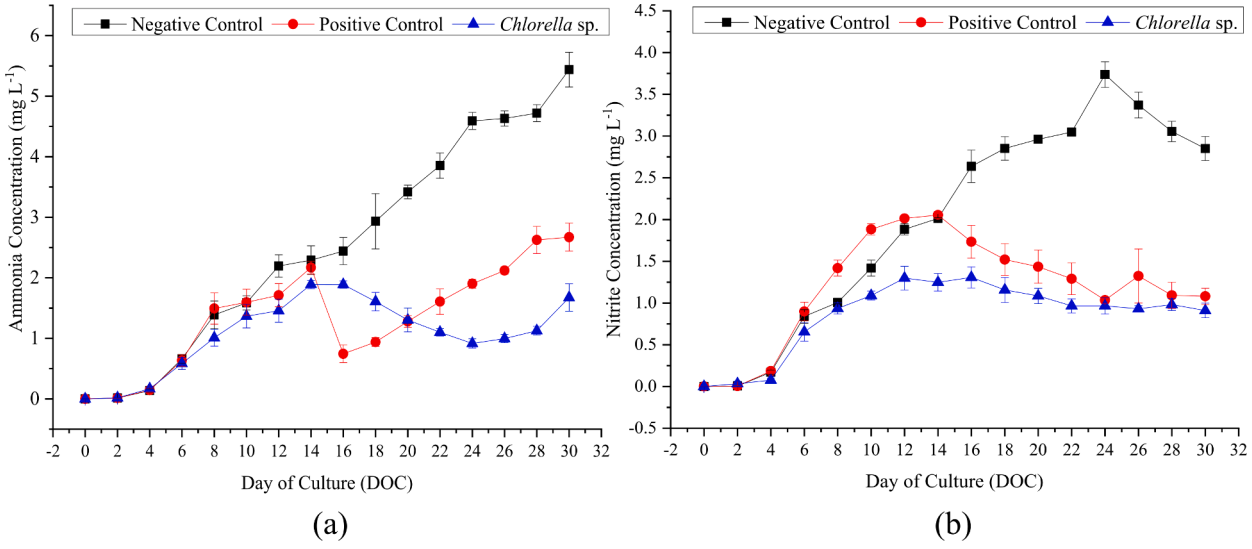

Fig. 5. The concentration of ammonia (a) and nitrite (b) in the white-leg shrimp culture, *L. vannamei* with and without microalgae addition throughout 30 days of experimental period. Error bars represent standard errors of the means (n = 3).

Table 2
Growth parameters of white-leg shrimp, *L. vannamei* in culture system for with and without the addition of microalgae, *Chlorella* sp.

	Negative Control	Positive Control	*Chlorella* sp.
Initial weight (g)	0.065 ± 0.01[a]	0.0611 ± 0.01[a]	0.064 ± 0.01[a]
Final weight (g)	0.639 ± 0.1[a]	0.593 ± 0.1[a]	0.954 ± 0.1[b]
Survival (%)	68 ± 2.52[a]	84 ± 3.51[b]	89 ± 8.14[b]
Feed Conversion Ratio (FCR)	2.25	2.71	1.44

Note: Different letters following mean values (±SE) in the same row indicate significant differences ($p < 0.05$).

croalgae of was helpful for the growth and feed conversion rate of white-leg shrimp. FCR is an essential for the new parameter in white shrimp cultivation because feed costs generally exceed 60% of the total cost of production which is particularly important for new farming techniques (Cuzon et al., 2004). On top of that, giving adequate feed might avoid abrupt water quality change. The FCR of *Chlorella* sp. tank is 1.44 which is in line with the previous studies that the FCR value of shrimp cultivation ranges from 1.2 to 2.5 (Venero et al., 2009).

Numerous studies have demonstrated that microalgae in a culture system have an excellent benefit for aquatic aquaculture system (Dong et al., 2022). The current findings indicated that adding of *Chlorella* sp. was conducive in enhancing *L. vannamei* growth performance when reared in culture system. Furthermore, *Chlorella vulgaris* was reported as a dietary supplement in shrimp production with rich in protein, vitamin, minerals, chlorophylls and other beneficial substances (Subramanian et al., 2015).

3.7. Central composite design of microalgae harvesting efficiency

For determination of harvesting study, filamentous fungus, *Aspergillus niger* was utilized to flocculate the excess green marine microalgae, *Chlorella* sp. UMT LF2 in culture system tank. Optimizing of the three independent variables was performed using a central composite design (CCD) with 20 runs of 5 central points. Results sufficiently suggest that the quadratic model was in good prediction of the experiments, and the terms in the model have a significant effect on the response. In addition, according to the significant p-value with $p > 0.0001$ and lack of fit that was determined to be not significant, the model was desirably fit to express the result. Therefore, regression model equation in equation (4) was developed considering the above factors. The CCD, as discussed earlier was used to establish the correlation between the selected variables to the harvesting efficiency.

The actual values of coded levels of different parameters: Dosage (A), pH (B) and mixing rate (C) are presented in Table 3 influence on harvesting efficiency of microalgae biomass, represented as Y_h, the response variable, has been investigated. The actual values of coded level '0' were fixed based on one-variable-at-a-time (OVAT) method. The equation in terms of coded factors can be used to predict about the response for given levels of each element. By default, the high levels of the factors are coded as +1 and the low levels are coded as −1. The coded equation helps identify the relative impact of the factors by comparing the factor coefficients.

$$Y_h = 89.51 - 1.57A - 3.19B - 3.98C + 1.65AB + 0.425AC - 3.775BC - 6.94A^2 - 6.90B^2 - 14.80C^2 \quad (4)$$

Where Y_h is the response, *A, B* and *C* is independent variables, A^2, B^2 and C^2 squared effects of the variables. A positive sign indicated that a variable's impact on harvesting efficiency was more potent at higher concentrations. In contrast, a negative sign indicated that a variable's influence was greater at lower concentrations (Duraiarasan, 2013).

In this study, the mixotrophic condition of *Chlorella* sp. with filamentous fungi for microalgal by fungal pelletization showed the best harvesting efficiency, 94.3% when an optimal condition was used; dosage 30 g L^{-1}, pH 7 and mixing rate 125 rpm (Table 3). The normal probability plot of residuals as illustrated in Fig. 6(a) shows some points scattered along the line demonstrating the normal distribution of residuals. The model's accuracy for each of the response variable was assessed by comparing the actual and predicted data similarity in the Actual vs. Predicted plot (Fig. 6(b)). The Residuals vs. Predicted values are plotted in Fig. 6(c) as a straight line. The plot displays a random scatter with a consistent range of residuals across the graph. The three diagnostic plots (Fig. 6(a–c)) show that the model has met the analysis of variance's presumptions and also illustrate the precision and applicability of RSM in optimizing the three parameters during the harvesting process.

Table 3
Central composite design of the variables with harvesting efficiency (%) as response.

Run	*A*	*B*	*C*	Y_h	
				Actual	Predicted
1	0	0	0	94.3	89.55
2	0	0	-α	57.2	54.3
3	0	+α	0	64.8	65.04
4	0	0	+α	42.4	39.8
5	0	-α	0	79.5	73.9
6	+1	+1	+1	51.4	49.95
7	0	0	0	86.8	89.6
8	0	0	0	89.7	89.6
9	0	0	0	93.4	89.6
10	−1	−1	+1	68.3	66.5
11	−1	−1	−1	63.9	67.7
12	0	0	0	89.05	89.6
13	−1	+1	-α	47.7	50.4
14	+1	−1	+1	55.3	57.1
15	+α	0	0	72.2	66.1
16	0	0	0	83.1	29.6
17	−1	+1	−1	68.5	64.3
18	-α	0	0	71.9	72.6
19	+1	−1	−1	59.3	60.4
20	−1	+1	−1	60.4	66.4

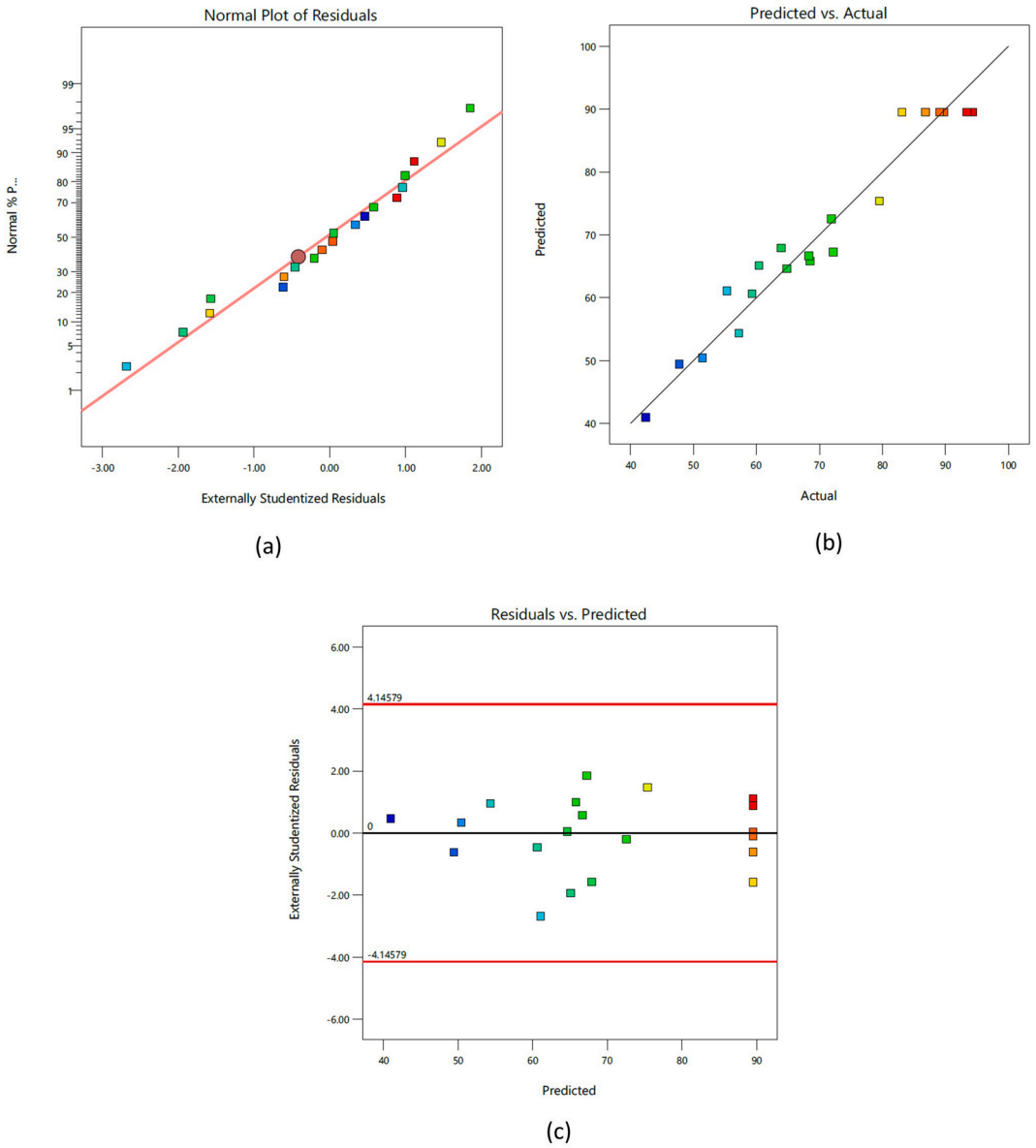

Fig. 6. Normal probability plot, residuals versus predicted plot, actual versus predicted plot of RSM quadratic model.

The three-dimensional contour plots shown in Fig. 7(a)–(c) indicates that presence of mutual interaction among the factors for the harvesting efficiency. The shapes of the contour plots indicate the significance of the interaction which an elliptical contour plot illustrates the significant effect of interaction. In contrast, a circular contour plot suggests that the interaction is insignificant. It can be seen that a more extensive response value was obtained with the changes in mixing rate, indicating the greatest effect on the response value (Fig. 7(b and c)). Fig. 7(a,c), shows the impact of dosage on the harvesting efficiency: at 30 g L^{-1}, the harvesting efficiency was maximum and while the dosage continued to increase, the efficiency was decreased. In contrast to Fig. 7(a) and (b), pH and dosage do not significantly affect harvesting efficiency, although the variable pH and mixing rate have a great influence. It shows that the pH correlated with the mixing rate in maximizing the harvesting efficiency. However, the continous increase of the mixing rate will decrease of the harvesting efficiency reaching 70% at a rate of 150 rpm.

In our previous study of harvesting single microalgae, *Chlorella* sp. by filamentous fungi, *A. niger,* the harvesting efficiency was positively correlated with the mixing rate (Mohd Nasir et al., 2019). However, high-speed mixing tends to break the flocs and excessive shearing force would in turn overcome the van der Waal's force resulting the coagulated cell's to redisperse again into the medium due to the cells restabilization (Choi, 2015). That was why this study's harvesting efficiencies at mixing rate of 150 and 167 rpm in this study were lower (<50) than in the same conditions at 125 rpm.

Fig. 8 shows the optimal combination of three parameters; dosage was derived in minimize, pH and mixing rate were derived by in range of the desirability function and by maximizing the desirability function of the harvesting efficiency. The optimization

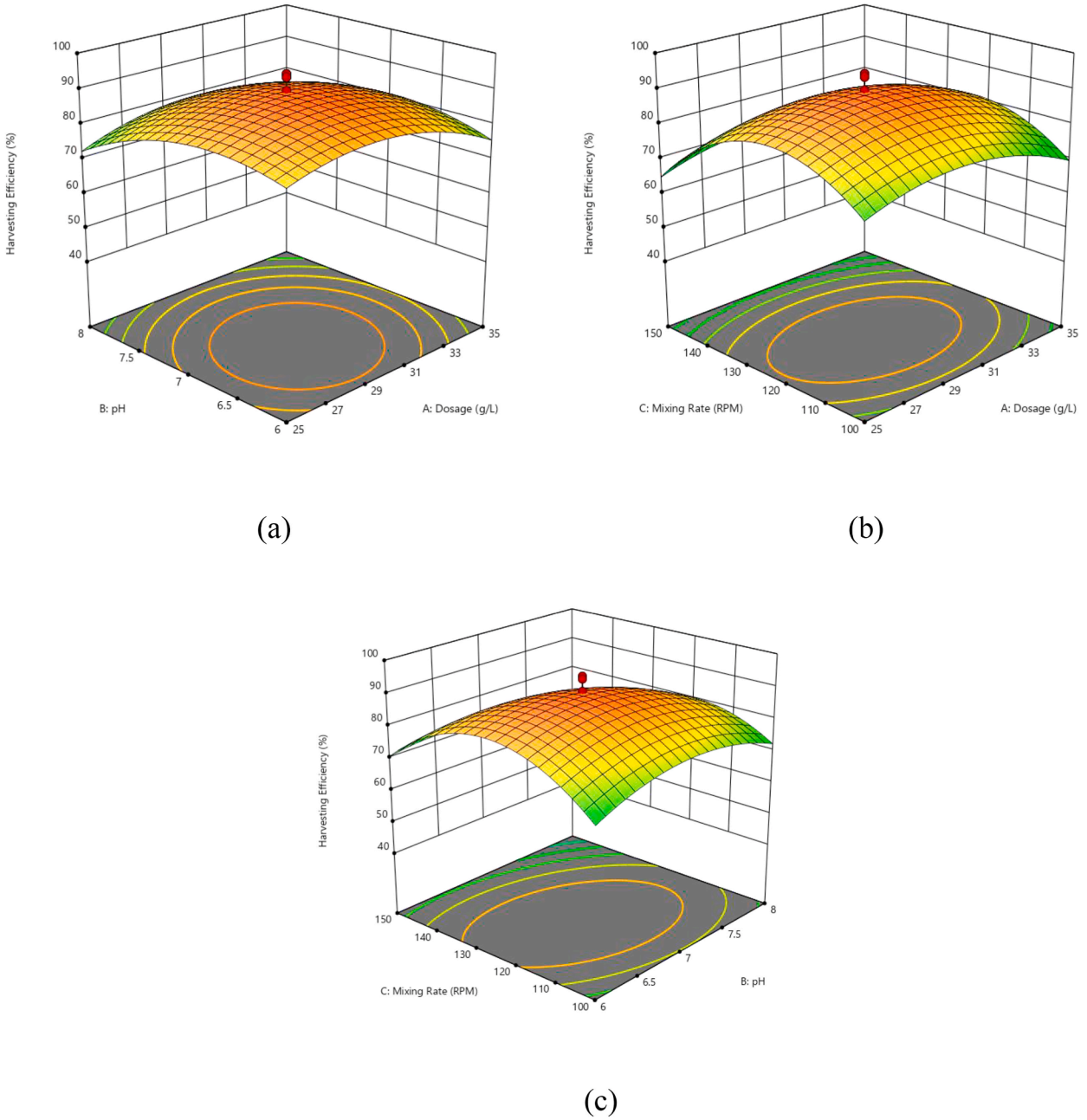

Fig. 7. Response surface and contour plot representing the harvesting process on various interactions between the three independent variables – Effect of Dosage and pH (a), Effect of Dosage and Mixing Rate (b), Effect of pH and Mixing Rate (c).

of the responses of the dosage, pH and mixing rate interaction is crucial for the operation of the harvesting process. The optimal combination of three parameters, 30 g L^{-1}, pH 6.8 and 122 rpm had the optimum parameters resulting in 90% harvesting efficiency with a desirability of 0.958 showed by solution 1. The confirmatory experiment, also known as the validation test showed a harvesting efficiency of 90% under optimal parameters compared with the harvesting efficiency of 90.05% obtained by the model. This demostrates the suitability and accuracy of the obtained solution provided by the CCD model.

4. Conclusion

The findings of this study indicated that the addition of *Chlorella* sp. successfully maintains a constant pH and enhance DO in water, limit the build-up of ammonia, nitrite, and orthophosphate in the culture system when added to 30-day intensive white-leg shrimp culture. Besides that, to increase the sustainability of wastewater treatment, microalgae also can transform nutrients (nitrogen

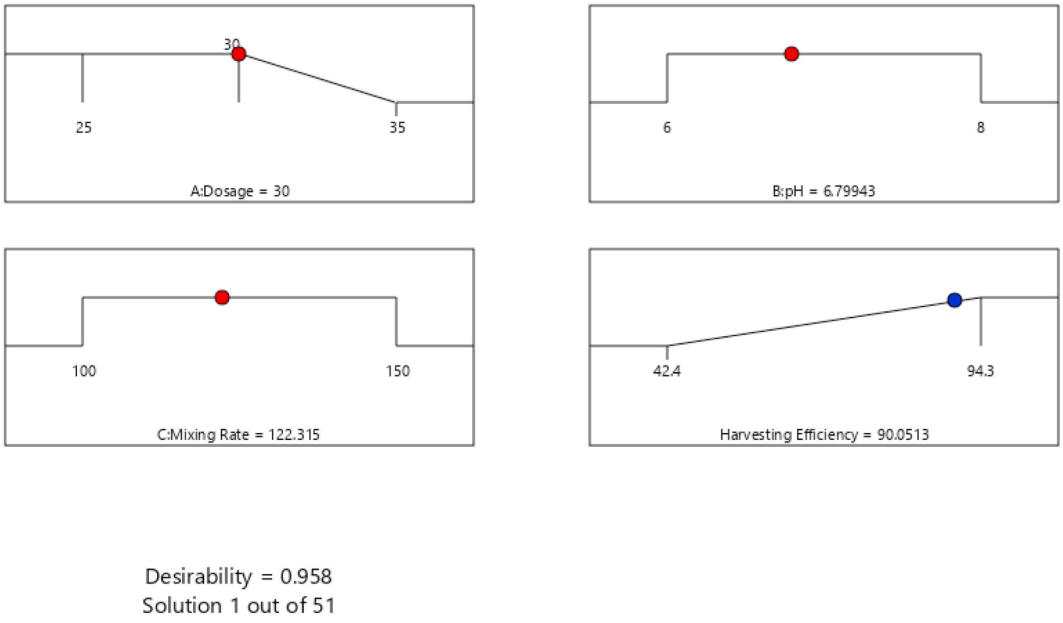

Fig. 8. Optimal parameters at the maximizing harvesting efficiency using desirability function.

and phosphorus) from wastewater into biomass and bioproducts. By enhancing the culture system, the addition of *Chlorella* sp. will reduce the operation cost by maintaining a water quality problem as well as can be meal supplementation to the shrimp development.

Credit author statement

Nurfarahana Mohd Nasir: Conceptualization, Methodology, Formal analysis, Writing - original draft. Ahmad Jusoh: Conceptualization, Writing - review & editing, Supervision. Hidayah Manan: review & editing, conduct on final submission. Nor Azman Kasan: Conceptualization, Writing - review & editing, Supervision, Funding acquisition. Amyra Suryatie Kamaruzzan: Supervision, Funding acquisition. Wan Azlina Wan Abdul Karim Ghani: Conceptualization, Writing - review & editing, Supervision. Setyo Budi Kurniawan: Investigation, Writing - review & editing. Fathurrahman Lananan: Investigation, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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