Bacillus amyloliquefaciens (IAE635) and their metabolites could purify pollutants, *Vibrio* spp. and *coliform* bacteria in coastal aquaculture wastewater

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Abstract: High-density aquaculture often causes the emission of polluted water to the marine environment in the coastal areas of China. To solve the aquaculture-related water quality problems, it is appropriate to adapt eco-friendly methods, such as using microbes and their metabolic products to purify polluted water. In this study, the purifying effects of *Bacillus amyloliquefaciens* (IAE635) metabolites (poly-γ-glutamic acid; PP) and IAE635 combined with their metabolites (MP) on turbidity, COD, NO_3^--N, NH_4^+-N, *Vibrio* spp. and *coliform* bacteria in coastal aquaculture wastewater were conducted in the lab and in situ ponds. The results showed that the removal of turbidity, COD, NH_4^+-N and NO_3^--N with PP and MP was more significant ($p < 0.05$). Compared to Control treatment (Co), the turbidity, COD, NH_4^+-N and NO_3^--N concentrations at 24th hour were evidently reduced by 86.6%, 87.5%, 83.3% and 58.0% for PP, 87.9%, 93.5%, 86.5% and 78.0% for MP, respectively. The populations of water pathogens under PP and MP were also significantly ($p<0.05$) removed compared with those of Co; at 24th hour, the *Vibrio* spp. and *coliform* bacteria were decreased by 68.7% and 66.3% for PP, 75.0% and 67.1% for MP, respectively. The water purifying effect of MP was slightly better than that of PP. In situ pond purification test demonstrated that MP significantly lowered the concentrations of turbidity, COD, NH_4^+-N, NO_3^--N and NO_2^--N, which was more effective than EM. A significantly higher ($p< 0.05$) γ-PGA concentration and the total bacterial population for MP compared to PP indicated that MP purifies the coastal aquaculture wastewater by both flocculation and microbial decomposition. The application of MP will benefit the aquaculture industry by providing a novel method for the removal of chemical pollutants and pathogens.

Keywords: coastal aquaculture, *Bacillus amyloliquefaciens* and their metabolites (MP), bio-flocculants, chemical pollutants, *Vibrio* spp., *coliform* bacteria, wastewater purification

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1 Introduction

With the recent rapid development of aquaculture in China, high-density and intensive breeding have become the most widely used approaches. For the intensive aquaculture, there are often residual feed, faeces and pathogens in the water[1-3]. Typically, it is characterized by increased nitrogen forms (NH_4^+-N, NO_2^--N and NO_3^--N), phosphates, suspended solids, BOD and COD as well as high populations of the opportunistic pathogen[2,4,5]. If the aquaculture water is untreated, it will result in the emission of nutrient-rich effluents that causes algae blooms, degradation of benthic communities, oxygen depletion and destruction of water quality[4,6]. Current practices for treating aquaculture wastewater include exchange with freshwater, sequencing batch reactors, wetland and filter systems[4,7,8]. However, the high cost of these methods decreases their widespread application.

In situ purification of wastewater in the aquaculture pond can decrease the quantity of water that should be exchanged, and therefore, it is a lower cost method with less discharge. Flocculation or coagulation is an effective method to quickly purify wastewater by using materials such as chitosan, polyacrylamide, or polymeric aluminium sulfate[9-11]. While these chemical flocculants have been shown to remove various toxins, most of them are not suitable for application in aquaculture with regard to food safety. Bioflocculants have been investigated for the removal of COD, nitrogen and organic pollutants of wastewater from brewing, meat processing, soy sauce brewing, and pharmaceutical and dairy production[12-14]. Additionally, for river water with low turbidity, a *Serratia ficaria* bioflocculant achieved removal efficiencies of 87.1% for COD and 84.2% for turbidity, which was better than that obtained with similar chemical synthetic flocculants[13,15,16]. Bioflocculants have been demonstrated to be effective in flocculating aquatic microbes as well. For example, the extracellular biopolymer produced by *Paenibacillus polymyxa* was shown to have a flocculating activity of up to 95% for algae[17].

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Moreover, the bioflocculant exuded by *Klebsiella pneumoniae* was used to remove *Acanthamoeba* cysts, an aquatic pathogenic amoeba, with a maximum removal efficiency of 84%[18]. A flocculant from another *Klebsiella* spp., *Klebsiella terrigena*, has been shown to remove *Cryptosporidium* oocysts from tap water with a removal efficiency of 62.3%[18].

Regarding the environmental problems caused by high-density aquaculture industry, it may be an appropriate approach for the application of microbes and their metabolic products to purify polluted waters. The study presented here examined the efficacy of MP as bioflocculant for treating the wastewater from an intensive shrimp aquaculture pond. As a biological purification method, the addition of MP would result in a reduction of COD, inorganic nitrogen and aquatic pathogens, which could assist for the healthy development of the aquaculture in coastal regions. Furthermore, the process described here, once scaled up, could provide an alternative method to high cost or chemically prohibitive aquaculture wastewater purification, which is of considerable significance to guarantee the sustainable development of the aquaculture industry in China.

2 Materials and methods

2.1 Microbes and medium

Bacillus amyloliquefaciens IAE635, which are capable of poly-γ-glutamic acid (γ-PGA) production, were isolated from coastal alkali-saline soil and obtained from the China Center for Type Culture Collection (accession number CCTCC 2013086). The γ-PGA culture medium of IAE635 was prepared using 35.3 g glutamate, 16.7 g citrate, 92.6 g glycerin, 1.2 g yeast extract, 1 g KH_2PO_4, and 0.5 g $MgSO_4$. The medium was amended with 100 mL distilled water, with a pH ranging from 6.0-6.3. Then, the prepared media were autoclaved for 20 min at 121℃. The concentration of γ-PGA produced by IAE635 fermentation was measured and determined to be 23.8 g/L[19].

The above fermented mixture (IAE635 and their metabolite γ-PGA) was reacted with the solution consisting of 2.6 g/L $Ca(OH)_2$, 5.1 g/L $CaCO_3$ and 13.8 g/L $CaCl_2$ by shaking at 150 r/min at 15℃ for 10 min. This mixture solution was stored at 4℃ and designated as IAE635 and their metabolite γ-PGA (MP).

Consistent with the concentration of γ-PGA produced by IAE635 fermentation, pure γ-PGA was dissolved in sterile tap water at a concentration of 23.8 g/L. This solution was also kept at 4℃ as the same as MP. The solution was designated as the pure γ-PGA solution (PP).

2.2 Experimental design

Water samples were collected from an intensive shrimp (*Litopenaeus vannamei*) aquaculture pond in Dongtai City, Jiangsu Province, China. The aquaculture water collection buckets were stainless steel cylinders and the collected water from different depths of aquaculture pond was filled by a bottom-sealed polymethyl methacrylate (PMMA) tube with a diameter of 10 cm and height of 120 cm. Table 1 indicates the initial properties of the aquaculture water before the purification experiments.

Three treatments were designed as presented in Table 2.

For each treatment, a 5 L aquaculture water sample was placed in the PMMA tube. Each treatment was replicated 3 times. After PP or MP addition, it was stirred with a 20 cm glass stick at 70 r/min for 10 s and then kept stationary for 24 h. Experiments were conducted in the greenhouse at temperatures of 29℃-40℃ (day) and 15℃-30℃ (night) with a natural photoperiod. To evaluate the purification effects of the aquaculture wastewater, samples were collected from the PMMA tube at a depth of 60 cm at 0.5^{th} h, 1^{st} h, 12^{th} h and 24^{th} h for each treatment. Some related water quality indicators (turbidity, COD, NH_4^+-N, NO_3^--N and populations of *Vibrio* spp., faecal *coliform* and total bacteria) were selected to determine for each treatment at four sampling time points.

Table 1 Main water quality parameters (Mean ±SD) of the study aquaculture pond

Water qualities	Concentrations
NH_4^+-N/mg·L^{-1}	1.02±0.14
COD/mg·L^{-1}	25.50±2.04
Turbidity/NTU	56.11±3.29
DO/mg·L^{-1}	7.11±0.54
NO_3^--N/mg·L^{-1}	25.54±1.26
Vibrio spp./10^2 cfu·mL^{-1}	13.2±0.85
Faecal *coliform*/10^2 cfu·mL^{-1}	7.20±0.63
Total bacteria (60 cm)/10^5 cfu·mL^{-1}	9.20±0.95
Total bacteria (119 cm)/10^6 cfu·mL^{-1}	1.60±0.11

Table 2 Experimental treatments

Treatment	Description
Co	Without adding PP or MP in the aquaculture water
PP	Adding PP to a final concentration of 2 mL/L in the aquaculture water
MP	Adding MP to a final concentration of 2 mL/L in the aquaculture water

In situ purification of intensive shrimp pond water was also conducted in Dongtai City by choosing an aquaculture farm located in coastal area, Jiangsu Province by which to compare the purifying effects of MP with EM, a commercial aquaculture water purification agent. Three treatments were set up as follows: (1) Control, without using any water purification agent in the aquaculture pond; (2) EM and (3) MP, 4000 mL of EM or MP was used per 667 m^2 of each aquaculture pond, EM or MP was mixed with aquaculture water at a volume ratio of 1:1000 and uniformly applied to the breeding pond via an electric spray. Water samples at the depth of 60 cm had been taken in three different places on the seventh day of each treatment. Numerical values of the water quality indicators for turbidity, COD, NH_4^+-N, NO_2^--N and NO_3^--N were determined and compared.

2.3 Water chemical properties determination

The turbidity of the water was measured using a WGZ turbimeter (Shanghai Licheng Technology Co., Ltd., China). The dissolved oxygen (DO) was measured using a dissolved oxygen analyser JPB-607 (Shanghai INESA Scientific Instrument Co., Ltd., China). The COD, NO_3^--N, NH_4^+-N and NO_2^--N levels were assayed using the method described by Fan et al.[7] and Travaini-Lima et al[8]. The γ-PGA concentration was measured using the method described by Ghosh et al[20].

2.4 Determinations of total bacteria, faecal *coliform* bacteria and *Vibrio* spp. in water

All bacterial populations are represented as colony forming units (cfu/mL). Water samples were collected from the pipes at depths of 60 and 119 cm. Total bacterial counts were examined according to the method described by Chen et al[19]. Briefly, coated ten-fold serial dilutions of the water sample were plated on beef peptone agar. The plates were incubated at 37℃ for 24 h, and the total colonies were counted. Faecal *coliform* bacteria were enumerated by filtering 100 mL of the water samples through a 0.45 μm membrane followed by inoculation on M-FC agar. These samples were incubated at (44.5±0.2) ℃ for 24 h prior to evaluation. Blue colonies on the M-FC agar were considered positive for faecal *coliform*[21]. *Vibrio* spp. were determined according to Kriem et al[22]. The spread-plate method was utilized with thiosulfate-citrate bile salts (TCBS) agar. The plates were incubated for 3 d at 20 ℃, and yellow-, green- or blue green-coloured colonies were considered positive for *Vibrio* spp.

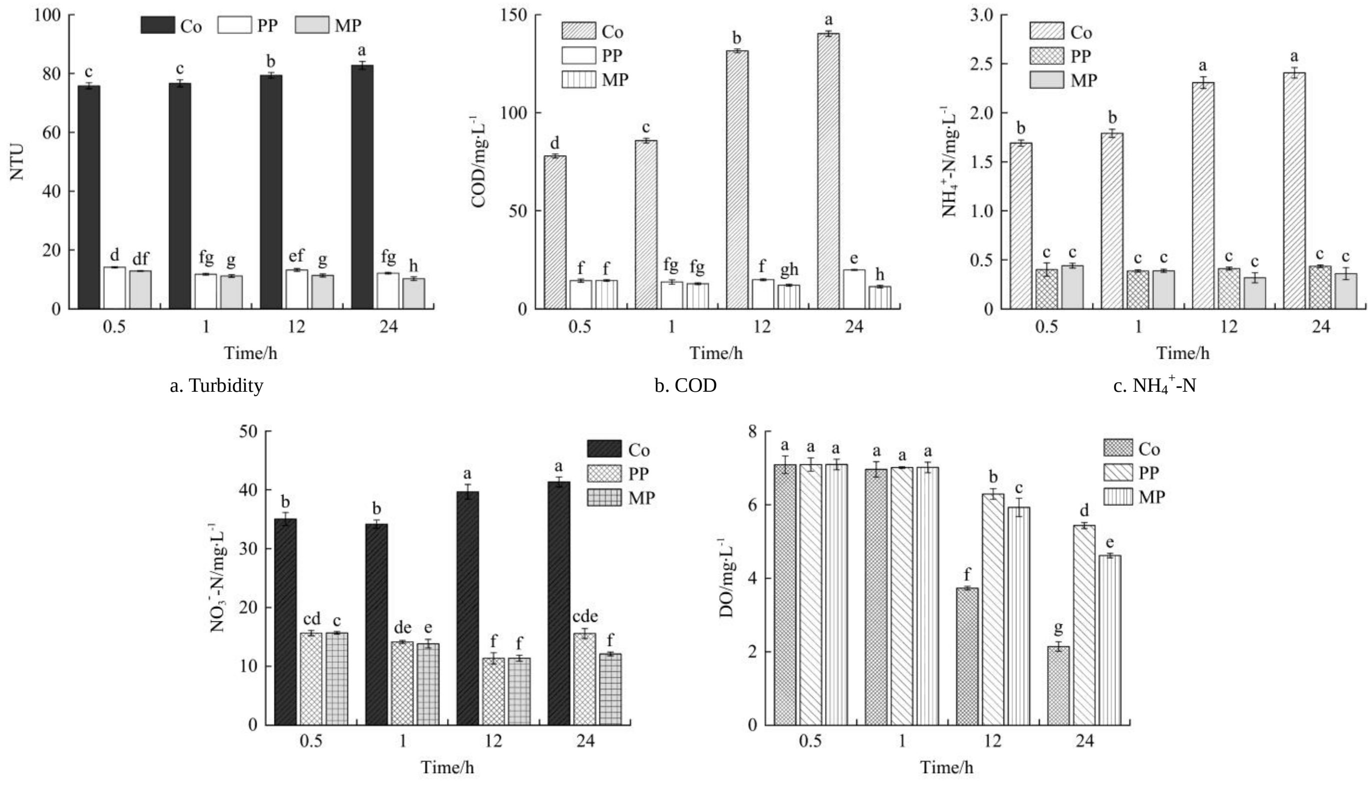
2.5 Statistical analysis

The experimental results were averaged over three replicates. The data were analyzed by using SPSS 18.0 software. Analysis of variance (ANOVA) was used in these tests, and the separation of means of the treatments was performed with Tukey's test (p<0.05) according to the methods of Ruxton et al[23]. The GraphPad Prism 6 software package was used to generate the graphs to present the experimental results.

3 Results

3.1 Changes in water quality under different treatments

Figure 1 shows that turbidity, COD, NH_4^+-N and NO_3^--N have a similar change trend with the reaction time for Co, PP and MP treatments. Generally, there was no evident change for above water quality parameters during 1st h, but significant (p<0.05) differences were observed at both 12th and 24th h. At 24th h, compared to the Co, turbidity, COD, NH_4^+-N and NO_3^--N were decreased by 86.6%, 87.5%, 83.3% and 58.0% in PP, while they were 87.9%, 93.5%, 86.5% and 78.0%, respectively in MP. The DO under all treatments showed no evident difference at 1st h (Figure 1e); however, a significant (p<0.05) decrease in DO was observed at 12th and 24th h. Compared to the PP and MP, the DO in the Co was lowered by 41.7% and 37.8% at 12th h, while it was reduced by 63.3% and 56.0%, respectively at 24th h.



a. Turbidity b. COD c. NH_4^+-N

d. NO_3^--N e. DO

Note: Means followed by the different lowercase letters are significantly difference at the 0.05 level (p < 0.05).

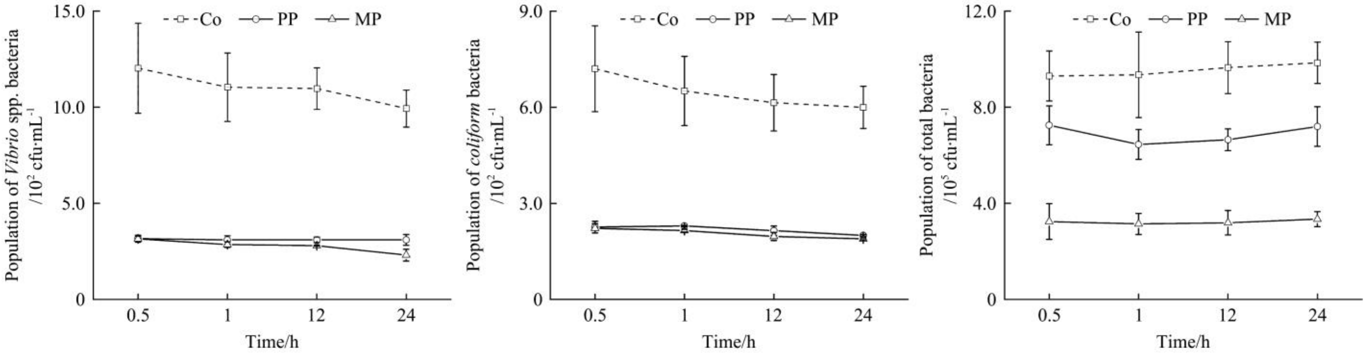
Figure 1 Effect of MP and PP on the (a) turbidity, (b) COD, (c) NH_4^+-N, (d) NO_3^--N, and (e) DO in aquaculture wastewater (60 cm samples)

3.2 Effects of MP and PP on *Vibrio* spp., faecal *coliform* and total bacteria（60 cm sample)

Figure 2 illustrates the *Vibrio* spp., faecal *coliform* and total bacterial colonies for the aquaculture water samples tested over a 24 h period. Compared to the Co, the populations of waterborne *Vibrio* spp., faecal *coliform* and total bacteria in the PP and MP were significantly (p<0.05) decreased at 30 min. Furthermore, this decrease (p<0.05) was maintained over the 24 h test period. At 24th h, *Vibrio* spp. and faecal *coliform* in PP were decreased by 68.7% and 66.3%, while they were reduced by 75.0% and 67.1% in MP (Figures 2a and 2b). As expected in 60 cm water layer, total bacterial colonies in PP were higher (p<0.05) than those in MP due to stronger bioflocculating activity for MP. The total bacterial populations in MP and PP were decreased by 66.3% and 26.5%, respectively, compared to those of Co at 24th h (Figure 2c).

3.3 γ-PGA concentration and total bacterial population in the bottom (119 cm sample)

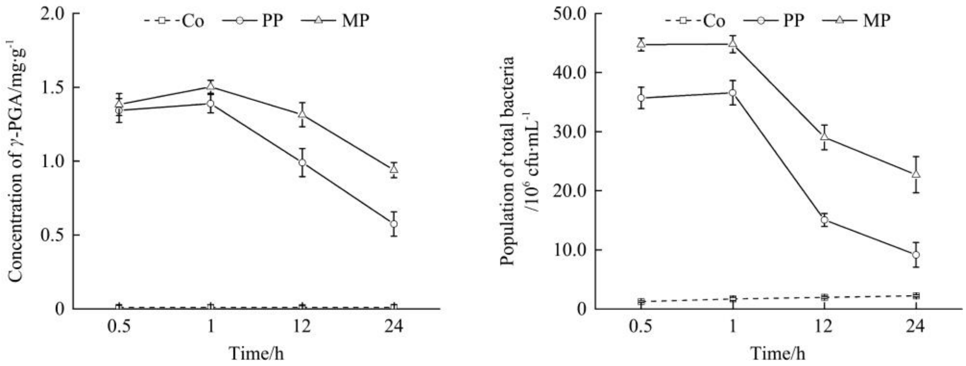
The γ-PGA concentration and total bacterial population of the bottom water (119 cm) were measured and shown in Figures 3a and 3b. Compared to γ-PGA concentrations examined at 30 min, they showed a 57.0% and 31.7% reduction for PP and MP at 24th h. Additionally, MP maintained a significantly higher (p<0.05) γ-PGA concentration than PP. Unlike the 60 cm sample, the total bacterial colonies of the 119 cm sample in both PP and MP, were significantly higher (p<0.05) than the Co. Furthermore, inconsistent with the 60 cm sample data, the number of isolated bacterial colonies from MP was significantly higher (p<0.05) than that from PP. The total bacterial colonies at 24th h in MP were 10.3 and 2.5 times higher than those of the Co and PP, respectively.



a. *Vibrio* spp. b. Faecal *coliform* c. Total bacteria

Note: The data are presented as the means ± SD.

Figure 2 Population of (a) *Vibrio* spp., (b) faecal *coliform* and (c) total bacteria from aquaculture wastewater (60 cm sample)



a. γ-PGA b. Total bacterial

Note: The data are presented as the means ± SD.

Figure 3 Concentration of (a) γ-PGA and (b) total bacterial population of the bottom water (119 cm sample)

3.4 In situ aquaculture pond water purification

As shown in Table 3, the spraying of MP into the shrimp pond could significantly lower the concentrations of chemical pollutants compared with the Control and EM treatments (p<0.05) on the 7th day. It indicated that turbidity, COD, NH_4^+-N, NO_3^--N and NO_2^--N in MP treatment were reduced by 74.1%, 65.2%, 60.1%, 73.7% and 78.8%, respectively, compared to Control treatment.

Table 3 In situ aquaculture pond water purification (Mean ± SD)

Treatment	NTU	Water qualities on the 7th day/mg·L⁻¹			
	Turbidity	COD	NH_4^+-N	NO_3^--N	NO_2^--N
Control	56.00±2.15^a	53.21±1.57^a	20.18±1.28^a	13.71±1.10^a	0.52±0.03^a
EM	43.57±2.30^a	42.12±3.15^a	18.23±2.04^a	11.26±0.87^a	0.41±0.01^a
MP	14.56±1.22^b	18.54±1.11^b	12.12±0.84^b	3.64±0.41^b	0.11±0.02^b

Note: The values followed by the different lowercase letters in a column are significantly different at the 0.05 level (p<0.05).

4 Discussion

Microbial reagents have been shown to be effective in removing COD and inorganic nitrogenous waste originated from the uneaten feed and excretion in aquaculture[4,24]. For example, EM used in the aquaculture water purification could transform NH_4^+-N or NO_3^--N to N_2 by nitrification and denitrification[5,6]. However, this natural purification process in high-intensity aquaculture is not completed prior to pollutants inflicting damage to aquatic organisms[14,25]. The results for MP as bioflocculant presented in this study over a 24 h experimental period are in line with previously reported data for chemical flocculants, such as polyacrylamide, aluminium and iron compounds[13]. The MP showed no bio-toxicity[26,27] and the removal rate of pollutants increased as the application amount of MP increased[5]. The removal of pollutants was increased as the extracellular peptide content of the IAE635 increased in PP treatment[28]. For MP treatment, it could be the increase in the concentration of starch in the IAE635[28,29]. In aerobic conditions, the nitrosation and nitrification effects of the *Bacillus* in the water were enhanced to result in a higher removal rate of nitrogenous waste[29]. In the previous study, the application of 2 mL/L PP/MP was recommended for aquaculture wastewater purification[5]. At the same application concentration, MP had a more satisfactory removal rate of pollutants than PP did.

Our results in this study indicated that the addition of 2 mL/L PP or MP to the aquaculture wastewater could significantly reduce the concentrations of turbidity, COD, NH_4^+-N and NO_3^--N. The evident reductions for PP treatment suspected that this purification effect was mainly due to the flocculation of -PGA, not primarily by the microbial processes[11,15]. However, the presence of the IAE635 provided a synergistic effect. The in-situ spraying results in this study also indicated that MP could rapidly remove the chemical pollutants compared with EM. It may prove that the purification effects of MP for the chemical pollutants are mainly attributed to the flocculation as the biofloculant, not to the single microbial degradation.

In addition, biotic pollutants such as bacterial pathogens must be considered when selecting aquaculture water purification methods. *Vibrio* spp. have been reported to be one of the most dangerous groups of pathogens, causing numerous aquatic diseases in aquaculture industry[1,7]. Furthermore, higher *Vibrio* spp. populations in the water produce a potential threat to the aquatic organisms[1,30,31]. Another bacterial pathogens of concern are *coliform* bacteria because they serve as the indicator of faecal contamination in the water[21]. This study found that the flocculation induced by γ-PGA was capable of lowering the amounts of *Vibrio* spp. and faecal *coliform* in aquaculture wastewater. It suggests that applications of PP/MP can also effectively decrease the overall use of antibiotics in aquaculture systems. The higher reduction of γ-PGA concentration collected from the 119 cm depth in PP-treated sample than in MP-treated sample may be a result of MP having contained the γ-PGA producing IAE635. Furthermore, our results suggested that IAE635 acted synergistically since MP appeared to purify the aquaculture wastewater better than PP. There existed the sufficient differences for the total bacterial population in different water layers and treatments. The results suggested that the increased population of total bacteria in the 60 cm depth water via the reaction time was not completely from the release of coagulation in the bottom water. It is assumed that the decrease of total bacteria in the bottom water via the reaction time could have resulted from microbial death or predation among microbes[16,24]. A relatively higher level of microbial heterogeneity in the aquaculture system often reduces the vulnerability of aquatic organisms to opportunistic bacteria such as *Vibrio* spp.[1,31] The increase in the PGA for MP compared to PP was attributed to the proliferation of IAE635 to produce, which presumably improved the aquaculture water quality.

5 Conclusions

The results of this study indicated that MP was effective in removing the chemical pollutants and dangerous pathogens from aquaculture water by flocculation and microbial decomposition. Specifically, compared to the Co, the removal efficiencies of turbidity, COD, NH_4^+-N, NO_3^--N, *Vibrio* spp. and *coliform* bacteria at 24th h were 87.9%, 93.5%, 86.5%, 78.0%, 75.0% and 67.1%, respectively. Additionally, there was a significant increase in water purity of the aquaculture system as a result of using MP compared to PP or EM. MP decreased the population of *Vibrio* spp. and *coliform* bacteria in the aquaculture wastewater better than PP did. The application of MP will benefit the aquaculture industry by providing an effective and sustainable method for the removal of chemical pollutants and pathogens. Therefore, this study provides a novel measure for treating high-intensity aquaculture wastewater and guidance on how to reduce the emission of coastal aquaculture wastewater to the marine environment.

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