ISOLATION AND CHARACTERISATION OF BIOFLOCCULANT-PRODUCING BACTERIA FROM MUD CRAB (*Scylla* sp.) AQUACULTURE PONDS

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Abstract: Mud crab is one of the most delectable and in-demand foods in the world, but there is a shortage of it because of the greater death rate of their seeds in the hatchery owing to disease, malnutrition, and poor water quality. The use of Biofloc Technology (BFT), a widely used, ecologically friendly technique that is effective for good nutrition, healthy cultures, and good water quality, has not yet been tried for mud crab farming. This study aimed to isolate and characterise potential Bioflocculant-producing Bacteria (BPB) from a mud crab, *Scylla* sp. aquaculture ponds to apply them as an inoculum of mud crab biofloc system. BPB was isolated from the water and sediment using the serial dilution, spread plate, and streaking plate methods on marine agar media, Yeast Peptone Agar (YPG) media, and enrichment media. Morphological, biochemical, and 16s rRNA molecular approaches were performed to screen the bacteria, where the flocculating activity of the bacterial species was determined using the kaolin clay suspension method. A total of 88 isolates were successfully found from both water (82 isolates) and sediment (6 isolates) samples. 18 bacteria isolates showed floc-forming characteristics such as slimy and milky appearance on YPG agar and enrichment media and were identified as *Bacillus cereus, B. tropicus, B. infantis, Vibrio alginolyticus, Priestia flexa,* and *Micrococcus luteus*. *B. tropicus* showed the highest (95% ± 0.135) flocculation activity, whereas *M. luteus* showed the lowest (74.81% ± 5.985) flocculation activity. Six prospective biofloc bacteria with increased flocculation activity were identified in this study, and these can be used in further studies on mud crab hatchery operations.

Keywords: Biofloc technology, BPB, 16s rRNA sequencing, flocculation activity, mud crab.

Introduction

Aquatic animal production including fishes, molluscs, and crustaceans was 82.1 million metric tons in 2018, with a 32% rise predicted by 2030 (FAO, 2020). Intensification of the aquaculture sector increases the production rate of culturing farms, while simultaneously addressing pollution issues, such as toxic nitrogen pollution caused by increasing stocking density and feed input. A study in Japan stated culturing each tonnes of fish generates 0.8 kg N and 0.1 kg of phosphorus (P), which is similar to the waste produced by 73 individuals per day (Suzuki *et al*., 2003).

Biofloc Technology (BFT) allows for pollution management by turning pollutants into biofloc aggregates, which culture organisms consume as food (Hargreaves, 2006). The toxic nitrogenous wastes are utilised in the BFT system by two types of bacteria: Functionally ammonia assimilating heterotrophic bacteria and nitrifying chemoautotrophic bacteria (Ebeling *et al*., 2006a). Aquaculture wastewater has

always been a problem for years and is looking for technological innovations to minimise or remove toxic effluents. These wastewater effluents are comprised of solid wastes generated from faces, uneaten feed, and dissolved wastes generated by the food metabolism in fish like nitrogen (N), and phosphorus (P). Flocculation is a less expensive, easier, and more successful way to remove colloids, suspended particles, and cell debris from these wastes (Zhang *et al*., 2012). Yu *et al*. (2009) identified flocculants and explained how they work. The study categorised flocculants into two groups: Synthetic and natural flocculants. Synthetic flocculants are more effective at treating water than natural ones, but they are not advised due to their greater cost and unfriendly nature to the environment. Despite being less effective than synthetic flocculants, natural flocculants are nonetheless advised due to their cost-and environmentally friendly properties.

Bioflocculant is a degradable flocculant made by combining bacteria, protozoa, fungus, and microalgae with other organic particles and the faces of cultured organisms (Hargreaves, 2013). It is dominated by heterotrophic bacteria, which can consume organic carbon and reduce ammonia-N levels through a nitrogen assimilation process, resulting in increased bacterial biomass in the system (Emerenciano *et al*., 2017). It maintains biofloc by controlling the carbon and nitrogen ratios in the system to support microbial growth. Avnimelech (1999) stated that utilising the heavy loads of inorganic chemicals of intensified aquaculture system resulting in the recycling of nutrients and water quality maintenance.

Extracellular Polymeric Substances (EPS) are secreted by microorganisms in the biofloc system, which aid in the synthesis of biofloc through bacterial cell aggregation in flocs, biofloc structure stabilisation, water retention, organic compound sorption for nutrient accumulation and enzymatic activity accumulation (Wingender *et al*., 1999; Laspidou & Rittmann, 2002). *Bacillus* sp., *Lactobacillus* sp., *Streptococcus* sp., *Staphylococcus* sp.,

Corynebacterium sp., *Serratia* sp., *Neisseria* sp., *Vibrio* sp., and *Klebsiella* sp. were identified from *Penaeus vannamei* pond as floc producers and among them *Staphylococcus* sp. was identified with the highest 829 mgL^{-1} extracellular protein (Kasan *et al*., 2016). *Bacillus megaterium* was discovered to have high flocculation activity in kaolinite and hematite solutions, with 90% in kaolinite and 85% in hematite (Devi & Natarajan, 2015).

Another study on *Cobetia* spp. determined with acidic polysaccharides as EPS found that all species of the genus had flocculation activity greater than 90% (Ugbenyen *et al*., 2012). *Pseudomonas sp.,* a flocculating bacteria isolated from an Egyptian wastewater treatment plant, was shown to have 99.89% flocculation activity (Hussien *et al*., 2018). Alias *et al*. (2022) stated that an effective biofloc producing bacteria (BPB), *Bacillus velezensis* isolated from the Langat River, Selangor, Malaysia showed 92.3% flocculation activity that was the highest among 34 isolates. The first commercial application of BFT in aquaculture has been recorded in the mid-1990s in Belize in North America and this technology is adopted mostly for catfishes, carp fishes, cichlids, and shellfishes (Vyas, 2020)

Among shellfishes, shrimp is the only group that is considered for culture in the biofloc system, and the technology has shown several pieces of evidence on the enrichment of nutrients profile and health status. *Litopenaeus vannamei* post-larvae have been reported to have 26% more weight gain, lower Feed Conversion Ratio (FCR), reduction of nitrogenous wastes, and lower virulence of *Vibrio haemolyticus* for Acute Hepatopancreatic Necrosis Disease (AHPND) (Kumar *et al*., 2020). Similar results for growth, feed conversion, and water quality were observed for the same species, *L. vannamei* in Mexico (Luis-Villaseñor *et al*., 2015). Like shrimp farming, mud crab culture is also a famous and economically profitable farming technique, but they are not still considered for culturing in biofloc system as experts suggested some reasons like cannibalism, higher labour cost, and complex operating system. Although

the mentioned reasons are not scientifically proven in any biofloc-based research, the experts have suggested them from the prospective analysis.

The zoea larval stages (zoea 1 through zoea 5) of mud crab can be cultured using biofloc technology because they don't exhibit cannibalism. Lower survivability and growth rate of mud crab larval stages are the main obstacles to the continuous seed supply and poor growth at grow-out mud crab farms (Jithendran *et al*., 2010; Rahman *et al*., 2019) and the researchers found nutritional imbalance (Holme *et al*., 2009), water quality (Li *et al*., 2008; Li *et al*., 2012), and diseases (Jithendran *et al*., 2010) are the main reasons behind the fact. A lot of previous studies already proved BFT as a promising tool to improve nutritional status, water quality, and immune system. So, the application of BFT in mud crab zoeal stages could be an effective method to minimise these hatchery operating obstacles. In order to establish biofloc technology in mud crab aquaculture, which has not previously been done, the goal of this study was to identify some viable bioflocculant-producing bacteria from the mud crab aquaculture system.

Materials and Methods

Sampling Site

The sampling site is a mud crab grow-out farm (N04°31.151 E103°27.197) that is located in Kerteh city in Terengganu state in Malaysia. The farm is located 118.09 meter eastern of Sungai Kertih. The mud crab farm was a semi-intensive farm with an area of 20,000 meter^2. Each of the pond size was about 400 meter^2. There are six rectangular ponds with a common inlet and outlet for all the ponds. Three out of six ponds were selected randomly for the sampling operation. The farm collects marine water during high tide. The sampling was conducted on 28th November 2021 and at that time the culture was in post-harvest phase.

Collection of Samples

Water and sediment samples were collected from three randomly selected ponds. The water sample was collected following protocols of WHO, (1997). Sterile glass sample bottle (Duran® laboratory bottle Sigma-Aldrich) of 500 ml was used to sample pond water. The glass bottle was rinsed with the pond water three times before collecting water sample. The
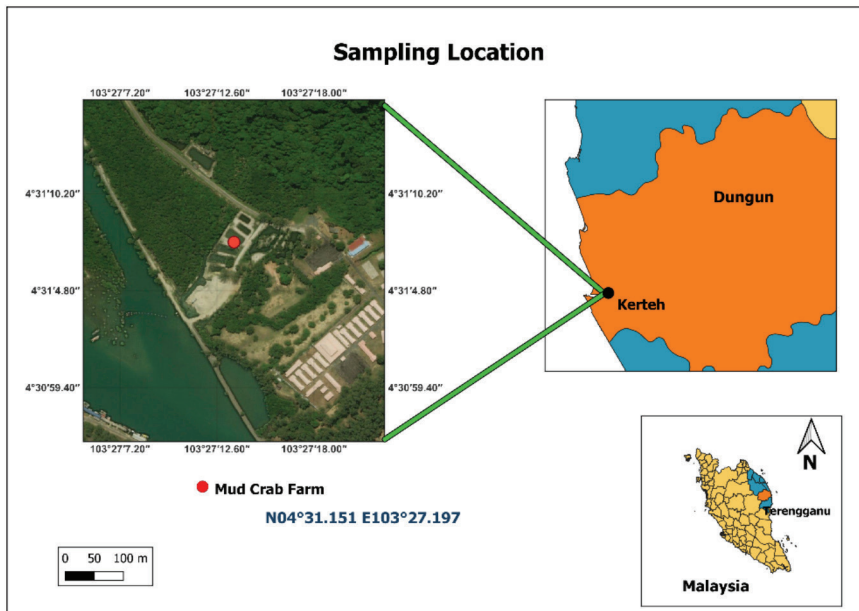

Figure 1: Location of the sampling site, mud crab grow-out farm located at Kerteh in Terengganu state of Malaysia

sediment sample was collected by following the protocols of EPA (2007). The sediment sample was collected using a Ponar grab (Ponar grab PG Aquatic Biotechnology) and collected in a sterile Ziplock bag and then stored at 4°C for further laboratory analysis. Both the water sample bottles and Ziplock bags were sterilised using 70% alcohol before each sampling operation to avoid any cross-contamination. The *in-situ* physicochemical characteristics (pH, DO, temperature, salinity) of water samples were determined using YSI multiparameter (Hydrolab Quanta, Germany), while total ammonia nitrogen, soluble reactive phosphate, nitrite-nitrogen were analysed following spectrophotometric method in the laboratory.

The Total Ammonia Nitrogen (TAN) was determined by following Parsons *et al.* (1984). A stock solution ammonia was prepared by mixing 3.67 g $(NH_4)_2SO_4$ in 1000 ml deionized distilled water. The 1,000 ppm ammonia was diluted with distilled water to 0.05, 0.1, 0.3, 0.5 and 1.0 ppm. 5 ml water samples were mixed with 0.2 ml phenol solution and then vortexed by using vortex machine (DLAB MX-S). 0.2 ml sodium nitroprusside was added and vortexed again. 0.5 ml oxidation reagent was added then and waited for 60 minutes for the reactions. The above-mentioned process was also conducted for the stock solutions and the absorbance value was taken for 640 nm (SHIMADZU UV Spectrophotometer UV-1800) for the stock and sample solutions. The concentration of ammonia was measured against the stock solutions standard graph. Phenol solution was prepared by dissolving 11.1 ml (10 g) phenol in 100 ml ethanol (95%). The oxidation reagent that was made by adding 100 ml alkaline reagent (20 g sodium citrate, and 1 g NaOH dissolving in 100 ml ddH$_2$O) with 25 ml sodium hypochlorite. The nitrite nitrogen (NO_3^- N) was also measured by following Parsons *et al.* (1984). The nitrite stock solution was made by dissolving 1.631 g KNO$_3$ in 1,000 ml ddH$_2$O. This 1,000 ppm nitrite solution was diluted with distilled water to 0.05, 0.1, 0.3, 0.5 and 1.0 ppm. Water sample (10 ml) was added with 0.2 ml sulphanilamide solution.

The solution was kept for 8 minutes and vortexed. 0.2 ml N-(1-napthyl)-ethylenediamine dihydrochloride (NED) solution was added and waited for 1 h before taking the absorbance at 543 nm. The stock solutions were also measured for making the standard graph by following the above-mentioned process. The Soluble Reactive Phosphate (SRP) was determined by following APHA, (1998). The stock phosphate solution was prepared 1,000 ppm by dissolving 1.432 g KH$_2$PO$_4$ in 1,000 ml ddH$_2$O. By diluting the 1,000 ppm phosphate solution with distilled water, 0.05 ppm, 0.1 ppm, 0.3 ppm, 0.5 ppm, 1 ppm phosphate solution was prepared. 5 ml water sample was mixed with 0.5 ml mixed reagent and waited for 1 h before taking the absorbance at 880 nm. The absorbance of the different stock solution was also taken to determine the phosphate concentration by making a standard graph after following the above-mentioned process.

Preparation of Different Media for Isolation and Screening of Biofloc-producing Bacteria

Marine nutrient agar (Zobell marine agar 2216) that is a commercial nutrient media specially for marine heterotrophic bacteria was prepared for the plate culture of bacteria by suspending 55.25 g in 1,000 ml of distilled water and then heated to boiling the solution for dissolving. Marine nutrient broth medium was also prepared by suspending 40.25 g in 1,000 ml distilled water. The preparation formula of YPG (Yeast Peptone Glucose) was glucose 10.0 g, peptone 2.0 g, urea 0.5 g, yeast extract 0.5 g, NaCl 0.1 g, MgSO$_4$.7H$_2$O 0.2 g, KH$_2$PO$_4$ 2.0 g, K$_2$HPO$_4$ 5.0 g, bacteriological agar 15.0 g and dissolved in up to 1000 ml distilled water (Chen & Zhao, 2003). The enrichment media preparation procedure was obtained from Che Hashim *et al*., (2019) by suspending glucose 10 g, urea 0.5 g, MgSO$_4$.7H$_2$O 0.2 g, 0.2g, KH$_2$PO$_4$, K$_2$HPO$_4$ 5 g, yeast extract 0.5 g in 1,000 ml filtered seawater. All media were autoclaved at 121°C for 15 minutes at 15 lb/inch^2 and the pH was maintained at 7.0 by adding 0.1 N HCl or 0.1 N NaOH.

Isolation and Screening of Biofloc-producing Bacteria

The water and sediment samples were processed for bacteriological assay immediately after they arrived at the laboratory. The bacteria from the water and sediment samples were isolated using marine agar according to Zaki *et al*. (2011). The water sample from three ponds was serially diluted with 0.9% saline water up to 10^{-3} in a 1 to 10 dilution series. 10 g of sediment from each sample was weighed and diluted using sterile phosphate buffer up to 10^{-5} dilutions. 0.1 ml of water and sediment solution was spread on a marine agar plate and kept at 30°C for 24 h. The bacteria colonies on the agar plate were examined after 24 h and streaked and re-streaked on another agar plate until the microscopic observations (Nikon ECLIPSE E200, 40X magnification) showed a single isolate. The aseptic control of this study was followed according to Burdass *et al*. (2009) to avoid any cross-contamination.

The screening of biofloc-producing bacteria from the isolates was carried out following the procedure by Che Hashim *et al*. (2019). For the glistening and slimy appearance on YPG agar, a loopful colony of the isolates was inoculated in 10 ml marine broth and then incubated for 24 h at 30°C. Then the bacteria solution from the broth was spread on YPG agar. Ropy colonies were examined in the enrichment media by inoculating the colony of isolates. For the observation of the floc forming attributes of the isolates, the YPG and the enrichment media were incubated at 30°C for 48 h. The biofloc-producing attributes showing isolates were termed as 'BP' before their further analysis. Bacterial colony characteristics and Gram staining test was done to isolate and identify the types of bacteria but for the characterisation, at the strain level, molecular analysis was conducted. The aseptic control of this study was followed according to Burdass *et al*. (2009) to avoid any cross-contamination.

16s rRNA Sequencing for the Identification of the Screened Potential Biofloc-producing Bacteria

For the genotypic sequencing of the screened potential bacteria, DNA was extracted using Qiagen DNeasy Blood and Tissue Kit. The extraction was done according to the kit manufacturer's protocols. Microbial full-length 16s rRNA sequence was amplified using the 27F (TTTCTGTTGGTGCTGATATTGCAGRGTT YGATYMTGGCTCAG) and 1492R (ACTTGC CTGTCGCTCTATCTTCTACGGYTACCTTG TTACGACTT) primers with Nanopore partial adapter on the primer 5' end (Matsuo *et al*., 2021). PCR was carried out using WizBio HotStart 2x Mastermix (WizBio, Korea) using the PCR condition of 95°C for 3 minutes followed by 35 cycles of 95°C for the 20 s (denaturation), 50°C for 20 s (annealing), and 72°C for 120 s (elongation).

The PCR products were visualised on gel and purified using SPRI Bead followed by index PCR using the EXP-PBC001 kit (Oxford Nanopore, UK). The barcoded libraries were pooled based on band intensity and size-selected using 0.5X vol of SPRI magnetic bead (Oberacker *et al*., 2019) removing fragments smaller than 500 bp. Quantification of the pooled barcoded amplicons used Denovix high sensitivity and an appropriate amount (~150 fmol) of the amplicons was used as the input for LSK110 library preparation (Oxford Nanopore, UK). Sequencing was performed on a Nanopore Flongle Flowcell for 24 hours. For the analysis of the obtained data, basecalling and demultiplexing (assignment of reads to respective samples) of the raw Nanopore reads used Guppy v5.0.7 "super accuracy mode". The demultiplexed reads were subsequently processed with NanoClust which performs read clustering, consensus generation, and abundance estimation. Taxonomic assignment of the sequence clusters used blastN and clusteres were searched against the latest GTDB release r202 16S rRNA database (trimmed to

only retain the V1-V9 hypervariable region) that is comprised of 254,090 bacterial and 4,316 archeal genomes organized into 45,555 bacterial and 2,339 archaeal species clusters. An OTU table was subsequently generated from the NanoClust cluster output table that can be used to infer sample purity. For phylogenetic analysis, the cluster sequences and their top blast hits were combined and aligned with MAFFT v7. A maximum-likelihood tree was constructed from the aligned nucleotide sequences using FastTree2 (-nt-gtr setting). The phylogenetic tree was constructed using iTOL: Interactive Tree of Life (embl.de) software.

Flocculation Activity of the Identified Biofloc-producing Bacteria

The flocculation activity of the identified bacteria was determined using kaolin clay suspension technique (Kurane *et al*., 2014) and was conducted following the procedure of Harun *et al*. (2018). Identified bacteria were cultured in an enrichment medium by incubating them in a shaking incubator (SI-600 Lab Companion Incubator Shaker) at 250rpm for 3 days at 30°C. The culture was then centrifuged using a centrifuge machine (Hettich Zentrifugen Universal 320) at 4°C for 30 minutes at 8,000 rpm. A liquid phase on the upper portion known as supernatant that was free of cells was used to determine the flocculation activity by adding 10 ml with 240 ml kaolin clay suspension. Five grams of Kaolin clay were dissolved in 1,000 ml distilled water and adjusted to pH by adding 0.1N HCl or 0.1N NaOH. After that jar test was carried out using the JLT6 Flocculation Tester (VELP SCIENTIFICA) and following three consecutive mixing at 230 rpm for 2 min, 80 rpm for 1 min, and 20 rpm for 30 min. The beakers were settled for 30 min before measuring the solution that was clarified at 550 nm using UV Spectrophotometer (Shimadzu UV-1800). Each bacteria species was measured in triplicates and a control treatment was also used by using 10 ml distilled water instead of supernatant (cell-free). The formula of flocculation activity from Harun *et al.* (2017) was used to determine the flocculation activity was:

$$\text{Flocculation Activity} = \frac{X-Y}{X} \times 100\% \qquad (1)$$

where,
X is the absorbance of control at 550 nm,
Y is the absorbance of the bacteria samples at 550 nm.

Data Analysis

The data obtained from the flocculation activity were analysed by using IBM SPSS Statistics 25.0 version software. One-way ANOVA at a 95% confidence level was conducted by obtaining comparative significance from Tukey HSD method.

Results and Discussion

Physico-chemical Parameters of Mud Crab Grow-out Ponds

The physical and chemical parameters of water (Table 1) at three selected mud crab grow-out ponds were determined on the field prior the sampling activities and in the lab for Total Ammonia Nitrogen (TAN), Soluble Reactive Phosphate (SRP), and nitrite-nitrogen. The water quality parameters determined for the three ponds are suitable in range except for the Dissolved Oxygen (DO) and pH (Table 2). The suitability of the study site ponds based on the previously published value for mud crab culture can be understood from the Table 2.

Screening and Identification of Potential Biofloc-producing Bacteria from the Water and Soil Sample

The bacteria were isolated from the water and soil sample after 5 successive streaking operations on the marine nutrient agar plate. A total of 88 isolates were found from the water and soil samples. In particular, the water sample was determined with 82 isolates and the other 6 isolates were isolated from the soil sample.

Table 1: Physico-chemical parameters including pH, salinity, DO, temperature, TAN, nitrite, and SRP of three mud crab grow-out ponds, labelled as Pond 1, Pond 2, and Pond 3. Value = mean ± standard error, and n = 3

Parameters	Pond 1	Pond 2	Pond 3
pH	7.213 ± 0.087[a]	7.087 ± 0.109[a]	7.263 ± 0.012[a]
Salinity (g/L)	29.203 ± 0.503[a]	27.234 ± 1.203[a]	28.233 ± 0.406[a]
DO (mg/L)	3.903 ± 0.443[a]	3.937 ± 0.172[a]	3.493 ± 0.423[a]
Temperature (°C)	28.567 ± 0.167[b]	30.567 ± 0.285[a]	29.333 ± 0.497[ab]
Total Ammonia Nitrogen (TAN) (mg/L)	0.067 ± 0.006[c]	0.255 ± 0.005[b]	0.316 ± 0.013[a]
Nitrite-N (mg/L)	0.018 ± 0.001[a]	0.0193 ± 0.0003[a]	0.029 ± 0.006[a]
Soluble Reactive Phosphate (mg/L)	0.105 ± 0.003[a]	0.108 ± 0.031[a]	0.0887 ± 0.004[a]

Table 2: Comparison of the previously studied and established suitable water quality parameters of mud crab culture pond (temperature, Salinity, pH, DO, TAN, Nitrite-N) value vs present study site parameters range with comments

Parameter	Optimum Water Parameters	Present Mud Crab Ponds	Comments
Temperature	25-35°C (Tahmid *et al.*, 2016)	28.5-30.5°C	Suitable
Salinity	15-30 g/L (Tahmid *et al.*, 2016)	27.23-29.20 g/L	Suitable
pH	7.5-8.5 (Syafaat *et al.,* 2021)	7.08-7.21	Slightly lower than the suitable range
DO	> 4 mg/L (Pedapoli & Ramudu, 2014)	3.49-3.90	Slightly lower than the suitable range
TAN	< 0.1 mg/L (Ganesh *et al.,* 2015)	0.067-0.316 mg/L	Pond 2 and pond 3 had a higher value than the suitable range (Result 3.1)
Nitrite-N	< 0.05 mg/L (Tahmid *et al.*, 2016)	0.018-0.029 mg/L	Suitable
SRP	0.1-0.2 mg/L (Coastal Aquaculture Authority, 2006)	0.088-0.108 mg/L	Suitable

Eighteen potential biofloc producing isolates were screened after the screening of the isolates through the YPG media and enrichment media and were encoded as BP-1 to BP-18 (Table 3) for the analysis of molecular identification by 16s rRNA sequencing. After the sequencing, the BLAST (Basic Local Alignment Search Tool) search of the sequences of the isolates showed six different species under four genera. All the isolates were identified as certain species when the coverage percentages were between 99-100%.

The identified four genera were *Bacillus, Micrococcus, Vibrio,* and *Priestia* and the species that matched with maximum identity percentage were *Bacillus cereus, Bacillus tropicus, Bacillus infantis, Micrococcus luteus, Vibrio alginolyticus,* and *Preistia flexa*. The Gram staining result showed all the species under *Bacillus* genera were gram-positive rod shape and the other three genera, *Vibrio, Micrococcus,* and *Priestia* were gram-negative but the shape of the species *Micrococcus luteus* was cocci, and the other *species Priestia flexa, and Vibrio alginolyticus* were rod-shaped. *V. alginolyticus* and *B. infantis* were found in both water and sediment and the other species were from only water sources.

Table 3: Number of potential biofloc-producing bacteria isolated from the water and the soil sample after culturing in marine nutrient agar and screening in YPG and enrichment media

Experimental Pond	Sample	No. of Isolates Screened by Marine Agar	No. of Isolates Screened by YPG and Enrichment Media
Pond 1	Water	18	8
	Sediment	3	1
Pond 2	Water	31	3
	Sediment	1	0
Pond 3	Water	33	5
	Sediment	2	1

According to Hargreaves (2013), biofloc is "a mixture of algae bacteria, protozoans and other kinds of particulate organic matter such as faces and uneaten feed in addition to some of zooplankton and nematodes, formed together to be an integrated and interdependent ecosystem". Chemo-autotrophic and heterotrophic bacteria are mainly the bacteria composition in the BFT system (Ebeling *et al*., 2006b). This study focused on the heterotrophic bacteria isolation, and identification as the co-culture of algae and heterotrophic bacteria is stated as the principle component of BFT (Crab *et al*., 2007; Ahmad *et al*., 2017). The identified bacteria were isolated from the water and soil of the mud crab farm just to ensure their effectiveness as biofloc producers and to avoid possible disease risks as they will be applied to the same group hatchery.

In this study, four species out of the six identified bacteria are reported non-pathogenic and the other two species are reported as pathogenic are *M. luteus* (Talpur *et al*., 2011) and *V. alginolyticus* (Gunasekaran *et al*., 2019). The isolates showed some characteristic morphologies of their colony and to evaluate the morphology some criteria like form, surface, color, elevation, size, and margins were observed (Table 4). All the colonies of the identified bacteria were found circular and shiny. White, red, and yellow were the colors shown by the colonies of the bacteria during culture. The red colony was found in the *B. infantis* strain, the yellow colony in the *M. luteus* strain,

and the white colony was observed in the rest four strains (*B. cereus, B. tropicus, P. flexa, V. alginolyticus*). The elevation was different for different bacteria strains. The raised elevation was found in the greatest number of the identified strains including *B. infantis, P. flexa,* and *V. alginolyticus*, the crateriform elevation in *B. tropicus*, the flat elevation in *B. cereus*, and the convex elevation in *M. luteus*. The margins of the colonies were entire for all the identified bacteria except *B. cereus* which showed an undulate margin. The size of the colonies ranged from 3-14 mm. The highest single colony size was observed in the species *B. cereus*, whereas *M. luteus* was found with the lowest, 3 mm single colony in size. The phylogenetic relationship of the different species was evaluated by MEGA 11.0.10 software and designated by iTOL v6 software (Figure 2).

The tree showed the relativeness of the identified bacteria with the bacteria already recorded in the NCBI library. The identified bacteria labelled as BP-1, BP-7, and BP-14 were matched 99.80-100% with the DNA sequences of *Vibrio alginolyticus* strain NBRC 15630 strain, BP-2, BP-5, BP-6, BP-13, BP-16, and BP-17 were matched 99.93-100% with *Bacillus tropicus* MCCC 1A01406 strain, BP-3, and BP-18 matched 100% with *B. cereus* IAM 12605 strain, BP-9, BP-10, BP-11, BP-12, and BP_15 were matched 99.78-99.85% with *Bacillus infantis* SMC 4352-1 strain, BP-4 matched 99.79% with *Micrococcus luteus* NCTC 2665

Table 4: Different biofloc-producing bacteria characterised by morphologies including their sources (S = sediment, W = water) colony attributes (form, surface, colour, elevation, size, margin), shape, gram reaction

Sample ID	Source	Form	Surface	Color	Elevation	Shape	Size (mm)	Margin	Gra Staining
BP-1	S	Circular	Shiny	White	Raised	Rod	4	Entire	(-)ve
BP-7	W	Circular	Shiny	White	Raised	Rod	4.5	Entire	(-)ve
BP-14	W	Circular	Shiny	White	Raised	Rod	4	Entire	(-)ve
BP-2	W	Circular	Shiny	White	Crateriform		7	Entire	(+)ve
BP-5	W	Circular	Shiny	White	Crateriform	Rod	5.5	Entire	(+)ve
BP-6	W	Circular	Shiny	White	Crateriform	Rod	6.5	Entire	(+)ve
BP-13	W	Circular	Shiny	White	Crateriform	Rod	6	Entire	(+)ve
BP-16	W	Circular	Shiny	White	Crateriform	Rod	7	Entire	(+)ve
BP-17	W	Circular	Shiny	White	Crateriform	Rod	7.5	Entire	(+)ve
BP-3	W	Circular	Shiny	White	Flat	Rod	14	Undulate	(+)ve
BP-18	W	Circular	Shiny	White	Flat	Rod	13	Undulate	(+)ve
BP-4	W	Circular	Shiny	Yellow	Convex	Cocci	3	Entire	(-)ve
BP-8	W	Circular	Shiny	White	Raised	Rod	3.5	Entire	(-)ve
BP-9	W	Circular	Shiny	Red	Raised	Rod	6	Entire	(+)ve
BP-10	S	Circular	Shiny	Red	Raised	Rod	6.5	Entire	(+)ve
BP-11	W	Circular	Shiny	Red	Raised	Rod	5	Entire	(+)ve
BP-12	W	Circular	Shiny	Red	Raised	Rod	5	Entire	(+)ve
BP-15	W	Circular	Shiny	Red	Raised	Rod	6.5	Entire	(+)ve

strain, and BP-8 was matched 100% with the DNA sequences of *Priestia flexa* NBRC 15715 strain.

The 16s rRNA sequencing data is derived by a high-throughput method that has the accuracy to lead the output reaching every taxonomic level of individual organisms (Johnson *et al*., 2019). In this study, the molecular analysis resulted in six different biofloc-producing bacteria species under three genera (Table 5). Three species, *B. tropicus, B. cereus,* and *B. infantis* were identified under the *Bacillus* genus, which accounted for 50% of the species identified. The genera *Bacillus* is one of the intestinal bacterial communities for mud crab. The intestinal microbial community for the green mud crab, *Scylla paramamosain* was investigated where three strains of *Bacillus* such as *B. pumilus* BP, *B. subtilis* DCU, and *B. cereus* HL7 were identified (Wu *et al.,* 2014).

The genus also reported abundant in the mud crab rearing system. Six *Bacillus* strains were such as *B. vallismortis* VITS-17, *B. sonorensis* N3, *B. tequlensis* TY5, *Geobacillus* sp. DB24, *B. mojavensis* SSRAI21, and *B. subtilis* A1 identified from a mud crab (*S. serrata*) in Indonesia (Hastuti *et al.*, 2021). The other studies related to the identification of biofloc forming bacteria resulted in various *Bacillus* sp. from different biofloc farms and culture ponds. Che Hashim *et al*. (2019) found three *Bacillus* species from the *Litopenaeus vannamei* biofloc farm and these were *B. cereus, B. subtilis, and B. pumilus*. Abd-El-Haleem *et al*. (2008) found all the bacteria in the Qatari ecosystem, Qatar were *Bacillus* species, and Kasan *et al*. (2016) extracted nine different biofloc bacteria including *Bacillus* sp. Manan *et al*. (2017) also found *Bacillus* sp. while analysing the biofloc composition of the biofloc from the hatchery of
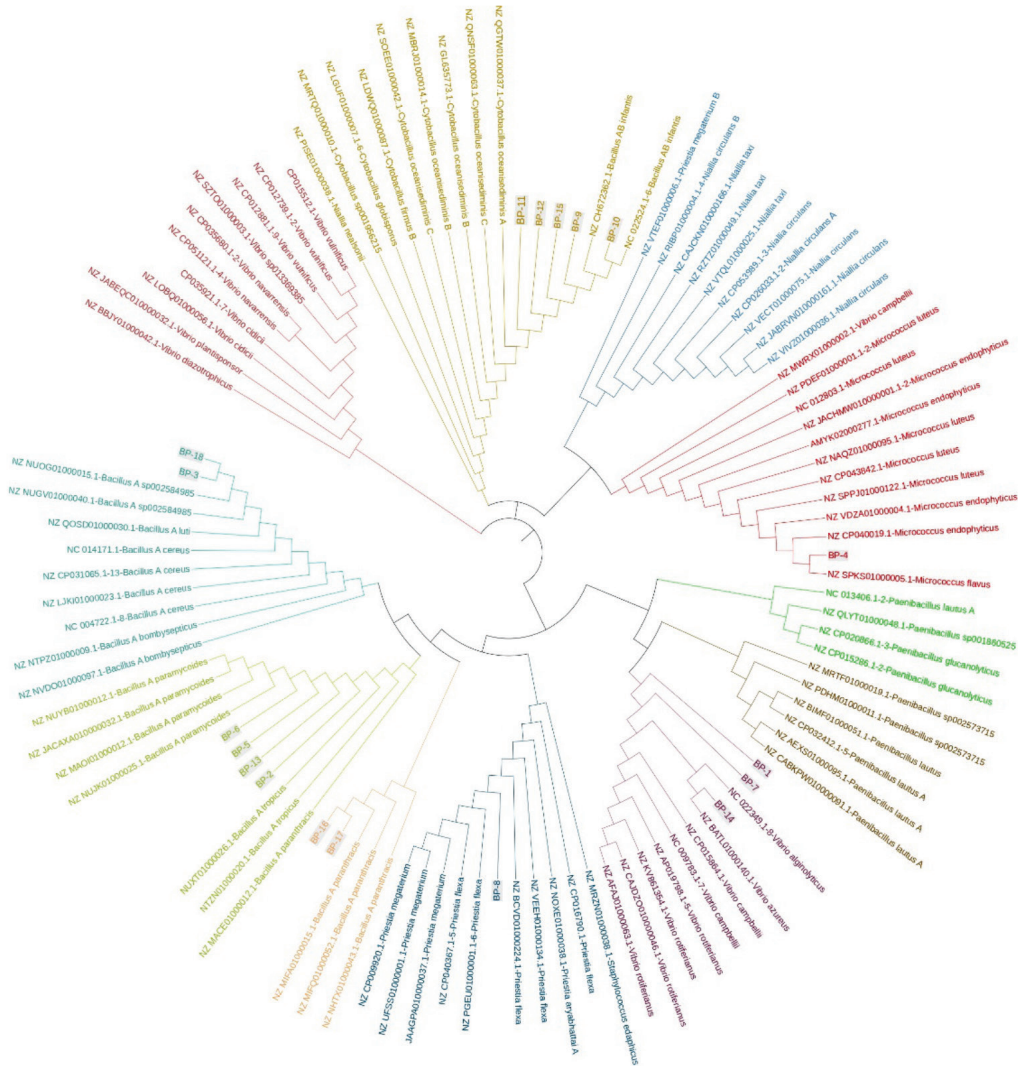
Figure 2: The phylogenetic tree of the identified 18 isolates was constructed by using MEGA and iTOL software and named (BP-1 to BP-18)

Penaeus vannamei. The other three identified species were *M. luteus, V. alginolyticus, and P. flexa*. The species *M. luteus* has already been established as an effective component of biofloc system as it was able to prevent *Vibrio harveyi* by showing vibrio lytic activity in biofloc system (Barcenal *et al*., 2015).

Biofloc has been established as a potential source of probiotics as it provides all the benefits of probiotics (Jamal *et al*., 2020) by holding beneficiary heterotrophic bacteria as

an important component and the evidence of it is highlighted in the study of Abd El-Rhman *et al*. (2009) where they found *M. luteus* showed *in-vivo* efficiency as an active probiotic by enhancing the growth performance and health status of Nile tilapia. The bacteria species *V. alginolyticus* has not been found as a component of biofloc system in a study till now but a study conducted by Shan *et al*. (2016) reported this species can reduce TAN (total ammonia nitrogen) and Nitrite-N from shrimp pond when

Table 5: Different biofloc-producing bacteria identified by 16s rRNA sequencing with their NCBI matched strain along with similarity and accession number

Sample ID	NCBI Matched Strain	Similarity	Accession Number
BP-1	*Vibrio alginolyticus* strain NBRC 15630	99.80%	NR_113781.1
BP-7		99.80%	NR_113781.1
BP-14		100%	NR_113781.1
BP-2	*Bacillus tropicus* MCCC1A01406	99.93%	NR_157736.1
BP-5		100%	NR_157736.1
BP-6		100%	NR_157736.1
BP-13		100%	NR_157736.1
BP-16		99.93%	NR_157736.1
BP-17		99.93%	NR_157736.1
BP-3	*Bacillus cereus* IAM 12605	100%	NR_115526.1
BP-18		100%	NR_115526.1
BP-4	*Micrococcus luteus* NCTC 2665	99.79%	NR_075062.2
BP-8	*Priestia flexa* NBRC 15715	100%	NR_024691.1
BP-9	*Bacillus infantis* SMC 4352-1	99.85%	NR_043267.1
BP-10		99.78%	NR_043267.1
BP-11		99.85%	NR_043267.1
BP-12		99.85%	NR_043267.1
BP-15		99.85%	NR_043267.1

the bacteria is added with sodium alginate beads. To date, there is not a single study about the potentiality of *P. flexa* as biofloc producer but it was previously known as *B. flexus* (Gupta *et al*., 2020) that has been well established as a probiotic (Ren *et al*., 2021).

Flocculation Activity of the Identified Biofloc-producing Bacteria

The flocculation activity of the six identified species (Figure 3) after the molecular sequencing was conducted by jar test analysis. The highest flocculation activity was shown by the species *B. tropicus* with 95% ± 0.135% followed by *B. cereus* with 93.7% ± 0.478% *Priestia flexa* with 90.4% ± 0.690%, *B. infantis* with 88.6% ± 1.425%, *V. alginolyticus* with 83% ± 3.356%, and *M. luteus* with 74.8% ± 5.985%.

After the One-Way ANOVA Tukey test, there were found no significant differences ($p > 0.05$) among the identified bacteria for flocculation activity. The standard acceptable value in Figure 3 shows 75% flocculation activity which was the lowest possible acceptable value for the flocculation activity to consider the bacteria as an effective biofloc producer.

The flocculation activity determination is a test that determines how effective bacteria are at flocculating kaolin clay that is suspended in a jar test by secreting Extracellular Polymeric (EPS) molecules. Many bacteria with flocculating ability have been identified as potential biofloc producers in earlier investigations (Table 6).

The flocculants production and the flocculation activity by the biofloc producing bacteria are influenced by the carbon-nitrogen sources and ratio (Crab *et al*., 2007; Kurane *et al*., 2014). The biofloc-producing bacteria were initially screened from the isolates by using the YPG media that provided peptone, urea, and glucose as a source of carbon and nitrogen. Bacteria consume urea as a source of nitrogen
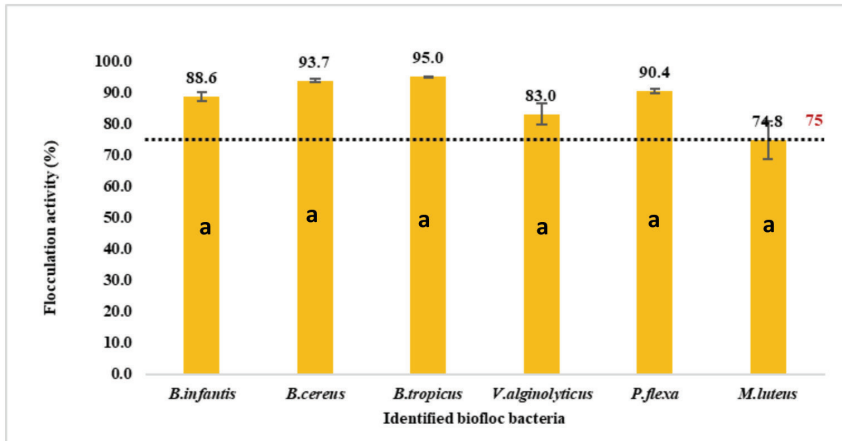
Figure 3: Flocculation activity of the six identified potential biofloc-producing bacteria (*B. infantis, B. cereus, B. tropicus, V. alginolyticus, P. flexa, M. luteus*) along with the ideal flocculation activity 75% for biofloc producers established by Abd-El-Haleem *et al.* (2008). Value = mean ± standard error (SE)

Table 6: Flocculation activity of the identified bacteria along with the previously studied biofloc producing bacteria

Bacteria Species	Flocculation Activity	References
Bacillus sp. QUST2	85	(Abd-El-Haleem *et al.*, 2008)
Bacillus sp. QUST6	81	(Abd-El-Haleem *et al.*, 2008)
Bacillus sp. QUST9	75	(Abd-El-Haleem *et al.*, 2008)
Bacillus megaterium	94.32%	(Luo *et al.*, 2016)
Bacillus enclensis	93%	(Ahmad Shukri et al., 2022)
Nitrareductor aquimarinus	86%	(Che Hashim *et al.*, 2019)
Pseudoalteromonas sp.	86%	(Che Hashim *et al.*, 2019)
Bacillus cereus	93%	(Che Hashim *et al.*, 2019)
Micrococcus luteus	74.8%	Present study
Vibrio algnolyticus	83%	Present study
Bacillus tropicus	95%	Present study
Bacillus cereus	93.7%	Present study
Bacillus infantis	88.6%	Present study
Priestia flexa	90.4%	Present study

showed the highest flocculation activity, 94.54% over the other nitrogen sources like ammonium sulphate, ammonium chloride, peptone, and yeast (Sheng *et al.*, 2006). Another study discovered soluble glucose as one of the carbon sources that provided better results for the water quality and growth performances of *L.vannamei* (Huang *et al.*, 2022). *Streptomyces* sp. showed higher flocculation activity for *Chlorella vulgaris* when the species used glucose as its carbon source (Li *et al.*, 2021). So, the YPG media screened bacteria must be the most effective in producing flocculating attributes and that was reflected in the flocculation activity of the identified bacteria.

Five out of six bacteria*, B. tropicus, B. cereus, B. infantis*, *P.flexa, V. algonolyticus* showed more than 80% flocculation activity and the highest flocculation activity was 95% showed by *B. tropicus*. *B. cereus* and *P. flexa* also showed more than 90% like 93.7% and 90.4%, respectively. The remaining species, *M. luteus* was determined with 74.8% flocculation activity and that value is lower than the standard value mentioned by Abd-El-Haleem *et al.* (2008) as the study considered those bacteria as biofloc-producing bacteria that showed flocculation activity > 75%. Although another study put the standard flocculation activity to 60% while reporting about six different biofloc bacteria as potential floc producers (Harun *et al.*, 2018). Despite the fact that this study discovered *M. luteus* to have lesser flocculation activity, Barcenal *et al.* (2015) found this species to be an efficient biofloc generating bacterium.

Conclusion

Biofloc-producing Bacteria (BPB) were successfully isolated and identified from a commercial mud crab grow-out farm. *B. tropicus, B. cereus, B. infantis, V. alginolyticus, P. flexa,* and *M. luteus* were identified as potential biofloc producing bacteria. In the kaolin clay suspension, *B. tropicus* had the maximum flocculation activity (95%) and *M. luteus* had the lowest flocculation activity (74.8%). *B. cereus, P. flexa, B. infantis*, and *V. alginolyticus* were also effective biofloc producers for their higher flocculation activity. The findings of this study are going to initiate the biofloc technology for the commercially important mud crab species hatchery operation that might be a potential solution for the enhancement of mud crab nursey operation. More research into the identification of extracellular polymeric compounds for the efficacy of these bacteria, as well as the use of the bacteria as inoculum in the biofloc system of the mud crab zoea stages, should be conducted in the future.

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