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To cite this article: Nurul Fakriah Che Hashim *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **246** 012007

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# Characterization of Marine Biofloculant-producing Bacteria Isolated From Biofloc of Pacific Whiteleg Shrimp, *Litopenaeus vannamei* Culture Ponds

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**Abstract.** Characterization of marine biofloculant-producing bacteria isolated from bioflocs of Pacific whiteleg shrimp, *Litopenaeus vannamei* culture ponds was prompted to explore the bacteria that enhanced bioflocculation process in aquaculture wastewater treatment. Certain marine bacteria were potentially secreted extracellular polymeric substances (EPS) which response to the physiological stress encountered in the natural environment that can act as biofloculants. This study aimed to identify marine biofloculant-producing bacteria isolated from biofloc; to evaluate their flocculating activities; and to characterize their protein in EPS. Phenotypic and genotypic identification of the bacteria including morphological and molecular approaches were employed, while their flocculating activities were examined via Kaolin clay suspension method and statistically analyzed. The EPS that acted as biofloculants were extracted using cold ethanol precipitation method. Protein concentration was determined by Bradford assay and protein profiling was finally completed with Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) method. Six species of marine bacteria known as *Halomonas venusta*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus pumilus*, *Nitratireductor aquimarinus* and *Pseudoalteromonas* sp. were successfully identified as biofloculant-producing bacteria. The highest flocculating activity was exhibited by *Bacillus cereus* at 93%, while *Halomonas venusta* showed the lowest record at 59%. All biofloculant-producing bacteria species showed different protein concentration that ranged between 1.377 µg/mL to 1.455 µg/mL. Several protein bands with different molecular weight that ranged between 16 kDa to 100 kDa were observed. This study revealed that all the identified bacteria species have high potential characteristics to initiate aquaculture wastewater treatment and may play important roles in bioflocculation process.

## 1. Introduction

Aquaculture is an industry that involves cultivation of freshwater and seawater organisms under controlled operations. However, application of effective technologies for wastewater treatment remains challenge in intensive aquaculture operations. High composition of uneaten fish feed and faeces in river or sea released by aquaculture operation can cause eutrophication problem [1]. Sludge contained debris, faecal materials and uneaten feed that settled in the bottom sediment can interfere with the interactions



of organisms at all biodiversity levels [2]. Therefore, to ensure long-term sustainability of aquaculture industry, environmental impacts must be minimized and alternative ways such as flocculation process need to be applied.

Flocculation offers an alternative method to overcome the problem of aquaculture wastewater effluent. It was reported as cheap, easy and effective technique to remove cell debris, colloids and suspended particles [3]. As compared to other conventional system, this method was volume independent to concentrate dead cells [4]. It functioned with the help of flocculant that will alter the nature of suspended particulate materials and enable them to form aggregates or small clumps [5]. Flocculants can be divided into synthetic and natural [6]. For wastewater treatment, synthetic flocculants are the best candidates for aquaculture industry. However, problems regarding their safety status to human health require alternatives flocculants that are more environmental friendly and harmless is crucial to be developed.

Alternatively, green technology metabolites known as bioflocculants which produced by microorganisms can acted similar function as synthetic flocculants to flocculate suspended particles, cells and colloidal solids [7]. Many microorganisms including algae, bacteria and fungi isolated from sludge and waste were reported to secrete extracellular polymeric substances. They are mainly consisting of high polymeric substances such as functional proteins, exopolysaccharide, polysaccharides, glycoproteins, protein, nucleic acid and cellulose [8,9]. In other industry, bioflocculants are also widely used as alternative treatment to remove inorganic solid suspensions, dye solutions, food and industrial wastewater [10]. From other previous studies, there were many bacteria have been reported to be involved in biofloc formation. A bacteria producing an extracellular biopolymer was isolated from contaminated medium and identified as *Bacillus licheniformis* [11]. *Paenibacillus* sp. CH11, *Bacillus* sp. CH15, *Herbaspirillum* sp. CH7 and *Halomonas* sp. were reported to produce biopolymer and have been evaluated as bioflocculants in the industrial wastewater effluents treatment [12]. A strain identified as *Vagococcus* sp. which secreted a large amount of biofloc agents was isolated from wastewater samples collected from Little Moon River in Beijing [13]. Other bacteria that have been reported as bioflocculant-producing bacteria were *Bacillus firmus* [14], *Citrobacter* spp. TKF04 [15], *Corynebacterium glutamicum* [16], *Enterobacter aerogenes* [17], *Nannocystis* sp. Nu-2 [18], *Bacillus subtilis*, *Bacillus licheniformis*, *Pacilomyces* sp., and *Nocardia amarae* YK [19], *Enterobacter agglomerans* SM 38, *Bacillus subtilis* SM 29 and *Bacillus subtilis* WD90 [20], *Bacillus cereus* B-11 [21], *Serratia ficaria* [22], *Lactobacillus delbrukii* sp.bulgaricus [23] and *Bacillus alvei* NRC-14 [24]. Therefore, the ultimate aim of this study was to characterize the most potential bioflocculant-producing bacteria involved in biofloc formation, particularly for aquaculture wastewater treatment.

## 2. Research Methods

### 2.1. Location of sampling site

Sampling of biofloc was carried out at Integrated Shrimp Aquaculture Park (iSHARP) Sdn. Bhd. It is located at Setiu, Terengganu (5°34'18.32"N, 102°48'25.86"E), about 30 km away from Universiti Malaysia Terengganu (UMT). iSHARP is a fully Integrated Aquaculture Park developed by Blue Archipelago Berhad specialized for Pacific Whiteleg shrimp, *Litopenaeus vannamei* farming in controlled conditions which operated since 2012. This farm is equipped with superintensive design, biosecurity and vis-à-vis location.

### 2.2. Collection of biofloc samples

Collection of biofloc samples were followed the standard operating procedures (SOP) prepared by iSHARP for biosecurity purpose. Before collection of bioflocs samples, ponds underwent a treatment process. From reservoir pond, sea water was pumped into treatment ponds. In these ponds, the sea water was treated with chlorine powder and left for three weeks to disinfect and kill all organisms. After treatment process, the sea water was distributed into culture ponds up to 1.5 metres depth. Before the culture ponds were stocked with selected post-larvae (PL) of *L. vannamei*, intensive aeration was

provided to eliminate all chlorine residues that have been used during treatment process and also to promote microalgae growth in the pond as live feed for PL. Biofloc samples were collected from 3 fully developed biofloc ponds. For each pond, a total of five litres of pond water containing biofloc samples was collected in pre-acid washed sampling bottles to eliminate contamination and was taken to laboratory for further analysis.

### *2.3. Media preparation*

The composition of marine nutrient agar included (per litre): 55 g of Difco marine agar in filtered deionized water. The production medium composed (per litre): 20.0 g of glucose, 5.0 g of  $K_2HPO_4$ , 2.0 g of  $KH_2PO_4$ , 0.3 g of  $NH_4(SO_4)_2$ , 0.5 g of urea, 0.5 g of yeast extract, 0.3 g of  $MgSO_4 \cdot 7H_2O$  and 0.1 g of NaCl in filtered deionized water [25]. The Yeast Peptone Glucose (YPG) medium composed (per litre): 10.0 g of glucose, 2.0 g of peptone, 0.5 g of urea, 0.5 g of yeast extract, 0.1 g of NaCl, 0.2 g of  $MgSO_4 \cdot 7H_2O$ , 0.2 g of  $KH_2PO_4$ , 5.0 g of  $K_2HPO_4$  and 15.0 g of bacteriological agar in filtered deionized water [26]. The medium for a slant included (per litre): 20.0 g of glucose, 5.0 g of  $K_2HPO_4$ , 2.0 g of  $KH_2PO_4$ , 0.3 g of  $NH_4(SO_4)_2$ , 0.5 g of urea, 0.5 g of yeast extract, 0.3 g of  $MgSO_4 \cdot 7H_2O$ , 0.1 g of NaCl and 20.0 of agar in filtered deionized water [22]. The enrichment medium (per litre): 10.0 g of glucose, 0.5 g of urea, 0.2 g of  $MgSO_4 \cdot 7H_2O$ , 5.0 g of  $K_2HPO_4$ , 2.0 g of peptone, 0.2 g of  $KH_2PO_4$  and 0.5 g of yeast extract in filtered seawater [27]. All media were adjusted to pH 7 and then sterilized by autoclaving at 121°C for 15 min.

### *2.4. Isolation and screening of bioflocculant-producing bacteria from bioflocs*

Samples of bioflocs were transferred into Imhoff cone for 24 hours to enable the biofloc to settle down. The settled biofloc samples were collected by siphoning out excess water. Biofloc that settled down in Imhoff cone was centrifuged at 6000 rpm for 3 minutes to obtain concentrated biofloc pellet. Isolation of bacteria from biofloc was performed by spread plate method of biofloc pellet on the surface of marine agar. Biofloc from each pond was plated in 3 replicates. Plates were incubated at 30°C for overnight. Single colonies with different morphologies from the cultured plates were inoculated onto new marine nutrient agar plates. The procedure was repeated until pure cultures were obtained. Screening of bioflocculant-producing bacteria was carried out using production medium and YPG medium. A loop of pure culture of isolates from marine nutrient agar plate with different colony morphologies were inoculated into 50 mL of production medium and spread on YPG medium. After incubation at 30°C for 48 hours, the isolates with ropy morphologies in production medium and mucoid colony morphologies in YPG medium were selected. Ropy colonies form long filaments when extended with an inoculation loop while mucoid colonies have a glistening and slimy appearance on agar plate [28]. The isolates were maintained on slant agar and kept refrigerated at 4°C for further phenotypic and genotypic identification.

### *2.5. Morphological observation and phenotypic characterization of bioflocculant-producing bacteria*

Morphological characteristics of bioflocculant-producing bacteria were performed by microscopic observations using Gram staining method. Phenotypic identification was fully carried out according to Bergey's Manual of Systematic Bacteriology to determine the taxonomy of isolated bioflocculant-producing bacteria as it provided descriptions and photographs of species and tests to distinguish among genera and species [29].

### *2.6. Genotypic identification of bioflocculant-producing bacteria with 16S rDNA sequencing*

High potential of bioflocculant-producing bacteria were further confirmed by genotypic identification through 16S rDNA sequencing. The Qiagen DNeasy Blood and Tissue Kit was used to extract bacterial DNA. It was conducted as per manufacturer's protocol. DNA was quantified using BioDrop  $\mu$ LITE (Isogen, Netherlands). All samples were measured in triplicates and the  $A_{260}/A_{280}$  ratio values were recorded. Quality of extracted DNA was checked through gel electrophoresis [30]. In this study, PCR involved a single set of primer that targets a specific gene that was used to detect an organism. Extracted genomic DNA from individual isolated bacterial strains was subjected to PCR amplification of 16S

rDNA using universal PCR primers, 27F and 1492R to amplify the 16S rDNA gene [31]. The sequences of primers used were; 27 Forward “5’-AGA GTT TGA TCC TGG CTC AG-3’ ” and 1492 Reverse “5’-ACG GCT ACC TTG TTA CGA CTT-3’ ”. PCR was carried out using commercial kit, GoTaq® PCR Core Systems (Promega, USA) for all DNA samples. All PCR reagents used for amplification of bacteria followed recommended reaction volumes and final concentrations provided by manufacturer. Each PCR mixture contained 0.25 µL of Taq polymerase, 10 µL of 10x PCR buffer, 3 µL of MgCl<sub>2</sub>, 1.5 µL of 200 nM of each primer, 1 µL of 200 nM of dNTP mix, 29.75 µL of distilled deionized water and 3 µL of DNA template (Qiagen, Hilden, Germany). Reactions was carried out in an Eppendorf Mastercycle gradient starting with a denaturation step for 5 minutes at 94°C, followed by 35 cycles with 1 cycle consisting of denaturation (94°C for 1 minute), annealing (55°C for 1 minute), elongation (72°C for 2 minutes) and a final extension step for 7 minutes at 72°C [32]. All PCR products were verified by agarose gel electrophoresis and visualized in gel documentation chamber. Only DNA samples with a single band and clear PCR product shown on agarose gel were selected to be purified and sequenced. Purification of PCR products was carried out using QIAquick PCR Purification Kit (Qiagen, 28104). The protocol followed manufacturer’s instruction. The amplified PCR products were sent to 1st Base Laboratory, Selangor-Malaysia for sequencing. Obtained 16S rDNA gene sequences were BLAST-analyzed at National Center for Biotechnology Information (NCBI); <http://www.ncbi.nlm.nih.gov/BLAST/> for similarity search.

#### *2.7. Determination of flocculating activity of bioflocculant-producing marine bacteria*

All identified bioflocculant-producing marine bacteria were cultured in enrichment medium [27]. Inoculum was prepared by incubated in SI-600 Lab Companion Incubator Shaker, with 250 rpm at 30°C for 3 days. The resultant culture broth was centrifuged using Hettich Zentrifugen Universal 320 at 8,000 rpm for 30 minutes at 4°C. The cell-free supernatants were used as produced bioflocculant to determine the flocculating activity of the bioflocculant-producing bacteria [13]. Flocculating activity was measured using a modified Kaolin clay suspension method [33]. Five gram of kaolin clay was suspended in 1 L of deionized water for preparation of 5.0 g/L of kaolin clay suspension. Kaolin clay suspension was adjusted to pH 7. For flocculating activity, 240 mL of kaolin clay suspension and 10 mL of bioflocculant solution (cell-free supernatant) were added into 250 mL beaker. By using JLT4 Jar/Leaching Tester Velp Scientifica, the flocculating activity was started with rapid mixing at 230 rpm for 2 minutes, followed by slow mixing for 1 minute at a speed of 80 rpm. The stirring speed was reduced to 20 rpm and stirring was continued for 30 minutes. Stirring apparatus was stopped and the samples in the beakers were allowed to settle for 30 minutes. The optical density (OD) of the clarifying solution was measured with Shimadzu UV Spectrophotometer UV-1800 at 550 nm. A control experiment was prepared using the same method but the bioflocculant solution was replaced by deionized water. The flocculating activity was calculated as follows;

$$\text{Flocculating activity: } [(B-A)/B] \times 100\%$$

which A and B were the absorbance at 550 nm for sample and reference, respectively.

##### *2.7.1. Statistical analysis*

Evaluation on flocculating activity of identified marine bioflocculant-producing bacteria was analyzed using Minitab 16.0 software. One-way ANOVA with grouping information by Tukey Pairwise Comparisons method and 95% confidence level was applied. Significant differences between the bacteria were determined at 0.05 level of probability.

#### *2.8. Characterization of protein composition in extracellular polymeric substances (EPS) produced by marine bioflocculant-producing bacteria*

Each species of bioflocculant-producing bacteria was cultured in enrichment medium at 250 rpm in orbital shaker for 3 days at 30°C for optimum extracellular polymeric substances (EPS) production [27].

### 2.8.1. Extraction of EPS from bioflocculant-producing bacteria

A total of 40 mL bioflocculant-producing bacterial culture was treated with 10 mL of 1N NaOH for 30 minutes at 4°C before extraction. 1N NaOH treatment was applied to give an effective recovery of EPS and to avoid destruction of EPS. After treatment, culture broth of bacteria was centrifuged at 20,000 rpm for 30 minutes at 4°C. After centrifugation process, two layers appeared and the cell-free supernatant layer was taken to extract crude EPS. EPS in the cell-free supernatant fluid was precipitated by addition of 3-volumes of ice cold 95% ethanol. The mixture was later left for 24 hours before it was centrifuged again at 10,000 rpm for 15 minutes (4°C). The precipitated EPS was collected on a Whatman filter paper (Grade 1: 11 µm) and precipitated again by addition of 3-volumes of ice cold 95% ethanol and dissolved in water at room temperature for further protein analysis.

### 2.8.2. Quantification of protein concentration in EPS

Protein in extracted EPS was analyzed for protein concentration by Bradford assay. Bovine Serum Albumin (BSA) was used to prepare a protein standard. Standard containing a range of 1 to 5 µg protein in 100 µL volume were prepared. For blank sample (0 µg/mL), distilled water and dye reagent were used. Each standard solution was pipetted into separate clean test tubes. 5 mL Bradford reagent (Bio-Rad) was added into the standard. The standard then was incubated for five minutes. The absorbance at 595 nm was measured. A standard curve was created by plotting the 595 nm values (y-axis) versus their concentration in µg/mL (x-axis). The same step was repeated for the samples. Finally, the concentration of samples was derived from the standard curve [34].

### 2.8.3. Protein profiling by SDS-PAGE

Protein composition in crude EPS was separated by SDS-PAGE. Preparation of sample loading buffer, non-continuous running buffer, isopropanol fixing solution, Coomassie Blue stain solution, resolving gel solution and stacking gel solution for SDS-PAGE were prepared with a slight of modification [35]. Polyacrylamide gel was cast using 4% stacking gel and 12% resolving gel. Protein marker, See All Blue Plus (Biorad) was used as protein marker. Probes were set at 80 volt power. The power was increased to 95 volt when the dye reached the resolving gel. SDS-PAGE was stopped when the sign of protein marker reached the foot line of the glass plate. The gel was rinsed with distilled deionized water for two or three times and then isopropanol fixing solution was poured on the gel and let for half an hour. The gel was stained with Coomassie Blue Staining for overnight. After that, the gel was destained with distain solution for overnight. At the end, the gel was washed with distilled deionized water with three to four changes over 2-3 hours. The protein band then was viewed using gel documentation system (Biorad).

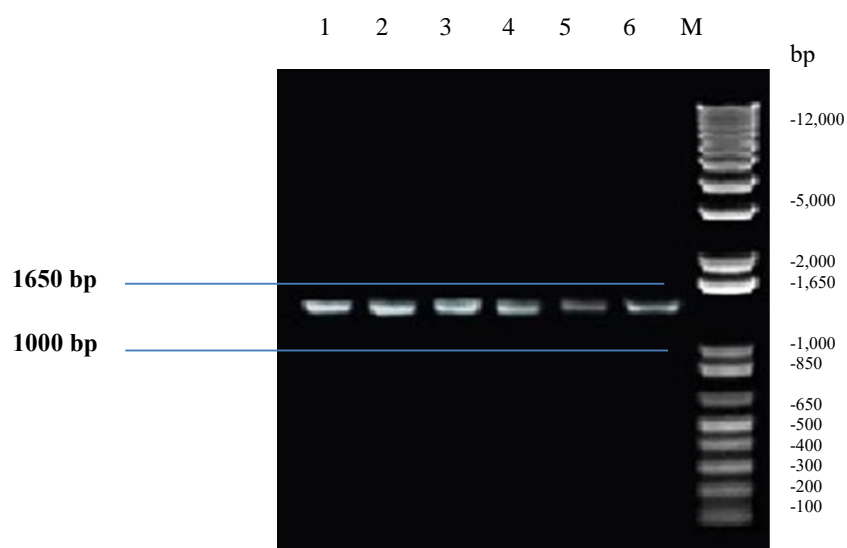
## 3. Results

### 3.1. Identification of bioflocculant-producing bacteria isolated from bioflocs

In this study, most of the phenotypic characteristics of the isolates were similar to those indicated by Bergey's Manual of Systematic Bacteriology. Based on biochemical characterization, the investigated isolates resembled two bacterial genera known as *Bacillus* and *Halomonas*. Two unsuccessfully identified genera were labelled as Unknown sp. 1 and Unknown sp. 2 (**Table 1**). **Table 2** showed the purity of the extracted genome from the high potential of bioflocculant-producing bacteria. The optimum purity ratio of extracted DNA was between 1.7 and 2.0 which means that no or less contamination occurred during the extraction process. All isolates showed an acceptable range of DNA purity and were used as templates in PCR amplification (**Figure 1**). According to the sequences evaluated in the public databases using BLAST search program on (NCBI) website (<http://www.ncbi.nlm.nih.gov/>), six species were identified as bioflocculant-producing bacteria. *Halomonas* sp. closely related to *Halomonas venusta*. *Bacillus* sp. 1, *Bacillus* sp. 2 and *Bacillus* sp. 3 closely related to *Bacillus cereus*, *Bacillus subtilis* and *Bacillus pumilus*, respectively. Unknown sp. 1 closely related to *Nitratireductor aquimarinus* while Unknown sp. 2 closely related to *Pseudoalteromonas* sp. (**Table 3**).

**Table 1:** Phenotypic characterization of marine biofloculant-producing bacteria isolated from biofloc**Table 2:**  $A_{260}/A_{280}$  ratio of biofloculant-producing bacteria rDNA

| Predicted genus       | Gram staining | Pigmentation            | Shape | Endospore staining | Catalase | Oxidase | Glucose fermentation | Mannitol fermentation | Lactose fermentation | Urease | Indole | Motility | Voges - Proskauer | Citrate | Nitrate reduction | Starch hydrolysis | Phenylalanine deaminase |
|-----------------------|---------------|-------------------------|-------|--------------------|----------|---------|----------------------|-----------------------|----------------------|--------|--------|----------|-------------------|---------|-------------------|-------------------|-------------------------|
| <i>Halomonas</i> sp.  | -             | Yellow                  | Rod   | -                  | +        | +       | +                    | +                     | -                    | -      | na     | +        | +                 | +       | +                 | na                | na                      |
| Unknown sp. 1         | -             | White                   | Rod   | -                  | +        | +       | +                    | -                     | -                    | +      | -      | -        | na                | +       | +                 | na                | na                      |
| Unknown sp. 2         | -             | White                   | Rod   | -                  | +        | +       | +                    | +                     | +                    | na     | -      | +        | na                | na      | -                 | na                | +                       |
| <i>Bacillus</i> sp. 1 | +             | Peach                   | Rod   | +                  | +        | -       | +                    | +                     | -                    | -      | -      | +        | +                 | -       | na                | na                | na                      |
| <i>Bacillus</i> sp. 2 | +             | White                   | Rod   | +                  | +        | -       | +                    | -                     | -                    | -      | -      | +        | +                 | -       | +                 | na                | na                      |
| <i>Bacillus</i> sp. 3 | +             | White                   | Rod   | +                  | +        | +       | +                    | +                     | +                    | -      | -      | +        | +                 | -       | -                 | -                 | na                      |
| Genus / Species       |               | $A_{260}/A_{280}$ ratio |       |                    |          |         |                      |                       |                      |        |        |          |                   |         |                   |                   |                         |
| <i>Halomonas</i> sp.  |               | 1.909                   |       |                    |          |         |                      |                       |                      |        |        |          |                   |         |                   |                   |                         |
| <i>Bacillus</i> sp. 1 |               | 1.856                   |       |                    |          |         |                      |                       |                      |        |        |          |                   |         |                   |                   |                         |
| <i>Bacillus</i> sp. 2 |               | 1.923                   |       |                    |          |         |                      |                       |                      |        |        |          |                   |         |                   |                   |                         |
| <i>Bacillus</i> sp. 3 |               | 1.853                   |       |                    |          |         |                      |                       |                      |        |        |          |                   |         |                   |                   |                         |
| Unknown sp. 1         |               | 1.939                   |       |                    |          |         |                      |                       |                      |        |        |          |                   |         |                   |                   |                         |
| Unknown sp. 2         |               | 1.906                   |       |                    |          |         |                      |                       |                      |        |        |          |                   |         |                   |                   |                         |



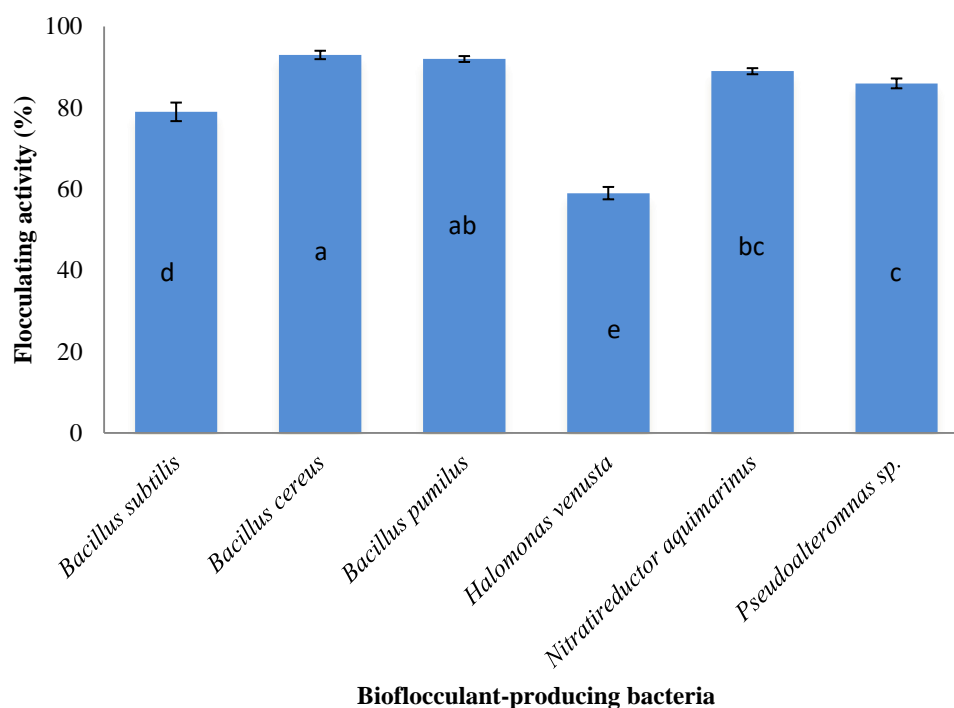
**Figure 1:** Amplification of ~1.5 kb fragment of PCR products from biofloculant-producing bacteria using 1492R and 27F primers. Lane 1: *Halomonas* sp., Lane 2: *Bacillus* sp. 1, Lane 3: *Bacillus* sp. 2, Lane 4: *Bacillus* sp. 3, Lane 5: Unknown sp. 1, Lane 6: Unknown sp. 2 and M: 1kb Plus DNA Ladder (Invitrogen)

**Table 3:** Sequencing of the 16S rDNA of biofloculant-producing bacteria isolated from biofloc according to the public databases on National Centre for Biotechnology Information (NCBI)

| Isolated genus        | Closest matching strain in NCBI            | Sequence similarity (%) | Accession number |
|-----------------------|--|-------------------------|------------------|
| <i>Halomonas</i> sp.  | <i>Halomonas venusta</i> NBRC101901        | 99                      | AB681589.1       |
| <i>Bacillus</i> sp. 1 | <i>Bacillus subtilis</i> YNA61             | 100                     | JQ039972.1       |
| <i>Bacillus</i> sp. 2 | <i>Bacillus cereus</i> MCCC1A06376         | 100                     | KJ812466.1       |
| <i>Bacillus</i> sp. 3 | <i>Bacillus pumilus</i> SH-B9              | 99                      | CP011007.1       |
| Unknown sp. 1         | <i>Nitratireductor aquimarinus</i> CL-SC22 | 99                      | HQ176466.1       |
| Unknown sp. 2         | <i>Pseudoalteromonas</i> sp. QY5           | 100                     | KP676699.1       |

### 3.2. The effectiveness of flocculating activity of identified biofloculant-producing bacteria

Flocculating activity of six identified species of biofloculant-producing bacteria isolated from bioflocs was conducted using Jar Test. Numerically, the highest flocculating activity was showed by *Bacillus cereus* with 93% followed by *Bacillus pumilus* with 92%. *Nitratireductor aquimarinus* showed 89% of flocculating activity and *Pseudoalteromonas* sp. showed 86% of flocculating activity. *Bacillus subtilis* recorded 79% of flocculating activity while *Halomonas venusta* showed lowest record, 59% of flocculating activity. According to statistical analysis using One-Way ANOVA, there was no significant difference ( $p < 0.05$ ) between *Bacillus cereus* (93%) and *Bacillus pumilus* (92%). Besides, there was no significant difference ( $p < 0.05$ ) between *Nitratireductor aquimarinus* (86%) and *Bacillus pumilus* (92%). There was also no significant difference ( $p < 0.05$ ) between *Nitratireductor aquimarinus* (86%) and *Pseudoalteromonas* sp. (86%). According to the statistic, *Bacillus subtilis* was significantly different as well as *Halomonas venusta* (**Figure 2**).





**Figure 2:** Flocculating activity of bioflocculant-producing bacteria isolated from bioflocs. Note that using grouping information by Tukey Pairwise Comparisons method and 95% confidence, if they do not share the same letter e.g (a, b, c, d, e) it means that they are significantly different. Error bars represented as standard deviation.

### 3.3. Characterization of protein composition in crude extracellular polymeric substances (EPS) from bioflocculant-producing bacteria

Characterization of protein composition in crude EPS from six species of bioflocculant-producing bacteria was analyzed in terms of concentration and molecular weight.

#### 3.3.1. Quantification of protein concentration in crude EPS of bioflocculant-producing bacteria

Each species of bioflocculant-producing bacteria showed different protein concentration (**Table 4**). The highest protein concentration in extracted EPS was produced by *Bacillus cereus* with 1.455 µg/mL followed by *Bacillus subtilis*, with 1.415 µg/mL. Protein concentration in extracted EPS from *Bacillus pumilus* was 1.403 µg/mL. Protein concentration in extracted EPS from *Pseudoalteromonas* sp., *Halomonas venusta* and *Nitratireductor aquimarinus* were 1.396 µg/mL, 1.388 µg/mL and 1.377 µg/mL respectively.

**Table 4:** Protein concentration in extracellular polymeric substances (EPS) from bioflocculant-producing bacteria

| Bioflocculant-producing bacteria | Protein concentration in EPS (µg/mL) |
|----------------------------------|--------------------------------------|
| <i>B. cereus</i>                 | 1.455                                |
| <i>B. subtilis</i>               | 1.415                                |
| <i>B. pumilus</i>                | 1.403                                |
| <i>Pseudoalteromonas</i> sp.     | 1.396                                |
| <i>H. venusta</i>                | 1.388                                |
| <i>N. aquimarinus</i>            | 1.377                                |

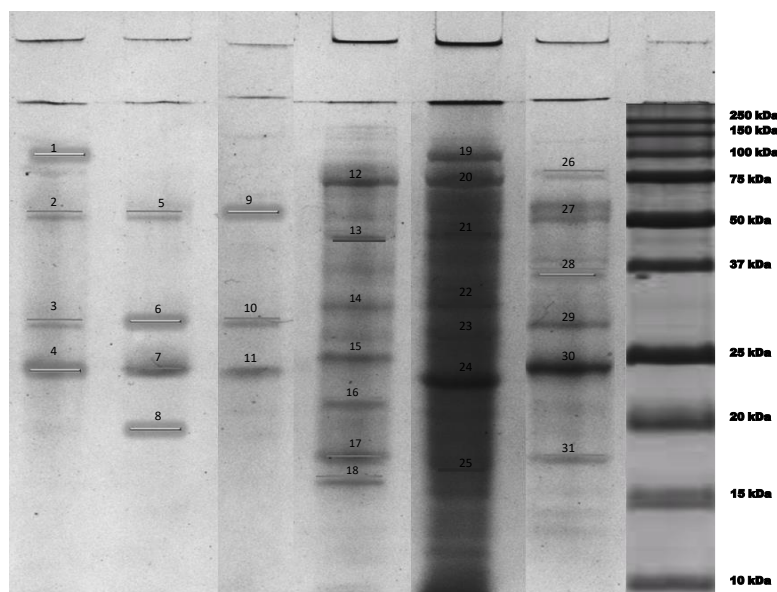
#### 3.3.2. Protein profiling by SDS-PAGE

**Table 5** showed the band of proteins that have been separated by 12% SDS-PAGE at 95V for 1 hour and 30 minutes. Precision PlusProtein™ All Blue Prestained Protein Standard (Biorad) was used as protein marker. Six species of bioflocculant-producing bacteria showed different bands with different molecular weight of protein, ranged between 16 kDa to 100 kDa (**Figure 7**).

**Table 5:** Protein profiling of marine bioflocculant-producing bacteria on SDS-PAGE

| Lane | Protein marker / Bioflocculant-producing bacteria | Estimated molecular weight (kDa) | Number of protein bands |
|------|---|----------------------------------|-------------------------|
| A    | <i>Nitratireductor aquimarinus</i>                | 24-100                           | 4                       |
| B    | <i>Halomonas venusta</i>                          | 19-55                            | 4                       |
| C    | <i>Pseudoalteromonas</i> sp.                      | 24-55                            | 3                       |
| D    | <i>Bacillus subtilis</i>                          | 16-75                            | 7                       |
| E    | <i>Bacillus cereus</i>                            | 17-100                           | 7                       |
| F    | <i>Bacillus pumilus</i>                           | 18-90                            | 6                       |

Lane A      B      C      D      E      F      M



**Figure 3:** SDS-PAGE profile of extracted EPS from biofloculant-producing bacteria under denaturing condition on 12% polyacrylamide gel. Lane A: *Nitratedirector aquimarinus*, Lane B: *Halomonas venusta*, Lane C: *Pseudoalteromonas* sp., Lane D: *Bacillus subtilis*, Lane E: *Bacillus cereus*, Lane F: *Bacillus pumilus*, M: Precision PlusProtein™ All Blue Prestained Protein Standard

## 4. Discussion

### 4.1. Identification of biofloculant-producing bacteria isolated from bioflocs

Conventional biochemical test was proven to have accuracy rate of more than 96%. Although biochemical test has been useful for bacteria identification, there were several limitations that need to be considered such as poor reproducibility and difficulties for large-scale applications [36]. The best way for identification of bacteria is through conventional biochemical test and 16S rDNA sequencing as no single test identification was proven to have 100% accuracy rate [37]. From this study, the result showed that *Bacillus* genus was the most common among the isolates. In previous studies, they were many bacteria of this genus that have been reported as biofloculant-producing bacteria. For example, *Bacillus licheniformis*, isolated from contaminated medium showed the ability to produce extracellular biofloculant while *Bacillus* spp. A56 and *Bacillus subtilis* were reported to produce proteinaceous biofloculants [11, 38, 19]. In other studies of characterization of microbial EPS, *Bacillus* sp. I-471 and *Bacillus subtilis* DYU1 were identified as biofloculant-producing bacteria [8,39]. In a study of decolourization of acid dyes, *Bacillus subtilis* and *Bacillus cereus* isolated from disposal site of tannery effluent were identified as biofloculant-producing bacteria [40]. In a study of role of extracellular polymeric substances in Cu(II) adsorption, the result indicated that the presence of biofloculant in EPS from *Bacillus subtilis* was significantly enhanced Cu(II) adsorption capacity [41]. Besides that, a biofloculant-producing bacteria known as *Bacillus toyonensis* strain AEMREG6 also has been isolated from sediment samples of a marine environment in South Africa [42]. Other genus of *Bacillus* identified as biofloculant-producing bacteria strains were *Bacillus subtilis* WD90, *Bacillus subtilis* SM29 [20], *Bacillus firmus* [14], and *Bacillus cereus* B-11 [21]. All these studies proved that genus of *Bacillus* is one of the most common isolated biofloculant-producing bacteria.

The genus of *Halomonas* bacteria also showed potential characteristic as biofloculant-producing bacteria. Biofloculants produced by *Halomonas* sp. were preliminarily evaluated as flocculating agents in the treatment of industrial wastewater effluents [12]. Besides, biofloculant-producing bacteria isolated from the bottom sediment of Algoa Bay, South Africa showed 99% of

similarity to *Halomonas* sp. Au160H based on 16S rRNA gene sequence. The nucleotide sequence was deposited as *Halomonas* sp. Okoh with accession number HQ875722 [27].

In a study of purification and characterization of EPS with antimicrobial properties from marine bacteria, *Pseudoalteromonas* sp. has been isolated from fish epidermal surface and has been identified as bioflocculant-producing bacteria [43].

In this study, Unknown sp.1 closely related to *Nitratireductor aquimarinus* when genotypic identification was conducted. *Nitratireductor aquimarinus* has been reported as a bioflocculant-producing bacteria isolated from biofloc of shrimp pond [44].

#### 4.2. The effectiveness of flocculating activities of identified marine bioflocculant-producing bacteria

Generally, there are factors to be considered in determining the difference of flocculating activity of specific species of bioflocculant-producing bacteria. The difference of flocculating activity of six identified species of bioflocculant-producing bacteria depends on the nature of EPS production during the bacteria growth.

In the present study, glucose, urea and peptone in YPG medium were used as the sources of carbon and nitrogen. It has been reported that carbon and nitrogen sources not only highly manipulate the bioflocculant production and bacterial growth but they also found to play significant roles in flocculating activity [45]. From a study of bioflocculant production, glucose was reported to be the ideal carbon source for bioflocculant production by bacteria, as it yielded about 87% flocculating activity compared to sucrose, fructose and starch, which yielded about 75%, 66% and 0% flocculating activities respectively [45]. Glucose was reported as the best carbon source to enhance the production of bioflocculants by *Halomonas* sp. V3a [46]. For nitrogen source, urea showed the optimal manufacture of bioflocculant and higher flocculating activity compared to peptone [45]. Urea was preferred nitrogen source for the cultivation of haloalkalophilic *Bacillus* sp. I-450 [8]. Peptone was found to be significant factor that affecting bioflocculant production by *Halomonas* sp. V3a [45]. Previous study of partial characterization and biochemical analysis of bioflocculants of *Halomonas* sp. isolated from sediment, the bioflocculant was optimally produced when glucose and urea were used as sources of carbon and nitrogen [27].

Initial YPG medium pH that was used for cultivation in this study was pH 7. pH tolerance is another important factor which determine the effectiveness of the bioflocculant in different polluted waters that have wide pH range [47]. The pH may affect product biosynthesis, cell morphology and structure, cell membrane function, ionic state of substrates, solubility of salts and uptake of various nutrients [48]. At low pH and high pH, similar effects have been observed where the absorption of H<sup>+</sup> ions tends to deteriorate the bioflocculant-kaolin complex formation process [49]. Maximum bioflocculant producing activity of *Bacillus cereus* and *Bacillus thuringiensis* was affected by pH between pH 7 to pH 8 [20]. However, these observations differ from the results of several studies which the maximum flocculating activity of *Bacillus* sp. F19, was observed at pH 2 while *Bacillus* sp. PY-90 was found to be actively high at acidic pH range between 3.0 to 5.0 [50, 51]. *Bacillus toyonensis* strain AEMREG6 exhibited above 60% of flocculating activity at medium pH of 5 [42]. Optimal pH values for the flocculating activity of *Halomonas maura* was pH 7.2 and pH 7.0 [52]. *Halomonas* sp. V3a also attained the highest flocculating activity at pH 7 [49]. In a study of partial characterization and biochemical analysis of bioflocculants of *Halomonas* sp., the bioflocculant was optimally produced with flocculating activity of 87% at pH 7.0 [27]. Most of *Bacillus* bacteria performed very well at acidic pH while *Halomonas* bacteria performed optimally at neutral pH.

Other factor is temperature where floc formation and floc size distribution caused by the hydrophobic interaction occurs reversibly in response to the change in temperature [53]. In this study, the temperature of bacterial culture was set up at 30°C for optimum production of bioflocculant. Maximum bioflocculant producing activity for *Bacillus cereus* and *Bacillus thuringiensis* was affected by temperature ranged between 30°C to 40°C and during growth period from 72 hours to 96 hours [20].

Optimum aeration and dissolve oxygen level during bioflocculant production also important for better bioflocculation performance. An increasing rate of oxygen transfer can enhance the rate of product

formation. Oxygen depletion can cause autolysis of the cells and eventually the formation of a hollow center. Aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product or by-product and oxygen [54]. To achieve the optimum performance of flocculation, during cultivation of six species of bioflocculant-producing bacteria for bioflocculant production, the orbital shaker was set at 250 rpm to ensure there was dissolved oxygen in the bacteria culture. Besides that, the observed flocculating activity might be due to partial enzymatic deprivation of the polymer flocculant in the late phases of cell growth [55].

#### 4.3. Characterization of protein composition in crude extracellular polymeric substances (EPS) from bioflocculant-producing bacteria

Bioflocculants produced by bioflocculant-producing bacteria were in form of crude extracellular polymeric substances (EPS). Determination of protein concentration in crude EPS is very important to prove that EPS were composed of protein. Protein composition in the crude EPS was believed to enhance the mechanism of bioflocculation. EPS was produced by microorganisms for various purposes in reaction to environmental stresses [56]. Most of bioflocculants by microorganisms were formed during their growth phase. For example, bacteria exploit the nutrients in the culture medium to synthesize high molecular-weight polymeric substances under the action of specific enzymes. Quantity and composition of protein in EPS have been shown to vary depending on bacterial strain and environmental stresses such as temperature, pH and ions [57]. Quantification of macromolecules within EPS indicated that proteins and carbohydrates are the major constituents with protein level escalating in EPS as growth proceeded from the exponential phase to the stationary phase [58].

Protein band profile on 12% polyacrylamide gel showed that all bioflocculant-producing bacteria species produced a variety of size and structure of protein in EPS. The ability of proteins to move through the gel is depending on their size and structure and relative to the pores of the gel. Large molecules migrate slower than small molecules and this movement created the separation of distinct particles within the gel. In this study, *Bacillus subtilis*, *Bacillus cereus* and *Bacillus pumilus* showed a quite intense of protein bands on SDS gel. The protein bands that appeared on SDS gel for *Bacillus subtilis*, *Bacillus cereus* and *Bacillus pumilus* were ranged between 16 - 75 kDa, 17 - 100 kDa and 18 - 90 kDa respectively. Extracted EPS from *Bacillus* genus usually are used as stabilizers, emulsifiers, binders, gelling agent and film formers. EPS from *Bacillus* genus had been an interesting topic because they are Generally Recognized as Safe (GRAS). Chemical compositions in EPS such as proteins, neutral polysaccharides, amphiphilic molecules and charged polymers that produced by wild-type *Bacillus subtilis* strains cultured under controlled laboratory conditions reveal a wide range of molecular weight with sizes ranging from 0.57 kDa to 128 kDa [58]. Most of proteins are found freely in the surrounding medium as they dissociated from cells and some are found within exopolymeric matrix. Proteins that composed by *Bacillus subtilis* also included the proteins that responsible for the extracellular enzymes discharge and protein export from the cytoplasm to the surrounding environment. Many proteins that secreted by *Bacillus subtilis* also involved in the degradation of molecules such as extracellular nucleic acids, phytic acid, lipids and glutathione [59]. In a study of production and characterization of EPS from bacteria isolated from pharmaceutical laboratory sinks, molecules, proteins and functional groups were found in the EPS produced from *Bacillus subtilis* using FTIR analysis [60]. Biopolymer flocculants named FQ-B1 and FQ-B2, produced by *Bacillus cereus* and *Bacillus thuringiensis* were precipitated by chemical elemental analysis and UV scan were performed for investigating the purified bioflocculant contained 2.56 µg/ mL (83.01%) and 1.78 µg/ mL (84.73%) of protein respectively [20]. In a study of glycoprotein bioflocculant, chemical analysis showed that purified bioflocculant produced by *Bacillus toyonensis* strain AEMREG6 was mainly composed of polysaccharide (77.8%) and protein (11.5%) [42]. Extracted bioflocculants from *Bacillus subtilis* can be used as an alternative agent to eliminate copper at lower concentrations but further study needs to be carried out on its actions mechanism, scaling up process and modifications to enhance its ability in order to make it more reliable for industrial utilization [61].

In this study, even though *Halomonas venusta*, *Pseudoalteromonas* sp. and *Nitratireductor aquimarinus* did not show very high concentration of protein in their extracted EPS, they still showed several prominent protein bands. *Halomonas venusta* showed four prominent protein bands that ranged between 19 - 55 kDa. It showed that protein was one of the main compositions in its biofloculants. This study was supported by a study of partial characterization of *Halomonas* sp. where chemical analysis revealed that biofloculant produced by *Halomonas* sp. was mainly polysaccharide and protein [27].

*Pseudoalteromonas* sp. showed three prominent protein bands that ranged between 24 - 55 kDa. It showed that protein was one of the components in its EPS. Previous finding on purification and characterization of EPS with antimicrobial properties from *Pseudoalteromonas* sp. has revealed that up to eight protein types of unknown proteins were detected within the EPS, with size of molecular weight ranging from 15.486 kDa to 113.058 kDa [43]. The *Pseudoalteromonas* sp. in the study also showed to produce the highest amount of EPS during the first 24 hours of culture.

The result obtained in the present study suggests that *Nitratireductor aquimarinus* is a potential biofloculant-producing bacteria. This bacteria produces four prominent protein bands that ranged between 24 - 100 kDa when analyzed using SDS-PAGE. However, there was no study of EPS characterization to indicate and support that its proteins from EPS can act as biofloculant.

## 5. Conclusion

Six species of marine bacteria were successfully identified as biofloculant-producing bacteria from bioflocs. They were closely similar to *Halomonas venusta*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus pumilus*, *Nitratireductor aquimarinus* and *Pseudoalteromonas* sp. The group of high flocculating activity was exhibited by *Bacillus cereus* (93%), *Bacillus pumilus* (92%), *Nitratireductor aquimarinus* (89%) and *Pseudoalteromonas* sp. (86%). *Bacillus subtilis* (79%) represented group of intermediate flocculating activity while *Halomonas venusta* (59%) was categorized as group of low flocculating activity. For protein characterization of crude EPS, all species of biofloculant-producing bacteria have different protein concentration that ranged between 1.377 µg/mL to 1.455 µg/mL with different banding patterns between three to seven bands at different molecular weight that ranged between 16 to 100 kDa.

It is recommended to further characterize on EPS produced by *Nitratireductor aquimarinus* especially in terms of function and structural using latest advanced methods such as nuclear-magnetic resonance (NMR) to characterize polysaccharide composition and high performance liquid chromatography (HPLC) to separate components of mixture from one another. The methods may assist in order to detect other complex compositions reported in EPS such as polysaccharides, nucleic acid, uronic acid, phospholipid and glycoprotein. The results would be an initial step towards the utilization and modification of EPS in future research in the production of valuable properties especially in aquaculture industry.

## 6. Acknowledgements

This project was supported by the Ministry of Education, Malaysia (MOE) under Fundamental Research Grant Scheme, FRGS (vot no. 59401). We also would like to thank iSHARP, Blue Archipelago Berhad, Setiu, Terengganu, Malaysia for *L. vannamei* aquaculture facilities. Finally, to all lab staffs at the Institute of Tropical Aquaculture (AKUATROP), Universiti Malaysia Terengganu who have major contributions throughout the study periods.

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