**Description of novel marine biofloculant-producing bacteria isolated from
biofloc of Pacific whiteleg shrimp, *Litopenaeus vannamei* culture ponds**

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Abstract:

Description of marine biofloculant-producing bacteria isolated from biofloc of
Pacific whiteleg shrimp, *Litopenaeus vannamei* culture ponds was prompted to
explore the bacteria that enhanced biofloculation 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.

Keywords: Natural flocculant, molecular identification, flocculating performance, extracellular polymeric substances, protein profiling

Importance:

Six species of marine bacteria isolated from biofloc of Pacific whiteleg shrimp, *Litopenaeus vannamei* culture ponds were identified as biofloculant-producing bacteria. Among those six species, *Bacillus cereus*, *Bacillus pumilus*, *Nitratireductor*

aquimarinus and *Pseudoalteromonas* sp. were highly potential to be used as booster for rapid formation of biofloc due to their high flocculating activities. Protein content in EPS of novel marine biofloculant-producing bacteria has beneficial consequences on degradation process of organic substances, denitrification of wastes and ions elimination from aquaculture wastes.

1. Introduction

Aquaculture is a huge industry that involves cultivation of freshwater and seawater organisms under controlled operations. However, application of effective technologies for wastewater treatment remains minimal in intensive aquaculture operations. High composition of uneaten fish feed and feces in river or sea released by aquaculture operation can cause eutrophication problem (Amirkolaie, 2011). Sludge such as debris, fecal materials and uneaten feed that settled in the bottom sediment can interfere with the interactions of organisms at all biodiversity levels (Yang et al., 2012). 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 (Zhang et al., 2012). As compared to other conventional system, this method was volume independent to concentrate dead cells (Salehizadeh & Shojaosadati, 2001). 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 (Newman, 2011). Flocculants can be divided into synthetic and natural (Yu et al., 2009). 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 (Zaki et al., 2011). 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 (Kumar et al., 2004; Feng & Xu, 2008). In other industry, bioflocculants are also widely used as alternative treatment to remove inorganic solid suspensions, dye solutions, food and industrial wastewater (Gao et al., 2009). 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* (Xiong et al., 2009). *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 (Lin et al., 2012). 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 (Gao et al., 2006). Other bacteria that have been reported as bioflocculant-producing bacteria were *Bacillus firmus* (Salehizadeh & Shojaosadati, 2002), *Citrobacter* spp. TKF04 (Fujita et al., 2001), *Corynebacterium glutamicum* (He et al., 2002), *Enterobacter aerogenes* (Lu et al., 2005), *Nannocystis* sp. Nu-2 (Zhang et al. 2002), *Bacillus subtilis*, *Bacillus*

licheniformis, *Pacilomyces* sp., and *Nocardia amarae* YK (Deng et al., 2005), *Enterobacter agglomerans* SM 38, *Bacillus subtilis* SM 29 and *Bacillus subtilis* WD90 (Rawhia et al., 2014), *Bacillus cereus* B-11 (Mao et al., 2011), *Serratia ficaria* (Gong et al., 2008), *Lactobacillus delbrückii* sp.*bulgaricus* (Gruter et al., 1993) and *Bacillus alvei* NRC-14 (Abdel Aziz. et al., 2011).

Therefore, the ultimate aim of this study was to characterize the potential biofloculant-producing bacteria involved in biofloc formation, particularly for aquaculture wastewater treatment.

2. Methodology

2.1 Location of sampling site

Sampling of biofloc was carried out at Integrated Shrimp Aquaculture Park (iSHARP) Sdn. Bhd (**Figure 1**). 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. Sampling activities were conducted from 25th June 2014 until 29th September 2014. Biofloc samples were collected from fully developed biofloc ponds. In this study, sampling activities were conducted once in every 10 days interval which involved various stage of biofloc formation. 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

Composition of marine broth contained (per litre): 37.8 g of Difco marine nutrient powder in filtered deionized water. The nutrient agar included (per litre): 55 g of Difco marine agar in filtered deionized water. The Yeast Peptone Glucose (YPG) medium composed (per litre): 10.0 g of glucose, 2.0 g of peptone, 0.5 g of urea, 2.0 g of yeast extract, 0.1 g of NaCl, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 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 (Ntsaluba et al., 2011). The production medium / enrichment medium (per litre): 10.0 g of glucose, 0.5 g of urea, 0.3 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 g of K_2HPO_4 , 2.0 g of peptone, 0.2 g of KH_2PO_4 and 2.0 g of yeast extract in filtered seawater (Cosa et al., 2011). The medium for marine slant agar included (per litre): 10.0 g of glucose, 5.0 g of K_2HPO_4 , 2.0 g of KH_2PO_4 , 0.3 g of $\text{NH}_4(\text{SO}_4)_2$, 0.5 g of urea, 2.0 g of yeast extract, 0.3 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g of NaCl and 20.0 of agar in filtered deionized water (Gong et al., 2008). All media were adjusted to pH 7 and then sterilized by autoclaving at 121°C for 15 min.

2.4 Isolation of bioflocculant-producing bacteria from bioflocs

Samples of biofloc 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. Concentrated pellet was diluted with saline solution. Isolation of bacteria from biofloc was performed by spread plate

method 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.

2.5 Screening and identification of bioflocculant-producing bacteria isolated from bioflocs

Screening of bioflocculant-producing bacteria was carried out using production medium and YPG medium. Bioflocculant-producing bacteria were identified through their appearances on solid medium (YPG medium) and liquid medium (production medium). Visual characterization based on ropy, mucoid and slimy was used for identification purposes. Ropy colonies form long filaments when extended with an inoculation loop while mucoid colonies have a glistening and slimy appearance on agar plate (Ortega-Morales et al., 2007). A loop of pure culture of each isolate from marine nutrient agar plate with different colony morphologies were inoculated into 50 mL of marine broth and incubated overnight at 30°C for mass production. After incubation, 1 mL of the culture was inoculated into production medium and 0.1 mL was spread evenly 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. The isolates were maintained on marine slant agar and kept refrigerated at 4°C for further analysis.

2.6 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 biofloculant-producing bacteria as it provided descriptions and photographs of species and tests to distinguish among genera and species (Black, 2005).

2.7 Genotypic identification of biofloculant-producing bacteria through 16S rDNA sequencing

All identified biofloculant-producing bacteria were further confirmed by genotypic identification through 16S rDNA sequencing.

2.7.1 DNA extraction of biofloculant-producing bacteria

Identification of microorganisms isolated from biofloc was carried out through molecular approaches. Qiagen DNeasy Blood and Tissue Kit was used to extract bacterial DNA. It was conducted as per manufacturer's protocol.

2.7.2 DNA quantification and qualification

DNA was quantified using BioDrop μ 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. Gel electrophoresis was conducted according to Mohamad (2014).

2.7.3 Polymerase chain reaction (PCR) amplification

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 (Yu et al., 2013) to amplify the 16S rDNA gene. 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 MgCl₂, 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
(Lane, 1991). 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.

2.7.4 DNA purifications and sequencing

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.

2.8 Determination of flocculating activity of bioflocculant-producing marine bacteria

All identified bioflocculant-producing marine bacteria were cultured in enrichment medium (Cosa et al., 2011). 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 (Gao et al., 2006).

2.8.1 Flocculating activity of bioflocculant-producing bacteria using Kaolin clay suspension method

Flocculating activity was measured using a modified Kaolin clay suspension method (Kurane et al., 1994). 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 experiment was repeated 3 times for each bioflocculant-producing bacteria. The flocculating activity was calculated as follows;

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

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

2.8.2 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.

2.9 Characterization of protein composition in extracellular polymeric substances (EPS) produced by marine bioflocculant-producing bacteria

Each bioflocculant-producing bacteria species was cultured in enrichment medium at 250 rpm in orbital shaker for 3 days at 30°C for optimum extracellular polymeric substances (EPS) production (Cosa et al., 2011).

2.9.1 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.

2.9.2 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 (Bradford, 1976).

2.9.3 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 following method described by Laemmli (1970) with a slight of modification. Polyacrylamide gel was cast using 4% stacking gel and 12% resolving gel. The 4% stacking gel was prepared using following reagents; 13.2% v/v of acrylamide/bis solution 37.5:1 (30% T, 2.67% C), 25.2% v/v of stacking buffer (0.5 M Tris-HCl pH 6.8), 1% v/v of Sodium Dodecyl Sulfate (10% w/v), 0.5% v/v of ammonium persulfate (10% w/v), 0.1% v/v of TEMED and the remaining volume was top up with distilled deionized water. The 12% resolving gel was prepared using

287 following reagents: 40% v/v of acrylamide/bis solution 37.5:1 (30% T, 2.67% C),
 288 25% v/v of resolving buffer (1.5 M Tris-HCl pH 8.8), 1% v/v of Sodium Dodecyl
 289 Sulfate (10% w/v), 0.5% v/v of ammonium persulfate (10% w/v), 0.5% v/v of
 290 TEMED and the remaining volume was top up with distilled deionized water. SDS-
 291 PAGE was started with the assembling of glass plate sandwich. Resolving gel
 292 solution was poured between the glass plates with a pipette and 1/4 of the space was
 293 left free for the stacking gel. The top of the resolving gel was carefully covered with
 294 0.1% SDS solution and left for 30 minutes until the resolving gel polymerized. A
 295 clear line has appeared between the resolving gel surface and the solution on top when
 296 polymerization was completed. Then the 0.1% SDS solution was discarded and gently
 297 washed with double-distilled water. The stacking gel solution was poured carefully
 298 with a pipette to avoid formation of bubbles. Combs were inserted and the gel was
 299 allowed to polymerize for at least 60 minutes. Combs were removed carefully. The
 300 gel was put into the electrophoresis tank. The tank was filled with fresh 1X Tris-
 301 glycine-SDS non-continuous running buffer (0.5 M Tris, 1.92 M Glycine, 1% w/v
 302 Sodium Dodecyl Sulfate, pH 8.3) to cover the gel wells. Samples were prepared by
 303 mixing with sample buffer (0.5 M Tris-HCl, 4% w/v SDS, 20% v/v glycerol, 10% v/v
 304 2-mercaptoethanol, 0.05% w/v bromophenol blue) at ratio 1:1 and were boiled for 10
 305 minutes before loaded into wells. Protein marker, See All Blue Plus (Biorad) was
 306 loaded into first lane followed by samples for the rest of lane. Probes were connected
 307 and 80 volt power supply was set. The power was increased to 95 volt when the dye
 308 reached the resolving gel. SDS-PAGE was stopped when the sign of protein marker
 309 reached the foot line of the glass plate. The gel was rinsed with distilled deionized
 310 water for two or three times and then isopropanol fixing solution was poured on the
 311 gel and let for half an hour. The gel was stained with Coomassie Blue Staining (0.1%

w/v Coomassie Brilliant Blue CBR-250, 50% v/v methanol, 10% acetic acid, 40% distilled water) for overnight. After that, the gel was destained with destain solution (10% v/v methanol, 10% v/v acetic Acid, and 80% v/v distilled water) 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

In this study, most of the phenotypic characteristics of the isolates were similar to those indicated by Bergey's Manual of Systematic Bacteriology (Boone et al., 2005). Based on biochemical characterization, the investigated isolates resembled two bacterial genera known as *Bacillus* and *Halomonas*. Two unsuccessfully identified genera were labeled as Unknown sp. 1 and Unknown sp. 2 (**Table 1**). Six different species that have been identified phenotypically were selected for further genotypic identification through 16S rDNA sequencing. **Table 2** showed the purity of the extracted genome from the bioflocculant-producing bacteria prior amplification of the DNA by PCR. The optimum purity ratio of extracted DNA was between 1.7 and 2.0 to ensure that no or less contamination occurred during the extraction process. All isolated bioflocculant-producing bacteria showed an acceptable range of DNA purity and were used as templates in PCR amplification (**Figure 2**). 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 from the composition of bacteria isolated from bioflocs. *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**).

3.2 The effectiveness of flocculating activity of identified bioflocculant-producing bacteria

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 3**).

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 biofloculant-producing bacteria

Each species of biofloculant-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.

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 biofloculant-producing bacteria showed different bands with different molecular weight of protein, ranged between 16 kDa to 100 kDa (**Figure 4**).

4. Discussion

4.1 Identification of biofloculant-producing bacteria isolated from bioflocs

From microscopic observation, three identified species were Gram-negative and three species were Gram-positive. The outer membrane of Gram-negative bacteria consists of lipopolysaccharide (LPS), protein and lipoprotein while cell wall of Gram-positive bacteria consists of a thick peptidoglycan layer (Nasir, 2014). The complexity of Gram-negative bacteria cell wall has resulted in better adaptation and survival in marine environment and the outer membrane especially LPS assisted in absorbing

nutrient under limited nutrient supply conditions (Nasir, 2014). All Gram-positive bacteria isolated in this study showed positive result in endospore staining test. The capacity to form endospore is unique to certain members of low G-C DNA bases of Gram positive bacteria such as from phylum Firmicutes (Traag et al., 2012). In this study, all species were rod-shaped. According to Thi et al., (2012), Gram-negative and rod-shaped bacteria were dominant in marine environment. Rod-shaped was more advantageous than coccus-shaped because it provides a higher surface-to-volume ratio. Therefore, it is more efficient in nutrient uptake in marine environment (Sjostedt et al., 2012).

In this study, biochemical test was used to identify bacteria species based on differences of biochemical activities of bacteria. It can be carried out conventionally or through commercial identification kit such as API system (Moraes et al., 2013). Commercial identification kit has been widely used as it is fast and its software databases mainly contain clinically important bacteria. It is very useful to identify small number of isolates especially for clinical samples. However, commercial identification kit did not perform well as compared to conventional biochemical test on bacteria species identification. Conventional biochemical test was proven to have accuracy rate of more than 96% while commercial identification kit has only 79 to 94% of accuracy rate because of limited number of tests in commercial identification kit that lead to low percentage of accurate identification (Janda & Abbott, 2002). In this study, species that showed positive result in catalase test indicated that they have the ability to degrade hydrogen through production of catalase (Cappucino & Sherman, 2001). All Gram-negative bacteria in this study contain cytochrome oxidase enzyme because they showed positive result in oxidase test. In carbohydrate fermentation test, species that showed positive result were able to ferment that type of

carbohydrate as a carbon source. According to Boone et al., (2005), every species of bacteria even in the same genus has different phenotypic characteristics. This explains why different phenotypic characteristics showed by *Bacillus* sp. in mannitol fermentation test where two species showed positive result while one species showed negative result. In urease test, isolates that showed negative results lack of urease enzyme because they were unable to hydrolyse urea to produce ammonia and carbon dioxide. Urease enzyme is produced by many different bacteria and is reported as virulence factor found in various pathogenic bacteria (Konieczna et al., 2012). In motility test, five species showed positive result. Most bacteria use flagella to move and will enable bacteria to detect and pursue nutrients. Motility is closely linked with chemotaxis which is the ability to orientate along certain chemical gradients (Josenhans & Suerbaum, 2002). In indole test, bacteria that lack enzyme tryptophanase unable to split indole from amino acid tryptophan resulted in no indole production. For Voges-Proskauer (VP) test, isolates that showed positive results can generate acetylbutanediol (ABD) from acetoin. In citrate test, isolates with positive results showed that they were able to utilize citrate as carbon source. This ability depends on presence of a citrate permease enzyme that helps in transport of citrate in the cell (Cappucino & Sherman, 2001). In nitrate reduction test, isolates that showed positive results produced nitrate reductase enzyme because they were capable of reducing nitrate (NO_3^-) to nitrite (NO_2^-). In phenylalanine deaminase test, isolates that showed positive results was able to remove amino group ($-\text{NH}_2$) from amino acid with the help of phenylalanine deaminase enzyme. They deaminated phenylalanine and converted it to keto acid, phenylpyruvic acid and ammonia. Isolates that gave positive results in starch hydrolysis test were able to produce extracellular enzymes such as α -amylase and oligo-1,6-glucosidase that hydrolyzed starch. 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 (Moraes et al., 2013). 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 (Janda & Abbott, 2002).

From this study, the result showed that *Bacillus* genus was the most common among the isolates. In previous studies, there were many bacteria of this genus that have been reported as bioflocculant-producing bacteria. For example, *Bacillus licheniformis*, isolated from contaminated medium showed the ability to produce extracellular bioflocculant while *Bacillus* spp. A56 and *Bacillus subtilis* were reported to produce proteinaceous bioflocculants (Xiong et al., 2009; Suh et al., 1997; Deng et al., 2005). In other studies of characterization of microbial EPS, *Bacillus* sp. I-471 and *Bacillus subtilis* DYU1 were identified as bioflocculant-producing bacteria (Kumar et al., 2004; Wu & Ye, 2007). In a study of decolourization of acid dyes, *Bacillus subtilis* and *Bacillus cereus* isolated from disposal site of tannery effluent were identified as bioflocculant-producing bacteria (Anuradha et al., 2014). In a study of role of extracellular polymeric substances in Cu(II) adsorption, the result indicated that the presence of bioflocculant in EPS from *Bacillus subtilis* was significantly enhanced Cu(II) adsorption capacity (Fang et al., 2011). Besides that, a bioflocculant-producing bacteria known as *Bacillus toyonensis* strain AEMREG6 also has been isolated from sediment samples of a marine environment in South Africa (Okaiyeto et al., 2015). Other genus of *Bacillus* identified as bioflocculant-producing bacteria strains were *Bacillus subtilis* WD90, *Bacillus subtilis* SM29 (Rawhia et al., 2014), *Bacillus alvei* NRC-14 (Abdel Aziz et al., 2011), *Bacillus* sp. CH15 (Lin et al., 2012), *Bacillus firmus* (Salehizadeh & Shojaosadati, 2002) and *Bacillus cereus* B-11 (Mao et

al., 2011). All these studies proved that genus of *Bacillus* was one of the most common isolated bioflocculant-producing bacteria.

The genus of *Halomonas* bacteria also showed potential characteristic as bioflocculant-producing bacteria. According to Lin et al., (2012), bioflocculants produced by *Halomonas* sp. were preliminarily evaluated as flocculating agents in the treatment of industrial wastewater effluents. Besides, a bioflocculant-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 (Cosa et al., 2011).

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 (Mohd Shahir Shamsir et. al., 2012).

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 (Nor Azman et al., 2017).

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. In this study, cultivation for production of EPS of six identified bioflocculant-producing

bacteria was performed following technique of Cosa et al., (2011). The difference of flocculating activity of six identified species of bioflocculant-producing bacteria probably 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 (Sheng et al., 2006). 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 (Sheng et al., 2006). Glucose was reported as the best carbon source to enhance the production of bioflocculants by *Halomonas* sp. V3a (He et al., 2009). For nitrogen source, urea showed the optimal manufacture of bioflocculant and higher flocculating activity compared to peptone (Sheng et al., 2006). Urea was preferred nitrogen source for the cultivation of haloalkalophilic *Bacillus* sp. I-450 (Kumar et al., 2004). According to He et al. (2009) peptone were found to be significant factors that affecting bioflocculant production by *Halomonas* sp. V3a. 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 (Cosa et. al., 2011).

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 (Wang et al., 2011). 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 (Fang & Zhong, 2002). At low pH and high pH, similar effects have been observed where the absorption of H⁺ ions tends to deteriorate the bioflocculant-kaolin complex formation process (He et al., 2010). Maximum bioflocculant producing activity of *Bacillus cereus* and *Bacillus thuringiensis* was affected by pH between pH 7 to pH 8 (Rawhia et al., 2014). However, these observations differ from the result of study carried out by Zheng et al. (2008) in 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 (Yokoi et al., 1995). *Bacillus toyonensis* strain AEMREG6 exhibited above 60% of flocculating activity at medium pH of 5 (Okaiyeto et al., 2015). Bouchtroch et al., (2001) reported optimal pH values for the flocculating activity of *Halomonas maura* was pH 7.2 and pH 7.0. *Halomonas* sp. V3a also attained the highest flocculating activity at pH 7 (He et al., 2010). 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 (Cosa et. al., 2011). Most of *Bacillus* bacteria performed very well at acidic pH while *Halomonas* bacteria performed optimally at neutral pH.

Other factor is temperature where flocs formation and floc size distribution caused by the hydrophobic interaction occurs reversibly in response to the change in temperature (Sakohara et al., 2000). In this study, the temperature of bacterial culture was set up at 30°C for optimum production of bioflocculant. According to Rawhia et al., (2014), 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.

Optimum aeration and dissolve oxygen level during biofloculant production also important for better bioflocculation performance. 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 (Selale, 2007). To achieve the optimum performance of flocculation, during cultivation of six species of biofloculant-producing bacteria for biofloculant 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 (Choi et al., 1998).

In this study, *Nitrateductor aquimarinus* shows relatively high flocculating activity comparable to *Bacillus pumilus* and *Pseudoalteromonas* sp. (**Figure 3**). According to Nor Azman et al., (2017), there is information available about the effectiveness of flocculating activity of *Nitrateductor aquimarinus* as biofloculant-producing bacteria.

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

Biofloculants produced by biofloculant-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 (Bhatia et al., 2013). Most of biofloculants 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 (Park & Novak, 2007). 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 (Omoike & Chorover, 2004).

Protein band profile on 12% polyacrylamide gel showed that all biofloculant-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. Many studies reported that extracted EPS from *Bacillus* sp. 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 (Omoike & Chorover, 2004). 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 (Tjalsma et al., 2004). In a study of production and characterization of EPS from bacteria isolated from pharmaceutical laboratory sinks by Nanda & Raghavan, (2007), molecules, proteins and functional groups are found in the EPS produced from *Bacillus subtilis* using FTIR analysis. The 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 (Rawhia et al., 2014). 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%) (Okaiyeto et al., 2015). 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 (Azmi et al., 2015).

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 bioflocculants. This study was supported by a study of partial characterization of *Halomonas* sp. where chemical

analysis revealed that bioflocculant produced by *Halomonas* sp. was mainly polysaccharide and protein (Cosa et. al., 2011).

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 (Mohd Shahir Shamsir et. al., 2012). 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 bioflocculant-producing bacteria. These bacteria produce 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 bioflocculant since no study claimed *Nitratireductor aquimarinus* as bioflocculant-producing bacteria.

5. Conclusion

Six species of marine bacteria were successfully identified as bioflocculant-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 bioflocculant-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.

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7. References

- Abdel-Aziz, S. M., Hoda, A. H., Foukia, E. M. and Nayera, A. M. A. (2011). Extracellular Metabolites Produced by a Novel Strain, *Bacillus alvei* NRC-14: 3. Synthesis of a Biofloculant that has Chitosan-Like Structure. *Life Science Journal*, 8(4): 883-890
- Amirkolaie, A.K. (2011). Reduction in the Environmental Impact of Wastewater Discharged by Fish Farms through Feed and Feeding. *Reviews in Aquaculture* 3(1): 19-26
- Anuradha, V., Stephy, L., Eppa, S. R., Nagarajan, Y., Mohamed Yacoob, S.A., Prathasarathy, V., Ramachandran, R., Peer, M., and Kalitha, P. (2014). Decolorization of Acid Dyes by *B.cereus* and *P.aeruginosa* isolated from Effluent of Dyeing Industry. *International Journal Pure Application Bioscience*, 2(3): 23-29
- Azmi, M. A., Norli, I., Farehah, Z. A., Ishak, S. A., Norfariha, M. N. S., and Azieda, A. T. (2015). Crude and Pure Biofloculants Produced from *Bacillus subtilis* for Low Concentration of Copper (Cu²⁺) Removal. *Iranica Journal of Energy & Environment*, 6(2): 103–110.
- Bhatia, D., Isabelle, B., Stéphane, S., François, B., Eric, D. V. H., Stéphanie, R., Piet, N.L. and Gilles G. (2013). Fluorescence detection to determine proteins and humic-like substances fingerprints of exopolymeric substances (EPS) from biological sludges performed by size exclusion chromatography (SEC). *Journal of Bioresource Technology*, 131: 159-165

671 Black, J.G. (2005). *Microbiology: Principles and Explorations*. (6th ed). USA: John
672 Wiley and Sons Inc.

673 Boone, D.R., Castenholz, R.W., Garrity, G.M., Brenner, D.J., Krieg, N.R. and Staley,
674 J.T. (2005). *Bergey's Manual® of Systematic Bacteriology*. Germany:
675 Springer.

676 Bouchotroch, S., Quesada, E., Del Moral, A., Llamas, I. and Béjar, V. (2001).
677 *Halomonas maura* sp. nov., a novel moderately halophilic, exopolysaccharide
678 producing bacterium. *Int. J. Syst. Evol. Microbiol.*, 51: 1625-1632.

679 Bradford, M. M. (1976). A rapid and sensitive method for the quantization of protein
680 utilizing the principle of the dye-protein binding. *Anal. Biochem.*, 72: 248-254.

681 Cappucino, J.G. and Sherman, N. (2001). *Microbiology: a laboratory manual*. (6th
682 ed). San Francisco, California : Pearson Education Inc.

683 Choi, C.W., Yoo, S.-A., Oh, I.-H. and Park, S.H. (1998). Characterization of an
684 extracellular flocculating substance produced by a planktonic cyanobacterium,
685 *Anabaena* sp. *Biotechnol. Lett.*, 20: 643-646

686 Cosa, S., Mabinya, L. V., Olaniran, A. O., Okoh, O. O., Bernard, K., Deyzel, S., and
687 Okoh, A. I. (2011). Bioflocculant production by *Virgibacillus* sp. rob isolated
688 from the bottom sediment of alga bay in the Eastern Cape, South Africa.
689 *Molecules*, 16(3): 2431–2442.

690 Deng, S., Yu, G. and Ting, Y.P. (2005). Production of a Bioflocculant by *Aspergillus*
691 *parasiticus* and Its Application in Dye Removal Colloid Surface. *B.*
692 *Biointerfaces*, 44(4): 179-186

693 Fang, Q.H. and Zhong, J.J. (2002). Effect of initial pH on production of ganoderic
694 acid and polysaccharide by submerged fermentation of *Ganoderma lucidum*.
695 *Process Biochemistry*, 37: 769–774.

696 Fang, L., Wei, X., Cai, P., Huang, Q., Chen, H., Liang, W. and Rong, X. (2011). Role
697 of extracellular polymeric substances in Cu(II) adsorption on *Bacillus subtilis*
698 and *Pseudomonas putida*. *Bioresource Technology*, 102(2): 1137–1141.

699 Feng, D.L. and Xu, S.H. (2008). Characterization of Biofloculant MBF3-3 Produced
700 by an Isolated *Bacillus* sp. *W. J. Microbiol. Biotechnol.*, 24: 1627.

701 Fujita, M., Ike, M., Jang, J.H., Kim, S.M. and Hirao, T. (2001). Bioflocculation
702 Production from Lower Molecular Fatty Acids as a Novel Strategy for
703 Utilization of Sludge Digestion Liquor. *Water Sci. Technol.* 44(10): 237-243

704 Gao, J., Bao, H., Xin, M., Liu, Y., Li, Q., & Zhang, Y. (2006). Characterization of a
705 biofloculant from a newly isolated *Vagococcus* sp. W31. *Journal of Zhejiang*
706 *University. Science. B*, 7(3): 186–92.

707 Gao, Q., Xiu-Hua, Z., Jun, M., Yi, Z. and Xue-Wei, D. (2009). Using *Ruditapes*
708 *philippinarum* conglutination mud to produce biofloculant and its
709 applications in wastewater treatment. *Bioresour. Technol.*, 100: 4996.

710 Gong, W.; Wang, S.; Sun, X.; Liu, X.; Yue, Q. and Gao, B. (2008). Biofloculant
711 Production by culture of *Serratia ficaria* and its application in wastewater
712 treatment. *Biores. Technol.* 99: 4668- 4674.

713 Gruter, M.; Leeflang, B.; Kuiper, J.; Kamerling ,J. and Vliegthart ,J. (1993).
714 Structural characterization of the exopolysaccharide produced by

715 *Lactobacillus delbrukii subspecies bulgaricus* grown in skimmed milk.
716 *Carbohydrate Research*, 239: 209 -226.

717 He, N., Li, Y., Chen, J. and Lun, S.Y. (2002). Identification of a Novel Biofloculant
718 From a Newly Isolated *Corynebacterium glutamicum*. *Biochem. Eng. J.* 11:
719 137-148

720 He, J., Zhen, Q., Qiu, N., Liu, Z., Wang, B., Shao. Z. and Yu, Z. (2009). Medium
721 optimization for the production of a novel biofloculant from *Halomonas sp.*
722 V3a' using response surface methodology. *Bioresour. Technol.*,100: 5922-
723 5927.

724 He, J., Zou, J., Shao, Z., Zhang, J., Liu, Z. and Yu, Z. (2010). Characteristics and
725 flocculating mechanism of a novel biofloculant HBF-3 produced by deep-sea
726 bacterium mutant *Halomonas sp.* V3a'. *World J. Microbiol. Biotechnol.*, 26:
727 1135-1141.

728 Janda, J.M. and Abbott, S.L. (2002). Bacterial identification for publication: when
729 enough is enough? *Journal of Clinical Microbiology*, 40(6): 1887-1891.

730 Josenhans, C. and Suerbaum, S. (2002). The role of motility as a virulence factor in
731 bacteria. *International Journal of Medical Microbiology*, 291: 605-614.

732 Konieczna, I., Zarnowiec, P., Kwinkowski, M., Kolesinska, B., Fraczyk, J., Kaminski,
733 Z., Kaca, W. (2012). Bacterial urease and its role in long-lasting human
734 diseases. *Current Protein and Peptide Science*, 13(8): 789-806.

735 Kumar, C.G., Joo, H.S., Choi, J.W., Koo, Y.M. and Chang, C.S. (2004). Purification
736 and characterization of an extracellular polysaccharide from haloalkalophilic
737 *Bacillus sp.* I450. *Enz. Microb. Technol.*, 34: 673-681.

738 Kurane, R., Hatamochi, K., Kakuno, T., Kiyohara, M., Kawaguchi, K., Mizuno, Y.,
739 Hirano, M. and Taniguchi, Y. (1994). Production of a bioflocculant by
740 *Rhodococcus erythropolis* S-1 grown on alcohols. *Biosci. Biotechnol.*
741 *Biochem.*, 58: 428-429.

742 Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the
743 head of bacteriophage T4. *Nature* 227: 680-685

744 Lane, D.J. (1991). 16S/23S rRNA sequencing in Nucleic Acid Techniques in
745 Bacterial Systematics, eds Stackebrandt E, Goodfellow M (Wiley, New York),
746 pp 115-175.

747 Lin, J. and Harichund, C. (2012). Production and Characterization of Heavy-Metal
748 Removing Bacterial Bioflocculants. *African Journal of Biotechnology*, 11(40):
749 9619-9629.

750 Lu, W.Y., Zhang, T., Zhang, D.Y., Li, C.H., Wen, P. and Du, L.X. (2005). A novel
751 bioflocculant produced by *Enterobacter aerogenes* and its use in defecating
752 the trona suspension. *Biochem. Eng. J.*, 27: 1–7.

753 Mao, Y., Tian, C., Zhu, J., Zhang, T. and Tong, L. (2011). Production of a novel
754 biopolymer by culture of *Bacillus cereus* B-11 using molasses wastewater and
755 its use for dye removal. *Adv. Materials Res.*, 230: 1119-1122

756 Mohamad, N.M., Jusoh, N.A., Htike, Z.Z. and Win, S.L. (2014). Bacteria
757 identification from microscopic morphology using Naive Bayes. *International*
758 *Journal of Computer Science, Engineering and Information Technology*, 4(2)

759 Mohd Shahir Shamsir, Ainul Masliana I., Wan Nur Atirah W. A. and Azmi R.
760 (2012). Purification and Characterization of Extracellular Polymeric

761 Substances (EPS) with Antimicrobial Properties from Marine Epibiotic
762 *Pseudoalteromonas* sp. *Jurnal Teknologi (Sciences &Engineering)*, 59: 57-61

763 Moraes, P.M., Perin, L.M., Silva, A. and Nero, L.A. (2013). Comparison of
764 phenotypic and molecular tests to identify lactic acid bacteria. *Brazilian*
765 *Journal of Microbiology*, 44(1): 109-112.

766 Nanda, A. and Raghavan, C. (2007). Production and characterization of
767 exopolysacharides (EPS) from the bacteria isolated from Pharma lab sinks. *Int.*
768 *J. PharmTech Res.*, 6(4): 1301-1305.

769 Nasir, H.M. (2014). Cultured Marine Bacteria Isolated from Sponges for Discovery of
770 Bioactive Compounds: *Department of Biology, Faculty of Science and*
771 *Technology*, Vol. Masters of Science, Universiti Malaysia Terengganu

772 Newman, S.G. (2011, March). Understanding Bioflocculation in Aquaculture
773 Producing System. *AQUA Culture Asia Pacific Magazine March/April*, 25-27,
774 Retrieved from <<http://www.aquaculturewithoutfrontiers.org>> 15th September
775 2014

776 Nor Azman Kasan, Nurarina Ayuni Ghazali, Mhd Ikhwanuddin and Zaharah Ibrahim,
777 (2017). Isolation of Potential Bacteria as Inoculum for Biofloc Formation in
778 Pacific Whiteleg Shrimp, *Litopenaeus vannamei* Culture Ponds. *Pakistan*
779 *Journal of Biological Sciences*, 20: 306-313.

780 Ntsaluba, L., Nwodo, U.U., Mabinya, L.V. and Okoh, A.I. (2011). Studies on
781 bioflocculant production by a mixed multure of *Methylobacteria* sp. Obi and
782 *Actinobacteria* sp. Mayor. *BMC Biotechnol.*, 13: 62.

783 Omoike, A. and Chorover, J. (2004) Spectroscopic study of extracellular polymeric
784 substances from *Bacillus subtilis*: Aqueous chemistry and adsorption effects.
785 *Biomacromolecules* 5: 1219–1230.

786 Ortega-Morales, B.O., Santiago-García, M.J., Chan Bacab, X., Moppert, E., Miranda-
787 Tello, M.L., Fardeau, J.C., Carrero, P., Bartolo-Pérez, Alex Valadéz-González
788 and Guezennec, J. (2007). Characterization of extracellular polymers
789 synthesized by tropical intertidal biofilm bacteria. *Journal of Applied*
790 *Microbiology*, 102: 254-264.

791 Okaiyeto, K., Uchechukwu, U. N., Leonard, V. M. and Anthony, I. O. (2013).
792 *Bacillus toyonensis* Strain AEMREG6, a Bacterium Isolated from South
793 African Marine Environment Sediment Samples Produces a Glycoprotein
794 Bioflocculant. *J. Microbiol. Biotech. Res.*, 3(3): 93-99.

795 Park, C., and Novak, J. T. (2007). Characterization of activated sludge exocellular
796 polymers using several cation-associated extraction methods. *Water Res.* 41:
797 1679–1688.

798 Rawhia, A. A., Mahmoud, N. El-Rouby, Hend, A. Abass and Zakia, A. Abo El-khier
799 (2014). Bioflocculants Produced by Bacterial Isolates from Egyptian soil,
800 Characterization and Application of Extracellular Bioflocculants and
801 Nanoparticles for Treatment of River Nile Water. *IOSR Journal of Pharmacy*
802 *and Biological Sciences*, 9(5): 103-114

803 Sakohara, S. and Nishikawa, K. (2000). Flocculation and compaction of highly
804 concentrated suspensions by using thermosensitive polymers. *Kagaku Kogaku*
805 *Ronbunshu*, 26: 298-304

806 Sakohara, S., Kimura, T. and Nishikawa, K. (2000). Flocculation mechanism of
807 suspended particles using the hydrophilic/hydrophobic transition of a
808 thermosensitive Polymer. *Higashi Hiroshima*, 26: 734-736

809 Salehizadeh, H. and Shojaosadati, S.A. (2001). Extracellular Biopolymeric
810 Flocculants-recent Trends and Biotechnological Importance. *Biotechnol. Adv.*,
811 19: 371-385.

812 Salehizadeh, H. and Shojaosadati, S.A., (2002). Isolation and Characterization of a
813 Bioflocculant Produced by *Bacillus furmus*. *Biotech. Letters*, 24: 35-40.

814 Selale, O. (2007). Investigation of the effects of dissolved oxygen concentration,
815 aeration and agitation on the morphology and rheology in submerged fungal
816 fermentation (master's thesis). Graduate School of Engineering and Science of
817 Izmir Institute of Technology, Turkey.

818 Sheng, Y. L., Zhang, Q., Sheng, Y. R., Li, C. B. and Wang, H. J. (2006). Screening
819 and flocculating properties of bioflocculant-producing microorganisms. *J.*
820 *Univ. Sci. Technol.Beijing*, 13(4): 289-292.

821 Sjostedt, J., Hagstrom, A. and Zweifel, U.L. (2012). Variation in cell volume and
822 community composition of bacteria in response to temperature. *Aquatic*
823 *Microbial Ecology*, 66: 237-246.

824 Suh, H., Kwon, G., Lee, C., Kim, H., Oh, H. and Yoon, B. (1997) Characterization of
825 bioflocculant produced by *Bacillus sp.* DP-152. *J. Ferm. Bioeng.*, 84: 108-112.

826 Tjalsma H., Antelmann, H. and Jongbloed, J.D.H. (2004). Proteomics of protein
827 secretion by *Bacillus subtilis*: separating the ‘secrets’ of the secretome.
828 *Microbiol. Mol. Biol. R.*, 68:207–233.

-
- 829 Thi, T.D., Dinh, Q.L., Dinh, T.Q. and Van, C.P. (2012). Isolation and identification of
830 marine bacteria from marine mud in Vietnam with antimicrobial activity.
831 *Journal of Vietnamese Environment*, 3(2): 71-75
- 832 Traag, B.A., Pugliese, A., Eisen, J.A. and Losick, R. (2012). Gene conservation
833 among endospore-forming bacteria reveals additional sporulation genes in
834 *Bacillus subtilis*. *Journal of Bacteriology*, 195(2): 253-260.
- 835 Wang, J., Wei, J. and Ma, H. (2011). Screening of a flocculant producing strain and
836 optimal conditions for production of bioflocculant. *Adv. Materials Res.*, 183:
837 829-833.
- 838 Wu, J. and Ye, H.F. (2007). Characterization and flocculating properties of an
839 extracellular biopolymer produced from a *Bacillus subtilis* DYU1 isolate.
840 *Process Biochem.*, 42: 1114-1123.
- 841 Xiong, Y., Wang, Y., Yu, Y., Li, Q., Wang, H., Chen, R., and He, N. (2009).
842 Production and Characterization of a Novel Bioflocculant from *Bacillus*
843 *licheniformis*. *Appl. Environ. Microbiol.* 76(9): 2778-2782.
- 844 Yang, Q., Kun, L., De-Xiang L., Xiao-Ming, L., Dong-Bo, W., Xian, L., Guang-
845 Ming, Z. and Xu, L. (2012). A novel bioflocculant produced by *Klebsiella* sp.
846 and its application to sludge dewatering. *Water Environ. J.*, 26: 560.
- 847 Yokoi, H., Natsuda, O., Hirose, J., Hayashi, S. and Takasaki, Y. (1995).
848 Characteristics of a biopolymer flocculant produced by *Bacillus* sp. PY-90. *J.*
849 *Ferment. Bioeng.*, 79(4): 378-380.

Yu, G.H., He, P.J. and Shao, L.M. (2009). Characteristics of extracellular polymeric substances (EPS) fractions from excess sludges and their effects on bioflocculability. *Bioresour. Technol.*, 100: 3193.

Yu, J., Zhou, X., Yang, S., Liu, W. and Hu, X. (2013). Design and application of specific 16S rDNA-targeted primers for assessing endophytic diversity in *Dendrobium officinale* using nested PCR-DGGE. *Appl. Microbiol. Biotechnol.*, 97: 9825-9836.

Zaki, S., Farag, S., Abu Elreesh, G., Elkady, M., Nosier, M. and Abd El Haleem, D. (2011). Characterization of bioflocculants produced by bacteria isolated from crude petroleum oil. *International Journal of Science and Technology*, 8(4): 831-840.

Zhang, J., Liu, Z., Wang, S., Jiang, P., (2002): Characterization of abioflocculant produced by the marine myxobacterium *Nannocystis* sp. NU-2. *Appl. Microbiol. Biotechnol.*, 59: 517-522.

Zhang, C., Cui, Y., Wang, Y. (2012). Bioflocculant produced from bacteria for decolorization, Cr removal and swine wastewater application. *Sustain. Environ. Res.*, 22(2): 129-134.

Zheng, Y., Ye, Z., Fang, X., Li, Y. and Cai, W. (2008). Production and characteristics of a bioflocculant produced by *Bacillus* sp. F19. *Biores. Technol.*, 99: 7686-7691.

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Figure 1: Location of sampling site, Integrated Shrimp Aquaculture Park (iSHARP) Sdn. Bhdin Setiu District, Terengganu, Malaysia (<http://www.earth.google.com,2016>)

Figure 2: Amplification of ~1.5 kb fragment of PCR products from biofloculant-producing bacteria using 1492R and 27F primers. Lane 1: *Halomonassp*, 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)

Figure 3: 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.

Figure 4: SDS-PAGE profile of extracted EPS from biofloculant-producing bacteria under denaturing condition on 12% polyacrylamide gel. Lane A: *Nitratireductor 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

886 **Table 1:** Phenotypic characterization of biofloculant-producing bacteria isolated from biofloc

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

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888 Keys: + = Positive, - = Negative, na = Not applicable

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892 **Table 2:** A₂₆₀/A₂₈₀ ratio of biofloculant-producing bacteria rDNA

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

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894 **Table 3:** Sequencing of the 16S rDNA of biofloculant-producing bacteria isolated from biofloc according to the public databases on
895 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

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

Biofloculant-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

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

Lane	Protein marker / Biofloculant-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

906 **Lane M** represented Precision PlusProtein™ All blue Prestained Protein Standard (Biorad)

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