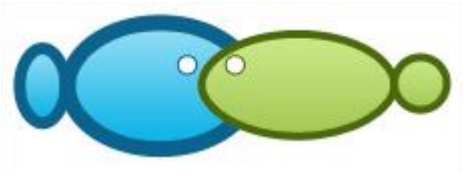

Isolation of bioflocculant-producing bacteria from *Penaeus vannamei* ponds for the production of extracellular polymeric substances

[1,2]Nor A. Kasan, [4]Mohd F. Amin Che Teh, [1]Nurarina A. Ghazali, [1]Nurul F. Che Hashim, [3]Zaharah Ibrahim, [4]Nakisah Mat Amin

[1] Institute of Tropical Aquaculture (AKUATROP), Universiti Malaysia Terengganu, Kuala Terengganu, Terengganu, Malaysia; [2] School of Fisheries and Aquaculture Sciences, Universiti Malaysia Terengganu, Kuala Terengganu, Terengganu, Malaysia; [3] Department of Biosciences and Health Sciences, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, Johor Bahru, Johor, Malaysia; [4] School of Fundamental Sciences, Universiti Malaysia Terengganu, Kuala Terengganu, Terengganu, Malaysia. Corresponding author: N. A. Kasan, norazman@umt.edu.my

Abstract. Bioflocculants are essential polymers with their flocculating activity depending on the characteristics of the secreted flocculants by bioflocculant-producing bacteria. However, the characteristics of bioflocculant produced by microorganisms were not investigated profoundly. In order to better understand these characteristics, determination of extracellular polymeric substances (EPS) from bioflocculant-producing bacteria were characterized in terms of protein concentrations. A total of 51 bioflocculant-producing bacteria isolates were screened from Pacific whiteleg shrimp, *Penaeus vannamei* culture ponds in Setiu, Terengganu, Malaysia. Screening of bioflocculant-producing bacteria were conducted through morphological approaches followed by protein extraction using Lowry assay method. The identified bioflocculant-producing bacteria includes *Corynebacterium* sp., *Klebsiella* sp., *Lactobacillus* sp., *Staphylococcus* sp., *Bacillus* sp., *Streptococcus* sp., *Vibrio* spp., *Neisseria* sp., *Serratia* sp. and *Yersinia* sp., with the highest protein concentration of 829 mg mL^{-1} were attained by *Staphylococcus* sp. The various amounts of EPS concentration produced by different species of bacteria were dependent on their specific population growth and growth rate. Therefore, the establishment of bioflocculant-producing bacteria isolated from biofloc which showed high tendency for EPS production were performed successfully.
Key Words: bioflocculant, flocculating activity, shrimp culture, protein concentration.

Introduction. Coastal area provides benefits to community that lives in coastal regions creating chance for ecosystem-based management, ecological restoration and enhancement of water quality (Crab et al 2012; Morrissey & Sumich 2012). For instances, coastal aquaculture farming for the marine organisms such as fish, crustaceans, mollusks and seaweed becomes public concerned toward the issues of sustainable aquaculture. While producing food for human consumption, sustainable aquaculture is able to maintain the natural resources with minimal ecological impacts (Martins et al 2010; Haslun et al 2012). However, the tendency of the occurrence of pathogens is typically increasing as the density of cultured organism increases (Ebeling et al 2005; Aji 2012). Thus, the discharge of wastewater from aquaculture operations is critically important in disposal and waste management.

Fundamental pre-treatment procedure for wastewater discharge in aquaculture system that contains fine particles usually involves flocculation process. Flocculation is the process where large agglomerates of particles in suspension or fine agglomerates are formed by coagulation through high weight molecular polymer materials (Tripathy & De 2006; Taşdemir & Taşdemir 2012). A larger size agglomerates formed by the coagulation are loosely bound, however the charges that carried by colloidal particles on their surface will stabilize the suspension (Lin & Harichund 2011). The addition of chemical flocculants

such as coal, bauxite, phosphate, potash sand, gravel, cement, soda ash, copper, silver, gold, beryllium, lead and zinc on the surface of colloidal particles can alter or dissolve material which facilitate the separation of solids by gravity (Tripathy & De 2006). Typically, the use of inorganic and organic commercial flocculating agents such as polyaluminium chloride and polyacrylamide are widely used in aquaculture industry for high performance and time-saving advantages (Sharrer et al 2009). However, recent concern on their application creates hazard effect toward the environment particularly for human consumption.

Alternatively, the use of microbial flocculants or known as bioflocculants will replace commercial inorganic and organic flocculants in aquaculture wastewater treatment with the occurrence of microorganisms such as bacteria, protozoa, algae and fungi. A naturally produced bioflocculant can be formed from a process called as bioflocculation. Bioflocculants are metabolites produced by microorganisms during their growth and it is substantially composed of high polymers such as cellulose, nucleic acid, glycoprotein, protein and extracellular polysaccharide (Zhang et al 2012). However, the mechanisms toward the formation of biofloc still remain elusive since the studies upon microbial communities are complex processes. Therefore, this study was prompted to improve better understanding of the bioflocculant excreted from bioflocculant-producing bacteria through protein extraction methods. The factors that contribute to the interaction mechanism of microorganisms in bioflocs formation were determined through extraction and characterization of the extracted bioflocculant. Consequently, better understanding on the characteristics of extracellular polymeric substances (EPS) in biofloc formation provides advantages in undergoing wastewater treatment process particularly in aquaculture industry.

Material and Method. The Pacific whiteleg shrimp, *Penaeus vannamei* farm is located in Setiu District, Terengganu, Malaysia with a total of 616 culture ponds which were operated by the Integrated Shrimp Aquaculture Park (iSHARP), Blue Archipelago Sdn. Bhd. (Figure 1).

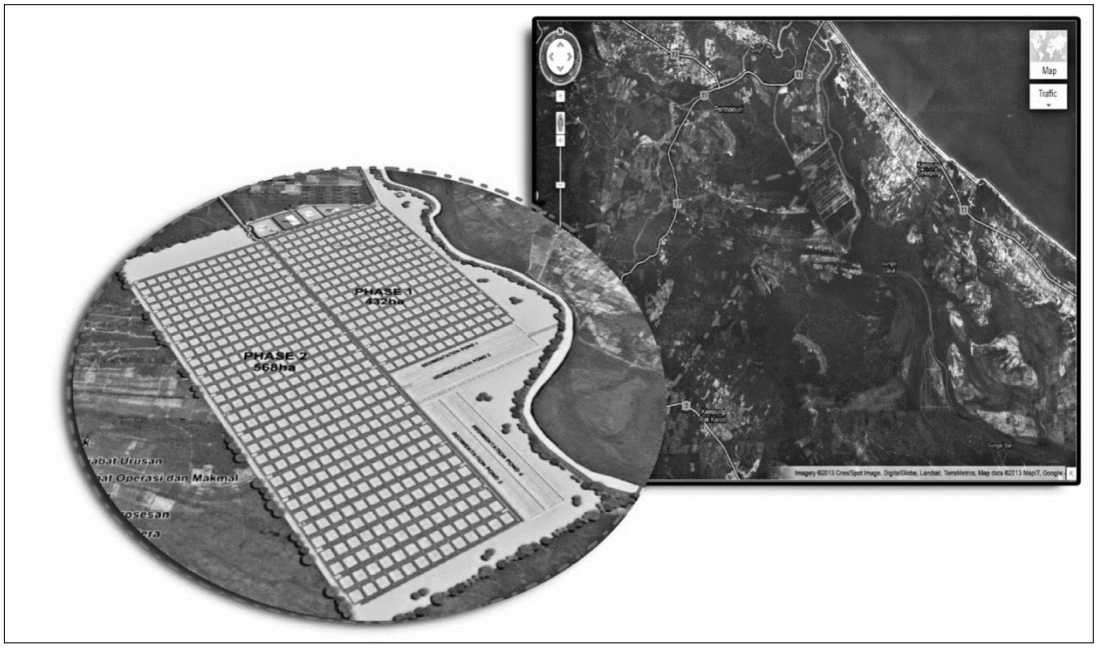

Figure 1. Location of the Pacific Whiteleg shrimp, *P. vannamei* culture ponds operated by Integrated Shrimp Aquaculture Park (iSHARP), Blue Archipelago Sdn. Bhd. at Setiu District, Terengganu, Malaysia.

The culture ponds were designed in modular basis which covers an area of 1000 hectares. This super-intensive shrimp culture ponds was developed into two phases which were Phase 1 and Phase 2. Phase 1 covers an area of 432 hectares, consisting of 216 ponds which estimated to produce a production capacity of 3,100 metric ton per

year. On the other hand, Phase 2 comprising a total of 568 hectares consisting of 400 ponds which are still under construction with an estimated production capacity of 5,600 metric ton per year. Each module of 24 ponds comprises of 20 production and 4 treatment ponds within 0.5 hectares in size.

All shrimp ponds and water distribution channels are fully lined with 0.65 mm High Density Polyethylene (HDPE) liners. This super-intensive shrimp farm has its own delivery canal for seawater consumption with seawater resources for the whole system is pumped from the South China Sea. Two modules share a discharge canal with seawater from the main supply channel, and the water is filtered through a series of 1000 µm and 250 µm filters prior entering the first treatment pond. It is estimated that this fully super-intensive shrimp culture ponds could produce the maximum production capacity of 8700 metric tonnes of shrimp per year.

Biofloc sampling and samples analysis. The sampling of bioflocs was conducted from three selected shrimp's pond at iSHARP, Setiu, Terengganu following standard operating procedures by Blue Archipelago Sdn. Bhd. Sampling events were chosen for a complete cycle of shrimp culture up to harvesting periods from the end of October 2013 until middle of January 2014. Three samples of biofloc were collected during the day of culture of 0 day, 30 days and 70 days, representing an early, middle and maturation of biofloc, respectively.

Three replicates consisting of two liters of water samples containing biofloc were collected from a shrimp pond using one liter Imhoff cone. This cone is standard equipment that was used to settle down the biofloc samples (Hargreaves 2013). The water samples containing biofloc were then settled down for overnight until it separated into two layers. The lower layer that forms sediment-like biofloc was further concentrated by centrifugation for three minute at 6000 rpm (Vijayalakshmi & Raichur 2002). The concentrated biofloc sample was further used for identification of pure culture and extraction of EPS in term of protein concentration.

Isolation and identification of bioflocculant-producing bacteria. All bacteria colonies in the concentrated biofloc samples were isolated using marine agar as the cultivation medium (Zaki et al 2011). The concentrated biofloc samples were streak on marine agar plates using inoculation loop for bacterial growth. All the plates were then incubated at 30°C for 24 hours. After 24 hours, different colonies which were grown on the plates were sub-cultured in zig-zag lines until the pure culture with different single colony of bacteria were obtained. Then, the pure cultures of bacteria were isolated by a series of re-plating. The different colony morphologies were isolated and maintained on marine agar slants as stocks. The stocks were kept in refrigerator at 4°C.

Identification of bioflocculant-producing bacteria was carried out to differentiate the major group of different colonies of bacteria, either Gram-positive or Gram-negative bacteria. All Gram-positive and Gram-negative bacteria were undergone a series of biochemical tests in order to identify the bacteria that potentially producing bioflocculant. In particular, the Gram-positive bacteria were tested for spore staining, acid fast test, catalase test, starch hydrolysis test, citrate test, strict anaerobes test, mannitol fermentation test, hemolysis test and bile esculin test while the Gram-negative bacteria were tested for oxidase test, indole test, MR-VP test, motility test, SIM test and urease test.

The screening of bioflocculant-producing bacteria was carried out using method as described by Abd-El-Haleem et al (2008). The pure isolates of bacteria were transferred into Yeast extracts Peptone Glycerol (YPG) medium which contains 10 gram peptone, 10 gram yeast extract, 20 gram glucose and 15 gram agar powder in a liter of deionized water at pH 6.5 (Chen & Zhao 2003). Bioflocculant-producing bacteria were screened based on their mucoid and ropy characteristics. The isolated strains were grown in 50 mL of YPG medium on a rotary shaker with 120 rpm per minutes at 25°C for three days. The bacteria suspension was prepared in mass culture for further use in protein extraction (Somasegaran et al 1992).

Extraction of extracellular polymeric substances from bioflocculant-producing bacteria suspension. After three days of incubation periods, broth cultures of bacteria were centrifuged under 20000 rpm for 20 minutes at 40°C. Free cells in supernatant containing EPS were separated by filtration through 0.22 µm filter membrane. The extractant was collected for protein analysis using Lowry method (Abu-Elreesh et al 2011). The amount of proteins in the samples were estimated by reading the absorbance at 750 nm of the end product of the Folin reaction against a standard curve of Bovine Serum Albumin (BSA) as standard protein solution.

Results and Discussion. A total of 147 isolates were successfully isolated from biofloc samples at different day of culture (DOC) of *P. vannamei* representing an early, middle and maturation of biofloc. There were 55, 37 and 55 isolates of bacteria identified for each DOC 0, DOC 30 and DOC 70, respectively (Figure 2). The isolated bacteria were sub-cultured and Gram staining procedures was further conducted in order to identify the bioflocculant-producing bacteria.

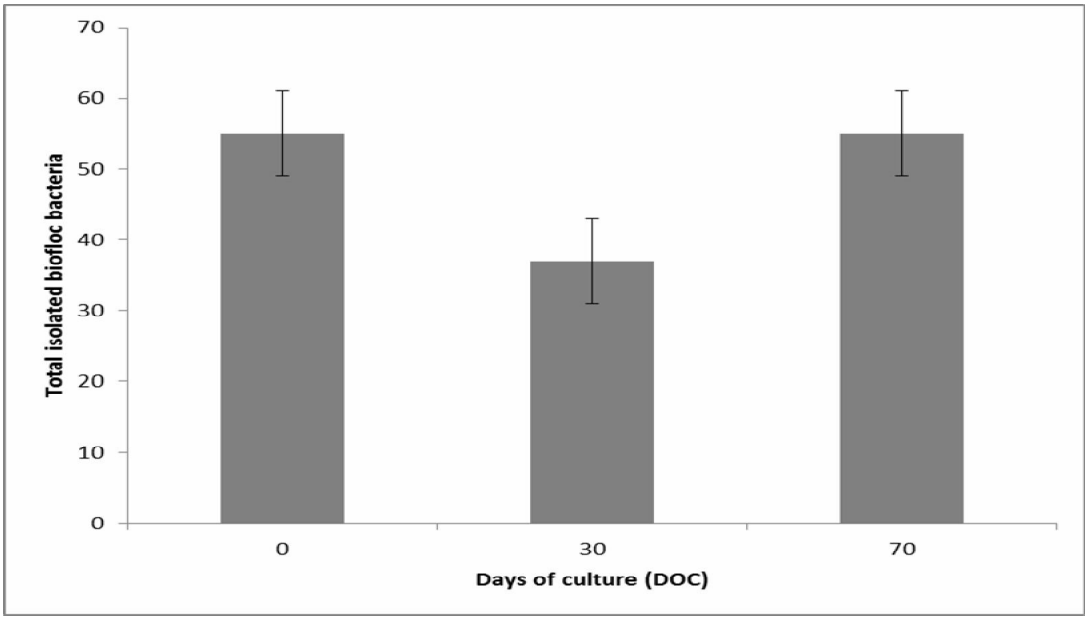

Figure 2. Total isolates of bacteria from biofloc samples for the day of culture (DOC) of DOC 0, DOC 30 and DOC 70 in *P. vannamei* culture pond.

The presence of bioflocculant-producing bacteria isolated from biofloc was recorded at different DOC in *P. vannamei* culture pond. After screening process using YPG medium, 51 bacteria isolates were identified to have high tendency for bioflocculant production (Table 1). Among the total number of bacteria isolated from biofloc samples, there were 9, 19 and 23 isolates of bioflocculant-producing bacteria were identified for each DOC 0, DOC 30 and DOC 70, respectively (Figure 3).

Table 1

The presence of Gram positive and Gram negative bioflocculant-producing bacteria for each DOC 0, DOC 30 and DOC 70

Day of culture (DOC)	*Type of bacteria*	*Number of bioflocculant-producing bacteria*		*Total number of bacteria isolates*
		Gram positive	*Gram negative*	
DOC 0	Bacilli	5	1	9
	Coccus	2	1	
DOC 30	Bacilli	4	8	19
	Coccus	7	0	
DOC 70	Bacilli	6	1	23
	Coccus	11	5	
Total		35	16	51

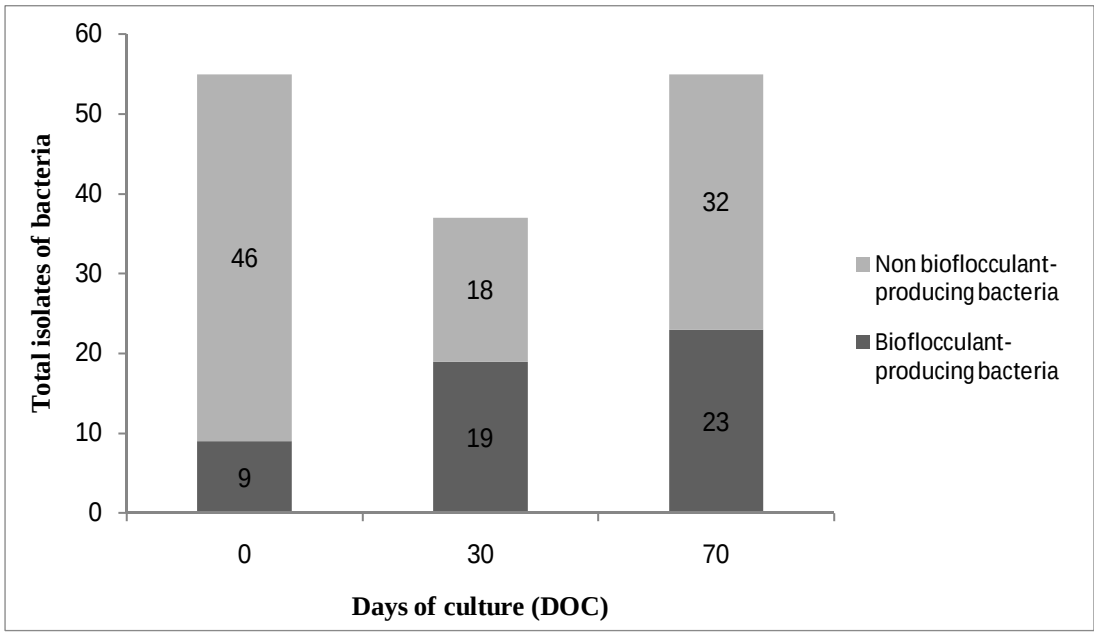

Figure 3. The total number of bioflocculant-producing bacteria and non-bioflocculant-producing bacteria isolates for each DOC 0, DOC 30 and DOC 70 from *P. vannamei* pond culture.

During DOC 0, the bacteria species distribution of bioflocculant-producing bacteria were dominated by *Staphylococcus* sp., *Corynebacterium xerosis* and *Corynebacterium kutscheri* (Figure 4). Only one species of *Neisseria* sp., *Klebsiella pneumoniae* and *Bacillus azotoformans* was successfully isolated during DOC 0.

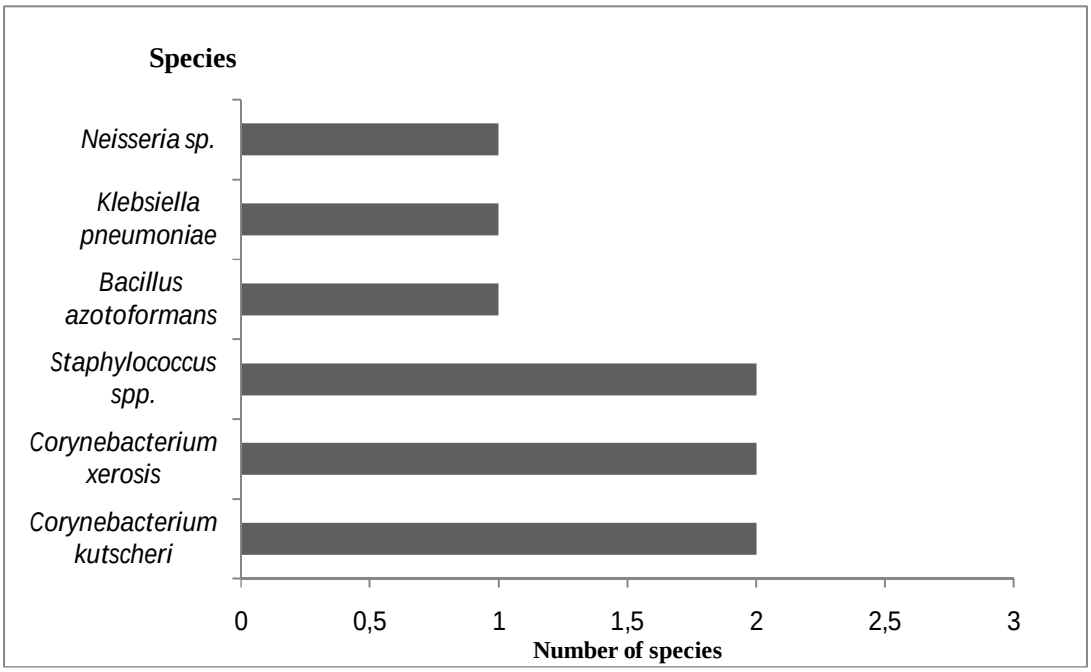

Figure 4. The bacteria species distribution of bioflocculant-producing bacteria from biofloc sample at DOC 0.

As compared to DOC 0, DOC 30 showed higher number of bioflocculant-producing bacteria isolates which were dominated by *Yersinia* sp. (6 species), *Staphylococcus* sp. (6 species), and followed by *Corynebacterium sp*. (4 species) and *Serratia sp.* (2 species). The lowest number of bioflocculant-producing bacteria isolate was *Streptococcus* sp. with only one species (Figure 5). As expected, the highest number of bioflocculant-producing

bacteria isolates were successfully identified during DOC 70 with total number of 23 isolates (Table 1). The most dominant species of bioflocculant-producing bacteria were *Streptococcus* sp. (5 species) and *Neisseria sp./Veillonella sp.* (5 species) followed by *Enterococcus* sp. (3 species) and *Staphylococcus sp./Micrococcus* sp. (2 species). The lowest number of bioflocculant-producing bacteria isolates were *Staphylococcus aureus* and *Vibrio* sp. with only one species, respectively (Figure 6).

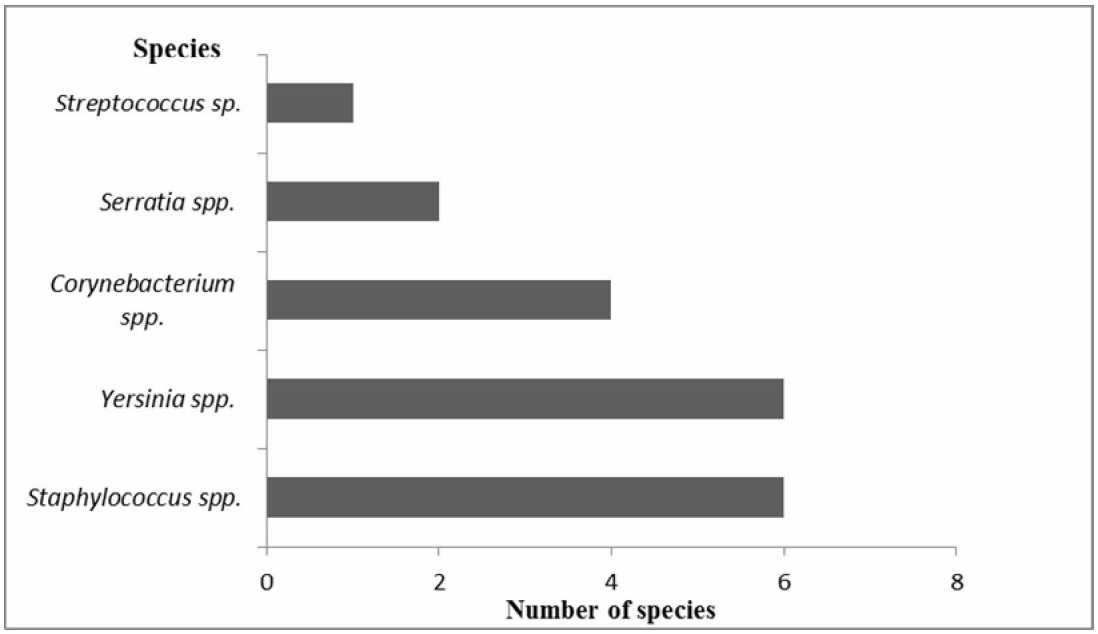

Figure 5. The bacteria species distribution of bioflocculant-producing bacteria from biofloc sample at DOC 30.

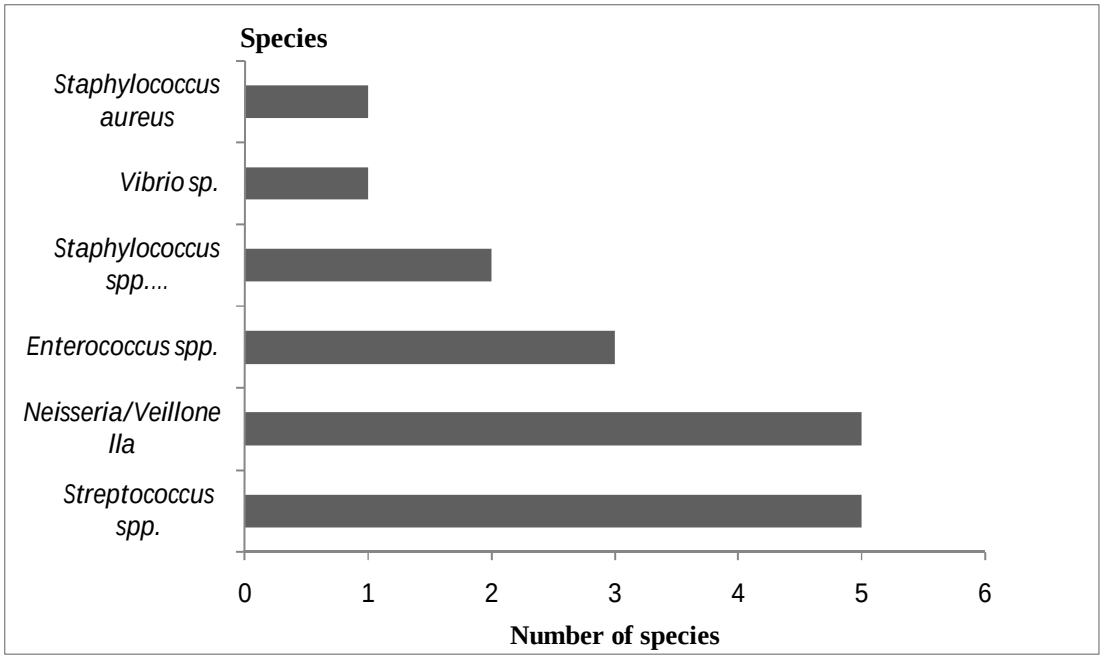

Figure 6. The bacteria species distribution of bioflocculant-producing bacteria of biofloc sample at DOC 70.

EPS production in terms of protein concentration from bioflocculant-producing bacteria was expressed as bioflocculant. These bioflocculants which were secreted by bacteria during their growth can induce solid particles in a liquid suspension to flocculate. Over 6 bioflocculant-producing bacteria isolated during DOC 0, *Staphylococcus* sp. showed the

highest total protein concentration with 785 mg mL^{-1}, while the lowest protein concentration was produced by *Corynebacterium kutscheri* with 635 mg mL^{-1}, respectively (Figure 7). Among all other bioflocculant-producing bacteria species, the protein concentration ranged between 600 and 800 mg mL^{-1}.

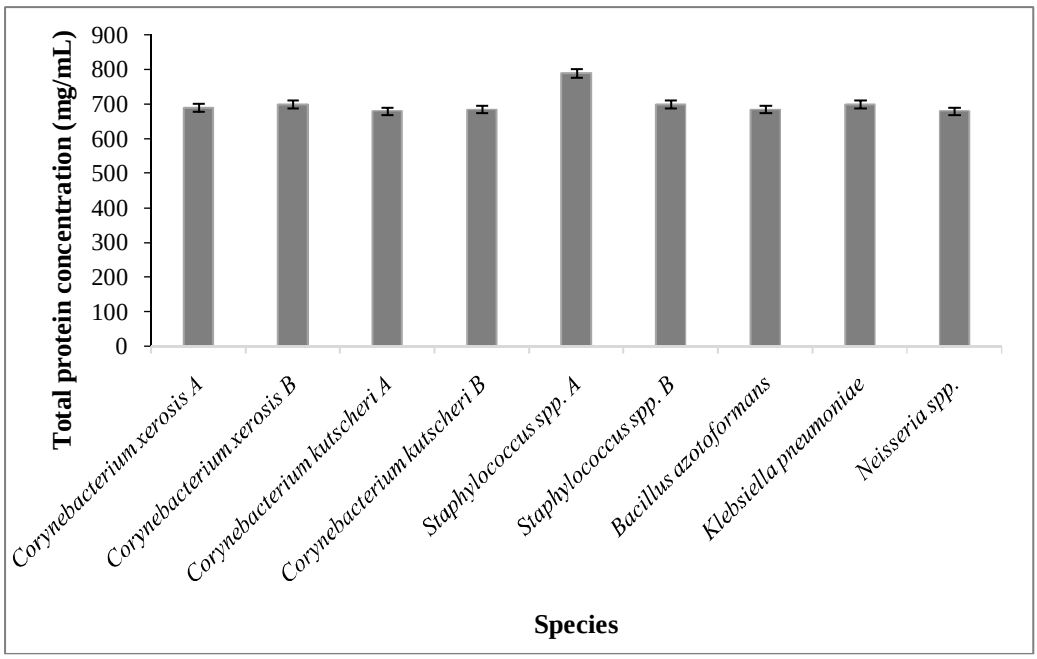

Figure 7. Total protein concentration produced by 9 bioflocculant-producing bacteria isolated during DOC 0.

During DOC 30, protein concentration of 5 bioflocculant-producing bacteria isolates showed that *Yersinia* sp. showed the highest protein concentration with 820 mg mL^{-1}, while the lowest protein concentration was produced by *Corynebacterium* sp. with 625 mg mL^{-1}, respectively (Figure 8). Over 6 bioflocculant-producing bacteria isolated from DOC 70, *Staphylococcus* sp. showed the highest protein concentration with 829 mg mL^{-1}, while the lowest protein concentration produced by *Enterococcus* sp. with 676 mg mL^{-1}, respectively (Figure 9). It was expected that the consumption of this microbial protein by the shrimp culture may contribute to their growth effectiveness.

A total of 147 of both non-bioflocculant producing and bioflocculant producing bacteria were successfully isolated from biofloc samples collected from *P. vannamei* farm operated by the Integrated Shrimp Aquaculture Park (iSHARP), Blue Archipelago Sdn. Bhd. Among the total number of bacteria isolated, there were 9, 19 and 23 of bioflocculant producing bacteria were identified during DOC 0, DOC 30 and DOC 70, respectively (Figure 3). Similar study was found that the bacterial communities were identified in every five consecutive days of sampling periods (Xia et al 2012). In addition, they also found that the Gram positive bacteria were more dominant as compared to Gram negative bacteria which was also indicated by our study (Table 1). Gram positive bacteria were the dominated species regardless of particulate control method (Haslun et al 2012). The Gram positive cocci bacteria were higher than Gram positive bacilli due to their ability to adapt in most marine environment and can be usually found in irregularly shaped clusters (Morrissey & Sumich 2012).

Up to 147 isolated bacteria from biofloc, there were 51 isolates with slimy and mucoid appearance were screened using yeast extract peptone glycerol (YPG) medium which characterized as bioflocculant producing bacteria (Abd-El-Haleem et al 2008). Morphologically, the colonies appearances for the pure culture strain were mucoid with cream-coloured, round, convex edge, smooth, viscous and about 1 mm diameter in size (Cosa et al 2011). There were 9 and 19 of bioflocculant-producing bacteria were screened at DOC 0 and DOC 30 which have high tendency for bioflocculant production. Throughout the sampling periods, DOC 70 showed the highest number of bioflocculant-

producing bacteria with 23 bacteria (Table 1). These were supported by Deng et al (2005) which stated that microbial bioflocculants are polymers produced by microorganisms during their growth overtime.

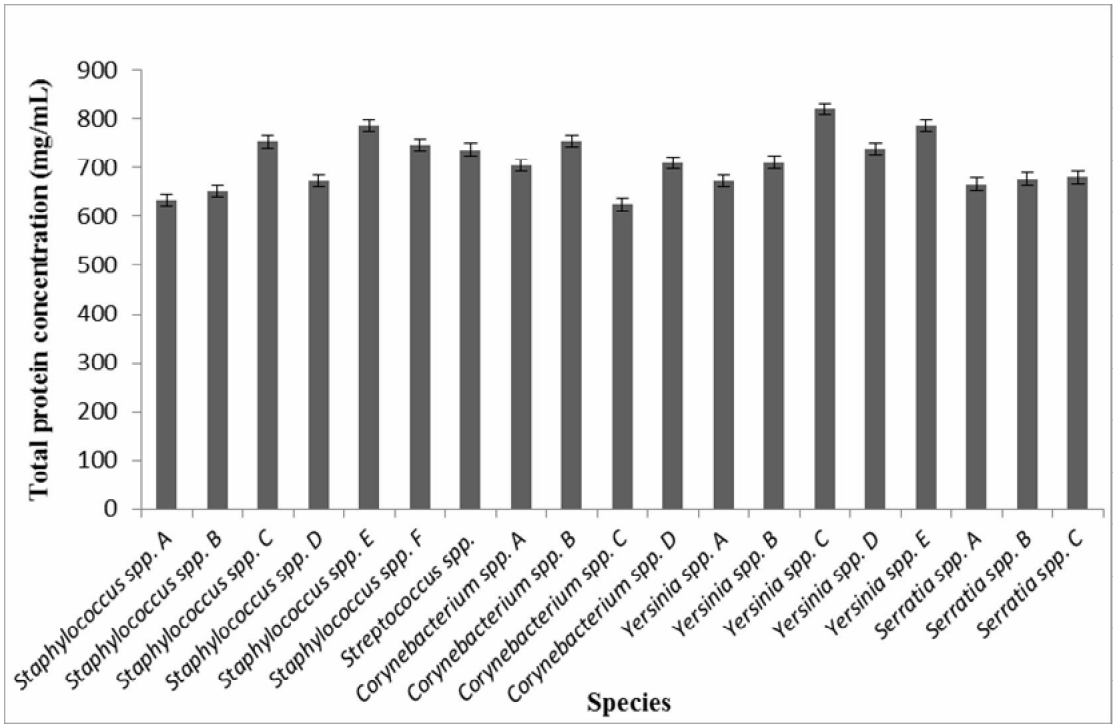

Figure 8. Total protein concentration produced by 19 bioflocculant-producing bacteria isolated during DOC 30.

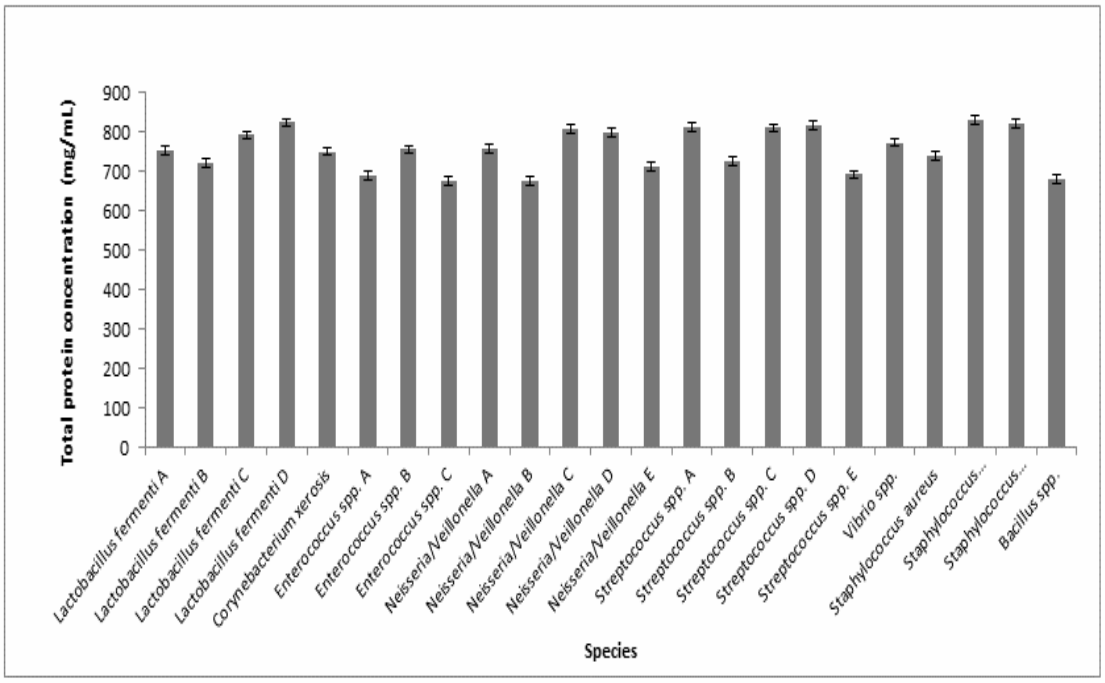

Figure 9. Total protein concentration produced by 23 bioflocculant-producing bacteria isolated during DOC 70.

In this study, EPS supernatant was used as its appear in significant amounts or even predominates in EPS preparations from pure cultures of bacteria (Wingender et al 1999). The purification of supernatant was properly taken into account to be accurate and potentially suitable produced product for human's consumption. Impurities of host cell

protein, deoxyribonucleic acid (DNA), adventitious and endogenous viruses, endotoxin, aggregates and other species must be removed (Liu et al 2010). Furthermore, most microorganisms produced bioflocculant during their growth periods. The conversion of simple substances in the environment into complex polymer via the respond of the bacteria can be used as bioflocculant. Therefore, the nutrients in the culture medium can be exploited by the bacteria to integrate high molecular weight polymers internally within their cell by specific enzymes, where these polymers can be released as capsule (Shih et al 2001; Abd-El-Haleem et al 2008). In this study, marine agar was used to culture the bacteria isolated from biofloc samples. The composition of marine agar mimics the seawater which will enhance the growth of bacteria abundantly.

Protein concentration of bioflocculant producing bacteria was determined by Lowry's method using bovine serum albumin (BSA) as a standard (Ugbenyen & Okoh 2013). Lowry assay method was most widely used to estimate the amount of protein in biological samples. BSA standard may be linearized over a wide concentration range by using a double reciprocal plot (Campbell 2006). Our finding showed that the protein concentration produced by bioflocculant-producing bacteria was increased commensurate with the DOC (Figure 7, Figure 8 and Figure 9). The amount of protein concentration produced proportionally with population the growth, thus the protein concentration are highly dependent on the bacterial growth rate (Kim et al 2000).

Marine environment has been a source of good and beneficial bacteria particularly as a consequence of the exclusive environmental conditions of marine habitats such as high pressure, low temperature and low nutrition (Zhang et al 2012). Thus, it is predictable that the marine environments, especially from marine biofloc samples could be developed as a good stockpile for organisms producing more environmental friendly bioflocculant (He et al 2002).

Conclusions. The establishment of bacteria isolation procedures from biofloc samples which showed high tendency for bioflocculant production were conducted successfully. There were different in total protein concentration throughout the DOC, which showed increasing amount of protein production once the biofloc was fully matured. It is suggested that the total protein concentration need to be correlated with the flocculating activity of each bacteria isolates in order to determine the most effective bioflocculant producer.

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Authors:
Nor Azman Kasan, Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia; School of Fisheries and Aquaculture Sciences, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia, e-mail: norazman@umt.edu.my
Mohd Fakhrul Amin Che Teh, School of Fundamental Sciences, Universiti Malaysia Terengganu, Kuala Terengganu, Terengganu, Malaysia, e-mail: fakhrulamin0905@gmail.com
Nurarina Ayuni Ghazali, Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia, e-mail: nurarinayuni@yahoo.com
Nurul Fakriah Che Hashim, Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia, e-mail: nuhahashim@yahoo.com
Zaharah Ibrahim, Department of Biosciences and Health Sciences, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia 81300, Skudai, Johor, Malaysia, e-mail: zaharah@fbb.utm.my
Nakisah Mat Amin, School of Fundamental Sciences, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia, e-mail: nakisah@umt.edu.my

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