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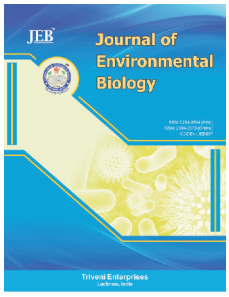

The potential of bioflocculant-producing bacteria as inoculum for biofloc based systems

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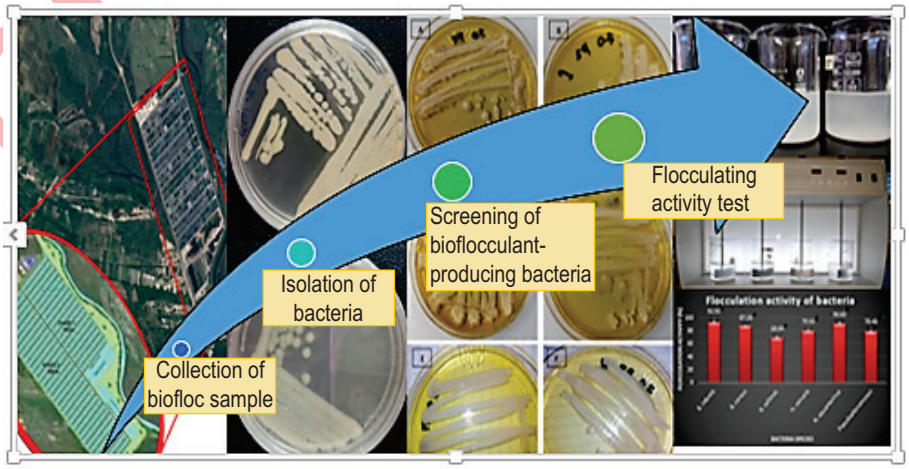
Abstract

Aim: Biological flocculants has been widely used around the world to replace the usage of synthetic flocculants for wastewater treatment. A new green technology using biological flocculant known as biofloc system was developed which offers zero water exchanged, low feed conversion ratio (FCR) and high nutritional profile. This study was conducted to determine the most potential bioflocculant-producing bacteria isolated from biofloc sample in Pacific Whiteleg shrimp, *P. vannamei* culture pond.

Methodology: Biofloc sample was collected using Imhoff cone and bacteria was isolated. The most abundant bacteria isolated throughout *P. vannamei* culture period was selected for screening of bioflocculant-producing bacteria using YPG medium and flocculation activity using floc-jar test.

Results: Screening of bioflocculant-producing bacteria showed that *B. infantis, N. aquimarinus, B. cereus, H. venusta, Pseudoalteromonas* sp., and *B. safensis were* characterized as highly mucoid and ropy colony morpologies. The highest flocculation activity with 93% was showed by *B. infantis* followed by *N. aquimarinus* (91%), *B. cereus* (87%), *H. venusta* (79%), *Pseudoalteromonas* (78%), while the lowest flocculation activity was showed by *B. safensis* with 69%.

Interpretation: As bacteria grew, extracellular polymeric substances (EPS) produced were involved in flocculation process. Each bacteria produced different EPS composition which differed their ability in flocculation process. Therefore, bacteria with high flocculation activity are potentially used as inoculum to increase flocculation process in biofloc production.



Introduction

Recently, aquaculture have expand rapidly to overcome dependable on fisheries resources and provide more than 50% of total fisheries production (FAO, 2010) with the expansion of aquaculture sector, issues on water quality management has also rised since aquaculture effluent can be high in dissolved nitrogen (Avnimelech, 2012). To overcome the problem, new and green technology of culturing technique using microorganisms are widely used such as Zero Exchanged Autotrophic Haterotrophic System (ZEAH) (Burford *et al.,* 2003; Burford *et al.,* 2004), microbial floc system (Avnimelech, 2007; Ballester *et al.,* 2010), single-cell protein production system (Avnimelech, 1989), suspended-growth system (Hargreaves, 2006) and active sludge system (Rakocy *et al*., 2004). Alternatively, biofloc technology (BFT) was first developed by Yoram Avnimelech in 1989 with the concept of bioreactor for single cell protein production (Avnimelech, 1989; Avnimelech, 2012).

The major concept of BFT is the addition of carbon that increase heterotrophic bacterial growth that causes aggregations of flocs, called bioflocs. Bioflocs are made up of algae, fungi, bacteria, diatom, protozoa, faeces, and uneaten feed that are held together in a loose matrix of mucus secreted by bacteria during their growth and bound by filamentous microorganisms or electrostatic attraction (Hargreaves, 2013). During the flocculation process, there are several steps that occur due to the electrostatic attraction of suspended particles, filamentous microorganisms, and loose matrix of mucus or extracellular polymeric substance (EPS) secreted by bacteria during their growth (Hargreaves, 2013). Commonly, fine particles of uneaten feed, faecal, and sludge are freely suspended in the water column and only settle down when flocculation occurrs (Crab *et al.,* 2012). The surface of most suspended particles in water have a negative charge and particles of the same charge will repel each other (Chui *et al.,* 2014). This action cause suspended particles to remain suspended in the water column. The use of coagulant assists in the flocculation process by electrostatic attraction. Coagulants have been used to stabilize the charge and allow clumping of suspended particles to form floc (Prakash *et al.,* 2014). Filamentous microorganisms are also involved in formation of biofloc where cilia from these microorganisms entrap suspended solids, as well as other substances. Attachments of filamentous microorganisms and other microorganisms on uneaten feed, fecal and sludge also promote the flocculation process (Crab, 2010; Dauda *et al.,* 2018).

Despite electrostatic attractions and filamentous microorganisms, a loose matrix of mucus or EPS are one of the most important intermediaries in biofloc formation. EPS have been shown to be more efficient in the flocculation process due to adsorption of surface charge being reduced by bioflocculants and thus, attracting particles to floc sufficiently as attractive forces become more effective (Li *et al.,* 2009). Bioflocculants are metabolites that are produced by bacteria during their growth. These metabolites are composed of polymerase substances such as extracellular polysaccharides, glycoproteins, functional proteins, nucleic acids and cellulose. These metabolites function as mucus or substrates in the aggregation of bioflocs (Gao *et al.,* 2006).

Naturally, bioflocs begins to develop after 30 days, with tformation of a green color due to the dominance of microalgae (Lara *et al.,* 2016). Afterwards, there is a transition process once the algae are hindered from sunlight. When the algae begins to crash, bacteria grow forming a brown bioflocs that are dominated by bacteria (Hargreaves, 2013). Brown bioflocs are more effective as compared to green bioflocs because bacteria are easier to control (Choo *et al.,* 2015). In addition, bacterial flocs are involved in nitrification of nitrogen, form microbial protein, and may act as probiotics to the cultured organisms (Simon, 2005).

The use of BFT offers many benefits to water quality as well as to the aquaculture organisms, such as growth or disease resistance (Xu and Pan, 2013; Dauda *et al.,* 2018). Therefore, bioflocs should be formed as early as possible although lag period can be up to 30 days. This can be reduced by introducing of bacterial innoculants as starter culture to promote biofloc formation. This study was conducted to understand the potential of bioflocculant-producing bacteria as inoculum to boost biofloc formation.

Materials and Methods

Collection of biofloc samples : Bacteria were isolated from biofloc in a white-leg *Litopenaeus vannamei* farm located in Setiu District, Terengganu, Malaysia which were operated by the Integrated Shrimp Aquaculture Park (iSHARP), Blue Archipelago Sdn. Bhd (Fig. 1). Bioflocs were collected from three selected shrimp's pond at iSHARP, Setiu, Terengganu following standard operating procedures by Blue Archipelago Sdn. Bhd. Three replicates consisted of two litres of water containing bioflocs, which were placed in an Imhoff cone overnight to settle the biofloc samples (Hargreaves, 2013). Sediment-like biofloc was further concentrated by centrifugation for 3 min at 6000 rpm (Vijayalakshmi and Raichur, 2002). The concentrated biofloc sample was used to identify pure cultures of bacteria and any associated flocculation activity.

Isolation of bacteria from bioflocs : Marine agar was used as cultivation medium for isolation of all bacterial colonies in the biofloc pellet (Zaki *et al.,* 2011). Biofloc pellets were then streaked onto marine agar and incubated for 24 hr at 30°C. After 24 hr, bacterial colonies on the incubation plate were sub-cultured in a zig-zag line. Then series of re-platings were conducted on isolated bacterial cultures until pure bacterial cultures were

Fig. 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

obtained. Isolated pure culture of bacteria were maintained on marine agar slants and kept in refrigerator at 4°C as a stock culture.

Screening of bioflocculant-producing bacteria : Screening of bioflocculant-producing bacteria were carried out following the method of Abd-El-Haleem *et al.* (2008). The pure isolates of bacteria were transferred into a Yeast Peptone Glucose (YPG) medium, which contained 10.0 g of glucose, 2.0 g of peptone, 0.5 g of urea, 0.5 g of yeast extract, 0.1 g of NaCl, 0.2 g of $MgSO_4.7H_2O$, 0.2 g of KH_2PO_4, 5.0 g of K_2HPO_4 and 15.0 g of bacteriological agar in one litre of deionized water at pH 7.0 using 1.0 M sodium hydroxide and 1.0 M hydrochloric acid and incubated at 35°C for 48 hr (Chen and Zhao, 2003; Zaki *et al.,* 2011). Isolated strains with highly mucoid and ropy colony morphologies in YPG medium were selected (Abd-El-Haleem *et al.,* 2008).

Enrichment medium: Screened bacteria were grown in an enrichment medium, which was prepared as seeding medium by mixing 10.0 g of glucose, 0.5 g of urea, 0.2 g of $MgSO_4.7H_2O$, 5.0 g of K_2HPO_4, 2.0 g of peptone, 0.2 g of KH_2PO_4 and 0.5 g of yeast extract in one litre of filtered seawater at pH 7.0 using 1.0 M sodium hydroxide and 1.0 M hydrochloric acid. This was incubated at 35°C for 48 hr using an incubator shaker at 120 rpm and 35 °C for 3 days (Zhang *et al*., 2012 modified by Cosa *et al*., 2011). The resultant culture broth was centrifuged at 8,000 rpm for 15 min and the cell-free supernatant was used for assessing flocculation activity.

Flocculating activity assay: Flocculating activity was measured using a modified kaolin clay suspension method (Kurane *et al.,* 1994). A concentration of a 5.0g l^{-1} kaolin clay suspension was prepared where 5.0g of kaolin clay powder was suspended in one litre of deionized water. The kaolin clay suspension was adjusted to pH 7.0 using 1.0 M sodium hydroxide and 1.0 M hydrochloric acid.

For the flocculating activity, 240 ml of kaolin clay suspension and 10 ml of bioflocculant solution (cell-free supernatant) were added into a 250 ml beaker. The flocculating activity assay was started with rapid mixing at 230 rpm for two min, followed by slow mixing for 1 min at a speed of 80 rpm using a JLT4 Jar/Leaching Tester Velp Scientifica. The stirring speed was reduced to 20 rpm and stirring was continued for 30 min. The stirring apparatus was stopped and the samples in the beakers were allowed to settle for 30 min.

The optical density (OD) of the clarifying solution at 3 cm below the surface was measured with a Shimadzu UV Spectrophotometer UV-1800 at 550 nm. A control flocculating activity assay was prepared using similar method, where the bioflocculant solution was replaced with deionized water. The

flocculating activity was calculated according to the following equation:

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

Where, A: absorbance reading of sample at 550 nm; B: absorbance reading of control at 550 nm

Statistical analysis: Flocculation activity of each bacterial sample was analysed using a one way-ANOVA, after prior confirmation of data homogeneity and normality. Significant differences between samples were determined at 0.05 level of probability. Statistical analyses were conducted using SPSS (2009) computer package.

Results and Discussion

Screening of bioflocculant-producing bacteria : From the bacterial sample collection obtained from biofloc in *L. vannamei* farm, six species showed positive flocculating activity. Six species of bioflocculant-producing bacteria showed a highly mucoid, cream colored, smooth, viscous, round, convex edge and ropy colony (Fig. 2) which indicated the production of bioflocculant substances during their growth when cultivated on YPG medium (Abd-El-Haleem *et al.,* 2008; Kasan *et al.,* 2015). All six species were identified as *Bacillus infantis*, *B. cereus*, *B. safensis*, *Halomonas venusta*, *Nitratireductor aquamarinus* and *Pseudoalteromonas*.

Flocculation activity : Flocculation activity ranged between 68% and 93% (Fig. 3), with the highest activity coming from *B. infantis*.

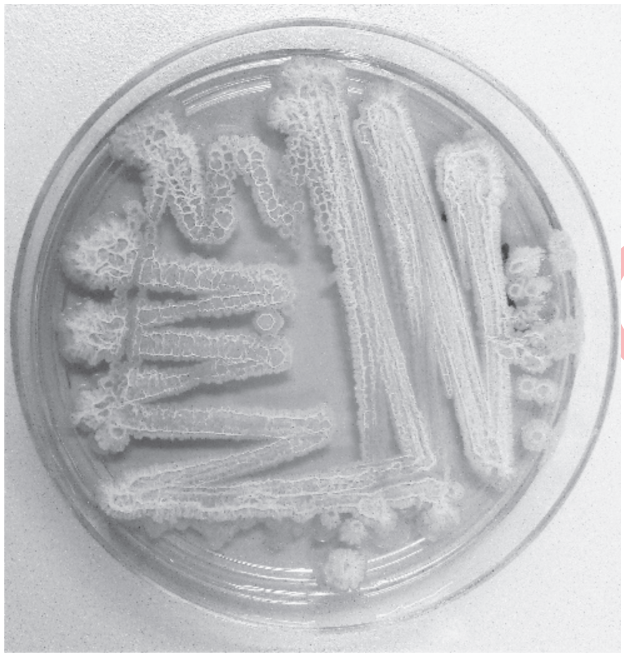


Fig. 2 : Bioflocculant-producing bacteria with highly mucoid and ropy colony

Flocculation process usually occurs in two ways, either by adhesion of ions or by adsorption of substances onto the mucus or EPS produced by bacteria (Brostow *et al.,* 2009). The highest flocculation acivity in *B. infantis* indicates a higher production or best type of EPS in order for the kaolin clay to be floced into suspended material in the floc jar test method. Indeed, most bacteria produce different types of EPS during their growth (Mikutta *et al.,* 2011). In this study, flocculation might have occured during the process by minerals being adsorped onto the clay by EPS substances secreted by the bacteria. This is because EPS was centrifuged from the cultured bacteria and then used in the floc jar test. Even though *B. infantis* produced the highest flocculation activity, other *Bacillus* species, that included *B. cereus* and *B. safensis* produced the flocculation activity of 87% and 69%, respectively, among the tested bacteria.

Even among *Bacillus* species, they are producing different EPS such as *B. subtilis* producing polysaccharidddes, *B. consortium* producing glycoprotein, *B. safensis* producing functional proteins and some other *Bacillus* species producing various EPS (Wu and Ye, 2007; Wang *et al.,* 1995). Although *B. infantis* showed the highest flocculation activity, *N. aquamarinus* showed the second highest flocculation activity, that was almost as high as *B. infantis* at 91% ($p<0.05$). This is possibly due to the potential of *N. aquamarinus* secreting EPS that are suitable to flocculate koalin clay. *N. aquamarinus* has been described as a nitrifying or nitrate-reducing bacteria (Labbé *et al.,* 2003). There is a lack of study on the application of *N. aquamarinus* as a potential probiotic, especially in aquaculture field, especially compared to *Bacillus* sp., that are well known to act as probiotic agents for cultured organisms (Touraki *et al.,* 2012; Ng *et al.,* 2014; Liu *et al.,* 2015). In addition to flocculation activity, *Bacillus* sp. can potentially offer another benefits such as nitrogen cycling in the cultured pond (Sorokulova, 2013; Yang *et al.,* 2011). However, *N. aqumarinus* are reportedly more effective at recycling nitrogen waste (Jang *et al.,* 2011) and therefore the implications of using *N. aquamarinus* should be explored.

The second and third lowest flocculation activity was produced by *H. venusta* (80%) and *Pseudoalteromonas* sp. (79%). *H. venusta* produce polysaccharides as their main EPS (Kumar *et al.,* 2004) and is a polymer that has a low charged density (Sam *et al.,* 2011), thus their ability to floc might only be enhanced in suspensions with high ionic strength. Li *et al.* (2008) showed that *Pseudoalteromonas* sp. were able to floc a variety of suspended solids such as kaolin clay, activated carbon, soil, magnesium oxide and aluminium oxide. Their ability to floc these materials may be enhanced in environments of high salinity and low water temperatures (Li *et al.,* 2008).

Screening of bioflocculant-producing bacteria and flocculation activity using isolated bacteria from biofloc samples were successfully conducted. There were six species of bioflocculant-producing bacteria that showed more than 60% of

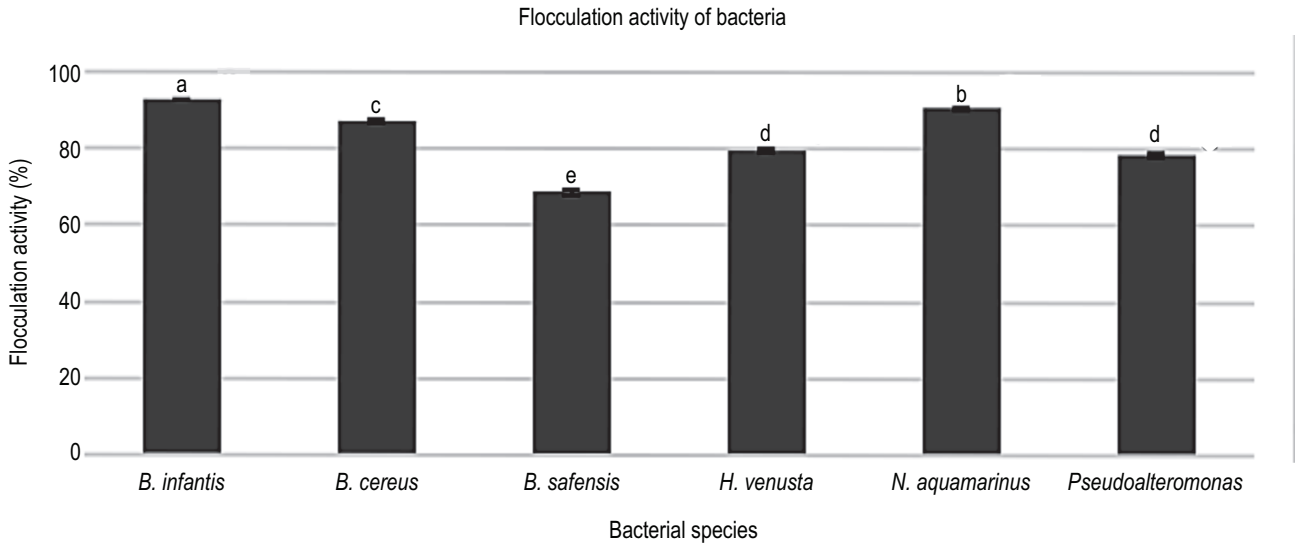


Fig. 3 : Flocculating activity of bioflocculant-producing bacteria consists of *B. infantis*, *B. cereus*, *B. safensis*, *H. venusta*, *N. aquamarinus* and *Pseudoalteromonas* sp. using floc jar test method

flocculation activity and were species specific that could be related to the type of EPS produced. The highest flocculation activity was found in *B. infantis,* which could be used to potentially reduce the start-up period for biofloc formation and potentially acting as a probiotic. It is suggested that futher studies characterize the EPS produced by bioflocculant-producing bacteria to better understand the efficiency of biofloc formation.

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