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Research Article
Isolation of Potential Bacteria as Inoculum for Biofloc Formation in Pacific Whiteleg Shrimp, *Litopenaeus vannamei* Culture Ponds

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Abstract

Background and Objective: A new green technology to reduce environmental damages while optimizing production of Pacific Whiteleg shrimp, *Litopenaeus vannamei* was developed known as "Biofloc technology". Microbial communities in biofloc aggregates are responsible in eliminating water exchange and producing microbial proteins that can be used as supplemented feed for *L. vannamei*. This study aimed to isolate and identify potential bioflocculant-producing bacteria to be used as inoculum for rapid formation of biofloc. **Materials and Methods:** For the purpose of this study, bacterial communities during 0, 30 and 70 days of culture (DOC) of *L. vannamei* grow-out ponds were isolated and identified through phenotypic and 16S rDNA sequences analysis. Phylogenetic relationships between isolated bacteria were then evaluated through phylogenetic tree analysis. One-way analysis of variance (ANOVA) was used to compare the differences of microbial communities at each DOC. **Results:** Out of 125 bacterial isolates, nine species of bacteria from biofloc were identified successfully. Those bacteria species were identified as *Halomonas venusta*, *H. aquamarina*, *Vibrio parahaemolyticus, Bacillus infantis*, *B. cereus*, *B. safensis*, *Providencia vermicola*, *Nitratireductor aquimarinus* and *Pseudoalteromonas* sp., respectively. Through phylogenetic analysis, these isolates belong to Proteobacteria and Firmicutes families under the genera of *Halomonas* sp., *Vibrio* sp., *Bacillus* sp., *Providencia* sp., *Nitratireductor* sp. and *Pseudoalteromonas* sp. **Conclusion:** In this study, bioflocculant-producing bacteria were successfully identified which are perfect candidates in forming biofloc to reduce water pollution towards a sustainable aquaculture industry. Presence of *Halomonas* sp. and *Bacillus* sp. in all stages of biofloc formation reinforces the need for new development regarding the ability of these species to be used as inoculum in forming biofloc rapidly.

Key words: Green technology, microbial communities, biofloc, *L. vannamei*, bioflocculant-producing bacteria, proteobacteria, firmicutes

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Growing human population that increases demand for food supply has led to intensive development of aquaculture industry worldwide, particularly in Asia. However, development of aquaculture industry has brought negative effects to the environment and natural sources through pollution of ground and surface waters by effluent discharge[1,2].

A new technology to reduce environmental damages and optimizing production on this industry has been developed known as "Biofloc technology" (BFT). The BFT is an efficient aquaculture system due to the ability of biofloc to continuously recycled and reused nutrients in the culture pond[3]. This is achieved by maintaining high ratio of carbon to nitrogen (C:N) in the water through addition of external carbon sources such as molasses or starch to stimulate heterotrophic bacterial growth that converts ammonia into microbial biomass[4]. The BFT are able to eliminate water exchange in aquaculture systems by maintaining optimum water quality as well as producing microbial protein that act as supplemented feed for aquatic organisms[5].

"Biofloc" is composed of aggregates of microorganisms including bacteria, fungi, microalgae, protozoans and rotifers and other types of particulate organic matter such as faeces and unused feed[6]. Bacteria as main component of biofloc were reported as bioflocculant-producing microorganisms that produced biopolymer substances that can flocculate suspended solids, cells and colloidal solids[7]. These biopolymers known as Extracellular Polymeric Substance (EPS) produced by microorganisms during their growth play an important role in flocculation process[8,9]. However, there is a lack of basic knowledge regarding microbial composition in biofloc[5,10]. As bacteria play significant roles in biofloc formation, a clear understanding on microbiological aspects mainly in microbial communities in biofloc is important for the effective design and successful operation of biofloc technology. Therefore, this study aimed to isolate and identify bacteria from biofloc through 16S rDNA sequences analysis which later can be used as potential inoculum for rapid biofloc formation.

MATERIALS AND METHODS

Biofloc sample collection: Biofloc sampling was conducted at the Integrated Shrimp Aquaculture Park (iSHARP), Blue Archipelago at Setiu, Terengganu (Fig. 1). The biofloc samples were collected from selected *L. vannamei* ponds during 0, 30 and 70 days of culture (DOC) as at this time, biofloc was observed to be formed. Two litres of biofloc samples was collected in pre-acid washed plastic bottles (1 L) and was taken back to laboratory for further analysis. The samples were then transferred into Imhoff cones to enable the biofloc to settle at the bottom of the cone for 24 h.

Preparation of biofloc samples and isolation of bacteria: The settled biofloc samples from Imhoff cone were transferred into a centrifuge tube for centrifugation at 6000 rpm for 3 min. The biofloc pellets were collected and was streaked onto marine agar as the cultivation medium. The cultures were then incubated for 24 h. The colonies which appeared on plates were purified by repeated streaking and Gram staining was performed to ensure the purity of the colony.

Identification of isolated bacteria based on phenotypic characterization: To identify the isolated bacteria, Gram staining was carried out according to standard microbiological protocol. The colonies were distinguished through visual observations of colony morphology. Individual colonies were characterized through commonly used biochemical test.

Genetic characterization and diversity analysis: Single colony of pure culture bacteria was grown in marine nutrient broth for 16-18 h. Cultures were then centrifuge at 9000 rpm for 15 min and the supernatant was decanted. The DNA extraction for bacteria using the Qiagen DNeasy Blood and Tissue Kit was conducted as per manufacturer's protocol.

Universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') described by Yu[12] were used to amplify the bacterial 16S rDNA gene. Polymerase Chain Reaction (PCR) was performed using GoTaq® PCR Core Systems (Promega, USA). All PCR reagents were used following recommended reaction volumes and final concentrations provided by manufacturer. The PCR amplification was performed in Veriti™ Thermal Cycler (Applied Biosystems, USA). The reaction conditions included an initial denaturation at 94°C for 1 min, followed by 35 cycles in series of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 90 sec, with a final extension at 72°C for 7 min[13]. The amplification product were separated by electrophoresis on a 1% agarose gel and purified using QIAQuick PCR purification kit (Qiagen, USA) according to manufacturer's protocol. The purified PCR products were sent to First Base Sdn. Bhd. for sequencing process.

Fig. 1: Location of the *L. vannamei* culture ponds under the management of iSHARP, Blue Archipelago Sdn. Bhd. at Setiu District, Terengganu, Malaysia[11]

Homologies of query sequences were searched at the National Centre for Biotechnology Information (NCBI) GenBank nucleotide database using Basic Local Alignment Search Tool (BLAST) through website[14]. The sequences were further subjected to Multiple Sequence Alignment (MSA) using ClustalX[15]. A phylogenetic tree using 16S rDNA sequences of isolated bacteria was constructed using Mega6 software (Version 6.06)[16].

Statistical analysis: All data were tested for homogeneity (O'Brien, Brown-Forsythe, Levene and Bartlett tests) and normality (Shapiro-Wilk test). When variances of these data were equal, parametric t-tests were performed. Differences of microbial communities with DOC were compared using one-way analysis of variance (ANOVA)[17]. One-way ANOVA was used because there was only one independent variable in this study which was number of bacterial species in each DOC. Statistically significant differences were accepted with α of 0.05. All statistical analysis was performed using JMP-IN (Version 4.0.3, S.A.S Institute Inc. Cary, USA).

RESULTS

A total of 10 bacterial isolates marked as SP1-SP10 were isolated from *L. vannamei* culture ponds. From these ten species, nine species were isolated from biofloc samples of *L. vannamei* pond. The colony characteristics of all isolated bacteria were analyzed. All colonies appeared as white to creamy in colour. The isolated bacteria observed to form mostly circular and smooth colonies on agar surfaces. All bacteria from phylum firmicutes were determined as Gram positive and spore forming while bacteria from phylum Proteobacteria were determined as Gram negative and non-spore forming.

To support this morphological and microscopic study, taxonomical identification was further investigated through molecular study and phylogenetic analysis. The 16S rDNA gene of 125 bacteria isolates of approximately 1500 bp were successfully amplified by PCR (Fig. 2). The 16S rDNA sequence data were subjected to a BLASTn search. The homology search results of bacterial strains resembling with existing DNA sequence database were identified (Table 1).

Bacteria SP1-SP7 were found to belong to group Proteobacteria showing 99-100% with *Halomonas* sp., *Vibrio* sp., *Nitratireductor* sp., *Alteromonas* sp., *Providencia* sp. and *Pseudoalteromonas* sp. genera, respectively. The analysis using BLASTn tool showed SP8, SP9 and SP10 belonged to Firmicutes branch showing sequence similarity of 98-100% with genera *Bacillus* sp. Phylogenetic tree also supported the blast analysis report and showed separate line of descent in the Proteobacteria and Firmicutes (Fig. 3).

In this study, biofloc was observed started to be formed in 30 DOC up to 70 DOC. The 0 DOC of shrimp culture showed the lowest number of bacteria species isolated with seven species while 30 DOC showed the highest species isolated with nine species (Fig. 4). The results showed that there was significant difference ($p<0.05$) of bacterial species in biofloc samples between DOC of *L. vannamei* culture periods.

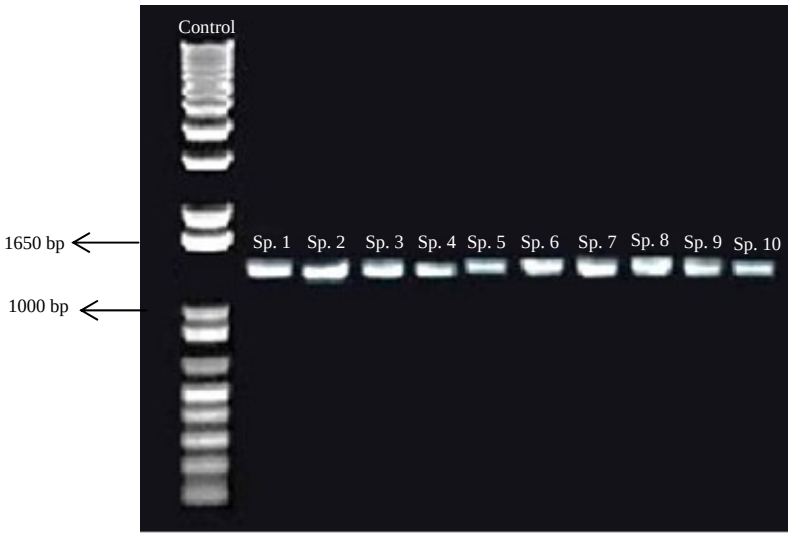

Fig. 2: Electrophoresis gel stained by ethidium bromide of 16S rDNA gene of PCR product using 1492R and 27F primers

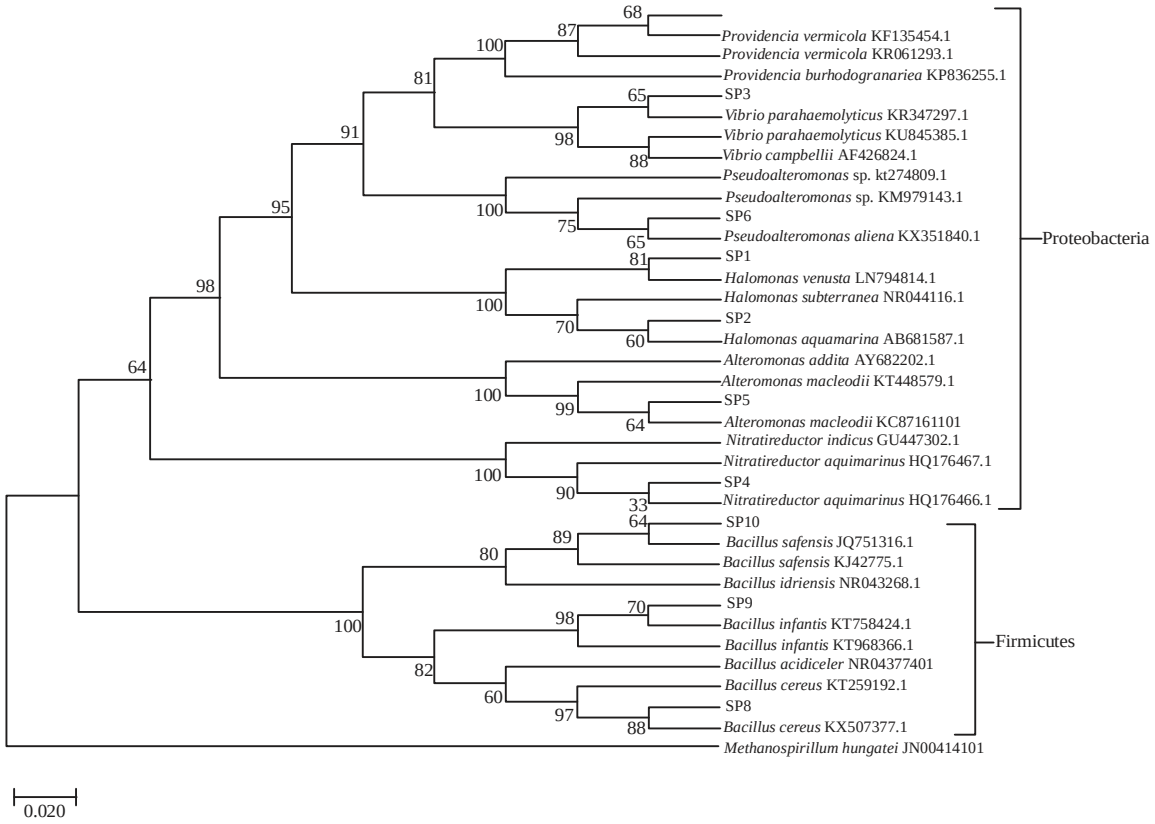

Fig. 3: Neighbour-joining phylogenetic tree of 10 bacterial species based on 16S rDNA sequencing depicting homology to closely related bacterial species

Note that bootstrap support values over 60% are shown. The scale bar indicates evolutionary distance

DISCUSSION

The findings of this study successfully provided substantial evidence that microbial composition in biofloc had enhanced water quality condition of *L. vannamei* culture. Microbial composition in 0 DOC showed the least bacteria species due to low concentration of organic matter and carbon sources where stocking of *L. vannamei* was not yet

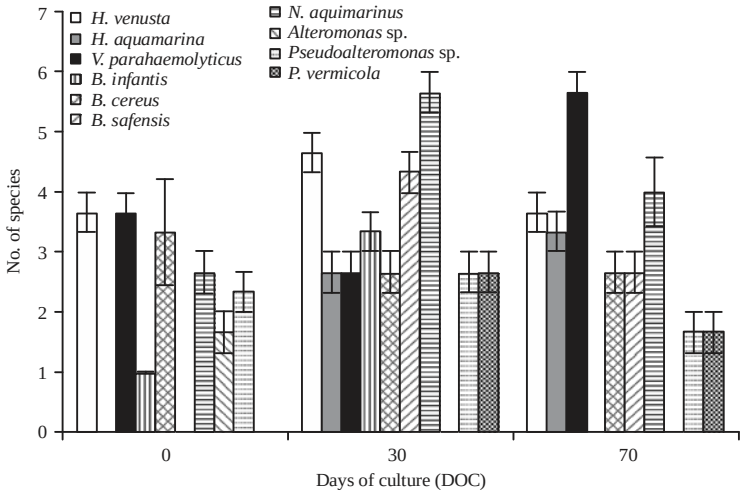

Fig. 4: Number of bacterial species isolated from biofloc of *L. vannamei* culture grow-out pond
Error bars represent standard errors

Table 1: List of bacteria isolates identified by 16S rDNA analysis deposited in NCBI, GenBank, USA with accession numbers

Strain Id	Isolates (GenBank accession number)	Nearest phylogenetic neighbour based on partial sequencing of 16S rDNA	Phylum	Identity (%)
SP1	KU955349	*Halomonas venusta*	Proteobacteria	99
SP2	KU955350	*Halomonas aquamarina*		
SP3	KU955351	*Vibrio parahaemolyticus*	Proteobacteria	100
SP4	KU955356	*Nitratireductor aquaimarinus*	Proteobacteria	99
SP5	KU955357	*Alteromonas* sp.	Proteobacteria	99
SP6	KU955359	*Pseudoalteromonas* sp.	Proteobacteria	100
SP7	KU955358	*Providencia vermicola*	Proteobacteria	100
SP8	KU955354	*Bacillus cereus*	Firmicutes	100
SP9	KU955353	*Bacillus infantis*	Firmicutes	100
SP10	KU955355	*Bacillus safensis*	Firmicutes	98

been introduced into the culture pond. As there was no biofloc was formed in 0 DOC, thus only bacteria that naturally present in the *L. vannamei* culture pond were isolated. Bacteria genera such as *Bacillus* sp., *Halomonas* sp., *Vibrio* sp., *Nitratireductor* sp., *Alteromonas* sp. and *Pseudoalteromonas* sp. that were successfully isolated and identified at 0 DOC were bacteria species that can be found in marine water[18-22]. Bacteria act as an efficient "Biochemical systems" degrader and metabolize organic residues[23]. In other words, they recycled nutrients efficiently in a form of organic and inorganic matter (unconsumed and non-digested feed, metabolic residues and carbon sources applied as fertilizers) into new microbial cells. Microorganisms that populate biofloc systems typically inhibit the natural aquatic systems and highly influenced by factors such as light intensity and concentration of organic matter[24]. This was proven where the presences of bacteria species during DOC 0 were also found in biofloc during 30 DOC.

The highest number isolated bacteria species from biofloc was at 30 DOC. The introduction of commercial shrimp feed that contains new bacteria species might have contributed to additional of four new bacteria species. For example, bacteria that were absent in BWT but exist in 30 DOC such as *H. aquamarina*, *Providencia* sp. and *B. cereus*. The presence of bacteria in commercial shrimp feed was partially depends on the ingredients used in feed formulation[25]. In this study, shrimp feed used were made from raw materials such as wheat flour, soybean, squid, fish meal and yeast. These raw materials were often contaminated with bacteria such as *Bacillus* sp. and *Vibrio* sp.[25]. On the other hand, bacteria species such as *Halomonas* sp. and *Providencia* sp. were reported as marine bacteria and can be found in marine environment[26].

Presence of bacteria genera of *Pseudoalteromonas* sp., *Bacillus* sp. and *Halomonas* sp. are beneficial for growth of shrimp culture as well as maintaining water quality of shrimp pond. *Pseudoalteromonas* sp. and *Halomonas* sp. have been used as probiotics in shrimps culture[27,28] while addition of *Bacillus* sp. has contributed in maintaining water quality by reducing total suspended solids in shrimp ponds[28]. In contrast, isolation of *Vibrio* sp. in shrimp pond and biofloc samples did not causing any outbreak of disease. Although *Vibrio* sp. has

been reported to cause diseases to shrimps, it was concluded that all *Vibrio* species was differ in virulence and must be present at certain threshold for disease to occur[29]. Acceptable level for total number of *V. parahaemolyticus* was below than 100 CFU mL^{-1} as reported by Shaari *et al.*[30]. In addition, presence of *Halomonas* sp. and *Bacillus* sp. has been found to have an inhibitory effect *in vitro* against *V. parahaemolyticus* and *V. harveyi* with antagonistic substances excretion[31]. This might improve immune system of *L. vannamei* as well as shrimp's resistance towards pathogen *V. alginolyticus*[32,33].

Interestingly, *Bacillus* sp. and *Halomonas* sp. were isolated in all DOC either from pond water or biofloc sample. Even though both genuses were dominantly exist in marine environment[34], both species have been reported to be a bioflocculant-producing microorganisms[34-36]. *Bacillus* sp. was reported to have a high flocculating activity of more than 90% and capable to produce polysaccharide bioflocculant[36] whereas *Halomonas* sp. was found to have flocculating activity of 95% and producing mainly polysaccharide bioflocculant[37,38].

The findings of this research was successfully provide substantial evidence as composition of heterotrophic bacteria which has the ability to produce bioflocculant are perfect candidate in forming biofloc which later can reduce water pollution. Up to date, there is a limited study on determination of microbial communities within biofloc. The uniqueness and biodegradability of microbial flocculants have prompted research into screening, characterization and structural identification of polymeric flocculants excreted by the microbes. The paucity of information on those aspects and further research is needed in order to develop better and environmentally safer alternatives as compared to the synthetic flocculants. Thus, those identified bacteria are highly potential to be used as inoculum in forming biofloc for sustainable aquaculture industry.

CONCLUSION

Nine species of bioflocculant-producing bacteria known as *Halomonas venusta*, *H. aquamarina*, *Vibrio parahaemolyticus*, *Bacillus infantis*, *B. cereus*, *B. safensis*, *Nitratireductor aquimarinus*, *Providencia vermicola* and *Pseudoalteromonas* sp. from biofloc of shrimp pond were successfully identified through 16S rDNA sequences analysis. Existence of heterotrophic bacteria genera such as *Halomonas* sp. and *Bacillus* sp., in biofloc showed high potential to be used as inoculums for rapid formation of biofloc towards sustainable aquaculture practices.

SIGNIFICANCE STATEMENTS

The findings of this study will benefit to the shrimp farmers around the world in implementation of biofloc technology that will minimize water exchange and enhance the growth of shrimp culture. The addition of isolated bioflocculant-producing bacteria as inoculum plays an important role to boost-up the formation of biofloc. Therefore, this sustainable aquaculture approach will maximize the overall production of shrimp to meet the market demands, while preserving environmental safety.

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