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Isolation of biofloc-associated bacteria from the rearing water of whiteleg shrimp, *Penaeus vannamei* in nursery tanks

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Abstract

We aimed to determine the water quality of the biofloc-based nursery tank system and to isolate and characterize biofloc-associated bacteria from the rearing water of the whiteleg shrimp, *Penaeus vannamei*, during the nursery production phase in response to recommendations to further study biofloc composition. Serially-diluted biofloc water samples from a shrimp nursery tank were spread onto Tryptic Soy Agar (TSA) plates, incubated for approximately 48 h at 28°C and observed for bacterial growth. Bacterial colonies that exhibited varying morphology were re-streaked onto fresh TSA plates to obtain pure colonies. Bacterial isolates were subjected to morphological tests following standard methods. Molecular characterization of the bacterial isolates was done by amplification and sequencing of the 16S rRNA. Water quality parameters including dissolved oxygen, pH, salinity, ammonia-N, nitrite-N and nitrate-N, were monitored twice daily using commercially available kits. The isolated bacterial colonies showed variations in the margin but were all similar in opacity. The colors of the bacterial colonies ranged from light yellow to white. *Staphylococcus*, *Pseudomonas*, and a putative *Bacillus velezensis* from the rearing water of a biofloc-based nursery tank of whiteleg shrimp were identified. During the one-month nursery production phase, levels of the nitrogenous wastes (total ammonia-N, nitrite and nitrate) were within acceptable levels required for shrimp farming.

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Introduction

Biofloc Technology (BFT) is an innovative aquaculture farming technology that involves carbon to regulate the carbon-to-nitrogen ratio in an aquaculture system, reducing harmful nitrogen concentrations, acting as a food source, and eradicating pollutants (Panigrahi *et al.*, 2018). Khanjani and Sharifinia (2020) emphasized in their study the value of Biofloc technology in improving aquaculture production, as well as how it could be used to cultivate important commercial species. In a biofloc technology system, the interaction of the planktonic and bacterial populations has an important role in the protein & energy requirements for the farming organisms of interest (Ayazo-Genes *et al.*, 2019). Biofloc system is composed of microbiomes which are essential to the improvement of the aquatic species present in the system (Crab *et al.*, 2010) and plays a central role in the management of water quality (Llario *et al.*, 2019). Also, Panigrani *et al.* (2019) said that the optimum number of bacteria is essential for the successful maintenance of the biofloc system. The estimation of microbial communities has always been the focus of the management of the biofloc system. Having an in-depth understanding of biofloc microbial communities has great importance in assessing further improvement on the biofloc system to yield a more successful outcome. There have been studies concerning the biofloc microbial composition in shrimp cultures. Jang *et al.* (2015) were able to identify 34 phyla, 69 classes, 138 orders, 217 families and 477 genera of bacteria in the biofloc system of shrimp, *Penaeus vannamei* tanks, and among these bacterial communities, *Chlorofexi* was identified as the most dominant group. In a study by Zhao *et al.* (2012) in kuruma shrimp (*Marsupenaeus japonicus*) biofloc systems, they were able to identify the bacterial species present in the bioflocs including *Proteobacterium*, *Bacillus* species and Actinobacteria. Other minor bacterial species, such as *Roseobacte*r sp. and *Cytophaga* sp. were also found. Cardona *et al.* (2016) identified bacterial phyla such as Proteobacteria, Bacteroidetes, Cyanobacteria, Euryarchaeota, Actinobacteria, Planctomycetes, Verrucomicrobia, Chloroflexi, and Crenarchaeota in a biofloc system of western blue shrimp, *Litopenaeus stylirostris*. Lastly, in a study by Manan *et al.* (2016), on Pacific white shrimp, *Penaeus vannamei*) culture, they were able to identify Gram-negative bacteria including *Vibrio fluvialis*, *Vibrio alginolyticus*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, and *Vibrio alginolyticus*, along with Gram-positive bacteria *Bacillus* sp., in the system.

In spite of the availability of studies on the characterization of bacterial communities in a biofloc system for shrimp culture, Panigrahi *et al.* (2019) suggested that both molecular and biochemical characterization of biofloc microorganisms, including heterotrophic-autotrophic organisms, are among the researchable issues and challenges for biofloc technology. Furthermore, Aravind *et al.* (2019) recommended further studies on the bacterial isolates obtained from biofloc systems as this allows a deeper understanding of the biofloc application. Dayal Syama (2019) added that characterization of biofloc microorganisms with a strong ability to regulate water quality might be an important subject for further study. These microorganisms could be used as a biofloc technology inoculum. The findings on this topic highlight the importance of further research into biofloc composition.

In order to bridge the gap of knowledge in this area, this study aimed to isolate and characterize biofloc-associated bacteria from the rearing water of the whiteleg shrimp, Penaeus vannamei, during the nursery production phase and to determine the water quality of the biofloc-based nursery tank system.

Materials and methods

Study site

The study was conducted in a shrimp farm (MARMI Agricultural Corp) located in Brgy. Balaring, Silay City, Negros Occidental. Brgy. Balaring is situated at approximately 10.8345, 122.9572, on the island of Negros. Sample collection for the study was done at the shrimp nursery tank of the farm (Fig.1) with an area of 450 m^2 and a depth of 1.2 m. The tank was round in shape, well aerated, provided with 2 1-horsepower paddlewheel aerators and was lined with high-density polyethylene (HDPE) to avoid contact with soil and contamination of the system (Caipang *et al.*, 2022).

Water sampling and collection

Two (2) liters of biofloc water samples were obtained from four (4) areas around the nursery tank during the daytime at 2 days post-stocking of whiteleg shrimp postlarvae. The collected samples were placed in clean and sterilized glass bottles and were then transported to the University of St. La Salle laboratory for further analyses. The water samples were stored in the refrigerator at 4°C to prevent degradation (Poulsen *et al.,* 2021).

Preparation of biofloc water samples and bacterial isolation

Biofloc water samples were subjected to a 5-fold serial dilution. The diluted samples were then spread into the prepared Tryptic Soy Agar (TSA) plates. The culture plates were then incubated in an inverted position for approximately 48 h at 28°C and observed for bacterial growth. Bacterial colonies that exhibited varying morphology were re-streaked onto fresh TSA plates to obtain pure colonies.

Characterization of bacterial isolates

The bacterial isolates were subjected to morphological tests following standard methods. Identification of the strains was primarily based on the phenotypic characters described in Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 2000). To facilitate molecular identification of the isolates, bacterial genomic DNA was extracted using a commercial kit from an overnight culture of the isolates in 5 ml Trypticase Soy Broth (TSB) following the procedures described by the manufacturer (Purelink Genomic DNA Mini, Thermo Fisher Scientific, California, USA). 16S rRNA was amplified using the eubacterial universal primers (Forward: GAGAGTTTGATCCTGGCTCAG; Reverse: CTACGGCTACCTTGTTACGA) of (Bianciotto *et al.*, 2003) in a 25 □L PCR reaction consisting of 2 □L (10-15 ng) of DNA as the template, 2 □L of each primer (5 pmol), 2.5 □L of 10 PCR buffer, 1.5 □L of 2 mM dNTP, 1□□L of 50 mM $MgCl_2$ and desired volume using distilled water. Polymerase chain reaction amplification was carried out following the PCR conditions described by Caipang *et al.* (2015). The PCR products were cleaned and sent for sequencing (Macrogen, Korea).

Sequenced data were aligned and analyzed to find the closest homolog of the bacterial isolates using the publicly available data of NCBI GenBank (blast.ncbi.nlm.nih.gov).

Monitoring of water quality

Water quality was monitored twice daily using commercially available kits. The parameters that were monitored included: dissolved oxygen, pH, salinity, ammonia-N, nitrite-N and nitrate-N.

Results

Characterization and identification of bacteria

Table 1 shows the characteristics that were observed from the colonies of the biofloc-associated bacteria. Most of the colonies are circular in shape and have flat elevations. The bacterial colonies showed variation in the margin but were all similar in opacity. The color of the bacterial colony ranged from light yellow to white. There were three (3) Gram-positive and one (1) Gram-negative bacteria identified in the study. In this study, 16S rRNA sequencing was used to identify the bacteria that are found in a biofloc system of *P. vannamei*.

As presented in Table 2, a total of four (4) bacterial species were identified based on a homology search of their 16S rRNA from Genbank. These included two *Staphylococcus* species, one *Pseudomonas* species, and *Bacillus velezensis*. Isolates A2 and A3 are putative *Staphylococcus* sp. based on their high similarity with various species of *Staphylococcus,* ranging from 88.60 to 89.25 % and 93.51 to 94.14%, respectively. Isolate A5 is a putative *Pseudomonas* species with 78.54 - 78.72% similarity, and isolate A6 with *Bacillus velezensis* at 99.72% similarity.

Table 1. Morphological characteristics of biofloc-associated bacteria from the rearing water of whiteleg shrimp nursery tank.

Bacteria sample	*Characteristics*					*Gram Stain*
	Shape	*Elevation*	*Color*	*Margin*	*Opacity*	
A2 	Circle	Raised	Light Yellow	Entire	Opaque	Positive (+)
A3 	Circle	Raised	White	Entire	Opaque	Positive (+)
A5 	Circle	Flat	White	Undulate	Opaque	Negative (-)
A6 	Irregular	Flat	Yellow	Lobate	Opaque	Positive (+)

Water quality

Table 3 presents the average values of the different water quality parameters in the nursery tank for one month. Total ammonia nitrogen (TAN), nitrite and nitrate were low in the nursery tank during the nursery phase. The dissolved oxygen showed an increase in concentration during the afternoon sampling. The pH values indicated that the water in the nursery tank was slightly basic during the one-month monitoring period.

Discussion

Schlaberg *et al.* (2012) and Lingojwar (2016) noted that isolates with > 99% identity are annotated at the species level; thus, A6 isolate is a putative *Bacillus velezensis* having an identity score of 99.72%. The identification of *Bacillus velezensis* in the biofloc system conforms with the findings of Tuan *et al.* (2021) and Manan *et al.* (2022) in their studies with shrimp. The presence of *B. velezensis* could have contributed to the maintenance of good water quality in the nursery tank. Thurlow *et al.* (2019) have observed reduced nitrate-nitrogen and total nitrogen levels in pond water treated with *Bacillus velezensis*. Moreover, the presence of *B. velezensis* in a biofloc system could also positively contribute to the health of the culture aquatic organisms.

A study by Khalid *et al.* (2021) evaluated the effects of dietary supplementation with the probiotic bacterium *Bacillus velezensis* on the growth and health of Pacific white shrimp.

Table 2. Bacterial isolates and their similarity with known sequences from the Genbank.

Bacterial Isolate	Closest species	Similarity (%)
A6	*Bacillus velezensis* strain FZB42 (NR_075005.2)	99.72%
A2	*Staphylococcus xylosus* strain JCM 2418 (NR_074999.2)	
	Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305 = NCTC (NR_114090.1)	88.60 -89.25%
	Staphylococcus edaphicus strain CCM 8730 (NR_113349.1)	
A3	*Staphylococcus saprophyticus* subsp. saprophyticus ATCC 15305 = NCTC 7292 (NR_074999.2)	
	Staphylococcus edaphicus strain CCM 8730 (NR_156818.1)	93.51 -94.14%
	Staphylococcus xylosus strain JCM 2418 (NR_113350.1)	
A5	*Pseudomonas gessardii* strain CIP 105469 (NR_024928.1)	
	Pseudomonas aylmerensis strain S1E40 (NR_169460.1)	
	Pseudomonas protegens strain CHA0 (NR_114749.1)	78.54 - 78.72%

The results showed that supplementation of *B. velezensis* effectively modulates the intestinal microbiota, promotes growth performance, improves disease resistance, and enhances the immune responses of *P. vannamei.*

For A2 and A3 isolates, their closest similarity is with *Staphylococcus* spp. However, the percentage similarity is less than 97%. As previously stated, isolates with 97% to 99% identity with known sequences in the database can only be annotated at the species level (Schlaberg *et al.*, 2012; Lingojwar, 2016). Thus, isolates A2 and A3 may potentially be novel species and require further characterization of these isolates to identify these isolates at the species level. Kurniawan *et al.*, (2020) investigated that *Staphylococcus* spp. possess biofloc-forming abilities and can modulate the virulence of pathogens.

In addition, Soares *et al.* (2021) demonstrated that *Staphylococcus* is a potent immunostimulant and bioregulator in the development of the biofloc system.

Table 3. Average values of water quality parameters in the nursery tank during the one-month rearing phase.

Water Quality Parameters	Values (Mean)	
Total Ammonia -Nitrogen (TAN)	0.1mg/L	
Nitrite (NO_2^{-1})	0.0mg/L	
Nitrate (NO_3^{-1})	0.0mg/L	
Dissolved Oxygen(DO)	AM	5.6ppm
	PM	6.3ppm
pH	AM	8.1
	PM	8.2

Legend: AM- Morning, PM-Afternoon.

Meanwhile, isolate A5 had 78.72% identity with *Pseudomonas* spp. suggesting that the isolated isolate could be a novel species under this genus. The presence of *Pseudomonas* may have also greatly contributed to ensuring that the water quality parameters in the biofloc system during the nursery phase are within the optimum range. Trung Tran *et al.* (2019) concluded that *Pseudomonas* is capable of heterotrophic nitrification and noted its importance with regard to ammonia oxidation. Their study found that there is a negative correlation between the abundance of Pseudomonas and TAN concentration. Dou *et al.* (2021) confirmed that *Pseudomonas fluorescens* is capable of efficient ammonia and nitrite removal in biofloc of shrimp culture ponds. Manan *et al.* (2016) added that *Pseudomonas* consumes the organic matter that settles in the pond culture, which are source of ammonia, and\converts them as useful protein food for shrimps through chemical processes. They are also capable of converting the nitrates into gaseous nitrogen, maintaining the water quality in a less toxic state. Water quality management is a top priority in shrimp farming, especially in ponds with higher stocking rates. Shrimp production and survival are affected when water quality deteriorates.

Fig. 1. Shrimp nursery tank.

The fitness or suitability of water for shrimp survival and growth is commonly defined as good quality water. The results in water quality imply that the nursery tank is in ideal condition to sustain the culture of white leg shrimp since it is shown that the concentration and values fall within the range that is considered safe for the shrimp culture. Maintaining an optimum quantity of dissolved oxygen in the biofloc tank is critical for shrimp survival and growth. Long-term stress from low oxygen concentrations reduces their susceptibility to disease and limits their growth. In most cases, shrimp stocks died in large numbers due to oxygen depletion (anoxia).

There are many factors that influence the amount of dissolved oxygen in biofloc tanks, including water temperature, respiration, and organic matter levels. According to Esparza-Leal *et al.* (2016), due to greater temperature, the oxygen level in the tank is generally low. Thus, the change in temperature explains the increase in dissolved oxygen during the afternoon. The fertility or potential productivity of pond water is also determined by its pH. Water with a pH of 7.5 to 9.0 is generally considered suitable for shrimp farming. Shrimp growth is inhibited when the pH drops below 5.0 (Rajkumar *et al.*, 2015). Low concentration levels of ammonia in a biofloc system are essential for the rearing of shrimp larvae. Lu and Serajuddin (2020) suggested that Ammonia nitrogen concentration is vital for shrimp aquaculture since this is toxic for the aquatic organisms at certain levels. Ammonium ions, such as nitrite and nitrate, are considered safe except at extremely high concentrations. The level of ammonia components in the nursery tank ranges at a very low concentration which implies that there may be the presence of bacteria that is capable of converting the ammonia from organic matter. The study of Putra *et al.* (2020) revealed that the concentration levels of ammonia, nitrite, and nitrate were significantly lower due to the presence of probiotic bacteria in the biofloc system that can change ammonia to nontoxic materials that are useful for growth.

Conclusion

The study identified *Staphylococcus*, *Pseudomonas*, and a putative *Bacillus velezensis* from the rearing water of a biofloc-based nursery tank of whiteleg shrimp. During the one-month nursery production phase, the levels of the nitrogenous wastes (total ammonia-N, nitrite and nitrates) were within the acceptable levels required for shrimp farming.

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