ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Biotechnology

ISSN 1682-296X
DOI: 10.3923/biotech.2018.135.141

Research Article
18s rDNA Sequence Analysis of Microfungi from Biofloc-based System in Pacific Whiteleg Shrimp, *Litopenaeus vannamei* Culture

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Abstract

Background and Objective: One of the major problem in Pacific Whiteleg shrimp, *Litopenaeus vannamei* farming industry is the accumulation of excess toxicant such as ammonia and nitrite. Biofloc technology served as an alternative solution to solve this problem by recycling the water for sustainable aquaculture management due to water scarcity, bio-security and bio-economy. Biological communities within biofloc are mainly composed of micro-organisms which plays an important role in biodegradation of organic material and removal of toxic contaminants. For the purpose of this study, molecular identification of microfungi isolated from biofloc was conducted. **Materials and Methods:** Isolation of microfungi composition was conducted by culturing the biofloc samples onto Potato Dextrose Agar (PDA) media which further identified molecularly using 18s rDNA sequences analysis. **Results:** The identified microfungi includes genus from *Aspergillus versicolor*, *A. niger*, *A. tamarii*, *A. flavipes*, *A. aculeatus*, *Penicillium citrinum*, *P. griseofulvum*, *Trichoderma virens* and *Pestalotiopsis microspora*. The most dominant microfungi were genus *Aspergillus* sp. followed by the presence of *Penicillium* sp. *Trichoderma* sp. and *Pestalotiopsis* sp. **Conclusion:** Existence of microfungi species such as *Aspergillus* sp. and *Penicillium* sp. in biofloc were perfect candidate in forming biofloc that could reduce water pollution towards a sustainable aquaculture industry.

Key words: Biofloc technology, *L. vannamei*, bio-security, bio-economy, 18s rDNA, microfungi

Citation: Nor Azman Kasan, Nurarina Ayuni Ghazali, Nurul Fakriah Che Hashim, Iswadi Jauhari, Ahmad Jusoh and Mhd Ikhwanuddin, 2018. 18s rDNA sequence analysis of microfungi from biofloc-based system in Pacific Whiteleg shrimp, *Litopenaeus vannamei* culture. Biotechnology, 17: 135-141.

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Increased in human population since past few years has led to intensive world development of aquaculture industry. In Asia, increase captured fisheries and population. However, the growth of aquaculture industry has significant impacts toward the environment and natural resources such as pollution of ground and surface waters by aquaculture wastewater discharge[1,2]. A new technology to reduce environmental damages and potentially to optimize production of aquaculture industry was developed known as "Biofloc Technology" (BFT).

The BFT is considered as an efficient alternative systems due to the ability of biofloc to continuously recycled and reused nutrients in the culture pond[3]. Furthermore, BFT are able to minimize water exchange and water usage in aquaculture systems by maintaining sufficient water quality, while producing microbial proteins that can be used as supplemented feed for aquatic organisms[4]. "Biofloc" is referred to the aggregation of micro-organisms and remaining particulate organic matter such as faeces and unused feed[5]. Apart from bacteria and microalgae, one of important micro-organism that composed biofloc is microfungi.

Microfungi can be considered as one of the most diverse eukaryotic organisms that play an important role for the decomposition of organic material[6]. Existence of filamentous microfungi in biofloc will strengthen and assist in forming larger and stronger biofloc[7]. Some species of microfungi such as *Aspergillus* sp. and *Penicillium* sp. were proven to be a bioflocculant producing micro-organisms with high flocculating activity[8,9] more than 90%. In addition, interaction and association of microfungi and other micro-organism within the biofloc can stimulate the growth and survival of shrimp as well as increase their resistance to disease[10].

Traditionally, microfungi can be identified using morphological approaches through comparison with known species which observed using compound microscope. However, these methods of identification are often problematic since several fungi genus are complex with some species that showed morphologically identical but belong to different species[11,12]. Unlike morphological identification that are time-consuming and require great skills, molecular identification have been known to be rapid, accurate and efficient for diagnosis, ecological works and also for phylogenetic relationships between species[13]. Molecular identification of microfungi up to species level has generally utilized the variable Internally Transcribed Spacer (ITS) regions of the rDNA since this gene is conserved enough to allow comparison across a wide range of fungal taxa[14]. To achieve the objective of this study, identification of microfungi isolated from biofloc samples of pacific whiteleg shrimp (Penaeus vannamei) culture pond was conducted using 18s rDNA sequences analysis.

MATERIALS AND METHODS

Biofloc sample collection: A few series of biofloc sampling were conducted at the Integrated Shrimp Aquaculture Park (iSHARP), Blue Archipelago, Setiu, Terengganu (Fig. 1). iSHARP was specialized in culturing pacific whiteleg shrimp, penaeus vannamei which was operated since 2012. The biofloc samples were collected from selected shrimp ponds during the day of culture (DOC) of 0, 30 and 70 days as its represent different stages of biofloc formation throughout the *L. vannamei* production cycle. Two litres of 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[15] to enable the biofloc to settle at the bottom of the cone within 24 h.

Preparation of biofloc samples and isolation of microfungi: The settled biofloc samples were transferred into a centrifuge tube (50 mL) by siphoning out excess water after 24 h, following centrifugation at 6000 rpm for 3 min. The biofloc pellet were collected and streaked onto Potato Dextrose Agar (PDA) following method by Wijedasa and Liyanapathirana[16]. Culture of microfungi were incubated at 28°C and observed for colony growth within 3-7 days for fungi identification. Isolation of different colonies by streaking method were carried out to obtain pure culture (Fig. 2).

Identification of microfungi isolates from biofloc sample using molecular approach

DNA extraction of microfungi isolates: All microfungi isolates were inoculated into 50 mL of potato dextrose broth under shaking conditions at 37°C for 5 days. The microfungi cells were harvested by centrifugation at 10,000 rpm for 15 min. The pellet was ground using liquid nitrogen to a fine powder using mortar and pestle. The tissue powder was then transferred into 15 mL test tube and the liquid nitrogen was allowed to evaporate. The DNA of each microfungi isolates from the pellet was extracted with a DNeasy Plant Mini Kit (Qiagen, Germany) following manufacturer's protocol. The purity of the extracted DNA was checked at 260 and 280 nm using BioDrop µLITE (Isogen, Netherlands) and stored at -20°C until further analysis.

Fig. 1: Location of Integrated Shrimp Aquaculture Park (iSHARP), Blue Archipelago at Setiu, Terengganu (http://www.earth.google.com)

Fig. 2: Pure culture of microfungi isolates after several sub-cultivation in PDA media

Table 1: Primers used for amplification of microfungi 18s rDNA

Primers	Sequences (5'-3')	T_{m^o} (°C)	Position
ITS1 (R)	TCCGTAGGTGAACCTGCGG	68.4	ITS region
ITS4 (F)	TCCTCCGCTTATTGATATGC	61.5	ITS region

R: Reverse, F: Forward, Tma: Melting temperature

18s rDNA amplification and sequencing technique: The extracted DNA of microfungi was subjected to amplification with a thermal cycler (VeritiTM Thermal Cycler, Applied Biosystems, USA) using specific primers (Table 1). Polymerase Chain Reaction (PCR) was carried out using commercial kit, *GoTaq*® PCR Core Systems (Promega, USA) for all DNA samples. The 50 µL reaction mixture contained 1.0 mM $MgCl_2$, 1.0X of 5X buffer, 200 µM PCR nucleotide mix, 20 µM of each primer, 1.25 µ of DNA polymerase and 0.5 µg of template DNA sample. The reaction involved initial denaturation at 95°C for 10 min, followed by 30 cycles in series of denaturation at 95°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 10 min[17]. A reaction mixture without DNA sample was used as a negative control and the PCR products were stored at -20°C. Following amplification, PCR products were analyzed by 1% agarose gel electrophoresis in 0.5 X TBE buffer (Promega, USA) and analyzed after staining with ethidium bromide (EtBr) solution[18].

Purification and sequence analysis: Purification of PCR products were carried out using QIAquick PCR Purification Kit (Qiagen, 28104) following manufacturer's protocol. The purified PCR products were sent to First Base Sdn. Bhd. for sequencing process. The results were then analyzed using Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Nine DNA sequences of microfungi species were successfully extracted which showed between 1.8 and 1.9 purity at 260-280 nm ratios. Amplification of 18s rDNA of all isolates using specific primer ITS 1 and ITS 4 resulted in amplicon bands of 500bp. The banding pattern produced by each species on 1% agarose gel were shown in Fig. 3. BLAST results concluded that the amplified microfungi sequences belongs to nine microfungi species with 99-100% similarity to NCBI sequences databases (Table 2). In this study, aggregation of microfungi in biofloc was started to form at DOC 30 up to DOC 70. This can also be observed by the lowest number of fungi species (5 isolates) was found at DOC 0 as compared to DOC 30 and DOC 70 of *L. vannamei* with 7 and 8 isolates, respectively (Fig. 4).

Table 2: Sequencing of the 18s rDNA gene of microfungi isolated from biofloc samples

Morphological identification	Closest matching strain in NCBI	Strain	Similarity (%)	GenBank accession number
Aspergillus sp. 1	*A. versicolor*	MA-229	100	KJ466864.1
Aspergillus sp. 2	*A. niger*	ASM-06	100	LC092112.1
Aspergillus sp. 3	*A. flavipes*	NA-X-39	100	KT833613.1
Aspergillus sp. 4	*A. aculeatus*	AM39	100	KP4148576.1
Aspergillus sp. 5	*A. tamarii*	LSRP-FS04	100	KR296875.1
Penicillium sp. 1	*P. citrinum*	-	99	KM491892.1
Penicillium sp. 2	*P. griseofulvum*	PRPX-FS14	100	KR296884.1
Trichoderma sp.	*T. virens*	JIXX-FM26	100	KR296867.1
Pestalotiopsis sp.	*P. microspora*	ZJ10-9L	100	FJ037745.1

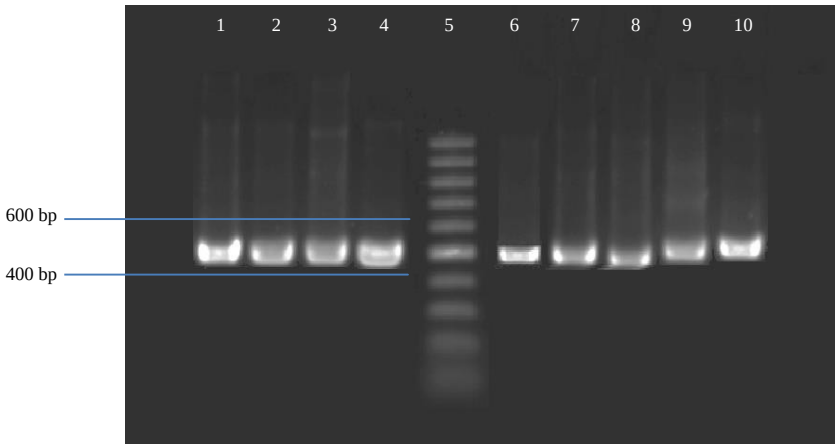

Fig. 3: Amplification of ~500 bp fragment of PCR product from fungi species using ITS1 and ITS4 primers. Lane 1: *Aspergillus* sp. 1, Lane 2: *Aspergillus* sp. 2, Lane 3: *Aspergillus* sp. 3, Lane 4: *Aspergillus* sp. 4, Lane 5: GeneRuler 100 bp DNA ladder, Lane 6: *Aspergillus* sp. 5, Lane 7: *Penicillium* sp. 1, Lane 8: *Penicillium* sp. 2, Lane 9: *Trichoderma* sp. and Lane 10: *Pestalotiopsis* sp.

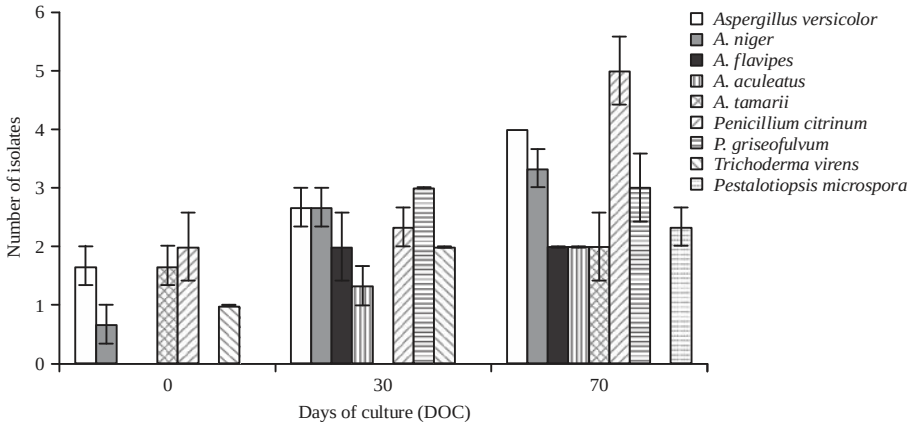

Fig. 4: Number of microfungi isolates for each DOC from biofloc samples of *L. vannamei* culture pond

DISCUSSION

In this study, 18s rDNA of microfungi isolates were successfully amplified using ITS primer pairs. Ribosomal DNA or rDNA has been widely used in identification of bacteria and eukaryotic organisms such as microfungi for many years. This was due to the fact that all micro-organisms possess their own genes and it was known to experience mutation at a slow rate over time[19]. The nuclear small sub-unit of ribosomal DNA (18s rDNA) regions are known to be conserved among microfungi, thus providing molecular basis in identification and phylogenetic analysis of fungi. The ITS specific primers were used as it targets the ITS regions of the rDNA gene complex of microfungi such as *Ascomycetes* sp., *Basidiomycetes* sp. and *Zygomycetes* sp.[20,21]. Since ITS region is non-coding and less conserved, it has been extensively used among closely distinguished related microfungi isolates[22].

Microfungi are saprophytic micro-organisms and rely on degradation of dead organic matter to obtain nutrients[7]. Least number of microfungi species was observed in DOC 0 might due to there was no biofloc was formed as no stocking of shrimp and low concentration of organic matter present in the pond[7]. In addition, this was supported by the most abundance of fungi species was observed in DOC 70 where there was high concentration of organic matter. As uneaten shrimp feed, faecal material and dead micro-organisms accumulate in biofloc during this period, concentration of organic matter increased[23], thus promoting microfungi occurrence in biofloc[7].

Filamentous microfungi such as those from genus of *Penicillium*, *Aspergillus* were reported to be used in sludge treatment and potentially to be applied for bio-flocculation process as it will assist in forming larger and stronger biofloc[7]. Interestingly, both species of *Penicillium* sp. and *Aspergillus* sp. were successfully isolated in each DOC during *P. vannamei* production cycle. This finding was supported by Da Silva *et al.*[24] which stated that both genera can be commonly found in shrimp pond. Since both genus of *Penicillium* sp. and *Aspergillus* sp. were found at every DOC, this was also proved that both genus were also present in biofloc. This might be due to the ability of *Aspergillus* sp. and *Penicillium* sp. play an important role as bioflocculant-producing micro-organisms[8,25,26]. Bioflocculant produced by *Penicillium* sp. has a high molecular weight biopolymer of over 3×10^5 Da[8]. High molecular weight is needed for a good flocculating efficiency in wastewater treatment as it involved stronger bridging and more adsorption points. Apart from having high flocculating activity (96%), *Penicillium* sp. also produce bioflocculant that contain groups of carboxyl- and hydroxyl- that are effective for flocculation[8]. On the other hand, *A. versicolor* and *A. niger* were able to produce bioflocculant with high flocculating activity more[27] than 80%. The produced flocculant was mainly consist of polysaccharide while purification of this bioflocculant showed presence of hydroxyl and carboxyl group[27]. Existence of this groups was reported to be a favourable groups for the bioflocculation process and stimulate high binding capacity[28].

The other species of microfungi known as *Trichoderma* sp. was found in DOC 0 and DOC 30. Existence of this species was expected, as *Trichoderma* sp. was classified as opportunistic fungi and can be found in various ecosystems[29]. However, this species was reported to be able to contaminate feed and food products[30]. Only one species of microfungi known as *Pestalotiopsis* sp. was found at DOC 70 of *P. vannamei* culture. This species was classified as endophytic fungi that was commonly found in tropical and sub-tropical ecosystems[31]. Existence of *Pestalotiopsis* sp. in biofloc was probably due to its ability to act as bioflocculant-producing microorganisms[32].

CONCLUSION

Nine species of microfungi from biofloc samples of *L. vannamei* culture pond known as *Aspergillus versicolor*, *A. niger*, *A. tamarii*, *A. flavipes*, *A. aculeatus*, *Penicillium citrinum*, *P. griseofulvum*, *Trichoderma virens* and *Pestalotiopsis microspora* were successfully identified through 18s rDNA sequences analysis. Existence of microfungi species such as *Aspergillus* sp. and *Penicillium* sp. in biofloc are perfect candidate in forming biofloc that can reduce water pollution towards a sustainable aquaculture industry.

SIGNIFICANCE STATEMENT

A clear understanding of microbiological aspects particularly on microbial communities in biofloc is important for the successful design and operation of biofloc technology. The main objective of this research was to identify microfungi isolated from biofloc-based system through molecular approach using 18s rDNA sequence analysis. Even though numbers of studies on biofloc application were conducted in recent years, the role of microfungi composition contributes to optimum condition for biofloc formation is remain largely unknown. Therefore, molecular identification of microfungi in biofloc aggregates is very crucial as it firmly strengthen the

previous reports. Knowledge on biological properties of microfungi involved in biofloc formation is necessary particularly when dealing with their high potential in aquaculture wastewater treatment. We hope that with this novel finding could be a breakthrough in the area of biofloc technology and any similar research.

ACKNOWLEDGMENTS

The project was fully funded by Fundamental Research Grant Scheme, FRGS (vot No: 59401), Ministry of Education, Malaysia. Special thanks to Mr. Leo Cababasay, shrimp farm manager of i-SHARP, Setiu Sdn. Bhd. and postgraduate students from AKUATROP, Universiti Malaysia Terengganu, MALAYSIA.

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