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CHARACTERIZATION OF MICROBIAL POPULATIONS IN *LITOPENAEUS VANNAMEI* (BOONE, 1931) BASED BIOFLOC SYSTEM USING DENATURING GRADIENT GEL ELECTROPHORESIS PROFILES OF 16S RRNA

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Abstract– Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene segments was used to profile microbial populations in different biofloc systems cultured with *Litopenaeus vannamei*. Biofloc tanks, filled with 350 L of water, were fed with sugarcane molasses (BFT_S), tapioca flour (BFT_T), wheat flour (BFT_W) and clean water as a control without biofloc. The extracted microbial community 16S rRNA fragment of the sample was amplified with GC-clamped F968 and R1378 and evaluated in the DGGE and the distinct bands were removed from the gel after the silver staining. The eluted DNA samples were re-amplified with the same set of primers but without GC clamp. Individual DGGE bands were sequenced and subjected to BLAST search on the GenBank nucleotide database National Centre for Biotechnology Information to identify the sequences with higher similarity. From sequence analyses, totally Fifteen isolates were found in biofloc and the bacteria containing the DNA fragments of Band 5, 8 & 9 were not found in the NCBI database so it will be considered to be a newly identified bacterial species. The results we obtained by using DGGE will be the basic information, to further study the microbes reaction involved in the nitrogen reduction in the biofloc system.

INTRODUCTION

Aquaculture systems mainly depend on the exploitation of autotrophic and heterotrophic microbial food webs. A heterotrophic food web consists of decomposition of organic matter by microorganisms, leading to the formation of assimilable detritus and inorganic nutrients. The detritus and associated microbes are directly consumed by the cultured animals or by other small animals on which the cultured species feed (Colman and Edwards, 1987; Moriarty, 1997). The heterotrophic food web consistently appears as a major contributor to the total production of the target animals (Schroeder, 1987). The fish assimilate only 15–30 % of the nitrogen added to the feed in a pond environment (Acosta-Nassar *et al*., 1994; Gross *et al*., 2000; Davenport *et al*., 2003), the remaining quantity is lost to the system as ammonia and organic-N in the form of faeces and feed residue. The organic-N in faeces and uneaten feed undergoes decomposition resulting in ammonia production. Therefore, a high protein level in fish feed contributes to the high concentration of ammonia in the water column which has detrimental to the cultured animals and needs to be minimised.

Microbial proteins were generated in ponds by microorganisms such as bacteria and protozoa under aerobic and anaerobic conditions by decomposing organic matter and uneaten feed. Organic matters decomposition process under the aerobic condition is faster than in anaerobic condition (Reddy and Patrick, 1975). The microorganisms play a major role with respect to natural productivity, nutrient cycling, water quality and the nutrition for cultured animals (Moriarty,

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1997; McIntosh *et al*., 2000). In many cases the microbes involved in the above process identified are limited. Only an estimated 20 % of the naturally occurring bacteria have been isolated and characterised so far (Muyzer *et al*., 1993). Selective enrichment cultures fail to mimic the conditions that particular microorganisms require for proliferation in their natural habitat. In such cases, the Molecular biological techniques have opportunities for the analysis of the species composition of microbial communities. It is based on electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants. In denaturing gradient gel electrophoresis DNA fragments of the same length but with different base-pair sequences can be separated by the GC content. Therefore the study envisages characterising the microbial community present in the biofloc using denaturing gradient gel electrophoresis.

MATERIALS AND METHODS

Experimental design

The experiment was carried out at Wet Laboratory of the Central Institute of Fisheries Education (CIFE), Versova, Mumbai, India. Fiberglass Reinforced Plastic (FRP) circular tanks of 500 L capacity with 0.98 m diameter, filled with 350 L of water were used for the experiment. Three treatments with a control in triplicates were set up using completely randomised design (CRD). Three treatments were fed with sugarcane molasses (BFT_S), tapioca flour (BFT_T) and wheat flour (BFT_W) for biofloc production. All the treatments and control were fed with shrimp pellet feed (Charoen Pokph (CP) and (India) Pvt. Ltd., India) having 34.5 % crude protein twice a day. The experimental tanks were well aerated (7 mg L^{-1}) by using a centralised aeration unit.

The seawater was pumped from the Aksa Beach (Mumbai, India) and it was stored in the 5000 L reservoir tank for a week to settle down. The seawater was diluted with freshwater to make a salinity of 25 g L^{-1}. The 350 L of diluted seawater (25 g L^{-1}) was filled in all the experimental tanks. The biofloc was produced using (25 g L^{-1}) in 500 L capacity tanks before stocking of the seeds. Sugarcane molasses, tapioca flour and wheat flour were used as a carbohydrate source for biofloc production. The sugarcane juice fermented for 2 days with yeast (*Saccharomyces cerevisiae*) were used as an input (sugarcane molasses) for biofloc production. The amount of NH_4-N released was estimated assuming that added carbohydrate contains 50 % carbon and that 50 % of the dietary protein input was converted to ammonia for that, 20 g of carbohydrate was added per gram of NH_4-N released. In consequence, 0.53 kg each carbon was applied for each kg of the 34.5% dietary protein feed administrated. Twelve-hour darkness and lightness of the photoperiod were maintained throughout the experimental period.

Seed stocking and management

The post larvae of *L. vannamei* (specific pathogen free) seeds were procured from Madha Hatchery located in Chennai, India. The seeds (0.15 ± 0.02 g) were stocked at the rate of 130 PL m^{-2} when bioflocs measured as floc volume and total suspended solids (TSS) was between 5-50 mL and 100 mg L^{-1} respectively. Dissolved oxygen in all experimental tanks was maintained at saturation level. CP pelleted feeds (1.8 - 3.0 mm) were used for feeding throughout the experiment. Feeding rate was at 1.5 % of the total stocked biomass daily and adjusted fortnightly after sampling of the animal. The feed was given in two equal quantities during 0800 and 1700 h in all the tanks. The shrimps were cultured for 60 days. When the pH of water dropped below 7.0, $NaHCO_3$ was added to raise the pH to 7.5.

Sample Collection

The biofloc samples were collected from the respective treatment tanks fortnightly during morning hours of the day between 8.00 and 10.00 am. The biofloc suspensions were taken in the Imhoff cone and allowed to settle for 30 minutes (Avnimelech, 2009). The settled biofloc suspension was transferred aseptically into sterile Uricol (Hi-Media Laboratories Limited, Mumbai, India) bottles, and stored in a refrigerated condition.

Enumeration of total heterotrophic bacterial load

Total heterotrophic bacteria (THB) were enumerated by using R2A agar medium{Enzymatic Digest of Casein (0.25 g), Protease Peptone (0.25 g), Acid Hydrolysate of Casein (0.5 g), Yeast Extract (0.5 g), Dextrose (0.5 g), Soluble Starch (0.5 g), Dipotassium Phosphate (0.3 g), Magnesium Sulfate Heptahydrate (0.05 g), Sodium Pyruvate (0.3 g), Agar (15 g) and Final pH: 7.2 ± 0.2 at 25 °C} from all the treatment tanks following spread plate method. The

inoculated plates were incubated at 37 °C for 48 h and it was expressed as colony forming unit per mL (CFU mL^{-1}).

DNA extraction from biofloc

1 mL of biofloc suspension was kept in Hi-Bead tubes (Hi-Media Laboratories Limited, Mumbai, India) and the lysozyme-SDS based phenol-chloroform method of extraction was followed with slight modifications; 500 µL of lysis 1 solution (0.15 *M* NaCl, 0.1 *M* EDTA; pH 8; 15 mg lysozyme/L) was added to the bead tube and mixed by horizontal vortexing for 2 minutes. It was then incubated at 37 °C for 1 hour in a water bath. After that, 500 µL of lysis 2 (0.1 *M* NaCl, 0.5 *M* Tris-HCl; pH 8; 12 % SDS) solution was added to it and incubated again for 1-2 hours at 60 °C in a water bath. The suspension was then centrifuged at 8000 g for 10 minutes in a Spinwin centrifuge (Tarsons Products Pvt. Ltd., Kolkata, India) and 1 mL of supernatant was recovered in separate vials. To this supernatant, 1 mL of saturated phenol (pH 8) was added, mixed and centrifuged at 8000 g for 8 minutes. The aqueous layer was recovered and an equal volume of chloroform was added, mixed and centrifuged for 5-6 minutes in Spinwin. After centrifugation, the aqueous layer was again transferred to separate tubes, and 2 volumes of absolute ethanol were added and centrifuged for 30 minutes at 4 °C at 8000 g in a microprocessor-based high-speed research refrigerated centrifuge (Eltek, Mumbai, India). The supernatant was decanted and pellets were washed with 500 µL of 70 % ethanol and centrifuged at 8000 g for 15 minutes using the centrifuge. Finally, the vials were decanted again and kept in a dry bath (SLM-DB-120, Bangalore Genei, Bangalore, India) at 60 °C for some time to evaporate the remaining ethanol. The DNA was then resuspended in 50 µL of nuclease-free water and stored at -20 °C.

Genomic DNA confirmation by Agarose Gel Electrophoresis

The elute was checked for the presence of genomic DNA using 1.5% agarose gel prestained with ethidium bromide (0.5 µg mL^{-1}). 1.0 µL of 6X loading dye was mixed with 10.0 µL of genomic DNA and loaded into the wells of the gel. 1.0 µL of loading dye was mixed with 5.0 µL 1kb DNA molecular weight marker and loaded into the wells. Electrophoresis was carried out in 1x TAE buffer at 70 volts for about 60 minutes. The gel was visualised and photographed using Bio-Rad Universal Hood II Gel Imager (Bio-Rad Laboratories, Hercules, California, USA).

PCR amplification using universal primers

PCR amplification of bacterial 16S rRNA genes was performed using universal primers (Eurofins Genomics India Pvt. Ltd., Bangalore, India) F968 and R1378 attached with GC clamp. The primer sequences, used in this study are given in Table 1 (Brons and Elsas, 2008). The components of PCR reaction are described in Table 2 and the reaction was carried out in a Quanta Biotech Thermo-cycler QB-96 (Quanta Biotech Ltd., Surrey, UK).

The PCR consisted of 30 cycles each of initial denaturation at 95 °C for 5 minutes, denaturation at 95 °C for 30 seconds, annealing at 61.3 °C for 30 seconds and extension at 72 °C for 1 minute, and a final extension for 15 minutes. The PCR products

Table 1. Primer sequences used in the study

Sr. No	Primer[a]	Sequence (52→32)
1.	With GC clamp F968"GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG - AACGCGAAGAACCTTAC[b]
2.	R1378	CGGTGTGTACAAGGCCCGGGAACG
3.	Without GC clamp F968	AACGCGAAGAACCTTAC

*a*F, forward primer; R, reverse primer; *b*52 GC clamp (up to hyphen).

Table 2. Components of PCR reaction

Sr. No	Reagents	Volume (50 µL)
1.	Master mix	25 µL
2.	Forward primer (100 picomole)	0.4 µL
3.	Reverse primer (100 picomole)	0.4 µL
4.	Template	3.0 µL
5.	Nuclease free water	21.2 µL

were examined by electrophoresis on 1 % agarose gel prestained with ethidium bromide and visualised in Bio-Rad Universal Hood II Gel Imager (Bio-Rad Laboratories, Hercules, California, USA) (Fig. 1).

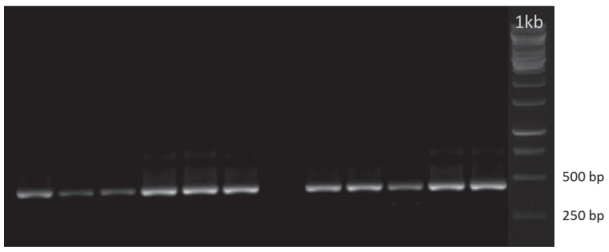

Fig. 1. Agarose gel electrophoresis picture of 410 bp DGGE amplicon obtained using GC- F968 and R1378

DGGE for PCR products

Denaturing gradient gel electrophoresis (DGGE) was performed with the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, California, USA) as per the manufacturer's instructions. PCR samples (10 µl about 500 ng), were loaded onto 1-mm thick 6 % (wt/vol) polyacrylamide: bisacrylamide gels in 1X TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA, pH 7.4). The polyacrylamide gels were made with denaturing gradients ranging from 40 to 55 % (where the 100% denaturant contained 7 M urea and 40 % (v/v) formamide (Bangalore Genei, Bangalore, India). The gels were run for 14-h at 60 V and 60 °C.

Silver staining of DGGE gel

The method described by Sanguinetti *et al*. (1994) was modified and followed to stain the DGGE gels. The stock solutions were prepared as follows: Fixing solution was prepared by adding 3 g of benzene sulfonic acid into the 100 mL of 24 % ethanol and stored in an amber colour bottle. The staining solution was prepared by dissolving 0.35 g of benzene sulfonic acid and 1 g silver nitrate in double distilled water and made up to 100 mL. Developing solution was prepared by dissolving 32.25 g of anhydrous sodium carbonate in double distilled water and made up to 250 mL.

All the working solutions were prepared from the stock solutions. The gels were first fixed with fixing solution (48 mL ethanol, 100 mL stock solution and 102 mL of double distilled water) for 30-45 minutes with gentle shaking. Then, the gels were stained with staining solution (50 mL stock solution and 200 mL double distilled water) for 30-45 minutes with gentle shaking in dark place. The gels were washed for 1 minute with double distilled water and the developer solution {(50 mL stock solution, 200 mL double distilled water, 250 µL sodium thiosulphate (20 mg mL^{-1}) and 250 µL formaldehyde} was poured into the tray. The first precipitate formed was quickly aspirated and the rest of the developer was added. After 5-10 minutes, the gels were soaked in 3 % acetic acid for 5-10 minutes to stop further development. The gel images were recorded with Bio-Rad Universal Hood II Gel Imager (Bio-Rad Laboratories, Hercules, California, USA) with white light settings.

Sequencing of excised DGGE bands

Distinct bands in all the lanes were marked, and bands which were common in a number of lanes were considered similar and only one band each from the similar positions of the polyacrylamide gel was eluted for sequencing. The prominent bands from the DGGE gels were excised with sterile coverslips and soaked overnight in 25 µL of nuclease-free water after little crushing. The PCR re-amplification was carried out using the same set of primers, but without GC clamp in a Quanta Biotech Thermo-cycler QB-96 (Quanta Biotech Ltd., Surrey, UK). The components of the re-PCR reaction are described in Table 3.

The re-PCR consisted of 30 cycles each of initial denaturation at 95 °C for 3 minutes, denaturation at 95 °C for 1 minute, annealing at 60.8 °C for 30 seconds and extension at 72 °C for 1 minute, and a final extension for 15 minutes. The reamplified PCR products were confirmed by electrophoresis on 1.6 % agarose gel prestained with ethidium bromide and visualized in a Bio-Rad Universal Hood II Gel Imager (Bio-Rad Laboratories, Hercules, California, USA) (Fig. 3). The re-PCR products were stored at -80 °C and directly send for sequencing to Banglore Genei, India. The partial bacterial 16S rRNA gene sequences were subjected to BLAST

Table 3. Components of re-PCR reaction

Sr. No	Reagents	Volume (30 µL)
1.	Master mix	20.0 µl
2.	Forward primer (100 picomole)	0.3 µl
3.	Reverse primer(100 picomole)	0.3 µl
4.	Template	1.0 µl
5.	Nuclease free water	8.4 µl

(www.ncbi.nlm.nih.gov/BLAST/) search on the GenBank nucleotide database NCBI (National Centre for Biotechnology Information) to identify the sequences with higher similarity.

Statistical analysis

All statistical analyses were performed using SAS v9.3 for Windows (Cary, North Carolina, USA). Water quality parameters and THB were compared by two-way repeated measures ANOVA with treatment as the main factor and sampling date as repeated measures factor. The analyses were run at 5% significance level.

RESULTS

Bacterial diversity

The biofloc was developed slowly in the initial stage with an adjacent area of the tanks. The bacteria developed by utilising the carbon source. The fast development of biofloc observed after the stocking of shrimps. It's due to the formation of bioflocculants by binding of faeces, feed and planktons with the carbon material. We observed that the density of the heterotrophic microbes in the biofloc was increased gradually after the 45 days. In 90th days the density of the microbes was high in all the treatment. The THB population during the experimental period in all the experimental groups, ranged from 6.0×10^2 to 3.87×10^8 cfu mL^{-1}. The treatment BFT_W and BFT_T showed significantly higher mean THB concentration than the control.

The 16S rRNA gene was PCR amplified using universal DGGE primers, F968 and R1378. The gel images obtained after DGGE are given in figure 2. The dominant bands appeared in the DGGE profiles bands were further subjected to re-PCR and the amplification was confirmed by running agarose gel electrophoresis (Fig. 3). From sequence analyses, totally Fifteen isolates were found and its presence in the biofloc was presented (Table 4). The sequences were deposited in the NCBI library and the accession number was obtained. DNA sequences of strains with more than 70% similarity to DNA sequences of Band 5, 8 & 9 were not found in the NCBI database. Therefore, the bacteria containing the DNA fragments of Band 5, 8 & 9 were considered to be a newly identified bacterial species.

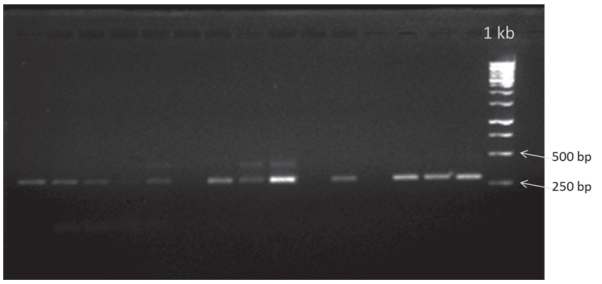

Fig. 2. DGGE gel showing the microbial diversity on the different treatment

DISCUSSION

Analysis of DGGE bands from biofloc of the different treatments revealed the existence of a

Table 4. Bacteria identified from different biofloc treatments and control

Sl. No.	Accession Number	Bp length	Taxonomy	Treatment			Control
				BFT_S	BFT_T	BFT_W	
1.	KF595288	371	Uncultured *Saccharophagus* sp	+	+	-	-
2.	KF595289	201	Uncultured *Sulfitobacter* sp	+	+	-	-
3.	KF595290	200	Uncultured *Sulfitobacter* sp	+	+	+	+
4.	KF595291	203	Uncultured *Ruegeria* sp	-	+	-	-
5.	KF595292	413	Uncultured bacterium	+	+	+	-
6.	KF595293	337	Uncultured *Pseudomonas* sp	+	+	-	-
7.	KF595294	203	Uncultured *Sulfitobacter* sp	+	+	-	-
8.	KF595295	297	Uncultured bacterium	+	+	-	-
9.	KF595296	281	Uncultured bacterium	+	+	-	+
10.	KF595297	205	Uncultured *Phaeobacter* sp	-	+	-	-
11.	KF595298	248	Uncultured *Vibrio* sp.	-	-	+	+
12.	KF595299	274	Uncultured gamma proteobacterium	+	+	+	-
13.	KF595300	215	Uncultured alpha proteobacterium	+	-	-	+
14.	KF595301	298	Uncultured gamma proteobacterium	+	+	+	-
15.	KF595302	203	Uncultured alpha proteobacterium	-	+	-	-

BFT_S, sugarcane molasses; BFT_T, Tapioca flour; BFT_W, Wheat flour.

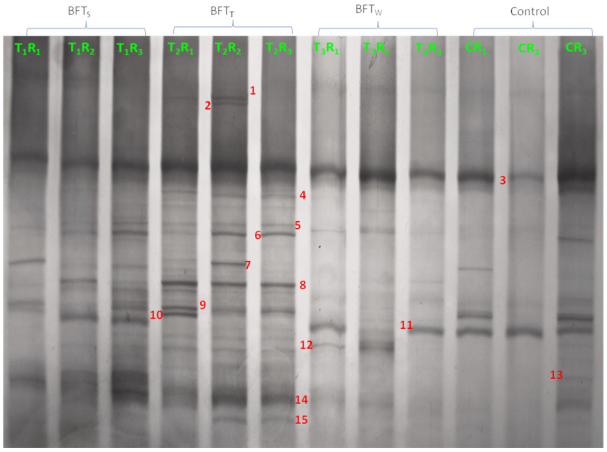

Fig. 3. Agarose gel electrophoresis picture of 300bp amplicon obtained after re-PCR of excised DGGE bands

richness of diverse bacterial community. The total number of bands visualised in a DGGE gel also provides an estimate of the bacterial community found in a biofloc treatment (Zhao *et al*., 2012). From sequence, analyses were five bacteria found to be the predominant in all the treatments.

Isolation and culture of the majority of the associated microflora in shrimp and other organism have proved difficult and therefore, research has focused on growth independent identification techniques (Lau *et al*., 2002) designed to monitor and identify particular microorganisms (Tendencia *et al*., 2006). DGGE is one of the growth independent techniques which are used for assessing the microbial diversity in biofloc (Zhao *et al*., 2012). Many bacterial communities were found but there is no *Bacillus*, *Proteobacterium* and *Vibrio* sp. could be identified. The distribution of dominant microorganisms and resilient ones in the biofloc system assures the competence of counteracting the effect of a sudden exposure to disturbance.

CONCLUSION

The biofloc system mostly dominated by the heterotrophic bacterial community which effectively involves reducing the nitrogen compounds to maintain the water quality. These bacteria colonise with detritus matter and form as biofloc which can effectively feed by shrimps for their growth. The bacteria identified from the biofloc by DGGE are responsible for colonisation. So augmenting this bacteria community can be a future alternative to individual probiotic culture for the aquaculture industry.

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