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Utilization of Complex Carbon Sources on Biofloc System and Its Influence on the Microbial Composition, Growth, Digestive Enzyme Activity of Pacific White Shrimp, *Penaeus vannamei* Culture

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Abstract

A two-month experiment was conducted to evaluate the effect of complex carbon sources on the biofloc system and its influence on *Penaeus vannamei* culture. Four sources of carbon viz. Tapioca flour (BFTf), Rice bran (BFRb), Wheat flour (BFWf), Rice Flour (BFRf), and biofloc were generated, the absence of CHO being considered as control (C). The experiment was carried out in 100L FRP tanks in triplicate, and the post-larvae (ABW: 0.11 g) were stocked @ 400 PL/m^3. Results revealed that the addition of complex carbon sources effectively reduces the TAN by 62-67%. The average body weight of shrimp in the rice flour and wheat flour treatments were significantly higher compared to control. Similarly, improved survival was observed in rice bran treatment (89%). Beneficial bacteria were isolated from all the treatments as well as control. Real-time analysis revealed significantly ($P<0.05$) higher expression of digestive enzyme-related genes compared to control the utilization of carbohydrates, exhibiting an encouraging trend. The complex carbon sources (BFRf) and (BFWf) have been effectively utilized, resulting in improved water quality, microbial diversity, growth performance, and enhanced digestive enzyme activity.

Introduction

The increasing human population in today's world demands high production of nutritious food. The aquaculture industry is one of the primary food-producing industries that play a significant role in food security, poverty alleviation, and affecting the economy in a positive manner. Shrimp aquaculture is ranked at eighth position production-wise and is second as far as global fish sales are concerned (Cai et al., 2017). The shrimp industry is a growing enterprise beset with problems such as excessive usage of water and land. Today, the biofloc technology in aquaculture utilizes limited water and land resources, consequent to which it can lead to an eco-friendly blue aquaculture industry (Bossier and Ekasari, 2017).

The main principle of the technology is the manipulation and maintenance of the C: N ratio in the culture environment (Chakrapani et al., 2021). Balancing carbon and nitrogen in the aquaculture medium will stimulate faster growth of the heterotrophic bacterial population. The nitrogen assimilation by heterotrophic bacteria converts the toxic ammonium into microbial protein (Avnimelech, 2009; Crab et al., 2012; Panigrahi et al., 2018). However, the conversion is faster due to the rapid growth of heterotrophic bacteria in the biofloc system (Mugwanya et al., 2021). Heterotrophic bacterial biomass yield per substrate unit was tenfold higher than

the nitrifying bacterial population (Hargreaves et al., 2006); this appears to be an economical way of removing the excess nitrogen. Further, the biofloc, a nutritious natural feed, reduces the cost of production by decreasing the percentage of protein in the feed (El-Sayed,2021). Hence it has a positive cascading effect in the culture system leading to improved water quality and growth performance.

Carbon and nitrogen ratio balancing is a crucial aspect of the biofloc system, and supplying a suitable carbon source determines the success of the technology. Several reports indicate that selecting appropriate carbon sources will determine the microbial community structure, function, and stability of biofloc (Hollender et al., 2002; Wei et al., 2016; Bakhshi et al., 2018; Romano et al., 2018; El-Sayed, 2021). While selecting the carbon sources, the following criteria need to be considered: nutritional composition, digestibility, economic viability, and continuous availability. The most commonly used carbon sources for biofloc development are molasses, glycerol, sugars such as dextrose, and agricultural by-products such as brans (Emerenciano et al., 2013: Dauda et al., 2017; Panigrahi et al.,2019b). Identifying new and locally available carbon sources is needed to develop biofloc technology. Complex carbon sources are reported to have a substantial impact on biofloc development and improved growth than when molasses is used (Mabroke et al., 2018). Since molasses and glycerol comprise simple sugars, complex carbon sources such as rice and wheat flour, rice bran, etc., which are made up of polysaccharides, have protein, lipids and other minor nutrients in addition to carbohydrates. The complex carbon sources can improve the nutritional values of biofloc and serve as additional food for the animals leading to improvement in body weight (Rajkumar et al., 2016; Verma et al., 2016).

On the other hand, simple carbon sources like glycerol can disperse quickly in the water and help reduce ammonia compared to complex carbon sources (Dauda et al., 2017). Hence, the simple carbon sources can be efficiently utilized by heterotrophic bacteria for multiplication resulting in e dispersion and quicker floc development. However, excess carbon source supplementation needs to be carried out in the culture tank. In contrast, the complex carbon sources of rice bran or wheat bran have complex nutrients which are decomposed slowly by heterotrophic bacteria. The slower dispersion of this carbon source aids in maintaining the floc formation in biofloc system. It also provides a stable response to the culture medium by reducing the nitrogen metabolites. (Khanjani et al., 2017). Hence, heterotrophic bacterial colonization is crucial in carbon sources, making them available to cultured animals. Heterotrophic bacterial population count and structure may vary depending on the carbon sources used for floc formation (Mabroke et al., 2018; Deng et al., 2018). This study replaced the complex carbon sources as a better alternative to the exhausted carbon sources. Our study evaluated the efficiency of different complex carbon sources supplementation on microbial community variation, growth, and survival of *Penaeus vanammei* cultured in the biofloc system.

Materials and Methods

Experimental Design

A 2-months experiment was carried out (triplicate) to evaluate the effect of complex carbon sources for biofloc generation and their influence on microbial colonization on *P. vannamei* culture. Four treatments viz. Tapioca flour (BFTf), Rice bran (BFRb), wheat flour (BFWf), and Rice flour (BFRf) were utilized for biofloc generation and compared to a control (C) that was devoid of any carbon source. The experiment was conducted in 100L FRP (Fiberglass reinforced tanks). For biofloc generation, the tanks were filled with disinfected seawater to which a concoction of biofloc inoculum was applied. Biofloc inoculum was prepared by fermenting the respective carbon sources with *Bacillus subtilis* (MTCC 2756) (10^9 CFU/ml) in sterile seawater (1 liter) for 24hrs after which it was filtered and added to the respective tanks. The animals (0.11±0.02 g) were stocked @ 400 PL/m^{-3}. The experiment was maintained with C: N ratio of 15:1 (Avnimelech, 1999), and animals were fed daily @ 10% of their biomass which was subsequently decreased to 1.5% at the end of the experiment. Continuous aeration was provided to facilitate proper mixing of the floc.

Water Quality Parameters

Physico-chemical parameters of the experimental water viz. temperature, pH, and salinity were recorded daily using a mercury thermometer, pH-Scan-Eutech instrument (Singapore), and hand refractometer, respectively. Measured Total ammoniacal nitrogen (TAN) using phenol hypochlorite method, nitrite (NO_2-N), nitrate (NO_3-N), Phosphate-P (PO4-P), total alkalinity, TSS, and chlorophylla every three days once (APHA, 1998). Settleable solids were measured using the Imhoff cone method daily.

Growth Performance

The shrimps were sampled once a fortnight. The average weight gain (AWG) (g), average daily growth g/day (ADG), specific growth rate (SGR), and survival (%) were measured as follows:

$$\text{Weight gain (\%)} = \frac{\text{Final weight (g) - Initial weight (g)} \times 100}{\text{Initial weight (mg)}}$$

$$\text{Specific growth rate (SGR) (\%)} = (\ln \text{final weight} - \ln \text{initial weight})/\text{Days of culture} \times 100$$

ADG (mg day-1) =Final weight (mg) - Initial weight (mg)
Experimental duration (days)

ADG: Average Daily Growth

Microbiological Analysis

Biofloc samples from the respective treatments were collected once in 10 days, and the heterotrophic bacterial community in the samples was enumerated by using different agar plates. The total heterotrophic bacterial population was enumerated by using Zobell marine agar (ZMA). The total *Vibrio* count and total *Bacillus* count were observed by using TCBS agar and HiChrome *Bacillus* agar, respectively (Himedia, India). In brief, biofloc samples from different treatments were serially diluted, spread on to respective agar plates, and incubated for 24-48 h at 32°C. After incubation, colonies with different morphology were chosen and stored at 4°C. Further, the genomic DNA was isolated (Genejet genomic DNA purification kit, ThermofisherScientific, India) from the respective bacterial culture, and the 16s rRNA gene was amplified. The PCR program used was as follows; initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 sec and extension at 72°C for 45 sec, with a final extension at 72°C for 10 min by using universal forward (27F) (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse (1492R) (5'-TACGGYTACCTTGTTACGACTT-3') primers. The PCR product was purified (Genejet PCR purification kit, Thermofisher Scientific, India) and outsourced for sequencing (Agrigenome labs Pvt Ltd., Cochin, India). Further, bacteria from each treatment were identified using a similarity search with the BLAST search engine, and phylogenetic analysis was carried out with MEGA. 10. Software.

Identification of the Planktonic Composition

The water samples were collected from all the tanks and concentrated to 50ml by filtering through a plankton net. The planktonic water samples were fixed in 5% formalin for further analysis (Pennak, 1978). The plankton was counted using a Sedgwick-Rafter counting cell. A 1ml sample was added into the rafter and kept undisturbed for 15 min for settling; later, the planktons were counted and represented as no of cells/liter (Asaduzzaman et al., (2010).

Expression of Digestive Enzyme and Metabolism-related Genes by Real-time Analysis

Animals (n=6) were collected at the end of the experiment, and total RNA from the hepatopancreas was isolated using the RNeasy Mini Kit (Qiagen, USA). The mRNA was converted into complementary DNA using the iScript 1st Strand cDNA Synthesis Kit (Bio-Rad, USA). The digestive enzyme and metabolism-related gene expression were performed by using a gene-specific primer as listed in Table. 1. The reaction mixture consists of the total reaction volume (20 µL) in each PCR tube of 10µL of 2X SYBR® Green qPCR master mix (Bio-Rad, USA), 1 µL each of forward and reverse primers (10 pmol), 1 µL of template DNA (30–60 ng) and 7 µL of PCR water. A negative control without a cDNA template was run to assess the overall specificity. Two-step PCR reaction was carried out with the following temperature cycle; holding stage at 95°C for 10 min (initial denaturation), 45 cycles of 00.15 sec at 95°C (denaturation) and 1 min at 60°C (annealing and extension) (Applied Biosystem's Real-Time PCR system StepOnePlus®). All the samples were analyzed in triplicate, and relative expression was calculated using

Table 1. List of primers used in the present study for mRNA expression of different digestive enzymes and metabolism-related gene expression in shrimp gut samples.

Gene	Primer sequence (5' – 3')	Accession no/Reference	Amplicon Size
Trypsin	F- TCCTCTCCAAGATCATCCAA R- GGCACAGATCATGGAGTC	Stephens et al., 2012	255bp
Chymotrypsin	F- GGCTCTCTTCATCGACG R- CGTGAGTGAAGAAGTCGG	Stephens et al., 2012	266bp
Cathepsin L	F-CTCAGGACGGTAAGTGTCG R-TTCTTGACCAGCCAGTAGG	Stephens et al., 2012	239bp
Cathepsin B	F-GGATGTAACGGAGGCTTC R-CTGTATGCTTTGCCTCCA	Stephens et al., 2012	212bp
α-Amylase	F-GGTAAACACTGACTCACGCC R-TTCACGTCTCCCTGGTACAC	AH013375.2	234bp
Pyruvate Kinase	F- ATCCTTGATGGTGCTGAC R-CCGTGTTCGTTGAGAAGT	EF102105.1	133bp
Fatty acid Synthase	F-TACGGAGAACCTAGTGGAAC R-CTACCGACGACGAAAAGTGA	HM595630	115bp
Triacylglycerol lipase	F-ACTGTCTCCTCTGCTCGTC R-ATGGTTTCTGGAATAGGTGTTT	XM_027365317.1	148bp
ß-actin	F-CAACCGCGAGAAGATGACAC R-TCGGTCAGGATCTTCATCAGG	GU732815.1	243bp

the comparative cycle threshold (CT) method (Pfaffl, 2001). CT is defined as the cycle number at which fluorescence reaches a set threshold value. The ß-actin gene expression was used as an internal control.

Statistical Analysis

The data were analyzed using SPSS (Version-17) software. One way ANOVA was deployed to compare the treatments and significance (P<0.05) was tested using Duncan's Multiple Range test. Unless otherwise specified, the significance was tested at 5% probability level.

Results

Assessment of Water Quality Parameters

The water quality parameters are presented in Table 2. There was no significant difference in temperature, pH, and salinity among the treatments and control. However, the level of NH_4-N NO_2-N, NO_3-N, and PO_4 significantly varied (P<0.001) between treatments and control. Besides, 62-67% of the reduced TAN level was observed in the treatments. In particular, BFWf showed the highest decrease of TAN level than control, but there was no significant difference between the treatments (P>0.05). All the treatments showed significantly higher values in TSS, settleable solids, and chlorophyll-a (P<0.001).

Growth Performance

Growth and survival of the shrimps after 60 days of rearing are depicted in Table 2. Shrimps reared in biofloc generated with different carbon sources showed encouraging results. The survival in treatments ranged from 81 to 89 %, and a significantly (P<0.05) higher survival was observed when compared to the control group (79%) (Table 3). The average bodyweight of the biofloc reared shrimp at the end of rearing (BFRf: 5.4g, BFWf: 5.06g, BFTf: 4.86g, BFRb:4.48g) was significantly higher than the control (4.05g) (Figure 1). Overall, the specific growth rate and average daily weight gain were significantly higher (P<0.01) in BFRf followed by other treatments (range from 0.079 to 0.088g) and control (0.066g), and their corresponding SGR varied between 6.0-6.4% day^{-1}(Table 3).

Microbiological Analysis

Microbiological analysis of the total viable count revealed higher values in biofloc treatments compared to control. Among the four different carbon sources used, BFRf showed a higher bacterial count (3.9×10^4), followed by BFWf (3.3×10^4). The total viable count of BFTf (2.9×10^4) and BFRb (2.9×10^4) was also on the higher side compared to control (1.9×10^4). The total *Vibrio* in different treatments appeared too low to facilitate counting compared to control (1.4×10^2). In addition, colonies were observed in HiChrome *Bacillus* agar, and

Table 2. Water quality parameters of biofloc-based shrimp culture and control groups. It represents the mean ± SD. Mean in the same row having different superscript differs significantly.

Parameter	Control	BFTf	BFRb	BFWf	BFRf	p value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Temperature(°C)	33^a±1.2	33^a± 1.2	33^a±1.4	32^a±1.2	33^a±1.3	NS
pH	7.7^a±0.0	7.6^a±0.1	7.6^a±0.0	7.5^a±0.1	7.7^a±0.1	NS
Salinity (ppt)	34.5^a±0.9	34.3^a±0.5	34.6^a±0.5	34.2^a±0.4	34.5^a±0.8	NS
Alkalinity $caco_3$(ppm)	134^a±2.8	154.1^b±7.7	148^b±8.4	150^b±8.4	157^b±2.1	0.001*
Nitrite (NO_2) (ppm)	0.64^d±0.5	0.36^a±0.3	0.51^c±0.5	0.42^b±0.5	0.40^{ab}±0.5	0.001*
Nitrate (NO_3) (ppm)	1.55^d±1.4	1.40^c±1.2	1.32^b±1.5	1.23^a±1.4	1.43^c±1.3	0.001*
Total Ammonia-nitrogen (ppm)	0.69^b±0.6	0.40^a±0.5	0.41^a±0.7	0.37^a±0.4	0.41^a±0.3	0.001*
Orthophosphate PO_4(ppm)	1.85^c±1.5	3.33^b±2.0	3.20^{ab}±2.0	3.88^a±1.3	3.06^c±2.3	0.001*
$Chlrophyll_a$ (µg L^1)	69.7^a±6.3	117.9^b±5.6	120.3^b±3.5	120.1^b±6.8	123.4^b±6.3	0.001*
Total suspended solids (mg L^{-1})	213.91^e±25.1	397.25^d±33.0	479.81^b±17.0	435.35^c±19.4	494.11^a±22	0.001*
Settleable Solids	5.6^a±1.82	21.4^b±5.0	21.6^b±4.84	21.8^b±3.45	21.7^b±6.54	0.001*

*P<0.001; NS, Not significant

Table 3. Growth performance parameters (mean ± SD) in control and biofloc systems supplemented with different carbon sources. Mean in the same row having different superscript differs significantly at (P≤0.05)

Parameters	Treatments				
	Control	BFTf	BFRb	BFWf	BFRf
Final body weight	4.05^a±0.4	4.86^b±0.6	4.48^{ab} ±0.5	5.06^{bc} ±0.3	5.4^c±0.8
Average daily Growth (g/day)	0.06^a±0.0	0.07^b±0.0	0.07^b±0.0	0.08^c±0.0	0.08^c±0.0
Specific growth rate (%/day)	6.0^a±0.2	6.3^b±0.2	6.1^{ab}±0.4	6.3^b±0.2	6.4^b±0.1
Survival %	79^a±1.8	84^b±4.2	81^{ab}±3.9	86^b±2.6	89^c±5.5

the values were BFTf: 1.9×10^2, BFRf: 1.2×10^2, BFRb: 1.2×10^2, BFWf: 1.0×10^4 and control: 0.8×10^2 respectively. Based on morphological and biochemical characteristics, initially, 55 bacterial isolates were selected from treatments and control. For further identification, 8 isolates, each were chosen from BFTf, BFRb, and BFWf treatments. Ten isolates from BFRf and 5 from control were also chosen.

Sequence Analysis of 16s rRNA Mitochondrial Gene

Sequence analysis of 16s rRNA revealed the identification of diverse species of bacterial population from each treatment. Strains of *Bacillus* were observed commonly in treatments and control, whereas strains of *Vibrio* (*V. parahaemolyticus* and *V. alginolyticus*) were observed only in control (Figure 6). The BFRf had more diversified *Bacillus* strains compared to other treatments. Three different photosynthetic bacterial strains belonging to the *Rhodobacteraceae* (BFT-AP31-33) family were only observed in this group (Figure 5.). In BFTf *Bacillus* strains, *Bhargavaea* and *Photobacterium* strains were also observed (Figure 2). In BFRb treatment, bacteria belonging to various genera were observed viz. *Bacillus*, *Pseudoalteromonas* (BFT-AP11), *Exiguobacterium* (BFT-AP11), and *Thalassolituus* (BFT-AP13) compared to other treatments and control (Figure 3). Isolates of the BFWf group belonged to two genera viz. *Bacillus* and *Staphylococcus* (Figure 4). All the identified sequences were processed and submitted to GenBank (Accession Number MK966343- MK966378; MK966415- MK966416).

Identification of the Planktonic Composition

Planktonic composition in the treatments and control was analyzed, revealing significant ($P<0.05$) differences between treatments and control. (Figure 7). Different groups of planktons viz. Rotifer, copepods, diatoms, cyanobacteria, microalgae, and nematodes were also identified. Overall, from the samples, 38 different species of planktons were identified, including *Chlorophyta, Bacteriophyta, Cyanophyta, Ciliophora, Rotifera,* and *Nematoda*. In control, an abundance of filamentous cyanobacteria and *nitzschia,* sp were observed. Nematodes, along with a considerable amount of cyanobacteria, were commonly found in all treatments and control. (Figure 8)

Expression of Digestive Enzyme and Metabolism-related Genes by Real-time Analysis

The expression of digestive enzyme-related genes was studied. Trypsin, chymotrypsin, cathepsin L, and cathepsin B genes belong to protein metabolism, while α-amylase, pyruvate kinase, triacylglycerol lipase, and fatty acid synthase genes belong to the carbohydrate and fatty acid metabolism. The genes for digestive enzymes were found upregulated in the biofloc treated group compared to the control, the differences being significant. (Figure 9a and9b). Among the treatments, higher fold expression was observed predominantly in BFRf (trypsin: 8.1 fold) (chymotrypsin: 10.2-fold) (cathepsin L: 5.1-fold) (cathepsin B and alpha-amylase: 4.8-fold) and (pyruvate kinase: 7.6-fold) followed by BFRb, BFWf, and BFTf.

Discussion

In the biofloc system, the carbon source is important to develop and maintain a zero-water exchange system. Though a simple carbon source like molasses effectively immobilizes ammonia, the exploitation of such a source would be high as we rely on a single source. This notion has provoked researchers to explore equally potential, cost-effective, and other locally available carbon sources. It has been revealed that complex carbon sources remove the ammonia slowly compared to simple carbon sources like molasses (Avnimelech, 1999; Ekasari et al., 2014). Many biofloc trials were conducted using simple carbon sources, which are predominantly monosaccharides. However, complex carbon sources such as wheat flour, rice flour, and wheat bran take time for breaking down into simple sugars before utilization. Although they take time, they do stay stable and aid in removing the nitrogen metabolites in the system compared to simple carbohydrates. (Wang et al., 2016; Khanjani et al., 2017). Our study showed significant improvement of water quality parameters in biofloc treatments while using different complex carbon sources. The TAN level reduction was observed in all the treatments compared to the control. The findings of our study are in agreement with those reported by Kuhn et al. (2009). It has been reported that TAN and the carbon sources used in the treatments were utilized by the bacteria to produce microbial floc and new cells. (Avnimelech, 1999). The reduced TAN level aids in promoting a better atmosphere in the culture system. Also, the appropriate level of settleable solids was maintained to retain optimum TSS levels. In our study, an increasing trend was discernible in TSS and settleable solids levels among the treatments. The heterotrophic bacterial populations effectively utilized the supplied complex carbon sources to produce biomass which was reflected in the bacterial count observed in each treatment. Our results corroborate the findings of Rajkumar et al. (2016), who reported similar observations. Abbaszadeh et al. (2019) reported that water quality levels on TAN and TSS were equally maintained in biofloc treatments using the spoilage date palm extract compared to molasses in *Penaeus vannamei* culture. In our study, complex carbon sources were well utilized for water quality maintenance and for assimilating the nitrogen metabolites. The findings in our study are in accordance with the results reported by Dauda et al. (2017). The authors observed that ammonia reduction and other water quality parameters like TSS were maintained well in rice bran

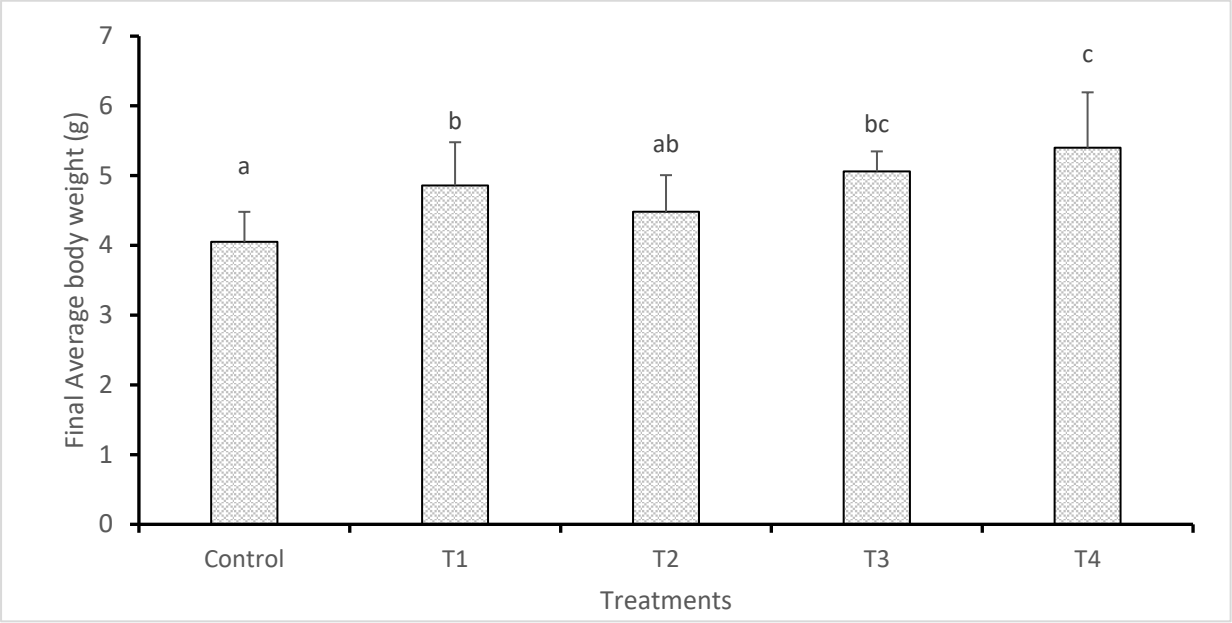

Figure 1. Mean values of body weight of *P. vannamei* in fifteen days interval of four treatments reared in biofloc system supplemented with different carbon source and control. Values are means (±SD) of three replicate tanks per sampling time in each group.

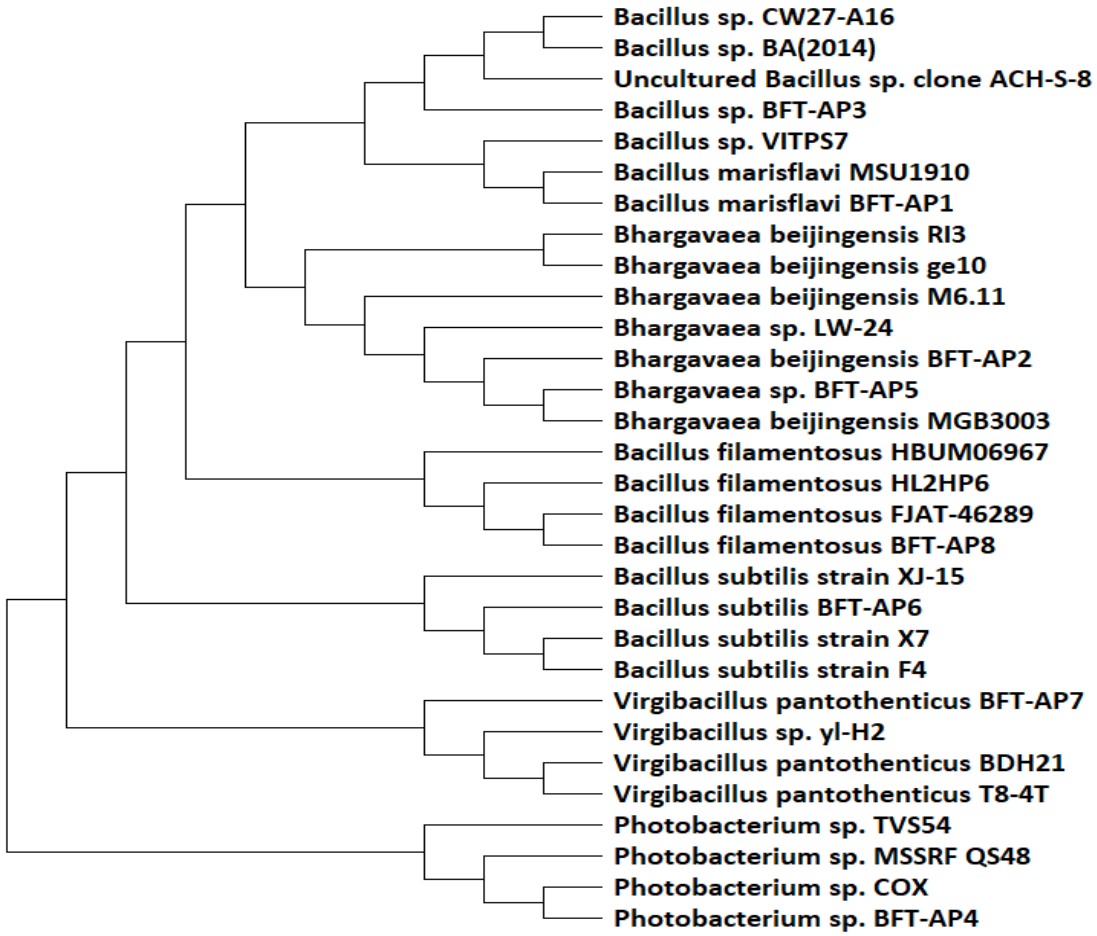

Figure 2. Phylogenetic relationship of identified strains from water samples of BFTf group (Strains names representing BFT-AP are the isolates of this present experiment)

compared with glycerol and sucrose in African catfish *Clarias gariepinus* culture under the biofloc system.

Biofloc and carbon sources significantly improved the growth performance of shrimp in all treatments compared to control. Notably, BFRf and BFWf showed improved growth compared to shrimp from other treatments. The addition of carbon sources is advantageous due to their nutritional composition. Panigrahi et al. (2019b) reported the nutritional composition of various carbon sources used in aquaculture. Their inclusion reduces the ammonia level and supports the growth of heterotrophic bacteria. (Panigrahi et al., 2019c). The diverse microbial community observed in each treatment shows that the host animal utilized the carbon source as a supplementary nutritive feed (Deng et al., 2018). Microbes can use various carbon sources, from simple to complex sugars (Thomsen 2005, Sujeet Kumar et al., 2017). Although rice flour, rice bran (high fiber content), and wheat flour comprise complex sugar with starch and polysaccharides (Zhou et al., 2007), they still render improved water quality, floc development, immunity, and growth. Rice bran is previously reported to give better shrimp yield by enhancing growth and survival and reducing FCR (Vilani et al., 2016). Rajkumar et al. (2016) found that the growth performance of *P. vannamei* was better when complex carbon sources such as wheat flour and tapioca flour were added rather than simple carbon sources such as molasses, a finding similar to what we observed in our study. Zhao et al. (2016) reported that supplying wheat bran (50%) with molasses (50%) for biofloc generation resulted in

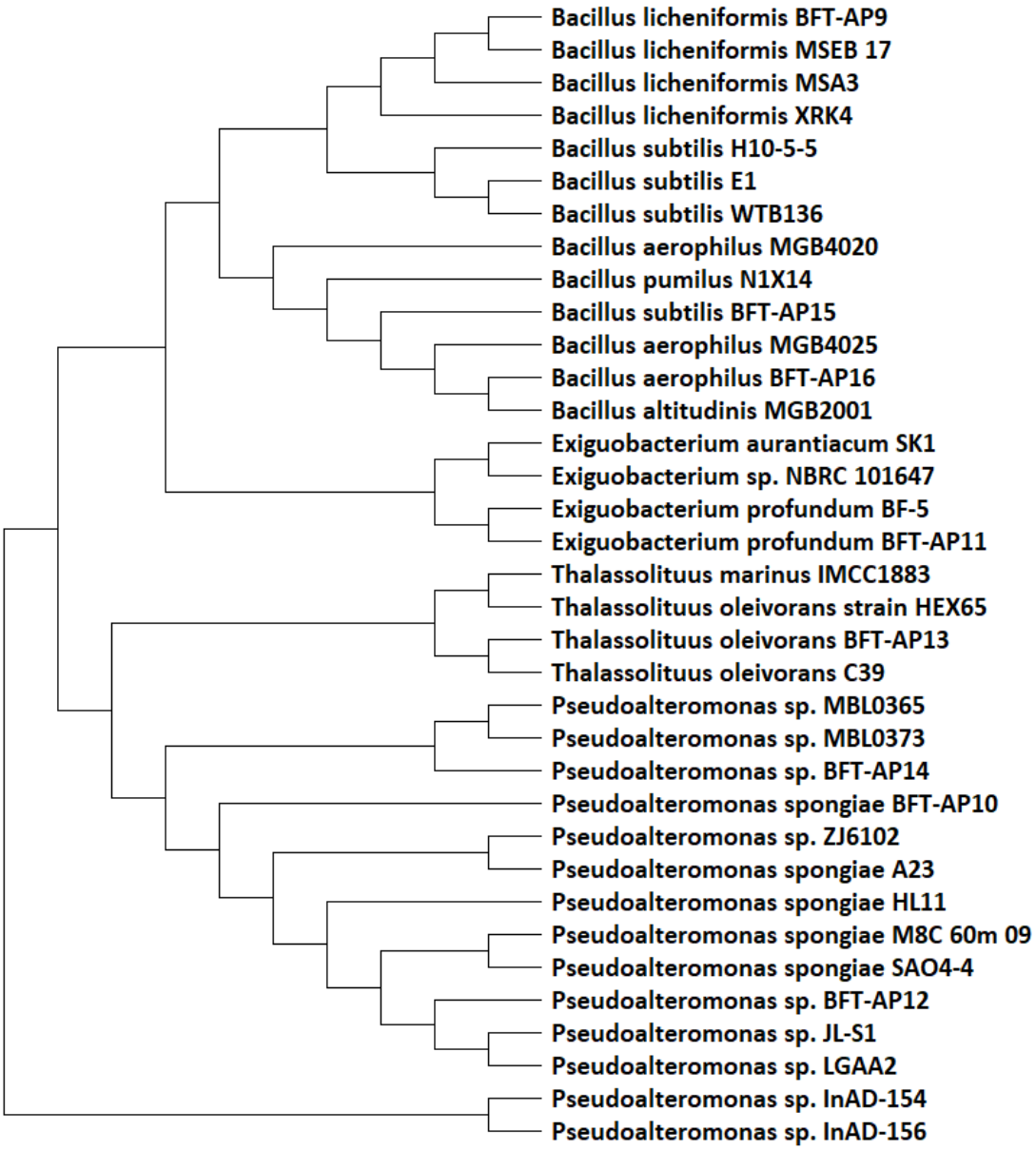

Figure 3. Phylogenetic relationship of identified strains from water samples of BFRb group (Strains names representing BFT-AP are the isolates of this present experiment)

improved weight gain and SGR in *P. vannamei* shrimp. Here again, the findings in our study corroborate those of Zhao et al. (2016).

In our study, microbial diversity was observed in response to the addition of complex carbon sources used for biofloc generation. Usually, the type of carbon sources will decide the bacterial abundance in the biofloc system (Deng et., 2018; Wei et al., 2020). Due to the nutritional values of complex carbon sources, the biofloc system harbored a more diversified bacterial abundance. In agreement with this observation, Mabroke et al. (2018) reported that the total bacterial count noticed in control and simple carbon sources is relatively low compared to complex carbon sources like wheat bran. On the contrary, the population of Vibrio in our study substantially reduced in biofloc treatments compared to control. The results align with those we reported in one of our previous studies (Panigrahi et al., 2018), who reported that the increasing C: N ratio in the biofloc system considerably eliminated the *Vibrio* population. While observing the different strains isolated from other biofloc groups indicated the strains of *Bacillus* to be common in all the treatments. *Bacillus strains* are ubiquitous, and many *Bacillus* strains have been used as probiotics in shrimp culture (Ahmad et al., 2017). The Bacillus genus is mainly observed in biofloc reared animals and the culture environment (Panigrahi et al., 2019c). Zhao et al. (2012) reported that *Bacillus* strains are observed primarily on biofloc groups, sometimes without the *Vibrio* group's complete absence. As a natural probiotic, *Bacillus is* effective against a wide range of pathogenic microbes because of

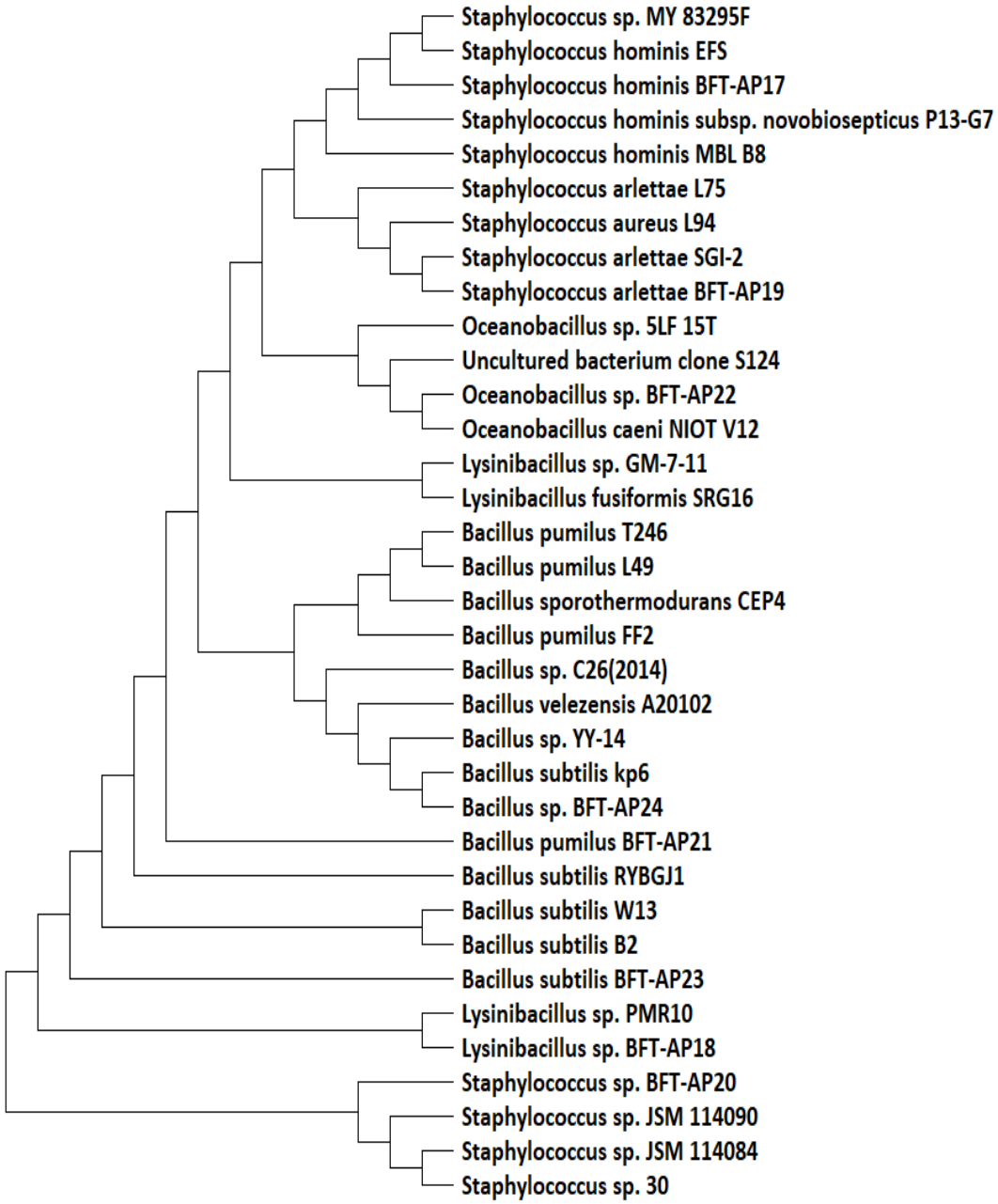

Figure 4. Phylogenetic relationship of identified strains from water samples of BFWf group (Strains names representing BFT-AP are the isolates of this present experiment)

its ability to produce spores and secondary metabolites (Harun et al., 2017; Bischoff et al.,2019; Soltani et al., 2019).The commonly used bacillus species in aquaculture are *Bacillus subtilis* (BFT-AP6) (Panigrahi et al., 2020), *Bacillus lichiniformis* (BFT-AP9) (Elsabagh et al.,2018), *Bacillus pumilus* (BFT-AP21)(Elsabagh et al., 2018), *Bacillus cereus* (BFT-AP27)(Reddy et al., 2018), *Bacillus megaterium* (BFT-AP28)(Hura et al., 2018) and *Bacillus amyloliquefaciens* (BFT-AP37) (Xie et al., 2013). *Bacillus sp* improves water quality, improves survival, immunity and enhances shrimp production (Hlordzi et al., 2020).

In contrast, only *Vibrio* was observed in the control group. The abundance of *Bacillus* may have a competitive exclusion in inhibiting the growth of *Vibrio* in treatments. In BFRb, treatment (BFT-AP 10 &14) *Pseudoalteromonas sp* was isolated. They produce extracellular enzymes with anti-bacterial activity (Holmström et al., 1999; Wang et al., 2018). These species were previously reported as a potential promotor of floc formation by aggregating the carbon sources and other organic and inorganic materials in the system (Harun et al., 2018; Hashim et al., 2018). In wheat flour-based treatment (BFT-AP17), *Staphylococcus horminis sp* was identified, improving immunity and increasing aquaculture production (Rajeswasri et al., 2016). Similarly, In BFRf rice flour-based treatment, mainly *Rhodobacteraceae bacterium sp* (BFT-AP31) *and Roseovarius sp* (BFT-AP32) were identified. These bacteria recycle the nutrients by utilizing organic and inorganic compounds, producing secondary metabolites, carbon monoxide oxidation,

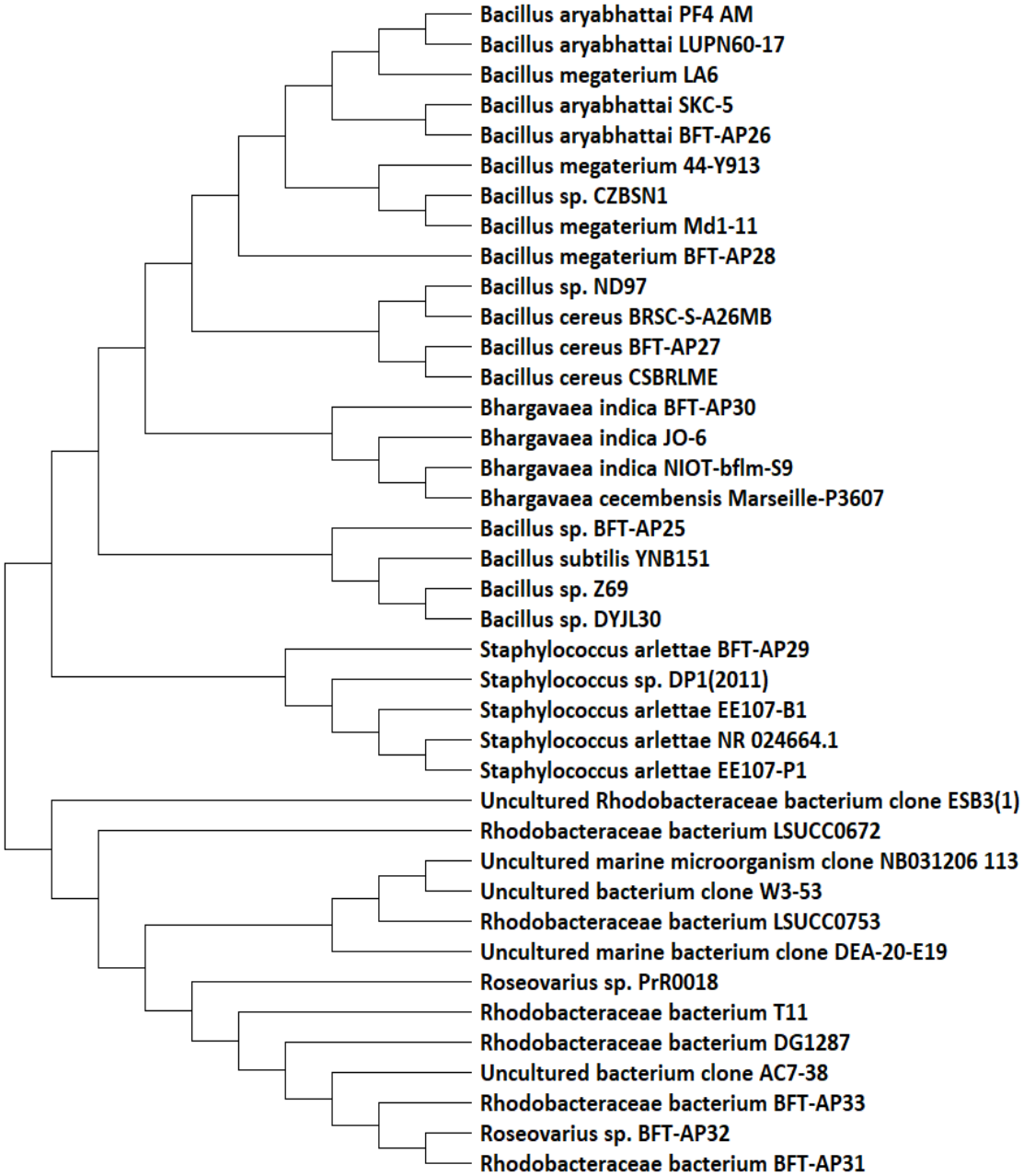

Figure 5. Phylogenetic relationship of identified strains from water samples of BFRf group (Strains names representing BFT-AP are the isolates of this present experiment)

sulfur oxidation and aerobic activity anoxygenic photosynthesis (Pujalte et al., 2014; Bischoff et al., 2019). They are usually associated with either micro or macroalgae in the high planktonic abundance (Ajani et al., 2018).

Bacteria, fungi, microalgae, protozoans, nematode, rotifer, ciliates, and detritus are the conglomerates in the biofloc system (Emerenciano et al., 2017). They maintain and control water quality, remove toxins, produce microbial biomass, improve the nutritive source and increase the candidate animal's immunity (Kim et al., 2014; Rajkumar et al., 2016; Panigrahi et al., 2018; El-Sayed. 2021). In aquaculture, microalgae are regularly used as feed for shrimps, their nutritional property and active involvement in various nutrient recycling levels are critical (Becerra-Dorame et al., 2011). The microbial population in biofloc treatments is diverse due to the addition of carbon sources (Ju et al., 2009; Panigrahi et al., 2019b; Sundaram et al., 2021). A similar trend was observed in

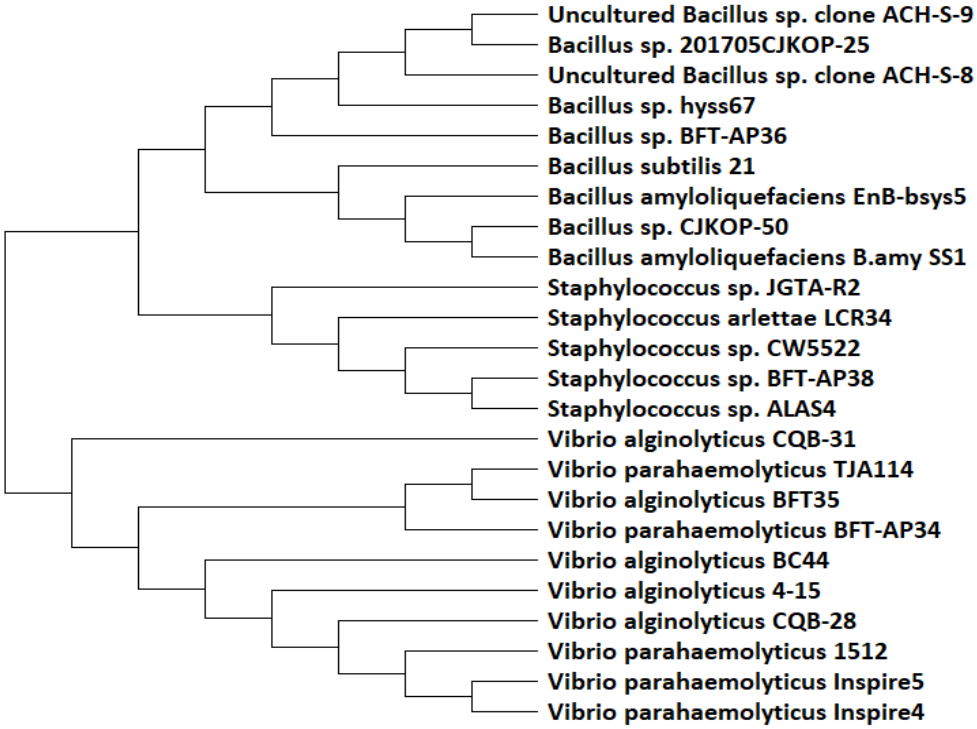

Figure 6. Phylogenetic relationship of identified strains from water samples of control group (Strains names representing BFT-AP are the isolates of this present experiment)

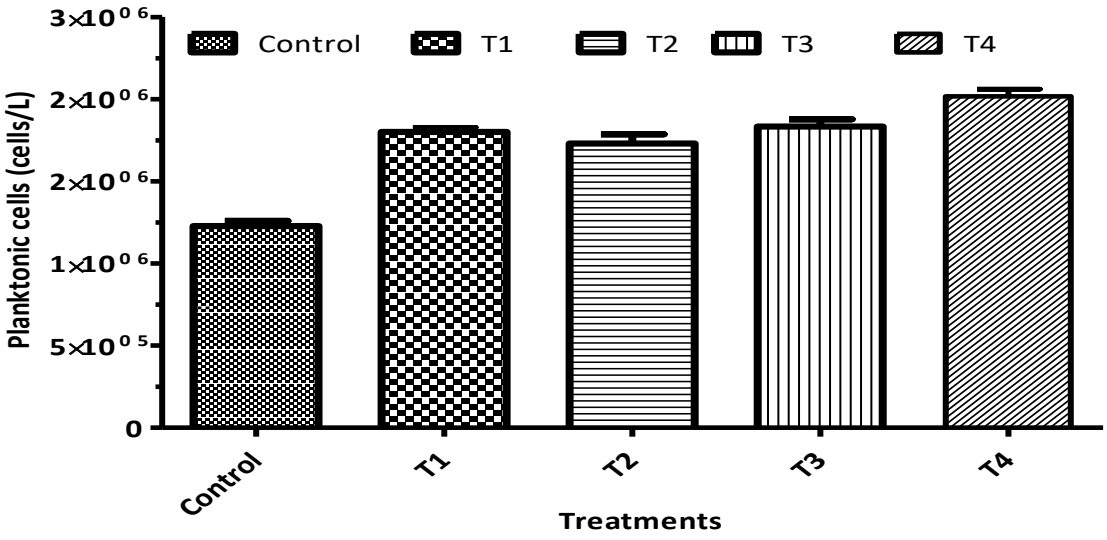

Figure 7. Mean value of planktonic counts present in different treatments (supplemented with different carbon source) and control. Values are means of three replicate tanks per sampling time in each group.

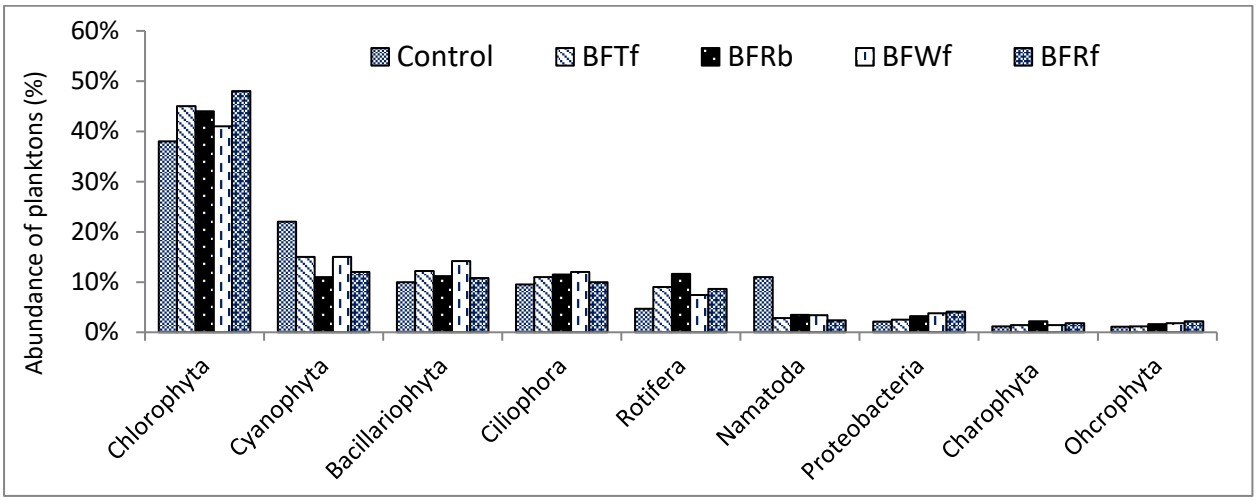

Figure 8. Relative abundance of planktons presents in different carbon source treated biofloc groups and control

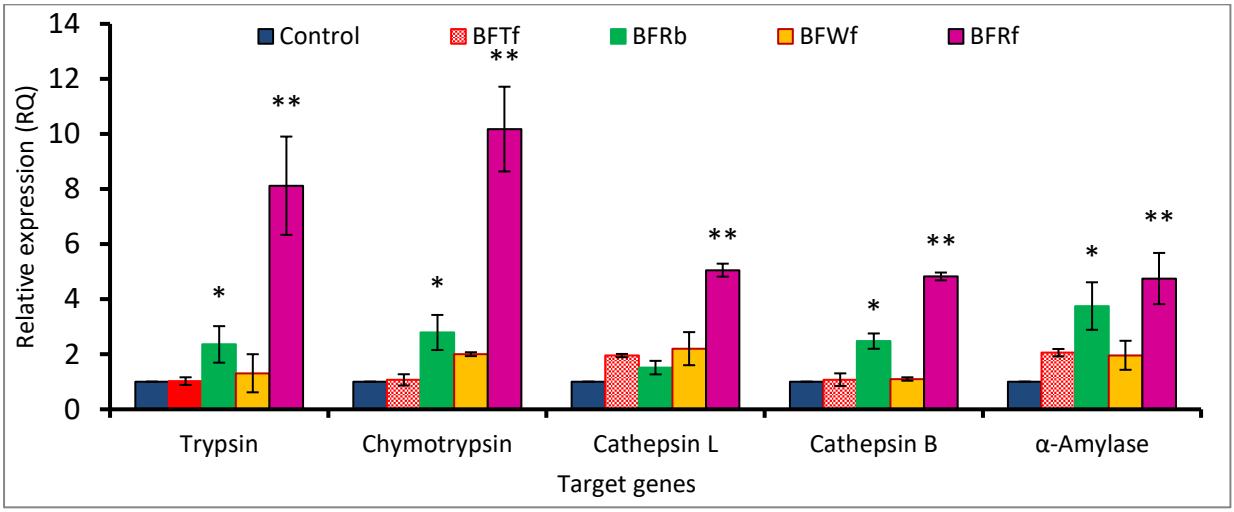

Figure 9a. Relative mRNA expression levels of Trypsin, Chymotrypsin, Cathepsin L, Cathepsin B, and α-Amylase gene in *P. vannamei* reared in biofloc system supplemented with varying complex carbon sources in comparison to that of the control as determined by real-time PCR. Five individual shrimps were analyzed from the control and each of the treatment groups. Data are means ± SD of gene expression in the different carbon source used treatments. Significant differences between different carbon source added groups are marked with *, **(P<0.05).

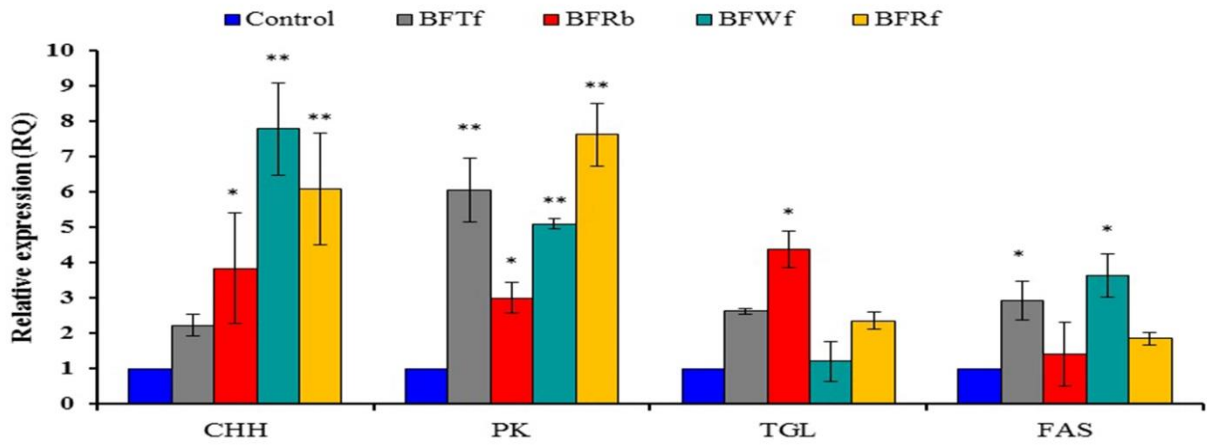

Figure 9b. Relative mRNA expression levels carbohydrate and Fatty Acid metabolism-related genes such as Crustacean Hyperglycemic hormone (CHH), Pyruvate Kinase (PK), Triacylglycerol lipase (TGL), and Fatty acid Synthase (FAS) gene in *P. vannamei* reared in biofloc system supplemented with varying complex carbon sources in comparison to that of the control as determined by real-time PCR. Five individual shrimps were analyzed from the control and each of the treatment groups. Data are means ± SD of gene expression in the different carbon source used treatments. Significant differences between different carbon source added groups are marked with *, ** (P<0.05).

our study. The biofloc treatments generated with different carbon sources were observed to be associated with highly diverse planktonic populations. Planktons serve as a nutritious source for the cultured species (Moss et al., 2001), whereas diatoms and ciliates were observed in low numbers. Rajkumar et al. (2016) and Ray et al. (2010), reported a similar result regarding the plankton community. In general, a higher percentage (41-48%) of chlorophytes was observed in all the treatments, including the control. Chlorophytes are the dominant microalgae observed in biofloc. Ray et al. (2010) also noticed a similar trend in that chlorophytes were highly propagated microalgae in *P. vannamei* culture. Likewise, Maicá et al. (2012) noted an abundance of chlorophytes and diatoms in *P. vannamei* biofloc culture. Lezama-Cervantes et al., (2010) observed that shrimp graze different species of cyanobacteria. Cyanophyta also plays a vital role in nutrient recycling, but many researchers have explained that higher profusion cyanobacteria could be toxic (Beccera-dorome et al., 2011). Simultaneously, Cyanophyta is a primary producer or decomposer in the food chain by incorporating nitrogen (Manan et al., 2016). In our study, the abundance of Cyanophyta and bacillariophyta are nearly similar, followed by a profusion of Rotifera and Ciliophora. Other essential species in biofloc are the Ciliates and Nematodes. Ray et al. (2010) reported that the presence of nematodes and ciliates is correlated. Nematodes and ciliates serve as rich live feed for the animals reared (Jiménez-Ordaz et al., 2021 Silva et al.,2021). Other zooplanktons such as Rotifera and Copepods are rich in nutrients that serve as a live feed in the biofloc system and play a vital role in the food chain in the aquatic system (Ballester et al., 2010; Asaduzzaman et al. 2010). Many bacterial species from proteobacteria were isolated in our study.

The effect of biofloc generated with complex carbon sources on the expression of genes related to digestive enzyme activity was studied. It was observed that shrimp cultured in biofloc exhibited upregulation of digestive enzyme-associated genes, the expression being exceptionally high in BFRf. Five genes viz. trypsin, chymotrypsin, cathepsin L, cathepsin B, Alpha-amylase were taken up for the study relating to the digestive enzymes. Among the five genes, trypsin and chymotrypsin are the mainly proteolytic enzymes present in the decapods, responsible for protein digestion (Muhlia-Almazan et al., 2003; Panigrahi et al., 2019a). Proteolytic enzymes can be significantly affected when there is low food availability or in the presence of non-nutritive food. Biofloc system ensures the continuous availability of food to host animals. Different beneficial bacteria utilize the availability of a higher ratio of carbon source, and the secondary metabolites produced by the bacteria stimulate the digestive enzyme activity of shrimps. Earlier, it was observed that the increasing C: N ratio might stimulate proteolytic and amylolytic activity in the digestive gland and stomach (Xu and Pan, 2012; Xu et al., 2013). *Penaeus vannamei* grown on mixed carbon sources of molasses, cornflour, and wheat bran increased protease, lipase, and amylase activity (Wang et al., 2016). Likewise, Becerra-Dorame et al. (2012) reported that *Penaeus vannamei* possess relatively high protease and amylase enzyme activity when reared in heterotrophic systems than in the autotrophic system. Hence, the availability of high carbon sources and C: N ratio provide a better environment and ensures the continuous possibility of food for the host species (Panigrahi et al., 2021). Accordingly, the digestive enzyme activity was found to be better in biofloc reared shrimp.

Simultaneously, the Crustacean Hyperglycemic Hormone (CHH) was found to be upregulated in biofloc treatments. The CHH functions as a regulator of glucose level in shrimp and participates in the metabolism of carbohydrates and fatty acids. It has an additional role in reproduction, osmoregulation, and ecdysis (Wanlem et al., 2011). Chung et al. (2010) reported that when crustaceans require extra energy, they switch to glycolysis, an anaerobic energy pathway modulated by CHH mainly during stress or when they tend to have low hemolymph glucose. The upregulated expression of CHH correlated with glucose is directly proportional to the digestion of carbon sources by the digestive enzymes (Chakrapani et al., 2021). In addition, pyruvate kinase is also upregulated in biofloc reared animals and acts as a key regulatory enzyme in glycolysis-mediated energy transformation. They catalyze the irreversible transphosphorylation of phosphoenolpyruvate to pyruvate and ATP (Valentini et al., 2002; Sánchez-Paz et al., 2008).

Lipid metabolism is an essential source of energy reserve, used when there is food scarcity for homeostasis and when animals encounter highly fluctuating environments. Lipid utilization varies depending on the changes in seasons (Martínez-Alarcón et al., 2020) and when the animals fight for survival in extreme conditions. Fatty acid synthase (FAS) and triacylglycerol lipase (TGL) are enzymes involved in lipid metabolism. The FAS is a multifunctional enzyme that catalyzes the synthesis of fatty acids (Chakrapani et al., 2021). Yang et al. (2011) reported that FAS counteracts against pathogenic white spot syndrome virus (WSSV) and *V. parahemolyticus* bacterial infection (Zuo et al., 2016). During homeostasis conditions, FAS maintains vitality. The upregulation of FAS and TGL indicates that it influences shrimp immunity and the utilization of lipids in the system (Chen et al., 2015), respectively. Subsequently, the enhanced digestive enzyme activity in the metabolism of carbohydrates, proteins, and lipids gives better performance. Complex carbon sources effectively affect ammonia assimilation and improve digestive enzyme activity by stimulating bio-active compounds in the biofloc system. Therefore, complex carbon sources are equally capable like any other simple carbon source.

Conclusion

Of late, biofloc technology is being developed as a sustainable culture practice in aquaculture. The supplementation of locally available carbon sources and their suitability to the biofloc system is an essential strategy for the success of this technology. The complex carbon sources were tested as substitutes for molasses. Results revealed the improved performance of shrimp in BFRf (rice flour) and BFWf (Wheat flour) complex carbon sources compared to control. Therefore, it could be concluded that the complex carbon sources such as rice flour and wheat flour are equally efficient in assimilating nitrogen metabolites, developing diverse microbial populations, providing better digestibility, enhancing growth, and increasing production in the biofloc system.

Ethical Statement

Ethics approval: The research undertaken complies with the current animal welfare laws in India. The study was undertaken with the approval of the statutory authorities of the Central Institute of Brackishwater Aquaculture, Chennai, India. The experimental animal Penaeus vannamei is not an endangered shrimp; the provisions of the Govt. of India's Wildlife Protection Act of 1972 are not applicable for experiments on this shrimp.

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Author Contribution

CS- drafting the manuscript**,** conducted experiment, performed sampling and analyzing the samples and data interpretation**, AP-** planning of the study, critical evaluation, data interpretation and fund mobility, **SJ-**Conducted Experiment**, MS-** Conducted experiment and provided valuable suggestions to the manuscript**, PE-** reviewed and provided valuable suggestions to the manuscript**, SRV-**analyzed the samples**, AK-**Sampling and data collection

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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