

Review Article

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Microbial Based Culture Systems: For Sustainable Shrimp Production

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ABSTRACT

Marine aquaculture is the fastest growing food producing sector with major contribution from carps in terms of volume and shrimp by value. Continuous development of aquaculture demands new alternative technologies to achieve sustainable production with low economic cost. The microbial based systems like biofloc technology (zero water exchange systems) and artificial substrate integrated (periphyton based) in normal culture systems represents the best methods of practice to get healthy shrimp production. The microbial based systems are eco-friendly aquaculture practices which can stimulate both heterotrophic or autotrophic groups of bacteria by maintaining carbon to nitrogen ratio (C:N) that can convert nitrogenous wastes into useful microbial biomass which can serve as natural food source for the cultured organisms. On other side, biofloc and periphyton helps in improving water quality by the removal of toxic nitrogenous substances from these systems. This technology is economically viable, eco-friendly, and socially acceptable.

Keywords

Microbial floc,
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Introduction

Aquaculture is the fastest growing food sector in the world, but today's industry utmost contest is to yield high quality animals at the economically viable cost. However, the existing intensive shrimp culture systems with more water replacements and feed wastage causing adverse environmental impacts like eutrophication, uncontrollable plankton

growth and others (Martinez-Porchas and Martinez-Cordova 2012). Shrimp culture is completely successful but industry needs to develop new technologies that will enhance the value and environmental sustainability (Kuhn *et al.*, 2010).

As an alternative to the existing intensive culture systems, biofloc technology (BFT) has been developed as an intensive rearing system

to make shrimp farming less cost effective and to increase the nutrient utilization efficiency, provide bioflocs as additional feed, reduce water use and effluent discharges and improve biosecurity (Wasielesky *et al.*, 2006). Bioflocs are heterogeneous mixture of bacteria, filamentous microalgae, protozoans, rotifers, worms and nonliving components. Bacterial growth increases when carbon source is added to the water with vigorous aeration at C/N ratio of 15:1 (Chamberlain and Hopkins, 1994; De Schryver *et al.*, 2008). Biofloc particles contain useful microbes like *Bacillus* sp., *Lactobacillus* sp. and *Vibrio* sp. (Anand *et al.*, 2014, Kumar *et al.*, 2015), bioactive compounds like carotenoids (Xu and Pan, 2013) and are known for probiotic (Crab, *et al.*, 2010) and immunostimulant (Anguilera-Rivera *et al.*, 2014) properties.

Microbial based culture systems are highly suited for shrimps because of its anatomical modifications that will permit them to eat up microbial floc material and assimilate microbial proteins, by that taking advantage of microbial biomass as a natural feed. Wasielesky *et al.*, (2006) reported that the natural productivity in BFT system provides natural in-situ food sources to the culture organism and enhance growth and survival. It is documented that *L. vannamei* mostly feeds on detritus and benthos (Cordova and Pena-Messina, 2005), consume microbial floc in biofloc systems^[12] and can be able to thrive well (Hargreaves, 2013). Growing this species under biofloc based system has shown to improve digestive enzyme activity, growth performances and survival (McIntosh, 2000; Tacon *et al.*, 2002; Buford *et al.*, 2004; Xu and Pan, 2012).

The probiotic effect of microbial floc can act on the *Vibrio* sp. both internally and externally and some external parasites of shrimp (Panigrahi *et al.*, 2014). The periodical addition of carbohydrates in the water is

known to select for polyhydroxy alkananoates (PHA) mobilization of bacteria in the form of bioflocs which can generate poly-β hydroxy butyrate (PHB), having antibiotic and immunomodulatory effects (Sinha *et al.*, 2008) and on consumption of these flocs by shrimp, PHAs degrades in the gut and contribute to antibacterial properties (Defoirdt *et al.*, 2007).

The microorganisms in the bioflocs and their cell metabolites can enhance innate immune system of shrimp and give protection against microbes (Smith *et al.*, 2003; Vazquez *et al.*, 2009). Panigrahi *et al.*, (2007) suggested that bioflocs are having some immune associated genetic materials like prophenoloxidase (PO), PO activation enzymes and serine proteinase1 thus implying immunomodulation in the shrimp. Kim *et al.*(2014) concluded that high total haemocyte count (THC) and PO activity in *L. vannamei* cultured in microbial based culture systems clearly showing significantly higher expression levels of proPO1, proPO2 and PPAE1 genes, which regulate the PO stimulation systems.

The use of artificial substrates is another technique to enhance shrimp yield. These substrates can give space for the growth of periphyton which contains heterogeneous groups of microorganisms which can act as natural food for the cultured organisms and maintained water quality (Langis *et al.*, 1988; Ramesh *et al.*, 1999; Thompson *et al.*, 2002). Some of the widely using natural substrates are bamboo, paddy straw, wood, sugar bagasse (van Dam *et al.*, 2002) and some artificial substrates like polyethylene and polypropylene screens, plastic bottles and certain commercial products (AquamatTM) have been used as in culture systems of fish (Azim *et al.*, 2004) and shrimp (Huchette *et al.*, 2000; Bratvold and Browdy, 2001; Richard *et al.*, 2009; Zhang, 2011). The substrate-based culture methods are also

known as periphyton-based culture systems and the periphyton on substrate is consists of heterogenous mixtures like algae, bacteria, fungi, protozoa, zooplankton, detritus and other invertebrates (Azim and Wahab, 2005). It can absorb nutrients such as ammonia and nitrite from water and maintained the dissolved oxygen (DO) and pH (Azim *et al.*, 2002; Dodds, 2003; Bender *et al.*, 2004; Schweitzer *et al.*, 2013).

The main aim of this review is application of microbial based culture systems in shrimp culture for sustainable production. The above information is mainly focus on the use of complex mixture of microorganisms such as bacteria, filamentous microalgae, protozoans, rotifers, worms and nonliving components as a natural food for the shrimp. Diverse studies have been conducted in this field with successful and useful results in shrimp culture. In addition to that, this review also gives basic knowledge about biofloc system to the students, researchers and industry people.

Suitability of BFT for Shrimp Culture

The basic factors in selection of species to be reared in a biofloc systems are includes tolerance to high suspended solids of water, physiological adaptations that permit them to feed on microbial biomass and digest (Milstein *et al.*, 2001; Crab *et al.*, 2012; Hargreaves, 2013). In the wild, vannamei ingest vast classes of phytoplankton, debris, macrophytes, small molluscs, crustaceans and zooplankton (Senanan *et al.*, 2009). A study by Kent *et al.*, (2011), revealed the capability of *L. vannamei* juveniles to feed on microalgae, reared in algal culture flasks. Thus crustaceans like shrimp and prawns are suitable species for BFT systems since they are detritivorous and opportunistic feeders which feed on bacteria, fungi and other decomposed material (Milstein *et al.*, 2001).

Coyle *et al.*, (2010) and Perez-Fuentes *et al.*, (2013) Shrimp prefer natural over commercial food as they feed continuously. Hargreaves (2013) specified that the cultured organisms like shrimps and tilapias have morphological modifications that permit them to feed on natural food i.e biofloc and can able to digest it. The ability of various species of shrimp and prawn to utilize biofloc, there by its positive effect on physiological functions and growth is well documented (Table 1).

Nutritional Composition of Biofloc

Bioflocs are the best nutritional resource, but it is depends on the type of systems, environments (Ju *et al.*, 2008a; Hargreaves, 2013). Intrinsically, bioflocs are eating by the reared organisms and start a nutrient recycling process within an aquaculture system. The nutritional worth of bioflocs to cultured organisms is reliant on many agents for instance food selection, both the capability to consume and digest it (Hargreaves, 2006). Looking at nutritional quality of bioflocs as shrimp food, different studies have been done. Biofloc contains adequate quantity of crude protein, essential lipids, carbohydrates and ash required for aqua feed (Crab *et al.*, 2010; Ballester *et al.*, 2010). Ballester *et al.*, (2010) reported biofloc Contains 30.4% of crude protein. This is accordant with 25-50% said by Hargreaves (2013). However, Ekasari *et al.*, (2014) presented 17.2-27.8% protein for biofloc harvested from shrimp culture systems, with Crab *et al.*, (2010) In the bacteriological food web, essential lipids and poly unsaturated fatty acids (PUFA) are mostly released from flagellated and ciliated protozoans groups and other cyanophyta (Zhukova and Kharlamenko, 1999), although majority of microbes are insufficient sources for PUFA (Meyers and Latscha, 1997). Crab *et al.*, (2010) reported that palmitic acids, palmitoleic acids and linoleic acids are the major group of fatty acids in biofloc.

In biofloc the fatty acid profiles may vary based on the carbon sources employed, the type of water used (fresh water, sea water) and the methods of production suggested by Emerenciano *et al.*, (2013). They also reported the presence of essential fatty acids such as linoleic acids, linolenic acids and arachidonic acids *etc.*, in biofloc. In biofloc 6-7.5% of lipid content is found by by Ekasari *et al.*, (2014) and it was within optimum range of 0.5-15% said by Hargreaves (2013). The major variations in nutritional properties of microbial biomass occur due to differences floc development (Rittmann and Mc Carty, 2001). For instance, bacteria could not develop on acetate and glycerol substrates but those can release huge amounts of exopolysaccharides, but are utilized by cultures organisms mostly spend for cell growth which can enhance the protein percentage in microbial biomass (Crab *et al.*, 2010). These studies reported that nutritional value of bioflocs depends on the Carbon : Nitrogen ratio and nature of substrates *etc.* Hence, the fundamental element for the development of biofloc is choice of carbohydrate source. Proximate compositions of microbial biomass in different studies are shown in Table. 2

Microbial Community in Biofloc

Biofloc development in outside system is very much profit oriented because of production of photoautotrophs during day time with light (Ray *et al.*, 2009; Avnimelech, 2012; Baloi *et al.*, 2013). and water colour may change from a green algal type to a brown bacterial type by increasing feeding rate, and calculated for microbial groups as colour index (Hargreaves, 2013). This colour change is one of the most important methods for reduction of ammonia to microbial groups, which can also demand more aeration (Hargreaves, 2013). The beneficial bacteria in the BFT are providing a natural food and maintain quality of water

(Avnimelech, 2012). In BFT, chemoautotrophic nitrifying bacteria, and heterotrophic ammonia assimilating bacteria are two important bacterial groups to maintain water quality (Ebeling *et al.*, 2006; Ray *et al.*, 2010a), with the actions high oxygen demand and low level of alkalinity, which needs more DO supply (Ray *et al.*, 2010a).

The pathway of nitrifying bacteria in culture system has been widely studied (Tal *et al.*, 2003; Michaud *et al.*, 2006; Crab *et al.*, 2007). The main principle is conversion of total ammonia nitrogen to nitrites and nitrite is converted into nitrate which is less toxic to fish (Peng and Zhu, 2006; Ray *et al.*, 2010b; Hargreaves, 2013) and simultaneously the removed TAN may developed it into cellular protein and it is providing natural food for rearing animals (Ebeling *et al.*, 2006; Ray *et al.*, 2010a). In addition to that, the rate of growth of heterotrophic bacteria is ten times more than nitrifying bacteria when sufficient organic carbon supplied, which may results in reduction of TAN levels and increment of microbial community and nutritional quality [63, 12, 13]. Some studies have been carried out on microbial community structure and composition of bioflocs in shrimp culture (Hargreaves, 2006; Crab *et al.*, 2012; Hargreaves, 2013). Some studies have been carried out on microbial community structure and composition of bioflocs in shrimp culture (Ju *et al.*, 2008a; Ballester *et al.*, 2010; Anand *et al.*, 2014; Ekasari *et al.*, 2014; Kumar *et al.*, 2015). According to Ju *et al.*, (2008) microbial biomass from *L. vannamei* cultured tanks having 24.6% of phytoplankton (dominated by diatoms like *Thalassiosira*, *Chaetoceros* and *Navicula*), 3% bacterial groups (two-third was G-ve and one-third of G+ve), a small quantity of protozoans (98% flagellates, 1.5% rotifers and 0.5% amoebae), 33.2% of dead material and the remaining ash (39.25%). It indicates that microbial biomass having heterogenous groups organisms.

Biofloc from *F. paulensis* cultured tanks were consists of heterotrophic bacteria, cyanobacteria, dinoflagellates, ciliates, flagellates and rotifers (Ballester *et al.*, 2010).

Anand *et al.*, (2014) The dominant microbial groups are *Vibrio* sps, *Lactobacillus* sps, *Bacillus* sps and fungi in biflocs. However, *Vibrio* sps. and *Bacillus* sps. dominated over *Lactobacillus* sps. The probiotic bacteria such as *Bacillus* sps. and *Lactobacillus* sps. in their gastrointestinal tract (GIT) of *L. vannamei* can leads to enhance digestive enzyme activity and immune response (Xu and Pan, 2013). The digestive enzyme secretions from *Bacillus* and *Lactobacillus* in the carbon addition systems shows better growth rate (Ringo *et al.*, 2012; Anand *et al.*, 2014). The microbial biomass in BFT can increase the digestive and assimilative capacity of shrimp resulted in best growth rate and survival (Anand *et al.*, 2014). In another study by Ekasari *et al.*, (2014) revealed revealed significantly lower ($P < 0.05$) total viable bacteria count (TBC) in the waters of conventional semi intensive system over the biofloc system. Kumar *et al.*, (2015) reported that molasses added treatment tank with highest level of TBC and *Vibrio* count than non-biofloc system.

Microbial role in stimulation of digestive enzyme activity

The microbial biomass in BFT plays a major role in stimulation of digestive enzymes activities in shrimps (Moss *et al.*, 2001; Xu *et al.*, 2012a) which could increase the growth rate of shrimp (Anand *et al.*, 2014). Xu and Pan (2012) The increment of protease and amylase enzyme activities in *L. vannamei* reared in BFT systems. The beneficial bacterial groups such as *Bacillus* sps. in the biofloc helps to modify the physiological and immunological status of the animal, by colonization of bacterial in the GIT (Zhao *et*

al., 2012; Xu and Pan, 2013). Studies in similar lines has shown increased level of digestive enzymes activity in fish and shrimp fed with probiotics, microalgae and periphyton supplemented diets (Lara-Flores *et al.*, 2003; Ziaei-Nejad *et al.*, 2006; Anand *et al.*, 2014). In shrimps microbial flocs shows positive effect towards the enzyme activity which can accelerate feed digestibility and utilization suggested by Xu and Pan (2014). The feeding of microbial floc at 4% and 8% level to the prawns, it can increase the growth performance and digestive enzyme activity of like amylase, cellulose and protease in BFT systems compared with control reported by (Anand *et al.*, 2014).

Microbial role in water quality maintenance

Microbial floc is another technology for keeping better water quality by the *in situ* action of living bacteria in the water, dominantly by heterotrophic group of bacteria (Avnimelech 1999 & 2012; Hargreaves, 2013). The supplementation of tapioca into *P. monodon* culture system can reduce the total ammonium nitrogen and $\text{NO}_2\text{-N}$ concentrations reported by Hari *et al.*, (2004 & 2006). The addition of tapioca in *M. rosenbergii* culture systems significantly decreases the TAN and $\text{NO}_2\text{-N}$ concentrations reported by Asaduzzaman *et al.*, (2008). Ray *et al.*, (2011) measured water temperature, DO, pH and salinity twice per day and TAN, nitrite and nitrate once a week in *L. vannamei* culture systems with two levels of biofloc *viz.*, low biofloc level (T-LS) and high biofloc level (T-HS) has shown higher TAN, NO_2 and lower NO_3 levels in T- LS than that of T-HS treatment. Raj Kumar *et al.*, (2015) studied in three different biofloc systems *viz.*, molasses, tapioca and wheat on the water quality, microbial floc composition and growth in *L. vannamei* revealed significantly less nitrogen species level in biofloc systems

than that of control system without biofloc. Luis-Villaseñor *et al.*, (2015) recorded water temperature, DO, pH, salinity twice daily and TAN, nitrite and nitrate at weekly intervals in *L. vannamei* culture showed significantly lower TAN, nitrite and nitrate levels in BFT system than control system. However, Effendy *et al.*, (2016) observed significantly higher ($P < 0.01$) values of NO_2 and NO_3 in semi biofloc system in comparison with semi intensive culture system of *Fenneropenaeus indicus*. Manan *et al.*, (2017) also conducted experiment on bioremediation process in biofloc technology of *P.monodon* and concluded that the microorganisms in biofloc act as natural bioremediation agents ensure that they can maintain optimum water quality for shrimp.

Microbial role in enhancement of growth in shrimp

The growth efficiency is the foremost important to the aqua farmers throughout the world. The benefits of biofloc towards growth and survival of shrimp has been reported by Hari *et al.*, (2004), Wisielesky *et al.*, (2006), De Schrivey *et al.*, (2008), Avnimelech and Kochba (2009), Krummenaeuer *et al.*, (2011), Xu and Pan (2012), Kim *et al.*, (2014), Kumar *et al.*, (2015), Luis-Villasenor *et al.*, (2015) and Effendy *et al.*, (2016). Shrimp are mostly detritus feeders (Focken *et al.*, 1998) that feeds on bacteria, fungi and decomposing material (Milstein *et al.*, 2001; Serfling, 2006), thus capable of utilizing biofloc as food. The biofloc can elevate the rate of nutrient ingestion, absorption, assimilation and it can give a source of cellular nutrition in vannamei sps. (Tacon *et al.*, 2002). The shrimp or fish has so many benefits by feeding on biofloc such as supply of various nutrients by the microbial community, increasing feed utilization and growth, stimulating digestive enzyme activities, lowering FCR and enhancing immune system (Burford *et al.*, 2004; Moss *et al.*, 2006;

Wasiolesky *et al.*, 2006; Ballester *et al.*, 2010; Jang *et al.*, 2011; Emerciano *et al.*, 2012; Xu and Pan, 2012; Perez-Fuentes *et al.*, 2013).

Xu and Pan (2012) reported more survival rate and better growth of vannamei in the BFT system with carbohydrate addition. Hargreaves (2013) reported that 20-30% of shrimp growth in biofloc based culture system is obtained from microbial proteins in the water. The rate of assimilation of protein and lipid in biofloc treatments showed a positive contribution of biofloc produced from waste material as a natural feed for the cultured organisms with lower FCR in the BFT systems (Megahed *et al.*, 2010; Zhao *et al.*, 2012; Xu *et al.*, 2012; Gao *et al.*, 2012). Xu *et al.*, (2012) reported that BFT-30% and BFT-35% crude protein (CP) feed tank with significantly higher weight gain and SGR in *L. vannamei* juveniles when compared to control (clear water and 35% CP feed) and no significant difference ($P < 0.05$) among BFT-25%, BFT-30% and BFT-35%. Anand *et al.*, (2014) evaluated the effect of biofloc under two carbohydrate sources (rice flour-R and molasses-M) and two levels protein diets (32% and 40%) in *P. monodon* juveniles has shown better ($P < 0.01$) growth rate in (40 + R), (40 + M) and (32 + R) treatments than control groups (32% and 40% protein diets without biofloc). Kumar *et al.*, (2015) found significantly higher ($P < 0.05$) weight gain and protein conversion rate in *L. vannamei* fed on 35% protein diet in biofloc systems with cornmeal as carbohydrate source than the control shrimp without biofloc. Effendy *et al.*, (2016) observed significant increase ($P < 0.01$) in growth of *Fenneropenaeus indicus* fed with 35% protein feed in biofloc systems when compared to clear water system.

Biofloc for shrimp Health Management

The presence of microbial biomass in culture system had been reported to enhance the health of the cultivated organism which may

be due to the inhibition of pathogens reported by Bricknell and Dalmo (2005). Bioflocs is a new technology for disease management with probiotic effect in contrast to conventional materials like antibiotic, antifungal, external probiotic and prebiotic supplementation (Emerenciano *et al.*, 2013). Further, bioflocs are reported to harbor huge microbes and bioactive compounds that shows positive effect on health of the shrimp (Xu and Pan, 2013).

Biofloc is also having amino sugars like glucosamine, muramic acid, bromophenols, phytosterols and pigments like carotenoids, chlorophylls and xanthophylls (Ju *et al.*, 2008a) they can elevate shrimp immune status by stimulating immune system (Linan-Cabello *et al.*, 2002; Burford *et al.*, 2004). In BFT system by regular supplementation of carbohydrate can enhance the growth of polyhydroxyalkanoate (PHA) aggregating microorganisms (Salehizadeh and Shojaosadati, 2001). Bacteria and the synthesized compounds might act as effective bio-control agents while providing the host with beneficial microbial balance in the gut (Emerenciano *et al.*, 2013).

Immunological response of shrimp in biofloc system

The crustacean having a nonspecific immune system with phagocytic and humoral contents, as a foremost defense (Lee and Soderhall, 2001). The common immunostimulants like peptide, lipopolysaccharides, probiotics, microalgae, carotenoids, vitamins and peptides (Devaraja *et al.*, 1998; Ringo *et al.*, 2012). They can elevate immunity power in shrimps and enhance capacity to eject pathogenic bacteria (Flegel, 1997; Nonwachai *et al.*, 2010). Ju *et al.*, (2008a,b) and Anguilera-Rivera *et al.*, (2014) suggested that the microbial flocs can confer benefits to the shrimp immune system which may be due to

the presence of carotenoids, retinoids, poly- β -hydroxybutyrate and exo-enzymes. Microorganisms has been used as immune-stimulants to enhance immune system and/or antioxidant status of shrimp, followed by increasing their resistance towards pathogenic bacteria (Ninawe and Selvin, 2009; Smith *et al.*, 2003; Becerra-Dorame, 2012). It has been reported that Acute Hepatopancreatic Necrosis Syndrome (AHPNS) an raising disease outbreak with heavy mortality rate in shrimp culture appeared to have lower incidence at BFT systems (NACA, 2012) which suggest that biofloc can have positive impact on immune-response of shrimp.

In shrimps, three different haemocytes, *viz.*, a granulocytes, granular and semi-granular haemocytes (SGH) has documented (Rodriguez and Le Moullac, 2000). The haemocytes perform different activities like phagocytosis, encapsulation, nodular aggregation (Soderhall and Cerenius, 1992; Vargas-Albores *et al.*, 1998) and production of Propo enzymes (Lopez *et al.*, 1996). In shrimp defence system granular cells plays an important role by their antimicrobial response (Chisholm and Smith, 1995) similarly a granulocytes act as a phagocytes, and involved in the production of agglutinins and antibacterial peptides (Rodriguez and Le Moullac, 2000).

The importance of total haemocyte count (THC) in disease resistance of shrimp has been documented (Rodriguez and Le Moullac, 2000; Ji *et al.*, 2009; Xu and Pan, 2013). Rodriguez and Le Moullac (2000) reported that low THC value in *L. stylirostris* became very sensitive to virulent *Vibrio alginolyticus*. In penaeids, THC from 20 - 40 x 10⁶ cells ml⁻¹ of haemolymph (Chang *et al.*, 1999). An increased levels of haemocyte count has been recorded after feeding shrimp with feed supplements such as probiotics (Rengpipat *et al.*, 2000), β -glucan (Lopez *et*

al., 2003), macroalgae and β -carotene supplemented diets (Supamattaya *et al.*, 2005). The microorganisms of biofloc ingested by the shrimp may release substances in the gastrointestinal tract that could potently stimulate the innate immunity and release more haemocytes into the circulation (NACA, 2012).

Xu and Pan (2013) significantly higher THC and phagocytic activity of the haemocytes in vannamei cultured in BFT cultures than control. The vannamei cultured in microbial floc tank with addition of 20% to 35% protein levels showed that there is no differences in total haemocyte count and immune status between treatment tanks showing that there is no effect by decreasing the protein level in the biofloc tanks (Xu and Pan, 2014). Ekasari *et al.*, (2014) found no significant difference in THC of vannamei shrimp rearing in biofloc tanks using various carbon sources. However, THC of vannamei from biofloc system having high values than control. Toledo *et al.*, (2014) also reported no significance difference in THC of *L. vannamei* juveniles fed on diets with three different dietary lipids grown in molasses based biofloc systems. Significantly higher THC, granulocytes and hyaline cells count was observed in haemolymph of *P. monodon* juveniles grown in rice flour based biofloc system compared to controls with no biofloc (kumar *et al.*, 2015). *F. indicus* juveniles grown in bifloc ponds have shown higher ($P < 0.01$) THC in haemolymph than that of without biofloc shrimp before and after cold challenge test (Effendy *et al.*, 2016).

In crustaceans, haemolymph components like protein, albumin, glucose, triglycerides and cholesterol levels reflects the nutritional quality of shrimp and it is high in shrimp fed on natural food (Gong *et al.*, 2000) or as a outcome of stress (Hall and Ham 1998; Lara-Flores *et al.*, 2007). In serum protein of shrimps around 90-95% of haemocyanin is

present (Depledge and Bjeregaard, 1989) and therefore its reduction can possibly affect specific immune proteins of that animal (Perazzolo *et al.*, 2002). The serum total protein concentration in tiger shrimp was reported to decreased after exposing to the higher level of nitrite nitrogen (Chen and Cheng, 1995).

An experimental study by Anand (2012) revealed significantly higher ($P < 0.05$) levels of serum protein in *P. monodon* juveniles fed on biofloc incorporated diets when compared the feed without biofloc component. Kumar *et al.*, (2015) reported significantly higher ($P < 0.01$) serum protein levels in monodon juveniles cultured in microbial floc systems than that of conventional system. Both semi-granular and granular cells are active in the proPO activity, which has a role in identification and defense in crustaceans (Rodriguez and Le Moullac, 2000; Chiu *et al.*, 2007). Phenoloxidase is an enzyme of the crustaceans that leads to melanisation of foreign cells to inactivate and suppress their multiplication in the host. Phenoloxidase is mostly produced by cell wall of bacteria such as β -1,3-glucans and lipopolysaccharides (LPS) (Perazzolo and Barracco, 1997; Sritunyalucksana and Soderhall (2000); Cerenius and Soderhall, 2012). Shrimps are consuming biofloc as a natural food in BFT systems (Crab *et al.*, 2012; Ekasari *et al.*, 2014) that increases in THC and PO activity which was confirmed in vannamei biofloc systems by Kim *et al.*, (2014) clearly showing significantly higher levels of proPO1, proPO2 and PPAE1 genes, which regulate the proPO activation systems. Higher PO activity in *L. vannamei* grown in the biofloc systems was also observed during both pre and post challenging test by injection with infectious myonecrosis virus (IMNV) than that of shrimp from the non-biofloc control system (Ekasari *et al.*, 2014). Kumar *et al.*, (2015) reported higher ($P < 0.05$) serum

phenoloxidase activity in monodon juveniles reared in BFT system than control system.

In crustaceans, lysozymes are the primary defenders against pathogens (Vazquez *et al.*, 2009). The invertebrate (*i-lyz*) lysozyme attacks both Gram-positive bacteria (Vocadlo *et al.*, 2001) and G-ve bacteria (Peregrino-Uriarte *et al.*, 2012). The *c-lyz* (like hen egg white lysozyme) has been defined in various crustaceans like *L. vannamei* (Mundo *et al.*, 2003), *P. Japonicas* (Hikima *et al.*, 2003), *P. chinensis* (Bu *et al.*, 2008), *P. monodon* (Supungul *et al.*, 2002; Tyagi *et al.*, 2007), *F. merguensis* (Mai and Hu 2009) and *L. stylirostris* (Lorgeril *et al.*, 2005; Mai and Hu, 2009). Vega *et al.*, (2006) reported antibacterial activity of *c-lyz* from *L. vannamei* against *V. alginolyticus*, *V. parahemolyticus* and *V. cholerae*. Chen *et al.*, (2015 reported decreased lysozyme activity in vannamei that dipped in seawater having 600 ppm *Gracilaria tenuistipitata* extract after 72 hrs of post ammonia (5 ppm) stress while in control the lysozyme activity significantly decreased ($P < 0.05$) after 24 hrs of exposure to 5 ppm of ammonia itself. Therefore, vannamei that received *Gracilaria tenuistipitata* extract showed slow decreased and fast recovery in lysozyme activity than to their actual values showing a positive immune response of lysozyme against ammonia stress.

Biofloc production and maintenance

Bioflocs are defined as aggregates (flocs) of algae, phytoplankton, free and attached bacteria, grazers of bacteria such as ciliates, flagellates, rotifers, nematods and copepods, living and dead material such as faeces and uneaten feed (Hargreaves, 2006; Crab *et al.*, 2009; Ray *et al.*, 2010b; Ballester *et al.*, 2010; Hargreaves 2013), yeast, cyanobacteria (Becerra-Dorame, 2012), colloids, organic polymers, cations and dead cells (De Schryver *et al.*, 2008) and inorganic particles (Burford

et al., 2003). Usual bioflocs are in unevenly manner, have a variety of distribution of particle size, are fine, easily compressible, and highly porous and are permeable to fluids (Chu and Lee, 2004). The development of biofloc in a new rearing culture tank requires inoculum such as soil or high microbial floc water from a previous production system (Gaona *et al.*, 2011). Avnimelech (1999) reported using of 20 g clay soil, 10 mg Ammonium Sulphate [(NH₄)₂SO₄] and 200 mg glucose (carbohydrate source) shaken for 12 h with 1L tap-water for the initial production of bioflocs. Similarly used 20 g of pond bottom soil in 1L of well aerated water containing 10 mg of (NH₄)₂SO₄ and 400 mg of different carbon sources *viz.*, tapioca, wheat, corn and sugar bagasse and were incubated for 24 h for initial floc development.

The main factor for the formation of microbial flocs is the capability of microbial cells to form microbial aggregates and quorum sensing property (De Schryver *et al.*, 2008). Another contributing factors are Carbon:Nitrogen ratio, type of carbon source and inorganic nitrogen, aeration, pH, temperature etc. The C:N ratio plays an important role in BFT systems (Hargreaves, 2013), ¹, and is an essential factor in development of inorganic nitrogen to microbial cells (Avnimelech, 1999; McIntosh, 2000; Hargreaves, 2013). The conversion rate of heterotrophic bacteria in general is 40-60%, carbon:nitrogen ratio of 10 or more in food is required for the growth of heterotrophic bacteria (Avnimelech, 1999). By increasing the C:N ratio, the heterotrophic bacteria can absorb ammonia from the water and converted into microbial biomass, containing protein (Hargreaves, 2013). Fontenot *et al.*, (2007) used four C:N ratios (5:1, 10:1, 20:1 and 30:1) in shrimp cultures by supplying molasses, and concluded that the C:N ratio of 10:1 gave the better results in

terms of high level of inorganic nitrogen removal from water. However, Luo *et al.*, (2014) reported that C:N ratio should be > 10:1. Hargreaves (2013) reported that C:N ratio 12-15:1 will support the heterotrophic bacteria. Further ^[67] reported with optimal ratio of C:N from 15-20:1.

In the case of high TAN levels Avnimelech (2012) suggested to apply 20 ppm of organic carbon to decrease TAN level by 1 mg/l. The supplementation of organic carbon in biofloc systems is very close to the feeding rate and protein content, or nitrogen content of the diet (Hargreaves, 2013). He reported that for every one kilogram of feed 30-38% protein added to the water, 0.5-1 kg of carbohydrate should be supplied to keep high C:N ratio.

Inorganic nitrogen and carbohydrate addition are primary materials for huge production of heterotrophic bacteria in BFT systems (Avnimelech, 1999; Ebeling *et al.*, 2006). Heterotrophic bacteria utilize inorganic nitrogen produced from feed, excretions or fertilizers (Ammonium Sulphate). The carbohydrate acts as a substrate for processing BFT and development of microbial floc (Avnimelech, 1999). Even though, selection of carbon source depends on its cost, easy availability, biodegradability and efficiency of bacterial assimilation. Various types of carbon sources used with different cultured species in BFT systems are presented in table.3.

The organic carbohydrate supplementation is related to the feeding rate and is generally added to water once, or twice in a day, after feeding (Avnimelech, 1999; Azim and Little, 2008; Xu *et al.*, 2012). Wheat powder was used as carbon because of its easy accessibility and making of best quality microbial floc (Azim and Little, 2008; Ballester *et al.*, 2010; Anand *et al.*, 2014; Raj Kumar *et al.*, 2015). Anand *et al.*, (2014) used 4.03 Kg of wheat

flour to develop 1kg of floc. Earlier, Kuhn *et al.*, (2009) reported 1 kg microbial floc production from 1.5 kg of sucrose in a bioreactor.

Raj Kumar *et al.*, (2015) reported that the addition of wheat flour could help to develop microbial floc and maintain water quality and increase shrimp yield over molasses, tapioca flour biofloc systems. Similarly, Anusha (2017) observed best floc composition in wheat flour produced biofloc when compared to tapioca, rice flour and molasses as carbon sources.

Suitability of Substrate based Biofloc Technology for Shrimp Culture

The artificial substrates are new methods employed in the growth of aquatic animals to elevate efficiency in rearing systems (Schweitzer *et al.*, 2013). Artificial substrates like polyethylene and polypropylene sheets, plastic bottles, nets and other materials (AquamatTM, Meridian, Calverton, MD, USA) have been produced and used under different culturing conditions, and reports of these experiments showing that these substrates can significantly increase the yield and survival of shrimps (Huchette *et al.*, 2000; Bratvold and Browdy 2001; Richard *et al.*, 2009; Zhang, 2011; Zhang *et al.*, 2014). Autotrophic microalgae and zooplankton present in periphyton of substrate based methods gives *in situ* food as microalgae components *viz*, diatoms, ciliates, flagellates, copepods and nematodes for rearing organism (Arnold *et al.*, 2005; Abreu *et al.*, 2007; Fernandes da Silva *et al.*, 2008; Audelo-Naranjo *et al.*, 2010). According to Burford *et al.*, (2004) epiphytes offering carbon and nitrogen (39-53%) to shrimp as they can be easily utilized by shrimp. In intensive cultures, substrates provide an extra surface area to the shrimp, which can decrease the competition for space and cannibalism

(Abdussamad and Thampy, 1994). The bottom living animals like shrimps are constrained to two dimensional space than three-dimensional space (Kumlu *et al.*, 2001; Otsoshi *et al.*, 2006) and have been reflects that the artificial substrates increase the space for living (Tidwell *et al.*, 1999; Arnold *et al.*,

2005; 2006; Zarain-Herzberg *et al.*, 2006). Addition of vertical substrate into shrimp culture environment helps in removal of suspended solids which can significantly reduce the organic pollutants load (Langis *et al.*, 1988; Hargreaves, 1998; Ramesh *et al.*, 1999).

Table.1 Studies on shrimp and prawn species as suitable candidates for biofloc culture systems

SL.No	Species	Reference
1	<i>Litopenaeus vannamei</i>	Buford <i>et al.</i> , 2004; Wasielesky <i>et al.</i> , 2006; Samocha <i>et al.</i> , 2007; Ju <i>et al.</i> , 2008a; Kuhn <i>et al.</i> , 2008, 2010; Krummenauer <i>et al.</i> , 2011; Emernciano <i>et al.</i> , 2012; Bauer <i>et al.</i> , 2012; Maica <i>et al.</i> , 2012; Ekasari <i>et al.</i> , 2014; Luis-Villaseñor <i>et al.</i> , 2015; Raj Kumar <i>et al.</i> , 2015; M. H. Khanjani <i>et al.</i> , 2016.
2	<i>Penaeus monodon</i>	Avnimelech and Mokady, 1988; Panjaitan, 2004.
3	<i>Penaeus setiferus</i>	Emernciano <i>et al.</i> , 2009.
4	<i>Farfantepenaeus paulensis</i>	Emernciano <i>et al.</i> , 2011a.
5	<i>Penaeus stylirostris</i>	Emernciano <i>et al.</i> , 2011b.
6	<i>Farfantepenaeus brasiliensis</i>	Emernciano <i>et al.</i> , 2012.
7	<i>Fenneropenaeus indicus</i>	Effendy <i>et al.</i> , 2016.

Table.2 Proximate analysis of Biofloc particles in different studies

Crude protein%	Carbohydrate %	Lipids%	Crude fiber%	Ash%	Reference
43.00	-	12.5	-	26.5	McIntosh <i>et al.</i> , 2000
31.2	-	2.6	-	28.2	Tacon <i>et al.</i> , 2002
12.0-42.0	-	2.0-8.0	-	22.0-46.0	Soares <i>et al.</i> , 2004
31.1	23.6	0.5	-	44.8	Wasielesky <i>et al.</i> , 2006
26.0-41.9	-	1.2-2.3	-	18.3-40.7	Ju <i>et al.</i> , 2008a
30.4	-	1.9	12.4	38.9	Ju <i>et al.</i> , 2008
49.0	36.4	1.13	12.6	13.4	Kuhn <i>et al.</i> , 2009
38.8	25.3	<0.1	16.2	24.7	Kuhn <i>et al.</i> , 2010
28.8-43.1	-	2.1-3.6	8.7-10.4	22.1-42.9	Maica <i>et al.</i> , 2012

Table.3 Various carbon sources tested in BFT systems

S.No	Carbon Source	Culture Species and Reference (s)
1	Acetate	<i>M. rosenbergii</i> (Crab <i>et al.</i> , 2010)
2	Cassava meal	<i>P. monodon</i> (Avnimelech and Mokady, 1988)
3	Cellulose	<i>O. niloticus</i> (Avnimelech <i>et al.</i> , 1989)
4	Corn flour	<i>Hybrid bass</i> (Milstein <i>et al.</i> , 2001); <i>Hybrid tilapia</i> (Asaduzzaman <i>et al.</i> , 2010)
5	Corn meal	<i>L. vannamei</i> (Luis-Villasenor <i>et al.</i> , 2015)
6	Dextrose	<i>L. vannamei</i> (Suita, 2009); <i>L. vannamei</i> Serra <i>et al.</i> , (2015)
7	Glycerol and glycerol+Bacillus	<i>M. rosenbergii</i> (Crab <i>et al.</i> , 2010)
8	Glucose	<i>M. rosenbergii</i> (Crab <i>et al.</i> , 2010)
9	Molasses	<i>L. vannamei</i> (Burflord <i>et al.</i> , 2004; Samocha <i>et al.</i> , 2007) ; <i>P. monodon</i> (Panjaitan, 2004); <i>P. monodon</i> (Kumar <i>et al.</i> , 2015)Serra <i>et al.</i> , (2015), <i>F. indicus</i> (Effendy <i>et al.</i> , 2016)
10	Rice flour	<i>P. monodon</i> (Kumar <i>et al.</i> , 2014) <i>P. monodon</i> (Kumar <i>et al.</i> , 2015) <i>L. vannamei</i> Serra <i>et al.</i> , (2015)
11	Sorghum meal	<i>O. niloticus</i> (Avnimelech <i>et al.</i> , 1999)
12	Soya hull	<i>Fenneropenaeus indicus</i> (Effendy <i>et al.</i> , 2016)
13	Starch	<i>O. niloticus x O.aureus</i> (Crab <i>et al.</i> , 2009); <i>Mozambique tilapia</i> (Avnimelch, 2007)
14	Sucrose	<i>L. vannamei</i> (Xu and Pan, 2014) and Xu <i>et al.</i> , (2013)
15	Tapioca	<i>L. vannamei</i> (Hari <i>et al.</i> , 2004); <i>M. rosenbergii</i> (Asaduzzaman <i>et al.</i> , 2008)
16	Tapioca by product	<i>L. vannamei</i> (Ekasari <i>et al.</i> , 2014)
17	Wheat flour	<i>O. niloticus</i> (Azim and Little 2008; Anusha, 2017); <i>P. semisulcatus</i> (Mohamed, 2010); <i>P. monodon</i> (Anand <i>et al.</i> , 2012); <i>L. vannamei</i> (Raj Kumar <i>et al.</i> , 2015)
18	Wheat bran + molasses	<i>F. brasiliensis</i> (Emerenciano <i>et al.</i> , 2012); <i>F. paulensis</i> (Emerenciano <i>et al.</i> , 2011b); <i>F. duorarum</i> (Emerenciano <i>et al.</i> , 2012); <i>F. paulensis</i> (Ballaster <i>et al.</i> , 2010)

Nutritional Value of Periphyton

Periphyton defined as a complex material attached on immersed substrates consists of phytoplankton, zooplankton, benthic organisms and detritus (Weitzel, 1979; Azim *et al.*, 2004). Microalgae produced on substrate are high sources of nutrient compounds such as fatty acids, amino acids, vitamins and pigments (Thompson *et al.*, 2002; Rajkumar and Kumaraguru-Vasagam,

2006; Khatoon, 2006). Protozoans and nematodes are major source of fatty acids [65]. Diatoms can give essential fatty acids and their lower fibre content can easily digested by shrimp (Zhukova and Kharlamenko, 1999). For example, *Penaeus* larva requires 44% of protein and natural food organisms plays an important role in growth (Colvin and Brand, 1977). The utilization of periphyton elevates the nutritional quality of shrimp (Audelo-Naranjo *et al.*, 2011) and it is also

depends on the type of substrate and submersion time. Ledger and Hidrew (1998) recorded 2-3% protein, 0.04-0.29% lipid, and 29-33% carbohydrate in periphyton developed on stones. Azim *et al.*, (2002) recorded that more protein from bamboo (32%) followed by kanchi (30%) and jute stick (13%). Keshavanath *et al.*, (2004) recorded 19.27-35.56% of protein in periphyton produced on bamboo. Becker (2007) reported 35-63% of protein, 10-57% of lipid and 2-22% of fat and some algae.

Water Quality in Substrate Systems

Periphyton on immersed substrate plays a major role in maintaining water quality in rearing ponds. Algae on substrate increase nitrification process and keep low levels of TAN and nitrite in water (Ramesh *et al.*, 1999; Thompson *et al.*, 2002; Khatoon *et al.*, 2009). Artificial substrate can reduce turbidity level of water and sufficient plankton in water (Asaduzzaman *et al.*, 2008; 2009a). Drenner *et al.*, (1997) used fish and periphyton for removing of nutrients from the water. Azim *et al.*, (2001) reported that suspended solids in water were caught on the substrate, could reduce ammonia and nitrate, produce oxygen, breaking down organic matter and enhance nitrification process. Periphyton present on the substrates can besides assimilating ammonia and nitrite, can also maintain optimum dissolved oxygen level, maintain pH of the water (Azim *et al.*, 2002; Dodds, 2003; Bender *et al.*, 2004; Schweitzer *et al.*, 2013). Arnold *et al.*, (2006) evaluated water quality parameters on weekly basis in rearing *P. Monodon* (PL15) for 56 days at two stocking densities ($1000/m^{-3}$ & $2000/m^{-3}$) with artificial substrates and without artificial substrates. Weekly reports shown that there is no significantly different ($P > 0.05$) in temperature, dissolved oxygen and pH among all tanks. The mean concentrations recorded by them for TAN, nitrite and nitrate with and without artificial substrates were 0.39 ± 0.17

ppm and 0.86 ± 0.15 ppm; 0.62 ± 0.09 ppm and 0.99 ± 0.34 ppm and 1.44 ± 0.09 ppm and 0.48 ± 0.13 ppm respectively. Zhang *et al.*, (2016) recorded significantly lower mean ($P < 0.05$) values of nitrite and nitrate in substrate based systems than the control in *L. vannamei* rearing water. However, no significant difference ($P < 0.05$) in mean values of TAN was observed between control and substrate groups.

Growth of shrimp in SBT Systems

The production output of *P.monodon* juveniles both in terms of growth and survival was significantly increased ($P < 0.05$) by keeping submerged substrates (Arnold *et al.*, 2006). Ballester *et al.*, (2007) reported higher ($P < 0.05$) final weight, survival and biomass in pink shrimp, *Farfantepenaeus paulensis* cultured in cages with polyethylene screen as substrates than that in cages without substrates. Uddin *et al.*, (2007) reported that *O. niloticus* (GIFT) in polyculture with the freshwater prawn (*Macrobrachium rosenbergii*) showed significantly higher growth, survival and yield of both GIFT and freshwater prawn in ponds with bamboo substrates than in the control ponds. Zhang *et al.*, (2010) reported significantly increased ($P < 0.05$) weight gain, survival and yield in *L. vannamei* with increase in number of substrates. Presence of substrates in the culturing systems has better growth of vannamei (Zhang, 2011). Similarly, Zhang *et al.*, (2014) has showed significantly higher ($P < 0.05$) growth and survival of vannamei juveniles in substrate (bolting silk net) based ponds than that in control pond without artificial substrate under winter indoor condition.

Immune Response of Shrimp in SBT System

Studies by Zhang *et al.*, (2010) observed significantly decreased ($P < 0.05$) activity of

non-specific immune parameters like phenoloxidase, antibacterial activity, lysozyme activity, peroxidase and hemolysin of *L. vannamei* juveniles reared in PVC aquaria with polypropylene fabrics screen substrates than those of control group. With an increasing substrates nos, the activity of these parameters were reduced, suggesting that submerged substrates can mitigate the negative impact of the more stocking density through providing enough living space for shrimp. The protein content of *L. vannamei* in both haemolymph and muscle, cultured in 1000 lit capacity cylindrical polyethylene tanks with Aquamats was more than that of control, due to the higher availability (and diversity) of the periphyton (Audelo-Naranjo *et al.*, 2012).

Periphyton Production in SBT Systems

The production of periphyton on immersed substrates is manipulated by different factors like accessibility of nutrients in the water, availability of light, depth of water, type of substrates etc. (Hay, 1991; Konan-Brou and Guiral, 1994; Keshavanath *et al.*, 2001). Nature and type of substrate plays a major role on periphyton development, ash free dry matter, pigments and algal colonisation on the substrate (Anderson and Underwood, 1994; Keshavanath *et al.*, 2001; Azim *et al.*, 2001; Danilov and Ekelund, 2001). The high periphyton biomass [dry matter (DM) and ash free dry matter (AFDM)] was noticed on bamboo substrate than jute stick, paddy straw and bagasse reported by (Keshavanath *et al.*, 2001; Rai *et al.*, 2008).¹ In marine environment the mean DM and AFDM recorded on substrates is 8.8 mg cm⁻² and 4.5 mg cm⁻² on mosquito screen after 30 days, however, 10 out of 13 experiments in freshwaters recorded below 4.5 mg cm⁻² of DM and below 1 mg cm⁻² of AFDM (Azim *et al.*, 2004; Richard *et al.*, 2009).

In conclusion, Biofloc technology (BFT) and periphyton based culture systems are alternative rearing systems to the existing intensive systems to make shrimp farming less cost effective and economically viable, environmentally sustainable. These systems are zero water exchange systems, so they can reduce water usage and effluent discharges and improve biosecurity. Bioflocs and biofilm (periphyton) are having complex mixture of substances such as bacteria, filamentous microalgae, protozoans, rotifers, worms and nonliving components all these materials are used by the shrimp as natural food. These systems can play a major role in sustainable aquaculture via good water quality and decrease in feed requirement, enhance immune response of shrimp and higher yield to achieve more profit in shrimp farming.

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