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Screening of the Bacterial Pathogens in Biofloc Technology based Aquaculture of the *Ctenopharyngodon idella*

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ABSTRACT

Great economic losses in fish aquaculture occur under the unhygienic conditions of the fishponds due to bacterial pathogens. Currently, Biofloc Technology (BFT) has proved successful in wastewater management as well as in controlling pathogenic loads. Since this technology has greatly supported marine fish, very scarce information is available for its successful implementation in freshwater fisheries. Furthermore, the pathogens specific to the carp cultures under the BFT system have not been studied yet. The unique attempt has been carried out in the Microbiology Lab of the Zoology Department of GC Women University, Faisalabad, Pakistan to screen bacterial pathogens in grass carp *Ctenopharyngodon idella* culture based on BFT utilizing agro-industrial wastes as a carbon source. The study confirmed the presence of bacterial isolates belonging to three genera namely Bacillus, Klebsiella, and Staphylococcus in water samples from three treatment groups. Bacillus species dominated over the pathogenic species i.e., Klebsiella and Staphylococcus in all treatment groups and is speculated to inhibit the harmful effects of Klebsiella and Staphylococcus species on the carp fish. This study is very important for the future designing of BFT based culture for freshwater fishes.

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INTRODUCTION

Monitoring and manipulating the microbial community in aquaculture environment hold great potential in improving water quality and controlling development of microbial infections. Because of the rapid increase in human population, there is also need of constant supply of high quality protein, which is fulfilled somehow by shellfish and finfish meat. Production of sea food can be obtained best from cultured species rather than capture fisheries. However, the problem of self-pollution and eutrophication in fish aquaculture is best controlled by microorganisms and probiotics (Tilia *et al*., 2016).

In fish culture, great economic and ecological losses are brought about by the pathogenic bacteria. The vibrios communities in the rearing system of Juvenile *L. vannamei* resulted in lesions in shrimp tissue (Rivera *et al.,* 2014). Bacterial pathogens use several mechanisms and synchronize with other cells to achieve microbial activities required for survival in host cells (Dong *et al*., 2007; Defoirdt *et al*., 2008). For healthy fish growth, aquaculture must be free of important bacterial pathogens like *Aeromonas salmonicida, Flexibacter columnaris, Flavobacterium branciophyla, Edwardsiella tarda* and *Flavobacterium psychrophilum*. Among the various bacterial diseases, Furunculosis, Edwardsiellosis, columnaris disease, bacterial gill disease and Coldwater disease cause major losses to carp fish culture (Sudheesh

et al., 2012).

Recently fish aquaculture has been facing a number of problems due to undesirable environmental impacts resulting from effluent discharge rich in inorganic nitrogenous compounds and organic matter. Biofloc technology has proved potentially sustainable aquaculture wastewater treatment (Bakar *et al.,* 2015). The system using BFT has minimal or null water exchange and nutrient cycling for microorganisms occurrence. Fish biomass and microorganisms, both favor consumption of alkalinity resulting in reduction of pH (Martinsa *et al*., 2016). It has proved to be an efficient tool to increase the resistance against large number of bacterial infections (Ekasari *et al*., 2015). It is reported that tissue lesions can be controlled and lowered downed through the introduction of microbial floc along with probiotics (Rivera *et al.,* 2014). Extremely small organization of the biofloc in closed hatchery fish culture revealed that through symbiotic process they acted as natural water stabilizers, decomposers (algae grazer) and utilized the organic matter loaded at bottom (heterotrophic bacteria) and transformed it to protein food which was later consumed by the shrimp in zero water exchange system (Manan *et al*., 2016).

Biofloc-based system recently faced several outbreaks of pathogenic bacteria. The use of antibiotics to overcome problem has not proven successful due to the development of antibiotic resistance in pathogenic bacteria (Defoirdt *et al*., 2011). However, many studies report the degradative and probiotic nature of the biofloc the for exclusion of pathogenic bacteria by the development of a competitive environment and transformation of nitrogenous compounds. An increase in the growth of heterotrophic bacteria along with probiotic bacteria inhibits pathogenic bacteria (Gutierrez *et al*., 2016).

Most studies of biofloc based system are taken on shrimps. Previous studies also showed that bioflocs contribute to fish health through immunostimulation (Ekasari *et al.*, 2014) and is independent of the C-source. However, recently Gutierrez *et al.* (2016) observed that C-source determine the type, quantity and community of the bioflocs which improves the health status of the fish. Very scarce information is available on pathogenetic types of bacteria inbioflocs-based carp culture system. Thus, the aim of the present study was finding the status of bacterial pathogens for *Ctenopharyngodon idella* culture utilizing agroindustrial waste as C-source in the fish feed.

MATERIALS AND METHODS

Collection of water sample

This study was held in the Microbiology lab.of Zoology department, GC Women University, Faisalabad. The water samples were collected from four different aquaria tanks (Table 1), each of control (C) and treatment groups (T1, T2 and T3) carrying seven *C. idella* fingerlings. The treatment groups contained fish fingerlings fed on microbial biofloc at 15:1 carbon to nitrogen ratio as T1 and the other with a 10% water exchange with biofloc feed as T2 while the T3 contained fish fingerlings fed on microbial biofloc + commercial fish diet (2.5% with zero water exchange. The bioflocculation was achieved by adding bannana peels as a carbon source. The control group aquaria contained fish feed on commercial feed (5% body weight) for a period of 60 days. These samples were collected in sampling vials for bacterial analyses. The first sampling was performed during the first week of the experiment while a second sampling was performed after 6 weeks.

Table 1. Aquaria for rearing C.idella fingerlings under different feed and water exchange conditions.

Experiment	Commercial feed (39% protein feed)	Bacterial Biofloc	Water exchange conditions
T1	0	5% body weight	0
T2	0	5% body weight	10 %
T3	2.5% body weight	2.5% bodyweight	0
C	5% body weight	0	100%

Isolation of the Bacteria

The water sample from four different aquaria under different feed and water exchange conditions were collected in clear sterilized glass vials. Their dilutions were then prepared. Later 0.1 ml of water sample (aquaria biofloc/control) was poured over the agar plates containing different media (Table. 2). This sample was then spread with the help of sterilized glass spreader. The plates were incubated overnight at room temperature 35-

37 ºC. The growth on the agar plates was observed and recorded (Benson, 1994).

Table 2. Composition of different media used for bacterial isolation from the rearing tanks of *C.idella.*

Trypticase Soy Agar		Eosine Merhylene Blue Agar (EMB)		Blood Agar	
Composition	(g/L)	Composition	(g/L)	Composition	(g/L)
Peptic digest of soybean meal	5.0	Peptic digest of animal tissue	10	Peptone	10
Sodium chloride	5.0	Dipotassium phosphate	2	Tryptose	10
Agar agar	15.0	Lactose	5	Sodium Chloride	5
Distilled water	1 liter	Sucrose	5	Blood	5%
		Eosin – Y	0.4	Agar agar	15
		Methylene blue	0.065		
		Agar agar	13.500	To the base medium, 5% sterile mammalian blood is added after autoclaving and before pouring onto the plates	
Final pH (at 25°C): 7.3±0.2		Final pH (at 25°C): 7.2±0.2		Final pH (at 25°C): 7.3±0.2	

Determination of Colony Forming Units (CFU)

Once the growth of the bacteria over the respective agar media plates was achieved, the colony-forming units (CFU/mL) of different bacterial isolates were counted with the help of the naked eye and magnifying glass and recorded.

Pure Culturing of the Bacteria

Each colony with distinct morphological characteristics was labeled and further processed for pure culturing using standard pure culturing techniques through successive quadrant screening on selective and nutrient agar plates.

Characterization and Identification of Bacteria

For the determination of colonial characterization, the subculture of the bacterium from each water sample was made on the nutrient agar plates, trypticase soya agar plates and eosin methylene blue agar plates. Colony texture, shape, size, color, elevation, consistency and margins of the colony were recorded (Table 2.2). Magnifying glass was used for visualizing fine characteristics of the colony (Benson, 1994).

The bacteria isolates were then identified upto the generic level by using different physicochemical test like Gram's staining, endospore staining, motility, catalase test and oxidase test following the protocols of. Hemolytic interpretation of Blood agar was also recorded.

RESULTS

The bacteria found in treatment groups T1, T2, T3 and control group C were isolated, enumerated and performed gram staining, endospore staining, motility test, catalase test, and oxidase test. The gram staining confirms that 62% of bacterial isolates were gram-positive and 38% of isolates were gram-negative rods. Further biochemical tests (Table 3.1) and their comparison with Bergey's manual confirmed the presence of three types of bacterial species belonging to genera Bacillus, Klebsiella, and Staphylococcus on TSA. Bacillus and Klebsiella were present in T1 and T2 while in T3 besides these two, Staphylococcus species were additionally present during 1st week of experiment. During 7th week of experiment, only species belonging to two types of bacterial genera namely Bacillus and Klebsiella were found. Bacillus species in all treatment groups increased exponentially, Klebsiella species further decreased and Staphylococcus species were eventually diminished when experiment proceeded towards 7th week (Fig. 3.1). EMB agar did't show any singal bacterial growth while blood agar petriplates showed gamma hemolysis for bacterial colonies isolated from water samples of all treatment groups.

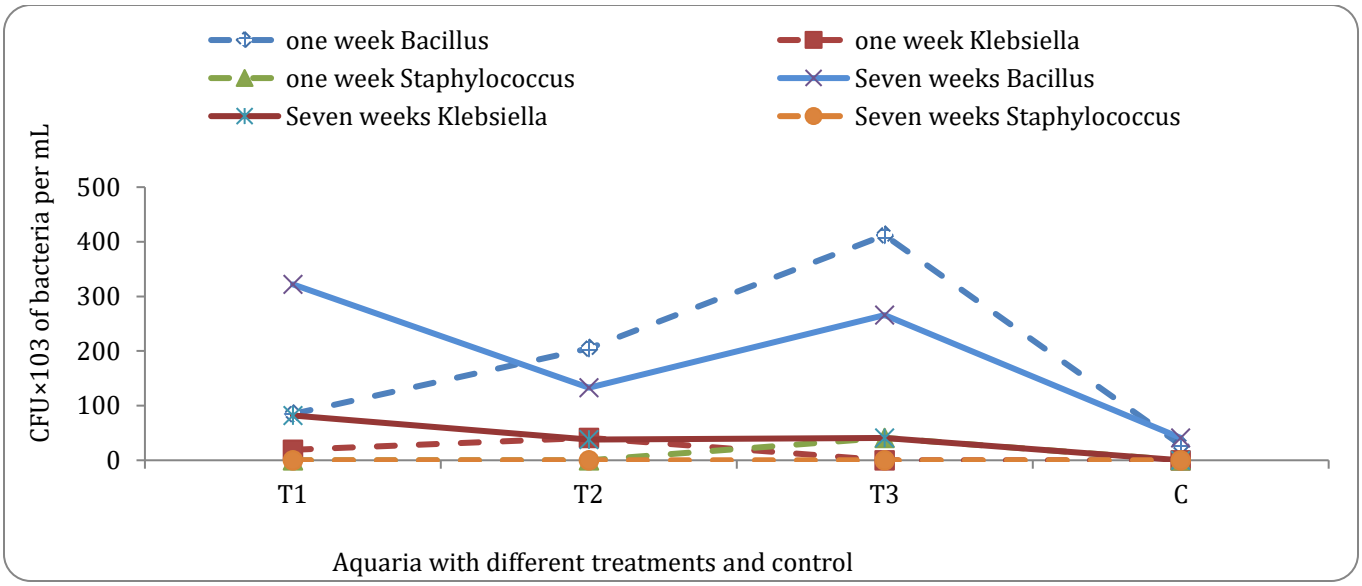

Figure 1. Comparison of CFU of bacterial isolates from all treatment and control group during 1st and 7th week of experiment.

It is obvious from the above shown figure that in T1 treatment group, the isolates ATM1, in T2 treatment group, the isolates BTM1, in T3 treatment group CTM1 and CTM2 isolates are with highest CFU during 1st week of experiment, while ATM11 isolates of treatment group T1, BTM22 isolates of treatment group T2, CTM11isolate of treatment group T3 during 7th week are with highest CFU. All these bacterial isolates after identification showed that these belonged to genus Bacillus. Besides these isolates, the isolates of T3, CTM2 identified during 1st week and the isolates CTM33 during 7th week are also belonging to genus Bacillus).

Table 2. Colonial Characteristics of the bacterial isolates on Trypticase Soya Agar after 24 hours of incubation at room temperature from rearing tanks of *C.idella.*

Experiment duration	Treatment/ control Aquaria	Isolate Code	CFU/mL	Shape	Size(mm)	margin	elevation	Texture/ appearance	Pigmentation	Optic properties
One Week	T1	ATM1	85×103	Circular	0.3	entire	Slightly raised	Smooth and shiny	white	opaque
		ATM2	19×103	irregular	0.5	undulate	Hilly	wrinkled and dull	Off-white	opaque
	T2	BTM1	205×103	Circular	0.3	entire	Slightly raised	Smooth and shiny	white	opaque
		BTM2	41×103	irregular	0.4	undulate	hilly	wrinkled and dull	Off-white	opaque
	T3	CTM1	369×103	Circular	0.3	Entire	Slightly raised	Smooth and shiny	white	opaque
		CTM2	41×103	nucleated	0.3	Entire	flat	Smooth and shiny	White	opaque
		CTM3	41×103	punctiform	0.1	Entire	flat	Smooth and shiny	white	opaque
	C	DTM1	28×103	Circular	0.4	entire	Slightly raised	Smooth and shiny	white	opaque
Seven Week	T1	ATM11	322×103	Circular	0.3	entire	Slightly raised	Smooth and shiny	white	opaque
		ATM22	82×103	irregular	0.5	undulate	Hilly	wrinkled and dull	Off-white	opaque
	T2	BTM11	133×103	Circular	0.3	entire	Slightly raised	Smooth and shiny	white	opaque
		BTM22	38×103	irregular	0.4	undulate	hilly	wrinkled and dull	Off-white	opaque
	T3	CTM11	152×103	Circular	0.3	Entire	Slightly raised	Smooth and shiny	white	opaque
		CTM22	38×103	irregular	0.4	undulate	hilly	wrinkled and dull	white	opaque
		CTM33	114×103	nucleated	0.3	Entire	flat	Smooth and shiny	White	opaque
	C	DTM11	41×103	Circular	0.4	entire	Slightly raised	Smooth and shiny	white	opaque

Table 3. Physicochemical properties of bacterial isolates from treatment group T1 on nutrient agar plates 48 Hours post-incubation at room temperature.

Experiment duration	Treatment/control Aquaria	Isolate Code	Shape and arrangement of the bacterial cells	Gram's Staining	Endospore Staining	Catalase Test Oxidase Test	Motility Test	Genus Identified
One Week	T1	ATM1	Short rods mostly in pairs	+ve	+ve	+ve -ve	Motile rods	Bacillus
		ATM2	Diplobacilli	-ve	-ve	+ve -ve	Non-motile	Klebsiella
	T2	BTM1	Short rods mostly in pairs	+ve	+ve	+ve -ve	Motile rods	Bacillus
		BTM2	Diplobacilli	-ve	-ve	+ve -ve	Non-motile	Klebsiella
	T3	CTM1	Short rods mostly in pairs	+ve	+ve	+ve -ve	Motile rods	Bacillus
		CTM2	rods	+ve	+ve(terminal)	+ve +ve	Motile rods	Bacillus
		CTM3	cocci(clumps)	+ve	+ve(centrall)	+ve -ve	Non-motile	Staphyloccocus
	C	DTM1	Short rods mostly in pairs	+ve	+ve(Terminal endospore	+ve -ve	Motile rods	Bacillus
seven week	T1	ATM11	Coccobacilli mostly in pairs	+ve	+ve(Terminal endospore	+ve -ve	Motile rods	Bacillus
		ATM22	Diplobacilli	-ve	-ve	+ve -ve	Non-motile rods	Klebsiella
	T2	BTM11	Coccobacilli mostly in pairs	+ve	+ve(Terminal endospore	+ve -ve	Motile rods	Bacillus
		BTM22	Diplobacilli	-ve	-ve	+ve -ve	Non-motile rods	Klebsiella
	T3	CTM11	Coccobacilli mostly in pairs	+ve	+ve(Terminal endospore	+ve -ve	Motile rods	Bacillus
		CTM22	Diplobacilli	-ve	-ve	+ve -ve	Non-motile rods	Klebsiella
		CTM33	rods mostly in pairs	+ve	+ve(Terminal endospore	+ve +ve	Motile rods	Bacillus
	C	DTM11	Coccobacilli mostly in pairs	+ve	+ve(Terminal endospore	+ve -ve	Motile rods	Bacillus

DISCUSSION

Biofloc technology (BFT) is one of the promising technique to raise fish in excess without major economy and environmental deterioration. The present research work was performed to assess one of the challenge to this technology i.e. pathogenic load in bioflocs which could be harmful for the fish. Screening of the bacteria on TSA agar plates in present study resulted in identification of two bacterial genera in treatment group T1, three bacterial genera in treatment groups T2 and T3 each and one genus in control group. The genus Bacillus was common to all experimental and control groups while the members of the genus Klebsiella and Staphylococcus were found only in the treatment groups i.e., T1, T2 and T3. The bacteria of Bacillus genus increased exponentially with increase of experimental time duration in all treatment groups but were almost same at end of experiment in number in control group.

The screening of the experimental (T1, T2 and T3) and control tanks (C) containing culture of *Ctenopharyngodon idella* for microbial analysis resulted in isolation of Bacillus, Klebsiella and Staphylococcus genera. Klebsiella has been reported as a potential pathogen of the fish and brings about injuries and mortalities. Dias *et al*. (2012) reported it as a causative agent of the injuries in nishikigoi carp. From the injured fish, *K. pneumonia* was isolated and identified biochemically. Kumar *et al*. (2010) observed mortalities in Moribund koi carp and *Cyprinus carpio* in a fish farm and found the Klebsiella spp. as one of the causative agents isolated and identified from those samples. Another study reported *Klebsiella pneumonia* as fish pathogens where injury symptoms were observed in fish of cyprinid family, nishikigoi carp. The pathogen was isolated and confirmed from the tissue sample pulverized from infected fish lesions. After that the same sample was plated on Blood agar and incubated for 24 hours at 37 °C temperature. By observing colonial characterization and physicochemical properties, *Klebsiella pneumonia* was identified (Seidler *et al*., 1978).

When the results of the bacterial isolation were compared between the samples from fish aquaria with experiment age of one week and those from the aquaria containing fish with experiment age of seven week, a dominance of *Bacillus* genus was observed overall which is indicative of

its competitive nature, enhanced survival and adaptability to the environment. Alfaragi and Alsaphar (2012) reported antagonistic behavior of the *Bacillus* spp. isolated from the *Cyprinus carpio* against fish pathogens just after 24 hours and inhibited the growth of *Aeromonas* spp. These findings clearly justify the dominance of *Bacillus* spp. with increase in the time of fish rearing in the present investigation.

Li *et al*. (2012) isolated Bacillus preparation from the grass carp *Ctenopharyngodon Idella* pond. By adding a mixture of species i.e., *Bacillus subtilis* and *Bacillus licheniform* containing 10^8 CFU/kg diet per seven days, enhanced immunity and antioxidant ability of the grass carp was observed (Li *et al*., 2012).

Third type of genus identified was Staphylococcus. Salty environment is best for growth of Staphylococcus and low activity level of water with reduced number of competing organisms (Tavakoli *et al.,* 2008). According to Herrero *et al*. (2003), *Staphylococcus aureus* did not appear as natural microflora part in fish aquaculture. But it was assumed that these pathogens contaminated the fish during capture and poor handling. As there is greater number of Bacillus probiotic species in treatment groups, it is not possible for Staphylococci to cause pathogenicity and infections in carp fish.

CONCLUSION

In biofloc based grass carp (*Ctenopharyngodon idella*) aquaculture, an increased bacterial load of Bacillus species was observed which exponentially dominated over pathogenic bacteria. As the experiment proceeded, the initial number of pathogenic bacterial genera Klebsiella and Staphylococcus were further decreased which indicates the development of a stable biofloc-based grass carp culture. The healthy physical status of the fish also assured the safety of BFT to be employed in carp aquaculture.

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