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ISOLATION AND CHARACTERIZATION OF PROBIOTIC BACTERIA FROM BIOFLOC FOR ENHANCING FISH GROWTH

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ABSTRACT

Biofloc technology (BFT) enhances water quality, fish growth, and immunity through diverse heterotrophic bacterial communities while reducing feed costs. This study isolated 22 probiotic strains, predominantly *Bacillus* species, from microbial bioflocs in Peshawar, Karak, and Bannu, Pakistan. Among these, Bacillus subtilis (PBSI-5), *Bacillus safensis* subsp. safensis (KBS3-8), and *Bacillus safensis* subsp. safensis (BBS4-5) were identified as the most promising due to their enzyme activities (amylase, cellulase, and protease). These strains significantly improved weight gain and specific growth rates in Mori (*Cirrhinus mrigala*). *Bacillus safensis* subsp. safensis showed the highest weight gain (31.02±1.24g) and % weight gain (82.98±3.32%). The study emphasizes the potential of these probiotics to enhance fish growth in biofloc systems and suggests further research on their immunological benefits and use as feed supplements. These findings offer promising applications for enhancing fish health, productivity, and sustainable aquaculture practices through optimized probiotic formulations.

Keywords: Aquaculture, *Bacillus, Cirrhinus mrigala,* Enhance growth, Microbial Bioflocs, Probiotic activities

INTRODUCTION

Biofloc technology (BFT) is used to reduce wastewater discharge, impede disease epidemics, and hold water free of pathogens, all of which aid in improving biosecurity on farms (**A Panigrahi *et al.*, 2020**). BFT is based on the development of macro aggregates, comprising of heterotrophic bacteria, phytoplankton, feed debris, organic matter and microorganisms which are capable of maintaining water quality **(Hargreaves, 2013**). It is a symbiotic system that comprises restricted aquatic animals, heterotrophic bacteria and cultured aquatic species. (BFT) is considered as an ideal option for sustainable and environmentally friendly aquaculture approach (**Shadrack *et al.*, 2023**). In a bioflocs system, heterotrophic bacteria convert the nitrogenous metabolites into potentially usable biomass. This technique has probiotic properties that can affect cultured fish species in general, but the addition of probiotic heterotrophic bacteria can boost up the beneficial effects of BFT **(de Souza *et al.*, 2014)**. Studies with Nile tilapia (*Oreochromis niloticus)* have revealed that augmentation of probiotics strains can enhance the survival, and feed conversion of reared fish species (**Wang *et al.*, 2005**). Previous researchers have utilized mixed cultures of bacteria (*Bacillus subtilis, Lactobacillus acidophilus,* and *Clostridium butyricum*) and yeast (*Saccharomyces cerevisiae*) for improving the immune modulatory system of *O. niloticus* resulting in improving resistance to *Edwardsiella tarda* infection (**Taoka *et al.*,** 2006). The use of *B. subtilis* has enhanced the innate immunity response and reduced the stocking density stress in Nile tilapia (**C.-H. Liu *et al.*, 2012**). The activity of digestive alkaline and acid proteases, as well as Nile tilapia growth, have been enhanced by the addition of the spores of three *Bacillus* species (*B. subtilis, B. licheniformis, B. cereus*) during larval development (**Boonthai *et al.*, 2011**).
To date, in Pakistan, few reports are available about the using probiotic strains to enhance the growth of reared species in biofloc systems. This gap highlights a significant opportunity for in-vitro screening strategies that could identify the beneficial impacts of probiotic bacterial strains on biofloc technology (BFT) systems. The current study aims to provide fresh insights into the exogenous use of heterotrophic bacteria in BFT, potentially increasing fish production and enhancing foreign export capabilities. In this investigation, probiotic bacteria were isolated from natural microbial bioflocs, then characterized and evaluated as inducers for fish growth in biofloc fish ponds. This research addresses the critical need for effective probiotic applications in BFT within Pakistan, offering a novel approach to improving aquaculture practices in the region.

MATERIALS AND METHODS

Sampling Sites

The environment of the sampling sites was entirely turbid and a natural microbial biofloc condition. Before sample collection, different water quality parameters, i.e., temperature, salinity, PH, and total dissolved solids, were checked. In the current research work, four water samples were collected from microbial bioflocs located in Peshawar (PBS1 and PBS2), Karak (KBS3) and Bannu (BBS4) Khyber Pakhtunkhwa Province Pakistan in sterile autoclave bottles. The water quality parameters of Peshawar biofloc included temperature (22.5°C), salinity (0.05%), PH (7.80), and total dissolved solids (645). In the same way for Karak biofloc, temperature (21.0 °C), salinity (0.03 %), PH (7.82), and total dissolved solids (621). Similarly, for Bannu biofloc, temperature (21.4), salinity (0.05 %), PH (7.68), and total dissolved solids (405) as shown in Table 1. All the water samples were brought into laboratory of Kohat University of Science and Technology Kohat and were kept in refrigerator at 4 for further use. All the water samples were brought into laboratory of Kohat University of Science and Technology Kohat and were kept in refrigerator at 4℃ for further use.

Table 1 Water quality parameters of microbial bioflocs of Peshawar, Karak and Bannu

Water quality parameters	Sampling sites			
	Peshawar 1 (PBS1)	Peshawar 2 (PBS2)	Karak (KBS3)	Bannu (BBS4)
Temperature	22.5 ℃	22.3 ℃	21.0 ℃	21.4 ℃
Salinity	0.05 %	0.06 %	0.03 %	0.05 %
PH	7.80	7.49	7.82	7.68
Total dissolved solid	645	675	621	405

Isolation, Culturing, and Phenotypic Attributes of Bacterial Isolates

Bioflocs water samples were serially diluted up.to 10^{-9} with peptone water in 15 ml test tubes and about 1000 ul of samples were added in each tube for serial dilution. Round about 100 ul for each sample were spread on a nutrient agar plate and incubated at 37 ℃ for about 24 hours. The plating experiments were performed in triplicate to ensure reproducibility, and negative controls (plates without inoculation) were included to confirm the absence of contamination. A standardized phenotypic key of Bergey's manual of systematic bacteriology was used to examine the shape (circular, wavy and dome), surface (rough and smooth) colour (white, creamy and creamy white) and size (small, medium, and large) of purified bacterial colonies **(Riker *et al.*, 1957**). Pure cultures of bacteria were selected after successful growth of microorganism and stored at -80 ℃ in 20% glycerol **(Lalloo *et al.*, 2007**). The quantity of viable bacteria cells in a sample is expressed as a colony-forming unit (CFU). The number of colony forming units (CFU) and cell viability was determined using the formula-below.
cfu / ml = no. of colonies x dilution factor / 25ml **(Waghmare *et al.*, 2018)**

Biochemical Profile of Bacterial Isolates of Microbial Bioflocs

Biochemical profile of bacterial isolates from microbial bioflocs were evaluated through the published protocols of Gram staining reagents included Crystal violet (primary stain), Gram's iodine solution (the mordant), ethanol (50:50 v:v) (the decolorizer), 0.1% basic fuchsin solution (the counterstain), and water (**Islam and Roy, 2018**), Endospore staining was performed by 5% (w/v) malachite green solution 0.5% (w/v) safranin solution. (**Donepudi, 2020**), Catalase was performed with the use of 3 % hydrogen peroxide (**L. Liu *et al.*, 2008**), Oxidase was treated with 1 % Kovács Oxidase reagent **(Poornima *et al.*, 2010**), For Simmon citrate, was used (citrate agar), and for Motility test culture grown on agar substrate (**Ariffin *et al.*, 2020**).

Molecular characterization of DNA extraction

A Phenol-Chloroform extraction protocol was used to extract genomic DNA from fresh broth cultures of isolates. **(Wright *et al.*, 2017**). PCR amplification was carried out with 0.5 µM of 16S rRNA (ribonucleic acid) universal primers 9 F 5' GAGTTTGATCCTGGCTCAG-3′ and 1510 R 5′ GGCTACCTTGTTACGA-3′, using 100 µL PCR tubes having 50 µL of reaction volume consist of PCR Master Mix (1X), DNA template and PCR water. An initial denaturation step at 95°C for 2 minutes was followed by 35 cycles of denaturation at 94°C for 50 seconds, annealing at 56°C for 40 seconds, and extension at 72°C for 2 minutes, with a final extension at 72°C for 7 minutes.1% (w/v) agarose gel was used to separate the amplified products. To view the clear bands, amplicons were visualized-to see the clear bands (Ahmed *et al.*, 2014).

Phylogenetic Analysis

The isolates16S ribosomal DNA (rDNA) sequences were analyzed phylogenetically using the Basic Local Alignment Search Tool (BLAST) and compared to sequences in the GenBank database of the National Centre for Biotechnology Information (NCBI). The sequences were compared to the reference taxa's sequences from public databases. The MEGA6 software's maximum likelihood method was used to construct the phylogenetic trees, and the parameter model was utilized to calculate evolutionary distances (**Kumar *et al.*, 2016**).

Probiotic Activities

Probiotic activities were performed in accordance with the standard protocol of amylase, cellulose, and protease activities. To observe the amylase activity, a single colony of the bacterial isolate was dotted onto the starch agar media and incubated for 24 hrs at 37℃. When Lugol's iodine solution (w/v) (1% iodine and 2% potassium iodide) was discharged on the colony a clear zone appeared, indicating that the bacteria could manufacture amylase. (**Poletto *et al.*, 2018**). For Cellulase Activity the single colony of bacterial isolate was marked on the surface of carboxymethyl cellulose (CMC= 1 g cellulose and 1.5 g agar). After one day of incubation, the growth of isolates was found on CMC media. In the next step, CMC agar plates were flooded with 2 % gram iodine and potassium iodide solution, and clear zones around the colonies were identified as a cellulose producers, and zones were measured using a ruler (**Sandra *et al.*, 2022**) and Skim milk agar (SMA= 2.5 g skim milk 1.5 g agar) was used to test bacterial isolates for the synthesis of extracellular protease and the presence of clear zones around the colonies on plates was evaluated since this shows the development of protease enzymes (**Riffel *et al.*, 2003**).

Experimental design for Biofloc

The experiments were conducted following approval from the Advanced Studies and Research Board (ASRB) in their 83rd meeting held on August 5, 2021, at Kohat University of Science and Technology (KUST). After obtaining the necessary approval, approximately 100 Cirrhinus mrigala (Mori) fish fingerlings, measuring 3-4 inches, were procured from Tanda Dam Fish Hatchery, Kohat. These fingerlings were then acclimatized for two weeks in fiberglass tanks at the Molecular Ecology and Conservation Lab of KUST. During the acclimatization period, the fish were fed without any experimental feed to ensure they adapted well to the laboratory conditions. The basic physio-chemical parameters of the water, including temperature, pH, dissolved oxygen, and ammonia levels, were measured weekly to maintain an optimal environment for the fish. At the time of stocking, the total number of fish, as well as the length and weight of each distinct fish in the tanks, were recorded meticulously to ensure accurate monitoring throughout the study **(Eyankware *et al.*, 2018)**.

Generation of Biofloc

All of the ingredients i-e. $CaCO_3$ (20 ppm), urea (20 ppm), and Single super phosphate were added to the fish tanks to established an autotrophic system. A mixture of Carbon sources consisting of millet, rice, wheat flour, corn, gram, and molasses were used 2 grams of each thoroughly mixed with each other. Fish tanks were comprised of probiotics i-e PBS1-5, KBS3-8, and BBS4-5, along with the carbon sources. Probiotic bacterial isolates were mixed in a half liter of autoclaved water, and melted carefully, and kept for 24 h for fermentation. To develop heterotrophic bioflocs, the fermented inoculum was administered 10 ml/tank in all of the treatment tanks for five days. The carbon source was present in the biofloc control, but probiotics were not added. In bioflocs treatments, 30% of the water was swapped once a week, while 50 % of the water was switched once a week in controls **(A Panigrahi *et al.*, 2020)**.

Probiotic Feeding

After flocculation and verifying the health status, 25 fish were distributed in all replicates, and probiotic feed was adjusted. Probiotic feed was given to the experimental group during the feeding experiment for 60 days (**Ekasari *et al.*, 2014)**.

Survival and Growth

At the time of stocking, the entire fish quantity and the biomass of each fish tank were recorded. Additionally, before stocking, fish from every bag were gathered and individually weighed. Every two weeks, 6–8 fish from each fish tank were removed and weighed separately in order to monitor growth and adjust food. After the two months of the experiment, fish fingerlings from each aquarium were taken, and weigh all of the fish fingerlings were weighed individually and recorded the entire number of fingerlings to calculate the average weight. The increase of biomass, weight gain (WG), specific growth rate (SGR %), weight gain percentage (WG %), and feed conversion ratio (FCR) were all assessed **(Zhou *et al.*, 2013)**. All this work was conducted under the supervision and approval of the Advanced Studies and Research Board (ASRB) at Kohat University of Science and Technology (KUST).

Biometry of the Samples

After 60 days of each treatment, growth parameters were recorded for the traits, including total length, weight gain, weight gain percentage (WG %), feed conversion ratio (FCR), and specific growth rate (SGR %) using the following formula;

FCR = Feed given (DW) / bodyweight gain (WW)
Weight gain (%) = (FW-IW) / IW) x100
SGR (%) = [ln (FW)-ln (IW) / N] ×100
Where,
FW = final weight, IW = initial weight, D = dry weight, WW= wet weight, ln= natural log and N= days of culture **(Akshaya Panigrahi *et al.*, 2019)**

Statistical Analysis

The current data were presented in the form of percent, average, and SD in triplicates. To know the difference between the groups the t-tests, tests were applied by using Statistics version 9. The p- value <.05 was considered significantly constant.

RESULTS

Sampling Sites

A total of 22 bacterial isolates were obtained from microbial bioflocs in Peshawar (34.0151º N, 71.5249º E), Karak (33.115269º N, 71.095535º E), and Bannu (32.986111º N, 70.604164º E) in Khyber Pakhtunkhwa, Pakistan, and were assigned specific codes as shown in Table 2. The bacterial isolates from the microbial biofloc of Peshawar 1 were represented as PBS1, microbial biofloc of Peshawar 2 as PBS2, microbial biofloc of Karak as KBS3 and microbial biofloc of

Bannu were designated as BBS4. Among those, 6 bacterial isolates were isolated from microbial biofloc of PBS1, 4 bacterial isolates were selected from microbial biofloc of PBS2, 7 bacterial isolates were isolated from microbial biofloc of KBS3 and 5 bacterial isolates were isolated from microbial biofloc of BBS4.

Table 2 Bacterial Isolates with their credential codes collected from Microbial Bioflocs of PBS1, PBS2, KBS3, and BBS4

Sampling sites	Isolates	No. of isolates	Credential codes of bacterial isolates
Peshawar 1	PBS1	6	PBS1-1, PBS1-2, PBS1-3, PBSI-4, PBS1-5, PBSI-6
Peshawar 2	PBS2	4	PBS2 -1, PBS2-2, PBS2-4, PBS2-6
Karak	KBS3	7	KBS3-1, KBS3-2, KBS3-3, KBS3-4, KBS3-7, KBS3-8, KBS3-9
Bannu	BBS4	5	BBS4-1, BBS4-2, BBS4-3, BBS4-4, BBS4-5

Phenotypic Attributes of Bacterial Isolates

Overall 22 bacterial isolates were isolated from different microbial bioflocs namely PBS1, PBS2, KBS3, and BBS4. These bacterial isolates were identified based on of their phenotypic attributes are shown in Table 3. Gram staining revealed that, all bacterial isolates from microbial bioflocs, namely PBS1, PBS2, KBS3, and BBS4, were found rod-shaped and Gram-positive. Furthermore, all bacterial isolates from PBS1, PBS2, KBS3, and BBS4 microbial bioflocs were observed as non-motile, while their colony shape was also evaluated revealing 13 bacterial isolates were in circular shaped, 6 were in wavy shape, and 3 were observed as dome-shaped. Similarly, the colony surface was also evaluated, which revealed that out of these 22 bacterial isolates, 8 bacterial isolates were had rough surface, while 14 bacterial isolates had smooth surfaces. In the same way, colony color for 22 bacterial isolates were evaluated revealing 10 bacterial isolates were in creamy white color, 8 bacterial isolates were white in color, 2 bacterial isolates were creamy in color and 2 bacterial isolates were yellow in color. Colony size was also evaluated which revealed that 12 bacterial isolates were of medium size, 7 bacterial isolates of large in size and 3 bacterial isolates were small in size.

The colony shape of 6 bacterial isolates from PBS1 microbial bioflocs was noted revealing 3 bacterial isolates were in wavy shape, 2 bacterial isolates were circular and 1 bacterial isolate was observed as dome shaped. Similarly, bacterial isolates from microbial biofloc PBS2 were evaluated revealing that, 3 bacterial isolates were circular, while 1 bacterial isolate was in a wavy shape. On the same pattern, from microbial biofloc KBS3, 5 bacterial isolates were in circular while 2 bacterial isolates were observed as dome-shape. Similarly, 5 bacterial isolates from microbial bioflocs BBS4 were observed revealing that 3 bacterial isolates were in circular, while 2 bacterial isolates were wavy shaped. Moreover, colony surface was also evaluated that showed out of 6 bacterial isolates from microbial biofloc PBS1, 4 bacterial isolates were on rough surface while 2 bacterial isolates were having smooth surface. Similarly, from microbial biofloc PBS2, 3 bacterial isolates had smooth surfaces while 1 bacterial isolate had a rough surface. In the same way, 7 bacterial isolates were isolated from KBS3 microbial biofloc revealing 5 bacterial isolates were showing smooth surface while 2 were showing rough surface. Furthermore, five bacterial isolates were isolated from microbial biofloc BBS4 that revealed, 4 bacterial isolates were having smooth surfaces while 1 bacterial isolate was showed a rough surface.

Colony color of bacterial isolates from microbial bioflocs including PBS1, PBS2, KBS3 and BBS4 was evaluated. It was observed that 6 bacterial isolates were observed from PBS1 revealing 2 bacterial isolates were yellow, 2 bacterial isolates were creamy in color, while 2 bacterial isolates were white. Similarly, for bacterial isolates from microbial biofloc PBS2, 3 bacterial isolates were creamy white in color while 1 bacterial isolate was white in color. Furthermore, from biofloc KBS3, 7 bacterial isolates were observed revealing, 4 bacterial isolates were creamy white in color while 3 bacterial isolates were white in color. On the same way, bacterial isolates from microbial biofloc BBS4 were also evaluated revealing, 3 bacterial isolates were creamy white in color while two bacterial isolates were white in color. Colony size of bacterial isolates from microbial bioflocs including PBS1, PBS2, KBS3 and BBS4 were evaluated showed, 6 bacterial isolates from PBS1, 4 bacterial isolates from PBS2, 7 bacterial isolates from KBS3 and 5 bacterial isolates from BBS4. Colony size of bacterial isolates from PBS1 was evaluated which showed 4 bacterial isolates were of medium size, while 2 bacterial isolates were of large size. Similarly, in bacterial isolates from microbial biofloc PBS2, 2 bacterial isolates were having small colony size while 2 bacterial isolates were having large colony size. On the same pattern from microbial biofloc KBS3, 5 bacterial isolates were in medium size, 1 bacterial isolate was in large size, while 1 bacterial isolate was in small size. In the same way, from microbial biofloc BBS4, 3 bacterial isolates were of medium colony size while 2 bacterial isolates were of large colony size as shown in Table 3.

Table 3 Phenotypic attributes of Bacterial Isolates from Microbial Bioflocs of PBS1,PBS2, KBS3 and BBS4

Bioflocs sampling areas	Bacterial Isolates	Cell shape	Gram staining	Motility	Colony shape	Colony surface	Colony colour	Colony size
	PBS1-1	rod	+	non-motile	Dome	Rough	yellow	medium
	PBS1-2	rod	+	non-motile	Wavy	Rough	yellow	large
Peshawar 1	PBS1-3	rod	+	non-motile	Wavy	Rough	creamy	medium
	PBS1-4	rod	+	non-motile	Wavy	Rough	creamy	large
	PBS1-5	rod	+	non-motile	circular	Smooth	White	medium
	PBS1-6	rod	+	non-motile	circular	Smooth	White	medium
	PBS2-1	rod	+	non-motile	Wavy	Rough	creamy white	small
Peshawar 2	PBS2-2	rod	+	non-motile	circular	Smooth	creamy white	small
	PBS2-4	rod	+	non-motile	circular	Smooth	creamy white	large
	PBS2-6	rod	+	non-motile	circular	Smooth	White	large
	KBS3-1	rod	+	non-motile	circular	Smooth	creamy white	medium
	KBS3-2	rod	+	non-motile	Dome	Rough	White	medium
	KBS3-3	rod	+	non-motile	circular	Smooth	creamy white	medium
Karak	KBS3-4	rod	+	non-motile	circular	Smooth	White	large
	KBS3-7	rod	+	non-motile	circular	Smooth	creamy white	medium
	KBS3-8	rod	+	non-motile	circular	Smooth	creamy white	small
	KBS3-9	rod	+	non-motile	Dome	Rough	White	medium
	BBS4-1	rod	+	non-motile	Wavy	Smooth	creamy white	medium
	BBS4-2	rod	+	non-motile	circular	Rough	White	medium
Bannu	BBS4-3	rod	+	non-motile	Wavy	Smooth	creamy white	large
	BBS4-4	rod	+	non-motile	circular	Smooth	White	medium
	BBS4-5	rod	+	non-motile	circular	Smooth	creamy white	large

Results of the Biochemical Tests

Different biochemical tests were conducted for bacterial isolates of microbial bioflocs, namely PBS1, PBS2, KBS3 and BBS4 in accordance with standard protocol. It was revealed that 22 bacterial isolates from microbial bioflocs PBS1, PBS2, KBS3 and BBS4 were spore-producing and catalase positive whil the oxidase test revealed that out of these 22 bacterial isolates, 13 bacterial isolates were oxidase negative while 9 bacterial isolates were oxidase positive. Similarly, a citrate test was conducted which revealed that out of 22 bacterial isolates, 19 bacterial isolates were citrate negative while 3 bacterial isolates were citrate positive.

Oxidase test has revealed that out of these 6 bacterial isolates from microbial biofloc PBS1, 5 bacterial isolates were oxidase negative while 1 bacterial isolate was oxidase positive. Similarly from microbial biofloc PBS2, all 4 bacterial isolates were oxidase positive. It was observed that out of 7 bacterial isolates from microbial biofloc KBS3, 4 bacterial isolates were oxidase positive while 3 bacterial isolates were oxidase negative while all 5 bacterial isolates from microbial biofloc BBS4 were oxidase negative. Citrate test has been evaluated which revealed all 6 bacterial isolates from microbial biofloc PBS1, were citrate negative while 3 bacterial isolates from microbial biofloc PBS2 were citrate negative while 1 bacterial isolate from microbial biofloc PBS2 was citrate positive. Similarly from microbial biofloc KBS3, 6 bacterial isolates were citrate negative and one bacterial isolate was citrate positive while 4 bacterial isolates from microbial biofloc BBS4 were citrate negative, and 1 bacterial isolate from microbial biofloc BBS4 was citrate positive as shown in Table 4.

Table 4 Biochemical profile of Bacterial Isolates from Microbial Bioflocs PBS1, PBS2, KBS3 & BBS4

Bacterial Isolates	Endospore Staining	Catalase Test	Oxidase Test	Citrate Test
PBS1-1	+	+	–	–
PBS1-2	+	+	–	–
PBS1-3	+	+	–	–
PBS1-4	+	+	–	–
PBS1-5	+	+	+	–
PBS1-6	+	+	–	–
PBS2-1	+	+	+	+
PBS2-2	+	+	+	–
PBS2-4	+	+	+	–
PBS2-6	+	+	+	–
KBS3-1	+	+	+	–
KBS3-2	+	+	–	–
KBS3-3	+	+	–	+
KBS3-4	+	+	–	–
KBS3-7	+	+	+	–
KBS3-8	+	+	+	–
KBS3-9	+	+	+	–
BBS4-1	+	+	–	–
BBS4-2	+	+	–	+
BBS4-3	+	+	–	–
BBS4-4	+	+	–	–
BBS4-5	+	+	–	–

(+) represents positive results while (-) represents negative results

Key for biochemical tests: Gram + indicates purple color, Gram – red color; endospores appeared bright green while vegetative cells as brown red or pink; Catalase + formation of bubbles; Oxidase + indicates purple color; Citrate + blue tint, citrate – green tint; Motility test indicates a zone of growth

Molecular Characterization of Bacterial Isolates, isolated from Microbial Bioflocs

The morphologically identified PBS1-5, KBS3 -8, BBS4-3, and BBS4-5 were successfully obtained as a pure culture from the enriched culture. On successful completion of the DNA extraction procedure, DNA was visualized on agarose gel for the confirmation of quantity and quality of DNA. Visibility of DNA was observed on Agarose gel electrophoresis to estimate the quantity and quality of DN as shown Figure 1.

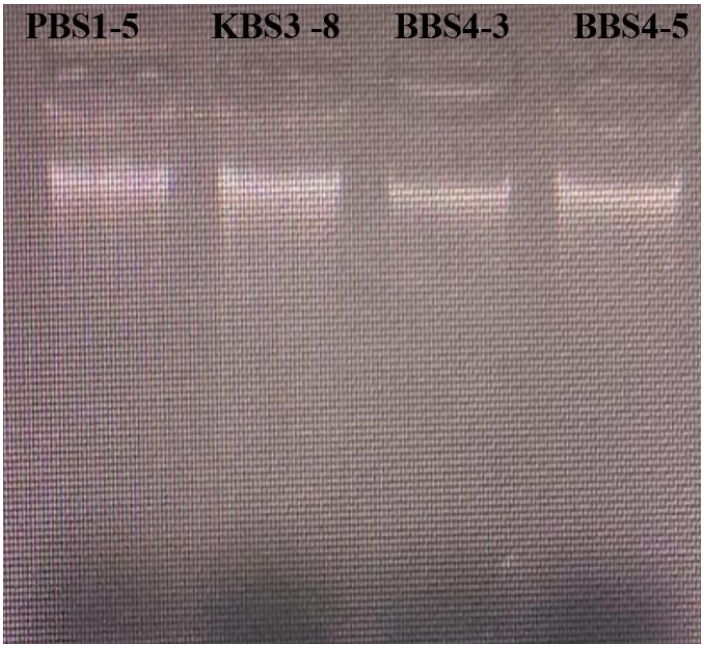

Figure 1 DNA Extraction of Bacterial Isolates on Gel

Amplification of I6S rDNA gene for bacterial isolates, isolated from microbial bioflocs including PBS1-5, KBS3-8, BBS4-3, and BBS4-5 was compared with 1 Kb ladder to confirm the band size of amplified PCR products. Electropharogram representing the amplifications of ~1400 bp 16S rDNA amplified product of bacterial isolates from various microbial bioflocs with primer pair comprising of 9 F 5' and 1510 R 3' are shown in Figure 2. The 16S rDNA amplified product of bacterial isolates from microbial bioflocs was sequenced and then the sequence analysis was performed by blasting these 16s rDNA gene sequences with the reference 16S rDNA accessible gene sequences in NCBI data bases. The results showed that the 16S rDNA gene of bacterial isolate PBS1-5 has 100 % similarity with other closely related *Bacillus subtilis* (NCIB 3610). Similarly the bacterial isolate KBS3-8 has a significant similarity of 99.78 % with 16s rDNA gene of *Bacillus safensis* (FO-36b). On the same pattern bacterial isolate BBS4-3 has shown significant 100 % similarity with the bacterial isolate *Bacillus safensis* (FO-36b). Moreover, bacterial isolate BBS4-5 has revealed a significant 100 % similarity with the bacterial strain *Bacillus safensis* (FO-36b) as shown in Table 5.

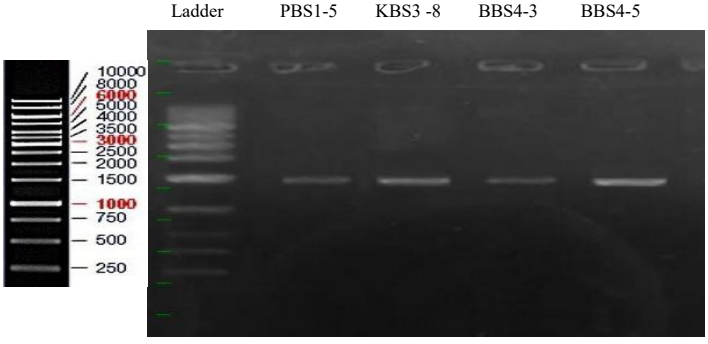

Figure 2 Electropherogram representing the amplification of 16S rDNA gene sequences for bacterial isolates of various microbial bioflocs

Table 5 Homology analysis of 16s rDNA gene sequences f Bacterial Isolates with closely related 16S rDNA gene sequences available in NCBI data bases

Name ID	Strain Name	Accession numbers of 16 S RNA gene	Closely related taxa identified by using Eztaxaon server data base(http://eztaxaon.e.ezbioc loud.net)	Sequence similarity (%) of r16S rRNA gene with closely related taxa	Top-hit taxonomy	Completeness (%)
PBS1-5	*Bacillus*	NCIB 3610	*Bacillus subtilis*	100	*Bacteria*; *Firmicutes*;*Bacilli*; *Bacillales*; *Bacillaceae*; *Bacillus*	63.2
BBS4-5	*Bacillus*	FO-36b	*Bacillus safensis subsp. Safensis*	100	*Bacteria*; *Firmicutes*; *Bacilli*; *Bacillales*; *Bacillaceae*;*Bacillus*;*Bacillus safensis*	62.4
BBS4-3	*Bacillus*	FO-36b	*Bacillus safensis subsp. Safensis*	100	*Bacteria*;*Firmicutes*;*Bacilli*; *Bacillales*; *Bacillaceae*;*Bacillus*;*Bacillus safensis*	63.2
KBS3-8	*Bacillus*	FO-36b	*Bacillus safensis subsp. Safensis*	99.78	*Bacteria*;*Firmicutes*;*Bacilli*; *Bacillales*; *Bacillaceae*;Bacillus;*Bacillus safensis*	63.2

Phylogenetic Analysis

A Maximum Likelihood (ML) relationship was carried out by using 16s rDNA gene sequences of bacterial isolates from microbial bioflocs with 38 closely related 16S rDNA gene sequences available in NCBI data bases as shown in Figure 2. A phylogenetic tree was constructed using the ML approach that was clustered into nine clusters with different percentages of similarities. Cluster I was comprised of 14 isolates including *Bacillus stercoris* (JCM 30051), *Bacillus tequitensts* (KCTC 13622), *Bacillus regosus* (SPB7), *Bacillus subtilis* (NCIB 3610), *Bacillus siamensis (*KCTC 13613)*, Bacillus velezensis* (CR-502), *Bacillus Nematocida* (B-16), *Bacillus amyloliquefaciens* (DSM 7), *Bacillus cabrialesii* (TE3), *Bacillus nakamurai* (NRRL B-41091), *Bacillus halotolerans* (ATCC 25096), *Bacillus mojavensis* (RO-H-1), *Bacillus cabrialesii* (TE3) and *Bacillus vallismortis* (DV1-F-3). In the same way, Cluster II was comprised of only one isolate *Bacillus subtilis* (PBS1-5), revealing an internal similarity of 85 % with their homolog cluster I. Similarly, Cluster III was also comprised of only one isolate *Bacillus spizizenii (*NRRL B-23049) revealing an internal similarity of 84 % showing close association with cluster I and cluster II in comparison to other clusters. Cluster IV was comprised of 8 isolates including *Bacillus haynesii* (NRRL B-41327), *Bacillus paralicheniformis (KJ-16), Bacillus glycinifermentans* (GO-13), *Bacillus licheniformis* (ATCC 14580), *Bacillus aerius* (24K), *Bacillus piscis* (16MFT21), *Bacillus sonorensis* (NBRC 101234) and *Bacillus swezeyi* (NRRL B-41294). revealing an internal similarity of 75% that was found to be more closely related to cluster I, cluster II, and cluster III. Cluster V was comprised of only one isolate *Bacillus atrophaeus* (JCM 9070). revealing an internal similarity of 97 % with their homolog clusters viz., cluster I, cluster II, cluster III, and cluster IV. Cluster VI was comprised of 9 isolates including *Bacillus zhangzhouensis* (DW5-4), *Bacillus pumitus* (ATCC 7061), *Bacillus australimaris* (NH71_1), *Bacillus safensis subsp. Safensis* (BBS4-5), *Bacillus safensis subsp. Safensis* (BBS4-3), *Bacillus safensis subsp. Safensis* (FO- 36b*)*, *Bacillus safensis subsp. Safensis* (KBS3-8)*, Bacillus xiamenensis* (HYC-10), and *Bacillus altitudinis* (41KF2b) reveal an internal similarity of 76 %. *Bacillus safensis subsp. Safensis* (BBS4-5), *Bacillus safensis subsp. Safensis* (BBS4-3), and *Bacillus safensis subsp. Safensis* (KBS3-8) were the isolates that were isolated from microbial bio flocs in the present study and were clustered in cluster VI, found to be more closely associated with clusters including cluster I, cluster II, cluster III, cluster IV, and cluster V showing monophyletic nature. Cluster VII was comprised of only two isolates including *Bacillus capparidis* (EGI 6500252) and *Bacillus gobiensis* (FJAT-4402) revealing an internal similarity of 100 % showing close association with clusters including cluster I, cluster II, cluster III, cluster IV, cluster V, and cluster VI. Cluster VIII and Cluster IX, both were comprised of only one isolate including *Bacillus salacetis* (SKP7-4) and *Bacillus acidicola (105-2)* respectively revealing an internal similarity. These two clusters were considered distant clusters in comparison to other clusters. The majority of the taxons in a phylogenetic tree were considered similar taxons revealing homology and monophyletic nature (Figure 3).

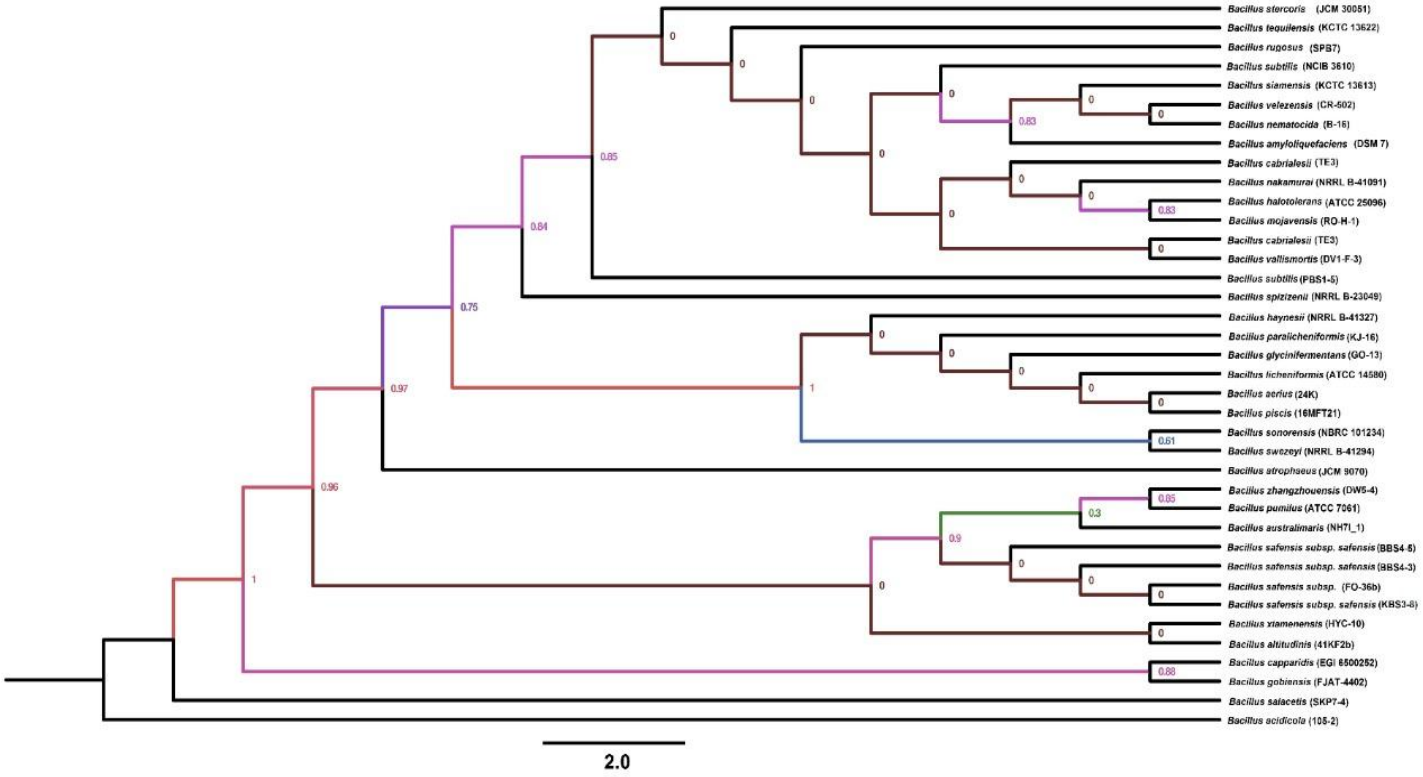

Figure 3 Maximum Likehood (ML) relationship of bacterial isolates based on 16S rDNA gene sequences with closely related 16s rDNA gene sequences available in NCBI data bas

Evaluation of Amylase Activity

The significantly highest amylase activity was observed for *Bacillus safensis subsp. Safensis* (20.2 ± 0.81 mm), followed by 18.3±0.73 mm for *Bacillus safensis subsp. Safensis* and 18±0.72 mm for *Bacillus subtilis* while significantly least amylase activity was observed for PBS2-4 (11.2±0.45 mm) followed by PBS1-6 (14.3±0.57mm) and KBS3-3 (15±0.6mm) as shown in Table 5. Few bacterial isolates including PBS1-1, PBS1-2, PBS1-3, PBS2-2, PBS2-6, BBS4-1, BBS4-2, BBS4-4, KBS3-2, KBS3-4, and KBS3-9 have not shown amylase activity on their respective media.

Evaluation of Cellulase Activity

The significantly highest cellulase activity was observed for *Bacillus safensis subsp. Safensis* (17.2±0.69mm), followed by (16.3±0.65mm) for KBS3-3, (16.2±0.65mm) for *Bacillus safensis subsp.Safensis* (15.2±0.61 mm) for KBS3-1, (14.4±0.58mm) for BBS4-1, (14±0.56mm) for *Bacillus subtilis,* (13.5±0.54mm) for PBS1-1, (13.2±0.53mm) for PBS2-2, (13±0.52mm) for KBS3-3, (12±0.48mm) for PBS1-4 and significantly minimum cellulase activity was observed for KBS3-9 and KBS3-4 (11.2±0.45 mm) as displayed in Table 5. Some of the bacterial isolates namely, PBS1-2, PBS1-3, PBS2-4, PBS2-6, BBS4-2, BBS4-4, KBS3-2, and KBS3-7 have not revealed cellulase activity on their corresponding media.

Evaluation of Protease Activity

On the same pattern protease activity of bacterial isolates was also noted which showed as slightly different from cellulase and amylase activities. The significantly highest protease activity was noted for *Bacillus subtilis* (21±0.84mm) followed by 19±0.76mm for KBS3-9 and 18.4±0.74mm for *Bacillus safensis subsp. Safensis* 18.3±0.73mm for KBS3-7, 17.4±0.7mm for KBS3-3 17.3±0.69mm for KBS3-1, 17±0.68mm for *Bacillus safensis subsp. Safensis*, 16.1±0.64mm for KBS3-2, 16±0.64mm for PBS2-1 and 14±0.56mm for BBS4-3 while significantly the lowest protease activity was observed for PBS1-6 and KBS3-4 (13.3±0.53 mm) as shown in Table 5. Few bacterial isolates such as PBS1-1, PBS1-2, PBS1-3, PBS1-4, PBS2-2, PBS2-4, PBS2-6, BBS4-1, BBS4-2, BBS4-4, and KBS3-4 probiotic bacterial isolate were not shown amylase activity on their relevant media.

Table 5 Evaluation of Amylase activity, Cellulase activity and Protease activity for Bacteria Isolates, isolated from Biofloc sample

Bacterial isolates	Amylase activity ± SD (mm)	Cellulase activity ± SD (mm)	Protease activity ± SD (mm)	P values
PBS1-1	0	13.5±0.54	0	0.4226
PBS1-2	0	0	0	-
PBS1-3	0	0	0	-
PBS1-4	16±0.64	12±0.48	0	0.1917
Bacillus subtilis	18±0.72	14±0.56	21±0.84	0.0129
PBS1-6	14.3±0.57	11.5±0.46	13.3±0.53	0.0039
PBS2-1	14±0.56	10.4±0.42	16±0.64	0.0145
PBS2-2	0	13.2±0.53	0	0.4226
PBS2-4	11.2±0.45	0	0	0.4226
PBS2-6	0	0	0	-
KBS3-1	17.1±0.68	15.2±0.61	17.3±0.69	0.0016
KBS3-2	0	0	16.1±0.64	0.4226
KBS3-3	15±0.6	16.3±0.65	17.4±0.7	0.0018
KBS3-4	0	11.2±0.45	0	0.4226
KBS3-7	12.4±0.5	0	18.3±0.73	0.1982
Bacillus safensis subsp.Safensis	20.2±0.81	17.2±0.69	18.4±0.74	0.0022
KBS3-9	0	11.2±0.45	19±0.76	0.2094
BBS4-1	0	14.4±0.58	0	0.4226
BBS4-2	0	0	0	-
BBS4-3	17±0.68	13±0.52	14±0.56	0.0066
BBS4-4	0	0	0	-
Bacillus safensis subsp.Safensis	18.3±0.73	16.2±0.65	17±0.68	0.0013

P<0.05 indicates significance

Note: The enzyme activity assays (amylase, cellulase, and protease) were performed in triplicate to ensure reproducibility. Negative controls (media plates without bacterial inoculation) were also included to confirm the absence of enzymatic activity from the media itself. The values are presented as mean ± standard deviation (SD). P-values were calculated using appropriate statistical tests, with P < 0.05 indicating significance.

Effects of Probiotics Supplementation

Effects of probiotics supplementation on Mori (*Cirrhinus mrigala*) in biofloc were evaluated as shown in Table 6. It was revealed that probiotics bacterial isolates have profound effects on weight gain, % weight gain, Feed conversion ratio (FCR) and Specific growth rate (SGR %). *Bacillus safensis* (probiotic bacterial isolate has shown a significant increase in weight gain of Mori (*Cirrhinus mrigala*) (31.02±1.24g) followed by *Bacillus subtilis* (30.9±1.24g), *Bacillus safensis* (22.29±0.89g) in comparison to control (15.3±0.61g). The % weight gain of *Cirrhinus mrigala* was significantly observed high for probiotic bacterial isolate *Bacillus safensis* (82.98±3.32 %) followed by *Bacillus subtilis* (81.78±3.27 %), *Bacillus safensis* (58.41±2.34 %) as compared to control (42.28±1.69 %). Similarly, FCR was observed highest for *Bacillus safensis* (2.09±0.08), followed by *Bacillus subtilis* (2.08±0.08), *Bacillus safensis* (1.57±0.06) in comparison to control (1.29±0.05) The SGR% was observed as significant for *Bacillus safensis* (0.93±0.04) followed by *Bacillus subtilis* (0.92±0.04), while least significant SGR% was observed for *Bacillus safensis* (0.71±0.03) in comparison to control (0.54±0.02).

Table 6 Effects of Probiotics Supplementation on growth parameters of Mori (*Cirrhinus mrigala*) in Biofloc

Growth Parameters	Control	*Bacillus subtilis*	*Bacillus safensis*	*Bacillus safensis*	P Value	95% Confidence Interval	
						Lower	Upper
Initial weight (g)	36.49±1.46	37.78±1.51	37.31±1.49	38.16±1.53****	0.0000	36.29	38.58
Final weight (g)	51.92±2.08	68.68±2.75***	68.33±2.73	60.45±2.42	0.0006	49.74	74.949
Weight gain (g)	15.34±0.61	30.9±1.24	31.02±1.24**	22.29±0.89	0.0072	12.81	36.941
% Weight gain	42.28±1.69	81.78±3.27	82.98±3.32**	58.41±2.34	0.0066	35.11	97.612
Feed conversion ratio	1.29±0.05	2.08±0.08	2.09±0.08**	1.57±0.06	0.0031	1.125	2.4
Specific growth rate (%)	0.54±0.02	0.92±0.04	0.93±0.04**	0.71±0.03	0.0037	0.478	1.072

P < 0.05 shows the significance ** significant, *** More significant, **** Most Significant

DISCUSSION

In the present study, a total of 22 bacterial isolates were isolated from different water samples of microbial bioflocs located in PBS1, PBS2, KBS3, and BBS4, Khyber Pakhtunkhwa, Pakistan. Out of these 22 bacterial isolates, 6 bacterial isolates were isolated from microbial bioflocs of PBS1, 4 bacterial isolates were isolated from microbial biofloc of PBS2, 7 bacterial isolates from microbial bio floc of KBS3, and 5 bacterial isolates were isolated from microbial biofloc of

BBS4. Phenotypic attributes were included as shape (Circular, wavy, dome) surface type (Smooth and rough), color (White, creamy, creamy white, and yellow), and size (Small, medium, and large) of bacterial colonies. All the bacterial isolates possessed major characteristics as Genus *Bacillus* such as Gram-positive rod-shaped, endospore-forming, and catalase positive but for oxidase and citrate tests they were mostly negative, and a few bacterial isolates were observed positive. The findings in our study agreed with those reported previously who have isolated Gram-positive *Bacillus spp*. from the animal gut and they have evaluated their probiotic potential. In the same pattern, three types of LAB such as *Bacillus*, *Lactobacillus,* and *Eubacterium* were isolated from the digestive tract of the catfish **(Diale, 2022**). By comparing the 16S rDNA sequences with the reference sequences available in DDBJ and GenBank, it was observed that the bacterial isolate PBS1-5 (Accession number NCIB 3610) had a similarity of 100 % with sequences of reference bacterial strain, *Bacillus subtilis*. Similarly, the bacterial isolate KBS3-8 (Accession number FO-36b) has revealed a significant similarity of 99.78 % with sequences of reference bacterial strain, *Bacillus safensis.* On the same pattern bacterial isolate BBS4-3 (Accession number FO-36b) has shown significantly 100 % similar with the bacterial isolate sequences of reference bacterial strain, *Bacillu*s safensis*.* Moreover, bacterial isolate BBS4-5 (Accession number FO-36b) has revealed significant similarity 100 %with sequences of the reference bacterial strain, *Bacillus safensis*. These outcomes aligned with the Maliehe *et al.*, which isolated a bioflocculant-producing bacteria from the NCBI website (http://www.ncbi.nlm.nih.gov/), which can be accessed using the BLAST search tool, was used to analyse the sequence. The bacteria were found to have a 95 % sequence similarity with accession number NR 133700.1 and were identified as *B. enclensis* **(Maliehe *et al.*, 2022**). In the present study, a phylogenetic tree was made using 16S rDNA gene sequences of bacterial isolates from microbial bio flocs with 38 closely related 16s rDNA gene sequences through the Maximum Likelihood (ML) approach, which clustered this taxon into nine clusters with different percentages of similarities. *Bacillus subtilis* (PBS1-5) and *Bacillus safensis subsp. Safensis* (BBS4-5), *Bacillus safensis subsp. Safensis* (BBS4-3), and *Bacillus safensis subsp. Safensis* (KBS3-8) were the isolates that were isolated from microbial bio flocs in the present study, which were clustered in cluster II and Cluster VI respectively. Cluster II was comprised of only one isolate *Bacillus subtilis* (PBS1-5) revealing an internal similarity of 85% with their homolog cluster I while Cluster VI was comprised of 9 isolates including *Bacillus safensis subsp. Safensis* (BBS4-5), *Bacillus safensis subsp. Safensis* (BBS4-3), *Bacillus safensis subsp. Safensis* (FO- 36b*)*, *Bacillus safensis subsp. Safensis* (KBS3-8) reveal an internal similarity of 76 % showing a monophyletic nature and close association with clusters including cluster I, cluster II, cluster III, cluster IV, and cluster V. The majority of the taxon in a phylogenetic tree were considered similar taxon revealing homology and monophyletic nature Recently, **Jang *et al.*, (2022**) have identified the impact of *Bacillus sp.* supplementation diet on survival rate and microbiota composition. The *Bacillus* sp. bacteria that were added to the diet were isolated from the intestines of wild glass eels and recognized by 16S rRNA sequencing analysis. *Bacillus sonorensis* NBRC 101234T (AYTN01000016), *B. haynesii* NRRL B-41327T (MRBL01000076), *B. licheniformis* ATCC 14580T (AE017333), and *B. paralicheniformis* KJ-16T (KY694465) all had 99.86, 99.59, 99.39, and 99.32% homology with the *Bacillus* species, respectively **(Jang *et al.*, 2022)**. In another report, **Naim and Mahboob (2020)** evaluated Bacillus spp by partial 16S rRNA sequence analysis. The Neighbour-Joining approach was used to create a phylogenetic tree and the Maximum Composite Likelihood method was used to calculate the evolutionary distances **(Naim and Mahboob, 2020).** Wu and Yang, 2020 have isolated probiotic bacteria from guppy Poeciliareticulata (Cyprinodontiformes: Poecilidae), and the 16S rRNA fragment of the isolates was characterized and depicted 100 % sequence identity. In the present study, it was observed that bacterial isolates were screened for probiotic activities such as amylase, cellulase, and protease. The significantly highest amylase production was observed for bacterial isolates as bacterial isolate *Bacillus safensis subsp. Safensis* has shown the highest amylase activity while the significantly least amylase activity was observed for PBS2-4. Previously probiotic *Bacillus* sp. isolated from garden soil has shown a distinct zone of starch hydrolysis (Nimisha *et al.*, 2019). Earlier, *Bacillus subtilis* gave a high yield of alpha-amylase after 48 hours of fermentation **(Raul *et al.*, 2014**). Cellulase assay was performed for bacterial isolates, which showed that 14 bacterial isolates were significantly cellulase producers that formed zones of inhibition on Carboxymethyl cellulase (CMC) agar medium while remaining 8 bacterial isolates did not show cellulase activity. The significantly highest cellulase activity was observed for *Bacillus safensis subsp. Safensis* followed by bacterial isolates KBS3-9 and KBS3-4 respectively. It was observed in earlier investigations that *B. subtilis was* isolated from agricultural and areas has shown the highest cellulase activity at pH 6.5 to 7.5 at 45℃ on 1.5 % Carboxymethyl cellulose (CMC) agar medium (**Vipul *et al.*, 2012)**. Similarly, in another study, *Paenibacillus* sp. strain B39 was isolated from poultry manure compost which has endoglucanase activity on the CMC substrate **(Wang *et al.*, 2005**). Besides amylase and cellulase probiotic activities, protease activities were also determined for bacterial isolates that have shown protease activities for 11 bacterial isolates while the remaining 11 bacterial isolates were observed as non-protease producers. The significantly highest protease activity was reported for *Bacillus subtilis* while significantly the lowest protease activity was observed for PBS1-6 and KBS3-4 respectively. It was found earlier that supplementation of *B. subtilis* in tilapia with a dose of 10^8 cfu/g diet has significantly increased protease and amylase activities in the intestinal homogenate after 4 weeks (H. Liu *et al.*, 2017). A similar result was also reported for a *B. cereus* strain, which was isolated from the soil **(Ayangbenro and Babalola, 2020**). Moreover, *B. licheniformis* strain 018 isolated from poultry farms has shown optimum protease production at 35°C **(Sanghvi *et al.*, 2016)**. In the current study, probiotic bacterial isolates i-e. *Bacillus subtilis*, *Bacillus safensis subsp. Safensis* and *Bacillus safensis subsp. Safensis* were evaluated for their probiotic potential in bio flocs on Mori (*Cirrhinus mrigala*) fish fingerlings. Analysis of growth parameters revealed that the probiotic bacterial isolates were significant for enhancing the weight gain, % weight gain, Feed conversion ratio (FCR), and Specific growth rate (SGR %) of Mori (*Cirrhinus mrigala*) fingerlings in bio flocs in their comparison to control. *Bacillus safensis subsp. Safensis* has shown a significant increase in weight gain, % weight gain, FCR, and SGR % of Mori (*Cirrhinus mrigala*) in comparison to the control. The findings in our study revealed that supplementation of bioflocs with beneficial probiotic bacterial isolates can improve the overall growth of Mori (*Cirrhinus mrigala*) fingerlings. Moreover, these probiotics supplementations were observed as significant for enhancement of the survival rate, improved water quality, reduced feed supplementation and enhanced immunity etc. Earlier, it was reported that *Bacillus cereus* was isolated from the intestine of the catfish, *Pangasius bocourti*, which has a probiotic effect on the growth after 60 days post-feeding **(Meidong *et al.*, 2018)**. Similarly, bighead carp, common carp, and grass carp were administered with *Bacillus spp*., which has shown considerable changes in FCR, SGR, and feed efficiency **(Ramzannejad *et al.*, 2021**). In another report, *Enterococcus faecalis* was supplemented as probiotics at 10^7 and 10^9 cfu g^{-1} diet for Javanese carp (*Puntius gonionotus*), which causes significant weight gain **(Tarkhani *et al.*, 2020)**. It was reported that the growth performance of *Labeo rohita* fish was increased with diets containing *B. subtilis*, *B. subtilis + L. plantarum* and *B. subtilis + P. aeruginosa + L. plantarum,* which has considerably improved impacts on the growth performance of experimental fish in comparison to the control group **(Giri *et al.*, 2014)**. Several studies have shown that *Bacillus* spp. could be used as probiotic bacteria to enhance immunity and resistance to pathogens in aquatic animals (Naresh *et al.,* 2014). The dietary supplementation of probiotic and bacterial cocktails was found to improve the gut immune response, morphology and microbial assemblage of the intestine in juvenile *Oreochromis niloticus* (**Yamashita *et al.*, 2017).**

CONCLUSIONS

It was revealed from the present study that the probiotic bacteria *Bacillus safensis subsp. Safensis*, followed by *Bacillus subtilis* and *Bacillus safensis subsp. Safensis*, produced significant impacts on the growth and survival of Mori (*Cirrhinus mrigala*) in bioflocs compared to the control. The probiotic bacterial isolate *Bacillus safensis subsp. Safensis* demonstrated superior growth performance compared to *Bacillus subtilis* and *Bacillus safensis subsp. Safensis* on Mori (*Cirrhinus mrigala*) in bioflocs. The practical implications of this approach are promising, as these strains can be cultured and applied on a larger scale to enhance sustainable aquaculture practices. Further studies focusing on large-scale application, optimization of dosage, and assessment of long-term effects are essential to fully harness the potential of these *Bacillus* strains in commercial aquaculture.

Future Recommendations

It is recommended that probiotic bacterial isolates *Bacillus safensis subsp. Safensis* and *Bacillus subtilis* can be applied on large scale i-e. hatcheries, big ponds etc. rather than a small scale to increase the production of fish. These probiotics bacterial isolates are further suggested for immunological studies and supplements in feed for other cultural fish to enhance digestion.

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Authors contribution: MD and AZ designed the experiments. AK performed the experiments and data analysis. MR, NA, SB, ER, AR and AK write and edit the paper. RN, MR, and AR reviewed the paper and validated the data. RN, MD and AZ supervised the project.

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