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Isolation, Identification, and Biofloc Production: Potential of Floc-Forming Bacteria Using a Novel Monoculture Approach and Medium

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Abstract

Biofloc is one of the suitable alternatives for decreasing food costs and increasing the immunity of cultured organisms against pathogens and diseases. This research aims to isolate, identify, and screen bioflocculant bacterial candidates to optimize practices for enhanced water quality management. Twelve bacteria were obtained from incubated shrimp pond water. These bacteria were tested for their ability to produce biofloc using a newly developed combination of Biofloc Standard Mineral Water (BSMW) and Biofloc Standard Medium (BSM) in a laboratory setting, utilizing an Imhoff cone. A novel medium was used for the first time for identifying biofloc-producing bacteria. Strain KBF-103 exhibited rapid biofloc production within 24 hours, making it suitable for pond water application. Strain KBF-103 was identified using molecular via 16S rRNA sequencing and shows a close relation with the *Bacillus cereus* strain.

Introduction

Aquaculture stands out as the most rapidly expanding segment within the realm of food production. It holds significant potential in enhancing global food security by supplying high-quality protein to the expanding population and offering livelihood opportunities to numerous individuals. In the twenty-first century, there is a growing acknowledgment of the crucial role played by the fisheries and aquaculture sectors in ensuring global food security and nutrition (FAO, 2022).

There are three types of aquaculture systems for the culturing of organisms in ponds, extensive which are characterized by low stocking densities and large pond areas, it relies on minimal external inputs. Semi-intensive; in this system, a balance is struck between stocking densities and pond sizes. Moreover, in this approach along with natural cycling, supplemental feed is also considered to increase production. The third is intensive aquaculture, representing a high-density cultivation system where ponds are closely managed, and external inputs, including feed and water treatments play an important role. The intensive approach is not applicable without biofloc technology. Biofloc technology changed the facet of intensive aquaculture with the purpose of attaining high productivity with a sustainable approach (Oddsson, 2020).

Biofloc is a congregation of beneficial microorganisms like heterotrophic bacteria, algae, ciliates, flagellates, fungi, nematodes, rotifers, metazoans, and detritus. Therefore, the bacterial community is predominantly heterotrophic rather than

autotrophic and denitrifying (Hargreaves, 2013). Sustaining the ratio of a high carbon-to-nitrogen will help to control biofloc. Moreover, to maintain water quality even without water exchange, Biofloc Technology (BFT) allows for high densities and biosecurity, maximises the use of nitrogen input in the form of feed, and ultimately produces a profitable system. By manipulating the C:N ratio to create a dense heterotrophic bacterial population, the system shifts from algae to bacteria as the dominant species and uses in situ bioremediation to preserve water quality. (Avnimelech, 1999). Studies reveal that in biofloc systems with a carbon-to-nitrogen ratio of 15–20:1, Lactobacillus species are more abundant than Enterococcus species (Islam, *et al*., 2021). Bacteria use carbon as a source of energy and N for their growth (Panigrahi, *et al*., 2019). At any one time, the biofloc system provides an extremely varied natural resource of protein and lipids that is easily accessible and abundant. (Avnimelech, 2007; Martinez-Cordova *et al*. 2014).

The heterotrophic bacterial population in the biofloc system contributes significantly to controlling ammonia concentration, crucial for maintaining a stress-free environment for shrimps in pond ecosystems. Effective nitrogen recycling, keeping Total Ammonia Nitrogen (TAN) below the safe level (1 ppm) with a high C:N ratio, ensures optimal conditions for shrimp well-being (Hargreaves, 2013).

For the past ten years, research and application have become more focused on the BFT system due to its potential to increase resource utilisation efficiency. Consequently, several advantageous aspects of the biofloc system have been discovered, including its nutritional value, the exogenous digestive enzymes it contributes, the possibility of pathogen it controls, and the immunostimulatory effects. (Betanzo-Torries et al. 2020; El-Sayed, 2021). Additionally, some research has indicated that the production of biofloc is mediated by microbial phenotypes that synthesis tiny membrane diffusible molecules (Chong *et al*., 2012; Hawver *et al*., 2016).

The intensive aquaculture approach, characterized by high stocking densities and meticulous management, poses significant challenges for farmers, particularly in terms of feed utilization and biofloc inoculum application. The substantial consumption of feed in intensive systems results in the accumulation of fecal matter and uneaten feed, leading to the buildup of sludge in ponds. This accumulation, coupled with the subsequent elevation of ammonia and nitrate levels, presents a potential threat to aquatic ecosystems, causing economic losses for aquaculturists. Additionally, the use of old pond flocs as inoculum introduces the risk of pathogenic and opportunistic bacteria, as highlighted by Aslam *et al*. (2022) in biofloc-based aquaculture systems.

The application of such inoculum to newly cultured ponds further amplify the risk, as limited control mechanisms exist for the regulation of beneficial and nonbeneficial organism growth. Bacterial pathogens identified in biofloc systems pose a substantial threat, capable of causing economic losses and harm to cultured fish and prawns. The high stocking densities inherent in confined spaces within intensive aquaculture systems create favorable conditions for the rapid spread of diseases, leading to widespread impacts on entire populations and significant economic losses. Furthermore, a critical gap in the existing literature pertains to the lack of a standardized and sustainable reproducible biofloc inoculum. Specifically, the absence of a standardized procedure for identifying mono bacterium bioflocculants tailored for application in biofloc technology (BFT) limits the advancement and adoption of this technology. Addressing these challenges is imperative for the sustainable development of intensive aquaculture systems, ensuring environmental integrity, economic viability, and the welfare of cultured aquatic organisms (Aslam *et al*., 2022; Panigrahi *et al*., 2019; Prasanthan *et al*., 2011; Takemura, *et al*., 2014; & Avnimelech, 2009).

This research aims to address this critical gap by focusing on the isolation, identification, and screening of mono bacterium bioflocculant candidates. By developing a standardized procedure for the identification of mono-bacterium bioflocculants. This study seeks to contribute valuable insights into optimizing BFT practices for enhanced water quality management and the promotion of immunostimulatory effects on cultured aquatic organisms.

Materials and Method

Sample Collection

Sample was collected from Konisa Shrimp Pond with a Latitude 18°17'27.2"N and longitude 83°17'44.9"E. Konisa village is located in Gajapathinagaram mandal of Vizianagaram district, Andhra Pradesh. The container rinsed twice with pond water and a 2L volume sterilized containers collected sample was sealed and transported immediately to the laboratory. The experiment was carried out during May 2022 to Aug 2023.

Physicochemical analysis of water: The water sample was tested for various parameters using established protocols. These parameters included temperature, turbidity, pH, Total Dissolved Solids (TDS), Electrical Conductivity (EC), ORP, total hardness, calcium hardness, magnesium hardness, alkalinity, ammonia, nitrite, sulphate, and phosphate. The testing was conducted using the BLE-C600 Smart Bluetooth 7 In 1 device and followed the Standard Methods For Examination of Water and Wastewater Methods (APHA, 1985).

Microbial quality analysis of the sample: Initial viable bacterial count was checked by taking of 50 µl of sample and spread on Nutrient Agar enriched with 3%

sodium chloride plate and as well TCBS by spread plate technique (Aneja, 2005).

Preparation of biofloc standard medium: A novel Biofloc Standard Medium (BSM) was developed, comprising Peptone (5.0 g), Sodium Chloride (5.0 g), Beef extract (1.5 g), Yeast extract (1.5 g), Glucose (10 g), and Distilled water (1L). The medium was subsequently sterilized by autoclaving at 121°C for 15 minutes. Henceforth, this medium is referred as BSM.

Preparation of biofloc standard mineral water (BSMW): BSMW was prepared to use clean, uncontaminated sea water (32-35 PPT), diluted with distilled water to obtain 1PPT (parts per thousand) salinity. Salinity was measured by using a Digital Salinity meter, (Model AR 8012). The alkalinity of the 1PPT water was adjusted to 160-200 ppm by employing the APHA 2320 B titration method and using sodium bicarbonate (APA, 2012).

Production of Biofloc Using Sample Water

A volume of one litre of water sample was collected in a sterilized Imhoff cone and then incubated with adequate aeration. The process involved the use of an aquarium air pump, specifically model AP-338, which has a capacity of 4 X 10 L/min. A transparent silicon tube with an internal diameter of 2.5 mm was utilized to provide air to the Imhoff cones. The aeration pipe was weighted correctly to ensure proper submersion. Furthermore, the ambient temperature of the room was carefully regulated to be 28±2°C, and no light was present to prevent the growth of photoautotrophic organisms. At first, a volume of 25-30 ml of BSM was added into the Imhoff cones. The visible examination of the sample for the formation of bioflocs was documented every 24 hours by pausing the aeration for 30 minutes, and the quantity of flocs formed was measured. The incubation period was extended until a biofloc with a volume of 5 to 10 ml was considered suitable for harvesting. If the amount of biofloc was less than 5 ml, it was deemed inadequate. In these instances, the incubation period was prolonged by introducing aeration and supplementing with 25-30 ml of BSM until the biofloc attained the desired volume.

Collection, Purification, Harvesting and Preservation of Biofloc

After attaining of suitable flocs, the Imhoff cones aeration stopped for more than one hour until the flocs settled completely at the bottom of the Imhoff cones. After settlement of flocs the supernatant (liquid above the sediment) was discarded, and flocs were transferred into centrifuge tubes and centrifuged at 3000 rpm for 15mins and washed twice with sterile saline water (0.9% sodium chloride). Then, the pellet was collected and labelled as first-generation floc accordingly. The first-generation flocs were preserved in saline water and refrigerator for further use.

Isolation, Purification, and Preservation of Bacteria

For Isolation of bacteria 1ml of harvested flocs was transferred into 15 ml centrifuge tubes and serial dilution of aliquots was done accordingly at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} For isolation of pure biofloc producing bacteria. Therefore 3rd, 4th and 5th dilution were used spread plate techniques (Aneja, 2005). Individual colonies were then purified by quaternary streaking method one or two times on nutrient agar plates to obtain distinct pure culture. All isolated bacteria were streaked on 1- 3% salted Nutrient agar media, and preserved with 20% (w/v) glycerol and kept at -20 °C for future use (Mahmmoud, 2020).

Screening of Biofloc Production by Isolated Bacteria

Preparation of inoculum: Bacterial cultures isolated from the first generation were screened for biofloc production. These cultures were cultivated overnight on Nutrient agar plate. A loopful of a fresh 15 to 18-hour-old culture was added to 10-15 ml of 0.5 to 3% sodium chloride Nutrient broth. This culture was agitated until the turbidity reached a specific range between 0.5 nm to 0.7 nm at 600 nm, using spectrophotometer (Go Direct SpectroVis Plus Spectrophotometer) indicating bacterial growth and activity. This activated bacterium was then ready for use as an inoculum for initiating biofloc formation.

Initiation of biofloc: Around 10-15 ml of activated bacterial inoculum was added to 1 litre of BSMW using Imhoff cones. Afterwards, 25-30 ml of BSM was added with sufficient aeration, and then the mixture was incubated. The process was iterated for a duration of 2-3 days, with the addition of 25-30 ml of BSM for every 24 hours until the desired bioflocs increased, below 5ml of floc was kept for additional 24-hour incubation.

Harvesting of biofloc: After incubation, visible observations of floc formation were recorded for every 24 hours. Adequate able Biofloc (10-15 ml) was harvested for microbiological analysis.

Microscopic Quality Observation of Biofloc

Physical parameters like colour was observed based on pigmentation, turbidity measured using TB100 Portable Turbidity Meter, and quantity of biofloc was measured after settlement of biofloc at the bottom of the Imhoff cone in ml. The floc was stained with crystal violet as well as unstained floc was also observed under a compound microscope. Microscopic observation of biofloc under different magnifications e.g. 4x, 10x, 40x and 100x was observed.

Phenotypic Characters of the Isolated Bacteria

Phenotypic characters like colony morphology, cell morphology, motility, endospore forming ability, and Gram's reactions were recorded. Biochemical tests like

string test, IMViC test (McDevitt, 2009), Amino acid decarboxylase tests and Carbohydrate fermentation tests was conducted (Reiner, 2012). Growth on different media such as Congo red agar, Bacillus selective media (Hi Media Pvt Ltd) was tested.

Molecular Identification Using 16S rRNA Gene

Nucleic acid was isolated from the test isolates and its quality was evaluated on 1% agarose gel, and a single band of high molecular weight DNA was observed. Fragments of 16S rRNA gene were amplified by using 16SrRNA-Forward 27F (5'AGAGTTTGATCCTGGCTCAG3') and 16SrRNA-Reverse 1492R(5'CGGTTACCTTGTTAC GACTT3') primers. A distinct PCR amplicon (a piece of RNA or DNA which is the source for amplification or replication reaction) band of 1,500 base pairs was observed and resolved on agarose gel and was purified to remove contaminants. By using BDT v3.1cycle sequencing kit on ABI 3730 X1 Genetic analyzer, forward and reverse DNA sequencing of PCR amplicon was carried out (Kimura, 1980). By using Aligner software consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data. 16S rRNA gene sequence was used to carry out BLAST with the nucleotide collection database of NCBI GenBank. Based on maximal identity score, the first ten sequences were chosen and aligned using the multiple alignment tool ClustalW. Phylogenetic tree and distance matrix was constructed by using Molecular Evolutionary Genetics Analysis (MEGA 10) (Kumar, *et al*., 2018).

Property Test of Identified Bacteria

Salt and Temperature Tolerance: All isolated organisms underwent testing for salt tolerance at varying concentrations, including 0%, 6%, 8%, and 10% NaCl. They were individually streaked on Nutrient agar plates containing different salt concentrations and incubated at 32°C. Growth observations were then recorded after 24 and 48 hours. Additionally, the isolated organisms were streaked on Nutrient agar plates and subjected to different temperatures, specifically 40°C, 45°C, 50°C, and 55°C. Growth observations were again recorded after incubation periods of 24 and 48 hours.

Enzymatic Production

Enzymatic assays, including the catalase test, oxidase test, starch hydrolysis, gelatine hydrolysis and hemolysin activity were conducted to assess the production of catalase, cytochrome-c, amylase, protease, and hemolysin activity, respectively as per the guideline outlined by Mastroianni (2016).

Result

The key physicochemical parameters observed in the sample water. Salinity was measured at 1 (ppt), while the EC recorded a value of 1750 µs/cm. The total dissolved solids (TDS) in the water sample were noted to be 630 (ppm), and the ORP was observed to be +98 millivolts (mV). The pH level of the water was found to be 7.8, indicating a near-neutral condition. Turbidity, a measure of water clarity, was recorded at 93 NTU, suggesting a moderate level of suspended particles. Further analysis revealed alkalinity levels of 224 ppm. Total hardness was measured at 384 ppm, with calcium hardness at 115 ppm and magnesium hardness at 269 ppm, indicating the presence of mineral ions in the water. Remarkably, ammonia, nitrite levels and phosphate were undetectable in the sample water, Sulfate was observed at 40 ppm.

Production of Floc Using Sample Water

After a period of 24 hours, the process of aeration was temporarily halted for a duration of 30 minutes, during which an amount of 3ml of floc was noticed. Following the period of observation, the incubation process was prolonged by introducing an additional 25-30ml of sterilized BSM at 48 hours of incubation, aeration was once again paused, resulting in the observation of 7ml of floc. This floc was then collected, purified, and subjected to screening in order to identify individual bacterial strains.

Isolation and Purification of Bacteria from First Generation Biofloc

Ater 48 hours of incubation 7 ml of floc was obtained. The floc was centrifuged and cleaned with sterile saline water twice. This floc was subject to serial dilution technique and aliquots of high dilution was plated on nutrient agar medium. After incubation pure culture of 12 bacterial strains were obtained by following general microbiological techniques. Which given code as KBF-101, 102, so on till KBF-112. These cultures were purified by quarternary streaking methods and preserved with 20% glycerol at -20°C for further research studies.

Screening of Isolated Bacteria for Biofloc Production

All 12 bacterial samples were screened for biofloc production using 1L Biofloc Standard Mineral Water (BSMW) with BSM. Among the isolates strain KBF-103 was showed potential to form flocs individually, the experiment was repeated by using 1% KBF-103 as inoculum confirmed the same results. Thus, isolate KBF-103 demonstrated the ability to form floc within 24 hours.

Flocs Production Using Isolated Bacteria (KBF-103).

The incubation of KBF-103 strain for 120 hours revealed its relationship with turbidity over this period. The experiment was done in duplicate, and the mean of the value was taken. The graph depicting floc production over 120 hours highlights three stages: initial floc formation in less than 24 hours (average volume 17.5ml), peak production at around 48 hours (average volume 27.5ml), and a subsequent decline. The decline post-48 hours may signify a bacterial death phase, impacting floc formation despite consistent nutrient addition Figure 1.

Simultaneously, turbidity levels were monitored, starting at 5 NTU and steadily increasing to 1000 NTU after 120 hours of incubation. Notably, as floc production declines, turbidity rapidly increases, indicating a correlation. The correlation coefficient of -0.40 suggests a medium negative correlation between declining floc production and rising turbidity. This may indicate that the decline in floc production contributes to the increase in turbidity (Figure 2)

Observation of KBF-103 Biofloc

The flocs were produced within 24 hours and some amount of the pure KBF-103 strain flocs were observed under microscope at different magnification as shown in Figure 3.

Microscopic observation at 4x: Under this magnification, the flocs appeared as tiny specks or dots scattered, Floc matrix comprised of extracellular polymeric structures that form microbial capsules was observed. This level of magnification provides a general overview of the distribution of bacteria within the sample.

Microscopic observation at 10x: At a closer magnification, individual bacteria remain indistinguishable, appearing as small, translucent dots. However, some basic shapes and arrangements, such as clusters or chains, become apparent. Larger bacterial aggregations can be observed, allowing for differentiation between different types based on their size and grouping patterns.

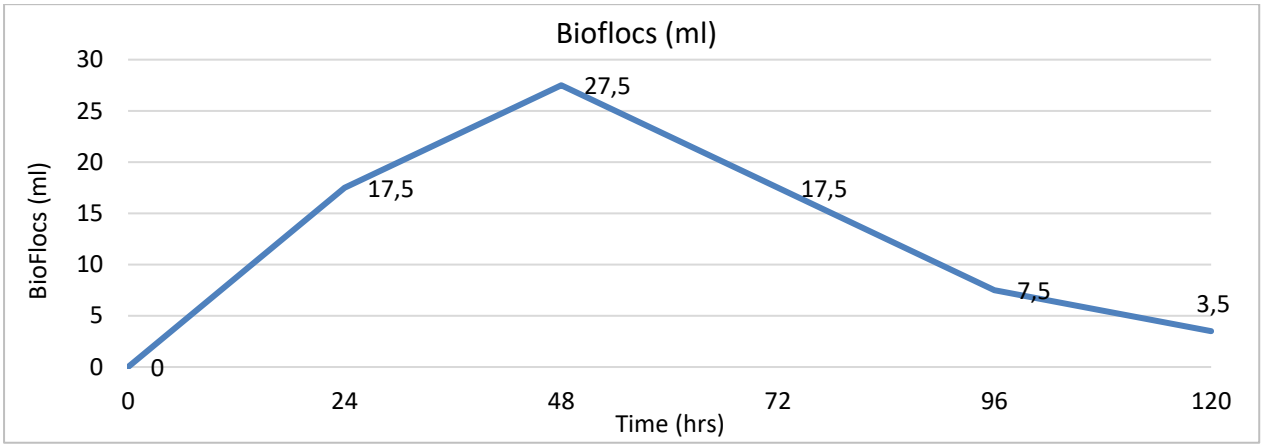

Figure 1. This graph illustrates the quantity of settled floc by KBF-103 over a period of 120 hours. The floc production reaches its peak at 48 hours and steadily decreases until 120 hours.

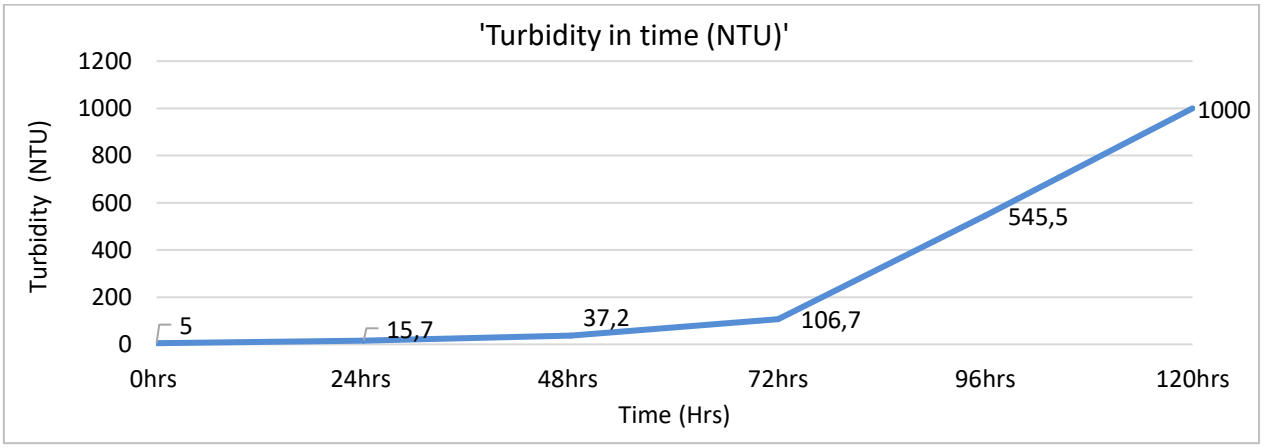

Figure 2. Turbidity generated by KBF-103 in media during a period of 120 hours. The graph illustrates that after 48 hours, there is a simultaneous decrease in flocs and an increase in turbidity.

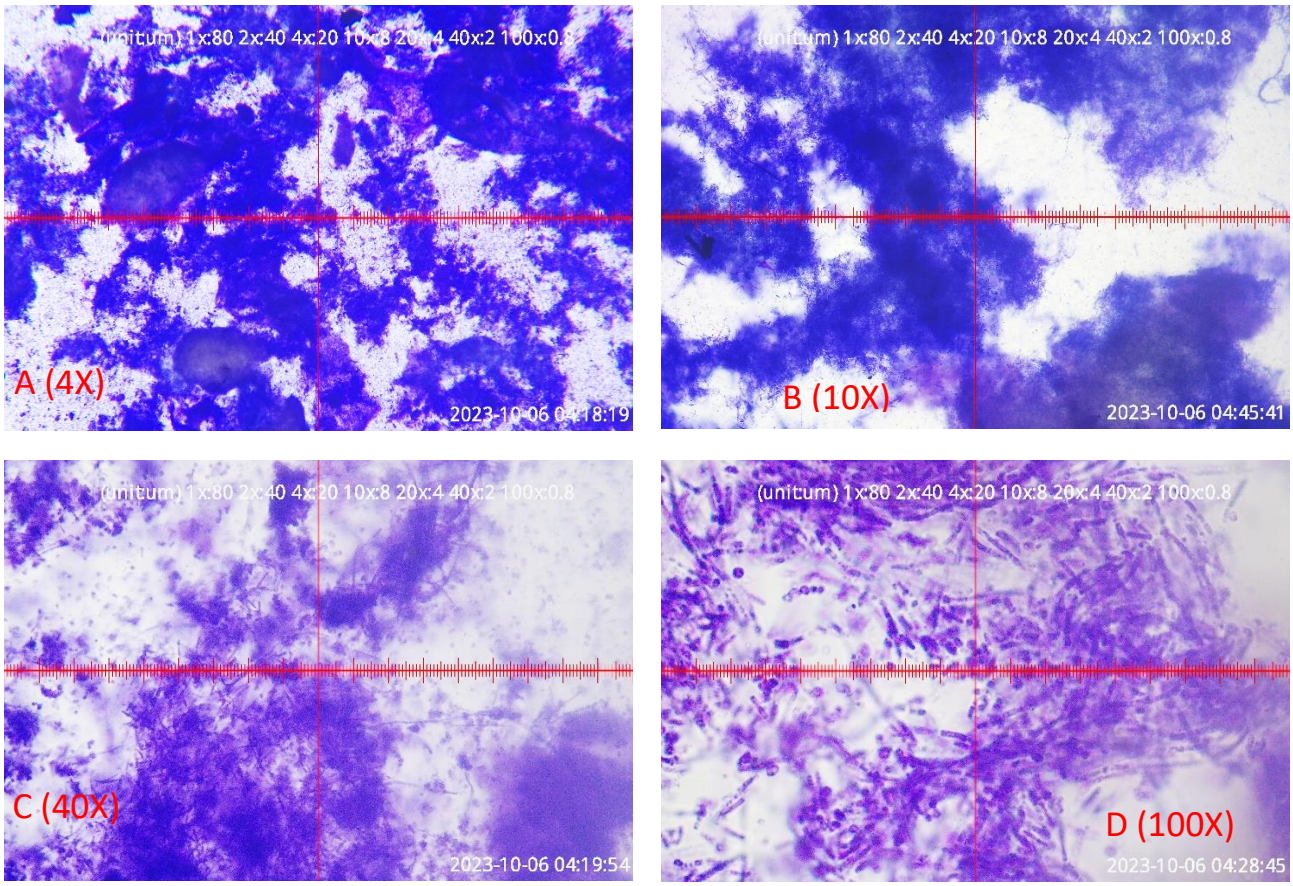

Figure 3. The biofloc generated by strain KBF-103 was examined using an Imhoff cone and checked under a microscope at various magnifications: a (4x), b (10x), c (40x), d (100x), and e. the settled KBF-103 flocs were found at the bottom of the cone.

Microscopic observation at 100x: Under this higher magnification, individual bacteria become distinguishable in much greater detail. Bacteria are observed as small, rod-shaped or spherical cells with distinct features. In the case of KBF-103 flocs, clear rod shapes and some internal spores are evident. No individual movements are observed, but this level of magnification allows for the potential identification of specific bacterial species based on their morphological characteristics. The increased magnification provides a clear and detailed morphological observation of bacterial cells.

Molecular Consensus data of KBF-103

GCTCAGGATGAACGCTGGCGGCGTGCCTAATACATG
CAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGT
TAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCC
ATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACC
GGATAACATTTTGCACCACATGGTGCGAAATTGAAAGGCG
GCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCG
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
TCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGC
GTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTT
AGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTG
ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAA
TTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCT
GATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGG
AAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATT
CCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAAC
ACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACT
GAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA
GAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAG
CACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTT
GACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCG
GGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAA
GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG
ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAG
GTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTA
GGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAAT
CGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCC
GAAGTCGGTGGGGTAACCTTTTGGAGCCAGCCGCCTAAG
GTGGGACAGATGATTGGGGTGAAGTCGTA

The sequence analysis revealed that biofloc producing bacterial isolate KBF-103 showed 100% 16S rRNA gene sequence similarity to Bacillus cereus strain AFS064220 (Nearest Accession No. OP986153.1) (Table 1). A phylogenetic tree was constructed using partial 16S rRNA sequences of isolated endophytic bacteria and representative bacterial type strain of related taxa generated by neighbor−joining method and presented in Figure 4.

Identification of KBF-103 Based on Biochemical Properties.

Different cultural and biochemical tests were carried out for the identification of bacteria and its metabolic activity, and the results were presented in Table 2.

The molecular analysis and biochemical results verify the identity of KBF-103 as *Bacillus Cereus*. Through meticulous examination of its genetic makeup and biochemical properties it has been confirmed that KBF-103 exhibits characteristics consistent with Bacillus cereus.

Salinity and Temperature Tolerance of KBF-103

The growth of KBF-103 bacterium exhibited a distinct response to salinity concentrations. Robust growth was observed on agar plates containing 0% and 3% sodium chloride, indicating the bacterium's preference for low salinity environments. However, growth was markedly inhibited on plates with 6% sodium chloride, suggesting a threshold for salinity tolerance. Further increases in salinity to 8% and 10% resulted in negative growth, indicating the bacterium's inability to grow under high saline conditions.

Regarding temperature sensitivity, KBF-103 displayed optimal growth at temperatures ranging from 32°C, to 50°C. At 55°C, however, bacterial growth was completely abolished, indicating thermal intolerance beyond this threshold.

Enzymatic Properties of KBF-103

Strain KBF-103 exhibited a positive catalase reaction with the addition of 3% H_2O_2 however, when para-aminodimethyl aniline oxalate solution was added, the strain was oxidase-negative. Gelatin hydrolysis yielded a positive result (4.5 cm proteolytic index), while Starch hydrolysis and hemolysin activity tests produced negative results for KBF-103.

Discussion

According to prior literature, this presentation marks the first report on the isolation of bacteria capable of producing biofloc. It is also the initial documentation of isolating Bacillus sp. using the innovative methodology, showcasing the ability to produce flocs within 24 hours in *in vitro* conditions. The isolated biofloc-producing bacteria belongs to the Bacillus genus, exhibiting high salt tolerance (6%) and high temperature (50° C) tolerance and the capacity to form spores resilient to harsh conditions. Notably, its unique advantage lies in the ability to produce flocs in

Table 1. Sequence producing significant alignment

Description	MaxS core	Total Score	QueryCover	E-value	Per. Ident	Nearest Accession
Bacillus cereus strain AFS064220	2747	2747	100%	0.0	100.00%	OP986153.1
Bacillus cereus strain AFS072025	2747	2747	100%	0.0	100.00%	OP986152.1
Bacillus cereus strain GE2B22	2743	2743	100%	0.0	99.93%	ON350770.1
Bacillus cereus strain AFS013435	2741	2741	100%	0.0	99.93%	OP986146.1
Bacillus cereus strain AFS068308	2736	2736	100%	0.0	99.87%	OP986148.1
Bacillus cereus strain AFS074395	2736	2736	100%	0.0	99.87%	OP986140.1
Bacillus sp.strain Firmi-7	2734	2734	99%	0.0	100.00%	MH683096.1
Bacillus sp.strain Firmi-37	2732	2732	99%	0.0	99.87%	MH683126.1
Bacillus pseudomycoides strain CN9	2724	2724	100%	0.0	99.73%	KY085988.1

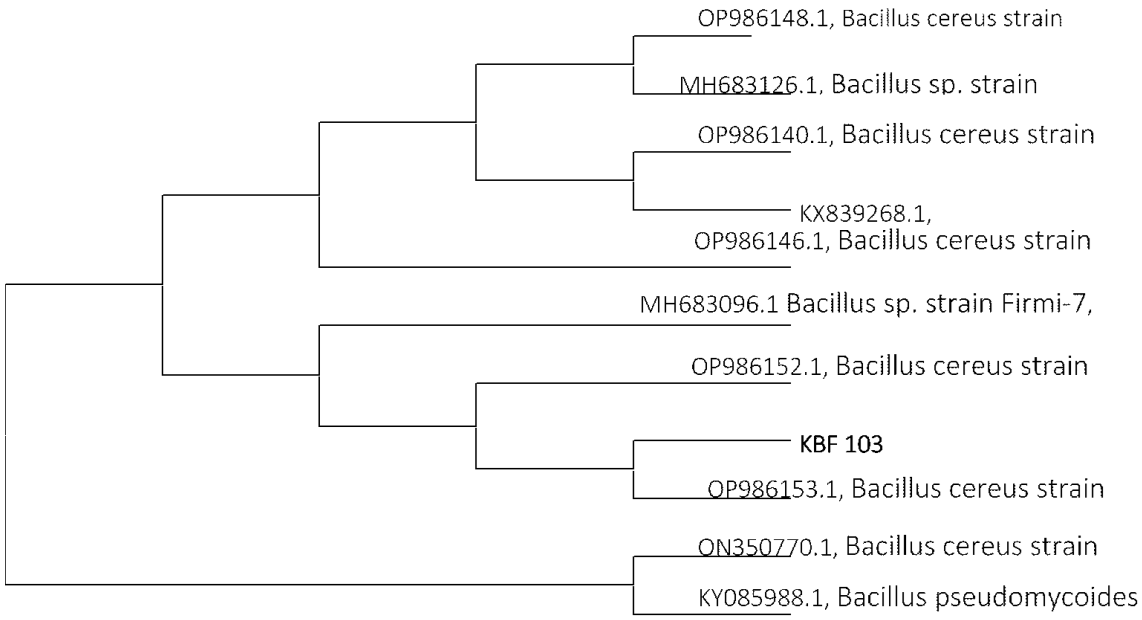

Figure 4. A phylogenetic tree was generated to illustrate the precise evolutionary placement of the type strain of *Bacillus cereus* strain AFS064220.

less than 24 hours, a characteristic not reported before. Additionally, the absence of hemolysis capability on blood agar suggests non-pathogenicity, enhancing the bacteria's favorable characteristics.

Hashim et al. (2019) discovered six kinds of bioflocculant bacteria: *Pseudoalteromonas sp., Nitratireductor aquimarinus, Bacillus pumilus, Bacillus cereus,* and *Halomonas venusta*. However, their evaluation was limited to YPG medium, and they did not explore the individual bioflocculent capabilities of these species. This highlights a gap in their research, leaving unanswered questions regarding the specific contributions of each bacterium to biofloc formation. In addition, Kumar *et al*. (2021) confirm that bacillus species play the main role in BFT system. This finding reinforces the importance of Bacillus species and confirms our findings. Nine species, including *Halomonas venusta, H. aquamarina, Vibrio parahaemolyticus, Bacillus infantis, Bacillus cereus, Bacillus safensis, Providencia vermicola, Nitratireductor aquimarinus, and Pseudoalteromonas* species, were suggested as possible inoculum for biofloc production by Kasan *et al*. (2017). But their research primarily focused on isolating bacteria present in biofloc at different time intervals within 0 to 70 days of culture. Notably they did not individually test these bacteria for biofloc production. According to Huang *et al*. (2022), the predominant phyla observed were Proteobacteria, constituting 45.8% of the community, followed by Bacteroidetes at 21.1%, Planctomycetes at 13.5%, Chlamydiae at 10.3%, and Firmicutes at 6.8%. This distribution highlights the diverse and abundant presence of these phyla within the microbial community. The main bacterial genera present in water biofloc included, *Tropicibacter, Saprospiracea, Planctomyces, Rhodobacteraceae, Ardenticatenia norank, Microbacterium, and Candidatus Alysiosphaera* (Xu *et al*., 2022) which none of them confirm the *Bacillus cereus* in Biofloc production.

The graph 3 suggests a dynamic change in water quality over the 120-hour monitoring period. Initially, the water was relatively clear, but floc levels and turbidity increased over time, indicating the introduction of suspended flocs. This change in water

Table 2. Biochemical properties of KBF-103

S.N	Parameter	Result	Reference (Bergey and Holt, 1994)	Remarks
Morphological observation				
	Colony	Filamentous, cloudy	Opaque, waxy	
	Gram's reaction	+	+	
	Cell shape	Rod, chain of 2 to 5,	Rod, chain	
	Endospore forming ability	Present (central, Oval)	**+**	
	Motility	-	**+**	
Biochemical Test				
	KOH	-	**-**	
	Catalase	+	**+**	
	Oxidase	-	**-**	
IMViC test				
	Indole	-	**-**	
	MR	+		
	VP	-	**+**	
	Citrate Utilization	+	**+**	
Carbohydrate fermentation test				
	Glucose	+	**+**	
	Fructose	+	**+**	
	Cellulose	+		
	Sucrose	+	**v**	
	Arabinose	-	**-**	
	Trehalose	-	**+**	
	Cellobiose	-	**v**	
	Lactose	-	**-**	
	Manose	-	**-**	
	Maltose	-	**+**	
	Salcin	-	**v**	
	Sorbitol	-	**-**	
	Inositol	-	**-**	
Amino acid decarboxylase test				
	Arginine	-	**v**	
	Lysine	-	**-**	
	Ornithine	-	**-**	
Selective media growth				
	Congo red Agar	Red color colonies		
	Bacillus Hi-Chrome Agar	Green colonies		
Enzymatic activities				
	Blood hemolysin Agar	-	**+**	
	Starch (Agar)	-	**v**	
	Gelatine (Agar)	+	**+**	
	Casien (Agar)	+	**+**	
	Esculin	+	**+**	
	Nitrate reduction	+	**+**	

The symbol "**+**" denotes positive characteristics, while "-" signifies negative attributes, and "V" represents variable outcomes across test.

quality could be attributed to various factors such bacterial growth, flocs disassociation, or changes in environmental conditions of bacteria will impose death. The correlation coefficient of flocs and turbidity in graph 1 and 2 clearly indicated negative and especially while the flocs start declining the turbidity increase tremendously. The increase in turbidity after the peak floc production indicates that flocs may be dissolving or breaking down, releasing suspended particles back into the water. Additionally, the growth of bacteria, potentially due to the availability of nutrients, could contribute to increased turbidity. Moreover, while there are flocs in cones the water is more clear rather than the disappearing of flocs.

The findings reveal the dynamic relationship between floc production and turbidity over a120-hours incubation period. The decline in floc production after the peak is associated with a rapid increase in turbidity, possibly due to floc dissolution and bacterial growth. This information is valuable for understanding the behavior of KBF-103 in floc formation and its impact on water turbidity. There is no research before regarding the issue. Further research may be needed to explore the factors influencing the bacterial death phase and its effects on floc production.

The observations suggest that bioflocs contain a complex mixture of bacterial cells. The ability to differentiate between individual bacterial species becomes more feasible as to increase the magnification.

The presence of clear rod-shaped structures with internal spores in KBF-103 flocs indicates the potential presence of specific bacterial species with distinct morphological features. There is no report about observation of flocs under 100x magnification and it's for the first time that reported.

Conclusion

The bacteria KBF-103, isolated for biofloc production, offers significant insights. Identified as *Bacillus cereus*, it displays robust salinity and temperature tolerance, forming flocs within 24 hours and producing spores for harsh conditions. It influences floc production and turbidity dynamics over 120 hours. Microscopic analysis reveals unique structures, indicating versatile energy production capabilities. These findings deepen understanding of KBF-103's role in biofloc formation and potential applications. Utilizing novel methods, this innovation standardizes biofloc production, enhancing aquaculture practices. Further research is needed to explore bacterial activity phases and turbidity dynamics comprehensively.

Ethical Statement

This research was carried out in compliance with relevant rules and regulations. Since there were no human participants, animal subjects, or sensitive data involved, approval from an ethical committee was not required. There are no ethical issues in this research

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Author Contribution

First: Autor: Conceptualization, Data curation, investigation, writing and reviewing original draft; and editing. Second author supervision.

Conflict of Interest

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

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