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Isolation of Flocculant Bacteria from The Gut of Tilapia in Mina Padi for Probiotic Application in Biofloc Technology

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ABSTRACT

Biofloc technology is a sustainable aquaculture system with considerable potential. The system operates through complex bacterial interactions, which require effective flocculant bacterial isolates to function optimally within the system. The present study aimed to isolate flocculant bacteria from the Mina Padi region in Banyumas, Indonesia, as candidate probiotics for fish farming using biofloc technology. Flocculant bacteria were selected using the kaolin suspension method, yielding three high-activity flocculant bacterial isolates as potential candidates. The flocculant bacterial isolates were successfully identified using 16S rRNA gene sequencing, namely *Bacillus altitudinis* NHA03, *Staphylococcus ureilyticus* NHB05, and *Kurthia gibsonii* NHC02. In the kaolin suspension test, peak flocculation activity was achieved at 72 hours, with values of 81.14% for NHA03, 80.44% for NHB05, and 89.67% for NHC02. The three isolates demonstrated stable performance across a pH range of 3-9 and a temperature range of 18-38°C. The highest flocculation activity was observed at 28°C, with NHC02 reaching 80%, followed by NHB05 at 75% and NHA03 at 65%. Regarding pH, NHB05 exhibited the highest activity at pH 3-5, NHA03 was optimal at pH 5, while NHC02 maintained consistent activity across all pH levels. Based on supporting tests, *Bacillus altitudinis* NHA03 and *Kurthia gibsonii* NHC02 were non-pathogenic, produced enzymes (protease, amylase, and cellulase), and demonstrated resistance to pH and temperature variations, indicating a strong capacity for environmental adaptation. *Staphylococcus ureilyticus* NHB05 demonstrated no extracellular enzyme activity and exhibited increased antibiotic resistance, making it less appropriate as a probiotic candidate. *Bacillus altitudinis* NHA03 and *Kurthia gibsonii* NHC02 can currently be recommended as probiotic candidates for use in aquaculture with biofloc technology based on their flocculation performance and other beneficial traits.

Keywords: *Bacillus altitudinis*, Biofloc technology, Flocculant bacteria, *Kurthia gibsonii*

INTRODUCTION

Biofloc is an environmentally friendly cultivation technology that can accommodate Nile tilapia fish (*Oreochromis niloticus*) at high densities, effectively increasing productivity (de Souza et al., 2019). In biofloc technology, the microbial community plays an important role in nutrient recycling by maintaining a high carbon-to-nitrogen ratio, thereby creating a large, balanced microbial community (Ahmad et al., 2017). Furthermore, some bacteria capable of producing extracellular products can form communities and suspended particles, thereby facilitating the formation of flocs or similar aggregates (Cai and Taylor, 2020). In biofloc cultivation, complex mechanisms and interactions among organic compounds, microalgae, and bacterial communities result in clumps that can be consumed by fish as feed (Jiang et al., 2019). To develop biofloc technology, it is necessary to characterize microorganisms with potential for rice-fish farming, including flocculant bacteria as floc-formers for biofloc applications.

Flocculant-producing bacteria are heterotrophic bacteria that are key to biofloc technology (Saeedi et al., 2025). Exopolysaccharide products produced by flocculant bacteria act as adhesives in forming aggregates or floating floc clumps during biofloc media cultivation. In addition, extracellular active compounds from flocculant bacteria can reduce turbidity and chemical oxygen demand in water (Kurniawan et al., 2021). Flocculant bacteria can effectively degrade cell residues and suspended particles in water, as waste from cultivation (Zhang et al., 2017). Several strains of flocculant bacteria have been widely used in the aquaculture industry, especially as waste management agents in cultivation,

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namely *Bacillus (B.) subtilis* (Jiang et al., 2019), *B. cereus*, *B. pumilis*, *Nitratireductor aquimarinus*, *Halomonas venusta*, *Pseudomonas* sp. (Che Hashim et al., 2019), and *Serratia marcescens* (Kurniawan et al., 2021).

Mina padi is an integrated cultivation system combining rice and fish, aiming to maximize the use of cultivation land. Mina padi cultivation has begun to develop in Banyumas, Indonesia (Anggraeni et al, 2025). Mina padi cultivation has a very complex environmental system. The amount of organic matter in waterways is affected by human interventions in rice production, such as fertilizer applications. This intervention promotes the growth of bacteria and microscopic plants (Palupi et al., 2023; Nurhafid et al., 2024). Based on this intervention, the presence of flocculant-producing bacteria is believed to have high abundance and diversity. The present study aimed to isolate flocculant-producing bacteria from the Mina Padi area in Banyumas, Indonesia, to develop them as probiotic isolates for fish farming using biofloc technology.

MATERIALS AND METHODS

Ethical approval

The present study was conducted with the approval of the Medical/Health Research Bioethics Commission, Faculty of Medicine, Sultan Agung Islamic University of Semarang, under protocol number 332/VIII/2024.

Study location

The present study was conducted from April to September 2024 using exploratory observation methods at the Aquatic Research and Microbiology Laboratory, Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University, Purwokerto, Indonesia.

Sampling location

The sample source used in the present study was obtained from sediments collected from the Mina padi cultivation area in Panembangan Village, Cilongok, Banyumas, Indonesia. Three sediment sampling points were taken in the same area, covering 50 m^2. These points included the inlet, middle, and outlet, at a depth of ± 5 cm from the sediment bottom surface. Sediment samples were stored in a coolbox at 4-8ºC, and the three samples were taken to the Research Laboratory of the Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University, Indonesia.

Inoculation

Bacterial inoculation was performed using the pour-plate agar technique. A total of 0.1 g of sediment was weighed using a digital scale (Hand counter, Japan) and then dissolved in sterile 0.9% NaCl (Fitriadi et al., 2023a). The dissolved sample was diluted to 10^{-5}, and the 10^{-3} to 10^{-5} dilution was cultured on tryptic soy agar (TSA; HiMedia, India). The culture results were incubated for 24 hours at 28ºC, as described by Ahn et al. (2019), with minor modifications. Ahn et al. (2019) used spread plating at 30°C to recover bacteria from distilled water and antiseptics, whereas the present study used pour plating at 28°C to isolate flocculant bacteria from sediment on TSA. Then, five isolates were randomly selected as representatives from each sampling block and were sterilized using the streak method on agar surfaces (Nurhafid et al., 2024).

Bioflocculant production

The stock bacteria from the previous stage, which involved isolating and purifying bacteria on the slant medium, were subsequently cultured in 5 mL of tryptone soy broth (TSB) liquid medium (Himedia, India). The culture media were incubated for 24 hours in a shaker incubator (IKA, Germany) at 28ºC and 150 rpm. Furthermore, 0.5 mL of the culture was added to 5 mL of sterile liquid culture media (TSB) and re-incubated in a shaker incubator at 28°C and 150 rpm for 24 hours (Zulkeflee et al., 2016). The culture sample was then centrifuged for one minute at 6000 rpm to separate the cells. The cell-free supernatant was used to determine the bacteria's ability to produce flocculants.

Flocculation activity screening and bacteria selection

Isolation and screening for bacterial bioflocculant activity were performed following the method of Kurniawan et al. (2021), with some modifications. Bioflocculant activity was determined by adding calcium chloride ($CaCl_2$; China) as a coagulant in the kaolin suspension (YUKAMI, Indonesia). The initial step was to dissolve the kaolin in distilled water at a concentration of 5 g/L. A total of 9.65 mL of kaolin solution was placed in a test tube. Then 0.25 mL of $CaCl_2$ (5 g/10 mL in distilled water) was added. Then vortexed for 20 seconds until the solution was completely homogeneous. After that, 0.1 mL of raw flocculant was added, and the mixture was homogenized by vortexing for 20 seconds. The treatment solution was then left at room temperature (28°C) for five minutes. The treatment solution without cell-free supernatant was prepared as previously described. As a control, the same procedure was performed without adding the cell-free supernatant; 0.1 mL of sterile TSB medium was used instead of the flocculant. Bioflocculant activity was assessed by measuring turbidity at 550 nm with a spectrophotometer (Biobase, China). The final turbidity in the control was determined by measuring the absorbance of the kaolin suspension without bacterial cell-free supernatant (sterile TSB was used instead of flocculant) at 550 nm (OD550), and the final turbidity in the sample was assessed by measuring the absorbance of the kaolin suspension containing bacterial cell-free supernatant at 550 nm (OD550). Bacteria were selected based on flocculation activity greater than 70%, and the bioflocculant activity was calculated using the following formula (Kurniawan et al., 2021).

Flocculant activity (%) = (Final turbidity in control – Final turbidity in sample) / final turbidity in control × 100

Characterization of flocculant-producing bacteria

The morphology of colonies and cells, catalase and oxidase tests, Gram staining, and pathogenicity on blood agar (HiMedia, India) were used to characterize flocculant bacteria.

Stability of bioflocculant activity

A stability test of bacterial bioflocculant activity was performed in liquid growth medium (TSB) at different temperatures (18ºC, 28ºC, and 38ºC) and pH levels (3, 5, 7, and 9) according to the method used by Zulkeflee et al. (2016), with some minor differences in pH and temperature range. Zulkeflee et al. (2016) focused on optimal bioflocculant production at 25-30°C and pH 7-8; however, the present study evaluated flocculant stability over a broader temperature (18-38 °C) and pH (3-9) range to assess robustness under non-optimal conditions. Bacterial bioflocculant activity was assessed in the kaolin suspension using the same procedure as the flocculation activity screening to evaluate its stability in producing flocculants under different environmental conditions.

Extracellular enzyme activity of flocculant-producing bacteria

The potential of flocculant-producing bacteria was tested for several abilities, including enzyme production (protease, amylase, cellulase) and inhibition of pathogenic bacteria such as *Aeromonas hydrophila (A. hydrophila).* Enzyme activity test was carried out using growth media enriched with substrates according to the enzymes being tested, including skim milk powder, starch, and carboxymethyl cellulose (Fitriadi et al., 2023a). Furthermore, antibacterial testing to inhibit pathogens was conducted using the agar diffusion method at a concentration of 10^9 (Ramos et al., 2012; CLSI, 2024).

Compatibility and antibiotic test

A compatibility test was conducted following the method of Fitriatin et al. (2020) to determine the commensal nature of bacteria in the environment. Three flocculant-bacterial isolates were cultured using the cross-streak method. Antibiotic testing was performed using the diffusion method on TSA medium (Himedia, India). Four antibiotics were tested, including tetracycline 30 mcg, chloramphenicol 30 mcg, ampicillin 10 mg, and lincomycin 10 mg (Himedia, India). The antibacterial activity of the bacteria against antibiotics was determined by the inhibition zone formed around the blank dish (Fitriadi et al., 2023b).

16S rRNA gene sequencing and identification

Identification of flocculant bacteria was performed by preparing liquid cultures, which were then purified using gDNA from the bacterial genome with the Genome Bacteria Mini Kit (GeneAid, Taiwan; Fitriadi et al., 2023a). The purification process adhered to the procedure outlined in the user manual. Then, after obtaining pure DNA, polymerase chain reaction (PCR) amplification was conducted using a pair of primers, 27f (5'-AGA GTT TGA TCC TGG CTC AG -3'), and 1392r (5'- GGT TAC CTT GTT ACG ACT T -3'(Fitriadi et al., 2023b). The PCR program was set according to the instructions in the kit manual, specifically for HS-RedMix PCR (Bioline, USA), as follows. The initial denaturation was conducted at 95°C for two minutes, followed by denaturation at 95°C for 20 seconds, annealing at 55°C for 20 seconds, extension at 72°C for 20 seconds, and a final extension at 72°C for five minutes, for a total of 35 cycles. Furthermore, DNA was visualized on an electrophoresis gel (Thermo Fisher Scientific, USA) with a 1500 bp band. The biofloculant bacteria sample was sequenced using Sanger sequencing.

Data analysis

The characteristics of three bioflocculant strains (NHA03, NHB05, NHC02) were analyzed descriptively and compared with previous studies as a reference for interpreting the magnitude and dynamics of flocculation activity, as well as with reports on aquaculture effluent and intensive ponds (Jiang et al., 2019; Kurniawan et al., 2021). Stability against pH and temperature was assessed by considering the tendency for optimal bioflocculant production under acidic conditions and the resistance of probiotic candidates to environmental variations (Lee et al., 1995; Mohamad et al., 2020).

RESULTS

The results of bacterial inoculation were obtained by randomly selecting five isolates from each sampling point (inlet, middle, and outlet), yielding 15 pure culture isolates. Furthermore, the isolates were tested for flocculant activity, resulting in 10 flocculant-forming bacterial isolates (Table 1). The bacterial flocculation activity test successfully yielded 10 isolates of floc-forming bacteria in kaolin suspension media. Based on test results across all isolates, flocculant-producing isolates exhibited variability in flocculant production, including differences in incubation time and flocculant activity. This variation might be attributed to strains originating from different source groups. However, descriptively, a higher number of flocculant-forming isolates was observed compared to non-flocculant isolates among the total isolates obtained from the rice-fish cultivation pond. Furthermore, three bacterial isolates with high flocculant activity were identified by isolate codes, including NHA03, NHB05, and NHC02, with values of 81.14%, 80.44%, and 89.67%, respectively, after 72 hours (Figure 1). Based on the average value as an indicator of optimal activity, bioflocculant activity indicated an optimal phase in floc formation, namely a 72-hour incubation period. After the 72-hour incubation period, floc-formation activity decreased. The most remarkable finding regarding the isolates' characteristics was the highest activity observed in isolate NHC02, which reached 89.67% after 72 hours.

Table 1. Flocculant activity of bacterial isolates from gut of tilapia cultured in tryptone soy broth medium

Biofloculant activity (%) **Isolate**	**24 hours**	**48 hours**	**72 hours**	**96 hours**
NHB04	65.88	77.15	69.87	69.87
NHA05	58.53	48.59	76.76	68.23
NHB01	41.71	40.61	78.95	67.92
NHC01	59.62	75.35	75.51	67.84
NHB02	63.38	52.97	76.45	67.53
NHB05	61.89	51.64	80.44	65.88
NHA02	56.34	48.12	76.06	55.09
NHC04	65.49	51.56	76.60	78.95
NHA03	63.07	67.06	81.14	68.86
NHC02	62.99	61.35	89.67	75.67
Average	59.89	57.44	78.15	68.58
Minimum	41.71	40.61	69.87	55.09
Maximum	65.88	77.15	89.67	78.95

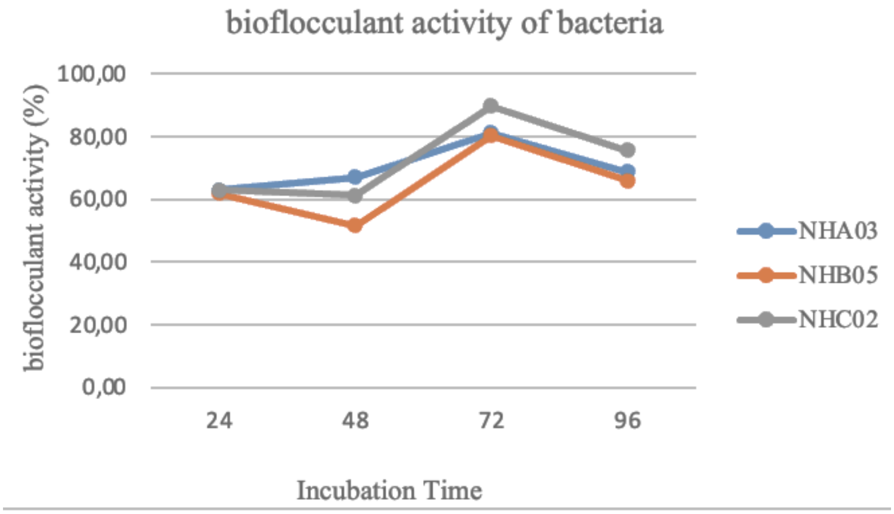

Figure 1. Bioflocculant activity of the three bacterial isolates from gut of tilapia in Mina Padi, Indonesia, namely NHA03, NHB05, and NHC02. The highest activity was shown by isolate NHC02, which reached 89.67% after 72 hours.

Three bacterial isolates with high flocculant activity were further characterized to determine their characteristics and taxa (Table 2). The results of the bacterial characteristic test demonstrated apparent differences under a microscope (Olympus, Japan) at 40x. The differences among isolates were clearly evident in the shapes, elevations, and edges of the colonies. Furthermore, the microscopic appearance of bacterial isolate cells exhibited very clear characteristics in the shape of the cells; for instance, NHA03 isolate was in the form of a long rod, NHB05 isolate was in the form of a coccus, and NHC02 isolate was in the form of a short rod (Figure 2). These characteristics indicated differences among colonies, as confirmed by 16S rRNA gene identification. Pathogenicity testing was conducted by observing bacterial hemolysis on blood agar. Three strains of flocculant bacteria demonstrated negative activity or were nonpathogenic, as indicated by their inability to grow on blood agar. Colony size was not quantified in this study and was therefore not included as a morphological parameter.

Table 2. Morphology and physiological characteristics of flocculant bacteria isolated from gut of tilapia in Mina Padi, Indonesia

Isolate **Characteristics**	**NHA03**	**NHB05**	**NHC02**
Colony form	Circular	Circular	Filamentous
Colony elevation	Raised	Pulvinate	Flat
Colony edge	Entire	Entire	Undulate
Colony pigmentation (28°C)	White	No pigmentation	White
Gram staining	Positive	Positive	Positive
Form of cell	Rod (bacillus)	Coccus	Rod (bacillus)
Potassium hydroxide test (KOH)	Positive	Positive	Positive
Catalase test	Positive	Positive	Positive
Oxidase test	Positive	Positive	Positive
Hemolytic test	Negative	Negative	Negative

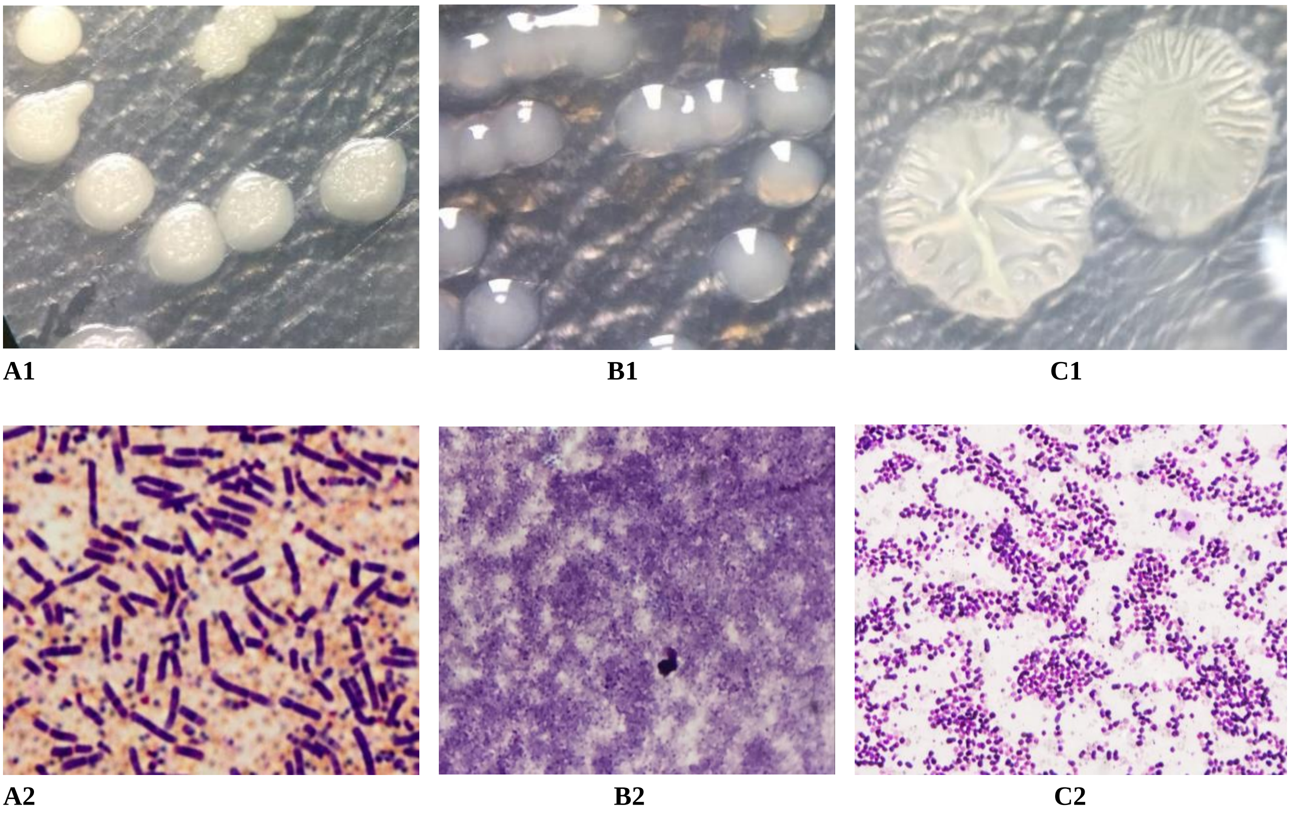

Figure 2. Microscopic and macroscopic morphology of bacterial colonies isolated from gut of tilapia in Mina Padi, Indonesia. Morphology of colonies on TSA medium; **A1**: NHA03 isolate, **B1**: NHB05 isolate, **C1**: NHC02 isolate, taken by authors. Morphology of cell using Gram staining; **A2**: NHA03 isolate, **B2**: NHB05 isolate, **C2**: NHC02 isolate, 1000x magnification

Bacterial isolates for flocculant production assessment were evaluated under two environmental conditions, namely temperature and pH. Testing the bioflocculant activity against different pH (3-9) and temperature (18°C-38°C) demonstrated that all three isolates (NHA03, NHB05, and NHC02) had high and adaptive flocculation abilities, with activity values ranging from 50% to 80%. Regarding pH variation, isolate NHB05 demonstrated superior activity, approximately 70% at pH 3 and 5, and remained stable above 60% at pH 7 and 9, exhibiting consistent flocculant activity across the tested pH range. Isolate NHA03 achieved optimal activity at pH 5 (65%), while NHC02 exhibited a consistent activity pattern (55-65%) across the entire pH range. In temperature testing, bioflocculant activity increased at 28°C, with NHC02 reaching the highest value (83%), followed by NHB05 (80%) and NHA03 (62%), while at 18°C and 38°C the activity decreased slightly but remained high. Since the isolates in this study were different strains, their tolerance capabilities varied. Under different pH conditions, distinct response patterns were observed among the isolates, with NHB05 exhibiting higher activity at acidic pH (3-5), NHA03 achieving optimal activity at pH 5, and NHC02 maintaining relatively consistent activity across the tested pH range.

Enzyme index analysis demonstrated a clear difference between isolates (Table 3). The NHA03 isolate had the highest proteolytic activity, moderate amylolytic activity, and weak cellulolytic activity, with no lipolytic activity. The NHC02 isolate demonstrated the strongest amylolytic activity, moderate proteolytic activity, and weak cellulolytic activity, also with no lipolytic activity. Conversely, NHB05 demonstrated no enzymatic activity across all categories. None of the isolates inhibited the growth of *A. hydrophila*. The current results indicated that NHA03 was more suitable for protein-rich substrates, NHC02 for starch-rich substrates, and NHB05 had limited potential. All isolates were negative for lipid substrate activity and did not act as antagonists against pathogens under the current test conditions.

Table 3. Characteristics of enzymatic activity produced by flocculant bacteria isolated from Mina Padi, Indonesia

Isolates	**Index activity**				**Inhibitory effect on *Aeromonas hydrophila***
	Proteolytic	**Amylolytic**	**Cellulolytic**	**Lipolytic**	
NHA03	+++	++	+	-	-
NHB05	-	-	-	-	-
NHC02	++	+++	+	-	-

+++: High enzymatic activity, ++: Medium enzymatic activity, +: Low enzymatic activity, -: Negative, no enzymatic activity

Compatibility testing indicated that the three bacterial isolates (NHA03, NHB05, and NHC02) could coexist in the same environment (Table 4), while antibiotic sensitivity testing revealed different resistance profiles (Table 5). The NHC02 had the largest overall inhibition zone (mean 15.83 mm; median 16.45 mm) and was the only isolate sensitive to lincomycin (23.0 mm), although it remained resistant to ampicillin and exhibited only moderate sensitivity to tetracycline and chloramphenicol. The NHA03 isolate demonstrated a moderate response to tetracycline and ampicillin (16.6-16.8 mm) but was resistant to chloramphenicol and lincomycin, without susceptibility. In contrast, NHB05 demonstrated dominant resistance to cloramphenicol, ampicillin, and lincomycin, with no ampicillin inhibition zone, and only moderate sensitivity to tetracycline, as reflected in the lowest mean inhibition zone value (6.28 mm). Overall, these findings highlighted NHC02 as the isolate with the most favorable sensitivity profile, showing sensitivity to lincomycin and resistance to only one antibiotic (ampicillin), compared to NHA03 and NHB05, which exhibited multiple resistances.

Table 4. Pairwise compatibility test of bacterial isolates on tryptic soy agar medium

Isolate	**NHA03**	**NHB05**	**NHC02**
NHA03	+		
NHB05		+	
NHC02			+

Compatibility: Positive (+) indicates the isolates did not inhibit each other's growth and can coexist.

Table 5. Antibiotic resistance and sensitivity characteristics of bacterial isolates of NHA03, NHB05, and NHC02

Isolate	**Antibiotic characteristic (mm)**			
	Tetracycline 30 mcg	**Chloramphenicol 30 mcg**	**Ampicillin 10 mg**	**Lincomycin 10 mg**
NHA03	16.6^{I}	12.6^{R}	16.8^{I}	2.6^{R}
NHB05	14.6^{I}	4.8^{R}	0^{R}	5.7^{R}
NHC02	18.8^{I}	14.1^{I}	7.4^{R}	23.0^{S}

R: Resistant (≤ 14.0 mm), I: Intermediate (14.1-18.9 mm), S: Sensitive (≥ 19.0 mm)

The 16S rRNA gene identification results revealed that three bacterial isolates, namely NHA03, NHB05, and NHC02, were identified as *Bacillus altitudinis* (*B. altitudinis*; 96.59%), *Staphylococcus ureilyticus* (99.90%), and *Kurthia gibsonii* (*K. gibsonii*; 100.00%; Table 6). The phylogenetic tree analysis confirmed the position of the taxon clade, and a bootstrap analysis with 1000 replicates demonstrated a reliable value of 70% (Figure 3).

Table 6. Identification of flocculant bacteria isolated from Mina Padi, Indonesia, based on the 16S rRNA gene

Isolate	Blast result	Identity (%)	Accession number of NCBI
NHA03	*Bacillus altitudinis* strain HQB232	96.59	KT758431.1
NHB05	*Staphylococcus ureilyticus* strain EE105-P1	99.90	MN581170.1
NHC02	*Kurthia gibsonii* strain SAU_AFB01	100.00	MN658386.1

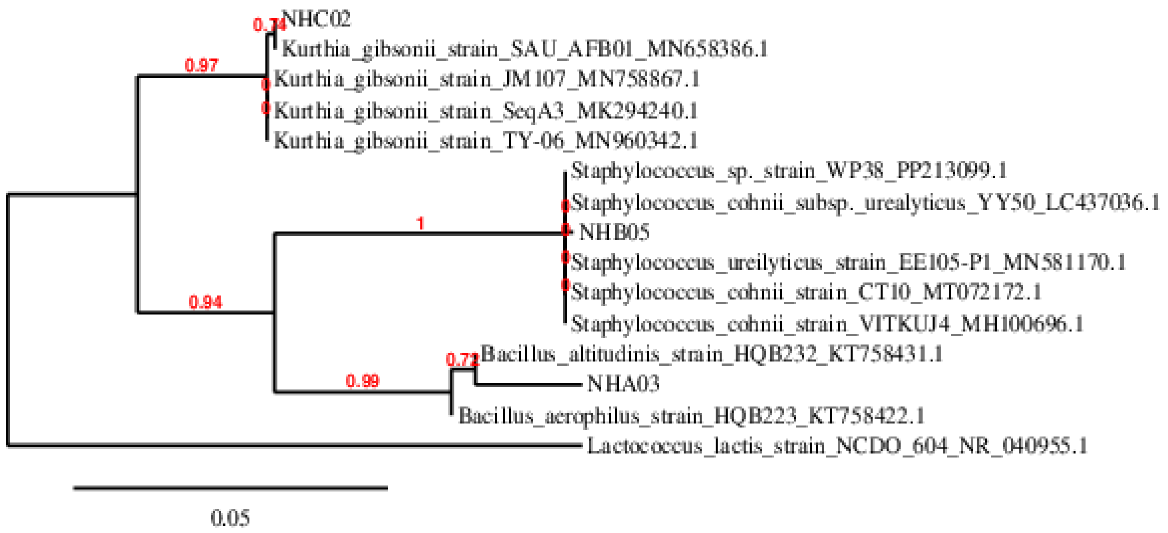

Figure 3. 16S rRNA phylogenetic tree of flocculant bacteria isolated from gut of tilapia in Mina Padi, Indonesia

DISCUSSION

Flocculant activity during the present study was considered very high compared to that reported by Kurniawan et al. (2021). Among the isolates, *B. altitudinis* NHA03 demonstrated promising probiotic characteristics. Previous studies have reported that *B. altitudinis* possesses beneficial probiotic properties, including functional and immunomodulatory potential (Jankoski and Carvalho, 2023). This discovery created opportunities to develop the *B. altitudinis* NHA03 isolate for use in biofloc technology. The bacterial isolates obtained in the present study have the potential to be utilized as probiotics. However, some studies have found that *Staphylococcus* sp. can serve as pathogens for fish (Çanak and Timur, 2020; Rajme-Manzur et al., 2023). Furthermore, many *Staphylococcus* sp. bacteria have been found to be resistant to different antibiotics, posing a risk to human health (Marijani, 2022; Rajme-Manzur et al., 2023). The isolation of *Staphylococcus* sp. from environmental waste indicated resistance to penicillin and oxacillin. The present study indicated that *Staphylococcus* sp. NHB05 exhibited non-pathogenic characteristics under the tested conditions. This observation aligns with the findings of Istiqomah et al. (2019), who reported that *Staphylococcus* sp. JC20, isolated from the digestive tract of *Octopus* sp., exhibited cellulolytic activity and was evaluated as a probiotic candidate. However, a minor inconsistency was observed in the enzymatic profiles, as NHB05 did not exhibit detectable extracellular enzyme activity in the present study. Such differences might be attributed to strain-specific variation and differences in ecological origin. *Staphylococcus ureilyticus* NHB05 is an important isolate to study more extensively, especially regarding its virulence genes and broader antibiotic resistance. According to Chauhan and Singh (2019) and El-Saadony et al. (2021), probiotic isolates should have beneficial abilities to the host and the environment. Consistent with previous studies, different bacteria isolated from aquatic environments have been reported to produce hydrolytic enzymes such as protease, amylase, and cellulase, which contribute to the biodegradation of organic compounds (Mohapatra et al., 2003; Facchin et al., 2013).

Furthermore, *K. gibsonii* demonstrated low resistance, making it a safe candidate for use as a probiotic. Nor et al. (2021) found that *K. gibsonii* can produce polypeptide biosurfactants to decompose textile waste in water. In addition, Mohamad et al. (2020) emphasized that measuring resistance to environmental temperatures and pH is an important test to assess the effectiveness and stability of probiotic isolates, consistent with the present experiment. *Kurthia gibsonii* NHC02 exhibited stable resistance to temperature and environmental pH, enabling it to survive in fish intestines and cultivation environments.

The enzymatic activities observed in *B. altitudinis* NHA03 in the present study are consistent with the findings of Bairagi et al. (2002), who reported that *Bacillus* sp. isolated from fish digestive tracts commonly produce extracellular enzymes such as protease and amylase, indicating a similar functional role in organic matter degradation and supporting the probiotic relevance of the present isolate. This observation was notably intriguing, as the detected activity is typically correlated with the stationary phase of bacterial growth. According to the present study, *B. altitudinis* NHA03 was highly stable across different pH and temperature conditions. Previous studies have indicated that *Bacillus* sp. played a beneficial role, especially in degrading organic compounds in water, thereby improving water quality (James et al., 2021). These findings align with the current results, as *B. altitudinis* NHA03 produced extracellular enzymes and maintained stable activity across different environmental conditions, thereby supporting its potential role in organic matter processing within biofloc systems. Previous studies have indicated that *B. altitudinis* 1.4 has beneficial and safe properties as a probiotic isolate, such as co-aggregation with pathogens and enhancement of the host's immune response (Jankoski and Carvalho, 2023). *Bacillus altitudinis* applied in feed exhibited substantial ability to improve growth performance, immunity, and resistance to *Streptococcus agalactiae* in tilapia (Van Doan et al., 2020).

Bacterial communities play an important role in biofloc technology because bioflocs contain high levels of organic material in the environment. These communities process the organic material so that it is not toxic to the environment (Panigrahi et al., 2022). Organic matter in the biofloc environment is required in sufficient amounts to support probiotic isolates in processing waste through nutrient and nitrogen cycles (Khanjani and Sharifinia, 2020; Manduca et al., 2020). Developing probiotic isolates is key to advancing biofloc technology, particularly floc-forming bacteria, also known as flocculant bacteria. When these bacterial isolates are used as probiotics in biofloc systems, they improve the nutrient cycle for farmed fish, as the floc acts as a natural food source in the environment (Kanwal et al., 2025). The findings of Arnepalli and Chapara (2025) are aligned with the present study, as both investigations demonstrated that bacteria isolated from aquatic environments possess floculant-producing capabilities suitable for biofloc applications, supporting the role of environmentally derived flocculant bacteria in biofloc system development. The role of probiotic isolates in enzyme production for biodegradation and maintenance of water quality, as reported by Nnenna et al. (2011) and James et al. (2021), aligns with the present study, where *B. altitudinis* NHA03 and *K. gibsonii* NHC02 exhibited extracellular enzyme production, underscoring their potential role in organic matter degradation in biofloc systems. The *B. altitudinis* NHA03 and *K. gibsonii* NHB05 isolates can be recommended as probiotic isolates in biofloc technology.

CONCLUSION

Bacillus altitudinis NHA03 and *K. gibsonii* NHC02 can produce several enzyme types (protease, amylase, and cellulase) and remain stable over a different range of temperatures (18-38°C) and pH (3-9), as demonstrated by flocculation activity measurements. *Staphylococcus ureilyticus* NHB05 exhibited no detectable enzymatic activity and was resistant to three of four tested antibiotics, thereby reducing its probiotic potential. Based on the characteristics of the bacterial isolates, *B. altitudinis* NHA03 and *K. gibsonii* NHC02 can be recommended as probiotic candidates for biofloc technology cultivation. However, NHB05 is not recommended due to the absence of extracellular enzyme production and an unfavorable antibiotic susceptibility profile. For further studies, *B. altitudinis* NHA03 and *K. gibsonii* isolates need to undergo *in vivo* testing to confirm their stability in biofloc system applications. Studies on *the K. gibsonii* isolate in aquaculture have been limited, so further investigation is needed to assess its safety as a probiotic candidate.

DECLARATIONS

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Authors' contributions

Ren Fitriadi, Petrus Hary Tjahja Soedibya, and Mohammad Nurhafid were responsible for the conceptualization and methodology. Mustika Palupi contributed to the investigation, project administration, and funding acquisition. Purnama Sukardi, Ufianah, and Laela Trianingtyas handled the data curation and funding acquisition. Philipus Uli Basa Hutabarat, Sata Yoshida Srie Rahayu, and Ahmad Musa provided resources, wrote the review, edited, and validated. All authors read and approved the final edition of the manuscript.

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Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

This article was written by the authors and has not been published elsewhere. The authors have checked the article for plagiarism and ensured that it is based on their original scientific results. No AI tools were used in conducting and preparing the present study.

Availability of data and materials

Data supporting the findings of the present study are available from the corresponding author upon reasonable request

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