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SCREENING AND ENZYMATIC–BIOCHEMICAL CHARACTERIZATION OF PROBIOTIC CANDIDATE BACTERIA ISOLATED FROM THE DIGESTIVE TRACT OF LAKE TOBA NILE TILAPIA (*Oreochromis niloticus*)

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ABSTRACT

Intensive Nile tilapia (*Oreochromis niloticus*) culture in floating net cages in Lake Toba has increased organic waste accumulation due to uneaten fish feed, contributing to water quality degradation, eutrophication, and mass fish mortality. Probiotics are considered a potential solution to improve feed digestibility to mitigate eutrophication and high concentration of dissolved organic matter. However, information on indigenous probiotic bacteria from the digestive tract of tilapia cultured in Lake Toba is limited, particularly regarding their enzymatic and biochemical characteristics. This study aimed to isolate, screen, and characterize enzyme-producing bacteria from the digestive tract of Lake Toba Nile tilapia as early probiotic candidates. Bacterial isolation was performed using serial dilution and culture on tryptic soy agar, followed by purification and morphological characterization. Enzymatic screening for amylolytic, proteolytic, and lipolytic activities was conducted using substrate-specific agar media, and selected isolates were identified using biochemical tests. Seven bacterial isolates were obtained, of which three exhibited extracellular enzymatic activity. Two isolates showed proteolytic activity with clear-zone diameters ranging from 17.4 $\pm$ 0.7 to 22.6 $\pm$ 0.7 mm, while one isolate demonstrated amylolytic activity with a clear-zone diameter of 1.57 $\pm$ 0.5 mm. No lipolytic activity was detected. Based on morphological and biochemical characteristics, the enzyme-producing isolates were identified as belonging to the genera *Micrococcus*, *Staphylococcus*, and *Streptococcus*. These results indicate that the digestive tract of Lake Toba Nile tilapia harbors indigenous enzyme-producing bacteria with potential as probiotic candidates. Further molecular identification, safety evaluation, and *in vivo* assessment are required prior to their application in aquaculture.

KEYWORDS: Lake Toba; mass mortality; organic waste; probiotic; tilapia

ABSTRAK: ***Skrining dan Karakterisasi secara Enzimatik–Biokimia Bakteri Kandidat Probiotik yang Diisolasi dari Saluran Pencernaan Ikan Nila** (Oreochromis niloticus) **Danau Toba***

*Budidaya ikan nila (**Oreochromis niloticus**) secara intensif dalam sistem keramba jaring apung di Danau Toba telah meningkatkan akumulasi limbah organik akibat adanya pakan yang*

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*tidak termakan, sehingga berkontribusi terhadap penurunan kualitas perairan, eutrofikasi, dan terjadinya kematian ikan secara massal. Probiotik dipandang sebagai salah satu solusi potensial untuk meningkatkan kecernaan pakan untuk mitigasi terjadinya eutrofikasi dan tingginya konsentrasi bahan organik terlarut. Namun, informasi mengenai bakteri probiotik indigenous yang berasal dari saluran pencernaan ikan nila yang dibudidayakan di Danau Toba masih terbatas, khususnya terkait karakteristik enzimatik dan biokimianya. Penelitian ini bertujuan untuk mengisolasi, menyeleksi, dan mengkarakterisasi bakteri penghasil enzim dari saluran pencernaan ikan nila Danau Toba sebagai kandidat probiotik tahap awal. Isolasi bakteri dilakukan menggunakan metode pengenceran bertingkat dan penanaman pada media tryptic soy agar, diikuti dengan pemurnian dan karakterisasi morfologi. Skrining aktivitas enzimatik meliputi aktivitas amilolitik, proteolitik, dan lipolitik menggunakan media agar dengan substrat spesifik, sedangkan isolat terpilih diidentifikasi melalui uji biokimia. Sebanyak tujuh isolat bakteri berhasil diperoleh, dengan tiga isolat menunjukkan aktivitas enzim ekstraseluler. Dua isolat menunjukkan aktivitas proteolitik dengan diameter zona bening berkisar antara 17,4 ± 0,7 hingga 22,6 ± 0,7 mm, sedangkan satu isolat menunjukkan aktivitas amilolitik dengan diameter zona bening sebesar 1,57 ± 0,5 mm. Aktivitas lipolitik tidak terdeteksi. Berdasarkan karakteristik morfologi dan biokimia, isolat penghasil enzim tersebut diidentifikasi berasal dari genus **Micrococcus**, **Staphylococcus**, dan **Streptococcus**. Hasil ini menunjukkan bahwa saluran pencernaan ikan nila Danau Toba mengandung bakteri indigenous penghasil enzim yang berpotensi sebagai kandidat probiotik. Identifikasi molekuler lebih lanjut, evaluasi keamanan, dan pengujian in vivo diperlukan sebelum aplikasinya dalam sistem akuakultur.*

KATA KUNCI: Danau Toba; ikan nila; kematian masal; limbah organik; probiotik

INTRODUCTION

Freshwater fish farming using floating net cages, or *keramba jaring apung* (KJA), has been well developed in Lake Toba and contributes to the livelihood of the local community around the lake (Garno *et al.*, 2020). However, the rapid expansion of floating net cages in Lake Toba, combined with its limited carrying capacity, has adversely affected the aquatic environment. High levels of waste from uneaten feed and fecal matter have been consistently observed, resulting in poorer water quality (Panjaitan & Manullang, 2022; Prasetya *et al.*, 2023).

It is well established that high concentrations of dissolved organic matter in fish-rearing media increase oxygen consumption during the aerobic biodegradation of these compounds. This results in a decline in dissolved oxygen concentration, after which anaerobic decomposition occurs, producing substances such as H_2S and ammonia and increasing toxic nitrogen and phosphorus (Ashari *et al.*, 2023). The accumulation of these toxic materials will cause mass mortality of fish and other aquatic organisms cultured in KJA and ponds (Chong, 2022). In Lake Toba, Garno *et al.* (2020) reported that BOD and COD values are regularly classified as high, exceeding Class I and Class II water quality thresholds. Their study suggested that the elevated BOD values in Lake Toba's waters likely originate from KJA's operations within the lake.

One of the primary causes of high concentrations of dissolved matter in water bodies is uneaten fish feed (Nathanailides *et al.*, 2023; Wisnu *et al.*, 2021). Preventing the accumulation of dissolved materials is challenging, given prevailing water quality conditions and fish-feeding behaviors. Moreover, it is concerning that, even after feeding, nutritional value may be lost due to the fish's low digestive capacity and limited digestive enzyme production (Nathanailides *et al.*, 2023). To mitigate eutrophication, improving feed digestibility and the production of digestive enzymes in fish by supplementing feed with bacteria that exhibit amylolytic, proteolytic, and lipolytic activities is recommended (Munguti *et al.*, 2020; Nathanailides *et al.*, 2023).

Tilapia is an omnivorous fish that produces protease, lipase, and carbohydrase enzymes (Mawardi *et al.*, 2023), and probiotic bacteria could improve these enzymatic processes. However, not all microbes living in the digestive tract of tilapia are capable of being probiotics; therefore, it is necessary to precisely identify the characteristics of probiotic bacteria found in the fish digestive tract (Thepnarong *et al.*, 2024). This approach ensures that native probiotics collected from the tilapia digestive tract function optimally, adapt to, and survive in the digestive tract of the same species (Kuebutornye *et al.*, 2020).

Research on the isolation of probiotics from the digestive tract of fish, including gourami (Dalahi *et al.*, 2014), catfish (Kurniasih *et al.*, 2014), and tilapia (Efendi & Yusra, 2014), has been widely published. Their application in feed has demonstrated significant effects on fish growth and feed efficiency. The findings of those studies underscore the importance of establishing a framework for the isolation, selection, and identification of probiotic bacteria from the digestive tracts of tilapia cultured in Lake Toba. Such a framework is needed due to limited specific information on the morphological and biochemical characteristics of probiotic bacteria in the digestive tract of tilapia cultured in floating net cages in the lake, particularly their amylolytic, proteolytic, and lipolytic activities. Therefore, this study aimed to determine the morphological and biochemical characteristics of bacteria from the digestive tract of tilapia cultured in Lake Toba, including the identification of isolates exhibiting amylolytic, proteolytic, and lipolytic activities.

MATERIALS AND METHODS

Sampling Location

Sampling was conducted in the waters of Lake Toba, North Sumatra, Indonesia, where Nile tilapia (*Oreochromis niloticus*) are intensively cultured in floating net-cage systems. Laboratory analyses, including bacterial isolation, enzymatic screening, and biochemical identification, were performed at the Fisheries Laboratory, Faculty of Fisheries, Dharmawangsa University, Medan, North Sumatera, Indonesia.

Sample Collection

Healthy Nile tilapia aged 2–3 months, weighing approximately 100–150 g, were randomly collected from floating net cages. Fish showing physical deformities (e.g., spinal or jaw deformities and fin erosion) or clinical signs of disease in accordance with standard fish health assessment criteria (e.g., skin lesions, hemorrhages, fin rot, abnormal swimming behavior, lethargy, or visible ectoparasites) were excluded. All samples were transported to the laboratory in sterile containers and placed in an insulated cooler with ice packs to maintain a stable temperature of approximately 4 ± 1°C until immediate processing in the laboratory (Linscott & Wang, 2023).

Experimental Design

This study used an experimental–descriptive exploratory design to isolate, screen, and identify probiotic candidate bacteria from the digestive tract of tilapia. The research was carried out in three consecutive stages: (1) field survey and sampling, (2) bacterial isolation and morphological characterization, and (3) screening of enzymatic activity followed by biochemical identification of selected isolates (Makuachukwu & George-Okafor, 2025).

Bacterial Isolation

A total of ten healthy Nile tilapia were used in this study. The digestive tract (stomach and intestine) of each fish was aseptically excised and homogenized in sterile physiological saline (0.85% NaCl) for 1 minute. A serial dilution was prepared up to 10^{-6}, and 0.1 mL of each dilution was spread onto tryptic soy agar (TSA; Merck, Germany) plates. The inoculated plates were incubated at 36°C for 48 hours. Bacterial colonies exhibiting distinct morphological characteristics were selected for further analysis (Firdus *et al.*, 2022).

Purification and Morphological Characterization

Selected colonies were repeatedly subcultured on TSA plates until pure isolates were obtained. Morphological characterization was conducted by observing colony shape, color, margin, and elevation. Cell morphology was examined by Gram staining under a binocular microscope (Olympus, Japan) to determine the Gram reaction and cellular morphology (Agustina *et al.*, 2022; Yulikasari *et al.*, 2024).

Screening of Enzymatic Activity

Pure bacterial isolates were screened for extracellular enzymatic activity, including amylolytic, proteolytic, and lipolytic activities. Amylolytic activity was tested using TSA (Merck, Germany) supplemented with 2% soluble starch and visualized by iodine staining (Merck, Germany); proteolytic activity was assessed on TSA supplemented with 4% skim milk powder (Oxoid, United Kingdom); and lipolytic activity was evaluated using TSA supplemented with 2% olive oil (Sigma-Aldrich, United States).

The formation of clear zones around bacterial colonies indicated positive enzymatic activity. The diameter of the hydrolytic zones was measured using a caliper and expressed in millimeters (mm).

Biochemical Identification

Bacterial isolates exhibiting positive enzymatic activity were subjected to biochemical characterization, including the catalase and oxidase tests, glucose and lactose fermentation tests, motility tests, spore-formation tests, and Ziehl–Neelsen staining. The biochemical profiles were compared with standard identification keys in Bergey's Manual of Determinative Bacteriology and Cowan and Steel's Manual for the Identification of Medical Bacteria to determine bacterial genus (Lee, 2023).

Data Analysis

Morphological and biochemical characteristics of bacterial isolates were analyzed descriptively. Enzymatic activity was quantified from the mean diameter of the hydrolytic clear zones and expressed as mean ± standard deviation. The identified probiotic candidate bacteria were classified according to their morphological, biochemical, and enzymatic profiles (Kurniasih *et al.*, 2014).

RESULTS AND DISCUSSION

Colony and Cell Morphology of Potential Probiotic Bacteria

Based on research conducted on bacteria found in the digestive tract (intestine) of tilapia, ten intestinal samples collected from tilapia yielded seven bacterial isolates that could grow and develop on TSA. Colony morphology was observed, including shape, edge, elevation, and color, which is illustrated in Table 1. Overall, the morphology of these colonies is nearly identical, differing only in shape, color, edge, and convex elevation. Each colony that showed predominant growth from the tilapia's digestive tract samples was transferred and re-grown on TSA media for 24 hours. The outcomes of the dilution and purification of bacteria isolated from the digestive tract of tilapia are displayed in Figure 1.

Conventional methods identify bacteria by enriching microbes to obtain a pure culture. From this pure culture, microbial identification is achieved by comparing the characteristics of the bacterial isolate with those of previously identified bacteria. If no bacterium shows 100% similarity, the focus shifts to the bacteria that exhibit the highest similarity. As a result, conventional identification techniques will always identify a previously known bacterium and struggle to recognize a new species.

Table 1 shows that the seven probiotic bacterial isolates exhibit similarities, characterized by entire edges and convex elevations. Isolates US2.3 and US3.1 exhibit a moderate-circular colony shape, while the other five isolates display a small-circular colony shape. Similarly, isolates US1.1 and US2.2 have yellowish-white and translucent-white colonies, respectively, whereas the other isolates have milky-white colonies (Figure 2).

Microbes exist in numerous forms. To study their growth, morphology, and physiological properties, it is necessary to isolate each microbe from others to obtain a pure culture. A pure culture consists of cells from a single species or strain of microbes. Depending on the type of microorganism involved, bacterial isolation can be performed using serial dilution followed by the spread plate technique to obtain discrete colonies from mixed microbial samples (Gupta, 2023).

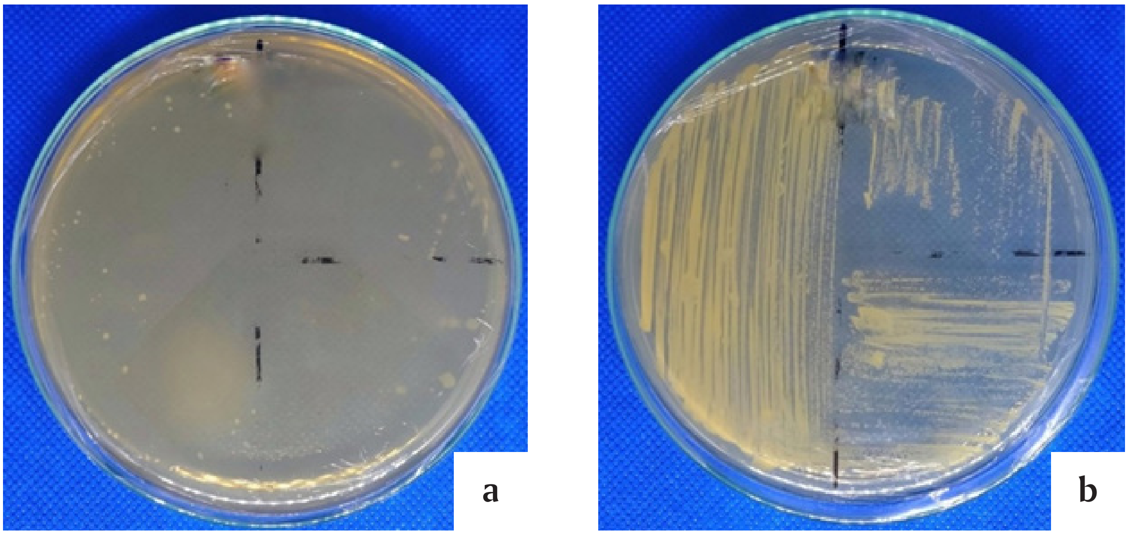

Figure 1. Bacterial colonies from the digestive tract of Nile tilapia grown on tryptic soy agar: initial isolation (a) and purified isolates (b)

Table 1. Morphological characteristics of bacterial colonies isolated from the digestive tract of Lake Toba Nile tilapia during probiotic screening

Isolate	Colony shape	Colony edge	Elevation	Color
US1.1	Small circular	Entire	Convex	Yellowish white
US1.2	Small circular	Entire	Convex	Milky white
US2.1	Small circular	Entire	Convex	Milky white
US2.2	Small circular	Entire	Convex	Translucent white
US2.3	Moderate circular	Entire	Convex	Milky white
US3.1	Moderate circular	Entire	Convex	Milky white
US3.2	Small circular	Entire	Convex	Milky white

Hydrolysis of Starch (Amylum), Fat (Olive Oil), and Casein (Protein) in Bacterial Isolates and the Resulting Clear Zone

The hydrolysis test of starch (amylum), fat (olive oil), and casein (protein) was conducted to assess the ability of bacteria to produce amylase, lipase, and protease enzymes, marking the initial stage in identifying bacterial isolates with probiotic potential for tilapia. This stage involved inoculating each bacterial colony onto media containing starch (amylum), fat (olive oil), and casein (protein). Seven isolates were selected as probiotic candidates based on their capacity to hydrolyze starch, fat, and casein. The results were indicated by the presence of clear zones around the isolates grown on enriched TSA (Table 2 and Figure 3).

Based on the results of proteolytic, amylolytic, and lipolytic activity tests conducted on the seven samples, three isolates were identified as potential probiotic bacteria. Isolates US1.1 and US1.2 exhibited hydrolytic activity on casein, resulting in clear zones around the isolates, and are therefore classified as proteolytic bacteria. Isolate US3.1 demonstrated activity in hydrolyzing starch, indicating that it is an amylolytic bacterium. However, no lipolytic bacteria were detected at this stage, as no clear zones formed around isolates inoculated onto lipid-substrate media. Typically, lipolytic bacterial isolates produce clear zones around their colonies (Ado *et al.*, 2025).

No lipolytic activity was detected in this study, as indicated by the absence of clear zones around the bacterial colonies on olive oil–supplemented agar. This negative result may

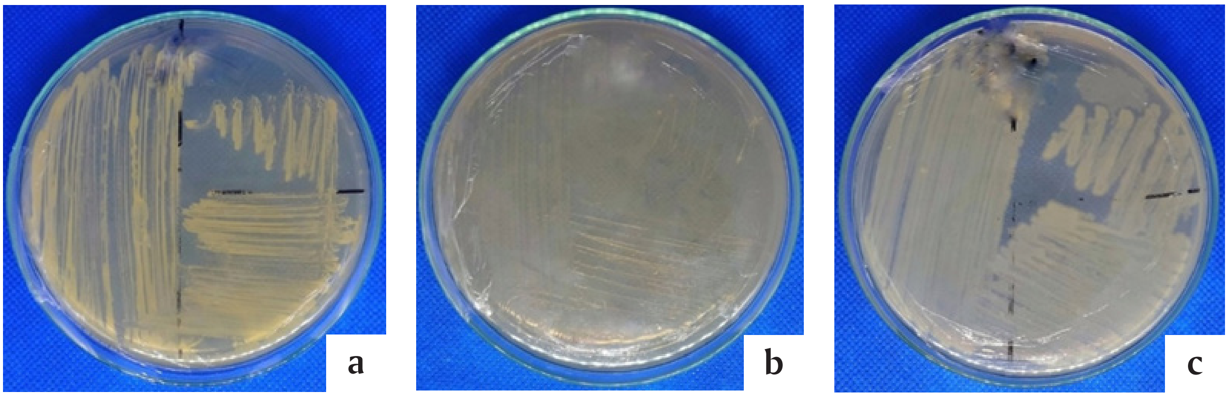
a b c

Figure 2. Colony color variation of bacterial isolates from the digestive tract of Lake Toba Nile tilapia: yellowish white (a), translucent white (b), and milky white (c)

Table 2. Enzymatic hydrolysis activity of bacterial isolates from the digestive tract of Lake Toba Nile tilapia assessed using starch, casein, and lipid substrates during probiotic screening

Isolate	Hydrolysis			Enzyme activity (mm; diameter of hydrolytic zone)
	Starch	Fat	Casein	
US1.1	-	-	+	22.6 ± 0.7
US1.2	-	-	+	17.4 ± 0.7
US2.1	-	-	-	-
US2.2	-	-	-	-
US2.3	-	-	-	-
US3.1	+	-	-	1.57 ± 0.5
US3.2	-	-	-	-

Note: no detectable hydrolytic activity (-); detectable hydrolytic activity (+).

be attributed to several factors. First, lipase-producing bacteria are often less prevalent in the digestive tract of Nile tilapia, where carbohydrate- and protein-degrading enzymes are more dominant due to the omnivorous feeding habit of the species (Bairagi *et al.*, 2002). Second, the qualitative plate assay using olive oil as a substrate has limited sensitivity, as lipase diffusion in solid media is relatively poor and clear zone formation may not be evident despite low-level enzyme production (Sirisha *et al.*, 2010). Furthermore, lipase expression is highly dependent on environmental conditions, including substrate concentration, incubation time, and pH, which may not have been optimal in the present screening assay (Al-Haidari *et al.*, 2021). Therefore, the absence of detectable lipolytic activity should be interpreted as a limitation of the preliminary screening method rather than definitive evidence of the absence of lipase-producing bacteria.

Measurements of the diameter of the hydrolytic clear zone showed that the proteolytic bacterial isolate US1.1 exhibited the highest extracellular protease activity, with an average clear zone diameter of 22.6 ± 0.7 mm. A clear zone of this size indicates significant proteolytic potential, as many proteolytic probiotic candidates in recent studies have demonstrated protein clear-zone diameters exceeding approximately 15–20 mm during qualitative testing for enzyme secretion. Proteolytic LAB strains showing clear zones > 20 mm are indicative of substantial protease production (Elkased, 2024). Protease production by probiotic bacteria is important because these enzymes degrade proteins in the host gastrointestinal tract, thereby improving nutrient digestion and feed utilization, which are desirable traits for effective probiotic strains in aquaculture systems. In comparison, isolate US1.2 had an average diameter of 17.4 ± 0.7 mm, slightly smaller than that of isolate US1.1. On the other hand, the isolated US3.1 showed amylolytic activity, with a clear zone. For isolates with amylolytic activity, the average hydrolytic zone diameter is 1.57 ± 0.5 mm.

The amylolytic activity observed in isolate US3.1 produced a relatively small clear zone diameter (1.57 ± 0.5 mm), indicating low extracellular amylase activity under the conditions of the qualitative plate assay. This limited zone formation may be influenced by several factors, including low enzyme secretion, diffusion constraints in starch-agar media, or suboptimal incubation conditions for amylase expression. Similar observations have been reported in preliminary screening studies, in which amylase-producing bacteria

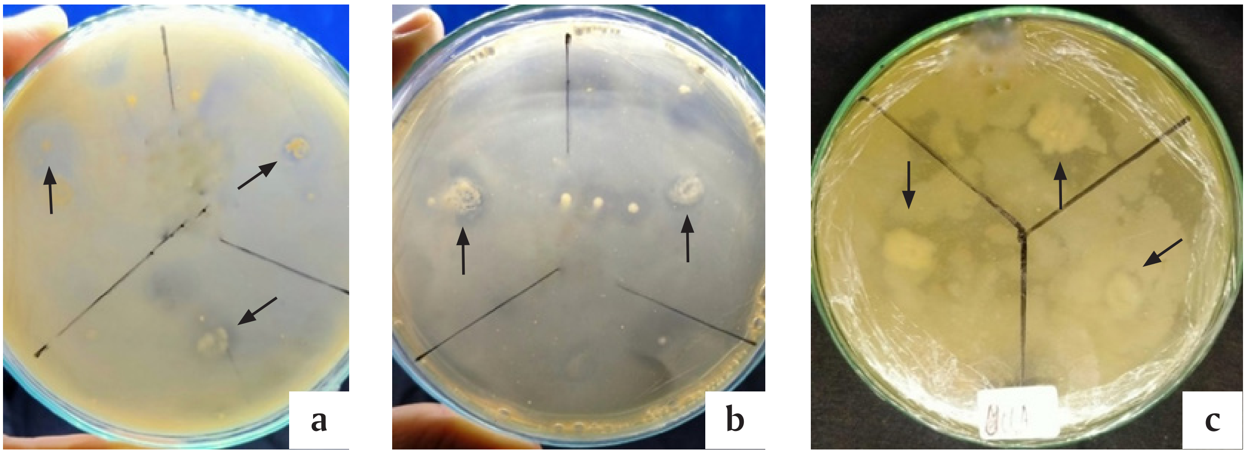

Figure 3. Clear-zone formation indicating proteolytic and amylolytic activities of selected bacterial isolates from the digestive tract of Lake Toba Nile tilapia during probiotic screening. Proteolytic activity of isolates US1.1 (a) and US1.2 (b), and amylolytic activity of isolate US3.1 (c)

exhibited small hydrolytic zones despite measurable enzymatic activity (Kusmiyati *et al.*, 2025). Therefore, the amylolytic activity observed in this study should be interpreted as an initial indication rather than a definitive measure of enzymatic efficiency.

Tests for starch, lipid, and casein hydrolysis were conducted to identify the presence of amylase, lipase, and protease activities in the bacteria. Starch and casein hydrolysis tests are crucial for selecting probiotic candidates. The activities of amylase, lipase, and protease enzymes can enhance the function of endogenous enzymes in the digestive tract of fish, suggesting that the presence of these bacteria may indirectly benefit their host. This aligns with the findings of Kusmiyati *et al.* (2025), who indicate that the action of protease, lipase, and amylase enzymes stimulates the production of endogenous enzymes by bacteria in the digestive tract. Therefore, the presence of these bacteria may indirectly benefit their host.

The starch hydrolysis test results are characterized by the formation of a clear zone after iodine application to bacterial isolates. This occurs because starch molecules are water-soluble and produce a blue color when mixed with iodine solution, but they create a clear zone during starch hydrolysis. This complex forms when amylose coils around the iodine molecule in a helical conformation. When the amylose polymer is cut into shorter lengths, the complex formed with iodine changes, causing the color to lighten and turn red or brown (Pesek & Silaghi-Dumitrescu, 2024). Based on this finding, only isolate US3.1 exhibited the ability to hydrolize starch, indicating the presence of amylase activity, which is capable of degrading starch into glucose, maltose, and dextrin.

A positive casein hydrolysis test is indicated by the formation of a clear zone around bacteria grown on skim milk agar. Based on this study's findings, isolates US1.1 and US1.2 could hydrolyze casein, indicating that these isolates possess protease activity that breaks down proteins into amino acids. According to Gurina & Mohiuddin (2020), protein hydrolysis yields individual amino acids, which can be used for protein synthesis, other cellular processes, or energy production.

Characterization and Identification of Potential Probiotic Bacteria

After testing the isolates for probiotic activity, morphological and biochemical tests were conducted. Based on the results of previous tests on seven isolates obtained from the digestive tracts of tilapia collected from Lake Toba, two potential isolates as proteolytic bacteria and one as amylolytic bacteria were identified. Furthermore, the three bacterial types exhibiting hydrolytic activity were examined at the genus level based on similarities to previously identified characteristics. Bacterial identification is performed using conventional techniques by comparing the bacteria to previously identified species. In the absence of an exact phenotypic match, bacterial isolates are identified based on the most closely related known species. As a result, conventional identification approaches inherently favor the recognition of previously described bacteria and have limited capacity to reveal novel species (Selim *et al.*, 2024). The results of the biochemical tests for the three bacterial isolates are presented in Table 3.

The characterization and identification of the three bacterial isolates revealed that isolate US1.1 exhibited Gram-positive cell morphology with a spherical (coccus) shape. Biochemical analysis showed that this isolate was motile, catalase-positive, oxidase-negative, and capable of fermenting glucose and lactose. Based on these characteristics, isolate US1.1 was identified as belonging to the genus *Micrococcus*. Isolate US1.2 exhibited characteristics largely similar to those of isolate US1.1, differing primarily in its oxidase reaction. Based on its biochemical profile, isolate US1.2 was classified as a member of the genus *Staphylococcus*. The characteristics of isolates US1.1 and US1.2 are consistent with the findings of Kurniasih *et al.* (2014), who reported that *Micrococcus* and *Staphylococcus* belong to the family Staphylococcaceae. The third isolate, US3.1, was identified as a member of the genus *Streptococcus* based on its biochemical characteristics. The results of Gram staining, cell morphology, and motility tests, which formed part of the bacterial identification process, are presented in Figure 4.

The results of morphological and biochemical analyses indicated that the bacterial isolates considered as potential probiotic candidates from the digestive tract of Nile tilapia belonged to the genera *Staphylococcus*, *Micrococcus*, and *Streptococcus*. Similar genera have previously been reported from the digestive tract of tilapia and other cultured fish species (Afrianto & Liviawaty, 2019; Kurniasih *et al.*, 2014), suggesting that these bacteria are commonly associated with the fish gut microbiome. Comparable findings were also reported by Ginting *et al.* (2018),

Table 3. Morphological and biochemical characteristics of probiotic candidate bacteria isolated from the digestive tract of Lake Toba Nile tilapia

Morphology & biochemical test	**Bacterial isolates**		
	US1.1	**US1.2**	**US3.1**
Cell morphology			
Gram	+	+	+
Shape	coccus	coccus	coccus
Biochemistry			
Ziel-Neelsen	-	-	-
Catalase	+	+	+
Glucose fermentative	+	+	-
Lactose fermentative	+	+	+
Motility	+	+	+
Oxidation	-	+	+
Bacteria genus	*Micrococcus*	*Staphylococcus*	*Streptococcus*

Note: (+) positive, (-) negative.

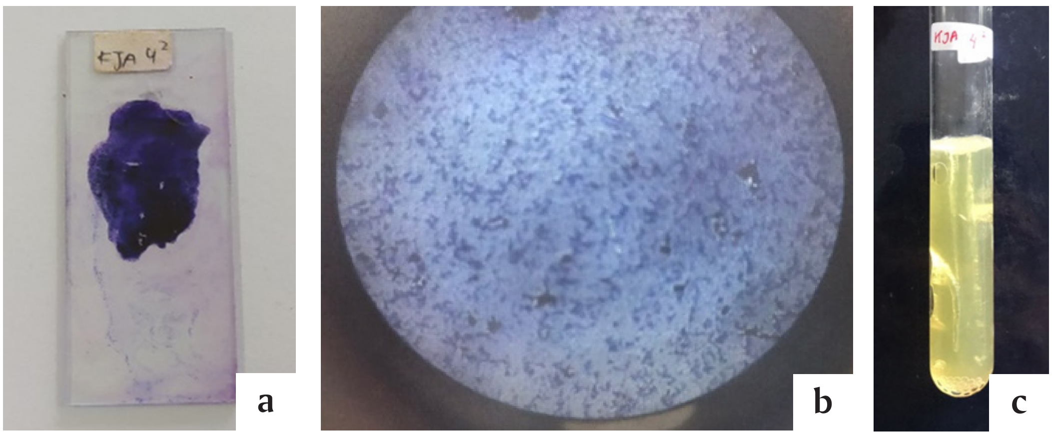

Figure 4. Gram-positive staining (a), coccoid cell morphology (b), and motility test results (c) of selected probiotic candidate bacterial isolates from the digestive tract of Lake Toba Nile tilapia

who identified *Staphylococcus*, *Micrococcus*, and *Lactobacillus* species as potential probiotic candidates in the digestive tract of milkfish (*Chanos chanos*).

Despite their frequent occurrence in the fish gut, it is important to note that certain species within the genera *Staphylococcus* and *Streptococcus*, such as *S. aureus*, *S. iniae*, and *S. agalactiae*, are well-documented fish pathogens with established virulence factors and disease associations in aquaculture systems (Maulu *et al.*, 2021; Wang *et al.*, 2022). Therefore, although the isolates identified in this study exhibited extracellular enzymatic activities associated with probiotic potential, they cannot yet be classified as probiotics. Additional safety evaluations, including hemolytic activity, virulence-associated traits, and antibiotic resistance profiling, are required to ensure biosafety prior to any *in vivo* application.

Isolate US1.1, identified as belonging to the genus *Micrococcus*, exhibited Gram-positive coccoid morphology with yellow-pigmented colonies and convex, circular edges. The cells appeared singly, in pairs, or in tetrads and demonstrated catalase-positive, oxidase-negative reactions, consistent with the characteristics commonly reported for *Micrococcus* species (Selim *et al.*, 2024; Tizabi & Hill, 2023).

Isolate US1.2 was classified within the genus *Staphylococcus* based on its Gram-positive coccoid morphology, creamy-white colonies, and biochemical properties, including catalase and oxidase positivity. These traits are consistent with previously described *Staphylococcus* species commonly isolated from aquatic environments and fish-associated microbiota (Selim *et al.*, 2024).

The third isolate, US3.1, was identified as a member of the genus *Streptococcus*. This isolate exhibited Gram-positive coccoid cells arranged in pairs or short chains, a morphology typical of *Streptococcus* species. The observed physiological characteristics are consistent with those reported for mesophilic streptococci commonly associated with aquatic organisms.

CONCLUSIONS

The probiotic bacterial candidates identified in this study consist of indigenous, enzyme-producing gut bacteria from the genera *Micrococcus*, *Staphylococcus*, and *Streptococcus*, isolated from the digestive tract of Lake Toba Nile tilapia. These bacteria were selected as early-stage probiotic candidates based on their ability to produce extracellular digestive enzymes, particularly proteases and amylases, which are associated with improved feed digestibility and nutrient utilization in aquaculture systems. However, this study is limited by the use of conventional morphological, biochemical, and qualitative enzymatic assays, which do not allow strain-level identification or precise evaluation of functional efficiency. In addition, genera such as *Staphylococcus* and *Streptococcus* include species known to be opportunistic or pathogenic in fish, highlighting the need for careful biosafety evaluation. Future studies should therefore apply molecular identification methods (e.g., 16S rRNA gene sequencing), quantitative enzymatic analyses, and comprehensive safety assessments, followed by *in vivo* feeding trials, to confirm the probiotic suitability and practical applicability of these bacterial candidates.

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AUTHOR CONTRIBUTION

ES: investigation, data curation, formal analysis, validation, writing original draft, and writing review and editing. DTA: investigation and writing review and editing.

DECLARATION OF COMPETING INTEREST AND USE GENERATIVE AI

The authors declare no competing interests.

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