Jurnal Sain Peternakan Indonesia
Available at https://ejournal.unib.ac.id/jspi/index
DOI: https://doi.org/10.31186/jspi.id.20.4.179-190

P-ISSN 1978-3000
E-ISSN 2528-7109
Volume 20 Issue 4 October-December 2025

Screening, Characterization, and Cultivation of Cellulase-Producing Bacteria as Probiotic Candidates for Poultry

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Revised: 2025-12-26, Accepted: 2025-12-27, Publish: 2025-12-31

ABSTRACT

Probiotics play an essential role in regulating gut microbiota and increasing feed digestibility in poultry. This study aimed to screen and characterize cellulase-producing bacteria as poultry probiotic candidates. Among the five isolates tested, isolates I5 and BP had cellulase activity, as indicated by clear zones surrounding the colonies on carboxymethyl cellulose (CMC) agar plates. Isolate I5 was more tolerant to low pH and 0.3% bile salts than isolate BP, indicating probiotic potential. Isolate I5 was selected for cultivating in tempeh wastewater-molasses medium (TM) and LB medium at 37 °C with shaking at 120 rpm. The results demonstrated that bacterial growth in TM medium was significantly lower ($p < 0.001$) than in LB medium. Importantly, bacterial growth in TM medium reached an optical density (OD_{600}) of 0.415 after 6 hours of incubation, indicating its adaptability to tempeh wastewater-molasses medium. These findings suggest that the TM medium promotes bacterial growth and proliferation, supports probiotic and enzyme production for use in poultry feed supplementation, and reduces cultivation costs. Therefore, the use of agro-industrial waste provides a cost-effective alternative for cultivating cellulase-producing probiotics, thereby contributing to value-added waste management, sustainable poultry production, and circular bio-economy practices.

Keywords: cellulase-producing bacteria, probiotic, poultry, tempeh wastewater-molasses medium, sustainable poultry production

INTRODUCTION

The poultry production sector has significantly contributed to efforts to improve nutrition for many people worldwide, particularly in Indonesia. Poultry is considered one of the most efficient livestock species at converting feed into valuable products, such as meat, milk, and eggs. Broiler chickens require 1.60-1.70 kg of feed to produce 1 kg of edible meat, while layer hens in the intensive production system using battery cages can convert 2,8 kg of feed into 1 kg of eggs (Nugraha et al., 2017; Sonaiya & Swan, 2004). In addition to feed conversion efficiency, poultry have relatively short production cycles and high reproductive rates compared with other livestock species. This makes the poultry sector a primary and highly affordable source of animal protein.

Feeding is considered the most crucial factor, significantly influencing the success of a poultry farming business and accounting for 60-80% of the total production expenditures (Sari et al., 2014). High feed-provision costs are a challenge in poultry farming. Therefore, locally derived feedstaff from agricultural and agro-industrial waste has potential as an alternative component in poultry feed, reducing feed costs and supporting sustainable livestock production (Ali et al., 2020). Nevertheless, local feed ingredients are limited by anti-nutritional factors, high crude fiber, and low protein content (Ababor et al., 2023; Anwar et al., 2025). Crude fiber and lignin represent the primary components of the plant cell walls, which account for up to 20% of plant-based poultry feed. These limitations affect the digestibility and availability of the nutrients for poultry production, leading to low growth performance and a higher feed conversion ratio (Kim et al., 2022).

Cellulase-producing bacteria are promising probiotics in the poultry industry, capable of degrading cellulose and modulating the gut microbiota. Cellulase plays an essential role as a biocatalyst that hydrolyzes cellulose into simpler molecules, including trioses, cellobiose, and glucose, thereby providing an energy source for better growth and health in poultry (Perim et al., 2024; Sutaoney et al., 2024; Wang et al., 2024). Consequently, the identification, characterization, and cultivation of cellulase-producing bacteria as probiotics for poultry serve as important approaches for developing feed additives as alternatives to antibiotic growth promoters (AGPs), thereby fostering a healthier

and more sustainable poultry production system (Anwar et al., 2025).

One prospective strategy to support sustainable agriculture-livestock systems is to cultivate probiotic bacteria using agro-industrial waste as the main growth substrate. Tempeh processing wastewater is a major by-product obtained during the tempeh processing, with approximately 24.28 liters of wastewater produced from 1 kg of processed soybeans (Pakpahan et al., 2021). Tempeh processing wastewater contains 0.47% crude protein, 4.06% total carbohydrate, 0.04% total fat, 94.55% water, and 0.88% ash (Sari & Rahmawati, 2020). Molasses is a by-product gained during the sugar refining process from sugar cane or sugar beets. Molasses from sugar cane contains 30-40% sucrose, 4-9% glucose, 5-12% fructose, 2-5% other carbohydrates, 7-15% ash, and 0.5-1.5% protein (Clarke, 2003). The combination of these agro-industrial wastes can provide a nutrient source for probiotic bacteria because both substances are biodegradable (Gifari et al., 2022).

The present study aimed to screen, identify, and characterize several probiotic bacterial candidates that produce extracellular cellulase enzymes, and then cultivate them using a medium composed of tempeh-processing wastewater and molasses. Adapting cellulolytic bacteria to grow well in culture medium derived from agro-industrial waste can improve their practical application in the poultry industry since soybean-derived products are a significant component of poultry feed. This strategy not only reduces the cost of preparing probiotic bacterial cultures but also reduces agricultural and agro-industrial waste. This sustainable approach aligns with the principles of a circular bioeconomy, which encourages greater economic value from waste and the use of more environmentally friendly microbial cultures. Therefore, we hypothesize that several bacterial isolates identified as Bacillus spp. and characterized for probiotic properties, including tolerance to low pH and bile salts, can produce extracellular cellulase and be effectively adapted to grow in agro-industrial waste-based culture media for poultry feed applications.

MATERIALS AND METHODS

Collection of Bacterial Isolates

A total of five bacterial isolates used in this study were obtained from the Laboratory of Biotechnology and Animal Product Processing at the Faculty of Animal Husbandry, University of Mataram (Indonesia). Three isolates (I5, I6, and I7) were isolated from the gastrointestinal tract of broiler chickens (Ali et al., 2020; Nurbaiti et al., 2016). The BP and BGT isolates were obtained from previous studies conducted by Ichsan (2004) and Gifari et al. (2022), respectively. Ichsan (2004) reported that BP isolates belonged to the genus *Bacillus spp*. All of these bacterial isolates have never been tested for their capability to produce cellulase enzymes.

Preparation of Culture Medium

Luria-Bertani (LB) medium was used as the primary culture medium. It contained 10.0 g/L of tryptone (Thermo Fisher Scientific, USA), 10.0 g/L of sodium chloride, and 5.0 g/L of yeast extract (Thermo Fisher Scientific). A total of 15.0 g of bacteriological agar (Thermo Fisher Scientific) was added to prepare 1.0 L of LB agar. Cellulase activity was tested using carboxymethyl cellulose (CMC) agar medium, following the protocol previously reported by Bairagi et al. (2002), with minor modifications. CMC-agar medium contained 10.0 g/L of sodium chloride, 5.0 g/L of yeast extract (Thermo Fisher Scientific), 10.0 g/L of tryptone (Thermo Fisher Scientific), 10.0 g/L of CMC powder, and 15.0 g/L of agar bacteriological (Thermo Fisher Scientific). The Sulfide-Indole-Motility (SIM) medium was prepared by weighing 36.23 g/L of SIM medium powder (Central Drug House, India), dissolving it in 1.0 L of distilled water, and dispensing it into reaction tubes. The starch agar medium was used to identify bacteria that produce amylase, an enzyme that breaks down starch. It contained 5.0 g/L of sodium chloride, 5.0 g/L of tryptone (Thermo Fisher Scientific), 1.0 g/L of yeast extract (Thermo Fisher Scientific), 10.0 g/L of starch powder, and 15.0 g/L of agar bacteriological (Thermo Fisher Scientific).

Plate Count Agar (PCA) was used to enumerate viable cells and assess survival under simulated gastrointestinal conditions. It was composed of 5.0 g/L tryptone (Thermo Fisher Scientific), 1.0 g/L yeast extract (Thermo Fisher Scientific), 1.0 g/L glucose, and 15.0 g/L bacteriological agar (Thermo Fisher Scientific). Simmon's citrate agar is used to test the ability of bacterial isolates to utilize citrate as the sole carbon source. A total of 24.2 g/L of powder of Simmon citrate agar was dissolved in 1 L of distilled water and dispensed into reaction tubes. The medium containing tempeh processing wastewater and molasses was prepared using

these materials as the primary carbon sources. The composition of the medium was 10% (v/v) tempeh processing wastewater, 5% (v/v) molasses, 8.5 g/L urea, 3.1 g/L KH_2PO_4, and 1.71 g/L $MgSO_4$. The tempeh processing wastewater was settled and filtered (0.8 µM) before being mixed with other medium components to prevent interference with absorbance measurements during bacterial growth. The pH was measured and adjusted to 7.2 with 2N NaOH. All media used in this study were sterilized by autoclaving at 121 °C for 15 minutes.

Preparation of Bacterial Preculture

Pure cultures of bacterial isolates stored in glycerol were streaked onto LB agar and incubated at 37 °C for 20 hours. Single colonies were selected and transferred into 5 mL of LB medium, then incubated at 37 °C with shaking at 120 rpm for 20 hours. Pure cultures in this liquid medium were used for further testing.

Selection of Cellulase-Producing Bacteria

The selection of cellulase-producing bacteria was conducted based on the ability of bacteria to generate extracellular cellulase enzymes around the bacterial colonies, which is indicated by the formation of a clear zone (Bairagi et al., 2002). A total of 20 µL of bacterial suspension, diluted to an optical density (OD600) of approximately 0.2, was applied to the surface of CMC-agar medium and incubated at 34 °C for 72 hours. After incubation, the CMC-agar medium was soaked in a 0.1% Congo red solution (w/v) and incubated at room temperature for 15 minutes. Clear zones formed after soaking in 1 M NaCl. The best isolates in producing cellulase enzymes were selected for further analysis.

Identification of the Selected Cellulase-Producing Bacteria

The identification of the selected bacterial isolate was carried out by following several steps, including morphological observations (form, elevation, and margin) and biochemical tests (Gram staining, catalase test, SIM test, citrate test, and starch hydrolysis test). For the starch hydrolysis test, 1% (v/v) Lugol's iodine was applied to the plates. A clear zone indicates amylase activity.

In Vitro Evaluation of Survival Rates under Simulated Gastrointestinal pH Conditions

The survival rates of bacterial isolates under simulated gastrointestinal pH conditions (pH 2.0 and 7.2) were determined using the procedure reported by Lin et al. (2006), with a slight modification. A single colony of each bacterial isolate was inoculated into 5 mL of LB medium and incubated at 37 °C with shaking at 120 rpm for 20 hours. 1 mL of preculture was transferred into 5 mL of phosphate-buffered saline (PBS) (pH 2.0 and 7.2), followed by incubation at 37 °C for 2 hours. 2N HCl was used to adjust the pH of PBS. The viable cells were examined using the pour plate method with PBS (pH 7.2). A total of 1 mL of the serially diluted sample was transferred to sterile plates, and PCA medium was gently added. After solidifying, the plates were incubated at 37 °C for 24 hours. The number of colonies ranged from 30 to 300. Each isolate was measured in triplicate. Survival rates (%) were calculated by dividing the bacterial population after pH treatments by the initial population and multiplying the result by 100.

Assessment of Bile Salt Tolerance

Bile salt tolerance was assessed by exposing 1 mL of 24-hour preculture to 5 mL of PBS (pH 2.0) and incubating at 37 °C for 1 hour without agitation. Following low pH treatment, the bacterial cells were harvested through centrifugation (13,000 rpm for 5 minutes), resuspended in 10 mL of liquid LB medium with or without the addition of 0.3% (w/v) bile salt (Thermo Fisher Scientific), and incubated at 37 °C for 3 hours, as previously described by Lin et al. (2006), with a slight adjustment. The surviving cells from each sample were evaluated in triplicate using the pour plate method as previously described. Bacterial tolerance to bile salts was assessed by comparing viable cell counts on PCA-agar medium. The percentage of bacterial tolerance to bile salt was determined by dividing the bacterial counts in 0.3% bile salt by the control and then multiplying by 100.

Cultivation of Cellulase-Producing Bacteria in Tempeh Wastewater–Molasses Medium

The bacterial precultures (100 µL) were inoculated into 4 mL of both tempeh wastewater–molasses medium (TM) and LB medium (control) at a standardized initial cell density ($OD_{600} \approx 2.5$). The cultures were incubated at 37 °C with shaking at 120 rpm. Bacterial growth was monitored spectrophotometrically by measuring optical density at 600 nm (OD600) at 3, 4, 5, and 6 hours, corresponding to regular 1-hour intervals from the start of growth measurement at 3 hours. Each measurement was performed in triplicate. Growth

rates were compared between media to evaluate the efficacy of the alternative formulation in supporting bacterial proliferation.

Data Analysis

The data obtained in this study were analyzed using Student's t-test to ascertain the differences in incubation durations and isolates. A paired t-test was conducted to compare values obtained from the same isolates under two conditions (with and without 0.3% bile salt). In contrast, an independent t-test was used to compare values between isolates. A *p*-value lower than 0.05 represented a statistically significant difference. Statistical analyses were conducted using JASP 0.95.4 software.

RESULTS AND DISCUSSION

Screening and Selection of Cellulase-Producing Bacteria

Local feedstaff derived from agricultural and agro-industrial waste has potential for use as feed components in the poultry industry, thereby reducing feed costs. Unfortunately, the high crude fiber and low protein content limit the utilization of local feedstaff. Poultry lacks endogenous enzymes that can digest non-starch polysaccharides (NSP), such as cellulose, so NSP digestion occurs biochemically in the crop and cecum, involving several microbes (Choct, 2015). Cellulase enzymes can be utilized to improve the digestibility of local feedstuff by hydrolyzing cellulose molecules into simpler molecules, such as trioses, cellobiose, and glucose (Wang et al., 2024). In the present study, cellulase activity was determined using a CMC-agar medium, followed by Congo red and NaCl soaking, as shown in Figure 1.

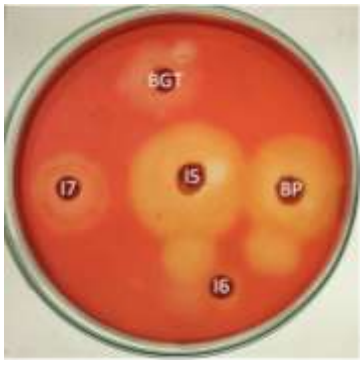

Figure 1. Detection of cellulase activity on CMC-agar plates. The plate was flooded with a 0.1% Congo red solution for 15 minutes, then washed with 1 M NaCl to visualize the cellulase hydrolysis zone. Clear zones surrounding the colonies (I5 and BP) indicate CMC substrate degradation, confirming extracellular cellulase activity.

The results of the cellulase activity test demonstrated that two bacterial isolates (I5 and BP) among five tested produced and secreted cellulase enzymes, as indicated by the formation of clear zones upon addition of 0.1% Congo red solution. Isolates I7 and BGT showed a lower ability to break down the CMC substrate, as presented in Figure 1. Moreover, no clear zones were observed for isolate I6, indicating that it is non-cellulolytic. Congo red has been reported to exhibit a high binding affinity for polysaccharides containing (1→4)-β-D-glucopyranosyl sequences, a feature of cellulose (Wood, 1980). The presence of the binding of Congo red to cellulose and its derivatives, such as CMC, is speculated to occur from a combination of electrostatic interactions and hydrogen bonding between dye azo (—N=N—) and amino groups and the cellulose fibres (Yamaki et al., 2005). Once cellulose is hydrolyzed by cellulase, releasing various low-molecular-weight sugars, such as glucose, Congo red loses its binding substrates, causing the red or purple complex to disappear. This subsequently changes the medium to colorless or slightly reddish, resulting in clear zones (Gupta et al., 2012).

Cellulolytic bacteria can extracellularly produce different cellulase complexes depending on the genes present and the carbon source used. The capacity of each bacterial isolate to degrade the CMC substrate suggests the variation in cellulolytic activity among the tested isolates. Previous studies have reported several bacterial species capable of producing cellulase enzymes, including *Bacillus amyloliquefaciens* DL-3 (Jo et al., 2008), *B. pumilus* EB3 (Ariffin et al., 2008), *B. subtilis* QY4 (Mushtaq et al., 2024), *B. coagulans* MA-13 (Aulitto et al., 2017), and *B. licheniformis* (Shyaula et al., 2023). Overall, isolates I5 and BP exhibit strong potential as cellulase-producing bacterial candidates for further characterization and have promising applications for enhancing fiber digestibility when used as probiotic feed supplements.

Identification of Cellulase-Producing Bacteria

Among five isolates tested on CMC-agar plates, two bacterial isolates (I5 and BP) exhibiting the largest clear zones were selected for

further identification. The identification of bacterial isolates was performed using morphological and biochemical tests, as described in Bergey's Manual of Determinative Bacteriology (Bergey & Holt, 1994), as shown in Table 1. The results demonstrated that both isolates were Gram-positive, rod-shaped, and catalase-negative, indicating that they are closely related to *Bacillus spp.* In contrast, Gonari et al. (2024) previously reported the isolation and identification of spore-forming, Gram-positive, catalase-positive Bacillus isolates from dairy products, soil, and livestock waste, indicating that biochemical differences may exist among *Bacillus* species from different sources. The SIM test showed that both isolates were indole-negative and did not produce hydrogen sulfide, but were motile, supporting their identification as members of the genus Bacillus.

Both isolates showed negative reactions for citrate utilization as the sole carbon source but hydrolyzed starch, consistent with the typical characteristics of *Bacillus* species. Similar characteristics were reported by Aulitto et al. (2017), who described *Bacillus coagulans* MA-13 as Gram-positive, catalase-positive, motile, negative for indole, starch-hydrolyzing, and producing the cellulase enzyme (endo-1,4-β-glucanase). Moreover, Lestari et al. (2020) Reported the biochemical properties of *B. subtilis* 1. A, which is Gram-positive, catalase-positive, indole-negative, citrate-negative, and motile. *B. subtilis* QY4 was observed to hydrolyze starch and cellulose (Mushtaq et al., 2024). Although molecular analysis was not performed in this study, the combined morphological and biochemical properties strongly suggest that both isolates are closely related to *Bacillus coagulans* and *Bacillus subtilis*.

Survival Rates under In Vitro Simulated Gastrointestinal pH Conditions

The bacterial isolates identified as *Bacillus* species with cellulose-degrading activity were subjected to *in vitro* simulated gastrointestinal pH conditions (pH 7.2 and 2.0) for 1 and 2 hours. The results demonstrated that isolate I5 had a higher survival rate at pH 7.2 for 1 hour than isolate BP ($p < 0.001$). Conversely, no significant difference was observed between the two isolates after 2 hours (Figure 2A**)**. The isolate I5 had survival rates of 100.84% after 1 hour of incubation and 97.42% after 2 hours at pH 7.2, while the isolate BP exhibited survival rates of 94.66% and 95.17% after 1 and 2 hours at the same pH (Figure 2A). Furthermore, the survival rates of both isolates did not differ significantly after 1-hour incubation at pH 2.0 (Figure 2B). The isolates I5 and BP had survival rates of 80.05% and 76.49%, respectively. In contrast, the results revealed a significant difference after 2-hour incubation ($p < 0.05$), with isolate I5 exhibiting a higher survival rate (65.69%) than isolate BP (62.18%), as shown in Figure 2B. These findings collectively demonstrated that the survival rates under *in vitro*-simulated gastrointestinal pH conditions gradually decreased while retaining a relatively high degree of adaptability.

Table 1. Morphological and biochemical properties of selected cellulase-producing bacteria

Parameters	Isolate properties	
	I5	**BP**
Morphological observation		
Form	Round	Round
Elevation	Convex	Convex
Margin	Entire	Entire
Color	White	White
Colony size	Moderate	Moderate
Biochemical tests		
Gram staining	Positive	Positive
Cell shape	Rod	Rod
Catalase	+	+
H_2S production	-	-
Indole	-	-
Motility	+	+
Starch hydrolysis test	+	+
Citrate	-	-

Note: (-): negative; (+): positive

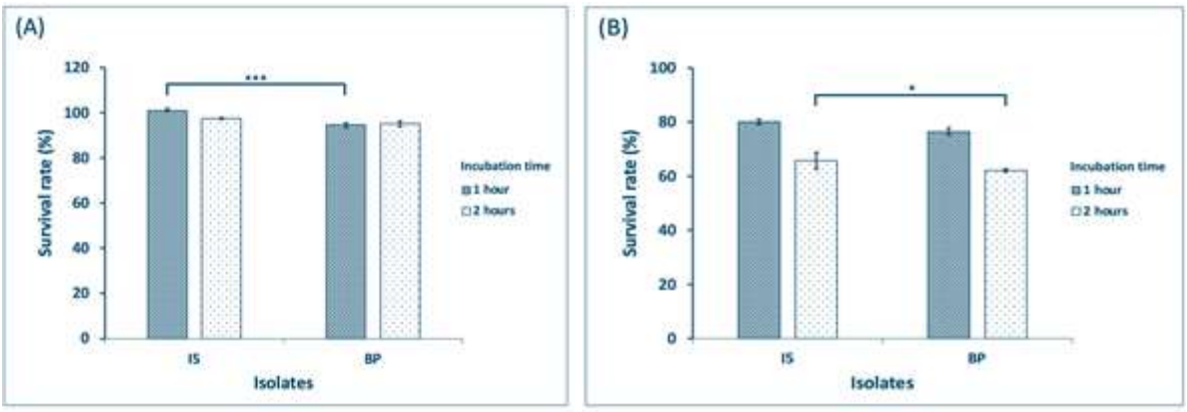

Figure 2. Survival rates (%) of the selected isolates with cellulose-degrading activity under *in vitro* simulated gastrointestinal pH conditions. (A) pH 7.2, (B) pH 2.0. Each value indicates the mean value ± standard deviation (SD) from three measurements. The *p*-values are <0.001 (***) and <0.05 (*).

Survivability and colonization of probiotic candidates under gastrointestinal pH conditions help evaluate their functional properties in poultry. Probiotics, which are administered orally to the host, must survive harsh conditions during ingestion and transit before reaching the intestines (Gharbi et al., 2019; Nemska et al., 2019). The pH of chicken gut ranges between 3.1 and 6.6 (Nkukwana et al., 2015), in which the gizzard and proventriculus have shown acidic conditions (pH < 3.0), and the caecum has the highest pH (almost neutral) (Sozcu, 2019). Both isolates exhibited high survival rates, exceeding 95% at pH 7.2 (Figure 2A) and 60% at pH 2.0 (Figure 2B) after 2 hours of incubation, indicating adaptability across a broad range of gastrointestinal pH conditions. Dabiré et al. (2022) reported that several *Bacillus* strains could survive at pH 2.0, with survival rates exceeding 35%. In addition, *B. cereus* F20 and *B. subtilis* F24 had survival rates of 49.56% and 44.91%, respectively (Dabiré et al., 2022). Another study reported that *B. subtilis* MG-1 demonstrated survival rates of 64.75%, 75.95%, and 77.73% at pH 2.0, 3.0, and 4.0, respectively (Ren et al., 2023). Furthermore, *B. subtilis* isolated from bee digestive tracts exhibited survival rates greater than 100% at pH 4.0 and 6.0 (Toutiaee et al., 2022). A survival rate of more than 50% in acidic conditions is considered high-acid resistance (Gharbi et al., 2019), suggesting that the strains are effective probiotics for poultry.

Variations in survival rates of *Bacillus* strains under acidic conditions may indicate that the acid tolerance is strain-specific. This likely occurs through combinatorial interactions between genetic and physiological mechanisms, which are common in acidophilic microorganisms. (Dabiré et al., 2022). The mechanisms of proton pumps also contribute to maintaining survival rates at low pH by expelling H^+ ions from the cell, generating interacellular compounds, and modifying cell wall structures to prevent H^+ ion entry (Sukmawati et al., 2024). Additionally, spore-forming bacteria, such as *Bacillus* species, can tolerate low pH environments and high temperatures. Spores can protect the cell and its genome from harsh external environments, subsequently germinate into a single active cell when the environment becomes favorable (Payne et al., 2024; Ren et al., 2023).

Tolerance to Bile Salt

The tolerance to bile salt is regarded as the subsequent vital criterion of probiotics for colonization and metabolic activity in the gastrointestinal tract of the host. In this study, the selected isolates tolerant to low-pH treatment were in vitro evaluated using liquid LB medium with or without 0.3% bile salt for 3 hours. The results showed that, after 3-hour incubation, bacterial counts in liquid LB medium containing 0.3% bile salt were significantly lower for both isolates than those in the control group (p < 0.001), as depicted in Figure 3A. The bacterial count of the isolate I5 was 6.45 Log CFU/mL in the control and decreased to 5.37 Log CFU/mL after exposure to 0.3% bile salt. Similarly, the isolated BP demonstrated the bacterial count of 7.83 Log CFU/mL in the control group and 5.69 Log CFU/mL in the 0.3% bile salt treatment.

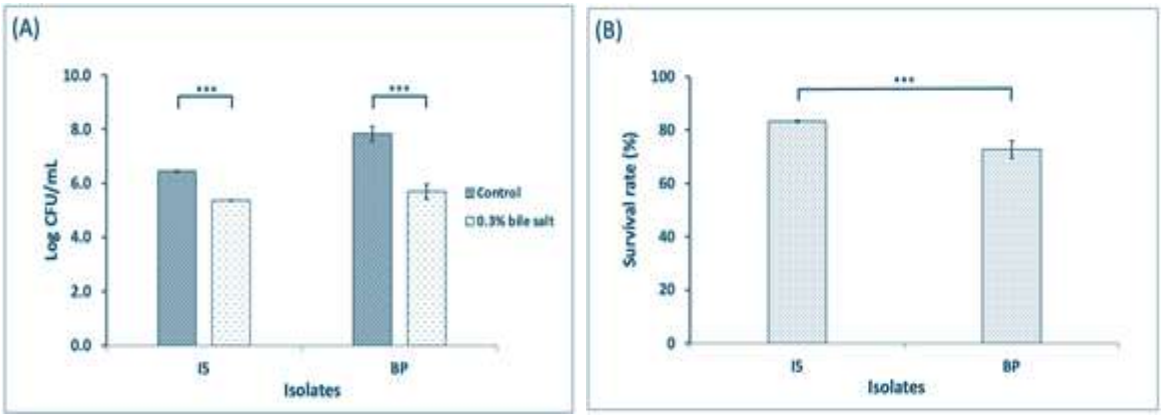

Figure 3. Tolerance to bile salt. (A) a comparison of bacterial counts (Log CFU/mL) in two different conditions: control (without bile salt) and 0.3% bile salt. (B) survival rates (%) at 0.3% bile salt. Each value indicates the mean value ± standard deviation (SD) from three replications. The *p*-value is <0.001 (***).

Comparing the two isolates, Figure 3B showed a significant difference ($p < 0.001$) between isolate I5 and BP, with survival rates of 83.24% and 72.71%, respectively, suggesting that both isolates are tolerant to 0.3% bile salt. These findings correspond to those reported by Dabiré et al. (2022), who found that various Bacillus strains had survival rates in 0.3% bile salt ranging from 52.69% to 87.91%. Moreover, Lee et al. (2012) reported survival rates of several Bacillus strains used as feed additives ranging from 82.77% to 93.10%.

Differences in probiotic tolerance to bile salts may be influenced by bile salt concentration, exposure duration, and strain (Anwar et al., 2025). The tolerance of *Bacillus* species to bile salts is associated with endospora formation, changes in cell wall structure, and bile salt hydrolase (BSH) activity. The BSH activity is frequently observed in the genus *Lactobacillus*, deconjugating bile salts in the gastrointestinal tract, thereby assisting the colonization and survival of probiotic bacteria (Risna et al., 2020). Although the presence of BSH enzymes in *Bacillus* strains is less investigated, screening studies have found that some *Bacillus* strains, including *B. coagulans*, *B. licheniformis* Me1, and *B. subtilis* Bn1, exhibited positive BSH activity in plate assays. Nevertheless, the authors further noted that molecular identification of the bsh genes and deeper mechanisms employed remain necessary (Nithya & Halami, 2013).

Cultivation of Cellulase-Producing Bacteria using Tempeh Wastewater–Molasses Medium

After determining the survival rates of cellulase-producing bacteria under *in vitro* simulated gastrointestinal conditions, isolate I5 was further selected for its high tolerance to low pH and 0.3% bile salts. Subsequently, this isolate was cultivated using a cost-effective medium composed of tempeh wastewater and molasses. Our results demonstrated that isolate I5 grew in tempeh wastewater–molasses medium (TM) after incubation at 37 °C with shaking at 120 rpm for 3, 4, 5, and 6 hours (Figure 4). Compared with LB medium as a control, bacterial growth in TM medium, as measured by optical density at 600 nm (OD_{600}), was significantly lower ($p < 0.001$) at all measurement durations. Interestingly, the OD_{600} reached 0.415 in TM medium after 6 hours, indicating that the TM medium is a suitable substrate for bacterial proliferation, although the growth was slower than in LB medium. These observations align with previous studies, demonstrating that the molasses-based medium can support the proliferation and development of *B. subtilis* (Kaleem et al., 2000). Similarly, Gifari et al. (2022) reported that a mixture of tempeh wastewater and molasses, as a primary substrate, supported the growth of bacteria that produce phytase enzymes.

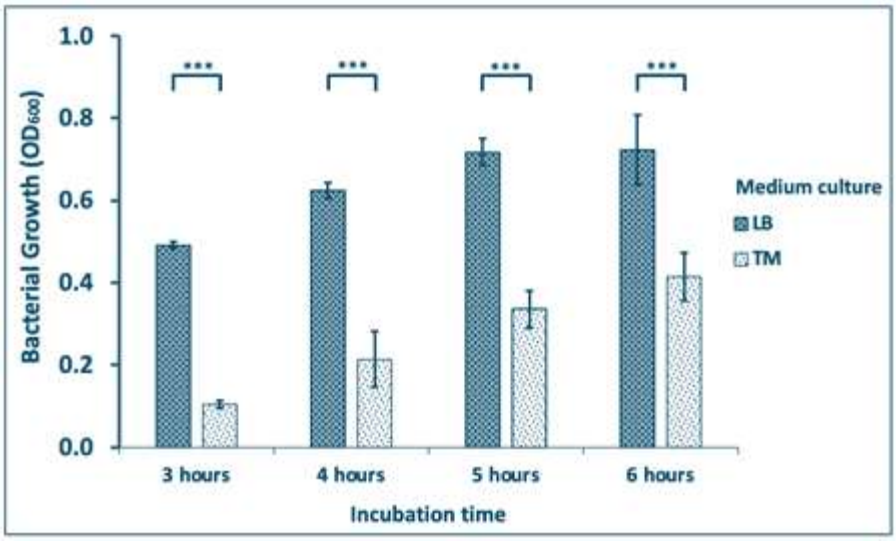

Figure 4. Bacterial growth, measured by optical density at 600 nm (OD600), in LB medium (control) and in tempeh wastewater–molasses medium (TM). Each value indicates the mean value ± standard deviation (SD) from three replications. The *p*-value is <0.001 (***).

This study emphasizes the importance of using agro-industrial waste as a nutrient source for cultivating beneficial microorganisms, including probiotics. Agro-industrial waste-based media are promising for producing *Bacillus* probiotics under monoculture conditions, providing an efficient and low-cost alternative for cultivating probiotic bacteria for use in poultry feed supplementation (Valle Vargas et al., 2024). The more beneficial bacteria can grow and proliferate, the more agro-industrial waste is degraded and recycled (Arbianti et al., 2018), thereby increasing agricultural good practices and ecological well-being. Moreover, leveraging locally sourced cellulase-producing bacteria offers a sustainable approach to enzyme acquisition, reducing reliance on commercial enzymes in poultry production and promoting the valorization of agricultural and agro-industrial by-products.

CONCLUSION

We successfully screened two bacterial isolates producing cellulase enzymes. Isolate I5 was identified as *Bacillus* spp. and exhibited high tolerance to low pH and bile salts, confirming its potential use as a poultry probiotic. This isolate was capable of growing and proliferating in tempeh wastewater–molasses medium, offering a sustainable method for probiotic preparation and enzyme acquisition for use in poultry feed supplementation. The use of such waste-derived medium components not only reduces production costs but also contributes to waste recycling and environmental sustainability, thereby advancing circular bioeconomy practices. Future studies should focus on cultivating and producing *Bacillus* spp. Using a tempeh wastewater–molasses medium in a bioreactor, followed by evaluation of probiotic stability and viability, in vivo assays, and feasibility for the scale-up and downstream processing.

Acknowledgment

The authors sincerely thank the Research and Community Service Institute (LPPM) of the University of Mataram for supporting the research through the PNBP Scheme under the contract number: 2397/UN18.L1/PP/2025. The authors also thank all staff at the Laboratory of Biotechnology and Animal Product Processing, Faculty of Animal Science, Universitas Mataram, Mataram, Indonesia, for any technical assistance.

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