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Characterization and Comparison of β-galactosidase Enzyme Activity in Yogurt Bacteria Isolated from Sheep's Milk Based on Lactose Hydrolysis

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Abstract. Milk consumption in Indonesia remains low, partly due to lactose intolerance caused by limited lactase activity. Although microbial β-galactosidase from lactic acid bacteria may improve lactose digestion, its activity in bacteria isolated from sheep milk yoghurt remains poorly characterized. This study aimed to isolate and assess β-galactosidase-producing bacteria from sheep milk yoghurt and determine their ability to hydrolyze lactose. Sheep milk was fermented using a commercial starter, followed by bacterial isolation through serial dilution and colony purification methods. Isolates were characterized by colony morphology, Gram staining and biochemical tests. Selected isolates were then tested for β-galactosidase activity using lactose-containing media, followed by cell permeabilization using ethanol and enzyme activity measurement using the GOD-PAP method based on the amount of glucose produced. Four bacterial isolates (BK1–BK4) were obtained, exhibiting lactic acid bacteria characteristics and presumed to belong to the genus *Lactobacillus* based on rod-shaped morphology, Gram-positive reaction, and homofermentative properties. All isolates were able to hydrolyze lactose, although at relatively low levels. The highest enzyme activity was observed in isolate BK1 at 0.034 U/mL with a hydrolysis percentage of 1.417%, followed by BK4, BK3 and BK2. Statistical analysis showed no significant differences among isolates ($p > 0.05$), indicating similar β-galactosidase production abilities. The low enzyme activity was presumed to be influenced by suboptimal permeabilization methods and reaction conditions that did not yet allow optimal substrate access to intracellular enzymes. Sheep milk yoghurt isolates show potential as β-galactosidase producers; however, extraction and reaction conditions require optimization to enhance activity.

Key words: β-galactosidase, sheep milk yogurt, lactose hydrolysis, enzyme activity

INTRODUCTION

Milk is an important source of nutrients, particularly protein, calcium and vitamin D, which play essential roles in growth and bone health (Saputra et al., 2024). However, milk consumption in Indonesia remains relatively low compared to other countries. Data from the national statistics agency indicate that milk consumption over the past five years has been relatively stagnant and remains below the standards set by international food organizations (Badan Pangan Nasional, 2025). One of the main factors contributing to this low consumption is the high prevalence of lactose malabsorption in the population.

Lactose malabsorption is caused by reduced activity of the lactase enzyme in the small intestine, which functions to break down lactose into glucose and galactose. As a result, lactose is not properly digested and instead fermented by the gut microbiota in the large intestine, producing gases and other compounds that can cause gastrointestinal symptoms such as diarrhea, bloating, and abdominal pain (Borralho & Marcos, 2025; Hammer *et al*., 2022). These symptoms are known as lactose intolerance, which often leads individuals to avoid milk consumption and may increase the risk of nutritional deficiencies (Putri et al., 2024).

One potential solution to this problem is the utilization of β-galactosidase, either in the form of supplements or through the consumption of low-lactose dairy products. This enzyme functions to hydrolyze lactose into glucose and galactose, making it easier to digest and absorb (Khusniati et al., 2015). β-galactosidase can be obtained from various sources; however, microorganisms are considered the most favorable due to their rapid growth,

production efficiency and ease of cultivation and modification. Therefore, microbial sources are more widely used for enzyme production compared to plant or animal sources (Raveendran et al., 2018).

β-galactosidase activity naturally occurs in fermented dairy products such as yogurt. During fermentation, microorganisms play an important role in lactose degradation and the production of bioactive compounds that are beneficial to health (Yerlikaya, 2023). Several lactic acid bacteria commonly found in yogurt and known to produce β-galactosidase include *Enterococcus lactis*, *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, and *Lactiplantibacillus pentosus* (Hassan et al., 2024; Vasudha et al., 2023). Besides bacteria, some fungi have also been reported to produce β-galactosidase. For instance, a study by Idris et al., (2024) showed that *Aspergillus niger* and *Geotrichum candidum* isolated from fermented sheep, goat and cow milk exhibited enzyme activities of 19.60 U/mL and 18.55 U/mL, respectively.

The characteristics and activity of microorganisms in fermented products are strongly influenced by the type of raw material used (Zhang et al., 2023). Sheep milk has higher nutrient content, especially protein, fat and lactose, than cow and goat milk. Its high lactose content makes it a suitable substrate to support microbial growth and enhance β-galactosidase production (Pandya et al., 2020). Considering its potential applications across various industries, particularly in the production of low-lactose dairy products, the unique fermentation environment of sheep milk is expected to support the growth of bacteria with enhanced β-galactosidase-producing capabilities. Although β-galactosidase-producing bacteria have been widely isolated from various fermented dairy products, information regarding the β-galactosidase activity of bacteria isolated from sheep milk yogurt remains limited. Therefore, this study aimed to isolate and phenotypically characterize bacteria from sheep milk yogurt and to compare their β-galactosidase activities based on their lactose-hydrolyzing ability. The findings of this study are expected to provide preliminary information on bacterial candidates with potential for the development of low-lactose dairy products.

METHODS

Isolation of Bacteria from Sheep Milk Yogurt

In this study, the primary sample consisted of 100 mL of sheep milk obtained from a local farmer in Banda Aceh. The sample was fermented into yogurt by adding 80 mL of a commercial starter culture (Biokul plain), containing *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium*. The fermented product was used as a source of bacterial isolates, which were selected based on colony characteristics for further identification and testing. Fermentation was carried out for 24 hours at 28°C (Sujono et al., 2019). Bacterial isolation was performed by serial dilution of the yogurt sample up to 10^{-3}. One milliliter of the diluted sample was poured onto Nutrient Agar *(HiMedia)* plates and incubated for 24 hours at 37°C. Bacterial purification was conducted by selecting three or more colonies and streaking the selected colonies onto fresh NA plates, followed by incubation at 37°C for 24 hours. After incubation, pure colonies were observed microscopically using Gram staining to determine Gram characteristics and bacterial morphology (Hayati et al., 2019).

Characterization of Bacterial Isolates

Bacterial characterization was carried out through both macroscopic and microscopic approaches, including morphological identification and Gram staining, as well as biochemical tests such as endospore staining, catalase test, fermentation type test, indole test, TSIA test

and mannitol utilization test. Endospore staining was performed using malachite green and safranin to detect the presence of spores. The catalase test involved the addition of 3% H_2O_2 to assess catalase enzyme production, indicated by bubble formation. The fermentation type test used MRSB (de Man, Rogosa and Sharpe Broth) medium with Durham tubes and was evaluated based on gas production during incubation. The indole test was conducted in SIM medium with Kovacs reagent to detect indole production. TSIA testing was carried out on Triple Sugar Iron Agar to evaluate sugar fermentation through changes in medium color after incubation. The mannitol test determined the isolates' ability to utilize mannitol as a carbon source. Observations were made based on changes in the media following incubation (Cappuccino & Welsh, 2018).

Isolation of β-Galactosidase Enzyme

Selected isolates for screening β-galactosidase-producing bacteria were inoculated into a production medium consisting of Nutrient Broth (*HiMedia*) supplemented with 2% lactose and incubated at 30°C, for 24 hours (Sitepu et al., 2020). The addition of lactose served to induce enzyme production and facilitate the selection of active β-galactosidase-producing strains (Vasudha et al., 2023). Each bacterial isolate was independently inoculated into five Nutrient Broth tubes to obtain five biological replicates, and each culture was subsequently used for the β-galactosidase activity assay. After incubation, the culture was centrifuged for cell permeabilization following a modified method by Bosso et al., (2020) The culture was centrifuged at 2.000 × g for 10 minutes at 4°C to separate the cells from the medium. The resulting cell pellet was washed once using disodium phosphate buffer. The pellet was resuspended in buffer, vortexed, and treated with 1 mL of 40% ethanol, then incubated at room temperature for 60 minutes. After that, the sample was centrifuged at 10,000 × g for 10 minutes at 4°C. The supernatant was removed, and the pellet was resuspended in 1 mL of phosphate buffer.

Lactose Hydrolysis Assay

This assay was performed to evaluate the capacity of β-galactosidase to hydrolyze lactose. The analysis was carried out indirectly by measuring the glucose concentration produced during the enzymatic reaction process. A total of 1 mL of permeabilized cell suspension was mixed with 1 mL of 5% lactose solution in buffer and incubated at 37 °C for 60 minutes. Glucose concentration was determined using the glucose oxidase–phenol aminophenazone (GOD-PAP) method (Glory Diagnostics) with blank, standard, and sample tubes. The blank tube contained only the GOD-PAP reagent and served as the reagent blank. The standard tube contained the GOD-PAP reagent and a glucose standard solution, whereas the sample tube contained the GOD-PAP reagent and the lactose hydrolysis reaction mixture. The reagent-to-standard and reagent-to-sample ratio was maintained at 100:1 according to the manufacturer's instructions. The mixtures were then incubated at room temperature for 10 minutes. The absorbance of the standard and sample was measured at 500 nm. The glucose standard solution used had a concentration of 100 mg/dL and was supplied as part of the GOD-PAP reagent kit (Glory Diagnostics).

Glucose concentration was calculated by comparing the absorbance value of the sample with that of the glucose standard solution, resulting in glucose concentration expressed in mg/mL. The obtained value was then converted into millimolar (mM). One unit of enzyme activity was defined as the quantity of enzyme required to generate 1 µmol of glucose per minute (Amin et al., 2023). The absorbance values obtained reflected the amount of glucose produced and could therefore be used to determine the reduction in lactose concentration.

Glucose concentration was calculated using the following formula:

$$\frac{\text{A sample}}{\text{A standard}} \times 100 = \text{mg/dl glucose}$$

Description:
A sample = absorbance of the sample
A standard = absorbance of the glucose standard

Enzyme activity was calculated as (Aslam et al., 2023):

$$Enzyme\ activity = \frac{\mu\text{mol glucose produced}}{V \times t}$$

Description:
V = Sample volume
t = incubation time

Lactose hydrolysis percentage was calculated using the following equation (Rosolen et al., 2015):

$$E_h(\%) = \frac{C_{\text{glu}} \times MM_{lac}}{Ci_{lac} \times MM_{glu}} \times 100$$

Description:
$E_h(\%)$ = Lactose hydrolysis percentage (%)
C_{glu} = Glucose concentration produced from lactose hydrolysis (g/L)
MM_{lac} = Molar mass of lactose (342 g/mol)
$C_{i\,lac}$ = Initial lactose concentration before hydrolysis (g/L)
MM_{glu} = Molar mass of glucose (180 g/mol)

Data Analysis

Data analysis was performed descriptively for bacterial characterization by comparing the characteristics of each isolate using the profile matching method based on Cappuccino and Welsh (2018). The β-galactosidase activity and lactose hydrolysis data were analyzed statistically. Data normality was assessed using the Shapiro–Wilk test. If the data were normally distributed, differences among isolates were analyzed using ANOVA (Analysis of variance. If the data were not normally distributed, the Kruskal–Wallis test was applied. The level of statistical significance was set at $\alpha = 0.05$. All statistical analyses were performed using IBM SPSS Statistics Version 25.

RESULTS AND DISCUSSION

Characterization of Bacteria from Sheep Milk Yogurt

Based on the results of this study, bacterial isolation from sheep milk yogurt produced four isolates coded as BK1, BK2, BK3, and BK4. Macroscopically, all isolates exhibited colony morphologies ranging from circular to irregular shapes, with entire to undulate margins, flat elevation, and cream to creamy white coloration, as shown in **Table 1**. The colony morphology of each isolate can be observed in **Figure 1**.

Table 1. Morphological Characterization of Bacteria from Sheep Milk Yogurt

No	Isolate	Macroscopic			
		Shape	Margin	Elevation	Color
1	BK1	Circular	Entire	Flat	Cream
2	BK2	Irregular	Undulate	Flat	Creamy white
3	BK3	Irregular	Undulate	Flat	Creamy white
4	BK4	Circular	Entire	Flat	Creamy white

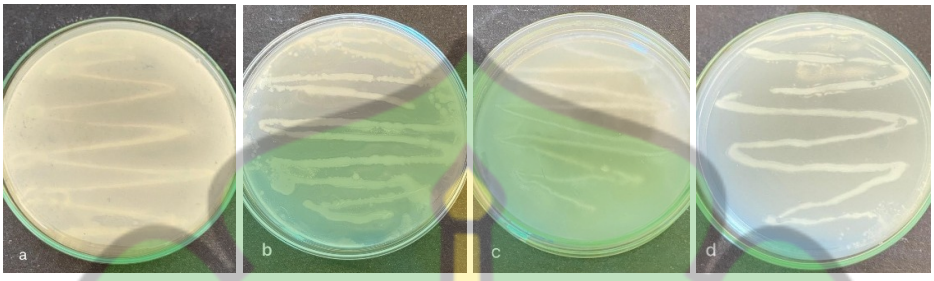

Figure 1. Colony Morphology of Bacteria from Sheep Milk Yogurt: (a) isolate BK1, (b) isolate BK2, (c) isolate BK3, (d) isolate BK4

Microscopic observation through Gram staining (Table 2 and Figure 2) showed that all four bacterial isolates were rod-shaped and purple in color. The purple coloration indicated that the bacteria were classified as Gram-positive. This characteristic is associated with the ability to retain the crystal violet stain due to the thick peptidoglycan layer in the bacterial cell wall. These results align with previous studies that have reported the predominance of Gram-positive bacteria in fermented dairy products (Taye et al., 2021).

Table 2. Microscopic Characteristics of Bacterial Cells from Sheep Milk Yogurt

No	Isolate	Microscopic		
		Shape	Colour	Gram reaction
1	BK1	Rod-shaped	Purple	+
2	BK2	Rod-shaped	Purple	+
3	BK3	Rod-shaped	Purple	+
4	BK4	Rod-shaped	Purple	+

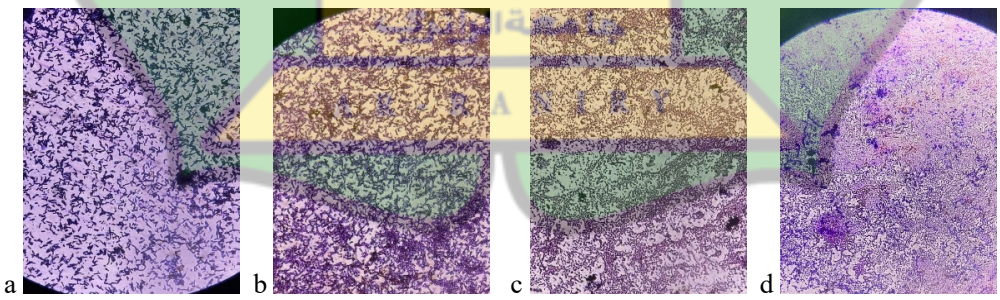

Figure 2. Gram Staining Results at 40× Magnification: (a) isolate BK1, (b) isolate BK2, (c) isolate BK3, (d) isolate BK4

Identification was continued with biochemical tests, which showed that all isolates were negative for endospore, catalase and indole tests (Table 3). The TSIA test results indicated that all isolates were able to ferment glucose, and isolate BK4 produced gas, as indicated by cracking of the medium. A positive result for glucose fermentation was marked by a color change in the medium, where the slant turned yellow and the butt remained red, indicating the

ability to utilize citrate as a carbon source. In the fermentation type test, all isolates were classified as homofermentative (HM), indicated by the absence of carbon dioxide gas production and showing that fermentation mainly produced lactic acid as the primary product (Medaando et al., 2024). In addition, all isolates showed negative results in the mannitol fermentation test, indicating that the isolates were unable to ferment mannitol.

Table 3 Biochemical Characteristics of Bacterial Isolates from Sheep Milk Yogurt

No	Characteristic	Isolates			
		BK1	**BK2**	**BK3**	**BK4**
1	Endospore	-	-	-	-
2	Catalase	-	-	-	-
3	Indole	-	-	-	-
4	Triple Sugar Iron Agar	K/A	K/A	K/A	K/A gas
5	Fermentation type	HM	HM	HM	HM
6	Mannitol	-	-	-	-
	Bacterial type	*Lactobacillus sp* BK1	*Lactobacillus sp* BK2	*Lactobacillus sp* BK3	*Lactobacillus sp* BK4

Note: K = alkaline (red), A = acid (yellow), HM = homofermentative

Based on the results of morphological characterization, Gram staining and biochemical tests referring to Cappuccino & Welsh (2018), the four isolates are suspected to belong to the lactic acid bacteria group of the genus *Lactobacillus*, characterized by Gram-positive, rod-shaped morphology, catalase-negative and mannitol-negative properties. This is further supported by the observed characteristics, namely rod-shaped cells, Gram-positive reaction, non-spore-forming ability and catalase-negative results, which are common features of lactic acid bacteria in fermented dairy products (Dewi et al., 2022; Hapsari & Mustakim, 2025).

Lactose hydrolysis percentage and enzyme activity assay of bacteria from sheep milk yoghurt

β-galactosidase is a hydrolase enzyme that catalyzes the hydrolysis of lactose into glucose and galactose by cleaving the β-glycosidic bond. This enzyme is widely produced by microorganisms, including bacteria and is generally inducible, meaning its production increases in the presence of lactose in the medium (Király et al., 2025). β-galactosidase activity can be determined through its ability to hydrolyze lactose, where the amount of glucose produced serves as an indicator of the degree of hydrolysis. In this study, glucose concentration was determined using the GOD-PAP method, which is specific for β-D-glucose. Enzyme activity is expressed in units (U), defined as the amount of enzyme that produces 1 µmol of glucose per minute (Eberhardt et al., 2021).

β-galactosidase in bacteria is generally intracellular, therefore, an extraction or permeabilization process is required to allow the substrate to access the enzyme. In this study, an ethanol permeabilization method was used to increase membrane permeability without significantly damaging the enzyme structure (Cheng et al., 2026). Ethanol was selected because it is relatively safe, readily available and has a lower environmental impact than other organic solvents. An ethanol concentration of 40% was chosen because it represents one of the optimum conditions identified through optimization using Response Surface Methodology (RSM), where the concentration range of 30–40% resulted in the highest percentage of lactose hydrolysis (Bosso et al., 2020).

The results showed that isolate BK1 produced the highest hydrolysis percentage of 1.417% with an enzyme activity of 0.034 U/mL, followed by BK4 with 1.205% and 0.029 U/mL, BK3 with 1.123% and 0.027 U/mL and BK2 with 0.398% and 0.010 U/mL (**Table 4**).

Table 4. Results of β-Galactosidase Enzyme Activity of Bacteria from Sheep Milk Yogurt and Lactose Hydrolysis Percentage

Bacterial Isolates	Percentage of Hydrolysis (%)	Enzyme Activity (U/ml)
BK1	1.417 ± 0.502	0.034 ± 0.012
BK2	0.398 ± 0.076	0.010 ± 0.002
BK3	1.123 ± 0.823	0.027 ± 0.020
BK4	1.205 ± 0.796	0.029 ± 0.019

Before comparing the isolates, the β-galactosidase activity and lactose hydrolysis percentage data were tested for normality using the Shapiro–Wilk test. All data were normally distributed and exhibited homogeneous variances ($p > 0.05$). Therefore, differences among the isolates were analyzed using one-way analysis of variance (One-Way ANOVA). No significant differences were observed in either lactose hydrolysis percentage or β-galactosidase activity among the four isolates (BK1, BK2, BK3 and BK4).

Statistical analysis showed no significant differences in β-galactosidase activity among the isolates tested. The percentage of lactose hydrolysis observed in all isolates was relatively low, indicating that the β-galactosidase activity obtained in this study had likely not yet reached its optimum level. Nevertheless, all isolates were able to hydrolyze lactose. This finding suggests that the isolates have the potential to be further developed as sources of β-galactosidase for the production of low-lactose dairy products. However, before practical application on a larger scale, improvements in enzyme activity through optimization of the production process and further evaluation are still required.

The low lactose hydrolysis and β-galactosidase activity observed in this study were likely associated with suboptimal growth conditions, including pH, temperature, medium composition, substrate concentration and incubation time (Zhang, 2025). In addition, the enzyme preparation method also influenced the measured enzyme activity. Previous studies have reported higher β-galactosidase activities using the ONPG assay (0.292 U/mL) or organic solvent extraction methods. For example, extraction using toluene, a toluene–acetone mixture and isoamyl alcohol yielded β-galactosidase activities of 0.3821 U/mL, 0.367 U/mL, and 0.03506 U/mL, respectively (Sari, 2024; Yuniarti et al., 2022). The low lactose hydrolysis and β-galactosidase activity obtained in this study may also have been influenced by the cell permeabilization method using 40% ethanol. Unlike extraction methods, which involve complete cell lysis and the release of intracellular enzymes into the solution, permeabilization only increases membrane permeability without completely disrupting the cell structure. Consequently, a portion of the intracellular β-galactosidase may not have been fully accessible to the substrate, resulting in lower measured enzyme activity (Kwon et al., 2021). (Gobinath & Prapulla, 2015) reported optimum permeabilization conditions for *Lactobacillus plantarum* using a mixture of 10% ethanol and 30% *n*-butanol for 10 min at 28°C. These findings indicate that the optimum permeabilization conditions depend on the microbial species; therefore, the parameters applied in the present study may not have been optimal for maximizing β-galactosidase activity.

Another factor that may have affected the enzyme activity measured in this study was the analytical method and the enzymatic reaction conditions employed, particularly the hydrolysis time and enzyme volume, which determine the amount of glucose produced. In this

study, lactose hydrolysis was performed at 37°C for 60 min using 1 mL of permeabilized cell suspension and β-galactosidase activity was determined indirectly by measuring the glucose released using the glucose oxidase–phenol aminophenazone (GOD-PAP) method. Aslam et al., (2023) reported that limited incubation time and suboptimal enzyme volume may result in incomplete lactose hydrolysis. Furthermore, because β-galactosidase activity was determined indirectly through glucose quantification using the GOD-PAP method, the measured activity was influenced by the hydrolysis reaction conditions. Therefore, further optimization of the permeabilization process, enzymatic reaction conditions, and the selection of an appropriate analytical method is required to obtain higher β-galactosidase activity. Such optimization should be tailored to the characteristics of the microorganism, as each microorganism possesses distinct optimum conditions for β-galactosidase production.

CONCLUSION

The bacteria isolated from sheep milk yoghurt showed characteristics of lactic acid bacteria, namely rod-shaped morphology, Gram-positive reaction, non-spore-forming ability and biochemical test results that were generally negative for catalase and indole, as well as homofermentative properties, suggesting affiliation with the genus *Lactobacillus*. The isolates' ability to hydrolyze lactose in this study was relatively low, with the highest enzyme activity observed in isolate BK1 at 0.034 U/mL and lactose hydrolysis of 1.417%. One-Way ANOVA showed no significant differences in either β-galactosidase activity or lactose hydrolysis percentage among the isolates ($p > 0.05$), suggesting that the four isolates have comparable potential for β-galactosidase production.

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