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Short Communication: Morphological, biochemical, and molecular identification of cellulolytic bacteria isolated from feces of endemic tropical herbivores

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Abstract. *Suharti S, Novrariani N, Wiryawan KG. 2023. Short Communication: Morphological, biochemical, and molecular identification of cellulolytic bacteria isolated from feces of endemic tropical herbivores. Biodiversitas 24: 4046-4051.* Indonesia has endemic herbivores that consume lignocellulose feedstuffs including grass, tree foliage, rice straw, and legume, indicating the presence of cellulolytic bacteria in their gastrointestinal tracts. Therefore, the aim of this study was to isolate and identify cellulolytic bacteria from the feces of tropical endemic herbivores, including anoa (*Bubalus depressicornis*), banteng (*Bos javanicus*), muntjak (*Muntiacus muntjak*), and Timor deer (*Rusa timorensis*). Bacteria were isolated using the serial dilution technique and screened on Carboxy Methyl Cellulose (CMC) media. The selected isolates were identified based on their morphological and biochemical characteristics, cellulase enzyme activity, and molecular identification of 16S rDNA. The result showed that a total of five bacterial isolates were isolated from feces of anoa, banteng, muntjak, and Timor deer. In addition, isolates exhibited characteristics of facultative anaerobes with gram-positive coccus, fermenting glucose, fructose, sucrose, starch, and cellulose. Based on cellulolytic index, isolates from anoa and banteng feces showed high cellulolytic activity with an index of about 1.2, indicating their potential as cellulose-degrading bacteria. Molecular identification and phylogeny analysis of cellulolytic bacteria isolates from anoa and banteng feces showed 100% similarities with *Enterococcus faecium*. Therefore, bacteria from feces of tropical endemic herbivores, especially anoa and banteng, possess cellulolytic activity and have potential as cellulolytic probiotic for ruminants that feed on forage-based diet. This is the first study to document the cellulolytic activity of anoa, and banteng feces.

Keywords: Cellulolytic bacteria, endemic herbivores, *Enterococcus* faecium, fecal microbes, natural resource

INTRODUCTION

The quality feed is essential for increasing ruminant livestock production and the main ingredient is forage, which contains high crude fiber, especially during the dry season. This high fiber content reduces nutrient digestibility, lowers fermentation by rumen microbes, and decreases the weight gain of ruminants. Mirzaeti-Alamouti et al. (2021) suggested that lamb rearing relies on pasture, providing insufficient live weight gain due to the lack of nutrients from natural grassland, particularly when the pasture quality is poor. Additionally, fiber digestibility is an important factor in regulating DMI in ruminants (Sousa et al. 2014). The foreguts of ruminants contain microbes, including bacteria, ciliate protozoa, anaerobic fungus, bacteriophages, viruses, and methanogens, enabling the digestion of various food items from plants, animals, and chemicals. The degradation of foods by rumen microorganisms is due to their predilection for certain feed forms and substrates (Faniyi et al. 2019), and cellulolytic bacteria are important in fiber degradation and fermentation in the rumen. Bacteria, mainly from the Fibrobacter and Ruminococcus genera, have been studied due to their vital role in the metabolic chain and their beneficial effects on host nutrition and gut health associated with the SCFA generation. However, a small number of bacteria have been isolated and characterized from mammalian large intestines due to technical challenges associated with their unique growth requirements in strictly anaerobic conditions (Froidurot and Julliand 2022)

Feed degradation and digestibility are increased by inoculating cellulolytic bacteria as probiotics. These probiotics include non-ruminant bacteria that adapt to ruminal circumstances and enhance the fermentation process. Furthermore, probiotics are microorganisms or components of microorganisms with beneficial effects on the host by controlling the intestinal flora to improve animal health (Castillo-González et al. 2014). The use of cellulolytic probiotics in ruminants improves performance, feed efficiency, microbial ecosystems, and the immune system. In addition, cellulolytic bacteria degrade crude fiber because they produce cellulase enzymes that break down the β-1,4-glycosidic links simultaneously during glucose fragment release (Jayasekara and Ratnayake 2019).

Indonesia is tropical country with endemic herbivores fed with lignocellulose feedstuffs including grass, tree foliage, rice straw, and legume. The ability of these herbivores to utilize forage-based feed indicates the presence of cellulolytic bacteria in their gastrointestinal tract. However, there is limited information about cellulolytic bacteria in endemic herbivores. Tropical

endemic herbivores such as anoa (*Bubalus depressicornis*), banteng (*Bos Javanicus*), muntjak (*Muntiacus muntjak*), and Timor deer (Flores-Miyamoto et al. 2005), have a higher ability to degrade fiber based on the forage consumed. The fiber utilization of these animals is due to the role of microbes in their gut, especially cellulolytic bacteria.

Cellulolytic bacteria isolated from the gastrointestinal tract have a high potential as a probiotic, though there are some difficulties in obtaining them from the gut. An alternative solution is to isolate microbes in feces. Previous studies reported various microbes in feces that can be isolated. According to Chantarasiri (2014), cow feces have strains that can be isolated and are similar to *Bacillus methylotrophic* bacteria, used in cellulose saccharification and bioethanol production. Lan et al. (2021) stated that cellulose-degrading microorganisms from feces of Sika Deer were mainly distributed in Proteobacteria, Firmicutes, Actinobacteria, and Ascomycota. Based on the analysis of 16S rRNA encoding genes, Missa et al. (2016) reported that bacteria from cattle feces have 5 different genera found in 12 isolates with high cellulolytic activity including *Pseudomonas* sp., *Acinetobacter* sp., *Bacillus*, *Stenotrophomonas*, and *Brachybacteria* sp.

The isolation of cellulolytic bacteria is necessary to identify candidates that can degrade feed containing high crude fiber, for use as a probiotic in ruminants. This study aimed to isolate and identify cellulolytic bacteria from feces of endemic tropical herbivores, including anoa, banteng, muntjak, and Timor deer. The cellulolytic activity and biochemical properties of the isolated bacteria were also estimated.

MATERIALS AND METHODS

Fecal sample collection

The fecal samples of anoa (A2), banteng (B1), muntjak (K1), and Timor deer (R1, R2) was obtained from Taman Safari Zoo, Bogor, West Java, Indonesia, samples were collected per rectum of each animal. Furthermore, the isolation procedure was approved by the Faculty of Animal Science, Institut Pertanian Bogor, Indonesia. The fecal bacteria were isolated from the fresh feces of 4 herbivores under anaerobic conditions by diluting feces with 3% glycerol liquid media and stored them at 4°C until analyzed.

Isolation and screening of cellulolytic bacteria

About 1 gram of the fecal sample was diluted with 9 mL NaCl 0.85%, and then 1 mL of the solution was put into a tube containing 9 mL of CMC broth media and homogenized. The tubes were incubated at 37°C for 24 hours. Additionally, CMC broth media consist of 10 mL of 1% Carboxy Methyl Cellulose (CMC), 3.7 g Brain Heart Infusion (BHI), 0.5 mL haemin, 0.05 mL resazurin, 0.1 g cysteine, and 100 mL aquadest (Ogimoto and Imai 1981). After incubation, 0.05 mL of the fecal solution was transferred into a diluent medium of up to 10-10 dilutions, cultured in CMC agar media, and incubated at 39°C for 24 hours. Bacterial colonies that grew in tubes were transferred to a new media to obtain pure cultures and stored in 80% glycerol media with a ratio of 3:1 (bacteria cultures : glycerol). Furthermore, Congo red agar method was used to analyze the activity of cellulolytic bacteria (Makowski et al. 2021). Bacterial colonies in CMC agar media were exposed to 0.3% Congo red for 20 minutes, then plates were washed with 1 M NaCl solution. Colonies showing a discoloration of Congo-Red were positive cellulose-degrading bacteria. The single pure colony with a maximum clearing zone was isolated for further screening. Then bacterial colony was cultured into a liquid medium and incubated for 96 hours. The liquid medium was centrifuged at 7000 g, then supernatant was removed, and the cell pellet was extracted for molecular identification.

Morphological and biochemical analysis of bacterial isolates

Isolates were identified morphologically (Gram staining, color, size of colonies), while microscopic observations included shape and size of bacteria cells. For biochemical examination, bacteria isolate were tested for their ability to ferment reducing sugars, including glucose, fructose, sucrose, starch, and CMC (Makowski et al. 2021).

Cellulolytic activity assay

Cellulolytic activity of bacterial isolates was estimated based on the formation of a clear zone around the colony on CMC media. This activity was measured using the clear zone diameter after CMC plate was poured with 1% congo red. In addition, cellulolytic index was calculated using the formula: CI= (diameter of zone – diameter of bacteria colony)/diameter of bacteria colony (Balla et al. 2022).

Molecular identification

Based on cellulolytic index, molecular identification of the two highest cellulolytic bacteria isolates was conducted at the Genetica Science Indonesia laboratory (www.ptgenetika.com). The isolates were identified on molecular level based on the 16S rDNA sequence, using primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3 ') and 1492R primer (5'-GGTTACCTTGTTACGACTT-3'), and PCR products at ~1400 bp. Genomic DNA extraction was carried out with PrestoTM Mini gDNA Bacteria Kit (Geneaid, GBB300), while PCR amplification was conducted using (2x) My Taq HS Red Mix (Bioline, BIO-25048). Meanwhile, the PCR product was sequenced using Bi-directional Sequencing. The final sequence result was used to identify bacteria using BLAST-N from NCBI. Also, the NCBI Blast Tree Method was used to analyze the Phylogenetic Tree – Neighbor Joining (Unrooted Tree).

RESULTS AND DISCUSSION

Morphological and biochemical characteristics of fecal cellulolytic bacterial isolates

A total of 5 bacterial isolates were isolated from feces of anoa (A2 isolate), banteng (B1 isolate), muntjak (K1 isolate), and Timor deer (R1 and R2 isolates). One isolate

each was obtained from anoa, banteng and muntjak, while 2 isolates were isolated from Timor deer. Table 1 showed that isolates were facultative anaerobes with gram-positive coccus.

All isolates had similar morphology, including gram staining, colony shape, and color, indicating that bacteria may be the same species. Salunke et al. (2012) reported similar morphological characteristics in bacteria isolated from Deer feces including gram-positive bacteria, white color colony, and oval in shape.

Bacterial isolates showed positive reactions in biochemical assay of reducing sugars, including glucose, fructose, sucrose, starch, and CMC (Table 2).

The results of reducing sugar analysis showed that all bacterial isolates fermented glucose, fructose, sucrose, starch, and cellulose. Meanwhile, B1 isolate showed the strongest cellulose (CMC) fermentation ability. This may be due to the superior ability to digest low-quality forages and the presence of cellulase enzyme to degrade cellulose. Cellulases hydrolyze glycosidic linkages in carbohydrate molecules by cleaving 1,4-glycosidic bonds between the glucosyl moieties in cellulose into their monomers. The hydrolysis is carried out by 3 main cellulases, including endoglucanases (endo-1,4-D-glucan hydrolases), exoglucanases (exo-1,4-D-glucan cellobiohydrolases), and glucosidases (-D-glucosidases) (Hua et al. 2022).

Cellulolytic activity of fecal cellulolytic bacterial isolates

Cellulolytic activity test showed that bacteria isolate from anoa (A2) had the highest cellulolytic index of 1.2, while isolate from muntjak (K1) had the lowest, which was 0.2 (Table 3). The clearing zone of selected isolates (A2: anoa, B1: banteng, K1: muntjak, R1, R2: Timor deer) was shown in Figure 1.

The clear zone indicated the hydrolytic activity of extracellular cellulase secreted by bacterial isolates. Based on cellulolytic index, isolates from anoa and banteng exhibited potential as cellulose-degrading bacteria with a value greater than 1.2. The ability of bacterial isolates to degrade carbohydrate fractions in forages was determined by the capacity to break down cellulose. About 3 cellulases combine to break down cellulose at different locations. The exoglucanases target non-reducing ends of cellulose or cellotetraose created by endoglucanase, producing cellobiose and cellotriose as products. Furthermore, endoglucanase breaks down the amorphous portions of cellulose, creating new chain ends, then β-glucosidases hydrolyze the compounds into glucose (Hua et al. 2022)

Sari et al. (2017) isolated cellulolytic *Enterobacter* from the rumen of Aceh cattle with an activity of ± 2.5 cm (11). According to Herdian et al. (2018), cellulolytic activity of lactic acid bacteria isolated from the gastrointestinal tract of Mentok (*Anas moschata*) varied from 1.67-3.22 cm.

Molecular of selected cellulolytic isolates

Based on molecular identification, cellulolytic bacterial isolates from anoa feces showed 100% similarity with *Enterococcus faecium, Enterococcus hirae, and Enterococcus* sp. (Table 4). Meanwhile, isolate from banteng feces had 99.86% similarity with *Enterococcus faecium, Enterococcus lactis, Enterococcus* sp., and 99.93 % with Bacterial strain IMAU11903 and IMAU11802 (Table 5).

Based on the phylogeny tree analysis, bacterial isolate from anoa feces/A1 (Figure 2) and banteng feces/B1 (Figure 3) had the closest relationship with *Enterococcus faecium.*

Table 1. Morphological characteristics of cellulolytic bacterial isolates from anoa, banteng, muntjak, and Timor deer fecal

Morphological characteristics	Fecal cellulolytic bacteria				
	A2 (anoa)	B1 (banteng)	K1 (muntjak)	R1 (Timor deer)	R2 (Timor deer)
Gram staining	Positive	Positive	Positive	Positive	Positive
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus
Colony shape	Rounded	Rounded	Oval, nucleated	Rounded, nucleated	Rounded
Colony color	Milky white	Broken white	Broken white	Broken white	White

Table 2. Biochemical characteristics of cellulolytic bacterial isolates from anoa, banteng, muntjak, and Timor deer fecal

Reducing sugars capability	Fecal cellulolytic bacteria				
	A2 (anoa)	B1 (banteng)	K1 (muntjak)	R1 (Timor deer)	R2 (Timor deer)
Glucose	++	+	++	+++	++
Fructose	+	+	+	+	+
Sucrose	++	++	+	+++	++
Starch	++	++	+	+	+++
CMC	+	+++	++	++	+

Note: +: low amount of reducing sugar, ++: moderate amount of reducing sugar, +++: large amount of reducing sugar

Table 3. Cellulolytic activity of bacterial isolates from anoa, banteng, muntjak, and Timor deer fecal

Isolates	Diameter of cellulolytic zone (mm)	Diameter of bacteria colony (mm)	Cellulolytic index
A2 (anoa)	22	10	1.20
B1 (banteng)	18	8	1.25
K1 (muntjak)	9	7	0.29
R1 (Timor deer)	15	7	1.14
R2 (Timor deer)	15	8	0.88

Table 4. BLAST results against NCBI database of cellulolytic bacteria isolate from anoa feces

Accession numbers	Species	Maximum score	Total score	Query cover	E value	Maximum identity
CP089319.1	*Enterococcus faecium* strain NMVRE-001	2510	15053	100%	0.0	100.00%
OL762478.1	*Enterococcus faecium* strain R12	2510	1510	100%	0.0	100.00%
CP089092.1	*Enterococcus faecium* strain V13-21-E11-012-001	2510	15025	100%	0.0	100.00%
CP085906.1	*Enterococcus faecium* strain SC1762-D	2510	15036	100%	0.0	100.00%
OK559667.1	*Enterococcus hirae* strain FUAD17	2510	2510	100%	0.0	100.00%
OK326390.1	*Enterococcus sp.* Strain T2-L-102	2510	2510	100%	0.0	100.00%
OK272450.1	*Enterococcus sp.* Strain Y3-M-102	2510	2510	100%	0.0	100.00%
OK272431.1	*Enterococcus sp.* Strain Y3-F-2	2510	2510	100%	0.0	100.00%
OK272430.1	*Enterococcus sp.* Strain Y3-F-1	2510	2510	100%	0.0	100.00%
OK272373.1	*Enterococcus sp.* Strain Y2-M-5	2510	2510	100%	0.0	100.00%

Note: (https://www.ncbi.nlm.nih.gov/nuccore/CP089319.1,OL762478.1,CP089092.1,CP085906.1,OK559667.1,OK326390.1,OK272450.1,OK272431.1,OK272430.1,OK272373.1)

Table 5. BLAST results against NCBI database of cellulolytic bacteria isolate from banteng feces

Accession numbers	Species	Maximum score	Total Score	Query cover	E value	Maximum identity
MN250794.1	*Enterococcus faecium* strain TSGB1181	2627	2627	100%	0.0	99.86%
MN007103.1	*Enterococcus faecium* strain DX5a	2627	2627	100%	0.0	99.86%
CP082267.1	*Enterococcus lactis* strain E483	2627	15712	100%	0.0	99.86%
JX420820.1	*Enterococcus* sp strain 14	2627	2627	100%	0.0	99.86%
MN660229.1	*Enterococcus lactis* strain KH16	2625	2625	99%	0.0	99.93%
MN060988.1	*Enterococcus faecium* strain MG89-2	2625	2625	99%	0.0	99.93%
MF893783.1	Bacteria strain IMAU11903	2625	2625	99%	0.0	99.93%
MF893780.1	Bacteria strain IMAU11802	2625	2625	99%	0.0	99.93%
KY283153.1	*Enterococcus sp* strain ZJ-16	2625	2625	99%	0.0	99.93%
KY283151.1	*Enterococcus sp* strain ZJ-14	2625	2625	99%	0.0	99.93%

Note: (https://www.ncbi.nlm.nih.gov/nuccore/MN250794.1,MN007103.1,CP082267.1,JX420820.1,MN660229.1,MN060988.1,MF893783.1,MF893780.1,KY283153.1,KY283151.1)

Figure 1. Clearing zone of selected isolates (A2: anoa, B1: banteng, K1: muntjak, R1and R2: Timor deer)

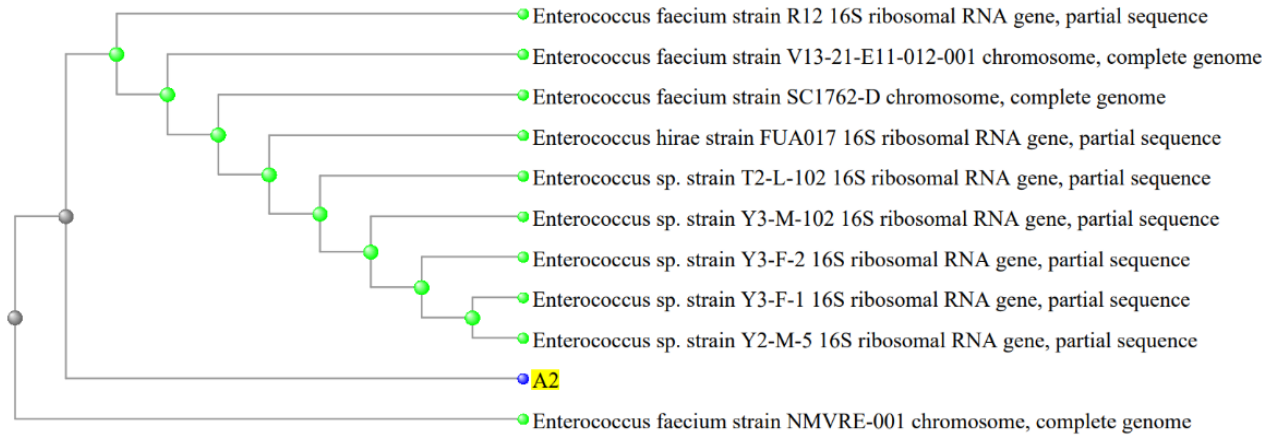

Figure 2. Phylogenetic tree of cellulolytic bacterial isolate from anoa feces (A1)

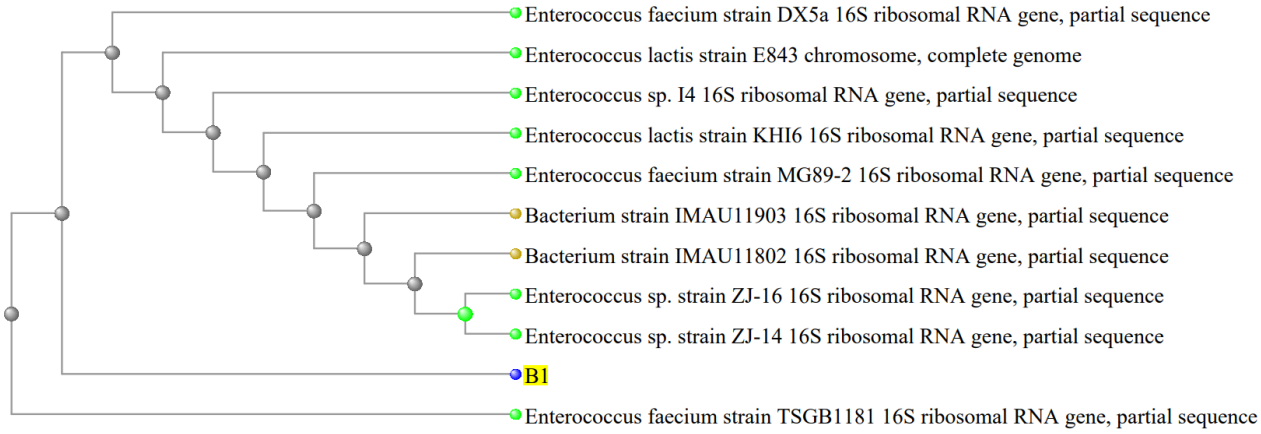

Figure 3. Phylogenetic tree of cellulolytic bacterial isolate from banteng feces (B1)

This study confirmed the presence of *Enterococcus faecium* in feces of anoa and banteng. Ramos et al. (2012) stated that *E. faecium* and *E. hirae* were the predominant enterococcal species isolated from cattle feces. Beukers et al. (2017) also identified *Enterococcus faecium* and *fecalis* from bovine feces. Furthermore, Zhao et al. (2021) reported that *E. faecium* and *E. fecalis* possess cellulolytic properties and enhanced cellulose degradation when added to the forage silage. The combined inoculation of these enterococci in different forages reduced the fiber content of resulting silages. Therefore, the results of present study indicated that *E. faecium* has cellulolytic properties.

Hanchi et al. (2018) reported that *Enterococcus* sp. isolated from bovine feces lacked virulence traits. However, *E. faecium* has shown potential as a probiotic candidate. The genus *Enterococcus* is a lactic acid bacteria (LAB) and has many important characteristics including multi-bacteriocin production. Additionally, bacteriocins from enterococci have antibacteria properties against Gram-positive and negative pathogenic bacteria (Lauková et al. 2017). Zommiti et al. (2022) stated that *E. faecium* strain 11181 was approved by The European Food Safety Authority (EFSA) Panel as a feed additive for fattening and enhancing the performance of various animals.

In conclusion, total 5 bacterial isolates were isolated from feces of anoa, banteng, muntjak, and Timor deer were facultative anaerobes with Gram-positive coccus, fermenting glucose, fructose, sucrose, starch, and cellulose. Based on cellulolytic index, isolates from anoa and banteng feces showed potential as cellulose-degrading bacteria. Molecular identification of cellulolytic bacteria isolates from anoa and banteng feces showed similarities with *Enterococcus faecium.*

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