Journal of Current Science and Technology, April - June 2025 Vol. 15 No. 2, Article 101

ISSN 2630-0656 (Online)

Cite this article: Boontanom, P., Phaparat, N., & Chantarasiri, A. (2025). Diversity of cellulolytic bacteria isolated from goat feces in rayong province, eastern thailand, and characterization of their endoglucanase activity. *Journal of Current Science and Technology*, *15*(2), Article 101. https://doi.org/10.59796/jcst.V15N2.2025.101

Journal of Current Science and Technology

Journal homepage: https://jcst.rsu.ac.th

Diversity of Cellulolytic Bacteria Isolated from Goat Feces in Rayong Province, Eastern Thailand, and Characterization of Their Endoglucanase Activity

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Received 13 November 2024; Revised 30 November 2024; Accepted 5 December 2024; Published online 25 March 2025

Abstract

Bacterial cellulases are crucial for breaking down cellulose, which is essential for various industries. These bacteria are found in the rumen of herbivores including domestic goats. Goat feces show potential as a source of cellulase-producing bacteria, but studies on these bacteria isolated from goat feces in Thailand remain limited. This study isolated and genetically identified cellulase-producing bacteria from goat feces in eastern Thailand. The cellulases produced by the most effective cellulase-producing bacterium were also characterized enzymatically. A total of 30 cellulase-producing bacteria were isolated and classified using PCR-RFLP analysis of the 16S rRNA gene. Thirteen different RFLP patterns were obtained through *Msp*I-*Alu*I digestion, belonging to nine bacterial genera: *Acinetobacter*, *Bacillus*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Exiguobacterium*, *Providencia*, *Pseudomonas*, and *Staphylococcus* (*Mammaliicoccus*). The predominant genera of the isolated cellulase-producing bacteria were *Escherichia*, *Exiguobacterium*, and *Corynebacterium*. Several of the isolated bacterial species had limited prior evidence of cellulase production. *Bacillus* sp. strain FMJ 1105 showed the highest cellulase activity using the CMC agar method and produced CMCase (endoglucanase) activity of 2.67 ± 0.06 U/mL. The optimum temperature and pH for CMCase activity were determined to be 50°C and pH 7.0, with a stability range of 25-70°C and pH 6.0-8.0 over 24 h of incubation. This study provides new insights into cellulase-producing bacteria isolated from goat feces in Thailand, contributing to the understanding of their enzymatic potential.

Keywords**:** *bacillus; cellulase-producing bacteria; CMCase; feces; goat*

1. Introduction

Cellulose is the major polysaccharide of plant cell walls and the most abundant component of plant biomass (Juturu, & Wu, 2014; Behera et al., 2017). This linear chain polymer comprises 1,4-glycosidic linkages between D-glucose (D-glucopyranose) subunits (Srivastava, & Mathur, 2023; Singh et al., 2024). Cellulose and its depolymerized glucose are valuable carbon sources for producing high-value chemicals such as biodegradable plastics, ethanol, sorbitol, ethylene glycol, and related chemical compounds (Yang et al., 2011, Juturu, & Wu, 2014; Yabushita et al., 2014; Wei, & Wu, 2017). Depolymerization of cellulose into glucose molecules is a key process for cellulosic biomass valorization (Jing et al., 2018; Zhou et al., 2021) by cellulase enzymes under mild conditions without generating toxic byproducts. Animals, plants, and various microorganisms are considered cellulase sources and producers (Siqueira et al., 2020; Dewiyanti et al., 2022).

Cellulases consist of three synergistic enzymes: endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Juturu, &

Wu, 2014; Narkthewan, & Makkapan, 2019; Siqueira et al., 2020; Boontanom, & Chantarasiri, 2021). They have several industrial applications, such as additives for the food industry, animal feed, decomposition of cellulosic residues for fertilizer production, juice extraction and clarification, the paper and pulp industry, paper recycling and de-inking, and the production of cellulosic bioethanol (Menendez et al., 2015; Siqueira et al., 2020; Ejaz et al., 2021; Dewiyanti et al., 2024). Cellulase is one of the most commercially utilized enzymes, with a market value of $1.68 billion in 2020 and a projected value of $2.45 billion in 2026 (Ranjan et al., 2023), accounting for 20% of the global enzyme market (Thakur et al., 2022). The high cost of enzyme production is the major impediment to using cellulases as biocatalysts in industrial applications (Sulyman et al., 2020) which is unsuitable for biofuel production (Singhania et al., 2021; Sutaoney et al., 2024).

Bacterial cellulases offer many advantages compared to cellulases from animals, plants, and fungi such as higher growth rates and genetic versatility (Menendez et al., 2015). The most commonly isolated cellulase-producing bacteria belong to the genera *Bacillus*, *Clostridium*, *Cellulomonas*, and *Pseudomonas* (Chantarasiri, 2020; Ejaz et al., 2021; Dewiyanti et al., 2022; Ikram et al., 2022). Only nine novel cellulolytic bacterial species have been discovered and reported in *the International Journal of Systematic and Evolutionary Microbiology* (Ejaz et al., 2021). Many studies have also attempted to isolate cellulase-producing bacteria from various environmental samples.

Cellulase-producing bacteria have been isolated from various sources, including animal guts and feces, compost, decayed plant material, organic matter, and soil (Photphisutthiphong, & Vatanyoopaisarn, 2019). Symbiotic microorganisms in the rumen of herbivores can hydrolyze and ferment cellulosic polymers (Song et al., 2017a). Ruminant herbivores have a unique organ called rumen, which is used in the digestion processes of cellulosic polymers through the action of enzymes produced by rumen cellulolytic microorganisms (Sultana et al., 2022). Therefore, the ruminal microbiome is an attractive source of symbiotic microbes and lignocellulolytic enzymes (Cheng et al., 2016; Thapa et al., 2023).

Goats are classified as small ruminants and grazing herbivores, and many cellulase-producing bacteria isolated from goat-related samples are strictly anaerobic. Previous studies isolated various anaerobic cellulase-producing bacteria from goat rumen and goat excreta using a cultivation-based method (Seo et al., 2013; Croos et al., 2019), while a metagenomic fosmid library constructed from the black goat rumen revealed a novel cellulase gene (Song et al., 2017b; Lee et al., 2018). However, reports on the isolation and genetic diversity of aerobic and facultative anaerobic cellulase-producing bacteria from goat-related samples, such as feces, are scarce.

Thailand has a population of over 380,000 domestic goats raised by smallholders (Pralomkarn et al., 2012). Therefore, to enhance our understanding of the species diversity and cellulolytic activity of cellulase-producing bacteria, this study isolated cellulase-producing bacteria from goat feces collected in Rayong Province, located in the eastern region of Thailand. The polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) were used alongside molecular analyses of the 16S rRNA genes to genetically identify the isolated cellulase-producing bacteria. The endoglucanase cellulase (CMCase) produced by the most effective cellulase-producing bacterium in this study was characterized enzymatically to determine its optimal conditions and stability across various temperatures and pH levels. This bacterium shows promise for biotechnological applications in related industrial fields.

2. Objectives

This study aims to isolate and genetically identify bacteria that produce cellulase from goat feces collected in Rayong Province, Thailand. It examines the diversity of these cellulase-producing bacteria. Additionally, the study focuses on identifying the most effective cellulase-producing strains for potential biotechnological applications. The most effective cellulase-producing bacterium, based on hydrolysis capacity, was enzymatically characterized to determine its endoglucanase cellulase (CMCase) activity under various conditions.

3. Materials and Methods

3.1 Collection of Goat Feces

Thirty samples of fresh and moist feces were collected from crossbred meat goats (Thai native × Anglo Nubian) in eight districts of Rayong Province, Thailand. The sample distribution was as follows: Mueang Rayong District (n = 10), Ban Chang District (n =2), Klaeng District (n = 1), Wang Chan District (n = 1), Ban Khai District (n = 3), Pluak Daeng District (n = 2), Khao Chamao District (n = 1), and Nikhom Phatthana District (n = 10). The samples were

gathered from droppings deposited on the ground by the goats on local farms, ensuring that the sampling process did not disturb any of the animals. Each fecal sample was stored in a sterilized plastic container at 4°C. The samples were processed for the isolation of cellulase-producing bacteria in the biosafety laboratory within 48 h.

3.2 Isolation and Cellulolytic Screening of Cellulase-Producing Bacteria from Goat Feces Samples

The feces samples were ground by a sterilized ceramic mortar and then diluted in sterilized water to obtain 1:100,000 dilutions. The feces dilutions were spread-plated on tryptone soya agar (TSA) (HiMedia, India) and incubated in a KB720 incubator cabinet (Binder, Germany) at 38.4°C for 24 h. The incubation temperature in this study was the mean rectal temperature of domestic goats (Arrigo et al., 2024). The bacterial colonies that grew on the TSA plates were selected based on morphological colony (pigmentation, shape, elevation, and margin of colony) and subsequently purified by the streak plate method on TSA. Each pure isolate was coded FMJ followed by the isolation number.

The cellulase-producing bacteria were screened by carboxymethylcellulose (CMC) agar and subsequently stained with iodine solution after incubation following the methodology described by Chantarasiri (2015). The isolates were enriched in 3 mL of soyabean casein digest medium (tryptone soya broth, TSB) (HiMedia, India) at 38.4°C for 24 h. One drop (5 µL) of each enriched broth was dripped on the center of the CMC agar. All agar plates were then incubated at 38.4°C for 48 h. After incubation, the plates were flooded with iodine solution for 10 min. The cellulase-producing bacteria were identified by the cellulolytic zone, that appeared around their colonies after iodine staining. The hydrolysis capacity (HC) of these bacteria was calculated by taking the ratio of the diameter of the cellulolytic zone to the diameter of the bacterial colony (Chantarasiri, 2020). All the experiments were conducted in triplicate and the HC values were averaged with standard deviations (SD).

3.3 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) Analysis of the 16S rRNA Genes

Each cellulase-producing bacterium was extracted to obtain purified genomic DNA using a commercial genomic DNA isolation kit (Bio-Helix, Taiwan). This DNA was then used as a template to amplify the 16S rRNA genes by the polymerase chain reaction (PCR) method. The PCR was conducted with a ready-to-use OnePCR reaction mixture (Bio-Helix, Taiwan), employing a universal forward primer (27F: 5′-AGAGTTTGATCMTGGCTCAG-3′) and a universal reverse primer (1492R: 5′-TACGGYTACCTTGTTA CGACTT-3′) for 35 amplification cycles. The amplification process followed the protocol described by Chantarasiri (2020) and was carried out in a Mastercycler Nexus Gradient thermal cycler (Eppendorf, Germany). The resulting 1,500 bp PCR products of the 16S rRNA genes were verified using a 1.5% (w/v) OmniPur agarose gel (Calbiochem, Germany).

The analysis of restriction fragment length polymorphism (RFLP) for the PCR products was conducted using the restriction enzymes *Msp*I and *Alu*I (New England Biolabs, UK). The enzymes were incubated in a rCutSmart Buffer (New England Biolabs, UK), following the methodology outlined by Chantarasiri (2020). The PCR products were digested with the restriction enzymes at 37°C for 12 h, after which the digestion reaction was terminated by heating the reaction mixtures to 80°C for 15 min, in accordance with the NEBcloner software version 1.14.0 protocol provided by New England Biolabs. The resulting restriction fragments were analyzed using a 3% (w/v) OmniPur agarose gel and visualized with the fluorescent reagent Novel Juice (Bio-Helix, Taiwan) on a UV transilluminator (Wealtec, Taiwan).

3.4 Identification of Cellulase-Producing Bacteria by Nucleotide Sequencing and Phylogenetic Analysis of the 16S rRNA Genes

The amplified 16S rRNA genes of each cellulase-producing bacterium were purified and sequenced using the services of Macrogen Inc. (Korea). The 1,300-1,500 bp nucleotide sequences of the 16S rRNA genes were aligned using the BLASTn suite based on the standard database and a megablast algorithm (highly similar sequences algorithm) from the National Center for Biotechnology Information (NCBI). The phylogenetic tree of all cellulase-producing bacteria was analyzed and generated by SeaView software version 4.7 (Laboratoire de Biométrie et Biologie Evolutive, Université de Lyon, France) with the neighbor-joining (NJ) method for 100,000 bootstrap replications. The generated tree was graphical editing by Figtree software version 1.4.4 (Institute of Evolutionary Biology, University of Edinburgh, UK). All nucleotide sequences of the 16S rRNA genes were deposited in the GenBank database

of NCBI under the following accession numbers: PQ141697, PQ141699, PQ141700, PQ141702, PQ141703, PQ141704, PQ141989, PQ141990, PQ142313, PQ142314, PQ142315, PQ142329, and PQ142330.

3.5 Activity Assays of the Partially Purified Cellulases from the Most Effective Cellulase-Producing Bacterium

The most effective cellulase-producing bacterium based on the HC value was *Bacillus* sp. strain FMJ 1105. This bacterium was cultured to prepare cellulases, following the methodology described by Chantarasiri (2020), in a CMC liquid medium under an aeration condition of a DURAN baffled flask with GL 45 thread (Schott-Duran, Germany) and shaking condition of a LSI-3016R incubator (Daihan Labtech, Korea) at 150 rpm at 38.4°C for 48 h. Crude cellulases were harvested from the CMC liquid medium as the cell-free supernatant using a Digicen 21R centrifuge (Ortoalresa, Spain) at 4,000 rpm for 30 min. The crude cellulase solution was partially purified and concentrated by Amicon ultra centrifugal devices with 10 kDa cut offs (Millipore, Ireland) and stored at 4°C.

The activity assay was determined based on the endoglucanase activity (CMCase activity). Endoglucanase is the most promising cellulase as it initiates the hydrolysis mechanism of the cellulose molecule and is used in various industrial applications (Mandeep et al., 2021). The CMCase assay was performed as previously described by Chantarasiri (2020) and Boontanom, & Chantarasiri (2021). This involved incubating 0.5 mL of partially purified cellulases with 0.5 mL of 2% (w/v) CMC in a 50 mM sodium phosphate buffer at pH 7.0. The incubation took place at 50°C for 30 min. The reducing sugars generated from the hydrolysis reaction of CMC were measured using the 3,5-dinitrosalicylic acid (DNS) method at a wavelength of 540 nm (Miller, 1959). The activity of CMCase was calculated based on a glucose standard curve. One unit (U) of CMCase is defined as the amount of endoglucanase cellulase needed to release 1 µmol of reducing sugars, expressed as glucose equivalents, per minute under the specified experimental conditions. All activity assays were conducted in triplicate.

3.6 Enzymatic Characterization of the Partially Purified Cellulases from the Most Effective Cellulase-Producing Bacterium

The partially purified cellulases from *Bacillus* sp. strain FMJ 1105 were evaluated for the temperature and pH conditions affecting CMCase activity. The assay for CMCase activity was conducted as described in the previous experiment. The optimum temperature and thermal stability of the partially purified cellulases were characterized following the methodology of Chantarasiri (2020). The optimum temperature range was determined as 25°C to 80°C in 50 mM sodium phosphate buffer (pH 7.0). Thermal stability was determined as the relative activity of CMCase after the partially purified cellulases were pre-incubated at varied temperature conditions for 24 h. The optimum pH and pH stability of the partially purified cellulases were characterized according to the methodology of Boontanom, & Chantarasiri (2021). The CMCase activity was assessed for optimal pH using various buffers at 50°C. The assay utilized the following buffers: a 50 mM citrate buffer (pH 4.0-6.0), a 50 mM sodium phosphate buffer (pH 6.0-8.0), a 50 mM Tris-HCl buffer (pH 8.0-9.0), and a 50 mM glycine-NaOH buffer (pH 9.0-10.0). To measure pH stability, partially purified cellulases were pre-incubated at 50°C for 24 h in the buffers described above, after which the relative activity of CMCase was evaluated. All activity assays were conducted in triplicate.

3.7 Data analysis

Data analysis included descriptive statistics and one-way ANOVA followed by Tukey's test with a 95% confidence level. All statistical analyses were conducted using R software version 4.4.1 (R Core Team, Vienna, Austria).

4. Results

4.1 Isolation and Cellulolytic Screening of Cellulase-Producing Bacteria from Goat Feces Samples

Thirty samples of goat feces were collected from the study areas in Rayong Province, Thailand. One hundred and seven bacterial isolates were isolated, and the colonies were purified. Most isolates were white and circular with an entire margin and convex elevation. The diameters of the isolated colonies ranged from 0.33 to 7.30 mm on TSA medium after 24 h of incubation. After screening on CMC agar, the 30 isolates were identified as cellulase-producing bacteria, with HC values ranging from 1.02 ± 0.01 (isolate FMJ 1502) to 3.78 ± 0.32 (isolate FMJ 1105), as shown in Table 1. Isolate FMJ 1105 was the most effective cellulase-producing bacterium in this study, as demonstrated by the cellulolytic zone on CMC agar after iodine staining, shown in Figure 1.

4.2 PCR-RFLP Analysis of the 16S rRNA Genes Amplified from the Cellulase-Producing Bacteria

The 16S rRNA genes of the isolated cellulase-producing bacteria were amplified using the PCR method, then digested with the *Msp*I-*Alu*I restriction enzymes, and electrophoresed on OmniPur agarose gel. The resulting RFLP profiles obtained from the 30 isolates are shown in Figure 2 with 13 different patterns observed. Pattern RFLP-F-01 was most commonly found in the cellulase-producing bacteria isolated from the goat feces samples, followed by patterns RFLP-F-03 and RFLP-F-06. All RFLP patterns were effective for categorizing the bacteria. Table 2 presents the various RFLP patterns and numbers of isolated cellulase-producing bacteria.

Table 1 Hydrolysis capacity (HC) values of thirty cellulase-producing bacteria isolated from goat feces samples in Rayong Province, Thailand

HC value	Bacterial isolates	Total number of bacterial isolates
1.00 - 1.99	FMJ 0102, FMJ 0202, FMJ 0303, FMJ 0401, FMJ 0801, FMJ 1001, FMJ 1003, FMJ 1404, FMJ 1502, FMJ 1601, FMJ 1603, FMJ 1604, FMJ 1703. FMJ 1802, FMJ 1902, FMJ 2201, FMJ 2404, FMJ 2505, FMJ 2603, FMJ 2902, FMJ 2903, FMJ 3002, FMJ 3003	23
2.00 - 2.99	FMJ 0302, FMJ 0602, FMJ 0604, FMJ 2301, FMJ 2402, FMJ 2403	6
3.00 - 3.99	FMJ 1105	1

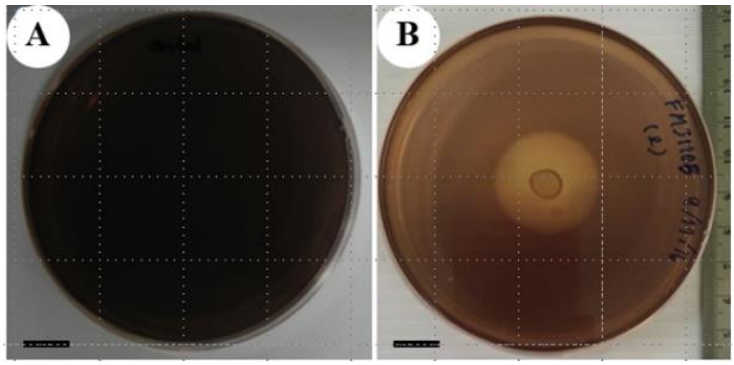


Figure 1 The cellulolytic area surrounding the bacterial colonies on CMC agar, observed after iodine staining. A. Non cellulase-producing bacterium. B. Cellulase-producing bacterium isolate FMJ 1105. Bar = 1 cm.

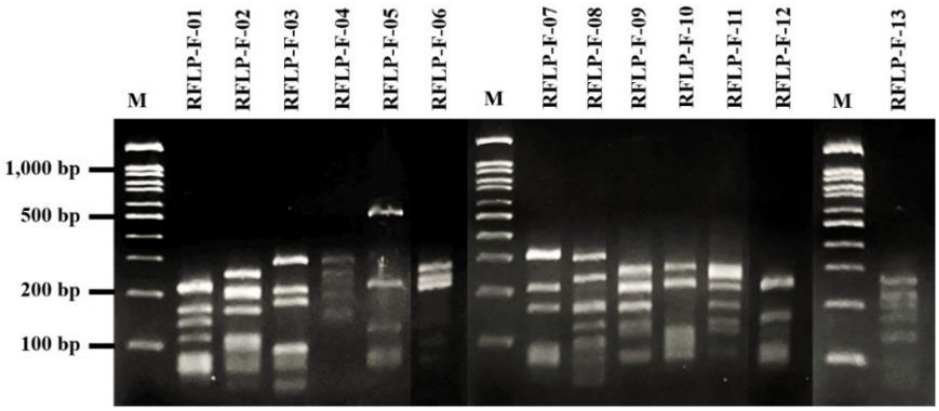


Figure 2 Thirteen RFLP patterns from PCR-RFLP analysis of the thirty isolated cellulase-producing bacteria. M denotes OneMark 100 RTU DNA molecular weight markers (Bio-Helix, Taiwan). This figure was constructed by connecting five OmniPur agarose gels

Table 2 Different RFLP patterns and numbers of the thirty isolated cellulase-producing bacteria obtained from goat feces.

RFLP patterns	Bacterial isolates	Total number of bacterial isolates
RFLP-F-01	FMJ 0102, FMJ 0202, FMJ 0303, FMJ 0401, FMJ 0801, FMJ 1001, FMJ 1003, FMJ 1902, FMJ 2902*, FMJ 3002	10
RFLP-F-02	FMJ 0302*	1
RFLP-F-03	FMJ 0604, FMJ 2201, FMJ 2403*, FMJ 2505, FMJ 2903	5
RFLP-F-04	FMJ 1604, FMJ 2404*	2
RFLP-F-05	FMJ 1105*	1
RFLP-F-06	FMJ 1404*, FMJ 1601, FMJ 1703	3
RFLP-F-07	FMJ 0602, FMJ 1502*	2
RFLP-F-08	FMJ 1603*	1
RFLP-F-09	FMJ 1802*	1
RFLP-F-10	FMJ 2301*	1
RFLP-F-11	FMJ 2402*	1
RFLP-F-12	FMJ 2603*	1
RFLP-F-13	FMJ 3003*	1

Remark: * The representative bacterial isolates for electrophoresis procedures on OmniPur agarose gel are shown in Figure 2.

4.3 Identification of Cellulase-Producing Bacteria by Nucleotide Sequencing and Phylogenetic Analysis of the 16S rRNA Genes Amplified from the Cellulase-Producing Bacteria

The representative cellulase-producing bacteria from each RFLP pattern were genetically identified, and their genomic DNA was extracted and amplified for 16S rRNA gene sequencing. Nucleotide sequence alignment of the 16S rRNA genes using the BLASTn suite revealed that the cellulase-producing bacteria isolated from goat feces samples belonged to nine genera including *Acinetobacter*, *Bacillus*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Exiguobacterium*, *Providencia*, *Pseudomonas*, and *Staphylococcus* (*Mammaliicoccus*) with a 98-99% identity and a 98-100% query coverage. All E values resulting from the BLASTn alignment were zero. The identity percentages and deposited GenBank Accession numbers of the isolated cellulase-producing bacteria are presented in Table 3.

A circular phylogenetic tree of the isolated cellulase-producing bacteria was constructed using the NJ algorithm with 100,000 bootstraps. The tree revealed three primary phylogenetic clades: Actinomycetota (formerly Actinobacteria), Bacillota (formerly Firmicutes), and Pseudomonadota (formerly Proteobacteria). The bootstrap values among representative bacterial strains downloaded from GenBank in these clades ranged from 40 to 100. The RFLP-F-06 pattern (FMJ 1404 as the representative isolate) was clustered with *Corynebacterium* in the Actinomycetota clade. The Bacillota clade comprised subclades *Bacillus*, *Exiguobacterium*, *Staphylococcus* (*Mammaliicoccus*), and *Enterococcus*. Within this phylogenetic clade, 6 RFLP patterns were identified including RFLP-F-03, RFLP-F-04, RFLP-F-05, RFLP-F-07, RFLP-F-08, and RFLP-F-13. The RFLP patterns RFLP-F-04 (represented by FMJ 2404), RFLP-F-05 (represented by FMJ 1105), and RFLP-F-13 (represented by FMJ 3003) were classified under the subclade *Bacillus*. The patterns RFLP-F-03 (represented by FMJ 2403), RFLP-F-07 (represented by FMJ 1502), and RFLP-F-08 (represented by FMJ 1603) were assigned to the subclades *Exiguobacterium*, *Staphylococcus* (*Mammaliicoccus*), and *Enterococcus*, respectively. Six RFLP patterns were assigned to the phylogenetic clade Pseudomonadota, namely RFLP-F-01, RFLP-F-02, RFLP-F-09, RFLP-F-10, RFLP-F-11, and RFLP-F-12. This phylogenetic clade encompassed the subclades *Escherichia*, *Pseudomonas*, *Acinetobacter*, and *Providencia*. The pattern RFLP-F-01 (represented by FMJ 2902) was categorized under the subclade *Escherichia*. The patterns RFLP-F-02 (represented by FMJ 0302) and RFLP-F-10 (represented by FMJ 2301) were classified under the subclade *Pseudomonas*. The patterns RFLP-F-09 (represented by FMJ 1802) and RFLP-F-11 (represented by FMJ 2402) were grouped under the subclade *Acinetobacter*, while the pattern RFLP-F-012 (represented by FMJ 2603) was clustered under *Providencia*. The circular phylogenetic tree depicting the cellulase-producing bacterial isolates is shown as Figure 3.

The isolated cellulase-producing bacteria were identified as closely related based on the BLASTn alignment results of the 16S rRNA nucleotide sequence and bootstrap values of the phylogenetic tree. *E. coli* (RFLP pattern RFLP-F-01) was the predominant cellulase-producing bacteria found in the goat feces samples, followed closely by *E. indicum* (RFLP pattern RFLP-F-03) and *C. stationis* (RFLP pattern RFLP-F-

06). All nucleotide sequences of the 16S rRNA genes have been deposited in the GenBank database of NCBI under the following accession numbers: PQ141697, PQ141699, PQ141700, PQ141702, PQ141703, PQ141704, PQ141989, PQ141990, PQ142313, PQ142314, PQ142315, PQ142329, and PQ142330.

Table 3 Identity percentages of the 16S rRNA gene sequences for the cellulase-producing bacteria isolated from goat feces.

RFLP patterns	Representative isolate	Closely related bacteria	GenBank Accession No. (Database)	Identity (%) *	GenBank Accession No. (Deposited)
RFLP-F-01	FMJ 2902	*Escherichia coli* strain ECIUP_13	MN094122.1	99.16	PQ141697
RFLP-F-02	FMJ 0302	*Pseudomonas luteola* strain SJC49	KX301304.1	98.81	PQ141699
RFLP-F-03	FMJ 2403	*Exiguobacterium indicum* strain REP307	MW344117.1	98.59	PQ141700
RFLP-F-04	FMJ 2404	*Bacillus amyloliquefaciens* strain BCRh10	KT153606.1	98.42	PQ141703
RFLP-F-05	FMJ 1105	*Bacillus* sp. strain M418	KJ944107.1	99.04	PQ141702
RFLP-F-06	FMJ 1404	*Corynebacterium stationis* strain AIK-1	KF975432.1	99.02	PQ141704
RFLP-F-07	FMJ 1502	*Staphylococcus sciuri* strain ISB2	KJ507203.1	98.90	PQ141990
RFLP-F-08	FMJ 1603	*Enterococcus hirae* strain MLG20-7	MT457687.1	99.38	PQ141989
RFLP-F-09	FMJ 1802	*Acinetobacter schindleri* strain M3	MG977679.1	99.14	PQ142314
RFLP-F-10	FMJ 2301	*Pseudomonas aeruginosa* strain C32	OQ405501.1	99.23	PQ142313
RFLP-F-11	FMJ 2402	*Acinetobacter radioresistens* strain B-Ace4	MK955373.1	98.51	PQ142315
RFLP-F-12	FMJ 2603	*Providencia rettgeri* strain HB14	KM659221.1	99.22	PQ142329
RFLP-F-13	FMJ 3003	*Bacillus pumilus* strain SpS06	OP810776.1	98.69	PQ142330

Note: * The results were analyzed using the BLASTn suite on May 25, 2024.

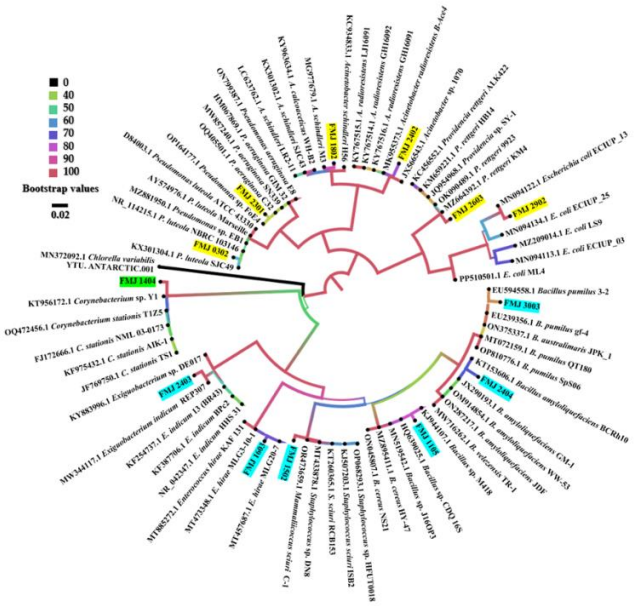
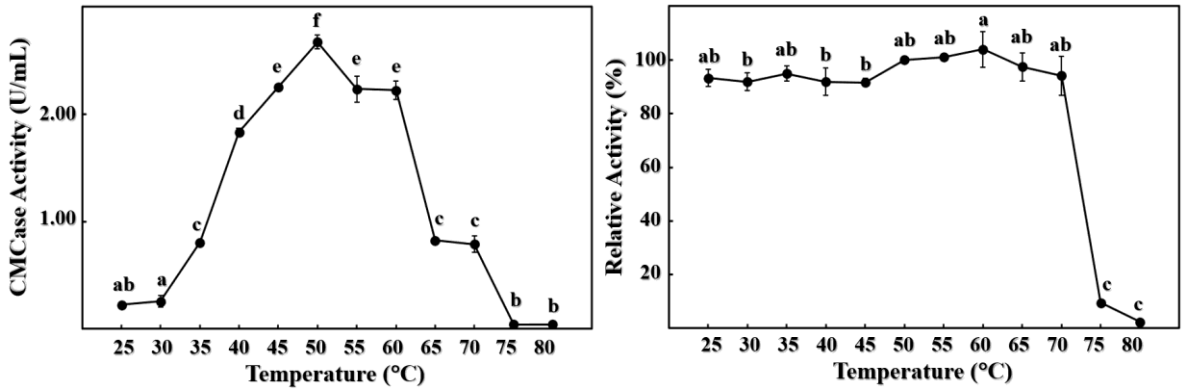
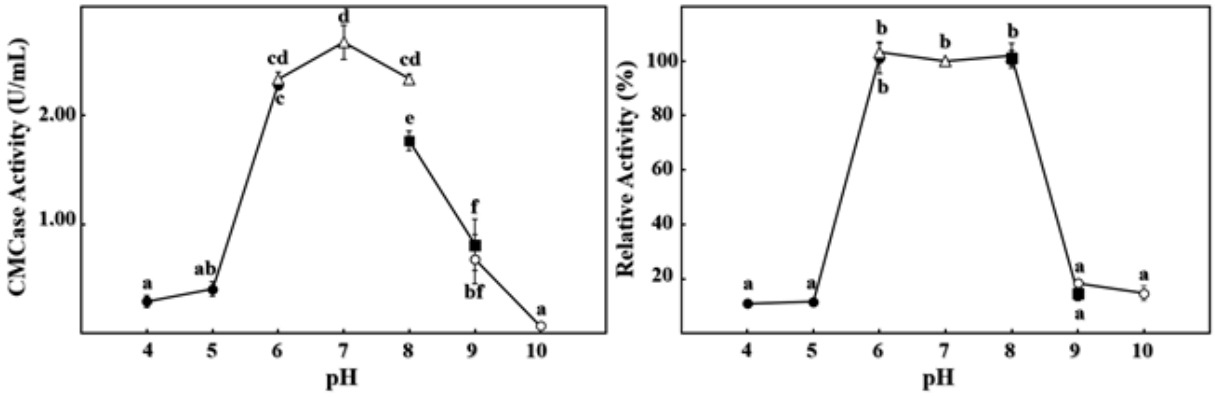


Figure 3 Circular phylogenetic tree of the isolated cellulase-producing bacteria using SeaView software version 4.7 and FigTree software version 1.4.4 based on 100,000 bootstrap replications



(A) **(B)**

Figure 4 (A) The optimum temperature and (B) thermal stability of CMCase activity from the partially purified cellulases of *Bacillus* sp. strain FMJ 1105. The error bars indicate the standard deviation of the triplicate measurements. Mean values followed by the same letter are not significantly different according to Tukey's test ($p < 0.05$)



(A) **(B)**

Figure 5 (A) The optimum pH and (B) the pH stability of CMCase activity from the partially purified cellulases of *Bacillus* sp. strain FMJ 1105. CMCase activity was assessed in a citrate buffer (●), sodium phosphate buffer (△), Tris-HCl buffer (■), and glycine-NaOH buffer (○). The error bars indicate the standard deviation of the triplicate measurements. Mean values followed by the same letter are not significantly different according to Tukey's test ($p < 0.05$)

4.4 Activity Assays and Enzymatic Characterization of the Partially Purified Cellulases from *Bacillus* sp. Strain FMJ 1105

Bacillus sp. strain FMJ 1105 was considered the most effective cellulase-producing bacterium based on its cellulolytic zone and HC value in a CMC agar experiment. FMJ 1105 was cultured in a CMC liquid medium for partially purified cellulases and examined for endoglucanase activity (CMCase activity). The activity assay results showed that *Bacillus* sp. strain FMJ 1105 produced partially purified cellulases at 2.67 ± 0.06 U/mL of CMCase under experimental conditions.

The partially purified cellulases extracted from *Bacillus* sp. strain FMJ 1105 were evaluated for CMCase activity under various temperature and pH conditions. The optimum temperature for CMCase activity was found to be 50°C ($p < 0.05$), while the optimum pH was determined to be 7.0, using a sodium phosphate buffer ($p < 0.05$). The results for the optimum temperature and pH for the partially purified cellulases are illustrated in Figures 4A and 5A, respectively. Furthermore, the partially purified cellulases remained stable at temperatures up to 75°C ($p < 0.05$) and within a pH range of 6.0 to 8.0 after 24 h of incubation ($p < 0.05$). The thermal and pH stability of the cellulase enzymes is shown in Figures 4B and 5B. Additionally, different buffer types were observed to influence CMCase activity at a pH of 8.0, as indicated in Figure 5A. However, the type of buffer did not significantly impact the stability of CMCase at the same pH value, as evidenced in Figure 5B.

5. Discussion

Cellulases are used in various industries, including agriculture, brewing, laundry, pulp and paper, and the textile industry (Ejaz et al., 2021). These enzymes are a topic of interest for both academic research and industries. Various cellulase-producing bacteria have been isolated from the rumen and feces contents of different ruminant animals, with potential applications in improving cellulose digestion or producing cellulase enzymes. Goat feces are suitable samples for isolating cellulase-producing bacteria because the enzymes associated with cellulose degradation are more abundant in goat feces than in other herbivores such as cattle and rabbits (Chen et al., 2024). Therefore, the isolation of cellulase-producing bacteria from goat feces can produce novel results.

This study assessed the current knowledge of cellulase-producing bacteria isolated from goat feces samples collected in Rayong Province, Thailand and discussed the cellulase characteristics of the most active isolated bacterium. A total of thirty cellulase-producing bacteria were isolated and classified using PCR-RFLP analysis. Thirteen different RFLP patterns were obtained, and the cellulase-producing bacteria were identified as belonging to nine genera including *Acinetobacter*, *Bacillus*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Exiguobacterium*, *Providencia*, *Pseudomonas*, and *Staphylococcus* (*Mammaliicoccus*). Previous reports on bacterial diversity in goat feces revealed the presence of pathogenic and nutrient cycling-related bacteria such as *B. subtilis*, *B. licheniformis*, *S. aureus*, *S. saprophyticus*, *E. coli*, and *Salmonella* sp. (Lu et al., 2021; Basnet, & Kilonzo-Nthenge, 2024).

Acinetobacter schindleri is an opportunistic human pathogen found in various natural environments, including soil and water (Kee et al., 2018). Another cellulase-producing *Acinetobacter*, *A. radioresistens* found in this study was first isolated from cotton that had undergone sterilization by gamma radiation (Wang et al., 2019) and is ubiquitous in soil and water environments (Verma, & Baroco, 2017). However, no previous studies have mentioned that these bacterial species can produce cellulases. This study identified the cellulolytic *A. schindleri* strain FMJ 1802 and *A. radioresistens* strain FMJ 2402 in goat feces for the first time, with slight activity shown by their HC values using the CMC agar method.

Bacillus is a genus of bacteria that is commonly found in various environments such as air, dust, soil, and water (Chantarasiri, 2020). *Bacillus* species are prolific producers of cellulase enzymes, secreted extracellularly in abundant amounts, thereby facilitating easy extraction and purification processes (Malik, & Javed, 2021). The isolation of cellulase-producing *Bacillus* species from herbivore feces has been the focus of several studies due to their potential applications in biotechnology. The isolated cellulase-producing bacteria in this study were closely related to *Bacillus* sp., *B. amyloliquefaciens*, and *B. pumilus*, with *Bacillus* sp. strain FMJ 1105 the most active with HC values of 3.78 ± 0.32 and CMCase activity 2.67 ± 0.06 U/mL under experimental conditions. The cellulase-producing *B. amyloliquefaciens* strain SS35 was isolated from rhinoceros dung and showed 0.079 U/mL of CMCase activity (Singh et al., 2013). A recent study of the feces from healthy Tibetan pigs reported an effective cellulase-producing *B. amyloliquefaciens* strain TL106 with a cellulase activity of 20.86 U/mL (Shang et al., 2022). *B. pumilus* is commonly found in soil and studies focusing solely on *B. pumilus* isolated from feces samples are limited. *B. pumilus* strain S124A was isolated from soil samples and identified as a cellulase-producing bacterium (Balasubramanian, & Simões, 2014).

Partially purified cellulases from *Bacillus* sp. strain FMJ 1105 were characterized for their CMCase activity under various pH and temperature conditions. The partially purified cellulases showed optimum CMCase activity at 50°C and stability between 25 and 70°C. The optimum pH and pH stability were found in neutral conditions between pH 6.0 and 8.0. The CMCase activity of *Bacillus* sp. strain FMJ 1105 was similar to that of other *Bacillus* cellulases isolated from various environments, showing a preference for pH levels between 5.0 and 8.0 and temperatures ranging from 45 to 60°C (Malik, & Javed, 2021; Listyaningrum et al., 2018). Experiments revealed that different buffers had diverse effects on the cellulase activity of *Bacillus* sp. strain FMJ 1105. The endoglucanase of *Bacillus* sp. strain FMJ 1105 could be used in the agriculture and food industries, which have neutral pH and mesophilic temperature conditions in the production processes.

Corynebacterium stationis, formerly known as *C. ammoniagenes*, is a type of facultative anaerobic bacteria that has been associated with diseases in humans and mastitis in cattle (Awadalla et al., 2022), and is found in the intestinal tracts of various animals (Kinose et al., 2024). *C. stationis* is used in the commercial fermentation of inosine monophosphate (IMP) and xanthosine monophosphate (XMP) (Kinose et al., 2024). *C. stationis* strains were the third highest cellulase-producing bacteria isolated from goat feces samples in this study. Three isolated *C. stationis* strains (FMJ

1404, FMJ 1601, and FMJ 1703) showed slight cellulase activity based on their HC values with CMC agar determination. No direct evidence presented in other studies suggested that *C. stationis* produces cellulases.

Enterococcus hirae is a zoonotic pathogen that causes infections in animals but is rarely isolated from human clinical samples (Bourafa et al., 2015), and is known to cause various infections in animals including endocarditis in chickens, diarrhea in rats, and mastitis in cattle (Gaudiano et al., 2023). *Enterococcus* species are ubiquitous in nature and have been isolated from the gastrointestinal tract of various animals including bovine and human (Beukers et al., 2017). A recent study revealed that *E. hirae* had unique genes associated with cellulose degradation (Zaidi et al., 2022).

Escherichia coli strains were the predominant cellulase-producing bacteria found in feces collected from Thai goats in this study. *E. coli* are well-known bacteria that are commonly found in the intestines and feces of warm-blooded animals, but their cellulose degradation capability has not been previously reported (Gao et al., 2016). A recent study found a cellulolytic *E. coli* strain in bovine rumen. *E. coli* strain ZH-4 isolated from bovine rumen in Mongolia showed an endoglucanase activity of 5.31 U/mL at pH 6.8 (Pang et al., 2017).

Exiguobacterium indicum strains were the second highest cellulase-producing bacteria in this study. *E. indicum* is a type of psychrophilic bacteria with strains found in aquatic environments (Chantarasiri, 2021). This bacterium has been isolated from poultry waste mixed soil (Solanki et al., 2021) and produces extracellular cellulases associated with the breakdown of cellulose into simpler sugars (Chantarasiri, 2021). Interestingly, members of the genus *Exiguobacterium* demonstrate a wide temperature tolerance, ranging from 4 to 50 °C, including strains isolated from cold environments (Tedesco et al., 2021). The isolated strains FMJ 0604 and FMJ 2403 exhibited cellulase activity based on their HC value of CMC agar determination, but few reports have investigated cellulolytic *E. indicum* and its cellulases (Chantarasiri, 2021).

Providencia rettgeri is commonly found in water, soil, and animal reservoirs (Sapkota et al., 2021) but cellulase-producing *P. rettgeri* has rarely been isolated from environmental samples. A previous study reported cellulolytic activity in another species, *P. vermicola* (Dini et al., 2024). Therefore, this study represents the first report of cellulase-producing *P. rettgeri* strain FMJ 2603.

Pseudomonas species are ubiquitous free-living bacteria usually found in moist environments such as soil and water and are prolific producers of enzymes like lipase, protease, amylase, cellulase, urease, and beta-glucosidase (Chauhan et al., 2023; Thongmee, & Sukplang, 2024). These enzymes can be used in various industrial applications. *Pseudomonas* strains have become important workhorses in biotechnology due to their diverse metabolic capabilities and ability to produce a wide range of enzymes. In this study, *P. luteola* strain FMJ 0302 and *P. aeruginosa* strain FMJ 2301 were considered cellulase-producing bacteria. *P. luteola* (formerly known as *Chryseomonas luteola*) is primarily found in environments with high moisture content (Ahmad et al., 2023), and is rarely considered a human pathogen (Barry, 2021). A previous report identified *P. luteola* as a cellulase-producing bacterium (Yazdansetad et al., 2015). *P. aeruginosa* is a ubiquitous bacterium found in various environments including soil, water, and the human body (Crone et al., 2020). This opportunistic pathogen can cause mastitis in dairy goats (Scaccabarozzi et al., 2015). Recent studies isolated cellulolytic strains of *P. aeruginosa* (Gunavathy, & Boominathan, 2015; Kamal et al., 2020; Chavda et al., 2023). Therefore, the ability of *P. aeruginosa* to produce cellulases presents significant opportunities for its application in environmental biotechnology.

Staphylococcus sciuri is a versatile bacterium with a wide ecological range covering domestic and wild animals, humans, and the environment (Zeman et al., 2017). In 2020, it was renamed *Mammaliicoccus sciuri* (Madhaiyan et al., 2020). This bacterium has recently been identified as a producer of pectinase (Sharma, & Wadhwa, 2023). Previous research indicated that several species in the *Staphylococcus* genus form a dominant group of cellulose-degrading bacteria, utilizing rice straw as a carbon source (Flimban et al., 2019). This study revealed that *S. sciuri* strains FMJ 0602 and FMJ 1502 are capable of producing cellulases. However, evidence from other studies that *S. sciuri* produces cellulases directly is lacking.

Finally, this study primarily examines bacterial diversity by directly isolating cellulase-producing bacteria from goat feces using culture-dependent methods. It is important to highlight that unculturable cellulase-producing bacteria, which could be identified through metagenomic approaches, are not included in this research. Consequently, further studies are necessary to assess the metagenomics of these unculturable bacteria.

6. Conclusion

In conclusion, goat feces can be a potential source for isolating cellulase-producing bacteria. Nine genera of cellulase-producing bacterial isolates were identified from goat feces in Rayong Province, Thailand based on the PCR-RFLP of the 16S rRNA gene. Cellulase-producing *E. coli* were the predominant bacteria found in this study. Several isolated cellulase-producing bacteria have limited mention in the literature, with no available evidence suggesting that they are significant cellulase producers. Therefore, more detailed research is needed to determine whether certain strains or environmental isolates possess cellulolytic capabilities. This study exhibited that *Bacillus* sp. strain FMJ 1105 was considered the most active cellulolytic bacterium, with CMCase active under meso-temperatures and stable under high temperatures. Moreover, its CMCase was active and stable under neutral pH conditions. Therefore, it is suitable for use in industries with mild production processes. However, further studies are required to assess molecular cloning and enzyme purification.

7. Acknowledgements

This research was funded by King Mongkut's University of Technology North Bangkok, Contract no. KMUTNB-66-BASIC-14. The authors wish to thank Kanokthorn Yingyong, Suchawadee Khaowsodsai, Piyanut Phutphong, and Chutikarn Jakawannorasing for providing samples and laboratory materials.

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