Macroscopic and Microscopic Isolation and Identification of Cellulolytic Bacteria from Mixtures of Coal and Beef Cattle

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Abstract. Cellulolytic bacteria are bacteria that can hydrolyze cellulose into smaller polysaccharides glucose units. Glucose is used as a source of energy and carbon for bacterial growth. This study aimed to determine the ability of isolates bacteria from feces of beef cattle and coal (bituminous, sub-bituminous, lignite) to produce cellulase enzymes based on the clear zone seen surrounding the colonies and characterization of cellulolytic bacteria isolates from cattle feces and coal. The cellulolytic activity of the bacteria was determined by their ability to degrade nutrient-carboxymethyl cellulose (Na-CMC) substrates. The result showed that six isolates tested were able to produce cellulase. BC2 isolate bacteria had the highest cellulolytic activity, 1.23, based on 48-h production time. Macroscopic identification bacteria isolates showed that colonies formed white with a slimy structure, irregular shape, rounded, rhizoid, and wavy. Smooth, branched edges. Microscopic identification of bacteria isolates showed five bacteria isolates from beef cattle feces, and their combinations were gram-positive and bacil shape, two of the isolate's bacteria coccus shape and one of which was a gram-negative group.

Keywords: Bacterial · Cellulolytic · Cellulolytic indexes · Coal · Feces

1 Introduction

In Indonesia, many ex-coal mining lands are reclaimed/greened and collaboratively used as beef cattle grazing land. In the ex-coal mining area, there is Coal Bed Methane (CBM), a natural gas derived from coal, dominated by methane gas, hydrocarbons, and non-hydrocarbon gases resulting from chemical, physical and biological processes. CBM comes from organic material (plants) through chemical and physical processes in the form of heat and pressure, continues until it turns into peat, and then coal is formed, during which organic material will produce H_2O, CO_2, CH_4, and other gases. CBM can also be formed from the activity of methanogenic bacteria in water trapped in coal, especially lignite. The types of coal are lignite, subbituminous, bituminous, anthracite. Starting from this, a study was conducted on the isolation and identification of methane-forming bacteria.

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Changes in organic material produce H_2O, CO_2, CH_4, and other gases known as biogas, so there are similarities in forming CBM and biogas. Both are formed from the activity of anaerobic bacteria. The process of converting organic material into methane gas consists of three stages: hydrolysis, acetogenic and methanogenic. This hydrolysis process decomposes complex organic materials (carbohydrates, proteins, fats) into more explicit materials (cellulosic, hemicellulosic, polypeptides, fatty acids). Previous research has isolated and identified bacteria present in beef cattle feces, lignite coal, sub-bituminous coal, and bituminous coal, 10 isolates were found, then from these 10 isolates in this study, a cellulolytic test was carried out, and the results of the selected isolates later will be used as a starter in the following research in the hydrolysis process as the initial stage of biogas formation. This study aimed to find isolates of cellulolytic bacteria from beef cattle feces and from various types of coal, which could later be grown and used as starters for biogas production in the pasture.

2 Materials and Methods

The materials used in this study were 10 isolates of freeze-dried bacteria obtained from beef cattle feces, lignite coal, sub-bituminous coal, and bituminous coal. Carboxy methyl cellulose agar, nutrient agar, 1% congo red, aquades, alcohol, lugol's solution, gentiana violet dye, fuchsin water. The tools used in this study were aluminum foil, autoclave, hot plate stirrer, spatula, caliper, bunsen, microscope, hocky stick, syringe, analytical balance, water bath, petri dish, label, osse, serum bottle, test tube, dispenser, thermometer.

The research method uses exploratory methods with descriptive analysis. This experiment was conducted to determine the cellulolytic index of isolates using NA-CMC media. The isolates tested were 10 isolates of freeze-dried bacteria originating from beef cattle feces, lignite coal, sub-bituminous coal, and bituminous coal. Repeated 3 times in each experiment. Cellulolytic index observations were carried out on day 1 and day 2 with incubation periods of 24 h and 48 h by measuring the diameter of the clear zone formed and the diameter of the bacterial colonies. The cellulolytic index is the ratio between the diameter of the clear zone and the diameter of the bacterial colony [9]. The results of bacterial isolation with selected cellulolytic index then identified the shape of the bacterial colony macroscopically and identified microscopically by gram staining.

3 Results and Discussion

3.1 Cellulolytic Index of Bacterial Isolates from Beef Cattle Feces and Coal

Essential the results of the cellulolytic index test obtained from 10 bacterial isolates from beef cattle feces and coal, 6 isolates of bacteria that have the ability to degrade cellulose were selected, the results of the isolation of these bacteria are presented in Table 1.

The results of the isolation of previous studies found 10 bacterial isolates with different morphology, bacterial isolates from beef cattle feces, lignite coal, sub-bituminous coal, bituminous coal, and a combination of coal and beef cattle feces. In addition, 6 bacterial isolates were obtained after the cellulolytic test that could grow on NA-CMC

Table 1. The average results of the analysis of the Cellulolytic Index of Bacterial Isolates from Beef Cattle Stool and Coal.

Sample	Cellulolytic Index 24 h incubation	Cellulolytic index 48 h incubation
BF	0.54 ± 0.05	0.65 ± 0.09
LC	0.12 ± 0.07	0.16 ± 0.03
SBC	0.29 ± 0.10	1.05 ± 0.03
BC_1	0.33 ± 0.03	0.91 ± 0.26
BC_2	0.98 ± 0.44	1.23 ± 0.07
BFC_2	0.08 ± 0.03	0.27 ± 0.2

Note: Cellulolytic Index Standard (Choi, 2005): Strong ≥ 2, Currently > 1 - ≤ 2, Weak ≤ 1
Information: Bacterial isolates from beef cattle feces = BF; Bacterial isolate from lignite coal = LC; Bacterial isolates from Sub-Bituminous Coal = SBC; Bacterial isolate from bituminous coal = BC; Bacterial isolates from Beef Cattle Feces and Coal = BFC.

media. This indicated that these bacterial isolates could decompose cellulose based on the resulting cellulolytic index value.

Bacterial isolates growing on NA-CMC media formed a clear zone, which indicated that these bacteria had cellulase enzymes and were able to degrade cellulose. This is in line with the opinion of Hidayah et al [6], which states that the potential of cellulolytic bacteria can be determined by the ability to secrete cellulase enzymes by testing the cellulolytic index based on the clear zone formed around bacterial colonies that grow on CMC media. After incubating for 24 h and 48 h, isolates grown on NA-CMC media were stained using 0.1% Congo red dye for 30 min. This was intended to emphasize the colonies and clear zones formed.

In Table 1 it can be seen that the average cellulolytic index of all bacterial isolates showed a weak ability because they had a cellulolytic index < 1, except for isolates of bacteria SBC1 and isolates of bacteria BC2, which had cellulolytic indexes of 1.05 ± 0.03 and 1.23 ± 0.07 including the moderate ability (cellulolytic index > 1 - 2), this is based on the opinion of Choi et al [3]. SBC1 and BB2 bacteria isolates that were incubated for 48 h produced cellulolytic index values in the medium category and higher than the ability of other isolates, it is suspected that these bacteria get nutrients that contain a lot of complex carbon, so they can produce more cellulolytic enzymes. This agrees with Driyo [4], who states that coal is composed of solid hydrocarbon compounds, and the composition is quite complex. Coal organic matter is composed of plants (tree bark, leaves, roots, and wood structure, with physical, chemical, and biological processes over a long period cellulose derived from plants will experience changes to lignite, sub-bituminous, bituminous, and anthracite coal). Each has a different hydrocarbon content. According to Ellin et al [5], coal has porosity, permeability, and fractures that can accommodate fluids and moisture that can grow bacteria between the coal pores.

Incubation time affects the value of the cellulolytic index. This agrees with Meryandini et al [7], they explained that the value of the cellulolytic index produced by bacteria could be increased by increasing the incubation time, thus allowing the enzyme

Table 2. Macroscopic Characteristics of Cellulolytic Bacterial Isolates from Cow Feces cut and coal.

Bacteria Isolate	Macroscopic Characteristics				
	Form	Color	Structure	Edge	Elevation
BF	Irregular	White	mucus	wavy	convex
LC	Irregular	White	mucus	wavy	hilly
SBC	Irregular	White	mucus	wavy	flat
BC_1	Circular	White	mucus	slippery	arise
BC_2	Rhizoid	White	mucus	forked	arise
BFC_2	Irregular	White	mucus	wavy	flat

Information: Bacterial isolates from beef cattle feces = BF; Bacterial isolate from lignite coal = LC; Bacterial isolates from Sub-Bituminous Coal = SBC; Bacterial isolate from bituminous coal = BC; Bacterial isolates from Beef Cattle Feces and Coal = BFC.

to degrade more substrates. According to Ambriyanto [1], the cellulolytic index is influenced by the pH, enzyme levels, and redox reactions.

3.2 Macroscopic and Microscopic Characteristics of Cellulolytic Bacterial Isolates from Beef Cattle Feces and Coal

The results of observations of selected bacterial isolates on macroscopic characteristics are presented in Table 2.

Based on the observations of shape, color, structure, edge, and elevation, the six isolated bacterial isolates had the same color and structure, which were white and slimy. However, they varied in the colony's shape, edge, and elevation. This is in accordance with Azizah et al. [12] that cellulolytic bacteria have macroscopic characteristics of circular shape, irregular, white color, wavy edges, elevation, blunt bulge, convex, and flat. In addition, Waling et al. [8] stated cellulolytic bacteria have red, cream, white, and yellow, irregular shapes, rounded, smooth edges, raised elevations, and convex. The results of observations of selected bacterial isolates on microscopic characteristics are presented in Table 3.

Based on the gram staining results, the microscopic characteristics of isolates BF, BC1, BC2, and BFC2 were bacilli-shaped, while isolates LC1 and SBC1 were coccus and all gram-positive isolates except LC1 gram-negative isolates. This is in line with the results of research by Ateng et al. [2], which stated that bacterial isolates obtained from organic waste had gram-positive characteristics, were spore-shaped and rod-shaped, and cocci produced cellulose enzymes that could break the beta-glucoside bonds in the cellulose chain. In line with Siska and Eko [11] stated that cellulolytic bacteria isolated from heaps of coffee husks have cream-colored characteristics, are gram-positive, do not spore, and are rod-shaped, and produce cellulase enzymes in the presence of a clear zone around the colony, with a high cellulolytic index value. According to Putri and Wardani [10], cellulolytic bacteria isolated from solid tapioca waste showed white colony, rod-shaped and gram-positive bacterial isolates.

Table 3. Microscopic Characteristics of Cellulolytic Bacterial Isolates from Cow Feces and coal

Bacteria Isolate	Microscopic characteristics		
	Cell Shape	Gram	Color
BF	Bacil	positive	purple
LC	Coccus	negative	red
SBC	Coccus	positive	purple
BC_1	Bacil	positive	purple
BC_2	Bacil	positive	purple
BFC_2	Bacil	positive	purple

4 Conclusions

In this study, 6 isolates of cellulolytic bacteria were obtained, namely isolates of bacteria FB, LC, BC1, and BCF with a weak cellulolytic index between 0.16 ± 0.03 - 0.91 ± 0.26 and isolates of bacteria SBC1 and isolates of bacteria BB2 with a moderate cellulolytic index. (1.05 ± 0.03 and 1.23 ± 0.07). Macroscopic characteristics of bacterial isolates, six isolated bacterial isolates had the same color and structure, which were white and slimy, but varied in shape, edge, and elevation of the colonies, while microscopically characteristic of isolates FB, BC1, BC2, and BFC2 were bacilli, while LC isolates, SBC1 shaped cocci, and of all gram-positive isolates except gram negative LC isolates.

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