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Research Article

BIODIVERSITY OF CELLULOLYTIC BACTERIA ISOLATED FROM FERMETODEGE FOR RUMINANT

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ARTICLE HIGLIGHTS

- Fermetodege as a source of cellulolytic bacteria that degrade cellulosic materials.
- Enhances ruminant feed by improving palatability and digestibility.
- Utilizes water hyacinth, reducing invasive plant spread and supporting ecosystems.
- Bioprospecting reveals new bacteria for effective fermentation starter development.

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ABSTRACT

Fermetodege is referred to as fermented ruminant feed made from rice bran, corn cobs, and heavy metal-free water hyacinth. Several factors influence both the fermentation process and the quality of the final products, including dominance, diversity, and evenness, of indigenous cellulolytic bacteria species. To increase the quality of fermented feed, there is a need to fully comprehend the influential factors. Therefore, this study aimed to assess factors influencing the biodiversity of cellulolytic bacteria isolated from fermetodege. Cellulolytic activity of the isolates was assessed through observation of growth on the carboxymethylcellulose (CMC) media. Simpson's species dominance, as well as Shannon-Weiner's diversity and evenness indices were also calculated. The results showed that cellulolytic activity of the bacteria isolates formed a clear zone after soaking the bacteria colonies in 0.1% Congo red and rinsing with 1 M NaCl. The Shannon-Weiner's diversity index was categorized as medium, with values ranging from 0.6849 to 1.8173. Observation on stable distribution of bacteria species showed evenness index between 0.7778 and 0.9983. Meanwhile, the Simpson's species dominance ranged from 0.1835 to 0.5082, showing that there was no dominant species. In conclusion, this study showed that fermetodege was a potential source of bacteria isolates and could be used as a fermentation consortium starter.

Keywords: *cellulolytic bacteria, diversity, dominance, evenness, fermetodege*

INTRODUCTION

Ruminant feed ingredients having high cellulose content are characterized by low palatability and digestibility levels (Isnawati 2020), showing the need for fermentation process to break down cellulose. Palatability is the characteristics of feed that influence the sensory response in animals and affects their appetite for feed (Aldrich & Koppel 2015). Some ingredients, such as rice bran, corn flour, and molasses, can act as palatants to mask unpleasant flavors and to improve feed palatability by enhancing the taste and smell of feed to make it more appetizing to animals and increase feed intake, resulting in weight gain and milk yield increase.

Digestibility refers to the number of nutrients absorbed by the organism. Commonly, it is calculated by subtracting the nutrients retained in the feces from the nutrients consumed (Atta *et al.* 2018). Many factors influence feed digestibility, such as the type of animals, the composition of the ingredient plant, and the difference in feed preparation. Animals with pre-gastric fermentation digestion, such as ruminant, has highly efficient digestion since the nutrients released are digested and absorbed in the gastric chamber and intestinal tract. Digestibility of ruminant feed is closely related to the chemical composition. A ruminant feed with high crude fiber content is poor in digestibility. However, grinding may increase absorbability because of the enhanced substrate accessibility for enzymatic action. Finer grinding increases the digestion rate in which the feed passes through the digestive tract. This leads to an increase in total feed intake because animals can consume more feed in a given amount of time (Ortolani *et al.* 2020).

Fermentation process can improve feed palatability and digestibility. According to Istiqomah *et al*. (2010), rice bran should be fermented using *Rhizopus* sp. to change the texture and flavor of feed ingredients and make the fermentation products more appetizing, easily digestible, and nutritious for the cattle. Generally, all fermented products usually contain compounds that are simpler and easier to digest than the original ingredients. These products are easily decomposed biologically and have higher nutritional value (Pakpahan & Restiani 2019).

During the extended dry season, particularly when forage is unavailable, fermented feed can be used to overcome the lack of food for animals. The production of these products can be carried out through various weeds, including water hyacinth and agricultural waste, such as corn cobs and rice bran containing high cellulose (Lardy *et al*. 2022). In this study, the mixture of corn cobs, rice ban, and water hyacinth is called fermetodege.

A widely recognized aquatic weed, namely water hyacinth, grows quickly and uncontrollably, disrupting aquatic ecosystem and causing siltation (Adanikin *et al*. 2017). The use of water hyacinth as one of fermetodege ingredients means preventing the wider spread of aquatic weed, reducing environmental pollution, and contributing to ecosystem preservation (Sivaramakrishnan *et al*. 2021).

The composition of lignocellulosic product includes 6.5% lignin, 16.4% cellulose, and 42.8% hemicellulose (Rezania *et al*. 2018). Proximate analysis showed that water hyacinth has 9.3% dry matter, 26.9% crude fiber, 12.4% ash, and 10.5% crude protein (Hossain *et al*. 2015). A significant source of raw materials for fermented feed preparation include rice bran, corn cobs, and agricultural by-products rich in lignocellulose (42.9% cellulose, 26% hemicellulose, and 22% lignin). According to Liu *et al*. (2017), the proximate analysis showed that rice bran comprises 23.21% crude fiber, 12.35% ash, 16.72% protein, and 52.87% carbohydrate. Corn cobs have significant lignocellulose content, consisting of 45.88% cellulose, 39.40% hemicellulose, and 11.32% lignin (Shariff *et al*. 2016). The proximate analysis showed that corn cobs contain 30.93% crude fiber, 2.26% ash, and 0.61% crude fat (Paynor *et al*. 2016).

Cellulolytic bacteria play an important role in degrading organic materials, such as lignocellulose, into simpler compounds during the production of fermented feed (Varma *et al*. 2015), which include *Bacillus brevis* (Tabssum *et al*. 2018) and *Burkholderia nodosa* NB1 (Tang *et al*. 2018). *Enterococcus*, *Klebsiella*, *Stenotrophomonas*, and *Microbacterium* isolated from sugarcane biomass have also been reported to show cellulolytic activities (Dantur *et al*. 2015). These bacteria are often obtained from the rumen of cattle and added to the cellulosic raw materials to produce a starter for ruminant fermented feed. Rumen is dominated by anaerobic bacteria, both facultative and obligate. Despite the numerous benefits, there is limited information on the use of bacteria for special fermented feed ingredients. Therefore, this study aimed to evaluate diversity, evenness, and dominance of indigenous cellulolytic bacteria species from fermetodege, which was made from water hyacinth, corn cobs, and rice bran. Bacteria isolates obtained from fermetodege can be regarded as a bioprospecting strategy to obtain a more effective source of fermentation starter, other than bacteria obtained from cattle's rumen.

The diversity, evenness, and dominance indices of indigenous bacteria isolated from fermetodege in this study are expected to provide useful reference for making effective consortium starter composition, for manufacturing cellulosic material-based fermented feed.

MATERIALS AND METHODS

Production of Fermetodege

Water hyacinth, rice bran, and corn cobs with a 1:1:1 ratio were used as raw materials to produce fermetodege. The corn cobs and water hyacinth were cleaned and cut into small pieces of 1-3 cm to increase surface area and enhance easy decomposition. The raw materials were then sun-dried to minimize the water content up to 30-40%, which is essential to prevent decay and optimize the fermentation (Farida *et al*. 2018). The raw materials were then cooked for 20-30 minutes, cooled, mixed evenly, and placed in a basket covered with banana leaves to create a microaerophilic condition (Fitrihidajati *et al*. 2015). In this study, there were four units of experiment.

Fermentation of Raw Materials

The fermentation process was carried out naturally using Solid-State Fermentation (SSF) method, which was affected by temperature,

water content, type of substrates, and presence of microbial bioactivity (Sadh *et al.* 2018). This method offers numerous advantages, including lower contamination risks, along with a straightforward, cost-effective fermenter design that enhances process efficiency and productivity (Leite *et al.* 2020). In this study, fermentation process was conducted under a microaerophilic condition. This condition offers several benefits, such as creating favorable conditions for obligate anaerobic bacteria and enhancing the growth of facultative anaerobic bacteria. The experimental units were placed at room temperature and observed daily for 15 days.

pH and Temperature Measurement

The measurement of pH and temperature was conducted at the top, middle, and bottom sections of the fermetodege. Average value from each replication were calculated to determine daily temperature and pH. For bacteria isolation data, feed samples were also taken each day (Isnawati 2019).

Cellulolytic Bacteria Isolation

Bacteria isolation was carried out daily for 15 days during fermentation process. A total of 10 g fermented feed samples were randomly collected at a depth of 10-15 cm from the fermenter. The sample was stored in a bacteria isolation tube and added with sterile distilled water until the final volume reached 100 mL and then, homogenized. The sample was then diluted to 10^{-5}, after which 1 mL was taken and cultured on 10 mL nutrient agar media using a pour plate method. After 24 hours of incubation, the grown colonies were characterized based on their morphology followed by enumeration. Each of bacteria isolates was recultured using the streak plate method. Subsequently, cellulolytic activity was determined based on their growth on carboxymethyl cellulose (CMC) media (Isnawati 2020). The isolated bacteria having cellulolytic activity were identified based on the forming of a clear zone around colony greater than > 1.0 cm (Gaur & Tiwari 2015) when dripped with 0.1% Congo red and rinsed using 1 M NaCl.

Identification of Bacteria Isolates

Bacteria isolates were identified by assessing physiological and biochemical characteristics with the Microbact Identification Kits (MicrobactTM GNB12A and 12B) and Bergey's Manual of Determinative Bacteriology (Holt 1994).

Data Analysis

Diversity, Evenness, and Dominance of Cellulolytic Bacteria Species

Based on a study of the microbial communities' succession (Liu *et al.* 2015) and fish population (Hanif *et al.* 2015), bacteria diversity was determined by using the Shannon-Wiener diversity, evenness, and Simpson dominance indices.

Shannon-Wiener diversity index was calculated by using formula:

$$H' = -\sum \left(\frac{ni}{N}\right) ln \left(\frac{ni}{N}\right)$$

where:

H' = Shannon-Wiener diversity index
ni = total number of species i
N = total number of all species.

The level of diversity is classified as:

- low when H' < 1
- moderate when 1 < H' < 3
- high H' > 3

The Evenness Index was calculated by using formula:

$$E = \sum \left(\frac{H'}{\ln S}\right)$$

where:

E = Evenness index
H' = Shannon-Wiener diversity index
S = number of species

Evenness is categorized as an unstable species distribution when the index is close to 0 and classified as stable when the index value is close to 1.

Simpson's dominance index was calculated by using formula:

$$C = \sum \left(\frac{ni}{N}\right)^2$$

where:

C = Simpson's dominance index
ni = total number of species i
N = total number of all species

Values of dominance index are ranged from 0 to 1 and categorized as:

- No dominant species in the community if C value is close to 0
- A dominating species in the community if C value is close to 1.

RESULTS AND DISCUSSION

Temperature and pH Changes during Fermentation Process

Measurement of temperature and pH were carried out during the fermentation process using Solid-State Fermentation (SSF) method for making fermented feed. The measurement showed differences in daily temperature and pH for 15 days of incubation with insignificant fluctuations (Figs. 1&2).

The highest temperature during fermentation process was 36.6 °C, while 30.2 °C was the lowest. Furthermore, the highest and lowest average pH were 7 and 6.8, respectively. These fluctuations showed the activity of cellulolytic bacteria in the degradation of raw materials. Temperature changes also affected the composition and activity of bacteria during the next day (Hansen *et al.* 2015). Meanwhile, variations in pH functioned as an indicator of the microbes' activity in producing organic acids (Dezam *et al.* 2017). Due to the insignificant fluctuations of temperature and pH during the fermentation process, bacteria enumeration was performed daily. Therefore, information related to the dynamics of each bacteria isolates numbers during the fermentation process can be obtained.

Cellulolytic Bacteria Isolates

Eight isolates were produced from the isolation and screening of cellulolytic bacteria, which were differentiated based on the morphological characteristics of each colony. The characteristics and identification data of each colony are presented in Table 1, while the total number of each isolate is presented in Table 2. Table 3 shows the calculation results for the Shannon-Wiener diversity, evenness, and Simpson's dominance indices of cellulolytic bacteria isolated from fermetodege.

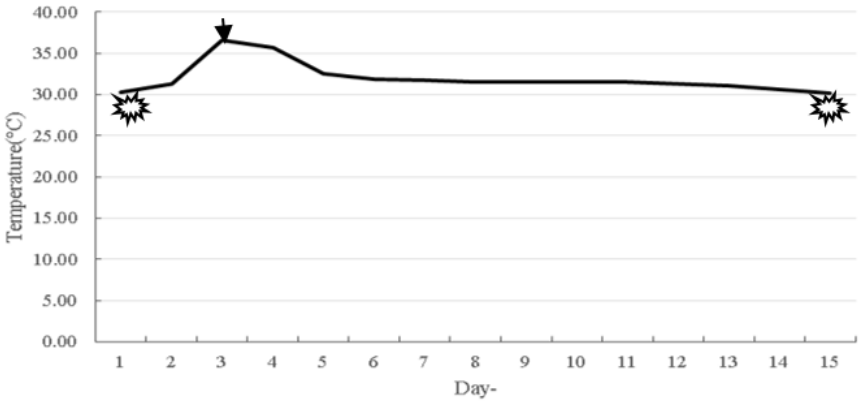

Figure 1 Changes in daily temperature of fermented feed during 15 days of fermentation process using the SSF method.

Notes: ▼ = the highest temperature occurred on the third day of the fermentation process;
✸ = the lowest temperature occurred at the beginning and at the end of the fermentation process.

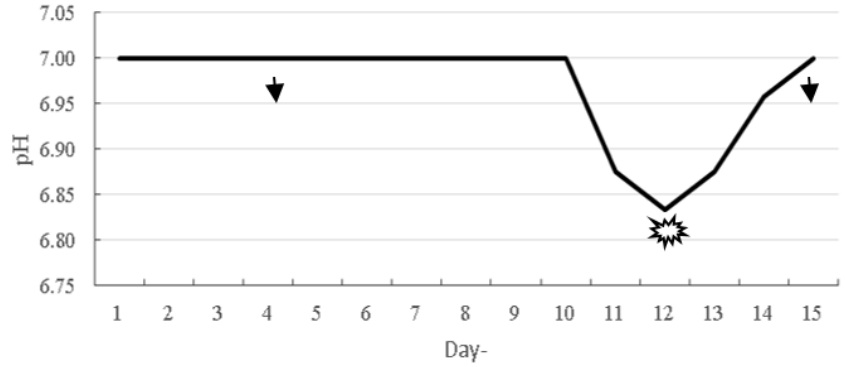

Figure 2 Changes in the daily pH of fermented feed during 15 days of fermentation process

Notes: ▼ = the highest pH occurred on the first day to the tenth and fifteenth day of the fermentation process;
✸ = the lowest pH occurred on the twelfth day of the fermentation process.

Table 1 Identification of cellulolytic bacteria isolated from fermetodege based on Microbact Identification Kits (MicrobactTMGNB12A and 12B)

Aspects	***Bacillus laterosporus* (B1) (70%)**	***Bacillus badius* (B2) (75%)**	***Bacillus pantothenticus* (95,3%) (B3)**	***Bacillus brevis* (B4) (65%)**	***Staphylococcus sciuri* (80%) (B5)**	***Bacillus stearothermophilus* (81,0%) (B6)**	***Burkholderia pseudomallei* (99,5%) (B7)**	***Enterococcus durans* (80%) (B8)**
Oxidase	+	+	+	+	+	+	+	+
Motility	+	+	+	-	+	+	+	+
Nitrate	+	-	+	-	+	-	+	+
Starch	-	-	+	-	+	+	_	-
CMC	+	+	+	-	+	+	_	+
Catalase	+	+	+	-	+	+	+	_
Lysine	-	-	-	-	+	-	+	+
Ornithine	-	-	-	-	+	-	+	+
H2S	-	-	-	-	-	-	-	-
Glucose	-	-	-	-	+	-	-	-
Mannitol	-	-	-	-	+	-	-	-
Xylose	-	-	-	-	+	-	-	-
ONPG	+	+	+	+	+	-	+	-
Indole	-	-	-	-	-	-	-	-
Urease	-	-	-	-	+	-	+	+
VP	-	-	+	+	+	+	+	+
Citrate	-	-	-	-	+	-	+	+
TDA	-	-	-	-	-	-	-	-
Gelatin	-	-	+	-	-	+	-	-
Malonate	-	-	-	-	+	-	-	-
Inositol	+	-	-	-	-	-	+	-
Sorbitol	+	-	-	-	+	-	+	-
Rhamnose	-	-	-	-	+	-	+	-
Sucrose	-	-	-	-	+	-	+	-
Lactose	-	-	-	-	+	-	-	-
Arabinose	-	-	-	-	+	-	-	-

Aspects	*Bacillus laterosporus* (B1) (70%)	*Bacillus badius* (B2) (75%)	*Bacillus pantothenticus* (95,3%) (B3)	*Bacillus brevis* (B4) (65%)	*Staphylococcus sciuri* (80%) (B5)	*Bacillus stearothermophilus* (81,0%) (B6)	*Burkholderia pseudomallei* (99,5%) (B7)	*Enterococcus durans* (80%) (B8)
Adonitol	-	-	-	-	+	-	+	-
Raffinose	-	-	-	-	+	-	-	-
Salicin	-	-	-	-	-	-	-	-
Arginine	-	-	-	-	+	-	+	+
Gram	positive	positive	positive	positive	Positive	Positive	Negative	positive
Cell shape	Rod	rod	rod	rod	coccus	Rod	rod	coccus

Notes: + = provide positive reaction; - = provide no reaction.

Table 2 The total number of each bacteria isolated from fermetodege

Isolates	Day-														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Bacillus laterosporus (B1)*	75.7	95	102.4	106	127	131	138.3	145	141.5	100	97.5	87.5	80.5	75.5	69.5
Bacillus badius (B2)			16.6	23.7	56.5	63.3	23.2		15.3		17.3		21.5	32.5	79.5
Bacillus pantothenticus (B3)				31.5	17.7		19.3						18.5	23.5	84.5
Bacillus brevis (B4)				25.5	37.5				24.3				20.3	31	
Staphylococcus sciuri (B5)								19.5					22	25.5	
Bacillus stearothermophilus (B6)*	58.5	62.3	70.6	65.5	66.5	91.5	98.5	112.5	115.3	110.3	100.5	97.5	88.5	80.5	80
Burkholderia pseudomallei (B7)				55.5							79.5			29	
Enterococcus durans (B8)		82.7	115.5								97.5	101.5	91		

Notes: * = bacteria species present throughout the fermentation process.

Table 3 Diversity, evenness, and dominance indices of cellulolytic bacteria isolated from fermetodege

Day-	Average number of bacteria	Shannon-Wiener diversity index	Evenness index	Simpson's dominance index
1	134.2	0.6849	0.9881	0.5082*
2	240.0	1.0841	0.9868	0.3428
3	305.1	1.2312	0.8881	0.3125
4	307.7	1.6425	0.9167	0.2198
5	305.2	1.4319	0.8897	0.2734
6	285.8	1.0561	0.9613	0.3616
7	279.3	1.1069	0.7985	0.3812
8	277.0	0.8916	0.8116	0.4439
9	296.4	1.0783	0.7778	0.3886
10	210.3	0.6919	0.9983*	0.5012
11	392.3	1.5020	0.9333	0.2322
12	286.5	1.0967	0.9983*	0.3346
13	342.3	1.7178	0.8828	0.2073
14	297.5	1.8173*	0.9339	0.1835
15	313.5	1.3838	0.9982	0.2512

Notes: * = the highest Diversity, Evenness and Dominance indices.

Fermentation process of organic materials consists of several phases, namely mesophilic and thermophilic reactions, cooling as well as maturation (Ishii *et al*. 2000; Yu *et al*. 2007). Bhatia *et al*. (2015) also included the latent phase, which occurred before the mesophilic reaction. Generally, fermentation process starts with the mesophilic phase shown by the increase of the temperature to 45 °C. Mesophilic bacteria activity leads to a rise in temperature and toward the thermophilic phase. Subsequently, the temperature rises higher than 45 °C, showing that the pile enters the thermophilic phase. Increased temperature facilitates the growth of thermophilic bacteria, where maximum metabolic activity is carried out using available nutrients. Depletion of certain nutrients leads to a decrease in the population of thermophilic bacteria and a reduction in metabolic activity, which results in a further reduction in temperature (40-45 °C) when the cooling phase begins. In the maturation phase, the temperature drops to the ambient level (20-30 °C). These phases cannot be determined based on the daily fluctuation of temperature shown in Figure 1. Nonetheless, the curve pattern showed the phases occurring in the fermentation process of this study.

Various physical and chemical changes occurred during fermentation. The changes were shown by the variations in temperature and pH, indicating the production of different microorganism community structures in each phase. Furthermore, bacteria, fungi, and actinomycetes were abundant in the aerobic degradation of solid waste (Bhatia *et al*. 2015).

Fermentation must be carried out by using selected microorganisms because each organic material requires specific enzymes produced by certain microorganisms (Boboesscu *et al*. 2014). For example, fungi *Rhizopus* can significantly lower the crude fiber content due to its lignocellulolytic enzymes (Belewu & Babalola 2009). Meanwhile, several *Bacillus* species produced protease and cellulase enzymes to break down complex organic matter in a plant (Oke *et al*. 2016; Sundarajan *et al*. 2011; Croos *et al*. 2019). The 8 species of cellulolytic bacteria, which had been isolated in this study were *Bacillus laterosporus, B. badius, B. pantothenticus, B. brevis, Staphylococcus sciuri, B. stearothermophilus, Burkholderia pseudomallei*, and *Enterococcus durans.* Certain numbers of these bacteria were also found in other fermentation processes of some materials (Ramos *et al*. 2011).

Bacillus brevis is often used due to its fast growth, low energy requirement, easy management, and ability to be genetically modified for producing cellulase enzymes that are resistant to alkaline conditions as well as high temperatures. Tabssum *et al*. (2018) showed that *B. brevis* was able to produce sugar from various cellulosic materials. Furthermore, the highest cellulase enzyme production of *B. brevis* was obtained by adding

0.5% yeast extract, 0.09% $MgSO_4$, and 0.03% peptone to the bacteria culture.

Liang *et al*. (2014) stated that 22 strains of bacteria have cellulolytic activity in the subtropical region of China and 36.36% belong to genus *Burkholderia*. Among these species, *Burkholderia nodosa* NB1 is known as a cellulase enzymes producer (Tang *et al*. 2018). *Burkholderia* sp. can also degrade lignin from single carbolic cellulosic materials (Akita *et al*. 2016) and produce extracellular protease (Vial *et al*. 2007). The enzymes generated by a blend of fungi and bacteria at the outset, facilitate the decomposition of organic compounds found in water hyacinth and corn cobs. The decomposition process results in feed that is more digestible by sheep. However, there were no significant variations in the weight gain observed among sheep fed with fermented feed at different levels of ration (Fitrihidajati *et al*. 2017).

Enterococcus is found in the digestive tract of various species of animals with forage as their main food source. Dantur *et al*. (2015) also showed that *Enterococcus* was found in sugarcane biomass along with other four genera of bacteria, namely *Klebsiella, Stenotrophomonas, Microbacterium*, and *Bacillus*. These genera showed the activity of endoglucanase including the cellulase group on cellulose substrates (Dantur *et al*. 2015).

Bacillus stearothermophilus is a rod-shaped, gram-positive, and facultative anaerobic bacterium, which is often found in soil and food. Most of these strains serve as probiotics often used in fish feed, while some are pathogenic in humans. Oke *et al*. (2016) stated that *B. stearothermophilus* produced endoglucanase enzymes to degrade lignocellulosic waste.

Cellulose degradation is caused by the synergistic action of three cellulase enzymes, which differ in terms of reaction mechanisms and structural properties. The three enzymes types are endoglucanase, exoglucanase, and -glucosidase (Lynd *et al*. 2002; Taherzadeh & Karimi 2008). Cellulase enzymes often catalyze the hydrolysis of 1,4-β-d-glycosidic bonds to produce glucose, cellobiose, and cello-oligosaccharides from cellulose and other cellulolytic materials. However, each enzyme family has different functions and structures. Endoglucanase randomly cleaves the internal glycosides bonds of cellulose to produce oligosaccharides of varying lengths, while exoglucanase cleaves the open ends to produce cellobiose. The two types of exoglucanase that have been identified include cellobiohydrolase I and cellobiohydrolase II. Specifically, cellobiohydrolase I acts from the reduced end of the substrate chain, while cellobiohydrolase II functions primarily at the end of the unreduced cellulose. β-glucosidase helps to hydrolyze cellobiose into glucose from the unreduced end. In this study, eight indigenous bacteria with cellulolytic activity were found, which could be used as raw materials from water hyacinth, corn cobs, and rice bran as energy and carbon sources. It was observed that variations in nutrients absorption from substrate and ecological factors influenced growth of colonies (Yu *et al*. 2015). Substrates containing cellulose are hydrolyzed by cellulase into glucose, serving as a significant source of carbon and energy for bacteria growth (Silva-Mendoza *et al*. 2020).

Based on the results, effective consortium starter was formulated from eight cellulolytic bacteria in fermetodege. However, the usable raw materials were not limited to water hyacinth, rice bran, and corn cobs to ensure the maintenance of variability and flexibility in the production of feed. Consortium starter from fermetodege bacteria isolates can be used for the manufacture of fermented feed from other cellulolytic materials, such as kitchen wastes or vegetable and fruit markets wastes, thereby enhancing supply of animal feed.

CONCLUSION

This study showed that eight bacteria isolated from fermetodege had cellulolytic activity. These bacteria were *Bacillus laterosporus, B. badius, B. pantothenticus, B. brevis, Staphylococcus sciuri, B. stearothermophilus, Burkholderia pseudomallei*, and *Enterococcus durans*. Cellulolytic activity of these indigenous bacteria should be further studied to determine the most potential bacteria. Therefore, to provide an effective starter formula to facilitate fermentation process of cellulolytic materials, recommendations were made for further studies on these bacteria consortiums.

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