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Activity of Cellulase and Ligninase Enzymes in a Local Bioactivator from Cattle and Buffalo Rumen Contents

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Abstract

Lignin is the main component of agricultural and plantation wastes, such as bagasse, straw and oil palm fronds. Lignocellulosic bonds in lignin, cellulose and hemicellulose can be broken down by enzymes. Numerous studies have utilised plantation waste as feed ingredients. Lignin is the limiting factor that affects the digestibility of this material. Therefore, the lignin content of plantation waste must be reduced before it is used as a feed ingredient. The use of local bioenzymes will be effective in breaking lignocellulose bonds. Thus, finding sources of enzymes that are easy to obtain, inexpensive to produce and effective as lignocellulose-degrading enzymes is necessary. This study aims to determine the activity of cellulase and ligninase enzymes in a bioactivator from rumen contents incubated for 7 days with different enzyme energy sources. The treatments included cattle and buffalo rumen contents added with molasses, palm frond, palm leaf extract and each enzyme. The parameters observed were the enzyme activities of cellulase, laccase, lignin and manganese peroxidase (MnP). Microbial identification was also performed. The results of statistical analysis showed insignificant differences ($P > 0.05$) amongst the parameters of the enzyme activities of cellulase (2.22–3.51 U/ml), laccase (10.62–20.11 U/ml), lignin peroxidase (1.74–4.93 U/ml) and MnP (2.40–7.06 U/ml). *Lactobacillus* sp. was identified through bacterial identification. Therefore, the live microbes discovered in the local microorganism solution originated from the study environment and not from the rumen contents.

Keyword: Enzyme activity, microbial morphology, cellulase, lignase, rumen microbes

1. Introduction

Animal feeding is a major factor determining the success of livestock businesses. Many studies have used agricultural and plantation by-products as substitutes for field grass in ruminant feed. These by-products mostly contain cellulose, hemicellulose and lignin that consist of lignocellulosic bonds. The digestibility of ruminant feed ingredients is influenced by the contents of lignin, cellulose and soluble substances.These rumen microorganisms could not for lignin degradation because they do not generate enzymes with ligninolytic activity (Pollegioni et al., 2015). Oil palm fronds as animal feed berries are low crude protein about 1.6%, high crude fiber content reaching 56.1%, and contain 79.27% ADF, 64.25% NDF, 12.91% cellulose, 15.34% hemicellulose and 15.34% lignins (Astuti et al., 2019; Tafsin et al, 2019). Lignin is a complex, heterogeneous phenylpropanoid polymer that accounts for 25%–30% of plant biomass. It is resistant to microbial degradation under natural conditions. Lignin and cellulose are the main plant components that are enzymatically degraded by bioactivators, such as the enzymes cellulase and ligninase. Ligninolytic microbial systems, which are primarily composed of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Glenn et al., 1983), have been used to improve digestibility and nutritional value. LiP and MnP are extracellular peroxidase enzymes that use H_2O_2 to degrade lignin, while laccase is a copper-containing enzyme that uses molecular oxygen to degrade lignin (Hattaka, 1994). Laccase is a metalloenzyme that can degrade lignin during the bio-catalysis process (Kameshwar and Qin, 2016). The use of commercial enzymes for animal feeding will need additional livestock operational costs. Therefore, because of it is necessary to find sources of natural enzymes that are cheap and easy to produce.

Using microbes for enzyme production has several advantages, including low production costs, short production times, high growth speeds, and easy control. The bacteria responsible for lignin degradation can be found in environments such as soil, digestive system of herbivora, wood-eating insects, effluents from paper industry, sludge, etc. (Brown and Chang, 2014; Tian et al., 2014). The contents of the rumen cattle contain crude protein, about 18.52 - 19.56%, Amino acids include lysine, leucine, alanine, asprate, arginine, valine, threonine and low methionine (Jovanović and Čuperlović, 1997). Rumen contents It also contains high crude fiber, calcium, phosphorus and magnesium (Agbabiaka et al., 2012; Elfaki and Abdelati, 2015). Rument microbes will produce enzymes in accordance with the given

substrate. For example, rument microbes will produce enzymes that degrade fiber when given straw and enzymes that break down tannins when given calliandra (Wina, 2005). Lignin and cellulose are the main plant components that are enzymatically degraded by cellulose and lignase. These enzymes are produced by bioactivators (Pandey et al., 2000). Molasses is the waste of sugar mills; that can use as a fermentation stimulant to increase the rate of silage acidification by providing fermentable sugars for the growth of Lactic Acid Bacteria (Guo et al., 2014).

Enzymes are biopolymer molecules that are composed of a series of amino acids in an ordered and fixed composition and chain arrangement. They are proteins that are produced and used by living cells to catalyse chemical reactions with a high level of specificity and increased reaction rates (Beilen and Li, 2002; Richana, 2002). Thus, enzymes have various advantages over conventional processes using chemicals. However, the main obstacle to the industrial application of enzymes is the high price of enzymes, and cannot be used repeatedly (Huey, 2008; Troger and Niranjan, 2010). Lignin-degrading or ligninolytic enzymes include laccase (polyphenol oxidase), LiP and MnP; all three are extracellular multienzymes that participate in lignin depolymerisation (Sánchez, 2010).

Cellulases are complex enzymes that gradually cut cellulose chains into glucose. Fungi, bacteria and ruminants produce cellulase. The commercial production of enzymes usually applies fungi or bacteria. Although the production of cellulases from ruminants has been underappreciated, cellulases originating from ruminants are being produced given their high availability. The rumen microbes are considered the most efficient microbial system in degrading lignocellulosic biomass (Flint et al., 2008), because they can produce enzymes from their environment and can be used in the feed and food industry, cellulose biofuels, and other industrial processes (Ribeirio et al., 2016). In contrast to commercial enzymes, cellulases have the potential to be produced from beef rumen fluid. Therefore, this study aims to determine the activity of cellulase and ligninase as a local bioactivator produced by incubating rumen contents with several different ingredients as the microbe substrate. This research can produce complex enzymes (cellulase, MnP, LiP and laccase) from waste materials that are cheap and easy to obtain.

2. Materials and methods

The materials used in this study were cattle and buffalo rumen contents, molasses, soybean soaking wastewater, oil palm fronds, oil palm leaves and some chemicals for enzyme activity measurement.

2.1. Bioactivator process

The rumen contents of cattle and buffalo were collected from an abattoir and placed in tubes. All the rumen contents were added with molasses and tofu soaking water. The treatments were the addition of palm leaves, palm fronds and a mixture of these materials, which had high lignin contents. Palm fronds and leaves were added to induce the bioactivator to produce ligninase. The rumen contents, molasses and tofu soaking wastewater were mixed at the ratio of 1:1:8 with 10% oil palm fronds and leaves. The tubes were tightly closed. Then, a hole was made in the middle and connected with a hose to a small bottle filled with water to catch fermentation waste. The tubes were incubated for as long as 10 days as shown in Figure 1.

Figure 1. Fermentation with the local bioactivator

2.2. LiP activity test

A total of 0.2 ml of enzyme filtrate (sample extract and phosphate buffer shaken for 1 h and then centrifuged for 10 min at 10000 rpm), 0.05 ml of 5 mM H_2O_2, 0.1 ml of 8 mM veratrine alcohol, 0.2 ml of 0.05 M acetate buffer (pH 3) and 0.45 ml of distilled water were added to a cuvette and then shaken. The absorbance of the solution at 310 nm was recorded at 0 and 30 min intervals. One unit of LiP enzyme activity was defined as the amount of enzyme that caused the conversion of 1 μmol (1μmol = 10^{-6} mol) veratril alcohol per minute (Tien and Kent, 1984).

2.3. MnP activity test

A total of 0.1 ml of 50 mM Na-lactate buffer (pH 5) was added with 0.1 ml of 4 mM guaiacol, 0.2 ml of 1 mM $MnSO_4$, 0.1 ml of 1 mM H_2O_2 and 0.3 ml of distilled water, as well as 0.2 ml of enzyme filtrates. The solution was then checked and read at the wavelength of 465 nm at 0 and 30 min (Leonowicz and Grzywnowicz, 1981).

2.4. Laccase activity test

A total of 0.4 ml of enzyme filtrate was added with 0.5 ml of acetate buffer (pH 5) and 0.1 ml of 1 mM 2′-azinobis-3-ethylbenzothiazole-6-sulphonic acid. Measurements using spectrophotometer were taken at 420 nm at 0 and 30 min (Wariishi et al., 1992).

Enzyme activity tests were conducted at the biotechnology laboratory of the Faculty of Animal Husbandry, Andalas University, Padang. Microbial morphology identification was performed at the Baso Veterinary Centre Laboratory, Bukit Tinggi. The analysis was continued by identifying the morphology of the fungi and bacteria present in the local bioactivator rumen contents. Microbes were identified on the basis of the results of the best enzyme activity evaluation. Samples were inoculated onto sodium agar (NA) medium for the identification of bacteria, and potato dextrose agar (PDA) was used as the medium for fungal/mould growth. The samples were diluted to the concentration of 10^{-1} –10^{-10}, and the selected isolates were further analysed on the basis of colony distribution

2.5. Experimental design

A 2 × 4 factorial randomised block design with three replications for each treatment was used in this study. Factor A was the type of rumen content: A1 = rumen cattle and A2 = buffalo rumen. Factor B was the microbial energy substrate: B1 = molasses, B2 = molasses + palm frond extract, B3 = molasses + palm leaf extract and B4 = molasses + palm frond and leaf extract. Enzyme activity data were processed by using analysis of variance. The bioactivator was identified by using the described method. Significant differences ($P < 0.05$) were further tested by using Duncan's multiple range tests.

The variables were observed through the isolation and identification of the bioactivator (fungi and bacteria) based on morphology and cellulase and ligninase activity tests on the crude bioenzymes in rumen content. Morphological data were obtained through descriptive analysis.

3. Results

3.1. Enzyme activity test

The activities of cellulose, laccase, LiP and MnP were tested. These enzymes are essential for lignin degradation. The average results of the enzyme activity test for the local bioactivator in rumen content are shown in Table 1.

Table 1. Average activity (U/ml) of the local bioactivator in rumen contents

Factor B	Factor A				Average
	B_1	B_2	B_3	B_4	
Cellulase enzymes					
A1	3.51	2.41	2.81	2.22	2.74
A2	3.44	2.52	3.31	3.64	3.23
	3.48	**2.46**	**3.06**	**2.93**	**2.98**
Lacasse Enzymes					
A1	20.11	16.40	11.70	15.91	16.03
A2	11.00	12.86	10.62	14.59	12.27
	15.55	**14.63**	**11.16**	**15.25**	**14.15**
Enzim Lignin Peroksidase					
A1	4.06	4.93	2.80	4.11	3.97
A2	2.61	1.74	4.16	3.80	3.08
	3.34	**3.34**	**3.48**	**3.95**	**3.53**
Manganese Peroxidase Enzymes					
A1	2.40	5.61	3.39	7.06	4.61
A2	6.86	4.17	3.18	5.18	4.85
	4.63	4.89	3.29	6.12	4.73

Note: A1 = cow rumen content, A2 = buffalo rumen content, B1 = rumen content only, B2 = rumen content and palm fronds, B3 = palm leaves and B4 = leaves and palm fronds

3.2. Identification of microbial morphology

The results of the chemical analysis are shown in Table 2. Microscopic and macroscopic analyses revealed that the bacterial colonies in Figure 2, the fungal colonies in Figure 3 and the bacterial isolates in Figure 4 had similar bacillus shapes.

Table 2. Chemical examination results for microbial isolates on NA media

No	Treatment	NA 1	NA 2
1.	Colony (color, shape, traits)	White	White
2.	Grams (morphology, spora)	+ bacteri	+ bacteri
3.	Aerobic / Anaerobic	A	A spora
4.	TSIA	M/K	M/K
5.	Gas	-	+
6.	H_2S	-	-
7.	catalase	+	+
8.	Oxidase	-	-
9.	Motility	+	+
10.	Indole	-	-
11.	Urea	+	+
12.	Citrate	-	-
13.	Lactose	-	+
14.	Glucose	-	+
15.	Sucrose	-	-
16.	Mannitol	-	+
17.	MR	+	+
18.	VP	+	+
19.	OF	-	+
20.	Nitrat	-	+
21.	Gelatin	+	+
22.	Genus	*Basillus, sp 1*	*Basillus sp 2*

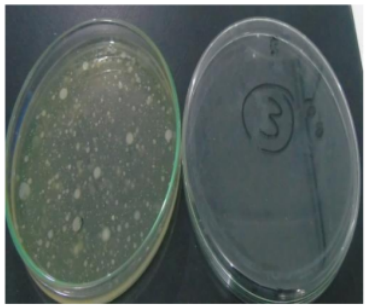

Figure 2. Bacterial colonies on 10^{-8}

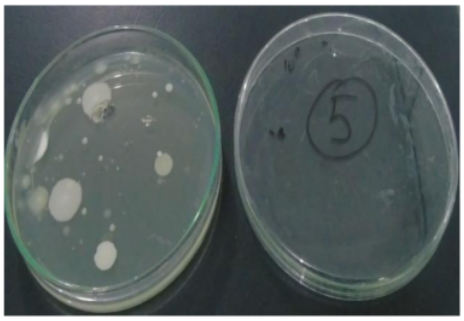

Figure 3. Fungal colonies on 10^{-5}

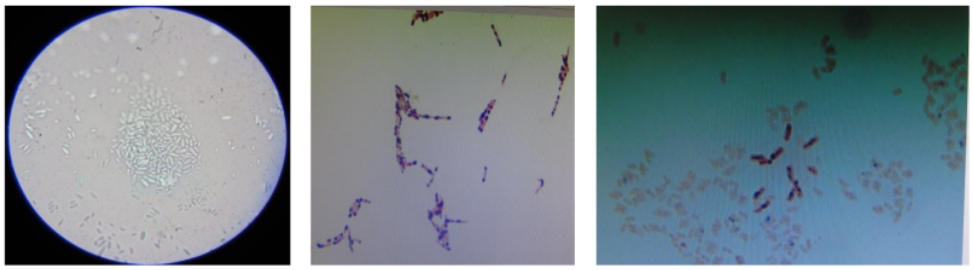

Figure 4. Staining of bacilli in the genus *Bacillus* in rumen contents on NA media

4. Discussion

4.1. Enzyme activity test

Statistical analysis showed that no interaction effect ($P > 0.05$) existed between the rumen source (cattle and buffalo) and microbe energy source on the activities of cellulase, laccases, LiP and MnP. The data in Table 1 show that LiP enzyme activity ranged from 2.46 U/ml to 3.48 U/ml. LiP enzyme activity in this study was higher than that in the work of Ilmi and Kuswytasari (2013), who found that LiP from *Gliomastix* sp. T3.7 had the enzyme activity of 0.06–1.022 in corn hump waste at pH 5 and 25 °C–35 °C. The data showed the rumen contents were the source of the bioactivator that was ingested and remained dominant in the form of fiber from forage consumed by livestock. For all treatments, the main energy source was molasses and soybean soaking water. Therefore, the main energy source in this work was very representative and supplied the energy needed by the microbes in the rumen to grow and exhibit high production. The average cellulase activity in this study was considerably higher than that in a previous work (Murtiyaningsih and Hazmi, 2017) that obtained the highest enzyme activity of 0.028279 U/m for cellulase from cellulolytic soil bacteria. This difference

was due to the large number of microbes in the rumen contents and the addition of molasses and tofu soaking wastewater that further increased microbial growth. Another study (Astuti et al., 2020) found 40 × 10^{12} total colonies in 1 ml of local bioactivator rumen contents mixed with molasses, soybean soaking water and palm oil fronds.

In this work, the average laccase enzyme activity ranged from 11.0 U/ml to 20.11 U/ml, LiP activity ranged from 1.74 U/ml to 4.93 U/ml and MnP activity ranged from 2.40 U/ml to 7.06 U/ml. Heterologous laccase production has been used for obtaining increased amount of the enzyme (Debnath and Saha, 2020). The activity of laccase and LiP enzymes in this study was much higher than the results of the study of Dimawarnita et al. (2019) who found LiP activity of 0.269 U/ml and laccase activity of 0.35 U/ml in Pleurotus ostreatus in oil palm empty fruit bunch media. Research of Fithri et al. (2020), Laccase could degrade lignin with apparent damage the lignocellulose substrate of corn cob and rice straw. Laccase, LiP and MnP activities of 2.02, 1.677 and 0.33 U/ml, respectively, were observed in sugarcane fermentation by 10% *Phanerochaete chrysosporium* because the microbes that thrived in the local activator were supplied with palm fronds and leaves with high lignin contents. Dhakar et al. (2014) maximum laccase production was observe as 28.2 U/l. Rumen microbes develop and produce enzymes on the basis of given feed (Astuti et al., 2021).

4.2. Identification of microbial morphology

The morphology of the fungi and bacteria present in the local bioactivator rumen contents was analysed. The microbes were identified on the basis of the best results of the enzyme activity evaluation.

Figure 2 shows that the bacterial colonies were circular, small, spread out and thin and had flat colony edges, a white colour and transparent structure. Morphological observation revealed that the fungal colonies had a circular colony shape, convex elevation, uneven edges, white colour and transparent structure (Figures 2 and 3). The shape of the colonies in this study was the same as that of the colonies reported by Yogyaswari et al. (2016), who found that the bacterial isolates from the rumen contents of Fries Holland cows formed white colonies. The microscopy observations demonstrated that all of the bacterial isolates were Gram-positive bacilli (Table 2 and Figure 4).

The Gram staining results for the isolates showed the bacteria were Gram-positive bacteria that were negative for H_2S content and positive for catalase content. Furthermore, microscopic and macroscopic analyses revealed that the bacterial isolates shared similar

bacillus shapes and belonged to two genera, namely, *Bacillus* sp. 1 and *Bacillus* sp. 2 (Table 2).

The bacterial identification results demonstrated that *Bacillus* sp. 1 was present in the local bioactivator comprising rumen content added with palm fronds and that *Bacillus* sp. 2 was present in the local bioactivator comprising rumen content added with fronds and palm leaves. In addition, the microscopic and macroscopic observations showed that the bacterial isolates in Figure 3 all had similar bacillus shapes. Some bacterial strains could lignin-degrading such as *Bacillus sp.CS-1, Bacillus pumilus, Bacillus atrophaeus, Bacillus sp, ect*. Kumar et al. (2021) state some bacterial strains could lignin-degrading such as *Bacillus sp.CS-1, Bacillus pumilus, Bacillus atrophaeus, Bacillus sp, ect*.

5. Conclusion

On the basis of the research results, the best bioactivator that can be used to improve feed quality was concluded to be rumen content mixed with molasses, soybean soaking water and oil palm fronds and leaves. It had the cellulase, laccase, LiP and MnP activities of 2.2, 15.91, 4.11 and 7.06 U/ml, respectively. Bacterial identification revealed that *Bacillus* sp. was present under the best treatment.

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