Artikel submit pertama

THE EVALUATION OF ENZYME ACTIVITY OF CELLULASE, LIGNINASE, AND IDENTIFICATION OF MICROBIAL MORPHOLOGY AT LOCAL BIOACTIVATOR FROM CATTLE AND BUFFALO RUMEN CONTENT WITH DIFFERENT ENERGY SOURCES

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ABSTRACT

This study aims to determine the activity of cellulase and ligninase enzymes on local bioactivator. This was based on the rumen contents of the rumen incubated for seven days with supplies of different sources of enzyme energy. The method used was a factorial design of 2 x 4 with three replications for each treatment. Factor A was the type of Animal (Cattle Vs Buffalo), factor B is the addition of an enzyme source supply material: B1 = Molasses, B2 = Molasses + palm frond extract, B3 = Molasses + palm leaf extract, B4 = Molasses + palm frond, and palm leaf extract. Also, the parameters observed were the activity of enzymes cellulase, laccase, lignin and manganese peroxidase, as well as microbial identification. The results of statistical analysis showed that the activity of the enzymes cellulase 2,22-3,51 U/ml, laccase 10,62- 20,11 U/ml, lignin peroxidase 1,74-4,93 U/ml, and manganese peroxidase 2,40-7,06 U/ml showed insignificant differences ($p > 0.05$). The results of microbial identification found the bacteria Lactobacillus sp. Based on these, it was concluded that the microbes discovered in the local microorganism solution live because of the study environment, not the microbes inherited from the rumen's contents.

INTRODUCTION

Animal feeding is a major factor in determining the success of the livestock business. Furthermore, many study uses by-products of agriculture and plantation as substitutes for the field grass. These by-products mostly contain lignocellulosic bonds that consist of cellulose, hemicellulose, and lignin, which cause the low nutritional value of these ingredients when directly fed as feed. Used of palm oil fronds as animal feed barried are low crude protein about 2.11%, high crude fiber content reaching 46.75% **[1]**(Murni et al., 2008). Astuti et al., (2019) reported the content of ADF 56,93%, NDF 78,05%, cellulose 21,91%, hemicellulose 15,34% and Lignin 15.34% [2]. Lignin is a polymer complex phenylpropanoids, heterogeneous, and 25-30% plant biomass. It is quite resistant to microbial degradation under natural conditions. Lignin and cellulose are the main components in plants enzymatically degraded by microorganisms such as enzyme cellulase and ligninase. Ligninolytic microbial systems have been used in improving digestibility and nutritional value. Primarily three enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase [3.4.5.6] (Glenn et al., 1983; Hatakka and Uusi-Rauva, 1983; Glenn and Gold, 1985), have been considered responsible for lignin degradation of animal feeds [7](Zadrazil, 1980). Lignin peroxidase (LiP) and manganese peroxidase (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 [8] (Hattaka, 1994).

The use of microbes for enzymes producer has several advantages, including low production costs, short production time, high growth speed, and ease to control. Factors such as fermentation medium, temperature, aeration, agitation, pH, and strains, which play a role in producing these enzymes, need to be optimized. The Cheaped and inexpensive Fermentation media is required since it is easy to obtain and produce expected enzymes for large production (Trismilah et al., 2003).

Many rumen contents on the slaughterhouse waste pollute the environment due to the rumen's abundant availability contents. It was being seen based on the slaughterhouse's number of slaughtered cattle and buffalo. Based on BPS Indonesia (2021) data, the average of Livestock Slaughtered at Slaughtering House (Abattoir) in 2019-2020 was 1069.614 heads of cows and 27,487 heads of buffaloes. Beef rumen fluid, apart from containing rumen microbes and enzymes secreted by rumen microbes, also contains food substances resulting from overhaul rumen microbes and enzymes and vitamins and minerals that dissolve in the rumen fluid. The cow's rumen content was 9.29% water; 8.45% crude protein; 1.23% crude fat;

33.53% crude fiber; 0.20% Ca; 0.45% P; 16.19% ash; and 31.60% NFE, and vitamin B12. The rumen contents have the potential as feed additives (Abbas, 1987). This slaughtering of livestock leaves the contents of the rumen untapped, only to be thrown away.

Rumen fluid deposits are rich in protein, amino acids, vitamins, and minerals. The composition of amino acids, minerals, vitamins, and enzymes also depends on the feed treatment (Budiansyah et al., 2011). The assumed rumen microbes would produce enzymes according to the given substrate. For example, when given straw, it will produce enzymes to degrade fiber and break down tannins when given a substrate of calliandra (Wina, 2005). The fiber content of Lamtoro leaf meal decreased by 53.640% with 100 ml/kg of rumen content enzyme extract (Fitriyani, 2011). Lignin and cellulose were the main components in plants that the enzyme cellulase and ligninase enzymatically degrades. These enzymes were produced by microorganisms (Pandey et al., 2000). Molasses are the waste of sugar mills, cheapest and easily as carbon sources rich in nutrients and minerals, and have the potential for microbial growth media (Anggraini et al., 2016).

Enzymes are biopolymer molecules composed of a series of amino acids in an ordered and fixed composition and chain arrangement. Enzymes were proteins produced and used by living cells to catalyze chemical reactions with a high level of specification and an increase in reaction rates (Richana, 2002; Beilen & Li, 2002). Thus, enzymes have various advantages over conventional processes using chemicals. However, the main obstacle for applying enzymes in the industry is the high price of enzymes, and the enzyme cannot be used repeatedly (Huey, 2008; Troger & Niranjan, 2010; Wibisono, 2010).

Cellulases are complex enzymes that gradually cut the cellulose chains into glucose. Fungi, bacteria, and ruminants produces cellulase. The commercial production of enzymes usually uses fungi or bacteria. The production of cellulase enzymes from ruminants has not been much appreciated, however, cellulases originating from ruminant is being produced given their large availability. Rumen fluid enzymes as an alternative technology are used in hydrolyzing crude fiber to increase the nutritional value of local feed raw materials (Pamungkas, 2012). Hartati's (2012) stated that cellulase enzymes have the potential to be produced from beef rumen fluid compared to commercial enzymes. Therefore, this study aims to determine the activity of cellulase and ligninase enzymes in local microorganisms with several different ingredients added. Additionally, microbes are considered to produce enzymes according to the food they get.

MATERIALS AND METHODS

The material used in this study was rumens content of cattle and buffalo, molasses, soybean water immersion, oil palm frond, oil palm leaf, and some chemistry for measuring enzyme activity.

The bioactivator process: The rumen content of cattle and buffalo were collected from the abattoir and placed in tubes. All the content of rumen treatments was added with molasses and tofu water immersion. The treatments were the addition of palm leaves, palm fronds, and a mixture of both, which had high lignin content. The addition of palm fronds and leaves is intended, hence, the microorganisms that develop are microorganisms capable of producing the ligninase enzyme. The contents of the rumen, molasses, tofu soaked wastewater were mixed in a ratio of 1:1:8, and 10% of the oil palm frond and leaf. The tube is tightly closed, after which a hole is made in the middle and connected with a hose to a small bottle filled with water to catch the waste of fermentation, then incubated for as long as ten days, as shown in Figure 1.

Figure 1. Fermentation Process local bioactivator

The method for enzyme activity test:

Test Activity of Lignin Peroxidase (LiP) Enzymes

A 0.2 ml of enzyme filtrate (extract sample and buffer pospat were shaken until 1 hour and then centrifuged for as long as 10 minutes with 10000 rpm), 0.05 ml of H_2O_2 5 mM, 0.1 ml of veratrine alcohol 8 mM, 0.2 ml of acetate buffer 0.05 M pH 3, and 0.45 ml of distilled water are added to cuvette and then shaken. The absorbance of the solution was at a wavelength of 310 nm for 0 and 30-minute intervals. One unit of enzyme activity in LiP is defined as the amount of enzyme that causes the conversion of 1 micromol (1µmol = 10-6 mol) veratril alcohol per minute (Syafrizal, 2007)

Test the Activity of Manganese Peroxidase (MnP) Enzymes

As much as 0.1 ml of 50 mM Na-lactate buffer pH five is added with 0.1 ml of 4 mMguaiacol, 0.2 ml of $MnSO_4$ 1 mM, 0.1 ml of H_2O_2 1 mM, and distilled water 0.3 ml, as well as 0.2 filtrates are added enzyme. The solution was then checked and read at a wavelength of 465 nm for 0 and 30 minutes (Leonowicz and Grzywnowicz, 1981)

Test the Activity of Lakase Enzymes

As much as 0.4 ml of enzyme filtrate plus 0.5 ml pH five acetate buffer of 0.5 ml and 0.1 ml of 1 mM ABTS. Then it was checked, and measurements were read to 420 nm wavelength. The reading was taken for 0 and 30 minutes (Wariishi et al. l., 1992).

Enzyme activity tests were conducted in the biotechnology laboratory of the Faculty of Animal Husbandry, Andalas University, Padang, and identification of microbial morphology was performed at the Baso veterinary center laboratory, Bukit Tinggi. The analysis was continued by identifying the morphology of fungi and bacteria present in the local bioactivator rumen contents. The identification of microbes was performed based on the results of the best enzyme activity evaluation. Samples were inoculated on Sodium Agar (NA) medium for identification of bacteria and Potato dextrose Agar (PDA) media as a medium for growing fungi/molds. The samples were diluted in 10^{-1} - 10^{-10}, and the selected isolates were further to be analyzed based on even colony distribution

Experimental design:

The factorial randomized block design 2 X 4 with three replications for each treatment used in this study. Factor A is the type of rumen content: A1 = Rumen cattle, A2 = Buffalo Rumen, Factor B supplies microbial energy: B1 = Molases, B2 = Molases + palm frond extract, B3 = Molases + palm leaf extract, B4 = Molases + extract of palm fronds and leaves. Enzyme activity data processing was performed using analysis of variance while identifying microorganisms using the described method. When the study resulted in a significant different affected ($p<0.05$), it will be further tested using duncan's multiple range tests,

The variables to be observed were the isolation and identification of microorganisms (fungi and bacteria) by morphology and cellulase and ligninase activity tests for crude bio enzymes of rumen content. The morphology data are shown in description analysis.

RESULTS AND DISCUSSION

Enzyme Activity Test

The enzymes activity test were cellulase enzyme, Lacasse Enzymes, Enzim Lignin Peroksidase, Manganese Peroxidase Enzymes. Furthermore, these enzymes are essential for lignin degradation. The average results of the enzyme activity test for local microorganisms in the rumen are shown in Table 1.

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

Factor B	Factor A				Rataan
	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 = contents of cow's rumen, A2 = contents of buffalo's rumen, B1 = contents of rumen only, B2 = contents of rumen and palm fronds, B3 = palm leaves, and B4 = leaves and palm fronds

The results of the analysis of statistics showed no interaction effect ($P > 0.05$) between the kind of rument resource (cattle and buffalo) with the sources of microbe energy on the activity of cellulase enzymes, laccases, Lignin Peroxidase Enzymes, and Manganese Peroxidase Enzymes. The data shows the rumen contents were used as a source of microorganisms that were ingested and still dominant in the form of fiber from forage consumed by livestock are given the main energy source in the form of molasses and tofu

soaking water for all treatments. Therefore, it is suspected that the main energy source shown is very representative, supplying the energy needed by microbes in the rumen to grow and develop to produce high value. This study's average cellulase enzyme activity result was much higher than Murtiyaningsih and M. Hazmi's (2017), measuring the activity of cellulase enzymes from cellulolytic bacteria from soil and getting the highest enzyme activity of 0.028279 U/ml. This was due to a large number of microbes in the rumen contents, then the addition of molasses and tofu soaking wastewater further increases microbial growth. Astuti et al. study (2020) found 40 x 10^{12} total colonies in 1 ml of local bio activator rumen contents mixed with molasses, soybean soaking water, and palm oil fronds.

The average laccase enzyme activity ranged from 11.0 to 20.11 U/ml, the mean lignin peroxidase enzyme ranged from 1.74 to 4.93, while the manganese peroxidase enzyme ranged from 2.40 - 7.06 U/ml. Laccase enzyme activity was much higher than Dimawarnita and Panji's (2018), which measured enzyme activity in Pleurotus ostreatus with media containing OPEFB 0.35 U/mL and lignin peroxidase (LiP) activity of 0.269 U/mL. Astuti et al. (2021) stated lignin enzyme activity of sugarcane fermentation by 10% Phanerochaete chrysosporium were laccase 2.02 U/ml, LiP 1,677 U/ml, MnP 0,33 U/ml. This was because the microbes that thrive in this local activator have been added to the palm fronds and leaves high in lignin content. Rumen microbes will develop and produce enzymes based on the feed given.

Identification of Microorganism Morphology

The analysis identifies fungi and bacteria's morphology present in the local bioactivator rumen contents. The identification of microbes was performed based on the results of the best enzyme activity evaluation.

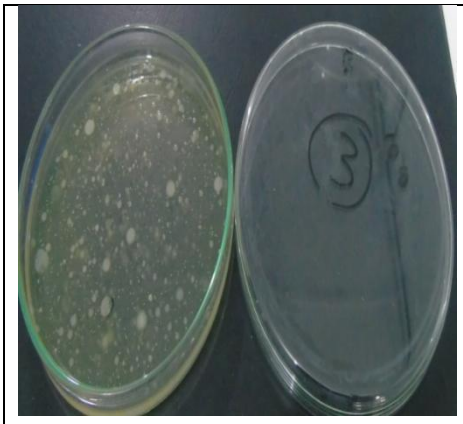

Figure 2. Bacterial Colonies on 10^{-8}

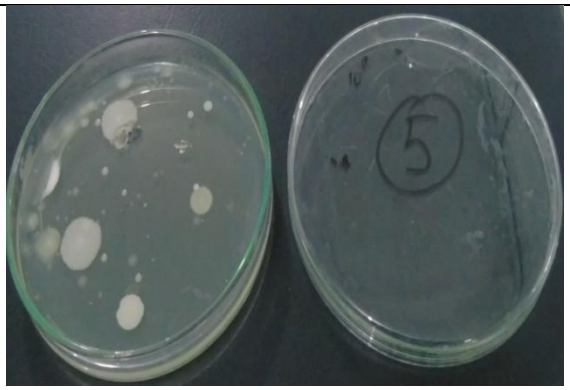

Figure 3. Fungal Colonies on 10^{-5}

Figure 2 shows that The Bacterial colonies are circular, small spread out, thin, the edges of the settlements are flat, white in color, and the structure is transparent. Morphological observations of fungal colonies showed circular colony shape, elevation convex, uneven edges, white color, and transparent structure (Figure 3). The results of the chemical analysis are shown in table 2.

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

No	Treatment	NA 1	NA 2
1.	Koloni(Warna, bentuk, Sifat)	White	White
2.	Gram (Marfologi, Spora)	+ bacteri	+ bacteri
3.	Aerobic / Anaerobic	A	A spora
4.	TSIA	M/K	M/K
5.	Gas	-	+
6.	H2S	-	-
7.	catalase	+	+
8.	Oxidase	-	-
9.	Motility	+	+
10.	Indole	-	-
11.	Urea	+	+
12.	Citrat	-	-
13.	Laktosa	-	+
14.	Glukosa	-	+
15.	Sukrosa	-	-
16.	Mannitol	-	+
17.	MR	+	+
18.	VP	+	+
19.	OF	-	+
20.	Nitrat	-	+
21.	Gelatin	+	+
22.	Genus	*Basillus, sp 1*	*Basillus sp 2*

The gram staining results on the isolates showed gram-positive bacteria, negative H2S content, positive catalase. Furthermore, in the microscopic and macroscopically observations,

bacterial isolates had similarities in all isolates bacillus shaped, and there were two genera, namely Bacillus sp one and Bacillus sp 2, Table 2).

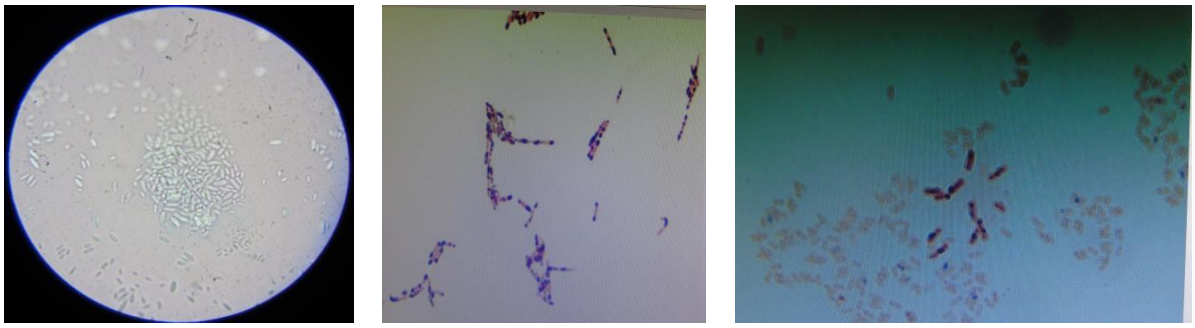

Figure 4. The image of local microorganisms staining the contents of the rumen in the form of bacilli on NA media with the genus *Bacillus sp*

The bacterial identification results showed Bacillus sp 1 in the rumen-filled moles added with palm fronds, and bacillus sp 2 in the local bioactivator rumen content isolates with added fronds and palm leaves. In addition, the microscopic and macroscopically observations showed that bacterial isolates in Figure 3 had similarities in all bacillus shapes.

RESULT

Based on the research results, it was concluded that the best bioactivator that can be used to improve the quality of the feed was rumen content mixed with molasses, soybean soaking water, leaves, and oil palm frond.

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Authors : Tri Astuti, Syahro A. Akbar, Nasir Rofiq, Novirman Jamarun, Nurul Huda, Ahmad Fudholi

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Abstract: * Needs minor revisions prior to the amendment of the main content. * An abstract is often presented separately from the article, so it must be able to stand alone. Hence the problem statement, aim, novelty and results of the study have to be included in. * The abstract can be more concise. * The factorial design and replications need not to be mentioned in Abstract, whereas the important results such as what type of animal or enzyme contribute significantly towards the resultant parameter (cellulase and ligninase enzyme activity) should be mentioned. Should edit properly on this. Introduction: * Describe more on the environmental issues together with treatment possibility and methods * Highlight novelty in last paragraph. * Kindly refer papers below as they are highly relevant to this report: * "A review on ammonia, ammonia-hydrogen and ammonia-methane fuels" * "Microalgae and ammonia: a review on inter-relationship" * "Optimization of hydrolysis-acidogenesis phase of swine manure for biogas production using two-stage anaerobic fermentation" * "Microalgae cultivation in palm oil mill effluent (POME) treatment and biofuel production" * Should update the references are quite a number are more than 20 years. Main body: * The main objective and novelty of this work is still deemed not highlighted enough. The authors should put in more efforts to revise the discussion properly in order to let the readers understand the importance of this work. * Kindly improve on the discussion. What is the significance of the results of the work? Include more relevant literatures. * Further enhance the discussion section, together with the results. * Are there not more recent references could be used in this study? * The authors should consider tabulate the factorial design parameters. * In Table 2, there are some terms seem unlike English. Please correct them accordingly. * Regarding the references, there are large number of them are not English references. Please correct them.	* The abstract was revised, which the main introduction to highlight this manuscript. * An abstract was presented separately from the article. Which the problem statement, aim, novelty and results of the study was included in. * The abstract was revised. * 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 .. * The introduction was revised, which the environmental issues together with treatment possibility and methods was included in. * Highlight novelty in last paragraph was added. "This research can produce complex enzymes (cellulase, MnP, LiP and laccase) from waste materials that are cheap and easy to obtain". * The main body was revised. * Little data currently exist of this study in literature to the best of the authors' knowledge. * Done * In Table 2 was revised. * Little data currently exist of this study in literature (English references) to the best of the authors' knowledge.

Conclusion: * This section is too short! * Kindly improve to include in more concise and significant results. * Should include some present challenges and possible routes to improve them. Describe them in more details. Papers for further reading: "Two-step thermodegradation kinetics of cellulose, hemicelluloses, and lignin under isothermal torrefaction analyzed by particle swarm optimization".	Conclusions was revised.
Reviewer #2 1. Highlights should be revised. Please check maximal no of highlights with no of word counts. 2. Why does the title meant on 'Local microorganisms'? Consider to use native or indigenous 3. Please improve the discussion as only two scopes were discussed. Much information is required. There is no discussion with comparison with the results from other literature. Discussion should be more comprehensive. So far, it could be observed that the methodology and introduction are more than the results and discussions. 4. Please amend the conclusion as currently the conclusion is only One sentence.	1. The highlights is revised. 2. The title was revised. 3. Done. 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 Mufidatul 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. 4. Conclusions was revised.

Acknowledgement

Authors also would like to thank the Reviewers and Editors due their appropriate and constructive suggestions as well as their proposed corrections, which have been utilized in improving the quality of this manuscript.

From: **Biocatalysis and Agricultural Biotechnology** <em@editorialmanager.com>
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Activity of Cellulase and Ligninase Enzymes in a Local Bioactivator from Cattle and Buffalo Rumen Contents

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Activity of cellulase and ligninase enzymes in local microorganisms from cattle and buffalo rumen content

--Manuscript Draft--

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Corresponding Author:	**Dr. Tri Astuti** Universiti Mahaputra muhammad Yamin, Indonesia
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Abstract:	This study aims to determine the activity of cellulase and ligninase enzymes on local bioactivator. This was based on the rumen contents of the rumen incubated for seven days with supplies of different sources of enzyme energy. The method used was a factorial design of 2 ×4 with three replications for each treatment. Factor A was the type of Animal (Cattle Vs Buffalo), factor B is the addition of an enzyme source supply material: B1 = Molasses, B2 = Molasses + palm frond extract, B3 = Molasses + palm leaf extract, B4 = Molasses + palm frond, and palm leaf extract. Also, the parameters observed were the activity of enzymes cellulase, laccase, lignin and manganese peroxidase, as well as microbial identification. The results of statistical analysis showed that the activity of the enzymes cellulase 2.22-3.51 U/ml, laccase 10.62-20.11 U/ml, lignin peroxidase 1.74-4.93 U/ml, and manganese peroxidase 2.40-7.06 U/ml showed insignificant differences (p> 0.05). The results of microbial identification found the bacteria Lactobacillus sp . Based on these, it was concluded that the microbes discovered in the local microorganism solution live because of the study environment, not the microbes inherited from the rumen's contents.
Suggested Reviewers:	Abdalbasit Adam Mariod, Dr Department of Biology, King Abd University, Saudi Arabia aalnadif@kau.edu.sa He is expert in this study and he has publish in high impact journals
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February 24, 2022

Professor Ching Hou, PhD

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Dear Professor,

I wish to submit a manuscript entitled “Activity of cellulase and ligninase enzymes in local microorganisms from cattle and buffalo rumen content” for possible consideration.

Finally I wish to affirm the manuscript has been prepared in accordance with instructions to authors. I also hereby affirm that the content of this manuscript or a major portion thereof has not been published in a refereed journal, and it is not being submitted for publication elsewhere.

Thank you very much and I shall wait for your kind response.

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Activity of cellulase and ligninase enzymes in local microorganisms from cattle and buffalo rumen content

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Wrong references citation format not in numbers. Please correct.

Answer: Thank you for your valuable comments. We have corrected of references citation format

Acknowledgement

Authors also would like to thank the Reviewers and Editors due their appropriate and constructive suggestions as well as their proposed corrections, which have been utilized in improving the quality of this manuscript.

Highlights

- The activity of cellulase and ligninase enzymes on local bioactivator is investigated.
- The activity of enzymes cellulase, laccase, lignin and manganese peroxidase, as well as microbial identification are observed.
- The enzyme activity test for local microorganisms in the rumen are presented.
- Chemical examination results for microorganism isolates on NA media are presented
- The microbes discovered in the local microorganism solution live because of the study environment, not the microbes inherited from the rumen's contents.

Activity of cellulase and ligninase enzymes in local microorganisms from cattle and buffalo rumen content

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Abstract

This study aims to determine the activity of cellulase and ligninase enzymes on local bioactivator. This was based on the rumen contents of the rumen incubated for seven days with supplies of different sources of enzyme energy. The method used was a factorial design of 2 ×4 with three replications for each treatment. Factor A was the type of Animal (Cattle Vs Buffalo), factor B is the addition of an enzyme source supply material: B1 = Molasses, B2 = Molasses + palm frond extract, B3 = Molasses + palm leaf extract, B4 = Molasses + palm frond, and palm leaf extract. Also, the parameters observed were the activity of enzymes cellulase, laccase, lignin and manganese peroxidase, as well as microbial identification. The results of statistical analysis showed that the activity of the enzymes cellulase 2.22-3.51 U/ml, laccase 10.62-20.11 U/ml, lignin peroxidase 1.74-4.93 U/ml, and manganese peroxidase 2.40-7.06 U/ml showed insignificant differences ($p > 0.05$). The results of microbial identification found the bacteria *Lactobacillus sp*. Based on these, it was concluded that the microbes discovered in the local microorganism solution live because of the study environment, not the microbes inherited from the rumen's contents.

Keyword: Enzime activity, microbial morphology, enzyme sellulose, enzyme lignase,rumen microbes

1. Introduction

Animal feeding is a major factor in determining the success of the livestock business. Furthermore, many study uses by-products of agriculture and plantation as substitutes for the field grass. These by-products mostly contain lignocellulosic bonds that consist of cellulose, hemicellulose, and lignin, which cause the low nutritional value of these ingredients when directly fed as feed. Used of palm oil fronds as animal feed barried are low crude protein about 2.11%, high crude fiber content reaching 46.75% (Murni et al. 2008). The oil palm fronds content of ADF 56,93%, NDF 78,05%, cellulose 12.91%, hemicellulose 15.34% and Lignin 15.34% (Astuti et al. 2019). Lignin is a polymer complex phenylpropanoids, heterogeneous, and 25-30% plant biomass. It is quite resistant to microbial degradation under natural conditions. Lignin and cellulose are the main components in plants enzymatically degraded by microorganisms such as enzyme cellulase and ligninase. Ligninolytic microbial systems have been used in improving digestibility and nutritional value. Primarily three enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Glenn et al. 1983). Lignin peroxidase LiP and manganese peroxidase (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).

The use of microbes for enzymes producer has several advantages, including low production costs, short production time, high growth speed, and ease to control. Factors such as fermentation medium, temperature, aeration, agitation, pH, and strains, which play a role in producing these enzymes, need to be optimized. The Cheaped and inexpensive Fermentation media is required since it is easy to obtain and produce expected enzymes for large production (Trismilah 2009).

Many rumen contents on the slaughterhouse waste pollute the environment due to the rumen's abundant availability contents. It was being seen based on the slaughterhouse's number of slaughtered cattle and buffalo. Based on data (Statistic 2020), the average of Livestock Slaughtered at Slaughtering House (Abattoir) in 2019-2020 was 1069.614 heads of cows and 27,487 heads of buffaloes. Beef rumen fluid, apart from containing rumen microbes and enzymes secreted by rumen microbes, also contains food substances resulting from overhaul rumen microbes and enzymes and vitamins and minerals that dissolve in the rumen fluid. The cow's rumen content was 9.29% water; 8.45% crude protein; 1.23% crude fat; 33.53% crude fiber; 0.20% Ca; 0.45% P; 16.19% ash; and 31.60% NFE, and vitamin B12. The rumen contents have the potential as feed additives (Abbas 1987). This slaughtering of livestock leaves the contents of the rumen untapped, only to be thrown away.

Rumen fluid deposits are rich in protein, amino acids, vitamins, and minerals. The composition of amino acids, minerals, vitamins, and enzymes also depends on the feed treatment (Budiansyah et al. 2010). The assumed rumen microbes would produce enzymes according to the given substrate. For example, when given straw, it will produce enzymes to degrade fiber and break down tannins when given a substrate of calliandra (Wina 2005). Lignin and cellulose were the main components in plants that the enzyme cellulase and ligninase enzymatically degrades. These enzymes were produced by microorganisms (Pandey et al. 2000). Molasses are the waste of sugar mills, cheapest and easily as carbon sources rich in nutrients and minerals, and have the potential for microbial growth media (Anggraeni, Isnaeni, and Toto 2016).

Enzymes are biopolymer molecules composed of a series of amino acids in an ordered and fixed composition and chain arrangement. Enzymes were proteins produced and used by living cells to catalyze chemical reactions with a high level of specification and an increase in reaction rates (van Beilen and Li 2002; Richana 2002). Thus, enzymes have various advantages over conventional processes using chemicals. However, the main obstacle for applying enzymes in the industry is the high price of enzymes, and the enzyme cannot be used repeatedly (Huey 2008; Troger, C. and Niranjan 2010; Wibisono 2010).

Cellulases are complex enzymes that gradually cut the cellulose chains into glucose. Fungi, bacteria, and ruminants produces cellulase. The commercial production of enzymes usually uses fungi or bacteria. The production of cellulase enzymes from ruminants has not been much appreciated, however, cellulases originating from ruminant is being produced given their large availability. Rumen fluid enzymes as an alternative technology are used in hydrolyzing crude fiber to increase the nutritional value of local feed raw materials (Pamungkas 2012). The cellulase enzymes have the potential to be produced from beef rumen fluid compared to commercial enzymes. Therefore, this study aims to determine the activity of cellulase and ligninase enzymes in local microorganisms with several different ingredients added. Additionally, microbes are considered to produce enzymes according to the food they get

2. Materials and methods

The material used in this study was rumens content of cattle and buffalo, molasses, soybean water immersion, oil palm frond, oil palm leaf, and some chemistry for measuring enzyme activity.

2.1. The bioactivator process

The rumen content of cattle and buffalo were collected from the abattoir and placed in tubes. All the content of rumen treatments was added with molasses and tofu water immersion. The treatments were the addition of palm leaves, palm fronds, and a mixture of both, which had high lignin content. The addition of palm fronds and leaves is intended, hence, the microorganisms that develop are microorganisms capable of producing the ligninase enzyme. The contents of the rumen, molasses, tofu soaked wastewater were mixed in a ratio of 1:1:8, and 10% of the oil palm frond and leaf. The tube is tightly closed, after which a hole is made in the middle and connected with a hose to a small bottle filled with water to catch the waste of fermentation, then incubated for as long as ten days, as shown in Figure 1.

Figure 1. Fermentation process local bioactivator

2.2. Test Activity of Lignin Peroxidase (LiP) enzymes

A 0.2 ml of enzyme filtrate (extract sample and buffer pospat were shaken until 1 hour and then centrifuged for as long as 10 minutes with 10000 rpm), 0.05 ml of H_2O_2 5 mM, 0.1 ml of veratrine alcohol 8 mM, 0.2 ml of acetate buffer 0.05 M pH 3, and 0.45 ml of distilled water are added to cuvette and then shaken. The absorbance of the solution was at a wavelength of 310 nm for 0 and 30-minute intervals. One unit of enzyme activity in LiP is defined as the amount of enzyme that causes the conversion of 1 micromol (1µmol = 10-6 mol) veratril alcohol per minute (Ming and Kent 1984).

2.3. Test the activity of Manganese Peroxidase (MnP) enzymes

As much as 0.1 ml of 50 mM Na-lactate buffer pH five is added with 0.1 ml of 4 mMguaiacol, 0.2 ml of $MnSO_4$ 1 mM, 0.1 ml of H_2O_2 1 mM, and distilled water 0.3 ml, as well

as 0.2 filtrates are added enzyme. The solution was then checked and read at a wavelength of 465 nm for 0 and 30 minutes (Leonowicz and Grzywnowicz 1981).

2.4. Test the activity of Lakase enzymes

As much as 0.4 ml of enzyme filtrate plus 0.5 ml pH five acetate buffer of 0.5 ml and 0.1 ml of 1 mM ,2'-azinobis 3-ethylbenzothiazole-6-sulfonic acid (ABTS). Then it was checked, and measurements were read to 420 nm wavelength. The reading was taken for 0 and 30 minutes (Wariishi, Valli, and Gold 1992).

Enzyme activity tests were conducted in the biotechnology laboratory of the Faculty of Animal Husbandry, Andalas University, Padang, and identification of microbial morphology was performed at the Baso veterinary center laboratory, Bukit Tinggi. The analysis was continued by identifying the morphology of fungi and bacteria present in the local bioactivator rumen contents. The identification of microbes was performed based on the results of the best enzyme activity evaluation. Samples were inoculated on Sodium Agar (NA) medium for identification of bacteria and Potato dextrose Agar (PDA) media as a medium for growing fungi/molds. The samples were diluted in 10^{-1} - 10^{-10}, and the selected isolates were further to be analyzed based on even colony distribution

2.5. Experimental design

The factorial randomized block design 2 × 4 with three replications for each treatment used in this study. Factor A is the type of rumen content: A1 = Rumen cattle, A2 = Buffalo Rumen, Factor B supplies microbial energy: B1 = Molases, B2 = Molases + palm frond extract, B3 = Molases + palm leaf extract, B4 = Molases + extract of palm fronds and leaves. Enzyme activity data processing was performed using analysis of variance while identifying microorganisms using the described method. When the study resulted in a significant different affected ($p<0.05$), it will be further tested using duncan's multiple range tests,

The variables to be observed were the isolation and identification of microorganisms (fungi and bacteria) by morphology and cellulase and ligninase activity tests for crude bio enzymes of rumen content. The morphology data are shown in description analysis.

3. Results and discussion

3.1. Enzyme activity test

The enzymes activity test were cellulase enzyme, Lacasse Enzymes, Enzim Lignin Peroksidase, Manganese Peroxidase Enzymes. Furthermore, these enzymes are essential for lignin degradation. The average results of the enzyme activity test for local microorganisms in the rumen are shown in Table 1.

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

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
	1.55	**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 = contents of cow's rumen, A2 = contents of buffalo's rumen, B1 = contents of rumen only, B2 = contents of rumen and palm fronds, B3 = palm leaves, and B4 = leaves and palm fronds

The results of the analysis of statistics showed no interaction effect (P> 0.05) between the kind of rument resource (cattle and buffalo) with the sources of microbe energy on the activity of cellulase enzymes, laccases, Lignin Peroxidase Enzymes, and Manganese Peroxidase Enzymes. The data shows the rumen contents were used as a source of microorganisms that were ingested and still dominant in the form of fiber from forage

consumed by livestock are given the main energy source in the form of molasses and tofu soaking water for all treatments. Therefore, it is suspected that the main energy source shown is very representative, supplying the energy needed by microbes in the rumen to grow and develop to produce high value. This study's average cellulase enzyme activity result was much higher than (Murtiyaningsih and Hazmi 2017), measuring the activity of cellulase enzymes from cellulolytic bacteria from soil and getting the highest enzyme activity of 0.028279 U/ml. This was due to a large number of microbes in the rumen contents, then the addition of molasses and tofu soaking wastewater further increases microbial growth. The research (Astuti et al. 2020) found 40 $\times 10^{12}$ total colonies in 1 ml of local bio activator rumen contents mixed with molasses, soybean soaking water, and palm oil fronds.

The average laccase enzyme activity ranged from 11.0 to 20.11 U/ml, the mean lignin peroxidase enzyme ranged from 1.74 to 4.93, while the manganese peroxidase enzyme ranged from 2.40 - 7.06 U/ml. Laccase enzyme activity was much higher than (Dimawarnita, Panji, and Faramitha 2019) which measured enzyme activity in Pleurotus ostreatus with media containing OPEFB 0.35 U/ml and lignin peroxidase (LiP) activity of 0.269 U/mL. The lignin enzyme activity of sugarcane fermentation by 10% Phanerochaete chrysosporium were laccase 2.02 U/ml, LiP 1,677 U/ml, MnP 0.33 U/ml. This was because the microbes that thrive in this local activator have been added to the palm fronds and leaves high in lignin content. Rumen microbes will develop and produce enzymes based on the feed given (Astuti et al. 2021).

3.2. Identification of microorganism morphology

The analysis identifies fungi and bacteria's morphology present in the local bioactivator rumen contents. The identification of microbes was performed based on the results of the best enzyme activity evaluation. Figure 2 shows that the bacterial colonies are circular, small spread out, thin, the edges of the settlements are flat, white in color, and the structure is transparent. Morphological observations of fungal colonies showed circular colony shape, elevation convex, uneven edges, white color, and transparent structure (Figure 2 and 3). The shape of the colony in this study showed the same results with the research (Yogyaswari, Rukmi, and Raharjo 2016) which found around and white colony shape from bacterial isolates rumen contents of Fries Holland. Based on microscopy observations of all bacterial isolates were gram-positive, and bacilli (Table 2 and Figure 4). The results of the chemical analysis are shown in Table 2.

The gram staining results on the isolates showed gram-positive bacteria, negative H2S content, positive catalase. Furthermore, in the microscopic and macroscopically observations,

bacterial isolates had similarities in all isolates bacillus shaped, and there were two genera, namely Bacillus sp one and Bacillus sp 2, Table 2).

The bacterial identification results showed Bacillus sp 1 in the local microorganism rumen-filled added with palm fronds, and bacillus sp 2 in the local bioactivator rumen content isolates with added fronds and palm leaves. In addition, the microscopic and macroscopically observations showed that bacterial isolates in Figure 3 had similarities in all bacillus shapes.

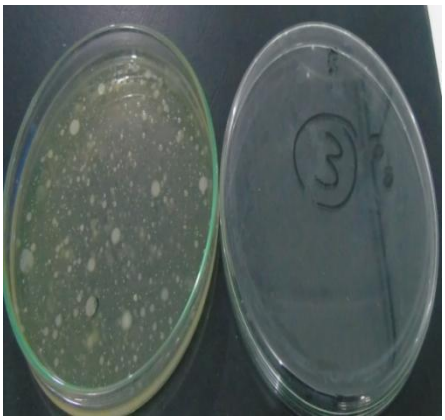

Figure 2. Bacterial colonies on 10^{-8}

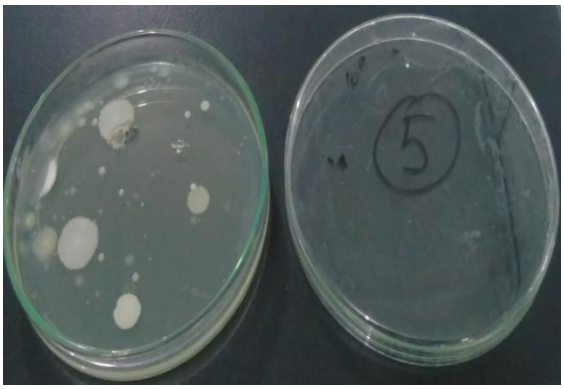

Figure 3. Fungal colonies on 10^{-5}

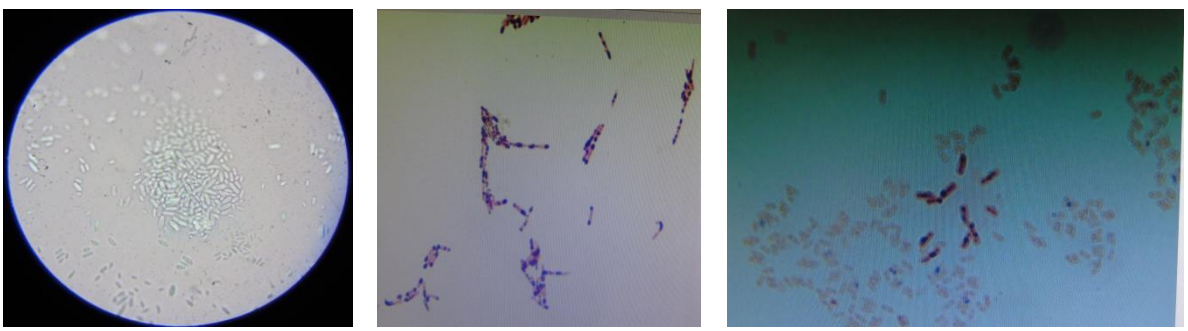

Figure 4. The image of local microorganisms staining the contents of the rumen in the form of bacilli on NA media with the genus *Bacillus sp*

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

No	Treatment	NA 1	NA 2
1.	Colony(Color, shape, Traits)	White	White
2.	Gram (Marfologi, Spora)	+ bacteri	+ bacteri
3.	Aerobic / Anaerobic	A	A spora
4.	TSIA	M/K	M/K
5.	Gas	-	+
6.	H2S	-	-
7.	catalase	+	+
8.	Oxidase	-	-
9.	Motility	+	+
10.	Indole	-	-
11.	Urea	+	+
12.	Citrat	-	-
13.	Laktosa	-	+
14.	Glukosa	-	+
15.	Sukrosa	-	-
16.	Mannitol	-	+
17.	MR	+	+
18.	VP	+	+
19.	OF	-	+
20.	Nitrat	-	+
21.	Gelatin	+	+
22.	Genus	*Basillus, sp 1*	*Basillus sp 2*

4. Conclusion

Based on the research results, it was concluded that the best bioactivator that can be used to improve the quality of the feed was rumen content mixed with molasses, soybean soaking water, leaves, and oil palm frond.

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Activity of cellulase and ligninase enzymes in local microorganisms from cattle and buffalo rumen content

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Abstract

This study aims to determine the activity of cellulase and ligninase enzymes on local bioactivator. This was based on the rumen contents of the rumen incubated for seven days with supplies of different sources of enzyme energy. The method used was a factorial design of 2 ×4 with three replications for each treatment. Factor A was the type of Animal (Cattle Vs Buffalo), factor B is the addition of an enzyme source supply material: B1 = Molasses, B2 = Molasses + palm frond extract, B3 = Molasses + palm leaf extract, B4 = Molasses + palm frond, and palm leaf extract. Also, the parameters observed were the activity of enzymes cellulase, laccase, lignin and manganese peroxidase, as well as microbial identification. The results of statistical analysis showed that the activity of the enzymes cellulase 2.22-3.51 U/ml, laccase 10.62-20.11 U/ml, lignin peroxidase 1.74-4.93 U/ml, and manganese peroxidase 2.40-7.06 U/ml showed insignificant differences ($p > 0.05$). The results of microbial identification found the bacteria *Lactobacillus sp*. Based on these, it was concluded that the microbes discovered in the local microorganism solution live because of the study environment, not the microbes inherited from the rumen's contents.

Keyword: Enzime activity, microbial morphology, enzyme sellulose, enzyme lignase,rumen microbes

1. Introduction

Animal feeding is a major factor in determining the success of the livestock business. Furthermore, many study uses by-products of agriculture and plantation as substitutes for the field grass. These by-products mostly contain lignocellulosic bonds that consist of cellulose, hemicellulose, and lignin, which cause the low nutritional value of these ingredients when directly fed as feed. Used of palm oil fronds as animal feed barried are low crude protein about 2.11%, high crude fiber content reaching 46.75%~~[1]~~ (Murni et al. 2008). The oil palm fronds content of ADF 56,93%, NDF 78,05%, cellulose 12.91%, hemicellulose 15.34% and Lignin 15.34%~~[2]~~ (Astuti et al. 2019). Lignin is a polymer complex phenylpropanoids, heterogeneous, and 25-30% plant biomass. It is quite resistant to microbial degradation under natural conditions. Lignin and cellulose are the main components in plants enzymatically degraded by microorganisms such as enzyme cellulase and ligninase. Ligninolytic microbial systems have been used in improving digestibility and nutritional value. Primarily three enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase~~[3]~~ (Glenn et al. 1983). Lignin peroxidase LiP and manganese peroxidase (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~~[4]~~ (Hattaka 1994).

The use of microbes for enzymes producer has several advantages, including low production costs, short production time, high growth speed, and ease to control. Factors such as fermentation medium, temperature, aeration, agitation, pH, and strains, which play a role in producing these enzymes, need to be optimized. The Cheaped and inexpensive Fermentation media is required since it is easy to obtain and produce expected enzymes for large production ~~[5]~~ (Trismilah 2009).

Many rumen contents on the slaughterhouse waste pollute the environment due to the rumen's abundant availability contents. It was being seen based on the slaughterhouse's number of slaughtered cattle and buffalo. Based on data~~[6]~~ (Statistic 2020), the average of Livestock Slaughtered at Slaughtering House (Abattoir) in 2019-2020 was 1069.614 heads of cows and 27,487 heads of buffaloes. Beef rumen fluid, apart from containing rumen microbes and enzymes secreted by rumen microbes, also contains food substances resulting from overhaul rumen microbes and enzymes and vitamins and minerals that dissolve in the rumen fluid. The cow's rumen content was 9.29% water; 8.45% crude protein; 1.23% crude fat; 33.53% crude fiber; 0.20% Ca; 0.45% P; 16.19% ash; and 31.60% NFE, and vitamin B12. The rumen contents have the potential as feed additives~~[7]~~ (Abbas 1987). This slaughtering of livestock leaves the contents of the rumen untapped, only to be thrown away.

Rumen fluid deposits are rich in protein, amino acids, vitamins, and minerals. The composition of amino acids, minerals, vitamins, and enzymes also depends on the feed treatment [8] (Budiansyah et al. 2010). The assumed rumen microbes would produce enzymes according to the given substrate. For example, when given straw, it will produce enzymes to degrade fiber and break down tannins when given a substrate of calliandra [9] (Wina 2005). Lignin and cellulose were the main components in plants that the enzyme cellulase and ligninase enzymatically degrades. These enzymes were produced by microorganisms [10] (Pandey et al. 2000). Molasses are the waste of sugar mills, cheapest and easily as carbon sources rich in nutrients and minerals, and have the potential for microbial growth media [11] (Anggraeni, Isnaeni, and Toto 2016).

Enzymes are biopolymer molecules composed of a series of amino acids in an ordered and fixed composition and chain arrangement. Enzymes were proteins produced and used by living cells to catalyze chemical reactions with a high level of specification and an increase in reaction rates [12,13] (van Beilen and Li 2002; Richana 2002). Thus, enzymes have various advantages over conventional processes using chemicals. However, the main obstacle for applying enzymes in the industry is the high price of enzymes, and the enzyme cannot be used repeatedly [14–16] (Huey 2008; Troger, C. and Niranjan 2010; Wibisono 2010).

Cellulases are complex enzymes that gradually cut the cellulose chains into glucose. Fungi, bacteria, and ruminants produces cellulase. The commercial production of enzymes usually uses fungi or bacteria. The production of cellulase enzymes from ruminants has not been much appreciated, however, cellulases originating from ruminant is being produced given their large availability. Rumen fluid enzymes as an alternative technology are used in hydrolyzing crude fiber to increase the nutritional value of local feed raw materials [17] (Pamungkas 2012). The cellulase enzymes have the potential to be produced from beef rumen fluid compared to commercial enzymes. Therefore, this study aims to determine the activity of cellulase and ligninase enzymes in local microorganisms with several different ingredients added. Additionally, microbes are considered to produce enzymes according to the food they get

2. Materials and methods

The material used in this study was rumens content of cattle and buffalo, molasses, soybean water immersion, oil palm frond, oil palm leaf, and some chemistry for measuring enzyme activity.

2.1. The bioactivator process

The rumen content of cattle and buffalo were collected from the abattoir and placed in tubes. All the content of rumen treatments was added with molasses and tofu water immersion. The treatments were the addition of palm leaves, palm fronds, and a mixture of both, which had high lignin content. The addition of palm fronds and leaves is intended, hence, the microorganisms that develop are microorganisms capable of producing the ligninase enzyme. The contents of the rumen, molasses, tofu soaked wastewater were mixed in a ratio of 1:1:8, and 10% of the oil palm frond and leaf. The tube is tightly closed, after which a hole is made in the middle and connected with a hose to a small bottle filled with water to catch the waste of fermentation, then incubated for as long as ten days, as shown in Figure 1.

Figure 1. Fermentation process local bioactivator

2.2. Test Activity of Lignin Peroxidase (LiP) enzymes

A 0.2 ml of enzyme filtrate (extract sample and buffer pospat were shaken until 1 hour and then centrifuged for as long as 10 minutes with 10000 rpm), 0.05 ml of H_2O_2 5 mM, 0.1 ml of veratrine alcohol 8 mM, 0.2 ml of acetate buffer 0.05 M pH 3, and 0.45 ml of distilled water are added to cuvette and then shaken. The absorbance of the solution was at a wavelength of 310 nm for 0 and 30-minute intervals. One unit of enzyme activity in LiP is defined as the amount of enzyme that causes the conversion of 1 micromol (1µmol = 10-6 mol) veratril alcohol per minute ~~[19]~~ (Ming and Kent 1984).

2.3. Test the activity of Manganese Peroxidase (MnP) enzymes

As much as 0.1 ml of 50 mM Na-lactate buffer pH five is added with 0.1 ml of 4 mMguaiacol, 0.2 ml of $MnSO_4$ 1 mM, 0.1 ml of H_2O_2 1 mM, and distilled water 0.3 ml, as well

as 0.2 filtrates are added enzyme. The solution was then checked and read at a wavelength of 465 nm for 0 and 30 minutes ~~[20]~~ (Leonowicz and Grzywnowicz 1981).

2.4. Test the activity of Lakase enzymes

As much as 0.4 ml of enzyme filtrate plus 0.5 ml pH five acetate buffer of 0.5 ml and 0.1 ml of 1 mM ,2'-azinobis 3-ethylbenzothiazole-6-sulfonic acid (ABTS). Then it was checked, and measurements were read to 420 nm wavelength. The reading was taken for 0 and 30 minutes ~~[21]~~ (Wariishi, Valli, and Gold 1992).

Enzyme activity tests were conducted in the biotechnology laboratory of the Faculty of Animal Husbandry, Andalas University, Padang, and identification of microbial morphology was performed at the Baso veterinary center laboratory, Bukit Tinggi. The analysis was continued by identifying the morphology of fungi and bacteria present in the local bioactivator rumen contents. The identification of microbes was performed based on the results of the best enzyme activity evaluation. Samples were inoculated on Sodium Agar (NA) medium for identification of bacteria and Potato dextrose Agar (PDA) media as a medium for growing fungi/molds. The samples were diluted in 10^{-1} - 10^{-10}, and the selected isolates were further to be analyzed based on even colony distribution

2.5. Experimental design

The factorial randomized block design 2 × 4 with three replications for each treatment used in this study. Factor A is the type of rumen content: A1 = Rumen cattle, A2 = Buffalo Rumen, Factor B supplies microbial energy: B1 = Molases, B2 = Molases + palm frond extract, B3 = Molases + palm leaf extract, B4 = Molases + extract of palm fronds and leaves. Enzyme activity data processing was performed using analysis of variance while identifying microorganisms using the described method. When the study resulted in a significant different affected ($p<0.05$), it will be further tested using duncan's multiple range tests,

The variables to be observed were the isolation and identification of microorganisms (fungi and bacteria) by morphology and cellulase and ligninase activity tests for crude bio enzymes of rumen content. The morphology data are shown in description analysis.

3. Results and discussion

3.1. Enzyme activity test

The enzymes activity test were cellulase enzyme, Lacasse Enzymes, Enzim Lignin Peroksidase, Manganese Peroxidase Enzymes. Furthermore, these enzymes are essential for lignin degradation. The average results of the enzyme activity test for local microorganisms in the rumen are shown in Table 1.

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

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
	1.55	**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 = contents of cow's rumen, A2 = contents of buffalo's rumen, B1 = contents of rumen only, B2 = contents of rumen and palm fronds, B3 = palm leaves, and B4 = leaves and palm fronds

The results of the analysis of statistics showed no interaction effect (P> 0.05) between the kind of rument resource (cattle and buffalo) with the sources of microbe energy on the activity of cellulase enzymes, laccases, Lignin Peroxidase Enzymes, and Manganese Peroxidase Enzymes. The data shows the rumen contents were used as a source of microorganisms that were ingested and still dominant in the form of fiber from forage

consumed by livestock are given the main energy source in the form of molasses and tofu soaking water for all treatments. Therefore, it is suspected that the main energy source shown is very representative, supplying the energy needed by microbes in the rumen to grow and develop to produce high value. This study's average cellulase enzyme activity result was much higher than ~~[22]~~ (Murtiyaningsih and Hazmi 2017), measuring the activity of cellulase enzymes from cellulolytic bacteria from soil and getting the highest enzyme activity of 0.028279 U/ml. This was due to a large number of microbes in the rumen contents, then the addition of molasses and tofu soaking wastewater further increases microbial growth. The research ~~[23]~~ (Astuti et al. 2020) found 40 $\times 10^{12}$ total colonies in 1 ml of local bio activator rumen contents mixed with molasses, soybean soaking water, and palm oil fronds.

The average laccase enzyme activity ranged from 11.0 to 20.11 U/ml, the mean lignin peroxidase enzyme ranged from 1.74 to 4.93, while the manganese peroxidase enzyme ranged from 2.40 - 7.06 U/ml. Laccase enzyme activity was much higher than ~~[24]~~ (Dimawarnita, Panji, and Faramitha 2019) which measured enzyme activity in Pleurotus ostreatus with media containing OPEFB 0.35 U/ml and lignin peroxidase (LiP) activity of 0.269 U/mL. The lignin enzyme activity of sugarcane fermentation by 10% Phanerochaete chrysosporium were laccase 2.02 U/ml, LiP 1,677 U/ml, MnP 0.33 U/ml. This was because the microbes that thrive in this local activator have been added to the palm fronds and leaves high in lignin content. Rumen microbes will develop and produce enzymes based on the feed given ~~[25]~~ (Astuti et al. 2021).

3.2. Identification of microorganism morphology

The analysis identifies fungi and bacteria's morphology present in the local bioactivator rumen contents. The identification of microbes was performed based on the results of the best enzyme activity evaluation. Figure 2 shows that the bacterial colonies are circular, small spread out, thin, the edges of the settlements are flat, white in color, and the structure is transparent. Morphological observations of fungal colonies showed circular colony shape, elevation convex, uneven edges, white color, and transparent structure (Figure 2 and 3). The shape of the colony in this study showed the same results with the research ~~[26]~~ (Yogyaswari, Rukmi, and Raharjo 2016) which found around and white colony shape from bacterial isolates rumen contents of Fries Holland. Based on microscopy observations of all bacterial isolates were gram-positive, and bacilli (Table 2 and Figure 4). The results of the chemical analysis are shown in Table 2.

The gram staining results on the isolates showed gram-positive bacteria, negative H2S content, positive catalase. Furthermore, in the microscopic and macroscopically observations, bacterial

isolates had similarities in all isolates bacillus shaped, and there were two genera, namely Bacillus sp one and Bacillus sp 2, Table 2).

The bacterial identification results showed Bacillus sp 1 in the local microorganism rumen-filled added with palm fronds, and bacillus sp 2 in the local bioactivator rumen content isolates with added fronds and palm leaves. In addition, the microscopic and macroscopically observations showed that bacterial isolates in Figure 3 had similarities in all bacillus shapes.

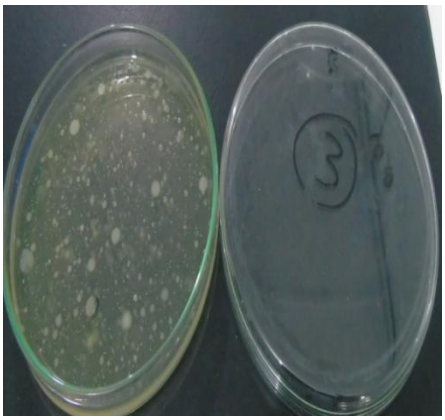

Figure 2. Bacterial colonies on 10^{-8}

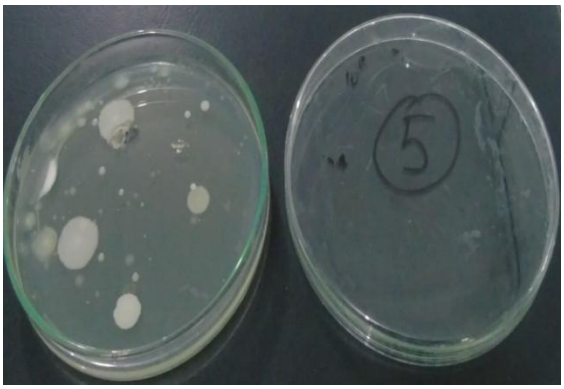

Figure 3. Fungal colonies on 10^{-5}

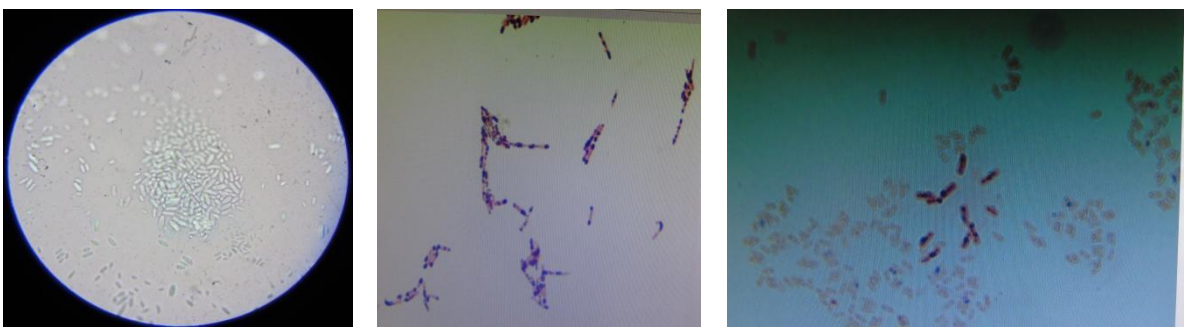

Figure 4. The image of local microorganisms staining the contents of the rumen in the form of bacilli on NA media with the genus *Bacillus sp*

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

No	Treatment	NA 1	NA 2
1.	Colony(Color, shape, Traits)	White	White
2.	Gram (Marfologi, Spora)	+ bacteri	+ bacteri
3.	Aerobic / Anaerobic	A	A spora
4.	TSIA	M/K	M/K
5.	Gas	-	+
6.	H2S	-	-
7.	catalase	+	+
8.	Oxidase	-	-
9.	Motility	+	+
10.	Indole	-	-
11.	Urea	+	+
12.	Citrat	-	-
13.	Laktosa	-	+
14.	Glukosa	-	+
15.	Sukrosa	-	-
16.	Mannitol	-	+
17.	MR	+	+
18.	VP	+	+
19.	OF	-	+
20.	Nitrat	-	+
21.	Gelatin	+	+
22.	Genus	*Basillus, sp 1*	*Basillus sp 2*

4. Conclusion

Based on the research results, it was concluded that the best bioactivator that can be used to improve the quality of the feed was rumen content mixed with molasses, soybean soaking water, leaves, and oil palm frond.

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Declaration of interests

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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% lign~~a~~ins (Astuti et al. 2019, Tafsin et al, 2018). 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.

~~The u~~Using~~e of~~ 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 (Jovanovic et al., 1997). Rumen contents It also contains high crude fiber, calcium, phosphorus

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and magnesium (Agbabiaka et al., 2012; Elfaki and Abdelati 2015). ~~Rumina~~ Rument microbes ~~would~~ will produce enzymes in accordance with the given substrate. For example, rument ~~inants~~ microbes ~~would~~ will produce enzymes that degrade fibere ~~e~~ 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 (van 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~~,; moreover, enzymes~~ and cannot be used repeatedly (Huey 2008; Troger, C. and Niranjan 2010; Wibisono 2010). ~~Lignin-degrading or ligninolytic enzymes include laccase (polyphenol oxidase), LiP and MnP; all three are extracellular multienzymes that participate in lignin depolymerisation (Sanchez, 2010).~~ Extracellular oxidative enzymes can attack and degrade lignin, by utilizing types of peroxidases including lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and dye remover, peroxidase (DyP) (Lambertz et al., 2016).

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), Rumen microbes are very good sources of fibrinolytic enzymes, carbohydrate-active enzymes that can degrade lignocellulose, 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 10 000 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 (Ming 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, Valli and Gold 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}

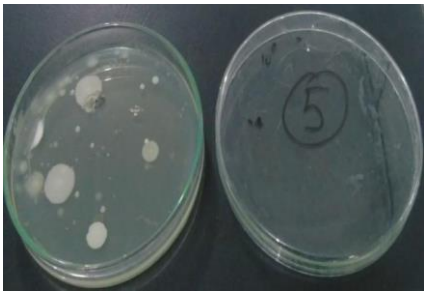

Figure 3. Fungal colonies on 10^{-5}

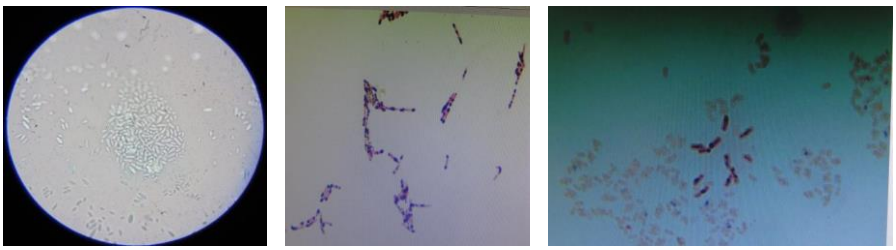

Figure 4. Staining of bacilli in the genus *Bacillus* in rumen contents on NA media

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 Mufidatul 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 fibere 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, et al., 2020). ~~The laccase enzyme activity in this work was considerably higher than the LiP activity of 0.269 U/ml and OPEFB activity of 0.35 U/ml found by a previous study (Dimawarnita, Panji and Faramitha 2019) on *Pleurotus ostreatus* in media.~~ 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.* (Longe et al 2016; Priyadarshinee et al 2016)

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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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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. A large amount of lignocellulose in agricultural by-products that are disposed of not only causes environmental damage but also loses materials that have the potential to be used in the production of paper animal feed, and other materials Sanchez, 2009).

.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; Wibisono, 2010). Extracellular oxidative enzymes can attack and degrade lignin, by utilizing types of peroxidases including lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and dye remover, peroxidase (DyP) (Lambertz et al., 2016).

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), Rumen microbes are very good sources

of fibrinolytic enzymes, carbohydrate-active enzymes that can degrade lignocellulose. 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 10 000 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*

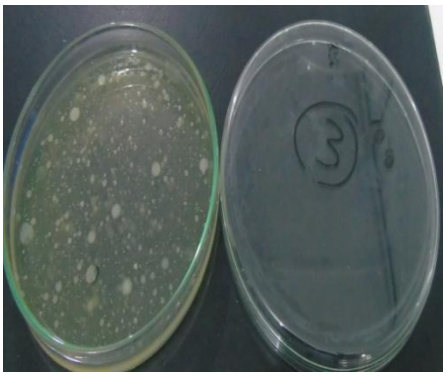

Figure 2. Bacterial colonies on 10^{-8}

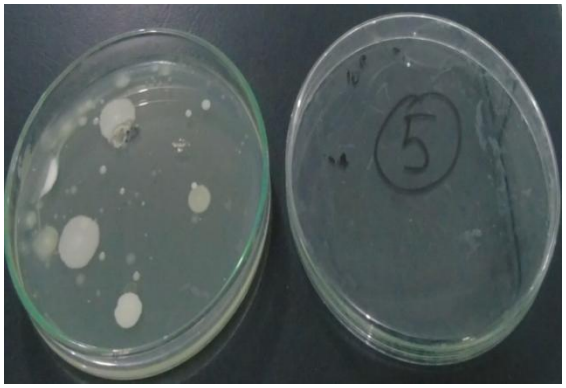

Figure 3. Fungal colonies on 10^{-5}

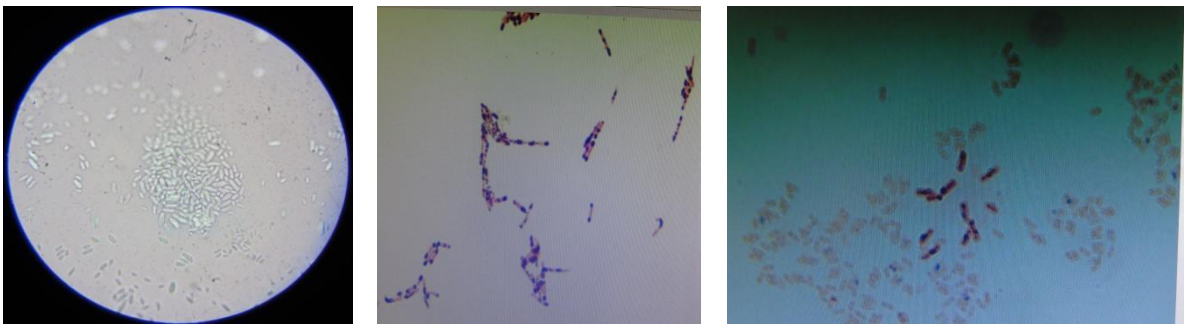

Figure 4. Staining of bacilli in the genus *Bacillus* in rumen contents on NA media

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. 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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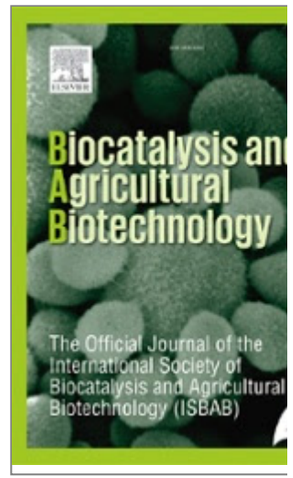

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Activity of cellulase and ligninase enzymes in a local bioactivator from cattle and buffalo rumen contents

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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.

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

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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, 1977). Rumen contents It also contains high crude fiber, calcium, phosphorus and magnesium (Agbabiaka et al., 2012; Elfaki et al., 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 et al., 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 Fig. 1.

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 (1 μmol = 10^{-6} mol) veratril alcohol per minute (Tien and Kirk, 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).

Fig. 1. Fermentation with the local bioactivator.

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.

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 Fig. 2, the fungal colonies in Fig. 3 and the bacterial isolates in Fig. 4 had similar bacillus shapes.

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 bac-

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.

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*

teria. 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).

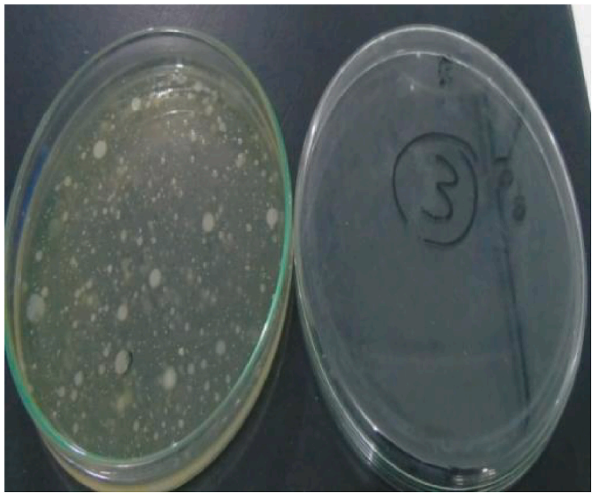

Fig. 2. Bacterial colonies on 10^{-8}.

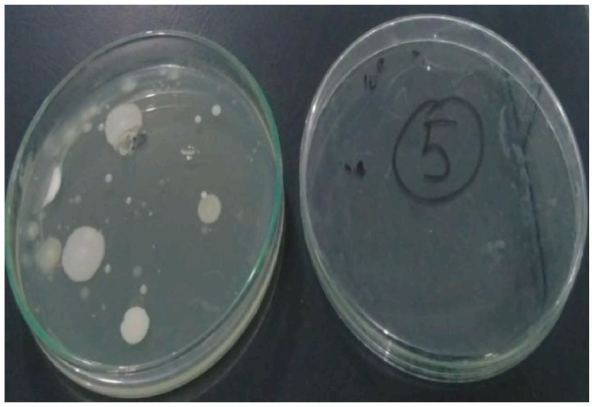

Fig. 3. Fungal colonies on 10^{-5}.

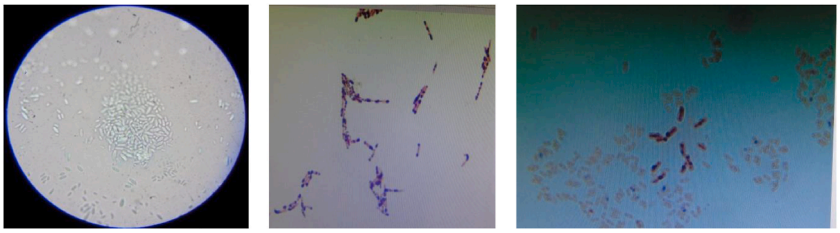

Fig. 4. Staining of bacilli in the genus *Bacillus* in rumen contents on NA media.

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.

Fig. 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 (Figs. 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 Fig. 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 Fig. 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.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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