The Activity of Cellulase and Ligninase Enzymes In Local Bioactivator From Cattle And Buffalo Rumen Content

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Abstract

Lignin is the main component of agricultural and plantation waste such as bagasse, straw, and oil palm fronds. Lignin, cellulose, and hemicellulose in lignocellulosic bonds can be broken down by enzymes. There are many types of research that utilize plantation waste as feed ingredients, but lignin is a limiting factor that affects the digestibility of these materials, so their content must be reduced before being used as feed ingredients. Using of local bio enzymes will be effective in breaking lignocellulose bonds. However, it is necessary to find sources of enzymes that are easy to obtain, inexpensive to produce, and effective as lignocellulosic degrading enzymes. This study aims to determine the activity of cellulase and ligninase enzymes on local bio activators. This was based on the rumen contents of the rumen incubated for seven days with supplies of different sources of enzyme energy. This treatment used rumen content of Cattle and Buffalo with the addition of Molasses, palm frond, palm leaf extract, and each other. 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 insignificant differences ($p > 0.05$) affect of all treatments for parametresthe 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. 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 studies use by-products of agriculture and plantation as substitutes for the field grass ruminant feeding. These by-products mostly contain lignocellulosic bonds that consist of cellulose, hemicellulose, and lignin. The digestibility of ruminant feed ingredients is influenced by the content of lignin, cellulose, and the anumber of soluble substances. The lignin content in forage forms a strong lignocellulosic bond so that it inhibits the digestibility of cellulose and hemicellulose, causing the absorption of nutrients to be not optimal. (Haryanti, 2009). Using of palm oil fronds as animal feed berries 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 bioactivator 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 bioactivator (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). Lignin-degrading enzymes (ligninolytic) consists of laccase (polyphenol oxidase), lignin peroxidase (LiP) and manganese peroxidase (MnP), all three are multi-enzymes extracellular which plays a role in the process of lignin depolymerization. (Sanchez, 2010). Lignin peroxidase (LiP) and manganese peroxidase (MnP) is an extracellular peroxidase enzyme that uses H2O2 in degrading lignin, while laccase is a copper-containing enzyme with use molecular oxygen to degrade lignin (Hattaka, 1994)

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 bioactivator be produced by incubation rumen content with added several different ingredients as the microbe feeding. This research can produce complex enzymes (cellulase enzymes, manganese, lignin peroxidase, laccase) derived from waste materials that are cheap and easy to obtain.

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 bioactivator that develop are bioactivator 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 tubes were 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 bioactivator 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 bioactivator (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

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 bioactivator in the rumen are shown in Table 1.

Table 1. Average Activity of Local Bioactivator in he 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

3.2. Identification of microorganism morphology

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.	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*

The microscopic and macroscopically observations showed bacteria colonies type on the figure 2, fungal colonies type on the figure 3, and bacterial isolates in Figure 4 had similarities in all bacillus shapes.

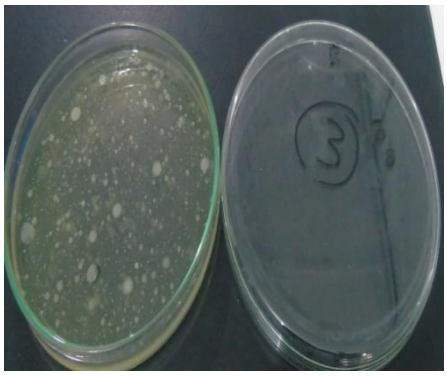

Figure 2. Bacterial colonies on 10^{-8}

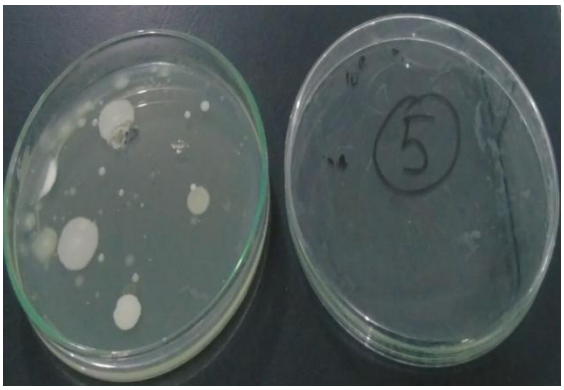

Figure 3. Fungal colonies on 10^{-5}

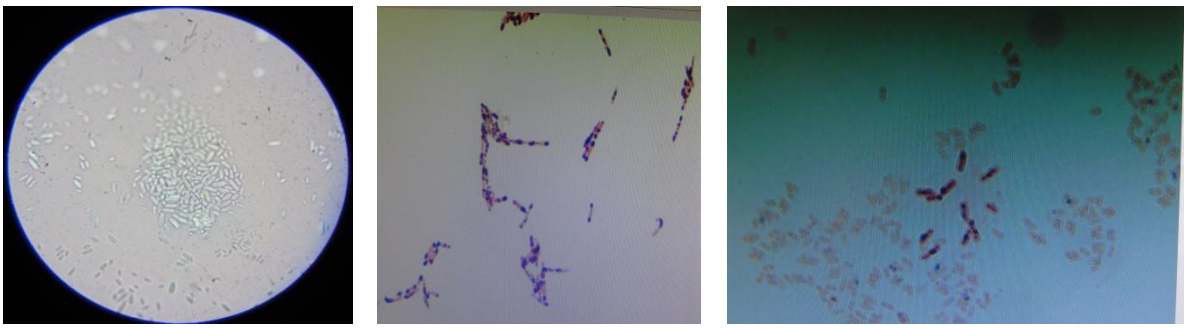

Figure 4. The image of a local bioactivator staining the contents of the rumen in the form of bacilli on NA media with the genus *Bacillus sp*

4. Discussion

4.1. Enzyme activity test

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 in Table 1 show that the activity of the lignin peroxidase (LiP) enzyme ranged from 2.46 to 3.48 U/ml. The activity of the LiP enzyme in this study was higher than that of Mufidatul and Kuswytasari (2013) who measured the activity of the LiP enzyme in Gliomastix sp. T3.7 on Hump Waste Corn and its enzyme activity 0.06-1.022 (pH 5, temperature 25-35). The data shows the rumen contents were used as a source of bioactivator 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, lignin peroxidase enzyme activity 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).

4.2. 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 H_2S 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*- 1 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.

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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.

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. with enzyme activities of Cellulase 2,2 U/ml, Lacasse 15,91 U/ml, Lignin Peroksidase 4,11 U/ml, Lignin Peroksidase 7,06 U/ml. The identification of bacteria on the best treatment found the presence of *bacillus sp*

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I am pleased to inform you that your manuscript has been accepted for publication.

My comments, and any reviewer comments, are below.
Your accepted manuscript will now be transferred to our production department. We will create a pro which you will be asked to check, and you will also be asked to complete a number of online forms re for publication. If we need additional information from you during the production process, we will cont you directly.

We appreciate you submitting your manuscript to Biocatalysis and Agricultural Biotechnology and ho will consider us again for future submissions.

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Kind regards,
Ching T. Hou
Editor-in-Chief

Reviewers and/or Editors' comments and Author Respond

Ms. Ref. No.: BAB-D-22-00223R1

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

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

Date : June 29, 2022

Reviewers and/or Editors' comments:

Reviewers Comments	Author Respond and Revision
Reviewer #1 Recommendation: Major revisions Reviewer Comments: General comment: This paper is regarding a research on the activity of cellulase and ligninase enzymes in local microorganisms from cattle and buffalo rumen content However, the writing and presentation of data is not of publication quality. The script in this current form can be revised to achieve publication quality. There are some clarifications needed to understand the processes carried out in this work. To conclude, this paper needs to be revised carefully before it can be considered in journal like BAB. Hope that comments below will be able to help to further improve the paper	
* Please make sure that the paper is checked by native English speaker, the language needs improvement.	*The manuscript is thoroughly revised, and all possible grammatical error has been corrected with improve English using proof reading service (KG support).
* Please check Guides for Authors to make sure it is followed strictly	* Done
* Language: There are some language errors (tenses, singular/plural) and incomplete sentences in the script. Please check the sentence structure, tenses and language carefully in the revised manuscript.	* The manuscript was corrected with improve English using proof reading service (KG support).
* Take note of unit spacing issue	* Done
Graphical abstract and highlights: * Only highlights are provided. Suggest to edit according to the GFA. Most of the points have exceeded the maximum allowable character.	* Done. The highlights was revised

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.