

# In vitro Clot Lysis Activity of Phenolic Compound Degrading Product From Lignin Sugarcane Baggase Using *Ochrobactrum sp.*

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## ABSTRACT

Sugarcane bagasse (*Saccharum officinarum L*) is a readily available waste product of cane sugar processing. The content of lignocelluloses in sugarcane bagasse is approximately 52.7% cellulose, 20% hemicelluloses, and 24.2% lignin. Lignin can be degraded by using microorganisms *Ochrobactrum sp.* Monomer derived from lignin degradation was fermented at pH 6, temperature 40 °C, for 5 days of incubation. Starter concentration was increase from 9.0% to 33.0%. Product was isolated from chloroform extraction and identified two major products as 2,6-di-tert-butyl-4-methylphenol and 2,6-di-tert-butylcyclohexa-2,5-diene-1,4-dione identification. Phenol and 4-hydroxybenzaldehyde was identified in trace amount. Both product shows capability to lysis the clots in vitro.

Key word: degradation; lignin; *Ochrobactrum sp.*; clot lysis activity

## INTRODUCTION

Sugarcane bagasse (*Saccharum officinarum L*) is a disposed of dry waste from sugar production. In about hundred tons of sugarcane-processed produces nearly 30 tons of wet bagasse. After undergo drying process can provide 40-50% weight [1,2]. This affect in the lifting of green-house emission to the environment. To overcome the issue, several strategy has been undertaken by applying the sugarcane bagasse conversion for production of bioethanol [3], biofuel [2,4], bio-briquettes [5,6], cellulose and nanocellulose [7,8], lignin [9,10], and other fine chemicals [11].

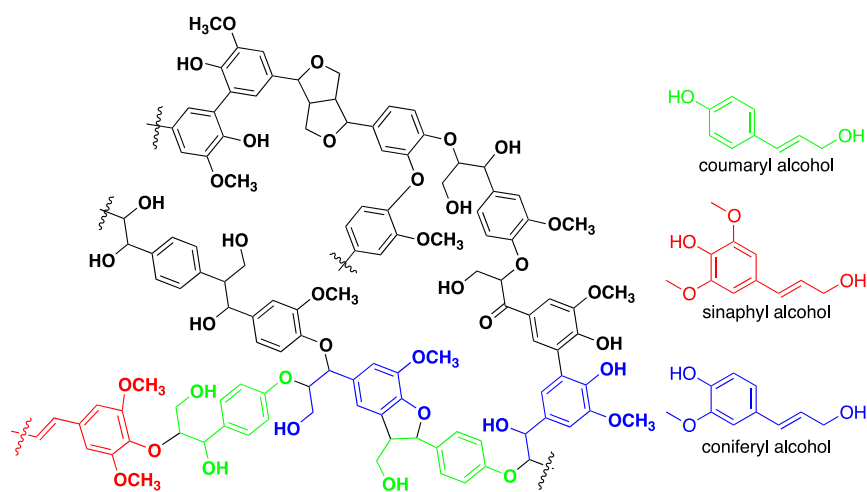
Sugarcane bagasse is composed of about 52.7% cellulose, 20% hemicelluloses, and 24.2% lignin. The cellulose part has been discussed for further reported [7,8,12–14], and this paper is disclosed recent strategy by applying of bacterial degradation process to produce lignin derivative product. Lignin is a natural polymer (Figure 1), which is mainly composed of phenyl propane or C9 unit. There are three kinds of C9 units contained in the lignin, i.e. coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol unit [15], and another paper-named composed of ferulate guaiacyl unit, syringyl unit, and p-hydroxyphenyl unit [16].

Degradation of lignin can produce derivative of lignin, some provides C9, C8, C7 unit and also C6 unit [17]. The method can be performed by hydrothermal [18], pyrolysis [4,6,17], chemical [19], enzymatic [20,21] hydrolysis and also bacterial degradation [10,22] process. The bacterial mediate lignin degradation requires the bacteria stable in acidic and rich-phenolic conditions. In most cases, phenolic compounds inhibit [23] the bacterial or microorganism

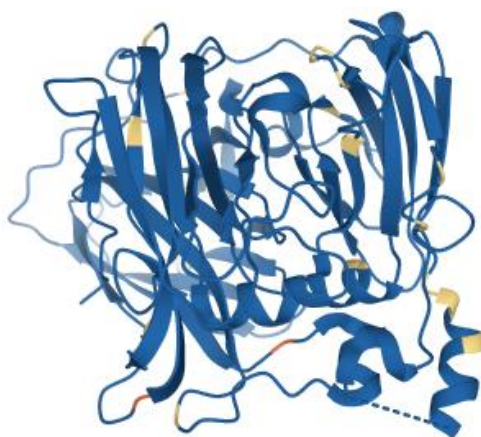
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growth. The species of *Ochrobactrum sp*, previously was reported to decompose lignin [10,22]. The mechanism involved the oxidation of  $\beta$ -aryl ether and biphenyl lignin dimer and also Cα-lignosulfonate to produce a low molecular weight of vanillic acid [22]. The gene side active involving of multicopper oxidase genes. The enzyme of the bacteria can be isolated and crystallized as *Ochrobactrum multicopper oxidase* (OCueO) with four-side coordination with mono copper centered attach with His495, His434, Cys490 and Met500 as axial ligand (Figure 2) [22]. Instead of vanillic acid, the recent investigation reports 2,6-di-tert-butyl-4-methylphenol and 2,6-di-tert-butylcyclohexa-2,5-diene-1,4-dione as the major product and 4-hydroxybenzaldehyde and phenol as the minor product. In addition, isolated of lignin degradation product was evaluated for clot lysis activity using bovine blood in vitro.



**Figure 1.** Structure of lignin, consist of coumaryl, sinapyl and coniferyl alcohol as carbon backbond.



**Figure 2.** Visualisasi of protein surface isolated from *Ochrobactrum sp* adopted from reference [22], which has function for lignin degradation. Image downloaded from PDB data, 6EVG.

Clot lysis activity relate to the ability of the compounds to dissolve or breakdown the blood clots or to hamper blood coagulation. The coagulation mechanism can involve the

activation, adhesion and aggregation of platelet and/or deposition and maturation of fibrin [24]. The activity compounds to lysis of clots, contribute ischaemic heart [25,26] and cardiovascular diseases [27]. Several reports give an antioxidative, antiplatelet, and anticoagulant activity for phenolic substituent [25,28] composed in the plants extract [29,30], food and fermented food [31,32].

## EXPERIMENT

### Chemicals and instrumentation

Chemicals used for research include sugarcane bagasse, toluene, ethanol, 5% NaOH, HCl 6M, chloroform, 3.1% sodium citrate, 2% calcium chloride, 100  $\mu$ L Streptokinase (SK), distilled water, MSM medium. The composition of the MSM used in this study was follow (g/L): NaNO<sub>3</sub> 4.0, NaCl 1.0, KCl 1.0, CaCl<sub>2</sub>.2H<sub>2</sub>O 0.1, KH<sub>2</sub>PO<sub>4</sub>.12H<sub>2</sub>O 3.0, MgSO<sub>4</sub> 0.2, FeSO<sub>4</sub>.7H<sub>2</sub>O 0.001; 2 mL trace element stock solution composed of (g/L): FeCl<sub>3</sub>.6H<sub>2</sub>O 0.08, ZnSO<sub>4</sub>.7H<sub>2</sub>O 0.75, CoCl<sub>2</sub>.6H<sub>2</sub>O 0.08, CuSO<sub>4</sub>.5H<sub>2</sub>O 0.075, MnSO<sub>4</sub>.H<sub>2</sub>O 0.75, H<sub>3</sub>BO<sub>3</sub> 0.15, and Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O 0.05.

The strain of *Ochrobactrum sp.* used was obtained from the culture collection of Indah Prihartini (Biotechnology Laboratory of Universitas Muhamadiyah Malang). This strain was identified by sequencing in 16 SrRNA.

Instrumentation applied for analysis include soxhlet apparatus, incubator, rotary evaporator, Buchner funnel, UV spectrophotometer (Shimadzu 1601), and GC-MS (Shimadzu QP-2010S).

### Lignin isolation from sugarcane bagasse

Sugarcane bagasse were first dried in sunlight and cut into small pieces ( $\pm$  1.5 cm). Then, it was grinded to pass a 1.0 mm sieves. The dried powder were extracted under soxhlet extraction process. A mixture of toluene/ethanol (2:1 v/v ratio) was used as solvent and circulating for 6 hours. The, the solid residue was dried in oven at 60 °C for 16 hours. A 100 g of the powder was soaked in 5% of sodium hydroxide solution for 6 hours, then acidified to pH 5.5 using hydrochloric acid solution 6.9 M. The solution was left to room temperature and concentrated by evaporation process left 20% of volume. Then, it was added ethanol 50 mL and stirred for 5 minutes. The liquid was filtered. This process was repeated three times, and the combining ethanol extract was evaporated under vacuum to produce lignin.

### Lignin degradation

The *Ochrobactrum sp.* bacterial cultures (72 hr) in the MSM pH 6 were inoculated into 1% (v/v) lignin as carbon source. The starter concentrations were varied (9.0; 17.0; 23.0; 29.0; and 33.0). It was kept in a shaker at 150 rpm at 40 °C for 5 days. The growth of bacteria was monitored by measuring the optical density at 620 nm wavelength. The solution culture was prepared for further analysis.

### Product isolation and identification

The degradation product of lignin were isolated from culture filtrate. The filtrate was extracted with chloroform, and further concentrated under vacuum rotary evaporator. Product was analyzed by using UV-Vis spectrophotometer and GCMS.

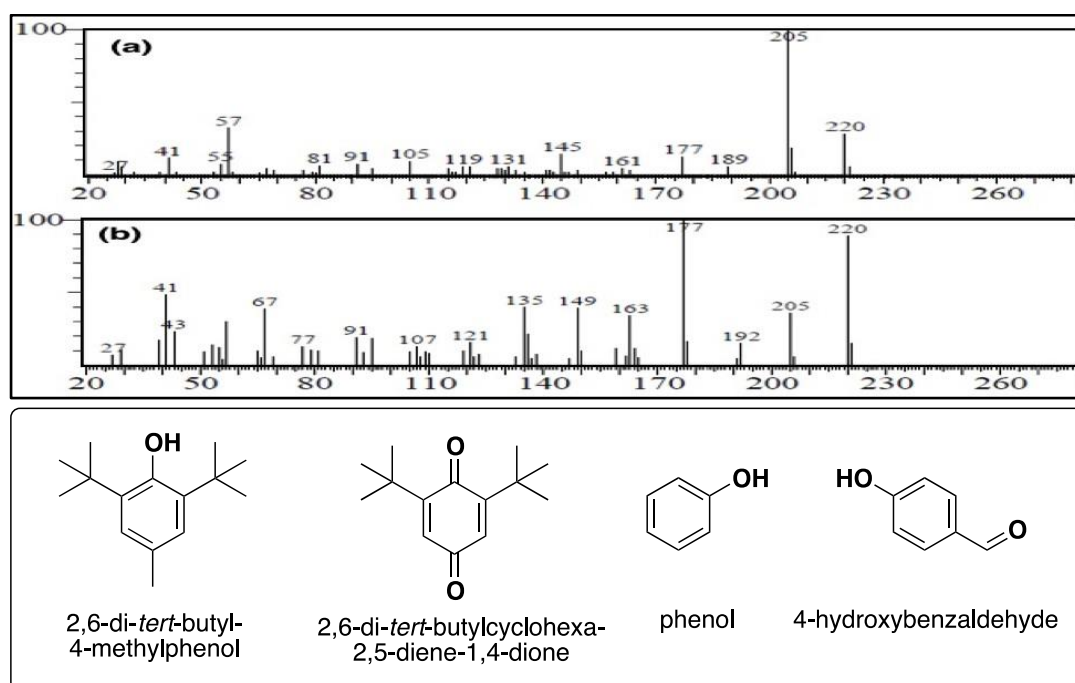
## Clot lysis evaluation

Cow blood was collected from local abattoir in Dau-Malang, Indonesia and immediately citrated using 3.1% sodium citrate solution. A 1000  $\mu\text{L}$  of blood in pre-weighed microcentrifuge tube was added with 200  $\mu\text{L}$  of 2% calcium chloride solution. Then, it was mixed well and incubated at 37  $^{\circ}\text{C}$  for 45 minutes for clot formation. The serum from a formed clot was aspirated out without disturbing the clot, and each of these tubes was weighed again to determine the weight of the clot (clot weight = weight of clot containing tube – weight of tube). Then, 100  $\mu\text{L}$  of aqueous lignin and lignin-degradation product was added separately into each microcentrifuge tubes containing the pre-weighed clot. In addition, a 100  $\mu\text{L}$  streptokinase (SK) (as a positive control) and 100  $\mu\text{L}$  of distilled water (as a negative control) were separately added to the control tubes. All tubes were then incubated at 37  $^{\circ}\text{C}$  for 90 minutes and observed for clot lysis. After incubation, the released fluid was removed, and tubes were weighed to measure different weight after clot disruption. The weight recorded before and after clot lysis was expressed as percentage of clot lysis.

## RESULT AND DISCUSSION

### Lignin degradation products

Gas chromatography-mass spectrometry were able to identify lignin degradation products based on comparison to the scanned mass spectra from sample (Figure 3) resulted toward the library mass spectra. The highest similarity index (SI) value from the two major products were identified as 2,6-di-*tert*-butyl-4-methylphenol and 2,6-di-*tert*-butylcyclohexa-2,5-diene-1,4-dione. Besides that, two minor products were also able to be identified in trace amounts as phenol and 4-hydroxybenzaldehyde. This result differs to previously reported for vanillic acid<sup>22</sup> as identification by using liquid chromatography in polar solvent.



**Figure 3.** Mass spectra of 2,6-di-*tert*-butyl-4-methylphenol (a), and 2,6-di-*tert*-butylcyclohexa-2,5-diene-1,4-dione (b). The chemical structure of the detected products.

Addition more bacteria to the reaction, can improve the degradation product. However, previous report [23] indicated the limitation for growth inhibition concentration. This can decrease product during fermentation process. Summary the effect of bacteria concentration toward product formation is tabulated in Table 1. Formation of product 2,6-di-tert-butyl-4-methylphenol increase from 1.31% to 10.65% by increasing of bacteria concentration from 9.0 to 29.0%. This product turns down to 7.94% with addition more starter to 33.0%. However, for product 2,6-di-tert-butylcyclohexa-2,5-diene-1,4-dione give improvement yield to 1.47% until concentration 33.0%. This product has not been identified at starter concentration 9.0%.

**Table 1.** Effect of starter concentration on product formation (fermentation for 5 days, pH 6 and temperature 40 °C)

Degradation product	Starter concentration (%)	Product percentage (%)
2,6-di-tert-butyl-4-methylphenol	9.0	1.31
	17.0	8.49
	23.0	8.99
	29.0	10.65
	33.0	7.93
2,6-di-tert-butylcyclohexa-2,5-diene-1,4-dione	9.0	-
	17.0	0.77
	23.0	0.62
	29.0	0.84
	33.0	1.47

### The clot lysis result

The activity of lignin and lignin-degraded product after fermentation using *Ochrobactrum sp.* was compared to the reference streptokinase and water as a control toward their ability to dissolve or lysis the clots of bovine blood. The result is depicted in Table 2. The lignin-degradation product after fermentation indicate capability to lysis the clot by  $34.50 \pm 3.23\%$ . This capability is a half time from the streptokinase ( $79.44 \pm 4.19\%$ ), but indicate a better capability than the lignin ( $18.45 \pm 8.81\%$ ) and water ( $13.43 \pm 2.89\%$ ) as control.

**Table 2.** The percentage of clot lysis cow blood samples treated with lignin, monomer lignin, streptokinase (positive control) and distilled water (negative control)

Evaluated sample	% clot lysis
Streptokinase 30.000 IU (control +)	$79.44 \pm 4.19$
Lignin	$18.45 \pm 8.81$
Lignin-degradation product 1.25 mg/mL	$34.50 \pm 3.23$
Distilled water	$13.43 \pm 2.89$

### CONCLUSION

To summarized, lignin-degraded product isolated from sugarcane bagasse lignin provide two major products named as 2,6-di-tert-butyl-4-methylphenol and 2,6-di-tert-butylcyclohexa-

2,5-diene-1,4-dione. The bacteria *Ochrobactrum sp.* did improve the major product with increasing the concentration. Moreover, these degradation products have capability to lysis the clots by  $34.50 \pm 3.23\%$  or a half time capability of streptokinase.

### CONFLICT OF INTEREST

Authors declare that no competing interest with publishing the manuscript.

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### REFERENCES

- [1] Restuti, D., Michaelowa, A. *Energy Policy*, **2007**, 35 (7), 3952–3966.
- [2] Sari, K. E., and Meidiana, C. *IOP Conf. Ser.: Earth Environ. Sci.*, **2019**, 314, 012028.
- [3] Alfian, M. M., Amin, M., Sholihul, H. M., and Aziz, M., *J. Appl. Eng. Sci.* **2020**, 18 (2), 262–266.
- [4] Pradana, Y. S., Hartono, M., Prasakti, L., and Budiman, A. *Energy Procedia* **2019**, 158, 431–439.
- [5] Navalta, C. J. L. G., Banaag, K. G. C., Von Adrian, O. R., Go, A. W., Cabatingan, L. K., and Ju, Y.-H., *Renew. Energy* **2020**, 147, 1941–1958.
- [6] Setter, C., Costa, K. L. S., de Oliveira, T. J. P., and Mendes, R. F., *Fuel Process. Technol.* **2020**, 210, 106561.
- [7] Candido, R., Godoy, G., and Goncalves, A. R., *Carbohydr. Polym.* **2017**, 167, 280–289.
- [8] Ferreira, F., Mariano, M., Rabelo, S., Gouveia, R., and Lona, L., *Appl. Surf. Sci.* **2018**, 436, 1113–1122.
- [9] Moubarik, A., Grimi, N., Boussetta, N., and Pizzi, A., *Ind. Crops Prod.* **2013**, 45, 296–302.
- [10] Xu, Z., Qin, L., Cai, M., Hua, W., and Jin, M., *Environ. Sci. Pollut. Res.* **2018**, 25 (14), 14171–14181.
- [11] Zhang, W., You, Y., Lei, F., Li, P., and Jiang, J., *Bioresour. Technol.* **2018**, 265, 387–393.
- [12] Shirkavand, E., Baroutian, S., Gapes, D. J., and Young, B. R., *Renew. Sust. Energy Rev.* **2016**, 54, 217–234.
- [13] Guerrero, E. B., Arneodo, J., Campanha, R. B., de Oliveira, P. A., Labate, M. T. V., Cataldi, T. R., Campos, E., Cataldi, A., Labate, C. A., and Rodrigues, C. M., *PLoS One* **2015**, 10 (8), e0136573.
- [14] Kumar, G., Sivagurunathan, P., Sen, B., Mudhoo, A., Davila-Vazquez, G., Wang, G., and Kim, S.-H., *Inter. Biodeterior. Biodegradation* **2017**, 119, 225–238.
- [15] Rico-García, D., Ruiz-Rubio, L., Pérez-Alvarez, L., Hernández-Olmos, S. L., Guerrero-Ramírez, G. L., and Vilas-Vilela, J. L., *Polymers* **2020**, 12 (1), 81.
- [16] Zhu, Y., Huang, J., Wang, K., Wang, B., Sun, S., Lin, X., Song, L., Wu, A., and Li, H., *Polymers* **2020**, 12 (1), 187.
- [17] Hasanah, U., Setiaji, B., Triyono, T., and Anwar, C., *J. Pure App. Chem. Res.* **2012**, 1 (1), 26.
- [18] Yedro, F. M., García-Serna, J., Cantero, D. A., Sobrón, F., and Cocero, M. J., *RSC Adv.* **2014**, 4 (57), 30332–30339.



- [19] O'Neill, E., Kawam, A., Van Ry, D., and Hinrichs, R., *Atmospheric Chem. Phys.* **2014**, *14* (1), 47–60.
- [20] Xu, C., Zhang, J., Zhang, Y., Guo, Y., Xu, H., Liang, C., Wang, Z., and Xu, J., *Int. J. Biol. Macromol.* **2019**, *141*, 484–492.
- [21] Wen, J.-L., Sun, S.-L., Yuan, T.-Q., and Sun, R.-C., *Green Chem.* **2015**, *17* (3), 1589–1596.
- [22] Granja-Travez, R. S., Wilkinson, R. C., Persinoti, G. F., Squina, F. M., Fülöp, V., and Bugg, T. D., *FEBS J.* **2018**, *285* (9), 1684–1700.
- [23] Kellock, M., Maaheimo, H., Marjamaa, K., Rahikainen, J., Zhang, H., Holopainen-Mantila, U., Ralph, J., Tamminen, T., Felby, C., and Kruus, K., *Bioresour. Technol.* **2019**, *280*, 303–312.
- [24] Francis, C., and Marder, V., *Annu. Rev. Med.* **1986**, *37* (1), 187–204.
- [25] Coull, B. M., Williams, L. S., Goldstein, L. B., Meschia, J. F., Heitzman, D., Chaturvedi, S., Johnston, K. C., Starkman, S., Morgenstern, L. B., and Wilterdink, J. L., *Stroke* **2002**, *33* (7), 1934–1942.
- [26] Meade, T., Ruddock, V., Stirling, Y., Chakrabarti, R., and Miller, G., *Lancet* **1993**, *342* (8879), 1076–1079.
- [27] Bridge, K. I., Philippou, H., and Ariëns, R. A., *J. Thromb. Haemos.* **2014**, *112* (11), 901–908.
- [28] Luceri, C., Giannini, L., Lodovici, M., Antonucci, E., Abbate, R., Masini, E., and Dolaro, P., *Br. J. Nutr.* **2007**, *97* (3), 458–463.
- [29] Zaman, R., Parvez, M., Jakaria, M., Sayeed, M. A., and Islam, M., *Res. J. Med. Plant* **2015**, *9* (3), 135–140.
- [30] Patel, D., Desai, S., Desai, A., Dave, D., and Meshram, D., *J. Pharmacog. Phytochem.* **2019**, *8* (3), 3916–3918.
- [31] Yoon, S.-J., Yu, M., Sim, G.-S., Kwon, S.-T., Hwang, J.-K., Shin, J.-K., Yeo, I.-H., and Pyun, Y.-R., *J. Microbiol. Biotechn.* **2002**, *12* (4), 649–656.
- [32] Mine, Y., Wong, A. H. K., and Jiang, B., *Food Res. Int.* **2005**, *38* (3), 243–250.