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OPTIMIZATION OF ENZYMATIC DELIGNIFICATION OF CASSAVA PEEL USING LACCASE ENZYME

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ABSTRACT
Cassava can be processed to produce cassava starch, cassava chips, and modified cassava flour (MOCAF). Cassava peel has cellulose and hemicellulose contents of 30 to 37% and 10 to 30%, respectively. Therefore, cassava peel has great potential as a raw material for cellulose. Cassava peel can be processed for cellulose production by enzymatic delignification. This study aims to optimize the enzymatic delignification of cassava peel using response surface methodology (RSM). Three process variables, such as incubation time(X1), concentration of enzyme (X2), and pH (X3), were evaluated. The yield of cellulose obtained is 18%. The higher content of cellulose obtained is 93.52%, under the conditions of time of incubation 6 days, laccase enzyme concentration of 3%, and pH of 3. The effect of the variable on cellulose content, for a single variable, only time of incubation (X1) has a significant effect on cellulose content (P-value < 0.05), while for interaction variables, X1 square, X2 square, and X1*X2 have a significant effect on cellulose content. The cellulose produced has great potential to be developed on an industrial scale to support the health industry, especially in the cosmetics sector.
Keywords: Cassava Peel, Cellulose, Enzymatic, Delignification, Laccase Enzyme.
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INTRODUCTION

Indonesia is a nation reliant on agriculture. One of the big-scale productions is cassava. Cassava can be processed to produce cassava starch, cassava chips, and modified cassava flour (MOCAF). Recently, cassava peel has not been used properly. Several efforts have been made to utilize cassava peel, such as for animal feed, food supplements, and most easily for making compost. Cassava peel is a material that has a lot of cellulose in terms of cellulose and hemicellulose, that known as lignocellulosic material. Cassava peel has cellulose and hemicellulose contents of 30 to 37% and 10 to 30%, respectively.[1,2] Therefore, cassava peel has great potential as a raw material for cellulose production. Cellulose can be processed further to produce nanocrystalline cellulose, cellulose acetate (CA), and carboxymethyl cellulose (CMC).[3-6] The isolation of cellulose from cassava peel begins with the delignification process, namely the removal of lignin from biomass. The delignification is an important process in the production of cellulose from biomass. The delignification can be conducted by several processes, such as alkali solvent, acid solvent, alkaline hydrogen peroxide (AHP), ionic solution, organic solvent, and enzymatic delignification.[7-14] Enzymatic delignification is a process that uses enzymes (mainly ligninolytic enzymes like laccases, lignin peroxidase, and manganese peroxidase) to remove lignin from lignocellulosic biomass. Compared to conventional chemical or mechanical methods, it offers several important advantages, such as: environmentally friendly, mild operational conditions, lower chemical usage, and potential for process integration. Enzymatic delignification will produce cellulose that can be applied in health and cosmetics. The cellulose can be processed to form cellulose microcrystalline (CMC) and cellulose nanocrystalline (CNC) by the milling process. CNC can be used as an active compound in sunscreen cream.[15-17] The enzymatic delignifications have been done by several researchers. The effect of the concentration of sodium hydroxide during the pretreatment process before enzymatic delignification (Cellic CTec-02 enzyme) has been studied by Yadaf *et al.*[18] The mechanism of the laccase enzyme in the process of delignification has been described by Haq *et al.*[19] This study aims to optimise the enzymatic

delignification of cassava peel using response surface methodology (RSM). The enzyme used is laccase. Three process variables, such as incubation time, concentration of enzyme, and pH, were evaluated. Fourier transform infrared (FTIR) spectrometry is used to analyze the functional group of cellulose in cassava peel. Scanning electron microscopy (SEM) is used to describe the morphology of the cellulose of cassava peel.

EXPERIMENTAL

Materials

Cassava peel was obtained from a local MOCAF industry at the Banjarnegara district (PT. Rumah Mocaf, Indonesia). Sodium hydroxide was purchased from Merck. Laccase enzyme was purchased from A&N Laboratory. Aquadest was purchased from Brataco.

Pre-treatment

The cassava peel was washed and dried in an oven at 105 °C for 6 h. The cassava peel was then ground using a milling machine to a size of -18 + 36 mesh. Pre-treatment of cassava peel was completed by diluting 100 g of dry cassava peel in 800 mL of 1 N sodium chloride solution. The mixture was then heated and stirred at a temperature of 90 °C for 90 min. The cassava peel was washed using demineralised water until neutral. The cassava peel was dried in the oven at a temperature of 105 °C for 3 h. The content of cellulose was analyzed using Chesson-Datta methods.[8]

Enzymatic Delignification

The enzymatic delignification process of cassava peel was conducted using the laccase enzyme. A total of 5 g of cassava peel after pre-treatment was diluted in 20 mL of 1% enzyme solution in the bottle. The mixture was incubated at room temperature for 1 day. After incubation, the cellulose was filtered and washed using hot water. The cellulose was dried in the oven at 105 °C for 3 h. The yield of the pre-treatment process and cellulose were calculated using Eqn.-1 and 2,

$$Y_{pre\text{-}treat} = w_1/w_0 \text{ x } 100\% \quad (1)$$

$$Y_{Cell} = w_2/w_0 \text{ x } 100\% \quad (2)$$

Where w_0 (g) is the dry weight of cassava peel, w_1 (g) is the dry weight of cassava peel after pre-treatment, and w_2 (g) is the dry weight of cellulose. Design of experiment is shown in Table-1.

Table-1: Experimental Design of Enzymatic Delignification

Variables	Range and Levels				
	+α	1	0	-1	-α
Incubation time, day (X1)	6.682	6	5	4	3.318
Enzyme concentration, % (X2)	4.682	4	3	2	1.318
pH (X2)	4.682	4	3	2	1.318

RESULTS AND DISCUSSION

Cassava peel (CP) is a lignocellulosic material. Based on Chesson–Datta Methods, the content of cellulose, hemicellulose, and lignin is 25.45%, 24.99%, and 17.62%, respectively. While the soluble water material reaches 23.55%. After the pre-treatment process, the content of total cellulose and lignin is 51.21% and 14.11%, respectively. During the pre-treatment process, the obtained yield of cassava peel pre-treatment (CP pre-treat) was 30%, which means the mass loss of cassava peel will be 70%. After the enzymatic delignification, it will be obtained yield of cellulose of 18%. The effect of incubation time on enzymatic delignification was observed in the range of 1 – 6 days. The optimum incubation time is 5 days, with the cellulose content of 90.12%. The increase of cellulose content reaches 75.98%, from 51.21% to 90.12%. The effect of the laccase enzyme was observed in the range of 1 – 5% with an incubation time of 5 days. The total cellulose content ranges from 90.12 to 93.08%. The optimum enzyme concentration is 3% with a cellulose content is 91.06%. While the effect of pH was observed in the range of 2 – 6 at the incubation time of 5 days and enzyme concentration of 3%. The total cellulose content ranges from 90.12 to 93.4%. The optimum pH is 4, with a cellulose content is 93.2%. The content of cellulose until 93% shows that cellulose obtained can be applied in health and cosmetics. Table-2 shows the cellulose content after the enzymatic delignification process.

Table-2: The Cellulose Content after the Delignification Process

Run	X1	X2	X3	Yield
1	0	0	0	91.12
2	1.682	0	0	93.52
3	0	0	0	91.2
4	1	1	-1	93.24
5	1	-1	-1	92.78
6	0	1.682	0	93.24
7	-1.682	0	0	85.64
8	0	0	-1.682	91.32
9	0	0	0	91.06
10	-1	-1	-1	88.82
11	0	0	0	91.06
12	-1	1	-1	90.28
13	1	1	1	93.2
14	0	0	1.682	92.9
15	0	-1.682	0	93.18
16	-1	-1	1	90.16
17	-1	1	1	87.28
18	0	0	0	91.06
19	0	0	0	91.06
20	1	-1	1	93.06

ANOVA and Model Fitting Analysis

Based on Table-2, the effect of the variables process on cellulose content was analyzed using the analysis of variance (ANOVA) method. Table-3 shows the ANOVA of each variable. For a single variable, only the time of incubation (X1) has a significant effect on cellulose content (P-value < 0.05), while for interaction variables, X1 square, X2 square, and X1*X2 have a significant effect on cellulose content.
Equation-3 shows the correlation of the cellulose content and the process variables using the second-order polynomial equation (coded value).

$$C_{Cell} = 91.1115 + 2.1229*X1 - 0.6540*X1^2 + 0.6294*X2^2 - 0.5825*X2*X3 \quad (3)$$

The value of R^2 of 0.94 shows that the correlation is suitable. Figure-1 shows the normal probability plot of coded variables vs cellulose content. Figure-3 shows the contour plot of coded variables vs cellulose content. Table-3 shows the ANOVA analysis.

Table-3: Analysis of Variance (ANOVA) of variables.

Term	Coef	SE Coef	P	Assignment
Coefficient	91.1115	0.2871	0.000	
X1	2.1229	0.1905	0.000	s
X2	-0.0527	0.1905	0.788	ns
X3	0.0906	0.1905	0.645	ns
X1*X1	-0.6540	0.1854	0.005	s
X2*X2	0.6294	0.1854	0.007	s
X3*X3	0.2405	0.1854	0.224	ns
X1*X2	0.2525	0.2488	0.334	ns
X1*X3	0.2375	0.2488	0.362	ns
X2*X3	-0.5825	0.2488	0.041	s

s = significant; ns = not significant

Figure-5 shows the structural analysis of cassava peel, the pre-treatment process, and enzymatic delignification. Laccase enzyme is well-known as a green catalyst. Laccase enzyme consists of three types of functional groups that bind to a copper ion as a center.

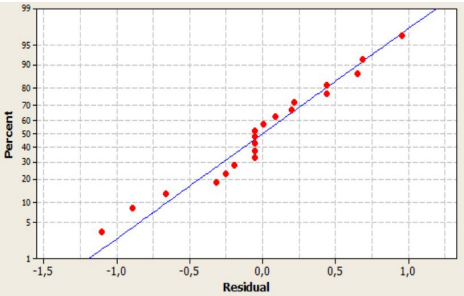

Fig.-1: Normal Probability Plot

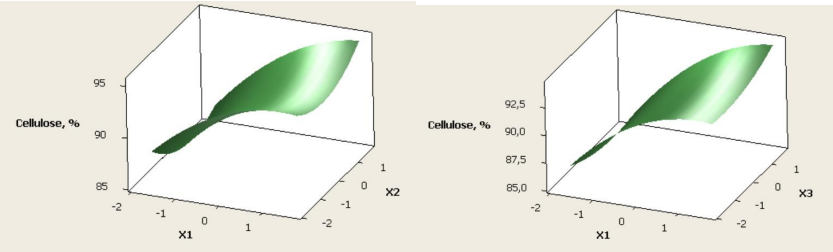

Fig.-2: Contour Plot of Coded Variables vs Cellulose Content

A type 1 copper (T1Cu) gives the protein its blue color. Another type is T2Cu and T3Cu, distributed between different binding sites. Three different kinds of copper functional groups reduce oxygen to water by forming a trinuclear cluster. A highly conserved internal electron transport mechanism keeps this special copper functional group structure intact by reducing one oxygen molecule to two water molecules.[19,20] During the lignin delignification process, lignin degradation depends on the cleavage of ether bonds, while carbon-carbon bonds are essentially stable. The lignin's ether bond cleavage is due to the presence of copper structures in the laccase enzyme, which form oxygen molecules (Fig.-6). The CP-pretreat consists of relatively thin and weak layers, meaning that the enzyme does not need to penetrate through a thick material, as in the case of wood. Also, the peel can be dispersed by moderate hydrodynamic shearing, rendering the material even more accessible.

Functional Groups of Cellulose

Figure-3 shows the FTIR spectra of cassava peel, cassava peel pre-treatment, and cellulose. There are seven main peaks, namely 3281 to 3288 cm^{-1}, 2932 cm^{-1}, 1621 cm^{-1}, 1421 cm^{-1}, 1320 cm^{-1}, 1151 cm^{-1}, and 1020 cm^{-1}. The hydroxyl groups (-OH) generated by chemically adsorbed water and surface primary hydroxyl groups are responsible for the peaks of 3281 to 3288 cm^{-1}. Methyl and methylene units' C-H stretching absorption is visible at the peak of 2932 cm^{-1}. The peak of 1621 cm^{-1} corresponds to the C = O stretching vibration of the carbonyl groups. While the peaks of 1421 cm^{-1}, 1320 cm^{-1}, 1151 cm^{-1}, and 1020 cm^{-1} correspond to the $–CH_2$ scissoring, C-H asymmetric, C-O antisymmetric, and C-O-C pyranose ring skeletal, respectively.[21,22] Table-2 shows the FTIR spectrum of several celluloses from various sources.

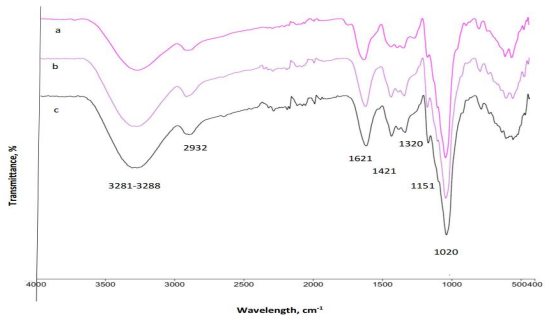

Fig.-3: FTIR Spectrum of Cellulose

Table-2: FTIR Spectrum of Several Celluloses From Various Sources

Raw Material	O-H Vibration	C-H Vibration	C=O Vibration	-CH_2 Vibration	C-H Vibration	C-O Vibration	C-O-C Vibration
Ficus Leaves[21]	3400	2890	1736	1419	1371, 1321	1157	1026
Coffee[22]	3350 to 3600	2901	1735	-	-	-	1040
Commercial Cellulose[23]	3391	2906	1760	-	1373	1162	1061
Sugarcane Baggase[24]	3451	2899	1644	-	1382	-	1060
Cassava peel	3281 to 3288	2932	1621	1421	1320	1151	1020

Morphology of Cellulose

In the pre-treatment process using sodium hydroxide solution, the soluble water materials and lignin will be dissolved in the water. Figure-4 shows the morphology of cassava peel, CP pre-treatment, and CP after enzymatic delignification at 1000x magnification. From Fig.-4, it is seen that the cassava peel contains a large amount of complex material. These complex materials are dissolved during pretreatment using NaOH solution. Based on the pre-treatment yield, the amount of complex material reaches 70%.

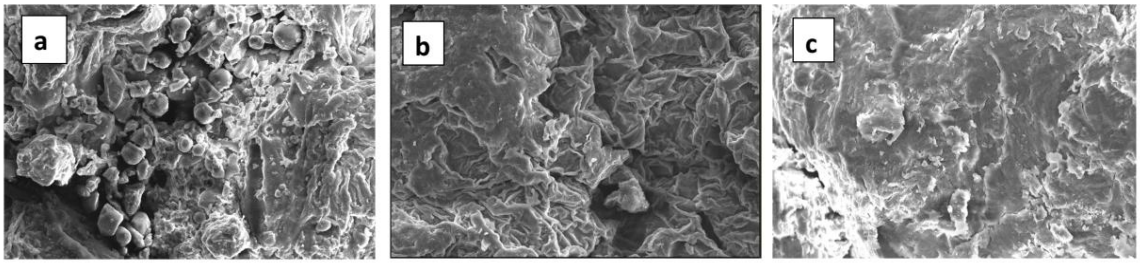

Fig.-4: Morphology of: (a) Cassava Peel; (b) CP Pre-Treatment; (c) Cellulose at 1000x Magnification

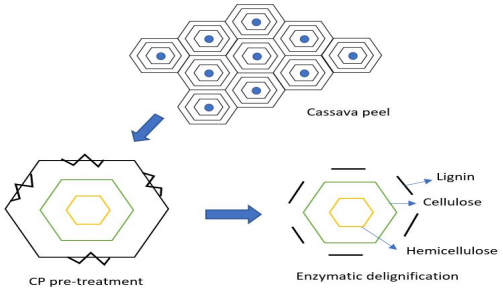

Fig.-5: Structural Analysis of Cassava Peel – Pre-Treatment Process – Enzymatic Delignification

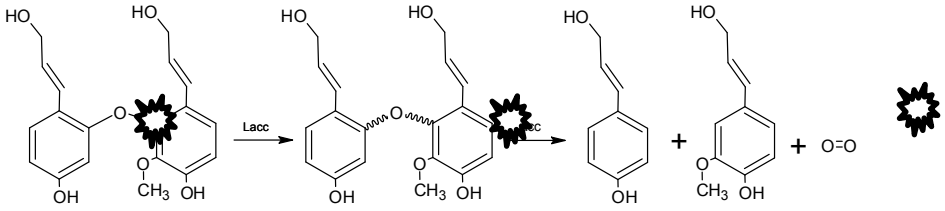

Fig.-6: Mechanism of Enzymatic Delignification using Laccase Enzyme

CONCLUSION

Cassava peel can be processed for cellulose production by enzymatic delignification. The yield of cellulose obtained is 18%. The higher content of cellulose obtained is 93.52%, under the conditions of time of incubation 6 days, laccase enzyme concentration of 3%, and pH of 3. The effect of the variable on cellulose content, for a single variable, only time of incubation (X1) has a significant effect on cellulose

content (P-value < 0.05), while for interaction variables, X1 square, X2 square, and X1*X2 have a significant effect on cellulose content. The cellulose produced has great potential to be developed on an industrial scale to support the health industry, especially in the cosmetics sector.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-Being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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