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Original Article

POTENTIAL OF CELLULASE OF *PENICILLIUM VERMICULATUM* FOR PREPARATION AND CHARACTERIZATION OF MICROCRYSTALLINE CELLULOSE PRODUCED FROM α-CELLULOSE OF KAPOK PERICARPIUM (*CEIBA PENTANDRA*)

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ABSTRACT

Objective: This study aimed to find psychochemical properties of microcrystalline cellulose (MCC) obtained from α-cellulose kapok pericarpium.

Methods: The cellulase activity was screened by clear zone and sugar reduction method. The enzyme from selected mold was purified by diethylaminoethyl (DEAE) chromatography. α-cellulose of kapok pericarpium was hydrolyzed using the purified cellulase enzymes. Microcrystalline cellulose (MCC) was identified by Fourier transform infrared (FTIR) spectrometry, and qualitative analysis test. The MCC samples were characterized for pH test, x-ray diffraction (XRD), and particle size analyzer (PSA).

Results: The optimum cellulase activity was shown by *Penicillium vermiculatum*. It's clear zone diameter around 3 cm and the cellulase activity was 67.73±0.25 mU/ml. The strongest cellulase activity was detected from 1st fraction (P1) out of 6 column fractions with optimum activity at 1.177±2 mU/ml. The optimal conditions for microcrystalline cellulose (MCC) preparation were at 50 ˚C, for 2 h, using 20 ml of acetate buffer pH 5 and 2 ml of cellulase enzyme. Microcrystalline cellulose (MCC) obtained at 78% w/w and its FTIR spectrum and x-ray diffractogram similar to reference while the pH of MCC was fulfilled requirements of The United States Pharmacopoeia 2007.

Conclusion: The use of purified enzyme of cellulase has succeded in microcrystalline cellulose (MCC) preparation and MCC yield obtained was 78% w/w, which showed similar characteristics to reference (Avicel PH 101)

Keywords: Microcrystalline cellulose, Enzyme purification, Kapok, Enzyme Hydrolyisis, Characterization

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INTRODUCTION

The kapok tree, *Ceiba pentandra*, formerly Bombacaceae family, is cultivated widely in southeast asia, as well as other parts of east Asia and Africa. The kapok fiber is an agricultural product obtained from the fruits of the kapok tree. Chemical compositions of kapok fiber are chemically composed of 64% cellulose, 13% lignin, and 23% pentosan [1]. Cellulose is the linear polymer of anhydroglucose and one of the most abundantly occurring natural polymers on earth. Cellulose is one of the complex carbohydrates consisting of 3000 or more glucose units. Cellulose derivatives are a class of natural polymers in which cellulose is swollen to form films with higher tensile strength and improved water vapor properties [2]. High cellulosic content of kapok fiber indicated its potential as source for microcrystalline cellulose (MCC), which is micro-sized crystalline part extracted from cellulose [3]. Microcrystalline cellulose (MCC) is an additional material that is used in the wide range of pharmaceutical, food, cosmetics, and other industries. Microcrystalline cellulose (MCC) is one of the most important tableting excipients due to its outstanding dry binding properties, enabling the manufacture of tablets by direct compression (DC) [4, 5] and it is most common excipient utilized for the production of pellets via extrusion spheronisation technique. In case of a drug having low solubility, MCC can give prolonged drug release profile due to the lack of disintegration of MCC based pellets [6]. The source of pharmaceutical excipient that can be obtained commercially is wood, but also it still used for various other purposes, such as making furniture, paper, tissue, and others. The use of wood in preparation microcrystalline cellulose is considered to be less effective because it reduces the number of trees on a large scale resulting in ecological imbalances. According to Rasha and Myasar (2018), drug formula that was prepared with MCC showed the shortest disintegration, flow properties ranged from good to fair [7].

Microcrystalline cellulose (MCC) can be synthesized through two different processes, namely the process of acid hydrolysis and enzymatic hydrolysis. The advantage of enzymatic hydrolysis is the methods of working at low temperature and low cost. In our previous study, the cellulolytic isolates from the soil have a high glucose concentration and it's needed 10% v/v crude enzyme extract for preparation of cellulose microcrystalline from water hyacinth, so it needs the more enzyme volume of crude extract enzym for preparation of microcrystalline cellulose [8]. To overcome high glucose concentration, purified enzymes are expected to reduce b-glucosidase activity which can degrade cellulose to glucose.

In this study, α-cellulose from kapok pericarpium was hydrolyzed using purified enzymes. Cellulase enzymes used was selected from a few molds which had the optimum cellulolytic activity from several molds. The fraction of the cellulase enzyme with the highest cellulase activity was used for hydrolysis. Microcrystalline cellulose (MCC) obtained were identified using Fourier transform infrared (FTIR), and qualitative analysis. The samples were characterized for pH test, x-ray diffraction (XRD), and particle size analyzer (PSA).

MATERIALS AND METHODS

Raw material

The raw material used in this study was kapok pericarpium powder obtained by Wahid's kapok farm, Pati district, Central Java province. The raw material was dried and homogenized at Balitro, Bogor city, West Java province.

Chemical material

The chemicals used in this study were Avicel PH 101 as a reference, nitric acid (Merck), acetic acid (Merck), sodium hydroxide (Merck), sodium hypochlorite (Merck), sodium nitrite (Merck), sodium sulfite (Merck), KBr powder (Merck), potato dextrose agar (DifcoTM), yeast extract (Himedia), peptone (DifcoTM), glucose (Merck), zinc chloride (Merck), aquadestillata (Merck), aquabidestillata (Otsuka).

Biological material

The molds used in this study were *Trichoderma reesei*, *Penicillium vermiculatum*, *Ganoderma* (University of Indonesia Culture Collection, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia), and *Phanaerochaete chrysosporium*(IPB Culture Collection, Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University).

Instruments

The instruments used in this study were incubator (Memmert), autoclave (Hirayama), hotplate stirrer (Corning), oven (Hotpack), particle size analyzer (Cillas 1190), spectrophotometry IR (Shimadzu), centrifugator (Kubota 6800), analytical scales (Acculab), scanning electron microscope (JEOL), x-ray diffractometer (Rigaku Smartlab), 3×25 cm DEAE-cellulose column (Sigma chemical), water bath (Memmert).

Isolation of α-cellulose kapok pericarpium

In 300 g of kapok pericarpium powder was dissolved in 4.02 L HNO_3 3.5%, followed by the addition of 40 mg sodium nitrite and heated at 90 ˚C for 2 h. The next procedure was continued according to previous research by Suryadi *et al.* [8].

Maintenance of culture

Vermiculatum the mold colonies were regrown from parent cultures planted in potato dextrose agar (PDA) medium. Incubation was carried out at 27 ˚C for 7 d.

Mold screening based on the clear and sugar reduction method.

For every 5 µl, crude enzyme extract was injected into 6 mm paper disc using micropipette in a petri dish containing CMC media for 3-7 d. 10 ml lugolwas used to make the transparent zone formation. The cellulase activity was measured using UV-Vis spectrophotometry [8].

Preparation of crude extract enzyme

The selected mold and *Trichoderma reesei* were re-grown to 5 PDA medium tubes. The spore suspension was made by adding 5 ml of aquabidestillata to each tube containing isolated culture in the agar medium and stirred the tube. For 5 Erlenmeyer 500 ml were inserted 5 ml of spore suspension, added 10 ml 0.1% tween 80 and nutrient broth solution. The mixture of solution was stirred at 150 rpm for 7 d at room temperature. After 7 d the solution was centrifuged at 3000 rpm for 10 min at 4 ˚C and the supernatant was taken for precipitation of endoglucanase [8].

Precipitation of endoglucanase with ammonium sulfate

For 1 liter of the supernatant solution was added slowly ammonium sulfate with constant stirring in the magnetic stirrer for 4 to 6 h at room temperature resulting in a saturation of 80-95%. After the desired percentage of saturation has been obtained, the next supernatant was centrifuged at 4000 rpm for 20 min at 4 °C until the brown precipitate was obtained. The precipitate was dissolved in 0.01 M phosphate buffer solution at pH 7.0 containing 0.1 M NaCl. Dark brown solutions were dialyzed at 4 °C for 72 h use the same buffer solution. Every 24 h buffer solution was replaced. The cellulase activity and protein were calculated using UV-Vis spectrophotometry [9].

DEAE column chromatography

DEAE-cellulose column chromatography was carried out according to the following procedure. The DEAE cellulose column was prepared after initial treatment with 0.5 N NaOH and 0.5 N HCl. This procedure was continued according to previous research by Megha *et al.* (2015) [9].

Optimization of pH and temperature for cellulolytic activity

For pH optimized 0,5 ml of the crude enzyme was preincubated without substrate, added buffer and adjusted to appropriate pH values (3, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0) respectively at 50 ˚C for 30 min, after which cellulose activity was determined. The procedure to determine the optimum temperature of the enzyme according to Ire *et al.* [10]. The cellulase activity was measured using UV-Vis spectrophotometry [8].

Optimization of hydrolysis time

The purified enzyme of selected mold and *Trichoderma reesei* were centrifuged at 3000 rpm 4 °C, for 10 min. For measurement, 1 g of α-cellulose was dissolved in 10 ml acetate buffer 0.1 M pH 5 and 0.2 ml of the enzyme supernatant was added. The solution mixture was incubated at 50 °C 160 rpm for 10 h and every 2 h the solution was taken and added DNS 1% 0.75 ml for measuring using UV-VIS spectrophotometry [9].

Optimization of hydrolysis concentration

For every 1 g of α-cellulose was dissolved in 20 ml 0.2 M acetate buffer pH 5 and added purified enzyme with some concentrations (0.4 ml, 1.2 ml, and 2 ml). The mixture of the solution was incubated at 50 °C 160 rpm for 2 h, 0.75 ml of DNS 1% was added and heated for 15 min and left to stand at room temperature. Absorbance was measured and calculated by the calibration curve equation [9].

Preparation of microcrystalline cellulose

An amount of 2 g of α-cellulose kapok pericarpium were dissolved in 20 ml acetate buffer (0.1 M, pH 5) and 2 ml of cellulase enzyme was added while stirring slowly. The mixed solution was stirred at 160 rpm at 50 ˚C for an hour above the water-shaking incubator. The procedure was continued according to previous research by Suryadi *et al.* [9].

Cellulose identification test on microcrystalline cellulose (qualitative analysis)

An iodine zinc solution was prepared by dissolving 20 g of the zinc-chloride and 6.5 g of potassium iodide in 10.5 ml of water. Add 0.5 g of iodine, and shake for 15 min. Added 10 mg of preparation in the watch glass and dissolved in 2 ml of zinc-chloride solution [11].

pH test

An amount of 5 g of MCC kapok pericarpium were dissolved in 40 ml aquadestillata and centrifuged for 20 min. The supernatant was taken for pH test using a standardized conductivity meter with a standard calibration of potassium chloride conductivity which had a conductivity of 100 µS per cm, then measured the supernatant conductivity and air conductivity used for test specimen purposes. Microcrystalline cellulose has a range of pH of 5.0 and 7.5 [11].

Identification of infrared spectrum

KBr powder has been dried at 105 ˚C for 24 h was weighed carefully±99 mg and added 1 mg of MCC. The mixture was crushed and mixed until homogeneous, then put into a disk. For 100 mg KBr were weighed for blank and made a baseline and scanned from 400 cm^{-1} to 4000 cm^{-1} wave number area [12]. The infrared (IR) spectrum of MCC was compared to the IR spectrum of Avicel PH 101.

Organoleptic test

The prepared samples were observed on a white base, including in shape or appearance, color, taste, and odor [13].

Analysis of particle size and distribution

The particle size and distribution of samples were analyzed according to previous studies by Suryadi *et al.* [14].

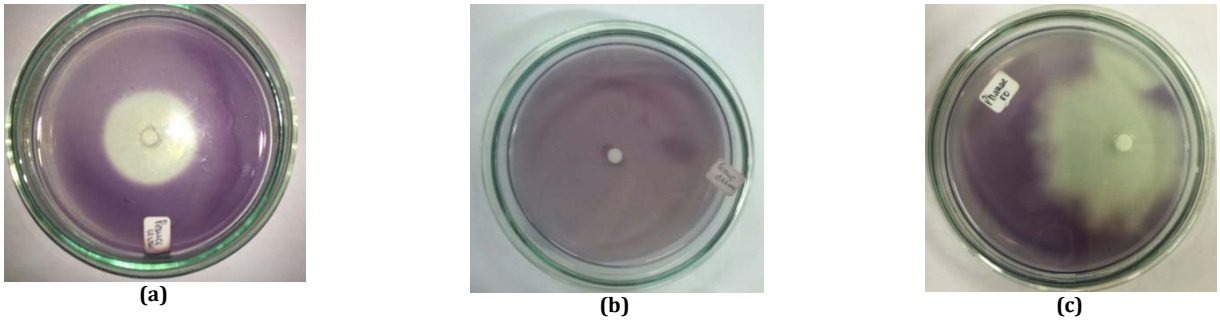
Analysis of X-RD

Crystalline property of samples was analyzed by XRD according to the study by Kips *et al.* [15].

RESULTS

Mold screening based on the clear zone and sugar reduction method

Based on the results of screening it can be seen that *Penicillium vermiculatum* has the greatest clear zone among the three molds. It's a clear zone around 3.00 cm (fig. 1).



(a) (b) (c)

Fig. 1: Results of cellulolytic activity of *Penicillium vermiculatum* (a), *Ganoderma* (b), and *Phanaerochaeta chrysosporium* (c) on CMC media each mold showed the clear zone diameter were 3.0 cm; 0 cm; 5.3 cm (respectively)

The cellulase activity was calculated using UV-VIS spectrophotometershowed that *Penicillium vermiculatum* had a low glucose level (48.79 ppm) with cellulase activity of 0.0677 U/ml. *Ganoderma* had high glucose level (0.8067 ppm) but the clear zone was not formed around the paper disc, and *Phanarochaeta chrysosporium* had high glucose level (75.67 ppm) with cellulase activity 0.1050 U/ml but it formed partial clear zone (Data not shown).

***Vermiculatum vermiculatum* purification of cellulase enzymes**

The results of the cellulase purification are shown in table 1. An amount of 15 ml precipitate enzyme has obtained after added ammonium sulfate. The precipitate enzyme was put into the DEAE column with gradient stepwise of 0.05 M NaCl, 0.1 M, and 0.2 M.

Table 1: Cellulase activity in crude enzyme extracts, ammonium sulfate fraction, dialysis, and fraction numbers from *Penicillium vermiculatum*

Penicillium	Volume (ml)	Total Cellulase activity units (mU/ml)	Total Protein (mg/ml)	Specific activity (U/mg)	Yield (%)
Crude enzyme extract	400	67.73±0.25	80.97±0.19	0.83±0	100
80-95% ammonium sulphate fraction	15	73.1±0.88	50.56±0.29	1.44±0.02	107.92
P1 (Dialysis)	6	1.177±2	42.94±0.48	27.40±0.33	1610.12
P2 (Buffer fosfat)	6	960.67±1.154	25.84±0.32	37.17±0.48	81.62
P3 (0,05 M NaCl)	6	549.67±1.52	24.77±0.36	22.18±0.26	57.21
P4 (0,1 M NaCl)	6	610.67±0.57	22.51±0.17	27.11±0.21	111.09
P5 (0,2 M NaCl)	6	640.67±1.154	12.45±0.40	51.45±1.56	104.91
P6 (0,2 M NaCl)	6	390.33±1.52	7.13±0.05	54.74±0.59	60,92

mean±SD (Standar deviation), n = 3., An amount of 6 ml purified enzyme were obtained from 400 ml of crude enzyme extract. The highest cellulase activity can be seen from the 1st fraction (fig. 2).

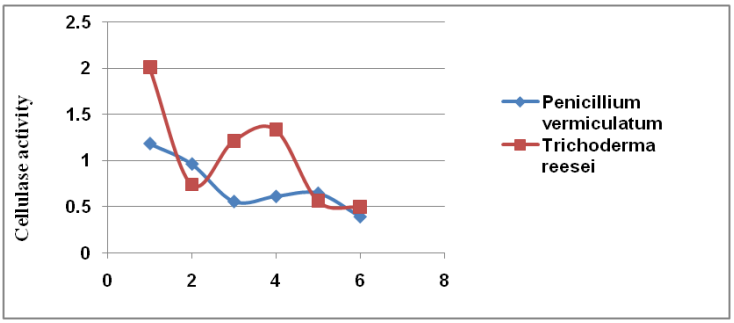


Fig. 2: Elution profile of DEAE column chromatography for the purification of cellulose

Optimization conditions of hydrolysis

Optimization condition of hydrolysis for MCC preparation were temperature, pH, enzyme concentration, and hydrolysis time. The optimum temperature and pH for hydrolysis were 55 ˚C and pH 5.5. The profile of temperature and pH were shown in fig. 3.

For 3 concentrations were tasted 0.4 ml, 1.2 ml, and 2 ml. The concentration of 2 ml purified enzyme in 20 ml of acetate buffer had higher cellulase activity. For the optimization of time for 10 h and every 2 h sampling was taken and tested using UV-Vis spectrophotometry, the result was shown (fig. 3) the second hour has lower glucose concentration than 4th, 6th, 8th and 10th h. The optimum condition was chosen as the optimum condition for MCC preparation by enzymatic hydrolysis.

Preparation of α-cellulose from the kapok pericarpium

An amount of 25 g α-cellulase or 16.67% were obtained from 150 g of kapok pericarpium powder (*Ceiba pentandra* (L.) Gaertner). The results of α-cellulose obtained were less than 64% due to washing and filtering so that some were left on the filter device and were wasted with the filtrate.

Preparation of microcrystalline cellulose by enzymatic hydrolysis

An amount of 3.8 g MCC or 78% w/w were obtained from 5 g of α-cellulose (fig. 4).

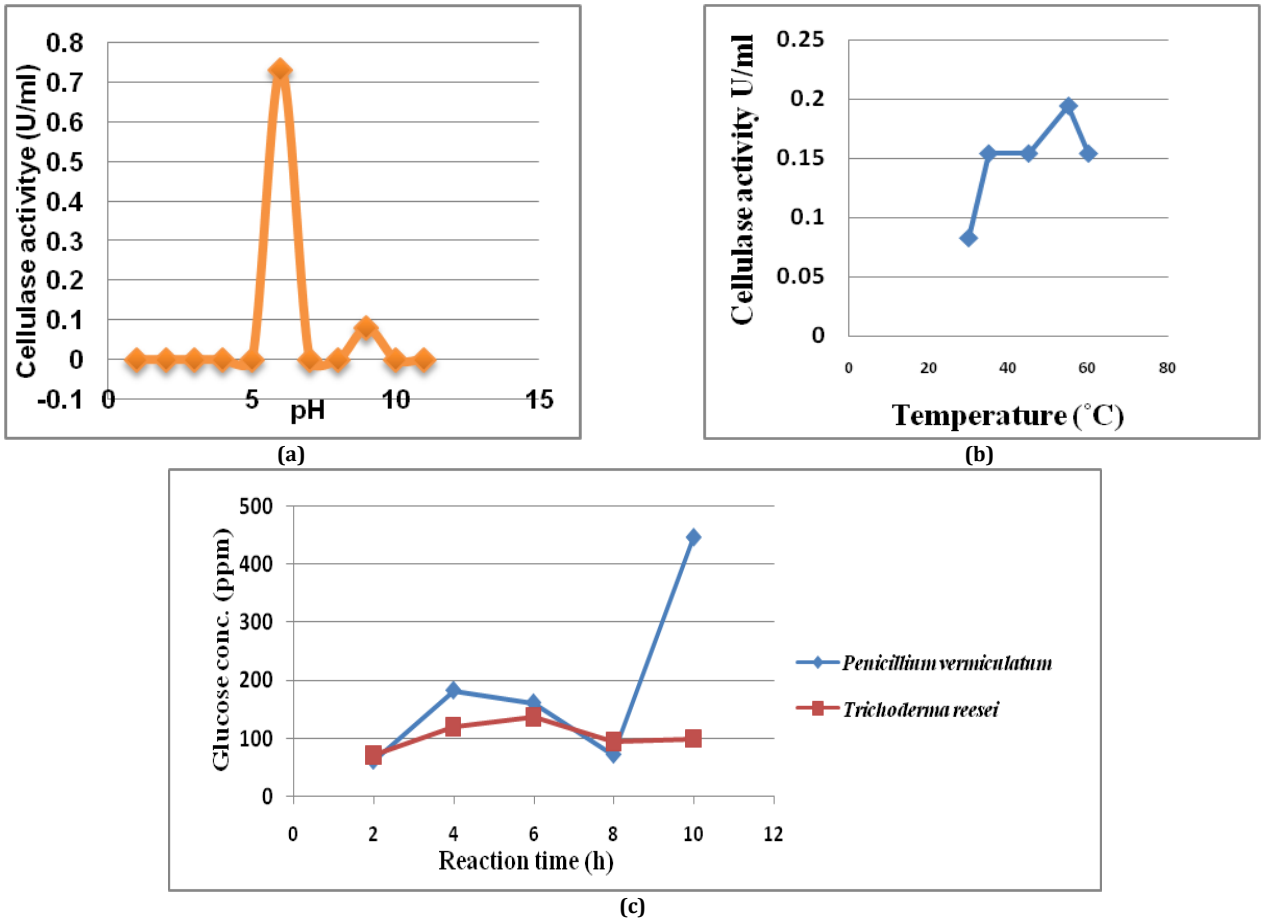


Fig. 3: Effect of pH on the cellulase activity of *Penicillium vermiculatum* (a), the effect of temperature on the cellulase activity of *Penicillium vermiculatum* (b), and the optimization duration of hydrolysis (c)

Fig. 4: α-cellulose of kapok pericarpium before hydrolyzed (a) and MCC of kapok pericarpium

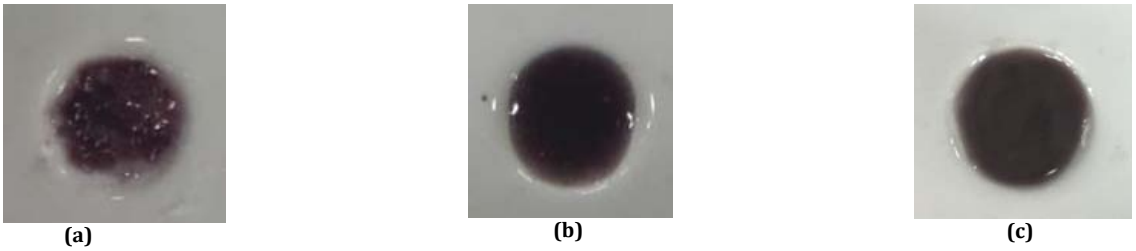


Fig. 5: Cellulose identification using ZnCl, after treated, with cellulase enzyme from *Penicillium vermiculatum* (a), with cellulase enzyme from *Trichoderma reesei* (b), and reference Avicel PH 101 (c)

Cellulose identification test on microcrystalline cellulose (qualitative analysis)

Microcrystalline cellulose was showed a color change to blue-violet when reacted with zinc chloride solution. The results confirm British pharmacopeia requirements.

pH test

Microcrystalline cellulose (MCC) of kapok pericarpium obtained has a pH of 7.0. The pH test result indicates that MCC of kapok pericarpium fulfills the requirements of The United States Pharmacopoeia 2007, which supernatant pH on ranges 5.0-7.5.

Analysis of Fourier transform infrared (FTIR)

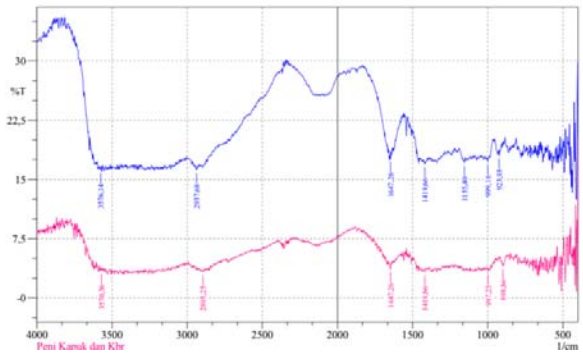


Fig. 7: The identification Fourier transform infrared (FTIR) results from Avicel Ph 101(blue) and MCC of kapok pericarpium (pink)

Based on the infrared spectrum, it can be seen some functional groups. Like at a wavelength of 3500 cm^{-1} a wide band was showed the presence of OH groups. In the 2895.25 cm^{-1} was shown a C-H aliphatic bond and in the wavelength 1647.26 cm^{-1} was frequencies of deformation vibrations of CH_2. The presence of glycosidic C-O-C was showed in the 1419.66 cm^{-1} band and 898.86 cm^{-1} was β-glycosidic. Based on literaturefor Avicel as a reference was showed the following vibration peaks of cellulose: 3445 cm^{-1}corresponding to intramolecular OH stretching, including hydrogen bonds, 2898 cm^{-1} due to CH and CH_2 stretching, 1650 cm^{-1} corresponding to OH from absorbed water, 1430 cm^{-1} due to CH2 symmetric bonding, 1375 cm^{-1} due to CH bending, 1330 cm^{-1} due to OH in-plane bending, 1161 cm^{-1} due to C-O-C asymmetric stretching, 1061 cm^{-1} due to C-O-C stretching, and 898 cm^{-1} corresponding to asymmetric (rocking) C1 (β-glycosidic linkage) out of the plane stretching vibrations [16].

Organoleptic test

Microcrystalline cellulose (MCC) of kapok pericarpium obtained was white, odorless, and tasteless.

Analysis of particles size and distribution

Microcrystalline cellulose of kapok pericarpium was hydrolyzed using cellulase enzyme of *Penicillium vermiculatum*, previously dried, crushed, and blended, 10% of the total particle size have a particle size of fewer than 13.06 µm, 50% of the total particle size have a particle size of fewer than 39.40 µm, and 90% of the total particle size has a particle size of fewer than 196.79 µm. The sample using enzyme cellulose of *Trichoderma reesei* showed results, 10% of the total particle size has a particle size of fewer than 12.63 µm, 50% of the total particle size has a particle size of fewer than 36.43 µm, and 90% of the total particle size has a particle size of fewer than 162.13 µm. Avicel PH 101 as standard showed results 10% of the total particle size has a particle size of fewer than 6.25 µm, 50% of the total particle size has a particle size of fewer than 15,61 µm, and 90% of the total particle size has a particle size of fewer than 24.00 µm. The particle size of samples that have been hydrolyzed with cellulase enzymes of *Penicillium vermiculatum* still in the range 0.01-200 µm.

Analysis of X-RD

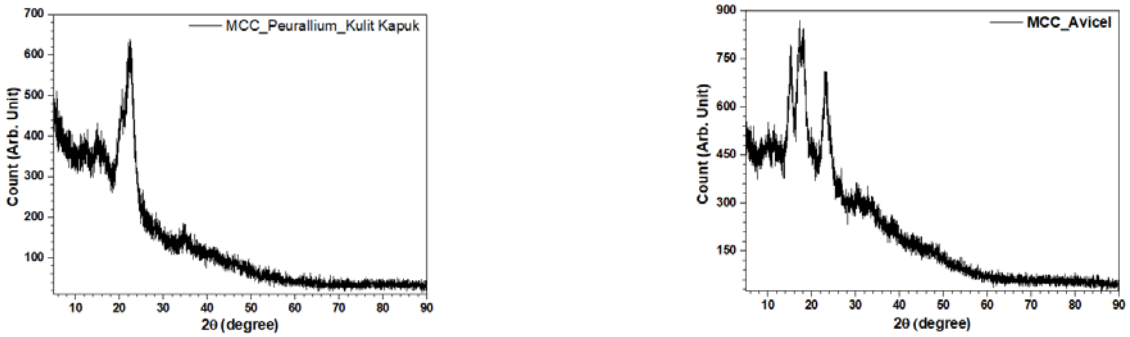


Fig. 8: Diffractogram of MCC kapok pericarpium powder from hydrolysis (a) and avicel PH 101

Based on x-ray diffraction (XRD) analysis, the reference diffractogram (Avicel PH 101) was showing a typical pattern, at 2θ (deg) =23.00 with an intensity 711 was showing a sharp peak that indicates the nature of crystalline. the sharp peaks on MCC kapok pericarpium were 2 peaks on 2θ (deg) value of 22.58 with intensity 634 and 21.85 with intensity 513, while the amorphous nature at 2θ (deg)=12.779 with intensity 418. The presence of the crystalline peak of the MCC kapok pericarpium was showed has the same diffractogram characteristics as Avicel PH 101.

DISCUSSION

The clear zones were formed around paper disc were caused by crude enzyme extract produce cellulase enzymes that are able to hydrolyze cellulose to glucose, it was monitored by iodine solution (Lugol reagent) as an indicator, when iodine was reacted with cellulose it produces a blue color, while with glucose were formed a clear zone. According to Zhang *et al.*, the dye was diffused into agar medium and absorbed by a long chain of polysaccharides that have the Beta-D-glucan chain [17]. Based on the results *Penicillium vermiculatum* has cellulase activity was formed clear zones, and *Phanaerochaetae chrysosporium* has cellulase activity, but it is not clearly formed. Whereas *Ganoderma* no clear zone is formed.

On purification of cellulase enzymes, the addition of salt in high concentrations is caused by the low salt concentrations can increase protein solubility, this is done by charged ions group of proteins and disturbed with the strength of strong electron was called salting in. Salt at high concentrations causes the H_2O molecule was bounded to the hydrophobic surface of the protein to bind with salt, the more H_2O molecules that bind to salt ions, the more proteins interact with each other so that they aggregate and precipitate. The precipitate that has been obtained was dialyzed using the visking tube to remove ammonium sulfate residue in the enzyme solution. Before dialysis, the visking tube was boiled in 2% $NaHCO_3$ solution containing 1 mmol EDTA for 10 min, then the dialysis bag was soaked in aquadestillate for twice in 10 min, and dried. Dialysis tubing is a semi-permeable membrane that allows as the movement of molecules below to cut off into and out of the dialysis bag. The tubing comes in a variety of sizes, usually with a M_r cut off approximately 12,000 but a tube with smaller and larger pore sizes can be obtained. Sodium bicarbonate and EDTA in preparation for visking tube were used to remove heavy metal ions [18]. The ion buffer in the column DEAE was exchanged with negative energy of protein, so the ion buffer with the positively charged protein will elute out of the column. When stepwise elution is performed, weakly bound proteins with the next matrix will be released and replaced

by salt ions in higher concentrations. According to Nooralabettuwhen salt concentration increases, ion salts such as Na^+or C^l-compete with proteins to bind with the surface charge of ion exchange resins and one or more of the bound proteins begin to elute and move down the column [19].

Based on the results of the study show the optimum temperature and pH for hydrolysis were 55 ˚C and pH 5.5. This is consistent with the literature that cellulase enzymes are stable at pH 4 to 6, and cellulase enzymes are stable at temperatures of 30 to 80 ˚C [10].

Addition of nitric acid in α-cellulose isolation of kapok pericarpium is caused by nitric acid is a good agent for the delignification process. Nitric acid can remove lignin in the form of nitrous lignin. However, nitric acid reacts very quickly with cellulose, so it is necessary to add sodium nitrite salt to prevent reaction with cellulose and the lignin degradation process can be accelerated. Furthermore, the addition of 1% Sodium sulfite is used to increase the delignification called sulfite pulping, where the dissolution of lignin is initiated by sulfonation of lignin which forms solid lignosulfate acid. This solid lignosulfate acid can dissolve with a hydrolysis reaction when it is warmed so that it can come out of the residue, during sulfonation most of the sulfonate groups attach to the lignin side chain which occurs within a few hours of the pulping process and causes lignin to dissolve [20]. Addition of sodium hydroxide in the concentration range between 1%-8% can reduce the lignin content from 60 to 90%. During the pulping process using a combination of sodium hydroxide and sodium sulfite during delignification, lignin and hemicellulose are degraded to alkali-soluble fragments [21]. In addition, the combination of these two solvents also causes xylan depolymerization without reducing cellulose [22]. Then, the addition of 17.5% sodium hydroxide was used to dissolve β-cellulose and ɣ-cellulose [23]. Sodium hypochlorite 3.5% is used for the process of whitening residues, hypochlorite has been used effectively in the sulfite pulping process where the stages also use alkali extraction carried out between two hypochlorite stages, the heating time using hypochlorite is around 30 min, but in some factories up to 3.5 h [24].

The results of preparation obtained from 5 g of α-cellulose were 3.8 grams of MCC kapok pericarpium or 78% w/w. Microcrystalline cellulose (MCC) produced was less because washed and filtered, fine particles of MCC left behind on the filter device and wasted with the filtrate.

CONCLUSION

As the conclusion the purified enzymes can reduce the glucose levels contained in the enzyme cellulase extract. Optimum conditions for enzymatic hydrolysis were at pH 5.5, temperature 50 ˚C, for 2 h. FTIR identification and the characteristic (XRD and PSA) of MCC kapok pericarpium were compared with reference (Avicel PH 101) showed that MCC had similar characteristics to reference and pH test showed that the samples was fulfilled requirements of The United States Pharmacopoeia 2007.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

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