J Young Pharm, 2017; 9(1) Suppl: s19-s23
A multifaceted peer reviewed journal in the field of Pharmacy
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Original article

Preparation of Microcrystalline Cellulose from Water Hyacinth Powder by Enzymatic Hydrolysis Using Cellulase of Local Isolate

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ABSTRACT

Introduction: Microcrystalline cellulose is one of the cellulose derivatives widely used in the pharmaceutical industry as an excipient in the manufacture of tablets. Unfortunately, most of these filler materials are still largely imported. On the other hand, Indonesia has huge potential to generate microcrystalline cellulose from agricultural waste or weeds such as oil palm empty fruit bunch and water hyacinth. The purpose of this study was to find a potential cellulolytic mold and raw material for preparation of microcrystalline cellulose by enzymatic hydrolysis. **Method:** The potential cellulolytic mold was obtained from rotted oil palm trunk, and alpha cellulose was prepared by digesting raw material powder by the alkaline condition. Cellulase enzymes are obtained through extraction from the cellulolytic mold and used to treat alpha cellulose at a concentration of 2, 6 and 10 % v/v, respectively. Then, the resulted microcrystalline cellulose was identified by SEM (Scanning Electron Microscope) and XRD (X-Ray Diffraction) and compared with reference Avicel pH 101. **Results:** The results showed that crude enzyme of isolated mold has better activity than Trichoderma reesei enzyme, which produced a lower concentration of glucose. **Conclusion:** Based on the comparison of crystal morphology and diffractogram pattern, water hyacinth has a great potential which showed crystalline characteristic similar to microcrystalline cellulose reference (Avicel pH 101)..

Key words: Water hyacinth, Microcrystalline cellulose, Cellulase, *Trichoderma reesei*.

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DOI: 10.5530/jyp.2017.1s.6

INTRODUCTION

Cellulose is a polymer of β-glucose with β-1-4 bonds between the units of glucose. Cellulose generally found in wood, cotton, and other lignocelluloses producing plants. Cellulose first isolated from wood in 1885 by Charles F. Cross and Edward Bevan. One of the cellulose derivatives often used in the pharmaceutical industry as an excipient in the manufacture of tablets by direct compression is microcrystalline cellulose (MC). Microcrystalline cellulose was first introduced in the early 1960s.[1] Microcrystalline cellulose serves as a binder, and a filler material as well as crushers and has shattered a relatively short time and can improve the flow properties of the granules.[2] The need of microcrystalline cellulose was supplied by importing from abroad that has an impact on the increase of price of drugs on the market.

Preparation of microcrystalline cellulose is generally used chemical processes by acid hydrolysis method, using a strong acid to remove amorphous part of cellulose to produce particles consisting of micro crystals.[3,4] However, chemical hydrolysis requires high activation energy and generates waste (acid, base, and organic compounds) which is less environmentally friendly. Current Research is being developed for a method of production of microcrystalline cellulose using cellulase enzymes. The process uses a controlled parameter, namely pH, temperature, and time.[5] Enzymatic hydrolysis has several advantages over acid hydrolysis. In the enzymatic hydrolysis, sugar degradation does not occur and can take place at low temperatures with high yield.[6]

On the other hand water hyacinth population that continues to grow each day and a huge number of oil palm empty fruit bunch (OPEFB) waste are becoming a problem that must be solved. Some utilizations of water hyacinth are: as art paper, as raw material for making bioethanol, particle board, and membranes,[7] while utilization of oil palm empty fruit waste is still limited. Based on its content, both sources of lignocellulosic materials has a high cellulose content composition. Water hyacinth has about 60% cellulose, 8% hemicellulose, and 17% lignin; while OPEFB waste has 41,30 – 46,50% cellulose, so they can be used to produce microcrystalline cellulose.[8-10] The purpose of this research was to find a potential cellulolytic mold and raw material for preparation of microcrystalline cellulose by enzymatic hydrolysis using crude enzyme of the potential isolate.

MATERIALS AND METHODS

Raw Materials and Microorganism water hyacinth raw material used in this study was obtained from *Balai PenelitianTanaman Rempah dan Obat* (Balitro), Bogor. Isolate of microorganisms derived from oil palm trunk, and stem of water hyacinth that had been rotted. *Trichoderma reesei* which obtained from collection of Sekolah Ilmu Teknologi Hayati (SITH), ITB, Bandung, was used as a reference culture. Medium of PDA was used for maintenance of the cultures.

Tools and Instruments

The tools used in this study were autoclave (Hirayama), oven (WTB Binder), analytical balance (Acculab), waterbath shaker, hotplate stirrer (Corning), pH meter (Eutech), sentrifugator (Kubota 6800), incubator (Memmert), vacuum oven (Hotpack), filter paper, ose, pipette volume and other glass wares commonly used in laboratories.

The instruments analysis used were UV-Vis spectrophotometer (Shimadzu), pH meter, Scanning Electron Microscope (JEOL), and X-ray diffractograf (Rigaku).

Chemicals

Chemicals used in this study were sodium hydroxide (Bratachem), nitric acid (Merck), ethanol (Merck), acetate buffer, urea, medium potato dextrose agar (Difco ™), media CMC order, media CMC liquid, congo red 0,1%, tween 80, buffer citrate phosphate pH 5, Dinitrosalycilic acid, ammonium sulfate (Merck), sulfuric acid (Merck), potassium dihydrogen phosphate (Merck), calcium chloride monohydrate (Merck), magnesium sulfate heptahydrate (Merck), aquabidestilata (Otsuka).

Screening of Cellulase Activity Based on Transparent Zone in CMC agar Medium

Screening of cellulase activity was based on a transparent zone in the CMC agar medium. To see the ability of fungi to degrade, each crude enzyme extract as much as 5 µL was injected to the paper disc using a micropipette in a petri dish containing media carboxy methyl cellulose (CMC), NaNO3, KH2PO4, KCl, MgSO4.7H2O, yeast extract, glucose and incubated at room temperature (27-28°C) for 3-7 days. The test was repeated twice for each isolate. Agar clear zone formation becomes clearer staining using Lugol.[11] In addition, the petri dishes are also grown *Trichoderma reesei* as a comparison/reference culture. Fungal isolates which showed cellulolytic activity can be seen from the the formation of a clear zone around the colony. Isolate of cellulolitic mold obtained was then selected by the ratio between the diameter of the clear zone and diameter of colony.

Preparation of Calibration Curve Standard Glucose

Glucose standard curve was created by making a series of glucose standard solution: 75 ppm, 90 ppm, 105 ppm, 120 ppm, 135 ppm and 150 ppm, which showed absorption between 0.2 to 0.8. Each glucose solution with a variety of different concentrations in distilled water was added with DNS 1% with a ratio of 1: 1. Each solution was then heated in a water bath at temperature of 100°C for 15 minutes and the solution was allowed to stand to room temperature. The absorbance was measured at a wavelength of 510-520 nm.

Crude Enzyme Cellulase Preparation and Its Selection with Sugar Reduction Method

A spore suspension was made by adding 5 mL aquabidest into test tubes containing slant culture of isolate in agar medium. Discratch the spores to blend with aquabidest. Then 10 mL of spore suspension in 0.1% tween 80 was added with nutrient broth solution, which composed of: yeast extract 200 mg. 300 mg peptone, $(NH_4)_2SO_4$ 280 mg, 200 mg KH_2PO_4, K_2HPO_4 200 mg, 200 mg $MgSO_4 \cdot 7H_2O$, CMC 1% 5 mL, and 0.1 M acetate buffer pH 5 to 200 mL. The solution mixture was stirred at 150 rpm for 120 minutes at room temperature. Then, a mixture of 30 mL of the solution was taken and centrifuged at 3000 rpm for 10 minutes 4°C

A spore suspension of fungal isolates were inoculated aseptically into each of the nutrient medium and incubated in the incubator with stirring for 72 hours (8 hours, 16,24,32,40,48,56,64, 72) at 50°C 75 rpm. Every 8 hours as much as 2 mL sample was taken and then centrifuged at 5000 rpm, 4°C for 15 minutes. Then, crude enzyme extract in the form of the supernatant was taken as 1 mL.

Screening process quantitatively using UV-Vis spectrophotometry. Volume ratio of cellulose substrate solution, an enzyme extract, and DNS 1% was 2: 1: 3. CMC as a substrate cellulose is dissolved in 0.2 M phosphate buffer pH 5. The enzyme extract as much as 0.4 mL taken and put into a test tube. Then, 0.8 mL of the CMC was added to the test tube and incubated at 40°C for 30 minutes. After that, 1.2 mL of 1% DNS was added into a test tube and then heated at 100°C water bath for 15 minutes. The test samples are allowed to stand until they reach the room temperature. The absorbance was measured at wavelength of about 510-520 nm (maximum wavelength), and glucose concentrations were calculated by equation of calibration curve.

Optimization duration of hydrolysis

Water hyacinth powder used here was powder that has delignified by heating in 17% NaOH, and used as substrates (alpha-cellulose). Crude enzyme extract of isolates and *T.reesei* were prepared by centrifugation at 3000 rpm 4°C, for 10 minutes. For the measurement, 1 gram of alpha cellulose hyacinth dissolved in 10 mL of 0.1 M acetate buffer pH 5 and added 0.2 mL of the supernatant enzyme. The solution mixture was incubated at 50°C 160 rpm for 12 hours with sampling every 2 hours and then added as much as 0.75 mL of 0.75 mL of 1% DNS. Then, the sample was heated for 15 minutes and allow to stand to room temperature. The absorbance was measured and calculated by equation of calibration curve.

Extraction of α-Cellulose and Preparation of microcrystalline cellulose by Enzymatic Hydrolysis

200 grams of hyacinth powder that has been sifted and then heated with NaOH solution 17.5% w/v for 5 hours at a temperature of 90°C. Furthermore, the suspension was filtered and washed with aquadestilata to achieve a pH of 6-7. After that it was dried in an oven at 60°C.[8] The powder isolated was alpha cellulose and keep drying before hydrolyzed to produce microcrystalline cellulose.

In the process of enzymatic hydrolysis, the enzyme used were varied by volume in order to see the optimum conditions in the preparation of microcrystalline cellulose. About 2 grams of α-cellulose hyacinth was dissolved in 20 mL of acetate buffer (0.1 M, pH 5) and 0.4 mL crude enzymes from isolates, and stirred slowly. The mixture of solution was stirred at 160 rpm 50°C for 1 hour. Then, the mixture was centrifuged at 10,000 rpm (at a temperature of 7 - 10°C for 20 minutes). The residu settles down with aquadest was washed to remove residual enzyme on cellulose and then dried.[5] The same processes were carried out using 1.2 mL and 2 mL crude enzyme of selected isolate.

The powder resulted was bleached using 20 mL H2O2 3% at a temperature of 80°C for 1 hour. After that, the bleaching powder is filtered and washed few times with aquabidest then dried in oven.

Analysis X-Ray Diffraction and Identification of Crystals by Scanning Electron Microscope (SEM)

Crystal Analysis was performed using XRD (Rigaku Miniflex 600) with a voltage of 40 kV, the power of 600 W, the electric current 15 mA and Cu radiation source at an angle $2\theta = 0 - 70$ °C.

For identification, samples were bonded with stable metal palladium, cleaned with a blower, and coated with gold and palladium in a pressurized machine, Dionspater 1492 x 102 atm. Samples were subsequently put into special room and then irradiated with electron beam powered 10 kV. The sample emit secondary electrons and electrons that bounce can be detected by a detector scientor which is then amplified by an electrical circuit that produces an image on a CRT (Catode Ray Tube). Shooting is done after selecting a specific part of the object (sample) and the desired magnification in order to obtain a good and clear photo.[12]

RESULTS AND DISCUSSION

Screening Cellulase Activity based on Clear Zone in CMC Media

Isolation of cellulolytic mold has already done in our preliminary study. In this study, screening for highest cellulase activity was carried out by

the method of cellulose degradation. The activity of cellulase is indicated in the formation of a clear zone around the colony of fungi. The sample of cellulases used were the supernatant crude enzymes from each isolate of mold. The medium used was medium agar CMC with Lugol staining at the end after incubation. CMC (Carboxymethyl cellulose) is a derivative of cellulose that can be used as a medium for testing the activity of cellulase. The addition of Lugol serves as dye to make it easier to see and measure the clear zone formed. Lugol reagent will be bound at 1,4-β glycoside on cellulose that will provide blue-black color. Meanwhile, transparent color showed that cellulose has been decomposed into monosaccharides that can not form a complex with iodine of lugol reagent. The ability to form clear zone on a specific media indicates that the fungus is able to produce the cellulolitic enzyme, cellulase.

From the results of measurements of clear zone, white isolate (IEP) and *Trichoderma reesei* (Figure 1A and 1C) showed a clear zone diameter of 0.25 cm and 0.15 cm, while black isolate (IH) which derived from weathered oil palm trunk (Figure 1B) showed higher cellulolytic activity with a clear zone of 1.12 cm. Other isolate with green pigment showed no activity (data not shown).

Preparation of Glucose Standard Calibration Curve

Standard solution of glucose 1500 mg / mL was used as the mother solution to make a standard solution of glucose each about 75 ppm, 90 ppm, 105 ppm, 120 ppm, 135 ppm and 150 ppm. Each of this solution was taken 1 ml and added 1 mL of 1% DNS (1: 1). The solution will showed the orange color after heating. After cooling down to room temperature, the absorbance was measured by spektrophotometer UV - Vis at wavelength of 510-520 nm.DNS is a reagent with redox reactions at the aldehyde group sugars and oxidized to carboxylate. Meanwhile DNS as an oxidant is reduced forming 3-amino-5-nitrosalicylic acid. This reaction is run under alkaline conditions. When there is a reducing sugar in a sample, the DNS solution that was originally yellow will react with reducing sugars, causing the reddish orange color will be more intense color when the concentration of sugar is higher. DNS reagent consists of dinitrosalicylic acid, Na-K tartrate (Rochelle salt), phenols, Na bisulfite, and NaOH. Na-K tartrate served to protect the reaction of dissolved oxygen. Phenol was used to increase the color intensity. Na bisulfite was used for stabilizing the color for their phenol. NaOH was used to achieve the basic conditions for the reaction to run and heating was needed to speed up the reaction. Based on spectrophotometry analysis, it was obtained an equation:

y=0,0058x-0,2111; *with linear regression coefficient* r=0,9963

Selection of cellulase with Sugar Reduction Method and Spectrophotometry

The results obtained from measurement of glucose concentration after treatment with different cellulase during 72 hour showed that all glucose concentrations rise and fall, not as stable as shown in Figure 2. Cellulase from *Trichoderma reesei* and white isolate (IEP) produced higher concentration of glucose in short time (8 hours) and kept stable enough until 56 hours. This suggested that the *Trichoderma reesei* and isolates IEP was not suitable for the purpose of this research because of high concentrations of glucose produced. Meanwhile, the desired result is a high cellulase activity with low enough concentration of glucose produced, which can be used to produce microcrystalline cellulose. From these results black isolate (IH) was selected for further experiments.

Extraction of Crude Enzyme and Optimization Duration of Hydrolysis

The addition of 0.1% between 80 as surfactant to help pull out the enzyme. The extraction of the enzyme is done by centrifugation at 3000 rpm for 10 minute at 4°C to separate the supernatant (crude enzyme extract) and the cells molds. The extraction process is done at 4°C for keeping the stability of enzyme.

An optimization stage of hydrolysis was carried out using hyacinth powder that has been delignified with 17.5% NaOH as the initial substrate of cellulose. The water hyacinth powder containing α-cellulose used as a raw material for the preparation of microcrystalline cellulose. Samples were incubated for 10 hours. Each sample was immediately added by DNS 1%, measured by spectrophotometry and put into a linear equation.

Preparation of α-Cellulose.

About 200 grams of powder hyacinth was delignified with 17.5% NaOH. The use of NaOH 17.5% was intended to separate the α-cellulose fraction (insoluble) from the soluble fraction (β and γ form). Furthermore, the suspension is filtered and rinsed with distilled water to achieve a neutral pH and eliminate the other impurities. Then, it was dried in a vacuum oven. The result is a light brown powder (data not shown).

Preparation of Microcrystalline Cellulose

Variation of enzyme concentration (2, 6, and 10 % v/v) was carried out by variation volume of crude enzyme added to 2 grams of alpha cellulose powder of hyacinth, and dissolved with 0.1M acetate buffer pH 5 to 20,0 ml. Cellulase enzyme is active on the conditions around pH 5. Reaction was run at 50°C for 1 hour by steering at 160 rpm to accelerate the process of homogeneous blend of solvent and extraction of the enzyme. Separation was done by centrifugation at 10,000 rpm, 7 - 10°C for 20 minutes to stop the enzymatic reaction. Sedimentation of hyacinth cellulose was filtered and rinsed with aquabidest few times to remove residual enzyme.

Bleaching was done by using 3% H_2O_2 at a temperature of 80°C for 1 hour. After that, the bleaching powder is filtered and washed few times with aquabidest, and finally dried in oven. The results of microcrystalline cellulose from water hyacinth can be seen in Figure 3.

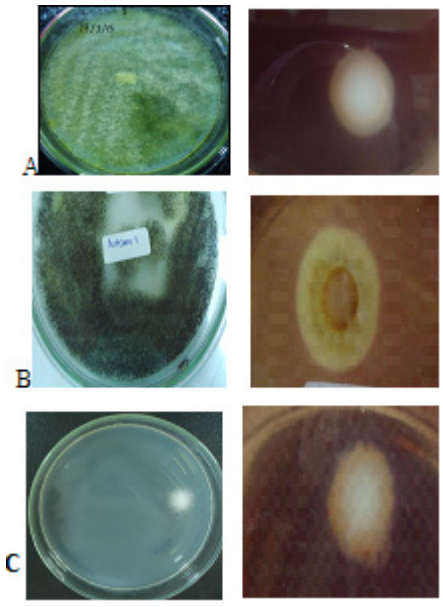

Figure 1: Colonies of Trichoderma reesei and isolates (left) and their cellulolytic activity (right). A, T.reesei; B, black isolate (IH) and C, white isolate (IEP).

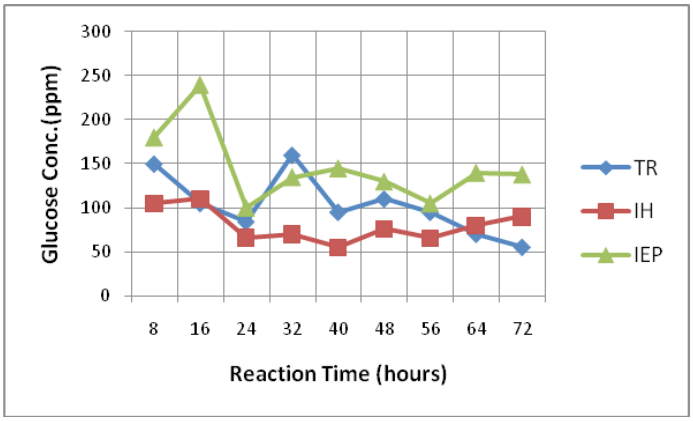

Figure 2: Glucose Concentration curve with DNS method using Trichoderma reesei (TR), Isolate IEP, and Isolate IH.

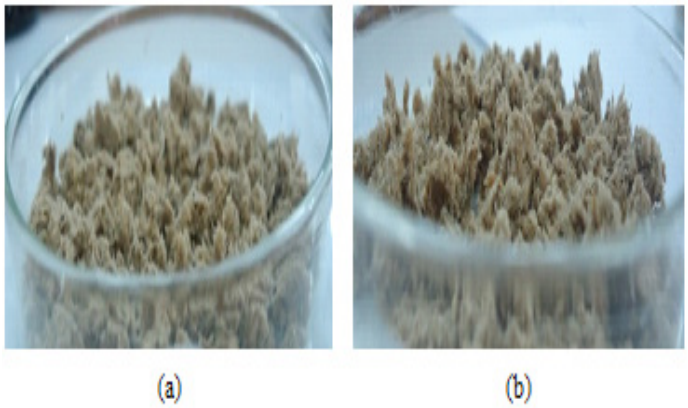

Figure 3: Microcrystalline cellulose powder from water hyacinth after Bleaching and hydrolysis with 6% (a) and 10% (b) crude enzyme of cellulase.

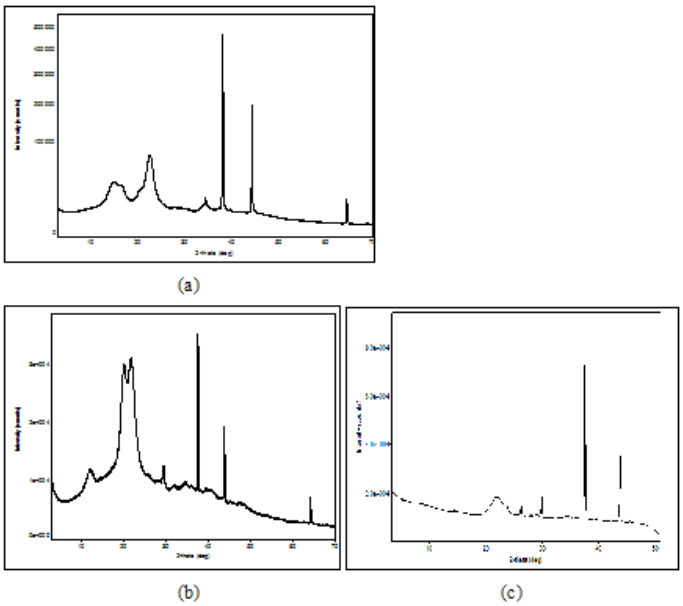

Figure 4: XRD diffractogram of Avicel pH 101 (a), Hydrolysis Results of Hyacinth powder (b) and Oil Palm Empty Fruit Bunch Powder (c).

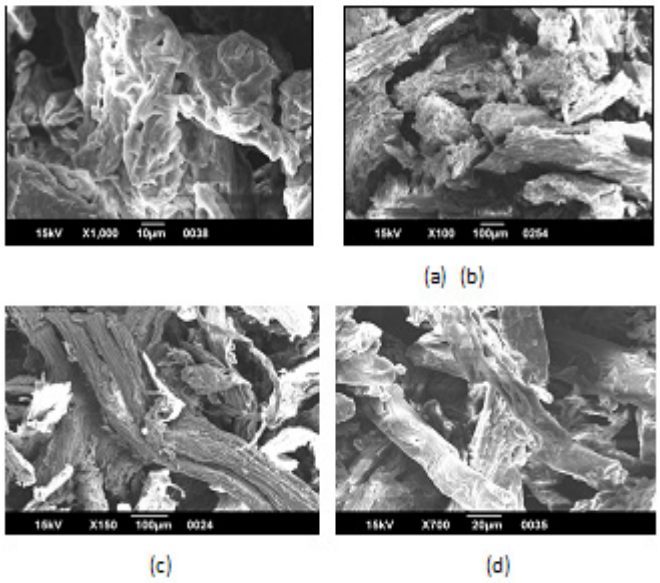

Figure 5: Result of SEM Analysis on Avicel pH 101, M=1000x (a), and Product of Hidrolysis with 2% (b), 6% (c) and 10% (v/v) (d) of crude enzyme (e); M= 700x

Even though the colour of hyacinth powder has changed after hydrolysis and bleaching process, but it still need to optimize the bleaching process to get the similar color with reference microcrystalline cellulose (Avicel pH 101). Other researchers proposed to use grinding or homogenizing again after enzymatic and bleaching process to find the suitable size and properties of microcrystalline cellulose.[5]

Analysis of crystal by X-Ray Diffraction (XRD)

XRD analysis was conducted in this study to see the shape of the crystal samples compared to reference, Avicel pH 101. In general, the polymer material of MCC is semi-crystalline. It means that MCC still contain the amorphous part beside the dominant crystalline parts. The X-ray diffractogram of crystalline polymers produce sharp peaks; while the amorphous polymers tend to produce a widened or blunt peak (see Figure 4).

In Avicel pH 101 that measured in this study (Figure 5.a) there is a typical pattern of a diffractogram, on 2θ value of 22.5 contained a sharp peak that shows the nature of the crystalline and on 2θ value of about 18 the blunt and widened peak or valley show amorphous nature. This diffractogram standard of microcrystalline cellulose is suitable with diffractogram microcrystalline reference reported by other researchers.[13] While the sample results of hydrolysis of hyacinth powder (Figure 5b) indicated 2θ value contained two sharp peaks 21.66 and 27. 57(twin peaks) but there is no blunt peak (amorphous form) at 2θ of 18, instead of valley between the peaks. Even though there is a little different in the pattern of each diffractogram, but they still has similarity in the properties of crystalinity, so they could still meet the criteria of the properties of microcrystalline cellulose standard that has a wide range of length chain 1000-1500 A, and the degree of polymerization between 200-300.[2,5]

The differences in the peak shape or diffract-o-gram pattern of sample hydrolysis of oil palm empty fruits bunch (Figure 5.c) with Avicel pH 101 indicating that it has a difference property of crystalline to Avicell reference, and also different size and degree of polymerization. Other possibility is product of microcrystalline from the sample of water hyacinth has properties of crystalline higher than standard Avicell pH101, as it showed less blunt or widened peak, and gave a value degree of crystalinity higher than standard Avicell pH 101, based on method of peak

Table 1. Comparison of Intensity Values peaks at 2 theta angle and Degree of Crystalinity (DC) between Avicel pH 101 and Sample Hydrolysis of Hyacinth

	Type of MCC			
	Avicel pH 101		Hydrolysis result	
2ɵ (deg)	22.54	18.5	21.66	16.5
Intensity	10000	3500	30000	10000
DC(%)	65		70	

height. (Table 1). Degree of crystalinity can be calculated based on ratio of the difference intensities of crystalline peak and non-crystalline peak to total intensity of crystalline peak.[14]

Analysis morphology of crystal by Scanning Electron Microscope (SEM)

In this study, there are three sample and one reference standard were analyzed using SEM namely: hydrolyzed sample of hyacinth powder with 2% (v/v) of the enzyme (Figure 5b), with 6% (v/v) of the enzyme (Figure 5c), and with 10% of enzymes (Figure 5d), as well as powder of Avicel pH 101 as a reference (Figure 5a). Based on the results of SEM analysis in figure above, it can be seen that microcrystals of cellulose has already formed on samples, even with low concentration of enzyme for hydrolysis. However, the samples look like still contained lignin or wood fibers. This is possibly due to imperfection in delignification process.

To solve this problem, the process can be corrected by improving extraction and delignification process, i.e by increasing NaOH concentration or increasing duration process, which can increase the degree of crystalline.

Based on the results of SEM analysis in Figure 5(d), it can be seen that hydrolysis of alpha cellulose with 10% v/v crude enzyme was nearly perfect to produce morphology of microcrystalline cellulose similar to reference Avicel pH 101.[2]

CONCLUSION

As the conclusion, among the isolates and *T. reesei* have been screened, black isolate (IH) was considered as the best source of crude cellulase with suitable activity for preparation of microcrystalline cellulose. The optimum condition of enzymatic hydrolysis with cellulase with the substrate of water hyacinth was on the first hour when the glucose concentration is low, with concentration of enzymes used as much as 10%(v/v). Based on the results of XRD and SEM analysis, there was a similar characteristic of crystalline between microcrystalline cellulose from hyacynth hydrolysis and reference Avicel pH 101.

ACKNOWLEDGEMENT

We would like to thanks Dean Faculty of Pharmacy, Universitas Indonesia and Head of Laboratory of Microbiology and Biotechnology for the facilities we used during our study.

CONFLICT OF INTEREST

This is a non-funding research work, that we declare there were no conflicts of interest.

ABBREVIATIONS USED

MC: Microcrystalline cellulose; **CMC:** Carboxy methyl cellulose; **DNS:** Dinitro salisilic acid; **XRD:** X-ray diffraction; **SEM:** Scanning electron microscope.

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Article History: Submission Date:21-12-16; Revision Date: 07-01-17; Accepted Date:21-01-17.
Cite this article: Suryadi H, Sutriyo, Sari HR, Rosikhoh D. Preparation of Microcrystalline Cellulose from Water Hyacinth Powder by Enzymatic Hydrolysis Using Cellulase of Local Isolate. J Young Pharm. 2017;9(1)Suppl:s19-s23.