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The Effect of Enzyme Concentration and Hydrolysis Time on the Yield of Microcrystalline Cellulose from Rice Straw

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Abstract: The purpose of this study was to determine the effect of enzyme concentration and hydrolysis time on the yield of microcrystalline cellulose, and to determine whether the results of microcrystalline cellulose examination met the requirements of the *British Pharmacopoeia* and *the United States Pharmacopoeia* . The *pretreatment* process was carried out chemically using 96% alcohol, sodium hydroxide and hydrogen peroxide. The hydrolysis process was carried out enzymatically using the cellulase enzyme produced by the fungus *Trichoderma viride* , with variations in enzyme concentration of 5, 10 and 15% v/v for 0.5, 1 and 1.5 hours respectively. The highest yield of microcrystalline cellulose was produced from the hydrolysis process with a cellulase enzyme concentration of 5% v/v for 1.5 hours. Based on the two-way ANOVA test, it is known that the enzyme concentration and hydrolysis time have no significant effect on the yield of microcrystalline cellulose. Examination of organoleptic, identification, pH, water solubility, drying shrinkage, and starch absence test showed that the resulting microcrystalline cellulose complied with the requirements of the *British Pharmacopoeia* and *the United States Pharmacopoeia.*

Keyword : Microcrystalline Cellulose, *Oryza Sativa* Linn, Hydrolysis, *Trichoderma Viride*, Cellulase.

INTRODUCTION

Microcrystalline cellulose or *microcrystalline cellulose* is widely used in the pharmaceutical, cosmetic, food and other industries. In tablet printing using the direct compression method, microcrystalline cellulose is used as a dry binder, tablet desintegrant, absorbent, filler, lubricant, and antiadherent. Microcrystalline cellulose has been used extensively as an additive in direct compression due to its good flow, compatibility, and compressibility (Ngozi, *et al.* , 2014; Haque, *et al.* , 2015).

Domestic demand for *microcrystalline cellulose (MCC) all comes from imports.* It is very relevant if our country starts to think about the domestic production of microcrystalline cellulose (Halim, *et al.,* 2002). The high price of commercial MCC is also a reason to find a cheaper source of microcrystalline cellulose, namely from agricultural waste.

Waste materials that can be processed into microcrystalline cellulose include: rice straw, bagasse, corn husks, sawdust, corn cobs, old newspapers and other materials containing cellulose (Haque, et al., 2015) . In this study rice straw waste was used because according to a survey, so far most of the rice straw was thrown away, only 4% was used for animal feed (Halim, *et al.,* 2002).

Rice straw is one of the most widely available, cheap, and renewable cellulose materials in the world. The compound content in rice straw consists of 32-47% cellulose, 19-27% hemicellulose and 5-24% lignin (Wang, *et al.,* 2015).

There have been many uses of rice waste in Indonesia, especially in agriculture, such as rice bran for fodder, rice husk for chicken coop base, and rice straw for fodder or mushroom growing media. Utilization for the industrial sector is still limited, even though some of the properties present in the waste, both physical and chemical properties can be further developed for the industrial sector. The chemical composition of rice shows that the cellulose content is quite high, namely around 40% (Halim, *et al.,* 2002).

Cellulose hydrolysis can be done chemically and enzymatically. Chemical hydrolysis can be carried out using acids, namely low concentrations of strong acids and high concentrations of weak acids (Oktavianus, 2013). Acid hydrolysis requires high activation energy and produces waste consisting of acids, bases and organic compounds that are not environmentally friendly (Suryadi, *et al.,* 2017). Enzymatic hydrolysis is carried out using cellulase enzymes. Cellulase catalyzes the hydrolysis of cellulose with three types namely: *endoglucanase, cellobiohydrolase, and β-glucosidase* (Li, *et al.,* 2009). The high price of cellulase enzymes due to the expensive process and pure raw materials has made researchers look for other ways to produce them. Cellulase enzymes can be produced by fungi and bacteria, including: *Trichoderma viride* (Li, *et al.,* 2009), *Aspergillus niger* (Kalyani, *et al.* , 2015), *Acetobacter cylinum* (Kulkarni, *et al* , 2012), and others.

In this study, the cellulase enzyme-producing microbe used was the fungus *Trichoderma viride* . This fungus is rich in endocellulase activity which selectively removes the amorphous part of cellulose, so it has the potential to hydrolyze cellulose into mycocrystalline cellulose (Braunstein, *et al.* , 1994).

The purpose of this study was to determine whether the enzyme concentration and hydrolysis time affected the yield of microcrystalline cellulose and to determine whether the obtained microcrystalline cellulose complied with the test requirements of the *British Pharmacopoeia* and *the United States Pharmacopoeia.* While the benefit of this research is to provide information and knowledge to the public about the utilization of rice straw waste which is expected to meet domestic demand for microcrystalline cellulose.

METHODS

The tools used were *Scanning Electron Microscope* (JEOLT 330A®), X-ray *Diffractometer* (PANanalytical XPertPRO®), *Fourier Transform Infrared Spectroscope* (PerkinElmer®), UV-Vis Spectrophotometer (Shimadzu Pharmaspec 1700 ®), optical microscope, *water laminar flow*, analytical balance, *hot plate* (CORNING PC-620D®), water bath, centrifuge (KENKO ®), oven, pH indicator, sieve *screener* , spirit lamp, Ose needle, Erlenmeyer flask, test tube, stir bar, measuring cup, glass beakers, pipettes, scissors, sterile cotton & gauze.

The materials used in the study were rice straw (*Oryza sativa* Linn), distilled water (Bratachem®), hydrogen peroxide (Bratachem®), sodium hydroxide (Merck®), *Trichoderma viride* T1sk, wheat bran, 70% alcohol (Bratachem®) , alcohol 96%, PDA (Oxoid®), zinc chloride, potassium iodide, iodine, dinitro salicylic acid (Himedia ®), Na-K tartrate, phenol, Na bisulfite, Na CMC, KH_2PO_4, $MgSO_4$ (Merck ®), K_2HPO_4 (Merck ®), CH_3COOH, CH_3COONa, $C_6H_8O_7 . H_2O$ and $C_6H_5O_7Na_3.2H_2O$.

The tools used are first washed and dried. Glassware that has a mouth covered with cotton wrapped in gauze, then wrapped in parchment paper. After that, it was sterilized in an autoclave at 121ºC and 15 lbs pressure for 15 minutes. Micropipette tips were arranged in a glass beaker, covered with aluminum foil, then sterilized in an autoclave at 121°C and 15 lbs pressure for 15 minutes. Spatel and Ose needles were sterilized in a flambier way over a spirit lamp flame for 20 seconds. The aseptic cupboard is cleaned of dust and sterilized by spraying 70% alcohol all over the inside of the cupboard. All work was carried out using aseptic techniques (Krisyanella, *et al.* , 2012).

RESULTS AND DISCUSSION

Results

1. Cellulase Enzyme

   The volume of cellulase enzymes obtained from one production in a 250 mL Erlenmeyer tube is ± 20 mL. The cellulase enzyme has an activity of 7.006 units/mL.
2. Production of α-cellulose

   The results of processing rice straw into α-cellulose, obtained an average yield of α-cellulose of 27.6%.
3. Yield of Microcrystalline Cellulose

   The highest yield of microcrystalline cellulose was obtained from the hydrolysis of α-cellulose with an enzyme concentration of 5% v/v for 1.5 hours. Yield produced from the hydrolysis of α-cellulose with various enzyme concentrations and hydrolysis times.
4. Results of Examination of Microcrystalline Cellulose

   The results of the examination of microcrystalline cellulose include organoleptic examination, drying shrinkage, identification, water solubility, pH test, starch test and flow properties test.
5. Surface Morphology Testing Data Using an Optical Microscope and *Scanning Electron Microscopy* (SEM)

   The results of testing the surface morphology of rice straw powder, α-cellulose, and microcrystalline cellulose using a microscope can be seen in Appendix 4. Further observations of the surface morphology of α-cellulose, and microcrystalline cellulose using SEM with magnifications of 750 and 1000 times.
6. *Fourier transform infrared spectroscopy* test data

   Microcrystalline cellulose and Avicel PH 102 infrared spectrum test data.
7. Crystal Index Testing Data Using X-Ray Diffractometer (XRD)

   The crystal index of α-cellulose and microcrystalline cellulose are 66.5% and 69.57%. XRD spectra of α-cellulose and MCC 5.

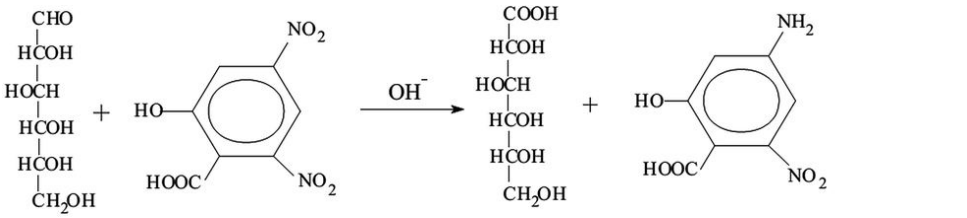
Discussion

The cellulase enzyme-producing fungus used in this study was *Trichoderma viride* T1sk isolated by Dr. Ir. Nurbailis, MS from the Faculty of Agriculture, Andalas University, Padang. The isolated and purified fungi came from the rhizosphere soil of banana plants in various areas in West Sumatra (Tanah Datar, Solok, and Padang Pariaman Regencies). *Trichoderma* spp. is a cosmopolitan fungus that can be found in various types of soil (Nurbailis, 2008).

Standard glucose solution of 250 ppm was used as mother liquor, then diluted to 100, 120, 140, 160, 180, 200, 220 ppm. Take 2 mL of each standard solution and add 2 mL of dinitrosalicylic acid *(* DNS) solution. The solution turns reddish orange after heating, so the absorbance can be measured using a UV vis spectrophotometer. In this study, the maximum absorption wavelength of DNS-glucose solution was 538 nm.

DNS is a reagent with a redox reaction on the aldehyde group of sugar and is oxidized to a carboxylic acid. DNS as an oxidant is reduced to form 3-amino-5-nitrosalicylic acid. This

reaction can occur under alkaline conditions. When there is reducing sugar in the sample, the DNS solution which is initially yellow in color will react with the reducing sugar causing a reddish-orange color that gets darker the higher the concentration. The DNS reaction with glucose can be seen in Figure 1.



glucose DNS gluconic acid **3-amino-5-nitrosalicyclic acid**

Figure 1. Reaction of glucose reduction with dinitrosalicylic acid (Xia, *et al,* 2015)

DNS reagents consist of dinitrosalicylic acid, Na-K tartrate, phenol, Na bisulfite, and NaOH. Na-K tartrate is used to protect the reaction from dissolved oxygen, phenol is used to increase the intensity of the color, Na bisulfite is used to stabilize the color that is formed, NaOH is used to reach alkaline conditions so that the reaction can occur, and heating is used to speed up the reaction (Suryadi, *et al.*, 2017).

Based on spectrophotometric analysis, the equation y = 0.106x + 0.029 is obtained with a linear regression coefficient R^2 = 0.993. From this equation, the activity of the cellulase enzyme was 7.006 units/mL. In a study conducted by Pandey, *et al.* in 2010 using the same fungi and carbon sources, lower cellulase enzyme activity was found, namely 0.73 units/mL. Gupta, *et al.* in 2014 conducted a similar study using *Trichoderma* sp. and cellulase enzymes were obtained with an activity of 9,700 units/g. This difference in activity may be due to differences in the sources and species of fungi used. In addition to the type of fungus, the carbon source also influences the activity of the cellulase enzyme. Hidayat, *et al.* in 2005 carried out the production of cellulase enzymes using *Trichoderma viride fungi* and rice straw carbon sources. The resulting cellulase enzyme activity was 1.52 units/mL. Gautam, *et al.* in 2010 carried out the production of cellulase enzymes using urban solid waste carbon sources, obtained cellulase enzyme activity of 2.31 units/mL.

Production of microcrystalline cellulose using materials containing cellulose. In this study, rice straw was used which was taken from the rice fields around the Limau Manis area, Padang City. The rice straw in question is the part of the rice stem where the roots, leaves and fruit have been removed so that the sample used is clean and does not mix with unwanted materials. Rice straw was cut ± 1 cm long and dried in the sun for ± 12 hours, drying aims to reduce the water content in the sample so that it can be stored for a long time without growing fungus. After drying, the rice straw was crushed by grinding using a grinder with the aim of forming the smallest size of rice straw to form powder and sieved using a 212 µm sieve.

Straw powder is weighed and processed into alpha cellulose by removing the lignin it contains (Halim, *et al.,* 2002). The straw powder was macerated with 96% alcohol for 24 hours to remove polar and non-polar compounds found in rice straw powder such as fats, waxes, carbohydrates (moosaccharides) and amino acids. The straw powder was then washed with hot water to develop the cellulose cells and to remove the remaining 96% alcohol solvent so that the impurity compounds were expected to be removed (Halim, 1999).

Lignin is one of the main components of rice straw besides cellulose, hemicellulose and ash. Lignin liberation from complex compounds is one of the important pre-treatments carried out before the hydrolysis process. This process is important before the hydrolysis of cellulolytic materials, because lignin is a strong wall attached to cellulose and hemicellulose fibers so that a plant becomes hard and can stand firm. Lignin can inhibit the penetration of

acids or enzymes before hydrolysis takes place (Gunam, 2010). Delignification was carried out with 3.5% sodium hydroxide at 100 $^{\circ}$ C for 2 hours. After delignification, *the pulp* is washed with distilled water until the pH is neutral, then dried in an oven at 60 ^{o} C. Low concentrations of sodium hydroxide can dissolve lignin and hemicellulose. Hemicellulose is a polymer composed of pentose and hexose sugar monomers. The cellulose obtained was then heated with 17.5% NaOH to obtain α-cellulose. Because the other parts of cellulose, namely β and γ cellulose are dissolved in 17.5% NaOH, while α-cellulose is not soluble (Gascoigne & Gascoigne, 1960; Stamp, 1964).

bleaching process is carried out using hydrogen peroxide (H_2O_2) with a concentration of 20%. The yield of alpha cellulose after bleaching was 18.23, 25.09 and 30.11%. The high concentration of H_2O_2 leads to the formation of OOH^- ions they form faster. OOH ion $^-$ which is formed from the addition of alkali which functions to hydrolyze lignin by breaking the Cα – Cβ bond on the lignin side of *the pulp molecule* . Termination of this lignin bond causes the chromophore groups in lignin to decrease and the cellulose content to increase.

Non-phenolic units constitute about 90% of the lignin structure. Hydrogen peroxide can break Cα – Cβ bonds in lignin molecules and is able to open lignin rings and other reactions. Hydrogen peroxide catalyzes an oxidation of the non-phenolic aromatic compound lignin to form an aryl cation radical. Hydrogen catalyzes the oxidation of non-phenolic lignin compounds by changing veratril alcohol to veradril aldehyde so that *the pulp* becomes white (Jayanudin, 2009).

The final stage of the production of microcrystalline cellulose is the hydrolysis process. In this study hydrolysis was carried out using cellulase enzymes extracted from the fungus *Trichoderma viride* T1sk. Enzyme concentrations used respectively were 5, 10, and 15% v/v with time variations of 0.5, 1, and 1.5 hours, respectively. Of all the variations in concentration and time, the highest yield was obtained at a concentration of 5% v/v and a time of 1.5 hours, namely 93.97%. Based on the results of the two-way ANOVA test, a significant value of 0.749 was obtained, which means that the difference in enzyme concentration and hydrolysis time did not significantly affect the yield of microcrystalline cellulose.

Microcrystalline cellulose produced in this study was compared with Avicel PH 102 using a *Fourier Transform Infrared spectrometer* . From the infrared spectrum it can be seen that the resulting spectrum has transmission peaks located at adjacent wave numbers. The spectrum of the resulting cellulose microcrystalline does not look very different from one another. Some literature says that the hydrolysis process occurs in amorphous areas, so that the ratio between crystalline areas and amorphous areas becomes higher (Halim, *et al.*, 2002).

XRD analysis was carried out to see the crystal index of the samples and compared with Avicel PH 101. The crystal index values of alpha cellulose and MCC obtained in this study were 66.5% and 69.57%. Meanwhile, the Avicel PH 101 crystal index according to a study conducted by Keshk and Haija (2011) is 74%. X-ray diffraction spectrum. In general, the nature of the microcrystalline cellulose polymer is semi-crystalline, which means that there are still amorphous parts besides the more dominant crystalline parts. The level of MCC crystallinity reported from several studies is 60-80% (Thoorens, *et al.*, 2014). Differences in the shape of the peaks or diffractograms of the samples with Avicel PH 101 indicated that the samples had different crystal properties and degree of polymerization with Avicel PH 101. These differences were associated with a different crystal lattice than the crystal structure of cellulose. In cellulose I, the cellulose chains are arranged parallel, while in cellulose II it shows an anti-parallel chain (Krassig, 1996).

Structure of alpha cellulose and microcrystalline cellulose produced and Avicel PH. The structure of alpha cellulose when compared to microcrystalline cellulose and Avicel PH 101 looks different, namely there are still many small structures consisting of fibers (fibrils). In the resulting microcrystalline cellulose, microcrystalline cellulose has been formed, but the

structure still looks like wood fibers. This may be due to imperfect *pretreatment process.* This problem can be overcome by increasing the concentration of NaOH during delignification or prolonging the duration of delignification (Suryadi, *et al.*, 2017).

CONCLUSION

From the results of the study it can be concluded that: 1. The highest yield of microcrystalline cellulose was obtained from hydrolysis with an enzyme concentration of 5% for 1.5 hours, namely 93.967%. 2. Enzyme concentration and hydrolysis time had no significant effect on the yield of microcrystalline cellulose (Sig>0.05). 3. The microcrystalline cellulose obtained met the requirements of *the British Pharmacopoeia* and *The United States Pharmacopoeia* for organoleptic, identification, pH, aqueous solution, drying shrinkage, and starch absence test, while the flow properties of microcrystalline cellulose 1 and 2 did not meet the requirements.

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