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SYNTHESIS AND FUNCTIONALIZATION OF MESOPOROUS SILICA MATERIALS TO IMPROVE ENZYMATIC CONVERSION OF CELLULASE

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ABSTRACT

Enzymaticactivity, reusability, and stability of immobilized cellulaseon modified mesoporous material with hexagonal mesostructured and different particle size (micro size and nano size) was studied. Cellulase is a group of enzymesthathydrolyzecelluloseintoglucose.Mesoporousmaterialswere first modified with various concentrations of VTMS (Vinyltrimethoxysilane). Various characterizations were conducted by using Transmission Electron Microscopy (TEM), nitrogen sorption and Fourier Transform Infrared Spectrometry (FTIR). Our study showed that the amount of VTMS affected the activity of immobilized cellulase enzymes. The research results suggest that hexagonal structure mesoporous silica material is promising as support for enzyme immobilization.

Keywords: mesoporous silica, immobilized enzyme, functionalization, enzyme activity.

INTRODUCTION

Begin in the mid-1970s, researches have focused on ethanol production from lignocellulosic materials. As we know that lignocellulose is raw material which is inexpensive, plentiful, and available on almost materials in this world, such as trees, grasses, corns, wastepaper, etc. Lignocellulose contains cellulose, a carbohydrate composed of β-linked glucose subunits; hemicellulose, a carbohydrate composed of xylose and other sugars; and lignin, a heterogeneous aromatic polymer [11]. Cellulose is converted into glucose by enzymatic hydrolysis using cellulase enzyme. Cellulase enzyme contains three main components: endo-1, 4-beta-D-glucanase, which lower the crystallinity of cellulose; cellobiohydrolase, which degrades cellulose; and beta glucosidase, which hydrolyzescellobiose and other oligomers [1]. Activity of cellulase is the most important factor in immobilized cellulase. Although the immobilized cellulose has a high loading but low activity, that cellulose cannot functioned as optimum as high activity. There has been ever increasing attention in mesoporous materials as interested research topics due to their functions, such as catalysts, drug delivery, sensors, adsorptions, immobilization, and optical devices. Begin on the discovery of M41S family of me soporous materials in 1992, research found some new materials that have a great potential to solve industrial problems, such as MCM-41, MCM-48, SBA-15, FDU-12, IBN-2, etc [5] SBA-15 has proved to be very promising for biomolecules separation and protein adsorption due to its large pore size and surface area to 300 Å and 700 m^2/g, respectively. SBA-15 has silica framework which iswell suited for the development of bonded and selective sorption phases, tight pore-size distribution and ordered structure [16]. Ying *et al* has successfully synthesized various mesoporous silica materials with different mesostructured in nano size. One of the products is IBN-4 which has similar mesostructured as SBA-15: hexagonal (one -channel like) yet different in size. [18]. the applications of SBA-15 as a support for enzymes or as a separation column material for proteins have been also reported. It has a much larger pore size and displays superior thermal, hydrothermal and mechanical stability. Takimoto *et al*. was succesfully immobilized cellulase enzyme on SBA-15/5.4 nm and the enzymatic activityof encapsulated cellulase was highly retained within SBA-15, which has a pore size slightly smaller than cellulase. Cellulase has a net positive charge at pH 4, and at the same condition, SBA-15 with 5.4 nm is negatively charged, which is indicating that cellulase can be adsorbed to the surface of the silica via electrostatic interactions [16]. There are many factors can affect the activity of immobilized enzyme, such as immobilized procedure, carrier, and soon. Different carriers can provide different external backbone for the enzyme molecules, which is expected to improve the stability of enzyme molecules at reaction conditions [16]. Many materials that can be used for immobilized enzyme, such as Ca-alginate, agar, K-Carrageen in, polyacrylamide, nylon, cellulose, polysulfone, alumina, silica, porousglass, ceramics, diatomaceous earth, clay, and bentonite [8]. Previous literatures showed that surface functionalization on mesoporoussilica is important factor for enzyme immobilization to maximize the interaction between the silica surface and enzyme. Hartono *et al.* synthesizedorgano-functionalized FDU-12 type silicas via the co-condensation TEOS with a suite of organosilanes, such as3-aminopropyltriethoxysilane (APTES), 3-mercaptopropyltrimethoxysilane (MPTMS), vinyltrimethoxysilane (VTMS), and phenyltrimethoxysilane (PTMS). Then, me soporous silica were tested in protein immobilization using bovineserum albumin and the cellulose-hydrolyzing enzyme cellulase. Cellulase enzyme is immobilized on organo-functionalized

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FDU-12 to determine its efficiency, activity, and stability. APTES-functionalized FDU-12 showed the highest adsorptionof proteins but also the lowest activity of immobilized cellulase. Cellulase immobilized on VTMS-functionalizedFDU-12 showed high efficiency, enzyme activity, and provided temporal enzyme stability [7]. Mesoporous silica with different surface functionalization and mesostructured performance for enzyme immobilization have been studied. However, to the best of our knowledge, the effects of particle size (micro size and nano size) in combination with surface modification are still limited. Nanosizemesoporous silica particles might support a better masstransfer for transportation of reactant and product, because its short channel. This short channel can also minimize unproductive zone within the pores.In this paper, we present the synthesis of micro size and nano size of hexagonal meso structured mesoporous silica) by using TEOS and followed by modification of the silica with VTMS. The particles are then used forcellulase immobilization. The performance of immobilized enzyme in terms of activity, stability, and reusability were also determined.

EXPERIMENTAL

Synthesis of nonfunctionalized and functionalized micron size and nano size of hexagonal mesostructured mesoporous silica materials

micron size (m-ms). Micron size mesoporous silica materials with hexagonal mesostructures were synthesized following the previous method with some modification. 4 g of P123 as a surfactant was added to 100 g water and 7.87 g HCl 37% and stirred at 35^{O}C overnight. Then, 8.53g of TEOS was added to solution and stirring was continued at this temperature for 20 hours. The synthesis continued by hydrothermal treatment in autoclave at130^{O}C for 24 hours. The product was separated and washed two times by methanol at 60^{O}C for 6hours to remove the surfactant and then dried at room temperature [19, 16].

Nano size (N-MS). Nano size mesoporous silica materials with hexagonal mesostructures were synthesized following the previous method with some modification. 0.5 g of P123 and 1.47 g of FC4 were dissolved into 80 mL 0.02 M HCl. 2 g of TEOS was added into solution and stirred at 35^{O}C for 20 hours. The synthesis continued by hydro thermal treatment in autoclave at 130^{O}C for 24 hours. The product was separated and was held two times by methanol at 60^{O}C for 6 hour store move the surface tant and then dried at room temperature [18]. Pure mesoporous silica was synthesized using TEOS as silica source and the various functionalized mesoporoussilicas were synthesized using a mixture of VTMS and TEOS by the ratio 1:5 and1:20.

Characterization

The morphology and structure of mesoporous silica samples were characterized by transmission electron microscopy (TEM). Theporesize, porevolume, and pore distribution were measured by nitrogen sorption analysis. Thevinylgroup existence on pure and functionalized mesoporous silica were determined by fourier transform infrared spectroscopy.

Cellulase immobilization

Firstly, pure and functionalized samples were degassed at 70^{O}C overnight. Then, various solution of cellulose ranging from 1-10 mg/m was madeto10mLcitratebuffer. Pipette 2mL each of the various cellulase solutions and incubated at 4^{O}C for 24 hours. 10 mg SBA-15 silicas were added into 8mL cellulase solutions and then incubated at 4^{O}C for 24 hours by water shaker. Pre-and post incubation enzyme concentrations were measured using Brad ford protein assay with BSA as the protein standard [12]. The cellulose loading (mgofcellulase/gofsilica) was calculated from the difference in pre- and postincubation enzyme concentration and plotted vs the cellulase equilibrium concentrationto givethe adsorption isotherm to measure using Langmuir equation [7].

Activity

The cellulose activity in pure and functionalized mesoporous silica was processed using a carboxy methyl cellulose (CMC) assay [5]. CMC is hydrolyzed by the cellulose enzyme that giving rise to glucose, which can be detected by DNS reagent [14]. DNS reagent was prepared by mixing 187.3mg of DNS, 302.6mg of sodium hydroxide, 544.6mg of potassium sodium tartrate, 145 mg of natrium thiosulfate, 2 drops of phenol, and 25 mL of distilled water. Then, 0.5 mL of CMC solution 1% wt was mixed with 0.5 mL of cellulase-containing solution (free enzyme, 100× dilution of commercial enzyme solution in citrate buffer; immobilized enzyme, 10 mg of pure and functionalized mesoporous silica after thecellulose immobilization process in 5 mL of citrate buffer) and hydrolisis process was taken for an hour. After that, 3 mL ofDNSreagent was mixed into solutions and boiled for 5 minutes until brown color of varying intensity obtained.Theconcentration of glucose, which was proportional to the cellulase activity, was measured by UV-vis absorbance band at 540 nm.

Reusability

The reusability of immobilized cellulase was determined by mixing 5mL of citrate buffer and 1mL of CMC solution into the cellulase-loaded samples of pure and functionalized mesoporous silica that has the highest activity. Then, the sample form the hydrolisis was taken after an hour and separated by centrifugation. The liquid part was added by 3 mL of DNS and boiled for 5 minutes

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until brown color obtained. The solid part was reused again to mix it with citrate buffer.

Stability

The stability of immobilized cellulase was tested by adding 8 mL of citrate buffer into thecellulase-loadedsamples of pure and functionalized mesoporous silica that has the highest activity. Then, the samples were stored at 24 hours. The concentration of enzyme in the remaining solution was determined by UV analysis.

RESULTS ANDDISCUSSIONS

Synthesis and functionalization

The synthesis process of pure and functionalized mesoporous silica has been prepared using tetra ethoxysilane (TEOS) as silica source that was mixed with pluronic123 (P123) as a surfactant. N-MS (nano mesoporous silicas) synthesized has similar processas M-MS (micron mesoporoussilicas), but Fluorocarbon 4 (FC4) was used to limit the growth of particle size. The functionalized mesoporous silicas used the ratio of VTMS: TEOSto1:5and1:20.Silicafunctionalization using vinylmoiety made a strong affinity for the cellulase and created a benign microenvironment for optimum enzyme activity [9]. Six samples have beensynthesized, three samples for micron size, and three samples for nano size. Micron size samples have three samples: M-MS for pure mesoporous silica; M-MSV5, and M-MSV20 for functionalized mesoporous silica. The ratio of VTMS: TEOS showed by "V5" means the ratio is 1:5 and "V20" means the ratio is 1:20. As similar with micron size, nano size samples have three samples and labelled N-MS for pure mesoporous silica; and N-MSV5, N-MSV20 for functionalizedmesoporous silica.

Characterization

Morphology of M-MS and N-MS. SBA-15 silica has a special character that makes SBA-15 different from the other mesoporous silica. SBA-15 silica has hexagonal structure and large pore diameter (between 2 and 30 nm) [15]. In other hand, IBN-4 has a similar mesostructured as SBA-15, but this particle has nanometer particle size [18]. Therefore, micron and nano size samples were characterized by TEM in order to know the morphology of each size as seen in Figure-1.

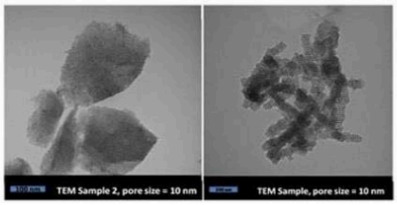

Figure-1. TEM images of micron-sized (left image) and nano-sized (rightimage).

Left image shows the TEM images for the micron mesoporous silica and right image for nanomesoporous silica it can be seen that both micron and nanomesoporoussilicas have hexagonal structure and ordered inshape.

Figure-2. The micron-sized particleenlargement.

Pore size, surface area, and pore volume of M-MS and N-MS. The nitrogen adsorption/desorption isotherms and pore size distribution curves are shown in Figures-3 and-4.

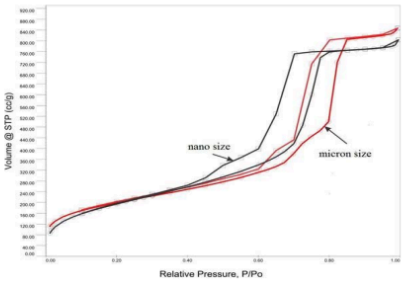

Figure-3. Micron and nanomesoporoussilicas adsorption/desorption isotherm curves.

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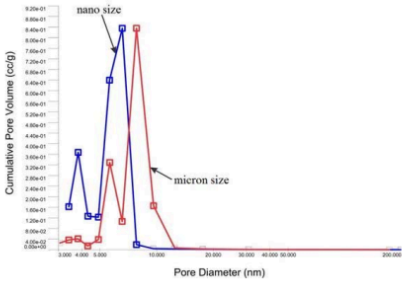

Figure-4. Micron and nanomesoporoussilicas BJH adsorptioncurves.

Figure-3 and -4 showed the analyzes of the nitrogen sorption isotherms of pure silica, both micron- and nano- sized. The micron silica has the BJH adsorption surface area of 508.5 m^2/g, pore volume of 1.2 cc/g, and pore diameterof 11.2 nm, whereas the nano silica has surface area of 408.8 m^2/g, pore volume of 0.7 cc/g, and pore diameter of 8.9 nm.As displayed in Figure-3, it can be seen that both silicas show type IV isotherms with clear hysteresis loops connected with capillary condensation in the mesopores [13]. Analyses of the nitrogen sorption isotherms for both micron and nanomesoporoussilicas showed the similar hystheresis peak for both of samples. The hystheresis is a typical for SBA-15 material that has a hexagonal structure. SBA-15 has a hexagonal structure that has cylindrical like in shape.

Vinyl modified M-MS and N-MS. Functionalized mesoporous silica contains vinyl group that help cellulase enzyme to bind in silica particle. Pure and functionalized mesoporoussilicas were checked byFTIR to know the vinyl group existence in silica particle. FTIR results were displayed in Figure-5.

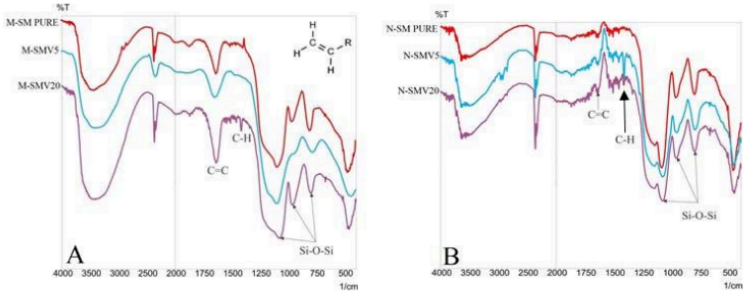

Figure-5. FTIR results for (A) micron size and (B) nano size.

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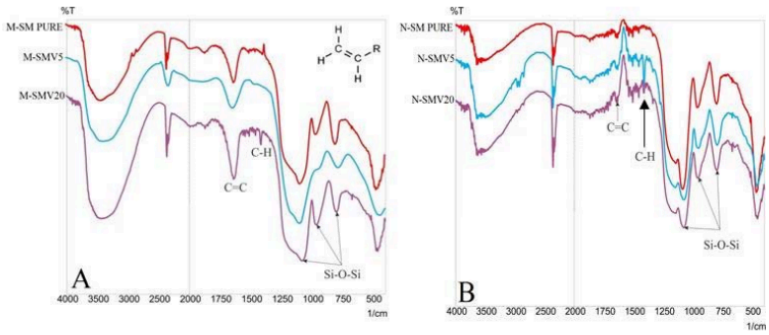

Figure-5. FTIR results for (A) micron size and (B)nano size.

From these Figures, both micron and nanomesoporoussilicas have similar curves. All of samples contain C=C, C-H, andSi-O-Si groups. In all the materials, the typical Si-O-Si waves around 1210, 1075, and 790 cm^{1} [10]. All vinylfunctionalizedsamples show spectra at around 1650 - 1400 which represent C=C and C-H respectively. There is no C-H group inpuremesoporous silica for every size, because C-H group means the vinyl existence in silica particle as seen in Figure-5 (A). It can be proven that functionalized mesoporous silica has successfully synthesized, because vinyl group already exist as C-H group in mesoporous silicas as seen in Figure-5.

Immobilized cellulase stability

There are two factors that need to be considered in terms of immobilized enzyme stability: operational and storagest ability. Operational stability refers to the extent to which immobilized enzymes maintain their catalytic activity, while storage stability is a term used to describe how well immobilized enzymes are protected from enzyme leakage [4]. This research applied the storage stability that takes seven daysperiod. Stability was done on both pure and modified silica material. Test for stability was performed by addingcitratebuffer into the cellulase-loaded samples of pure and functionalized mesoporoussilicas. The result was showed on Table-1 saysthatmodified mesoporous silica on nano-sized had better stability due to the modification on their surfaces.

Table-1. Stability data of nano pure andN-MSV5.

	NanoPure	N-MSV5
Loading amount (mg of cellulase/g ofsilica)	80.310	82.820
Released enzymes (mg ofcellulase)	12.547	7.528
Remaining enzymes (mg ofcellulase)	67.762	75.291
Remaining percentage(%)	84.375	90.909

As shown on Table-1, modified mesoporous silica with vinyl groups has a better stability than pure mesoporous silica because evinyl groups can hold the cellulose in the silica. The vinyl functionalized mesoporous silica showed high enzyme loading capacity and activity. The main reason for the high loading amount of cellulose is the hydrophobic interaction between cellulose and silica, as the protein has a high affinity to hydrophobic surfaces [2, 3]. The hydrophobicity of vinyl functionalized silica gives rise to a benign microenvironment for cellulase, beneficial to maintaining cellulase conformation and flexible mobility of the active sites [7]. Because of the better data of stability, the modified mesoporous silica was continued to reusability test. From this stability test, it can be concluded that vinyl-modified silica has better ability to hold enzymes compared to unmodified silica.

Immobilized cellulase activity

Test on the activity was done by CMC assay which was hydrolyzed on mesoporous silica then tested byDNSmethod. Activity of immobilized enzyme was measured by comparing both functionalized micron-sized and functionalized nano-sized silicas (M-MSV5, M-

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MSV20, N-MSV5, N-MSV20). Based on activity analysis from those four samples, N-MSV20 had the highest activity in comparison to the other modified silica. As comparison, the activity of all other functional samples were determined as percentage against N-MSV20 (Table-2).

The results suggest that nano-sized silica had better capability to convert cellulose to glucose compared micron-sized silica. The functionalization of mesoporous silica has an important part in determining the activity of immobilized enzymes. The functional groups on the functionalized surfaces created a specific space, which can bind enzymesand/ordirect enzyme attachment onto the material [3]. Nano size silica had better activity in comparison to micron sizesilicamaterial due to its shorten channel length as shown in our TEM result (Figure-1). The channel length are almost tentimesshorter than the micron particle. Shorter channel length lowers the possibility of pore blocking, and also improve themassdiffusion inside the particle. As results, interactions between immobilized enzymes and substrates can besignificantlyenhanced.

Table-2. Activity data of micron and nanomesoporous silica.

Sample	**Loading amount** (mg of cellulase/g ofsilica)	**Percentage activity** (%)
M-MSV5	70.9	34.93
M-MSV20	49.9	58.46
N-MSV5	49.9	78.20
N-MSV20	49.9	100

Immobilized Cellulase Reusability

We choose micron and nano-sized V5 for the reusability due to the previous stability test which usedM-MSV5sample. The reusability was tested by mixing buffer solution and CMC into the cellulase-loaded samples. Thepercentageof first activity and second activity were based on 100% activity of N-MSV20. The results showed that the immobilized cellulose could be used for 2cycles without significant loss of activity. After 2 cycles, micron-sized still retained 70% of its initial activity. Table-3 shows that micron-sized V5 had analmostsimilar performance with nano sized V 5(Table-3).

Table-3. Reusability data of micron and nanomesoporous silica.

	M-MSV5	**N-MSV5**
Loading amount (mg of cellulase/g ofsilica)	70.984	49.952
Percentage activity 1(%)	34.93	78.20
Percentage activity 2(%)	24.44	43.52
Percentage reusability(%)	69.98	55.65

CONCLUSIONS

Cellulase enzyme was successfully immobilized onto vinyl functionalized mesoporous silica. Vinylfunctionalization supports the stability of enzymes that attached on the silica material. The vinyl groups in VTMS-modified materials made a strong binding for the enzyme and created a benign microenvironment to enhancedenzymeactivity. Different concentration on vinyl groups in mesoporous silica affects the activity ofthe immobilized enzymes. The synthesis of micron and nano-sized silica showed that particle size had significant effect towards the activity of the enzyme. Enzyme immobilization within both micron-sized particle and nano-sized particle had a reasonable value of activity and reusability.

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