

# VINYL FUNCTIONALIZED CUBIC MESOPOROUS SILICA NANOPARTICLES AS SUPPORTING MATERIAL TO ENHANCE CELLULASE ENZYME STABILITY

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## VINYL FUNCTIONALIZED CUBIC MESOPOROUS SILICA NANOPARTICLES AS SUPPORTING MATERIAL TO ENHANCE CELLULASE ENZYME STABILITY

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### ABSTRACT

Mesoporous silica materials have emerged as promising platform as supporting materials for enzyme immobilization. However, a significant enhancement of cellulase enzyme activity entrapped inside silica pores remains a challenge. In this paper, we report cellulase enzymes immobilization on vinyl functionalized cubic mesoporous silica materials with different particle sizes and different vinyl concentration. Silica materials possess cubic mesostructured with two different sizes: micron size (around 8  $\mu\text{m}$ ) and nano size (around 300 nm) were made by using  $\text{F}_{127}$  surfactant at different acid concentrations. In addition, vinyl functionalization at different concentrations was also developed through co-condensation method. Vinyl functionalized nanoparticles showed significant improvements of enzyme activity, stability and reusability compared to the micron size particles. The increase of vinyl concentration with its hydrophobic characteristics within silica materials induced a high loading amount of cellulase enzymes. Vinyl functionalization created benign microenvironment for cellulase enzymes to perform its high activity. We found the benefit of using the vinyl functionalization and also the application of nanoparticles, compared to common large particle cubic mesoporous silica materials.

**Keywords:** enzyme immobilization, cellulase enzyme, mesoporous silica, nanoparticles, surface modification.

### 1. INTRODUCTION

Over the past few decades, high demand for energy and fossil fuel depletion [1] has given huge interest in looking for alternative energy sources. [2] There are some alternative fuels such as bioethanol and biodiesel to replace fossil fuel as non-renewable substance in the world. The most interesting and abundant organic [3-6] materials in the biosphere is cellulose which convert energy and chemicals. [6] Cellulose can be founded easily from corn or sugar cane. [7] Cellulose consist of 1, 4-beta glycosidic bond linked glucose units as the main component of lignocellulosic biomass. [1] Production of ethanol with high conversion use enzymatic process with cellulase as a raw material need two-step process: first, mixture of cellulose and cellulase enzyme breaks down the cellulose chains and produce glucose, then converted into ethanol. The largest problem of using cellulose in industrial application is the high cost of enzyme. There is an approach to increase the affectivity of enzymatic process with immobilized enzyme into solid supports to protect the enzyme so the enzyme can be reused. [8] There are many scientist that use polymer as solid carrier for enzyme immobilization.

Mesoporous silica has many advantages such as narrow pore size distribution, uniform and tunable pore size, and high surface area with facile surface functionalization. [8, 9] Mesoporous silica can be used for many applications such as drug delivery [9-12], fluorescence biological probes, water purification, bio-imaging [13], and enzyme immobilization [1, 2, 14, 15]. Fan *et al.*, has firstly synthesized ordered mesoporous

silica (FDU-12) with cubic-mesostructured with three-dimensional connectivity. [8] Its pore structure (3-D) and large pore size present a better material than MCM-41 and SBA-15 because FDU-12 gives better mass transfer and reduce pore blocking. [8] But, in enzyme immobilization in mesoporous silica needs large pore size<sup>16</sup> because the size of cellulase enzyme ranging from 24 to 74 Å (when spherical) or from 13 x 79 Å<sup>2</sup> to 42 x 252 Å<sup>2</sup> (when ellipsoidal). [17] Therefore, many scientists have investigated mesoporous silica for enzyme immobilization with different pore sizes. [8, 16, 18] FDU-12 with very large pore size up to 27nm provides an excellent candidate to adsorption studies. Mesoporous silica with large pore size allow more ready diffusion of the substrate to the active sites of enzymes to get higher enzyme activity. [8, 16]

Increasing affectivity of enzyme immobilization, mesoporous silica with organo-functionalized gives higher loading amount and activity than non-functionalized mesoporous silica. [19, 20] Many researches have tried different organic compounds to modify mesoporous silica used vinyl ( $\text{CH}=\text{CH}_2$ ) [21] and thiol ( $-\text{SH}$ ) in SBA-15 and FDU-12. In the previous study, Hartono *et al.* showed that vinyl-cubic mesoporous silica (FDU-12) gives the best activity than the other organosilane. [8] Organosilane functionalization reduce the pore size as the increasing amount of organosilane. [22-25] Functionalized mesoporous silica is got by two approaches: co-condensation and postsynthesis grafting. [23, 26, 27] Synthesis conditions obtained via the latter method is important because the synthesis temperature and hydrothermal treatment may affect the pore structure



distribution and the pore size. [28, 29] its need lower synthesis temperature to expand the pore size and also pore extensions agents such as 1, 3, 5-trimethylbenzene (TMB). It is difficult to immobilize the enzyme into mesoporous silica from the previous studies. [30, 31]

Recently, cubic mesoporous silica with different size in micron size (FDU-12) and nano size (IBN-2) has been made. [32] Over the past decades, mesoporous silica in micron size has been used for enzyme immobilization, but there are some weakness such as the mass transfer inside the pore less efficient than the nano size. To the best of our knowledge, there is no research that synthesized cubic mesoporous silica with different size in micron and nano size and study to explore the synergy between the uses of silica with different concentrations of vinyl-organosilane. We used low synthesis temperature (20°C) and high hydrothermal treatment temperature (up to 130°C) to produce 3-D cubic mesostructured with large entrance pore (5-6nm) and cavity size (9-10nm). In addition, we studied about the effects of different concentrations of vinyl-organosilane and different size materials. Nanosized mesoporous silica material, either functionalized or non-functionalized show a better performance in terms of cellulase loading amount and maintaining cellulase activity.

## 2. EXPERIMENTAL SECTION

### Chemicals

Triblock poly (ethylene oxide)-b-poly (propylene-oxide)-b-poly (ethylene oxide) copolymer (EO<sub>106</sub>PO<sub>70</sub>EO<sub>106</sub>, pluronic F<sub>127</sub>, M<sub>n</sub> = 13400), tetraethoxysilane (TEOS, 99%), 1, 3, 5-trimethylbenzene (TMB), Vinyltrimethoxysilane (VTMS, 98%), Bovine Serum Albumin (BSA, 98%), carboxymethyl cellulose (CMC), and *Aspergillusniger* cellulose solution were purchased from Sigma-Aldrich Pty. Ltd., Australia. 3, 5-Dinitrosalicylic acid (DNS, 98%) and sodium metabisulfite (98%) were obtained from Widya Mandala Catholic University Laboratory, while sodium hydroxide (97%), hydrochloric acid (32%), and potassium chloride (99.8%) were purchased from Ajax Finechem. Cationic Fluorocarbon Surfactant (FC<sub>4</sub>), Phenol (C<sub>6</sub>H<sub>6</sub>O), C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>Na.3H<sub>2</sub>O, Ethanol (C<sub>2</sub>H<sub>6</sub>O).

### Synthesis of pure and vinyl functionalized of cubic mesoporous silica in nano and micron size

In this synthesis of micron sized cubic mesoporous silica (MS), 1 g of pluronic F<sub>127</sub> and 5 g of KCl were mixed in 60 mL of 2 M HCl at 20° C (*synthesis temperature*) and stirred for 30 min. Then, add 1.6 g of TMB and stirring was continued until 6 hours at synthesis temperature. After it, 4 g of TEOS was added and continued stirring for 24 h at 20° C. The solutions after were synthesized then removed to an autoclave and heated at 130° C (*hydrothermal temperature*) for 24 h of hydrothermal treatment. The product was filtered and

dried at 50° C in 0.4 atm, then washed two times using a mixture of methanol 150 mL and hydrochloric acid (HCl) 5 mL at 50° C for 6 h each for calcination to remove the surfactant and another pore enlarging agent. In the term to make a nano sized cubic mesoporous material silica (NS), 0.5 g of F<sub>127</sub> and 1.4 of FC<sub>4</sub> mix in the solutions of 5 g KCl and 60 mL of 2 M HCl for 24 h before 0.5 g of TMB was added and stirring continued for 4 h, then 3 g of TEOS was mixed into the solutions and was stirred for 24 h. The further step was the same step as micron sized cubic mesoporous silica. Both pure cubic mesoporous silica (MS-pure and NS-pure) was synthesized with only TEOS, while the functionalized cubic mesoporous silica were synthesized using a mixture of TEOS and VTMS as organosilane in different molar ratio VTMS: TEOS (1:5, 1:10, 1:20). The vinyl-functionalized cubic mesoporous silica were named as MS-V<sub>5</sub>, MS-V<sub>10</sub>, and MS-V<sub>20</sub> for micron size and NS-V<sub>5</sub>, NS-V<sub>10</sub>, and NS-V<sub>20</sub> for nano size. In another studies, calcination process was done by using ethanol to remove the surfactant and the enlarging pore agent. This method can remove only 80% of surfactant respectively. But, by using methanol as a solution, the amount of surfactant that can be removed is 90%. The calcination proves that the pores are available for enzyme or protein immobilization. The NS has a shorter length than MS, with this size the contact of enzyme in the pores will increase because there is less died zone in the pores.

### Bovine Serum Albumin (BSA) adsorption

Pure and vinyl-functionalized cubic mesoporous silica materials began to degas overnight at 120°C. BSA was mixed in citrate buffer (0.01M, pH 4.8) as stock solutions of BSA with various concentration in a range from 0.1 to 2 mg.mL<sup>-1</sup>. To the tune of 0.01 g of cubic mesoporous silica materials was dissolved in 5 mL of each BSA solution, and the suspensions were incubated for 24 h at low temperature (4°C) and 200 rpm shaking speed in water shaker. The suspensions were separated by centrifugation for 20 min in 20°C and 4750 rpm. There are two concentrations of BSA: pre- and post-incubation which were determine by using characteristic UV-vis. The absorbance of BSA is 595 nm and the linear part of a C<sub>BSA</sub>-A<sub>280 nm</sub> as standard curve from the adsorption experiments. The difference amount of BSA in the pre- and post-solution after adsorption was considered equal to the amount of BSA adsorbed in the cubic mesoporous silica materials. BSA adsorption isotherms were constructed via combinatorial analysis of BSA solution equilibrium concentration and BSA loading of the materials (mg of BSA/g of sample), and the data points were fitted using a Langmuir equation.

### Cellulase immobilization, activity, stability, and reusability

Commercially *Aspergillusniger* cellulose solid was used as enzyme source and various dilutions were prepared using citrate buffer (pH = 4.8). Initially, immobilization enzyme on various concentrations of cubic



mesoporous silica were began with all samples degassed at 120°C overnight. 0.01 gram of silica was then incubated in 8 mL buffer in shaking water bath for 24 h in 4°C of each sample of the various enzyme solutions. The samples were filtered and centrifuged. Pre- and post-incubation enzyme concentrations ( $C_0$  and  $C_i$ , respectively) were determined using a Bradford Protein Assay with BSA as a standard protein. In the last, the loading amount of enzyme (mg of cellulase/g of sample) was calculated from the difference in pre and post-incubation enzyme concentration. Then, the data are plotted vs the cellulose equilibrium ( $C_{eq}$ ) to give the adsorption isotherm and fitted using a Langmuir equation.

Activity from cellulase enzyme that immobilized to cubic mesoporous silica material can be determined by carbomethyl cellulase (CMC) assay. First, make citrate buffer solution (pH = 4.8) by mix 76.267 mL  $CH_3COOH$  and 169.63 grams  $C_2H_3O_2Na \cdot 3H_2O$  in 3000 mL aquades. Making DNS reagent by mix 10.6 grams 3,5-dinitrosalicylic acid (DNS), 19.8 grams NaOH, 30.6 potassium sodium tartar, 7.6 mL phenol, 8.3 sodium metabisulfite, 1416 mL  $H_2O$ . Then, 0.01 gram nanoporous silica, 0.5 mL CMC solution (1% wt) mixed with 0.5 mL of cellulase, 5 mL buffer solution and then the hydrolysis reaction was processed for 1 hour. After that, 3 mL of DNS reagent mix with the solution and the mixture was boiled for 5 minutes until a red-brown color of varying intensity was gotten ( $\lambda_{max} = 540nm$ ). Then determine the highest concentration of glucose (C) from standard curve of glucose (absorbance vs C). Blank experiments also needed to verify that non-functionalized and vinyl-functionalized cubic mesoporous silica without immobilized enzyme did not produce glucose. It is prove that the activity was obtained from immobilize enzyme in materials.

After the highest activity is found, stability can be determined by take 0.01 gram of cubic mesoporous silica materials of the highest activity and mix with 5 mL cellulase to 50 mL citrate buffer. Keep it in 4°C for 7 days. Every one day take a sample and measure the absorbance to determine the concentration of enzyme with standard curve of BSA.

The highest activity of cubic mesoporous silica will centrifuge and mix with 0.5 mL CMC solution (1% wt) mixed with 0.5 mL of cellulase, 5 mL buffer solution and then the hydrolysis reaction was processed for 1 hour. After that, 3 mL of DNS reagent mix with the solution and the mixture was boiled for 5 minutes until a red-brown color of varying intensity was gotten ( $\lambda_{max} = 540 nm$ ). The reusability process is done for 3 times of recycle.

#### Characterization

Transmission electron microscopy (TEM) images were collected on a JEOL 1010 electron microscope at an electron beam acceleration voltage of 100 kV. Nitrogen sorption isotherms of the samples were obtained using a Quantachrome Quadrasorb SI analyzer at 77 K. Samples were degassed overnight at 120 °C in a vacuum prior to all

$N_2$ -sorption measurements. The surface area was calculated according to the Brunauer-Emmett-Teller (BET) model using data in the relative pressure range  $p/p_0$  0.05-0.25. The total pore volume was calculated from the  $N_2$  amount adsorbed at maximum  $p/p_0$  (0.99). The pore size distribution was calculated according to the BJH method i.e., cavity and entrance pore sizes were determined from the adsorption and desorption branches, respectively. Fourier Transform Infrared Spectroscopy (FTIR) images were collected from Shimadzu/FTIR-8400S at maximum rate of scanning.

### 3. RESULT AND DISCUSSIONS

#### Vinyl-functionalized mesoporous silica with Large Pore

Enzyme immobilization requires specific carriers which enables efficient mass transfer and intensive contacts between enzymes and substrates. Since the main purposes of enzyme immobilization is to maintain enzyme activity, thus carriers that can accommodate these requirements are highly sought after. Cubic mesostructured mesoporous silica materials have unique advantages compared to 2 dimension hexagonal structures in terms of better mass transfer and more resistance to pore blockage. This makes cubic mesostructured mesoporous silica as a preferable choice to perform any chemical reactions within their pores.

Two mesoporous silica materials with similar cubic mesostructured but different particle size (micron size: 4-8  $\mu m$  and 100 - 300 nm) have been successfully synthesized. Result of Nitrogen Sorption analyzes of non-functionalized cubic mesoporous silica samples in micron and nano size are display in Figure-1 and Figure-2. Its show invariably type IV isotherms with type H2 hysteresis loops that indicated of mesoporous materials with a cubic mesostructured. [33] The relatively sharp increase of the isotherm adsorption at the end of the  $p/p_0$  range confirm there is a capillary condensation and proves the uniform large pore structure of cubic mesoporous silica. The hysteresis indicates that there is a different of pore size and cavity size in the materials and confirm a cage like mesostructured material. The entrance pore size and cavity distributions also show large pores in all materials. For MS-pure have 10 nm cavity size and 4.6 nm entrance pore size. NS-pure has 10.4 nm cavity size and 3.8 nm entrance pore size.

Vinyl functionalized and non-functionalized cubic mesoporous silica material were prepared and characterized as described in the experimental section. Vinyl group ( $C=C$ ) affect the structure of the cubic mesoporous silica matrix and its formation differently. Hartono et al., showed that non-functionalized and vinyl functionalized cubic mesoporous silica has similar four main peaks, that reflect an ordered, cubic (3-Dimensional) structure ( $Fm3m$ ). [28] But with vinyl functionalized cause a reduction in cavity size and pore size. In some materials, there is a decrease of peak intensity that indicates less



ordered mesostructured. From the previous studies [8], showed that co-condensation synthesis of vinyl and TEOS still give a cubic type mesostructured with insignificant disruptions of the ordered structure.

Decreasing pore size of vinyl functionalized cubic mesoporous silica materials cause by the strong hydrophobic interactions between TMB and Vinyl group. It is happened in the micelle core and causes micelle swelling (make large pores of the material). The interaction lead of extraction in the fractions of the TMB from the micelle and VTMS functionalization give bigger cavity size compared to other organosilanes. [8] Due to this result, the different concentration of VTMS: TEOS (1:5; 1:10; 1:20) were prepared.

From Figure-3 and Figure-4 displayed the FTIR spectra result of the cubic mesoporous silica samples (MS and NS) non-functionalized (pure) and vinyl functionalized in different concentrations. The peaks indicating to the C-H stretching and bending vibration of  $F_{127}$  appear in  $1460\text{ cm}^{-1}$  respectively. A new bends at  $1637\text{ cm}^{-1}$  indicates the vibrations of vinyl ( $\text{C}=\text{C}$ ) group. In non-

functionalized (pure) cubic mesoporous silica whether in micron or nano size there is a peak at  $1600\text{ cm}^{-1}$  that indicates the rest of surfactant that still bind in the materials. In all materials, the typical of Si-O-Si bends around  $790\text{ cm}^{-1}$  and  $1210\text{ cm}^{-1}$  indicates the formation of all condensed silica network. The others peak at  $950\text{ cm}^{-1}$  also present the Si-OH groups which is non-condensed. This is an agreement with the result from the previous study of FTIR. [21]

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displays the TEM images of MS-pure samples that absorbs from 2 different directions (perpendicular and parallel) to the post. The result confirms the large pores of cavity and pore sizes. A relatively ordered mesostructured also can be seen in these Figures. The length of MS is longer than NS. It is indicates that FC-4 (fluorocarbon) can limit the growth of micelle and produce shorter length of materials. Therefore the TEM result is in agreement with the result of nitrogen sorption measurements because FDU-12 maintains a cubic mesostructured.

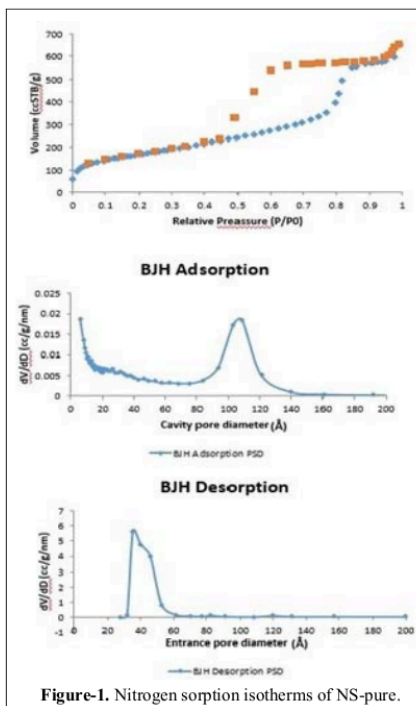


Figure-1. Nitrogen sorption isotherms of NS-pure.

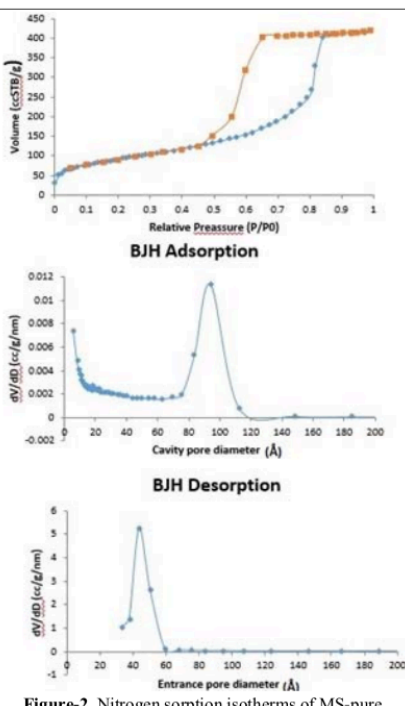


Figure-2. Nitrogen sorption isotherms of MS-pure.





### Enzyme immobilization

The immobilization of cellulase enzyme were done at pH 4.8 using citrate buffer, which is close to isoelectric point of cellulase: 4.75 [34]. It's also the optimum pH range of free cellulase enzyme to get the maximum activity.

**Table-1** summarizes the result of cellulase immobilization of cubic mesoporous silica with open and blocked pores. This result indicates that the loading of cellulase enzyme with open pores is better than blocked pores. It show that cellulase absorption in MS with open pores can immobilized approximately 60% and 40% for NS into the pores. It is happened because the enzyme more penetrates into the pores than on the surface. Loading capacity of cubic mesoporous silica with blocked pores is

smaller than open pores because there is no hydrophobic interaction between VTMS and cellulase in MS-pure and NS-pure. Both vinyl functionalized and non-functionalized cubic mesoporous silica possessed a negative charge. [8] It is important to know that cellulase has 3 large enzymes (CBH, EG and cellobiase) [4], in size range from 24 to 74 Å (when spherical) or from 13 x 79 Å to 42 x 252 Å (when ellipsoidal). [17] Each of these enzyme has own isoelectric point. From the previous study, EG and CBH can be classified into different families (e.g., EG I, EG V, CBH I, etc.) that has unique cubic structure and a specific isoelectric point. [35] The majority of enzyme cellulase has more negative charge than positive charge so it is match with non-functionalized and functionalized cubic mesoporous silica charge.

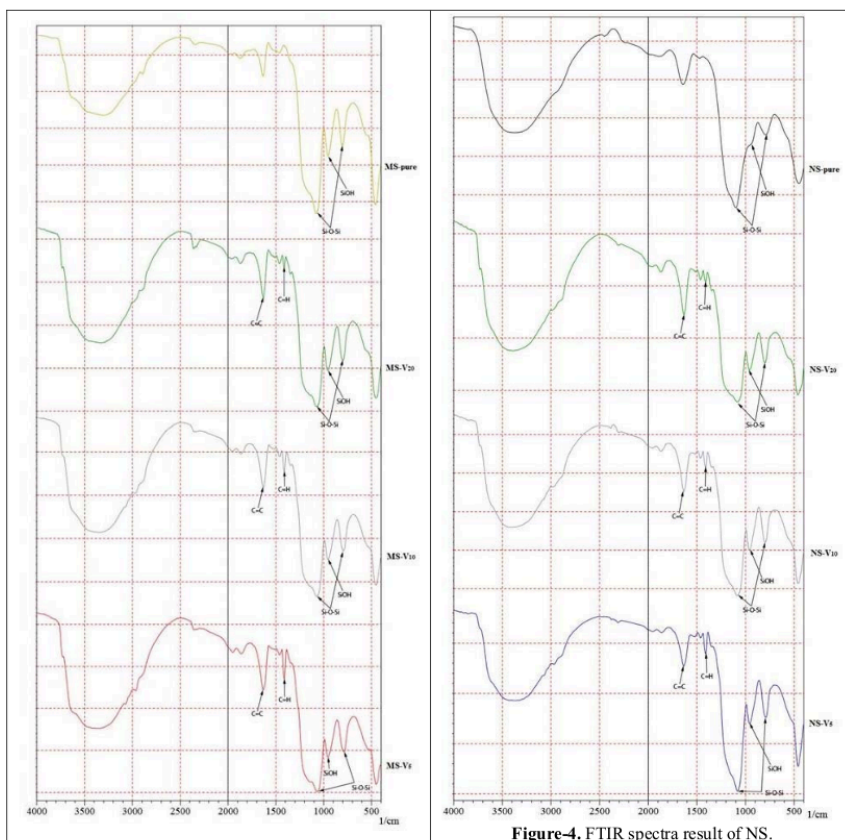


Figure-4. FTIR spectra result of NS.



Figure-3. FTIR spectra result of MS.

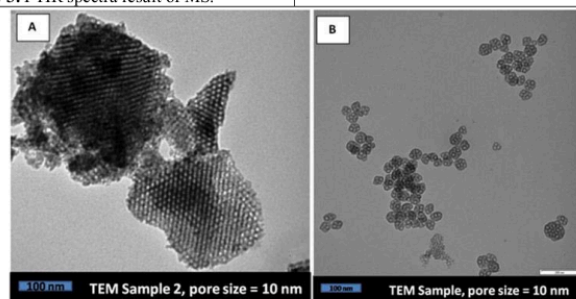


Figure-5. TEM images of MS-pure samples.

Table-1. Cellulase loading capacity of non-functionalized (MS-pure and NS-pure) with open and blocked pores.

| Sample                   | Loading capacity at pH 4.8, $q_e$<br>(mg of cellulase / g of silica) |
|--------------------------|--|
| MS-pure                  | 13   |
| MS-pure w/ blocked pores | 3  |
| NS-pure                  | 9  |
| NS-pure w/ blocked pores | 4.8  |

Tables 2 and 3 show the result of loading cellulase enzyme in cubic mesoporous silica is showed. The data in these two tables show that nano-size has a better loading amount of cellulase in each material. In MS-V<sub>5</sub> also have the biggest loading amount whether in nano size and micron size. It happened because the influence of pore size and surface characteristics affect the ability of cellulase to penetrate the pores. It also proves that with increasing amount of VTMS can increase the loading amount too. This is happens because of the hydrophobic interactions between certain protein domains and the organosilane (vinyl). [24, 25]

#### Activity

The activity from enzyme cellulase in MS-pure and functionalized cubic mesoporous silica was tested using Carboxymethyl Cellulose assay as described in experimental section. Activity means the amount of glucose that can be converted by cellulase materials (MS and NS).

From Table-2 and Table-3 non-functionalized and functionalized material with different initial cellulase loading were tested, the functionalized material affected in material adsorption capacity and activity of immobilized enzyme. The functional groups (vinyl C=C) on the modified surface create microenvironment, which can attracts enzymes and or direct enzyme attachment onto the

solid support [25]. In this research, MS-pure showed the lowest activity to convert cellulose (CMC) into glucose. On the other hand, MS-V<sub>5</sub> and NS-V<sub>5</sub> showed the highest enzyme activity in micron and nano size. In this case, to prove that the silica material MS-V<sub>5</sub> and NS-V<sub>5</sub> can convert cellulose into glucose, the material must have enough entrance pore. Figures 6 and 7 shows that the MS-V<sub>5</sub> has 10 nm of cavity and 4 nm of entrance pore, and the NS-V<sub>5</sub> has 10 nm of cavity and 4 nm of entrance pore. It also show that high enzyme loadings did not always correlate with high enzyme activities.<sup>19</sup> But, with higher concentration of VTMS, it can increase the activity of the materials.

The hydrophobic interaction between cellulase and the VTMS increase the loading amount of cellulase, as the protein has a high affinity to hydrophobic surface.<sup>19,25</sup> Non-functionalized cubic mesoporous silica also gives high cellulase activity, because the low degree of hydrogen bonding that affect in support and cellulase has no detrimental effect on the mobility of the enzyme's site. [8]

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Table-3 clearly show the superiority of nanosized particles compared to the micron size. We believe this is mainly related to the short channel length of interconnected cubic mesostructured within the nanosized mesoporous silica materials. The relatively large of cellulase enzyme size in



relation with the pore size hindrance distribution of substrate (cellulose) homogeneously along the pore channel. The use of micron size mesoporous silica materials with its long channel increases the possibility to form inactive size along the channel. Some parts can be blocked thus leaves the enzymes totally inoperative. On the other hand, the short length reduces the formation of inactive site along the pores channel. Its short channel enables highly efficient contact between enzyme and substrate (cellulose). The efficient contact of enzyme and

cellulase is the main prerequisite to gain a high conversion rate. The benefit form nanosizedmesoporous silica materials in synergy with the vinyl functionalization further increased enzyme loading amount and enzyme activity. The increase of enzyme loading clearly increase enzyme loading and activity, yet, one needs to be careful, since the excessive increase of vinyl loading might cause significant pore size reduction which can affect enzyme loading and activity.

**Table-2.** Cellulase activity before and after immobilization in MS.

| Sample  | No. Sample  | Loading capacity, $q_e$ (mg of cellulase / g of silica) | Activity compared to free enzyme (%) |
|---------|-------------|---|--------------------------------------|
| MS-pure | Free enzyme |   |                                      |
|         | 0.000       | 0.000   | 0.000                                |
|         | 1.000       | 5.550   | 0.671                                |
|         | 2.000       | 5.850   | 0.755                                |
|         | 3.000       | 8.010   | 1.416                                |
|         | 4.000       | 9.830   | 3.020                                |
|         | 5.000       | 10.650  | 6.292                                |
|         | 7.000       | 12.960  | 7.550                                |
| MS-V20  | Free enzyme |   |                                      |
|         | 0.000       | 0.000   | 0.000                                |
|         | 1.000       | 6.980   | 0.839                                |
|         | 2.000       | 12.030  | 1.416                                |
|         | 3.000       | 12.870  | 3.020                                |
|         | 4.000       | 13.560  | 5.663                                |
|         | 5.000       | 17.840  | 6.606                                |
|         | 7.000       | 18.010  | 10.067                               |
| MS-V10  | Free enzyme |   |                                      |
|         | 0.000       | 0.000   | 0.000                                |
|         | 1.000       | 5.670   | 1.416                                |
|         | 2.000       | 9.540   | 2.697                                |
|         | 3.000       | 10.870  | 3.775                                |
|         | 4.000       | 11.440  | 4.404                                |
|         | 5.000       | 19.970  | 16.989                               |
|         | 7.000       | 20.230  | 18.875                               |
| MS-V5   | Free enzyme |   |                                      |
|         | 0.000       | 0.000   | 0.000                                |
|         | 1.000       | 7.130   | 2.328                                |
|         | 2.000       | 9.760   | 2.517                                |
|         | 3.000       | 10.530  | 5.663                                |
|         | 4.000       | 14.210  | 7.550                                |



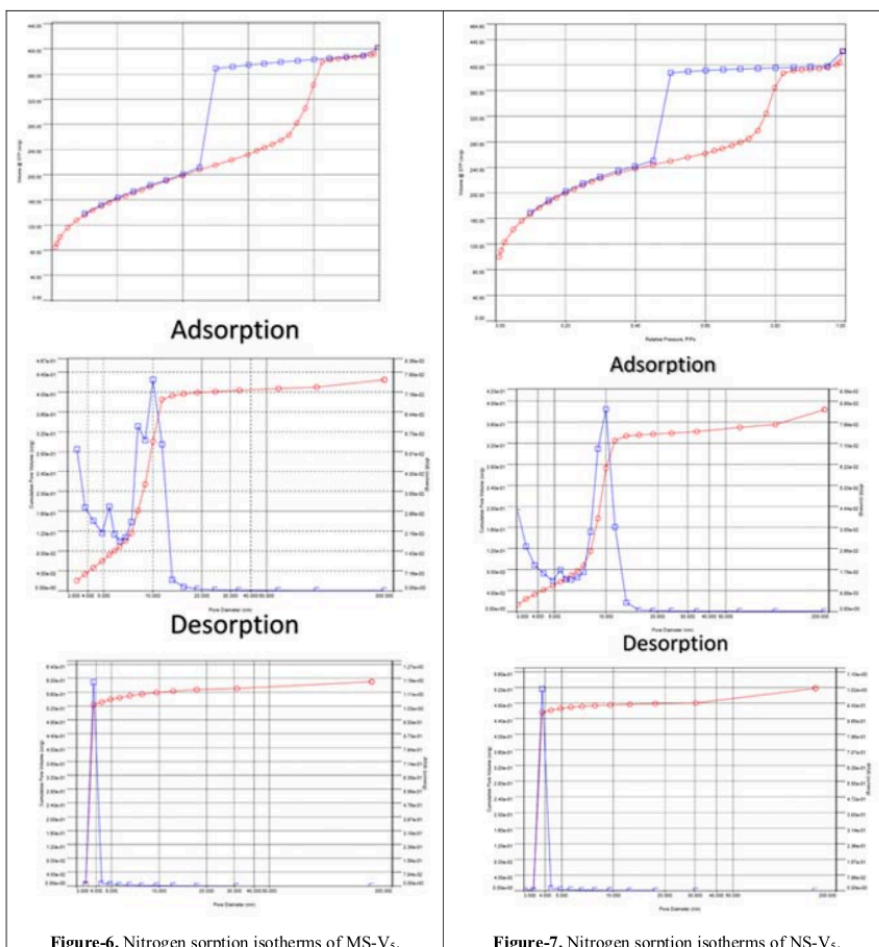


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|  |       |        |        |
|--|-------|--------|--------|
|  | 5.000 | 20.430 | 24.538 |
|  | 7.000 | 22.110 | 30.546 |

**Table-3.** Cellulase activity before and after immobilization in NS.

| Sample  | No. Sample  | Loading capacity, $q_e$ (mg of cellulase / g of silica) | Activity compared to free enzyme (%) |
|---------|-------------|---|--------------------------------------|
| NS-pure | Free enzyme |   |                                      |
|         | 0.000       | 0.000   | 0.000                                |
|         | 1.000       | 5.710   | 1.416                                |
|         | 2.000       | 5.740   | 1.888                                |
|         | 3.000       | 11.070  | 4.719                                |
|         | 4.000       | 10.120  | 6.606                                |
|         | 5.000       | 15.690  | 8.400                                |
|         | 7.000       | 16.430  | 10.067                               |
| NS-V20  | Free enzyme |   |                                      |
|         | 0.000       | 0.000   | 0.000                                |
|         | 1.000       | 6.010   | 1.941                                |
|         | 2.000       | 6.230   | 2.157                                |
|         | 3.000       | 14.110  | 3.775                                |
|         | 4.000       | 15.130  | 5.285                                |
|         | 5.000       | 19.970  | 6.795                                |
|         | 7.000       | 21.210  | 18.875                               |
| NS-V10  | Free enzyme |   |                                      |
|         | 0.000       | 0.000   | 0.000                                |
|         | 1.000       | 6.990   | 2.097                                |
|         | 2.000       | 10.010  | 3.356                                |
|         | 3.000       | 11.310  | 8.305                                |
|         | 4.000       | 13.490  | 14.157                               |
|         | 5.000       | 21.330  | 24.538                               |
|         | 7.000       | 21.960  | 37.752                               |
| NS-V5   | Free enzyme |   |                                      |
|         | 0.000       | 0.000   | 0.000                                |
|         | 1.000       | 7.890   | 3.775                                |
|         | 2.000       | 8.630   | 7.550                                |
|         | 3.000       | 14.840  | 10.382                               |
|         | 4.000       | 21.610  | 18.875                               |
|         | 5.000       | 23.980  | 49.077                               |
|         | 7.000       | 24.130  | 56.627                               |

Figure-6. Nitrogen sorption isotherms of MS-V<sub>5</sub>.Figure-7. Nitrogen sorption isotherms of NS-V<sub>5</sub>.

#### Stability and reusability of cubic mesoporous silica with cellulase

There is a factor in immobilization cellulase enzyme into cubic mesoporous silica: storage stability. [8] The storage stability means how much cellulase enzyme that still bind with the materials (enzyme leakage). [36] The stability test were done in 3-days period that shown in Table-4. In general, cubic mesostructured silica with higher cellulase enzyme amount has bigger chance of enzyme leakage. In MS-pure and NS-pure, the amount of loss cellulase enzyme is bigger than organo-functionalized

MS and NS. It is because the silanol group on the surface of MS-pure and NS-pure has weak interaction with the enzyme which is hydrogen bonding. But, with VTMS functionalized the loss of cellulase enzyme is less because there are two bonding: hydrogen and hydrophobic.

Cubic mesoporous silica also can be reused for several times. Table-5 shows that vinyl functionalized MS and NS still gives high activity than the non-functionalized MS and NS. So, vinyl functionalized is not only increase the stability but also the reusability.



**Table-4.** Stability of cellulase enzyme immobilized on MS pure, NS pure, MS-V5 and NS-V5.

| Sample  | No. | Co    | Ceq (1) | Ceq (2) | Ceq (3) |
|---------|-----|-------|---------|---------|---------|
| MS-pure | 1   | 0.104 | 0.096   | 0.100   | 0.179   |
|         | 2   | 0.117 | 0.105   | 0.110   | 0.242   |
|         | 3   | 0.121 | 0.114   | 0.121   | 0.280   |
|         | 4   | 0.124 | 0.118   | 0.128   | 0.186   |
|         | 5   | 0.511 | 0.500   | 0.507   | 0.635   |
|         | 7   | 0.521 | 0.508   | 0.549   | 0.725   |
| MS-V5   | 1   | 0.104 | 0.094   | 0.100   | 0.104   |
|         | 2   | 0.117 | 0.100   | 0.104   | 0.121   |
|         | 3   | 0.121 | 0.109   | 0.114   | 0.128   |
|         | 4   | 0.124 | 0.112   | 0.124   | 0.141   |
|         | 5   | 0.511 | 0.485   | 0.576   | 0.594   |
|         | 7   | 0.521 | 0.495   | 0.497   | 0.521   |
| NS-Pure | 1   | 0.104 | 0.096   | 0.100   | 0.121   |
|         | 2   | 0.117 | 0.107   | 0.114   | 0.141   |
|         | 3   | 0.121 | 0.109   | 0.124   | 0.162   |
|         | 4   | 0.124 | 0.117   | 0.138   | 0.183   |
|         | 5   | 0.511 | 0.493   | 0.518   | 0.725   |
|         | 7   | 0.521 | 0.503   | 0.556   | 0.680   |
| NS-V5   | 1   | 0.104 | 0.094   | 0.097   | 0.100   |
|         | 2   | 0.117 | 0.102   | 0.107   | 0.114   |
|         | 3   | 0.121 | 0.105   | 0.110   | 0.117   |
|         | 4   | 0.124 | 0.103   | 0.114   | 0.124   |
|         | 5   | 0.511 | 0.481   | 0.487   | 0.500   |
|         | 7   | 0.521 | 0.493   | 0.494   | 0.500   |



**Table-5.** Reusability of MS pure, NS pure, MS-V5 and NS-V5.

| Sample  | No. | Act (1) | Act (2) | Act (3) |
|---------|-----|---------|---------|---------|
| MS-pure | 1   | 0.087   | 0.090   | 0.093   |
|         | 2   | 0.075   | 0.081   | 0.072   |
|         | 3   | 0.029   | 0.042   | 0.046   |
|         | 4   | 0.016   | 0.022   | 0.028   |
|         | 5   | 0.012   | 0.013   | 0.017   |
|         | 7   | 0.006   | 0.008   | 0.019   |
| MS-V5   | 1   | 0.015   | 0.017   | 0.021   |
|         | 2   | 0.009   | 0.010   | 0.013   |
|         | 3   | 0.007   | 0.009   | 0.010   |
|         | 4   | 0.005   | 0.007   | 0.009   |
|         | 5   | 0.002   | 0.003   | 0.003   |
|         | 7   | 0.001   | 0.001   | 0.002   |
| NS-Pure | 1   | 0.046   | 0.052   | 0.055   |
|         | 2   | 0.038   | 0.043   | 0.046   |
|         | 3   | 0.012   | 0.013   | 0.014   |
|         | 4   | 0.009   | 0.010   | 0.012   |
|         | 5   | 0.004   | 0.005   | 0.007   |
|         | 7   | 0.004   | 0.004   | 0.006   |
| NS-V5   | 1   | 0.012   | 0.014   | 0.017   |
|         | 2   | 0.010   | 0.012   | 0.015   |
|         | 3   | 0.005   | 0.006   | 0.008   |
|         | 4   | 0.002   | 0.003   | 0.004   |
|         | 5   | 0.001   | 0.002   | 0.003   |
|         | 7   | 0.001   | 0.001   | 0.001   |

#### 4. CONCLUSIONS

Vinyl functionalized cubic mesostructured mesoporous silica nanoparticles at different organosilane concentration have been successfully synthesized through co-condensation method. TEM images indicate the formation of ordered cubic mesoporous silica for both micron size (MS) and nano size silica particles (NS). These results are in agreement with the nitrogen analyses. Both silica materials (MS and NS) have similar pore size and vinyl concentrations. Functionalized and Pure/un-functionalized MS and NS had similar loading amount of cellulase enzyme. Yet, NS showed significantly improved cellulase activity, stability and reusability compared to MS at similar loading amount. NS with its short interconnected [24] pores had a better mass transfer of substrate: cellulose. This condition leads to higher interaction between substrate (CMC) and cellulase enzymes. As a result a significant improved activity of cellulase enzyme can be achieved.

The increase of vinyl concentration within cubic mesoporous silica network either for micron size or nano size increase the enzyme immobilization, activity, stability, and reusability. This research confirmed the significant benefit from vinyl functionalized NS in maintain high performance of cellulase enzyme. We believe that this type of material can be also useful for other biomolecules adsorption with various applications.

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