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Synthesis of mesoporous silica with different pore size for cellulase immobilization: pure physical adsorption

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To discuss the physical adsorption mechanism of the adsorption process of cellulase, a commercial enzyme cocktail sauced from Acremonium, was immobilized in mesoporous silica with various pore sizes by pure physical adsorption in this study. Mesoporous silica materials with 17.6 nm and 3.8 nm pore size (hereafter referred to as Ms-17.6nm and Ms-3.8nm, respectively) were synthesized in the manner of a seeded-growth method. Other available mesoporous silica denoted as H-32 and diatomite were also used as sorbents. Then the sorbents were characterized via Small-angle X-ray scattering (SAXS), transmission electron microscope (TEM), scanning electron microscope (SEM), Barrett-Emmett-Teller (BET) method and Brarrett-Joyner-Halanda (BJH) method to confirm their mesostucture. Furthermore, adsorption ability of different sorbents and enzymatic activities of immobilized cellulase were studied. The adsorption amounts exhibited a clear correlation with the pore size of the sorbents; i.e., the adsorption amounts of MS-17.6nm (410 mg/g) with the pore size was similar to the long axes of cellulase molecule was higher than that for MS-3.8nm (315 mg/g) with the pore size approximated to the short axes of cellulase (which was realized at 50 °C). Besides, the adsorption behavior of diatomite (with pore size about 200 nm) revealed a periodicity because the pore size was significantly larger than cellulase molecules. In the meantime, the pore size was suggested to be a critical factor for enzymatic activity of the cellulase. When the average pore size of MS-3.8nm just matched the short axes of cellulase molecules, immobilized cellulase preserved active sites of cellulase intactly and showed the best activity (i.e. 63.3% of free cellulase activity at 50 °C). Consequently, pore size of the sorbents had a significant influence on cellulase immobilization.

Introduction

Fuel energy production from renewable biomass has been considered as a strategic way to solve the problems of fossil fuel depletion and environmental pollution. Lignocellulosic biomass, such as forestry and agricultural waste, is one of the most promising sources to generate biomass-based energy.[1–5] Cellulose, which consists of 1, 4-beta glycosidic bond linked glucose units, is the main component of lignocellulosic biomass. Since the hydrolysate of cellulose, glucose is easily converted into ethanol, butanol, or other fuels and fine chemicals, researchers have intensively investigated the way to convert cellulose into glucose [5–8] Three main methods concluding physical, chemical, and biological process have been applied to cellulose conversion.[3-5] However, physical and chemical processes usually require extreme treatments, such as requirement of high pressure, high temperature or strong acids. These methods are often energy intensive, and might produce undesirable by-products.[7–10] On the contrary, enzyme-assisted processes for cellulose conversion are usually performed in relatively mild reaction conditions with high specificity, which has been considered as an alternative "green" approach.[11,12]

For this "green" approach, cellulase, which is a general term for a group of enzymes that hydrolyze cellulose, has been intensively studied due to its significant function in converting cellulosic biomass to glucose.[13,14]. Therefore enzyme cost reduction is one of the critical factors to decrease the cost of cellulosic biofuel production. Since free cellulase has a low stability and cannot be efficiently recovered and reused, if these problems can be solved, the cost of enzymatic hydrolyzation may be reduced potentially To overcome these problems, immobilization methods, ultrafiltration systems, aqueous two-phase systems and modification of cellulase have been studied.[15-19] These reports suggested that immobilization of cellulase may be a promising way of increasing the efficiency of enzyme utilization.[20,21]

During the past two decades, extensive research has been carried out on the immobilization of cellulase on inorganic materials. Unfortunately, immobilized cellulase on the surface of inorganic material, such as amorphous silica or magnetic nanoparticles, could not retain the full activity of free cellulase. Among these various inorganic materials, mesoporous silica have been intensively

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investigated for their potential application as delivery vehicles for small-molecule drugs, DNA, and proteins, owing to their uniform pore size, large surface area, and high accessible pore volume.[22-26] Chang et al.[27] synthesized two mesoporous silica with different pore size and surface area, for physical adsorption and chemical binding of cellulase. Meanwhile, the reaction conditions, including temperature, time, and amount of cellulase for cellulosic hydrolysis, were optimized. The results showed that the cellulase chemically linked to mesoporous silica exhibiting carboxyl groups and a large pore size could achieve an effective cellulose-to-glucose conversion exceeding 80% yield and excellent stability.

To create more suitable immobilization of cellulase by using mesoporous silica, it is of great importance to understand the factors that influence the immobilizing behavior of proteins within mesoporous silica. It has been found that two factors may greatly influence the immobilization properties. The first is the surface characteristics of mesoporous silica and proteins. The surface charges of sorbents and the proteins must be complementary, because it is generally accepted that the electrostatic interaction between protein and mesoporous silica is one of the most important factors that influence adsorption and desorption.[28] The second is the size of mesopore, or more specifically, the pore size with relative to the protein molecule size. To absorb cellulase, the pore size of the mesochannels should be sufficiently large for ''comfortable'' entrapment of biomolecules.[29] Kisler et al.[30] have demonstrated that the adsorption amounts in MCM-41 materials depended strongly on the adsorbing molecular size relative to the pore size for a range of biomolecules. Nevertheless, Takimoto et al.[31] encapsulated the cellulase molecules using mesoporous silica SBA-15 and the enzymatic activities of cellulase immobilized onto mesoporous silica of various pore sizes were studied. The encapsulated cellulase on SBA-15 with a pore size of 8.9 nm gave the highest enzymatic activity, despite not having largest pore size and maximum adsorption amout. Herein, a new theory is proposed for pore size, not the larger the better it gets -- larger pore size may achieve an excellent initial adsorption amount but following desorption and perishing operation stability, meanwhile, the molecular dense arrangement, caused by abundant adsorption would prevent conformational flexibility of cellulase and finally result in enzymatic activity lost. Although there have been several reports focusing on physical adsorption of enzymes with mesoporous silica as sorbents,[23] it is a lack of information to use mesoporous silica for cellulase adsorption.

To investigating the physical adsorption mechanism of the adsorption process, we discussed several factors as follow: two mesoporous silica with different pore size of 17.6 nm and 3.8 nm (MS-17.6nm and MS-3.8nm, where 'MS' means the mesoporous silica) were synthesized, meanwhile, a series of available mesoporous silica denoted as H-32 and diatomite were also used as sorbents. In addition, the effect of morphology of different sorbents, immobilization temperature and amounts of sorbents on the adsorption amounts were exhaustively studied. We also investigated the enzymatic activity of the cellulase immobilized on different sorbents.

Experimental

Materials

All chemicals were of analytical grade and used without further purification. Carboxymethyl cellulose sodium salt (CMC, nacalai tesque), sodium acetate (NaAc, nacalai tesque), acetic acid (nacalai tesque), hexadecyltrimethylammonium bromide (CTAB, nacalai tesque), ethyl acetate (EtAc, nacalai tesque), sodium silicate meta (nacalai tesque), methanol (nacalai tesque), hydrochloric acid (nacalai tesque), H-32 (mesoporous silica, AGC Co.), diatomite (Huali Co.), GLU C Ⅱ (glucose kit, Wako) and acremonium cellulase (Meiji Seika Pharma Co.). Deionized water was used throughout the experiments.

Synthesis of mesoporous silica

In the experimental procedure, mesoporous silica denoted as MS-17.6 nm was prepared by a seeded-growth method.[32] A mixed solution was prepared by dissolving 5.0 g CTAB and 6.0 g $Na_2O{\cdot}SiO_2$ in 90 mL of aqueous solution at room temperature (25 °C). Then, 6.4 mL of ethyl acetate was added, and the mixture was stirred for 5 min and allowed to stand at room temperature for 6 h. After this period of aging, the mixture was stirred at 90 °C for 48 h in an oil bath. Then, the heating was stopped and the suspension was cooled naturally to room temperature. Finally, the product was collected by centrifugation and CTAB templates were completely extracted by Soxhlet extractor with acidic methanol solution (pH 1.4) for 10 h.

MS-3.8 nm was synthesized as follows. Briefly, 4.2 g CTAB and 3.5 g $Na_2O{\cdot}SiO_2$ were added to 170 mL of aqueous solution and stirred for 30 s at room temperature. After a clear solution was obtained, 3.2 mL of ethyl acetate was subsequently added to the system. The reaction was allowed to proceed for 48 h at 80 °C. After removing the CTAB templates from the as-synthesized materials by heating them at 550 °C for 3 h, the mesoporous silica was collected.

Immobilization of cellulase on sorbent

The immobilization of cellulase on sorbents was carried out according to the following procedures. In a typical adsorption experiment, a certain amount of sorbents were added to the cellulase solution (100 mg cellulase, 25 mL of 10 mM NaAc buffer with pH value 5.0) in a vessel covered to prevent evaporation. Then the mixture was stirred at a given temperature for 24 h to establish the adsorption equilibrium. The immobilized cellulase on sorbents was collected by centrifugation at 10000 × g for 10 min following washed by NaAc buffer for 3 times. The amount of cellulase molecules remaining in the supernatant was determined by the method of Bradford[33] using boving serum albumin as the standard. The amount of cellulase immobilized was calculated using the formula

$$(x/m)_a=(C_i-C_e)\times V_a/W,$$

Where $(x/m)_a$ is the amount of cellulase immobilized per unit weight of sorbent ($mgmg^{-1}$), C_i is the initial concentration of protein ($mgmL^{-1}$) in cellulase solution, C_e is the equilibrium concentration of protein ($mgmL^{-1}$), V_a is the solution volume (mL), and W is the sorbent weight (mg).

To investigate the effect of sorbents morphology on immobilization, the immobilization was carried out by adding 250 mg of different sorbents to 25 mL of 4 mg/mL cellulase solution and

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stirring for 24 h at 50 °C. Then, the amount of cellulase immobilized on different sorbents was measured by UV-Vis spectroscopy. To investigate the effect of temperature on immobilization, 25 mL of 4 mg/mL cellulase solution was added to MS-17.6nm powder (250 mg). The mixture was stirred at 50 °C, 25 °C and 4 °C for 24 h. To investigate the effect of sorbents amounts on immobilization, experiments involving adsorption of cellulase into MS-17.6nm and MS-3.8nm were conducted for a range of initial input of sorbents from 150 mg to 250 mg in given concentration of cellulase at 50 °C for 24 h. To keep the concentration of cellulase as a constant, the mass of cellulase was 100 mg dissolved in 25 mL buffer.

Enzymatic hydrolysis of the cellulose

Cellulase activity was determined as the amount of released glucose after cellulose degradation. 200 μL of cellulase immobilized on sorbents (containing 0.5 mg cellulase) or free cellulase solution (2.5 mg/mL) was mixed with 200 μL of CMC solution (2%) and then incubated at 50 °C for 30 min. After that, the mixture was boiling for 5 min to stop the hydrolysis reaction. One International Unit (IU) of cellulase activity is defined as the amount of cellulase that hydrolyzes CMC to produce 1 μmol glucose per minute.

After hydrolysis, the mixture was centrifugation at 10000 × g for 10 min to remove immobilized cellulase. And then the supernatant was used in glucose analysis via the glucose kit of GLU C Ⅱ.

Characterization

Nitrogen (N_2) sorption isotherms were measured with a Micromeritics Tristar 3000 analyser at 77 k. The Barrett-Emmett-Teller (BET) method was utilized to calculate the surface areas. The pore volume and pore size distributions were calculated from the desorption branch of the isotherms using the Brarrett-Joyner-Halanda (BJH) method. Solution UV-vis spectra were achieved by UV spectrophotometer (Quawell Q5000) under 595 nm wave length. Small Angle X-ray Scattering (SAXS) experiments were performed on a Kratky compact small-angle system equipped with a position-sensitive wire detector (OED 50M from MBraun, Graz) containing 1024 channels of width 53.6 μm. CuKα radiation of wavelength 1.542 Å was provided by a Seifert ID 3000 X-ray generator operated at 50 kV and 40 mA. The analysis of size, shape and particle distribution of the samples was carried out using TEM (Tecnai G2 F30) at 100 kV and SEM (Hitachi S-4300) at 15 kV. For this purpose, dispersions of sample nanoparticles were pipetted onto carbon-coated copper grids.

Result and discussion

For the physical adsorption mechanism, synthetic mesoporous silica MS-17.6nm and MS-3.8nm and commercial materials H-32 and diatomite were used as the sorbents. The effect of morphology of sorbents, immobilization temperature and the adding amount of sorbents were studied.

Characterization of mesoporous silica

The morphological and structural characterization of different mesoporous silica and available porous silica were performed by N_2 adsorption-desorption analysis, small-angle XRD,TEM and SEM. Fig. 1 represented the small-angle X-ray scattering patterns of MS-17.6nm and MS-3.8nm, respectively. It can be seen that the SAXS pattern of MS-17.6nm (Fig. 1a) exhibited a well-pronounced (100)

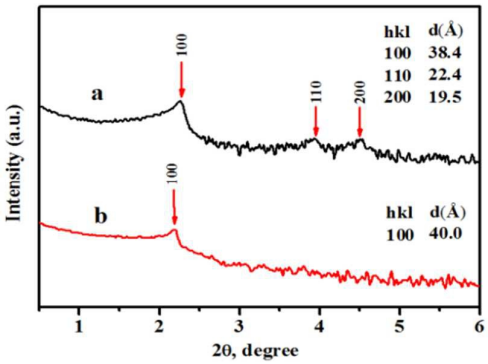

Fig. 1. Small-angle X-ray scattering patterns for (a) MS-17.6nm and (b) MS-3.8nm.

diffraction peak with a d spacing of 38.4 Å, as well as two small (110) and (200) peaks associated with a 2D hexagonal mesostructure (p6mm), confirming the highly ordered mesostructure of the silica host. [34] For the MS-3.8 nm, SAXS pattern showed a (100) diffraction peak, as you have said, MS-3.8nm have a similar mesostructure to MS-17.6nm. But, the intensity of (100) peak is weak, and there are no other two peaks, indicating that the 2D hexagonal structure of MS-3.8nm is incomplete. This can be associated with low temperature and dilute solution or the short hydrocarbon chains of the organic template during synthesis.[35-37] In addition, a part of 2D hexagonal structure of MS-3.8nm may be destroyed. So, MS-3.8nm is less ordered mesostructure than MS-17.6nm.

Further evidence for mesostructure was provided by the TEM images as presented in Fig. 2, which were representative of mesoporous silica prepared with CTAB. According to the TEM image of MS-17.6nm (Fig. 2c), there was some curved lattice fringes with a d spacing of 36.9 Å, which showed well-ordered mesopore structures viewed along the [001] directions, suggesting that the mesoporous silica was a highly ordered mesostructure. Furthermore, the pores and lattice fringes of MS-3.8nm could be observed directly from Fig. 2d, the pore size and d spacing was both

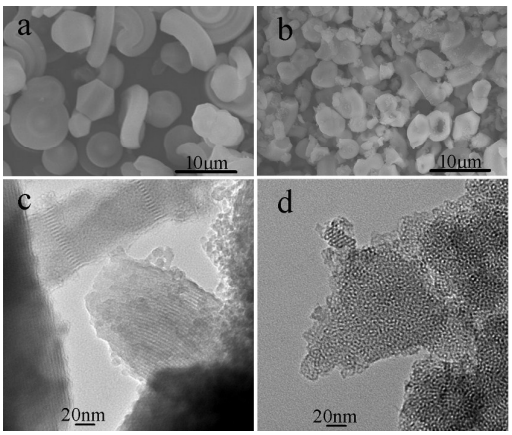

Fig. 2. SEM and TEM images of mesoporous silica: (a, c) MS-17.6nm and (b, d) MS-3.8nm.

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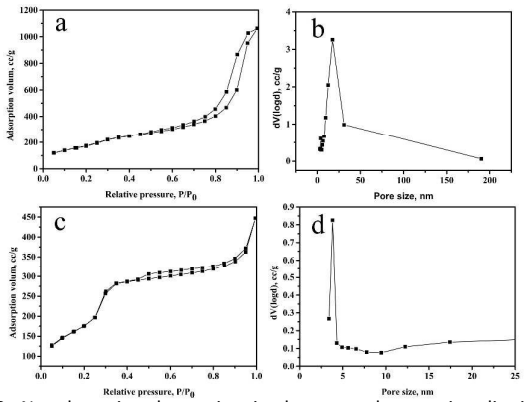

Fig. 3. N_2 adsorption-desorption isotherms and pore size distribution curves of mesoporous silica with different pore size: (a, b) MS-17.6nm and (c, d) MS-3.8nm.

about 38.2 Å. Meanwhile, it can be observed clearly that MS-3.8nm was composed of accumulation by flaky silica. Both of the results agreed well with the value of (100) crystal plane calculated by small-angle XRD analysis respectively. The particle size of mesoporous silica was shown in Fig. 2a and Fig. 2b: MS-17.6nm (4-12 μm) and MS-3.8nm (1-7μm). Besides, the N2 adsorption isotherms of the materials and their corresponding (BJH) pore size distributions determined from desorption branch were also exhibited in Fig. 3. As displayed in Fig. 3a, the adsorption-desorption isotherm of MS-17.6nm possessed a type Ⅳ curve with H1 hysteresis loop at relative high P/P0 according to the IUPAC classification,[38] suggesting a mesoporous structure. The specific surface area, average pore diameter, and total pore volume of MS-17.6nm were calculated to be 474.7 m^2g^{-1}, 17.6nm and 1.5 cm^3g^{-1}, respectively. The pore-size distribution analyzed by BJH method was shown in the inset, which displayed a sharp peak at 17.6 nm. For comparison, MS-3.8nm with specific surface area of 105.2 m^2g^{-1}, average pore diameter of 3.8 nm and total pore volume of 0.3 cm^3g^{-1} was also measured by BET method (Fig. 3c). As can be seen, a typical type Ⅳ curve with H4 hysteresis loop was observed, indicating the mesoporous structure which was possibly attributable to the accumulation by flaky silica. These situations were consistent with the SAXS and TEM results.

Immobilization of cellulase on different sorbents

Since chemical immobilization may extremely likely occupy the activity sites of cellulase, leading difficulty for cellulase to change conformation, the cellulase was immobilized on different sorbents by pure physical adsorption without any chemical bond or covalent bond. The cellulase catalytic activity is essentially correlated with its conformation. To obtain a good activity, the cellulase must be immobilized in such a way that it can easily change conformation. Nevertheless, there are various factors crucial to the immobilization, such as morphology of sorbents, reaction temperature, amount of sorbents, and reaction time. To obtain appropriate immobilization to let cellulose change its conformation freely, the effect of morphology of sorbents was determined first.

Effect of morphology

As shown in Fig. 4, the largest adsorption capacity appeared in the case of MS-17.6nm, 325.5 mg/g (cellulase/ sorbents), almost 1.2

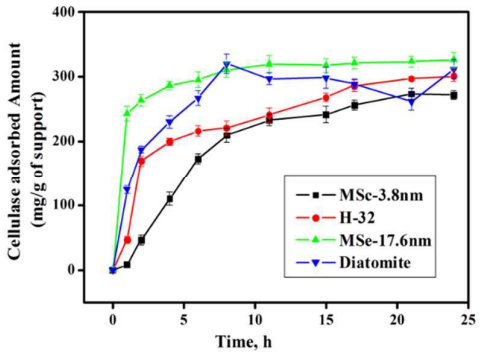

Fig. 4. Cellulase adsorption amount on different sorbents which are given in the inset.

Table 1 Adsorption amounts of cellulase and physicochemical property of mesoporous silica.

Mesoporous silica	Pore diameter (nm)	BET surface area (m^2g^{-1})	Total pore volume (cm^3g^{-1})	Adsorbed amounts (mg g^{-1})
H-32	25	232.5	0.77	301.0
MS-3.8nm	3.8	105.2	0.30	271.7
MS-17.6nm	17.6	474.7	1.51	325.5
Diatomite	200	13.0	0.11	311.5

times more than that adsorbed by MS-3.8nm. As for the MS-3.8 nm, H-32 and diatomite, the adsorption amount after 24 h was about 271.7, 301.0 and 311.5 mg/g, respectively, slightly lower than that of MS-17.6nm. Among these sorbents, it was surprising that the adsorption behavior of diatomite revealed a periodicity. The maximum adsorption amount in the circle 320.0 mg/g was obtained at 8 h, and the minimum adsorption amount in the circle 261.3 mg/g was obtained at 21 h, then the intact adsorption circle was calculated as 26 h. These situations may be attributed to the different morphology of sorbents. To further explain, the adsorption amounts and physicochemical properties of mesoporous silica were shown in Table 1.

As the results of previous analysis showed, cellulase molecule was an elongated object, and the long and short axes were ca. 124 Å and ca. 37 Å.[39] Obviously, the pore diameter of diatomite was significantly larger than the size of cellulase molecule, cellulase molecules can be easily adsorbed into the pores with loose and disordered arrangement due to capillary action. Meanwhile, when the system established adsorption equilibrium, loose cellulase molecules without immobilization were washed out from the pores under stirring. This kind of circulation generated the unique periodicity of the adsorption behavior for diatomite. The geometrical dimension of cellulase molecule and sorbent was another factor affecting immobilization. Takahashi[40] and his co-workers indicated that the mesoporous material which had the pore size close to geometrical dimension of cellulase would show better adsorption capacity. Although the sorbent of MS-3.8nm had the pore size of 3.8 nm, very approximate to the short axes of cellulase, the sorbent exhibited the minimum adsorption amount. This would be attributed to the pore space was blocked with some

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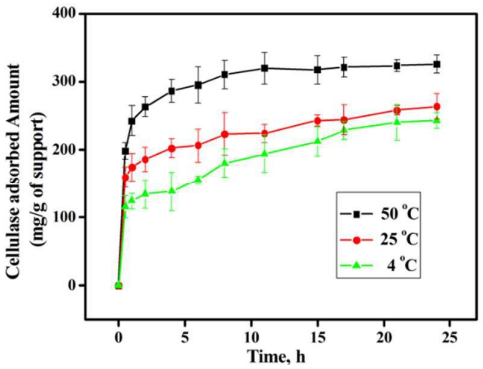

Fig. 5 Comparison of the cellulase adsorption on MS-17.6nm for different temperatures which are given in the inset.

cellulase molecules. The cellulase molecules that stuck in the entrance of pores made the diffusion of cellulase into the pores difficultly. However, the sorbent of MS-17.6nm, of which the pore size was slightly larger than the long axes of cellulase molecule, showed the maximum adsorption amount. After cellulase being adsorbed into the pores, there was appropriate space for molecules not to escape but adjust as dense and ordered arrangement. As far as H-32 (pore size = 25 nm) is concerned, its adsorption amount (301 mg g-1) relatively approached that of MS-17.6nm (325.5 mg g-1), this phenomenon was because the adsorption mechanism of H-32 was same as that of MS-17.6nm.

Therefore, pore size slightly larger than the long axes of cellulase molecule was essential for cellulase immobilization.

Effect of temperature

The temperature profiles of the adsorption amounts of cellulase using MS-17.6nm were measured (Fig. 5). As far as we are aware, there have been no reports on the relationship between temperature and adsorption amounts in MS-cellulase system, possibly due to the fact that most of the proteins are easy to be denatured. 25 mL cellulase (4 mg/mL) solution was added to MS-17.6nm powder (250 mg). The mixture was stirred at 50 °C, 25 °C and 4 °C for 24h. It was shown that the reaction system at 50 °C reached adsorption equilibrium after 11 h. It was because MS-17.6nm had reached adsorption saturation after 11 h. It was not favourable for further loading of cellulase due to the extremely high space hindrance in mesopores. Meanwhile, the adsorption amounts of cellulase under 25 °C and 4 °C were increasing as a function of time over the whole time range, the maximum equilibrium adsorption amount cannot be observed within 24 h in our experiments. In addition, at the given time, both the adsorption amounts and rate of cellulose in MS-17.6nm increased along with temperature increase. This was in accordance with the cellulase immobilization process we suggested above. Temperature is an important factor of adsorption efficiency. It is because adsorption behaviour of cellulase molecules is an endothermic process.[41] Therefore, increasing temperature may promote immobilization of cellulase into mesoporous silica with high efficiency, and it may be anticipated that, the amounts of cellulase immobilized at 25 °C and 4 °C may be further increased if enough long contact time offered. Thus, subsequent experiments used a temperature of 50 °C for immobilization.

Effect of amounts of sorbents

From an economic point of view, determining the optimal amount of sorbent makes it possible to use the minimum amounts of sorbents to immobilize cellulase while maximizing the glucose yield. Experiments involving adsorption of cellulase into MS-17.6nm and MS-3.8nm were conducted for a range of initial input of sorbents from 150 mg to 250 mg at 50 °C for 24 h. To keep the concentration of cellulase as a constant, the mass of cellulase was 100 mg dissolved in 25 ml buffer.

As summarized in Fig. 6, adsorption curves of MS-17.6nm towards contact time showed a little difference. The saturated adsorption amounts of cellulase were about 325, 410, 260 mg/g at contacting time of 17, 8 and 6 h, where the sorbent amounts were 250, 200 and 150 mg, respectively. Different conclusion was drawn when MS-3.8nm was used (Fig. 7). The adsorption amounts were about 270, 315, 315 mg/g at contacting time of 21, 21 and 17 h, where the sorbent amounts were 250, 200 and 150 mg, respectively. The results shown that MS-17.6 was more effective to adsorbed cellulase than MS-3.8, which was due to a regular microstructure, large pore size and surface area. The kinetically

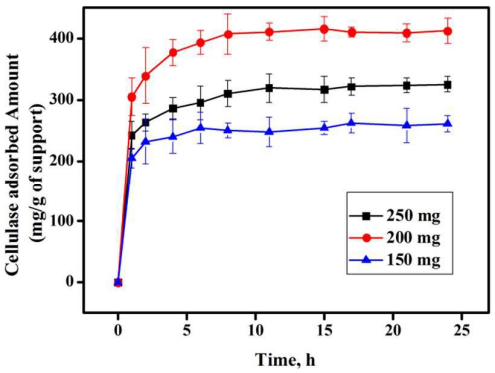

Fig. 6 Comparison of the cellulase adsorption on MS-17.6nm for different amounts of sorbents which are given in the inset.

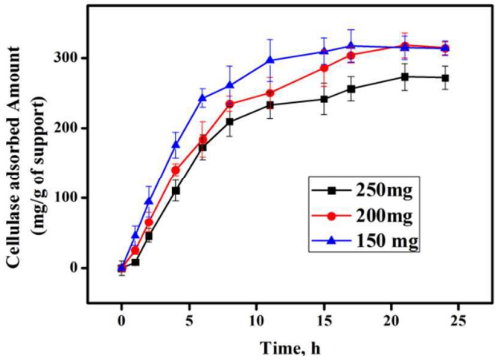

Fig. 7 Comparison of the cellulase adsorption on MS-3.8nm for different amounts of sorbents which are given in the inset.

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adsorption rate not only depended on the microstructure of sorbent, but also related to the sorbent amounts.

In the case of physical adsorption, the physical adsorption process may contain surface adsorption and pore adsorption. The size of a single cellulase was shown in Fig. 9, and the average pore size of MS-3.8 was about 3.8 nm. So surface adsorption was dominated during the adsorption process. Therefore, in a certain concentration of enzyme solution, the less adsorbent, and the higher enzyme concentration in the relative microenvironment, the faster adsorption rate and the more fully adsorption it got. So that, the adsorbed amount of average per gram of carrier was higher. Therefore, when the addition amount of MS-3.8 was 150 mg, the adsorbed amount of average per gram of carrier was the largest, and it was the smallest when the addition amount was 250 mg.

Compared to MS-3.8, MS-17.6 had a larger pore size (17.6 nm) and surface area (474.7 m^2/g), hence there were more surface adsorption and pore adsorption in MS-17.6. When the amount of adsorbent was 150 mg, the enzyme concentration in the micro-environment was higher and the adsorption rate was faster. However, the average pore size of MS-17.6 was similar to the size of a single cellulase. It may block a part of pore in a rapid adsorption, which would hinder the further adsorption of the channel and thus increase the ratio of surface adsorption. Finally, the adsorbed amount was not high. When the amount of adsorbent was 200 mg, the enzyme concentration in the micro-environment was low, and the probability of clogging of the channel became smaller. The carrier can fully adsorb cellulase by pore adsorption and surface adsorption, and finally obtain the largest adsorbed amount. When the amount of adsorbent was too much (250 mg), the carrier also could fully adsorb cellulase by pore adsorption and surface adsorption, but the adsorbed amount would be decreased because of the lower relative concentration of cellulase. So, for MS-17.6nm, there is an optimal value between sorbent amount and concentration of cellulase.

Activities of the different immobilized cellulase

The cellulase immobilized on different sorbents all showed activities toward cellulose hydrolysis. Significant differences between relative enzymatic activities have been nevertheless found depending on the type of sorbents.

Fig. 8 showed the effect of temperature on the specific activity of the immobilized cellulase using different sorbents. Each immobilized or free cellulase used for the reaction had the same cellulase content 0.5 mg, in comparing the activities of the cellulase. Intriguingly, the shape of the temperature profile of cellulase immobilized on MS-3.8nm was very similar to the specific activity curve of free cellulase and displayed a high specific activity (i.e. 63.3% of free cellulase activity at 50 °C) which could be concluded that the acremonium cellulase immobilized on MS-3.8nm maintain preserved the original morphology of free cellulase extremely. In addition, cellulase immobilized on MS-3.8nm retained a higher level of specific activity at elevated temperature (i.e. 67.0% of free cellulase activity at 60 °C) in comparison to the other hydrolysis temperature. This result illustrated that the immobilization of cellulase on MS-3.8nm increased the stability of free cellulase with regard to higher temperature. As described above, sorbent of MS-3.8nm had the pore size of 3.8 nm, very approximate to the short

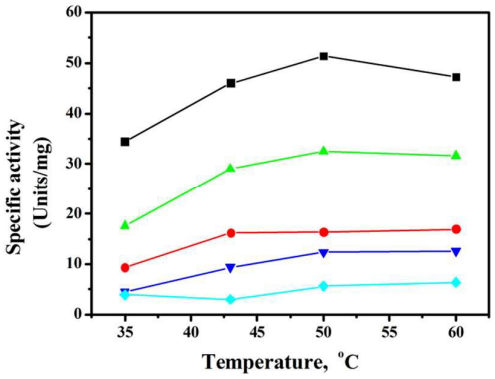

Fig. 8 The effect of temperature on the specific activity of immobilized cellulase. Cellulase was immobilized on MS-17.6nm (▼), MS-3.8nm (▲), H-32 (●), and diatomite (◆). Free was no immobilized cellulase (■) as positive control.

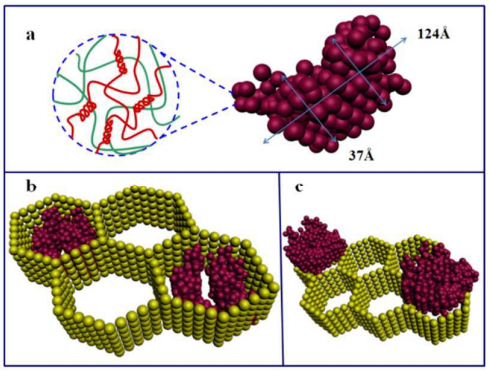

Fig. 9 Structural model of a cellulase molecule (a) and image models of immobilized cellulase in MS-17.6nm (b) and MS-3.8nm (c) using a computer schematic model.

axes of cellulase molecules, then the molecules stucked in the entrance of pores. Therefore, active sites of cellulase molecules were preserved.

As the adsorption amounts increased, specific activity decreased gradually, cellulase immobilized on H-32 and MS-17.6nm exhibited specific activity 35.8% and 26.6% of free cellulase activity at 60 °C, respectively. This may be attributed to the cellulase immobilization process we suggested previously, sorbents MS-17.6 nm which have average mesopores size matched long axes of cellulase molecules showed the maximum adsorption amount. When cellulase was adsorbed into the pore, there was appropriate space for molecules not to escape but adjust as dense and ordered arrangement. However, the dense and ordered arrangement would prevent conformational flexibility of cellulase, as we know the cellulase molecules need conformational change in the interaction process between cellulase and substrate. So, MS-17.6nm revealed a lower specific activity than H-32. And the image models of adsorption mechanism for MS-17.6nm and MS-3.8nm was exhibited in Fig. 9.Meanwhile, both of MS-17.6nm and H-32 retained a higher level of specific activity at elevated temperature in comparison to the

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other hydrolysis temperature, by contrast, it was obvious that specific activity of free cellulase decreased when temperature higher than 50 °C.

In the case of diatomite, a significant difference in the specific activity was observed, which revealed the lowest specific activity i.e. 13.5% of free cellulase activity at 60 °C. This may be contributed by the lamellar structure of diatomite: immobilized cellulase molecules were embedded between the lamellas, which prevented cellulase from contacting with substrate. However, it was still obvious that the specific activity of cellulase immobilized on diatomite demonstrated better thermal stability: as temperature increasing specific increased.

These findings illustrated that mesoporous silica were favorable for stabilizing cellulase and repetitive application. Immobilized cellulase showed high specific activity in thermal treatment when the enzyme molecules were adsorbed to MS-3.8nm, which showed relatively small adsorption amounts and had average mesopore sizes matched the short axes of cellulase molecules. Immobilization preserved the catalytic specificity toward cellulose-to-glucose, which is the best way to immobilize cellulase with a stable efficiency.

Conclusions

In conclusion, the loading efficiency in this case exhibited a clear correlation with the pore size of mesoporous silica on which the enzymes was immobilized; i.e., the adsorption amounts of MS-17.6nm (410 mg/g) with the pore size was similar to the long axes of cellulase molecule was higher than that for MS-3.8nm (315 mg/g) with the pore size approximated to the short axes of cellulase (which was realized at 50 °C). Furthermore, the sorbents with larger pore size showed an optimum amount of 200 mg in 4mg/mL cellulase solution, moreover the smaller pore size sorbents possessed the highest loading efficiency with the usage amount of 200 mg as well as 150 mg under the same conditions. Besides, the adsorption behavior of diatomite revealed a periodicity because the pore size was significantly larger than cellulase molecules. In the meantime, the pore size was suggested to be a critical factor for enzymatic activity of the cellulase. When the average pore size of MS-3.8nm just matched the short axes of cellulase molecules, immobilized cellulase preserved active sites of cellulase intactly and showed the best activity (i.e. 63.3% of free cellulase activity at 50 °C). Consequently, the results successfully demonstrated the expected theory -- the mesoporous silica with pores slightly larger than the long axes of cellulase molecule would obtain a relative large adsorption amount. However, sorbents with pores approximate to the short axes of cellulase molecules would preserve active sites of cellulase. Herein, cellulase immobilization on MS-3.8nm having an appropriate pore size would be useful and applicable to industrial processes and other applications, especially certain environmentally useful enzymatic reactions such as the decomposition of cellulosic biomass.

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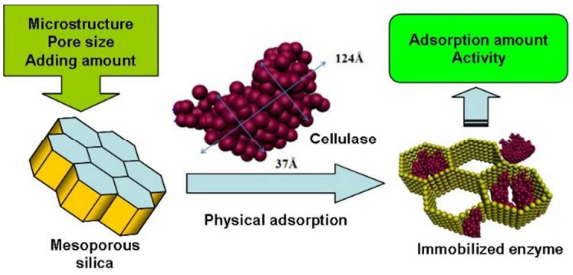

Studying the physical adsorption mechanism and with high adsorption amount in the mesoporous silica immobilized cellulase process.